



22 **Abstract**

23

24 The role of the highly-conserved 'DRY' motif in the signaling of the CB<sub>1</sub> cannabinoid  
25 receptor (CB<sub>1</sub>R) was investigated by introducing single, double and triple alanine  
26 mutations into this site of the receptor. We found that the CB<sub>1</sub>R-R3.50A mutant displays  
27 a partial decrease in its ability to activate heterotrimeric G<sub>o</sub> proteins (~80% of wild-type  
28 CB<sub>1</sub>R (CB<sub>1</sub>R-WT)). Moreover, this mutant showed an enhanced basal β-arrestin2  
29 recruitment. More strikingly, the double mutant CB<sub>1</sub>R-D3.49A/R3.50A was biased  
30 toward β-arrestins, as it gained a robustly increased β-arrestin1 and β-arrestin2  
31 recruitment ability compared to the wild-type receptor, while its G protein activation was  
32 decreased. In contrast, the double mutant CB<sub>1</sub>R-R3.50A/Y3.51A proved to be G protein-  
33 biased, as it was practically unable to recruit β-arrestins in response to agonist stimulus,  
34 while still activating G proteins, although at a reduced level (~70% of CB<sub>1</sub>R-WT).  
35 Agonist-induced ERK1/2 activation of the CB<sub>1</sub>R mutants showed good correlation with  
36 their β-arrestin recruitment ability but not with their G protein activation or inhibition of  
37 cAMP accumulation. Our results suggest that G protein activation and β-arrestin binding  
38 of the CB<sub>1</sub>R are mediated by distinct receptor conformations and the conserved 'DRY'  
39 motif plays different roles in the stabilization of these conformations, thus mediating both  
40 G protein- and β-arrestin-mediated functions of CB<sub>1</sub>R.

## 41 **1. Introduction**

42

43 Seven transmembrane receptors (7TMRs) constitute the largest family of plasma  
44 membrane receptors. Most of their intracellular effects are mediated via direct coupling to  
45 heterotrimeric G proteins. To understand the molecular details of 7TMR activation and G  
46 protein coupling, identification of key structural elements regulating these processes is  
47 critically important. Using mutational analyses as well as recent high resolution X-ray  
48 crystal structure data, such structural features have been extensively mapped  
49 (Venkatakrishnan et al. 2013). Among these, the conserved Asp-Arg-Tyr (DRY) motif,  
50 located at the beginning of the second intracellular loop (ICL2), seems to play a central  
51 role both in the activation and the G protein coupling of class A (rhodopsin-like) 7TMRs  
52 (Rasmussen et al. 2011). Nevertheless, the exact nature of this regulatory role is still not  
53 completely understood. For instance, although the Arg residue (R3.50) is suggested to  
54 directly interact with the G protein  $\alpha$  subunit in the active 7TMR conformation, its non-  
55 conservative mutations in many cases fail to impair G protein coupling of the receptor  
56 (Fanelli et al. 1999; Rhee et al. 2000; Rovati et al. 2007). Furthermore, Asp (D3.49) is  
57 believed to stabilize inactive receptor conformation by forming a salt-bridge with the  
58 neighboring R3.50 (Scheer et al. 1996; Scheer et al. 1997; Ballesteros et al. 1998;  
59 Ballesteros et al. 2001; Li et al. 2001), however, its mutations can also result in  
60 completely diverse phenotypes, depending on the investigated receptor (Rovati et al.  
61 2007). Therefore, the exact role of the DRY motif obviously shows receptor-specific  
62 differences, and its detailed analysis for a particular 7TMR seems reasonable.

63 Besides G proteins,  $\beta$ -arrestins are also able to directly bind to the intracellular surface of  
64 an activated 7TMR, leading to the desensitization and internalization of the receptor  
65 (Shenoy and Lefkowitz 2011). Moreover, receptor-bound  $\beta$ -arrestins can also serve as a  
66 starting point for G protein-independent signaling pathways, such as the activation of the  
67 p42/44 mitogen-activated protein kinase (MAP kinase) cascade or Src kinases (Wei et al.  
68 2003; DeWire et al. 2007).

69 Many data suggest that the  $\beta$ -arrestin-bound conformation of 7TMRs may differ from the  
70 one mediating their G protein activation, a fact being implicitly exploited by several  
71 functionally selective 7TMR ligands as well as by functionally selective 7TMR mutants,  
72 which are able to induce  $\beta$ -arrestin recruitment without affecting G protein coupling or  
73 vice versa (Reiter et al. 2012). However, in the lack of a high resolution crystal structure  
74 describing a 7TMR in its  $\beta$ -arrestin-bound form, relatively little is known about the  
75 receptor-arrestin binding interface. According to the prevailing idea, arrestins utilize two  
76 distinct sites to bind to 7TMRs, one of which is a ‘phosphorylation sensor’, recognizing  
77 Ser/Thr-phosphorylated C-terminus of the receptor (Gurevich and Benovic 1993;  
78 Gurevich and Gurevich 2006). The other site is a so-called ‘activation sensor’, which  
79 recognizes the active 7TMR conformation, independently of receptor phosphorylation  
80 (Gurevich and Gurevich 2006). The 7TMR elements constituting the docking site for the  
81 arrestin ‘activation sensor’ are less understood. The second intracellular loop (ICL2),  
82 beginning with the DRY motif, has been proposed to play such a role (Huttenrauch et al.  
83 2002; Marion et al. 2006). Furthermore, complementary roles for the DRY motif and  
84 receptor C-terminus in the regulation of  $\beta$ -arrestin binding have been described (Kim and  
85 Caron 2008). In addition, mutations of R3.50 in many cases results in basal  $\beta$ -arrestin

86 binding and subsequent constitutively desensitized phenotype of 7TMRs (Barak et al.  
87 2001; Wilbanks et al. 2002). Thus, the conserved DRY motif seems to be involved not  
88 only in G protein coupling, but also in  $\beta$ -arrestin binding of 7TMRs.

89 The CB<sub>1</sub> cannabinoid receptor (CB<sub>1</sub>R) belongs to the 7TMR superfamily. The signaling  
90 pathways originating from CB<sub>1</sub>R are mediated mainly via heterotrimeric G<sub>i/o</sub> proteins,  
91 and include inhibition of cAMP production, activation of GIRK potassium channels,  
92 inhibition of Ca<sub>v</sub> calcium channels, and activation of MAP kinase cascades (Turu and  
93 Hunyady 2010). Moreover, CB<sub>1</sub>R shows basal G protein activation and constitutive  
94 internalization under diverse cellular conditions (Letierrier et al. 2006; McDonald et al.  
95 2007; Turu et al. 2007). Like most other 7TMRs, CB<sub>1</sub>R also recruits  $\beta$ -arrestin following  
96 activation, which leads to the desensitization and internalization of the receptor  
97 (Kouznetsova et al. 2002; Daigle et al. 2008; Gyombolai et al. 2013). The binding  
98 between  $\beta$ -arrestins and CB<sub>1</sub>R is relatively weak, and the affinity of the receptor for  $\beta$ -  
99 arrestin2 ( $\beta$ -arr2) is substantially higher than that for  $\beta$ -arrestin1 ( $\beta$ -arr1) (Gyombolai et  
100 al. 2013). Furthermore,  $\beta$ -arr1 recruitment of CB<sub>1</sub>R appears to be agonist-dependent  
101 (Laprairie et al., 2014; Flores-Otero et al., 2014). Interestingly, in addition to canonical G  
102 protein-mediated intracellular effects, recent data suggest the existence of  $\beta$ -arrestin-  
103 mediated, G protein-independent signaling of CB<sub>1</sub>R, i.e. the p42/44 MAPK (ERK1/2)  
104 activation of the receptor seems to be at least partly mediated by  $\beta$ -arrestins (Ahn et al.  
105 2013a; Mahavadi et al. 2014).

106 Via these cellular events, CB<sub>1</sub>R is involved in the regulation of many important  
107 physiological and pathophysiological processes, such as memory, learning, pain  
108 sensation, metabolic regulation, or the regulation of vascular tone (Pacher et al. 2006).

109 Moreover, several natural and synthetic cannabinoid ligands are known to stabilize  
110 distinct active CB<sub>1</sub>R conformations, i.e. prove to be functionally selective (Glass and  
111 Northup 1999; Mukhopadhyay and Howlett 2001; Ahn et al. 2013a). Thus, investigation  
112 of the structural elements responsible for G protein- and  $\beta$ -arrestin-mediated CB<sub>1</sub>R  
113 functions has a major physiological and pharmacological impact. Accordingly, a number  
114 of studies have aimed to identify such regulatory motifs of CB<sub>1</sub>R. A detailed  
115 computational model based on the crystal structure of the  $\beta_2$ -adrenergic receptor-G $\alpha_s$   
116 complex, combined with mutational data, suggested that distinct residues in the ICL2 and  
117 ICL3 regions of the CB<sub>1</sub>R may be involved in the stabilization of the active, G $\alpha_i$ -coupled  
118 receptor conformation (Shim et al. 2013). Two other recent studies analyzed the role of  
119 several intramolecular salt-bridges, which may stabilize inactive, partially active and  
120 fully active CB<sub>1</sub>R conformations (Ahn et al. 2013b; Scott et al. 2013). According to this  
121 model, D3.49 and R3.50 residues form salt-bridges with K4.41 and D6.30, respectively,  
122 which (together with a D2.63+K3.28 salt-bridge) may keep the receptor in a partially  
123 active conformation under basal conditions.

124 Less is known about the structural features governing the  $\beta$ -arrestin binding of CB<sub>1</sub>R. The  
125 C-terminal Ser/Thr phosphorylation of the receptor seems to play a role, since alanine  
126 mutations of these residues impaired agonist-induced  $\beta$ -arrestin recruitment and  
127 subsequent internalization of CB<sub>1</sub>R (Daigle et al. 2008).

128 Although the above studies clearly provide important insights into the molecular details  
129 of CB<sub>1</sub>R function, none of them assessed the role of the DRY motif in CB<sub>1</sub>R function  
130 directly, i.e. through mutational analysis. More importantly, none of the available studies  
131 have aimed to identify  $\beta$ -arrestin-regulatory motifs of CB<sub>1</sub>R other than the receptor C-

132 terminus. Therefore, our goal was to analyze the role of the conserved DRY sequence in  
133 the G protein activation and  $\beta$ -arrestin binding of CB<sub>1</sub>R. We introduced single, double  
134 and triple alanine mutations into this site of CB<sub>1</sub>R and applied functional assays directly  
135 measuring G protein activation,  $\beta$ -arr2 recruitment and intracellular signaling of wild-  
136 type and mutant CB<sub>1</sub>R variants.

137

## 138 **2. Materials and Methods**

139

### 140 *2.1. Materials*

141

142 The cDNA of the rat vascular CB<sub>1</sub>R was provided by Zsolt Lenkei (Centre National de la  
143 Recherche Scientifique, Paris). cDNAs of human  $\beta_1$  and  $\gamma_{11}$  G protein subunits were  
144 purchased from the Missouri S&T cDNA Resource Center (Rolla, MO).  $\beta$ -arr2-eGFP  
145 cDNA was kindly provided by Dr. Marc G. Caron (Duke University, Durham, NC).  
146 Molecular biology enzymes were obtained from Fermentas (Vilnius, Lithuania) and  
147 Stratagene (La Jolla, CA). Fetal bovine serum (FBS), OptiMEM, Lipofectamine 2000,  
148 and PBS-EDTA were from Invitrogen (Carlsbad, CA). CHO-K1 and HeLa cell lines were  
149 obtained from ATCC (American Type Culture Collection, Manassas, VA).  
150 Coelenterazine h was from Regis Technologies (Morton Grove, IL). WIN55,212-2, 2-  
151 arachidonoylglycerol and AM251 were from Tocris (Bristol, UK). Cell culture dishes  
152 and plates for BRET measurements were from Greiner (Kremsmunster, Austria). Anti-  
153 pERK1/2, anti-ERK1/2 and HRP-conjugated anti-rabbit and anti-mouse antibodies were

154 from Cell Signaling Technology Inc. (Beverly, MA). Unless otherwise stated, all other  
155 chemicals and reagents were from Sigma (St. Louis, MO).

156

### 157 *2.2. Plasmid constructs and site-directed mutagenesis*

158

159 The mVenus-tagged rat CB<sub>1</sub>R (CB<sub>1</sub>R-mVenus) was created by exchanging the sequence  
160 of eYFP in CB<sub>1</sub>R-eYFP (kindly provided by Zsolt Lenkei (Centre National de la  
161 Recherche Scientifique, Paris)) to the sequence of mVenus using AgeI and NotI  
162 restriction enzymes.  $\alpha_0$ -Rluc and YFP- $\beta_1$  constructs were created from  $\alpha_{0A}$ -CFP (kindly  
163 provided by Dr. N. Gautam (Azpiazu and Gautam 2004)), and  $\beta_1$ , respectively, as  
164 described previously (Turu et al. 2007).  $\beta$ -arr2-Rluc was constructed as described  
165 previously (Turu et al. 2006). Plasma membrane-targeted mVenus (MP-mVenus) was  
166 constructed as described previously (Varnai et al. 2007). Plasma membrane-targeted  
167 super Renilla luciferase (MP-Sluc) was generated from MP-mVenus by replacing the  
168 mVenus coding sequence with the cDNA of super Renilla luciferase (Woo and von  
169 Arnim, 2008). The EPAC-based BRET sensor was constructed as described previously  
170 (Erdelyi et al. 2014). Mutations in the DRY motif of CB<sub>1</sub>R or CB<sub>1</sub>R-mVenus were  
171 inserted by the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA)  
172 according to manufacturer's suggestions. Sequences of all constructs were verified using  
173 automated DNA sequencing.

174

### 175 *2.3. Cell culture and transfection*

176



177 CHO or HeLa cells (passage numbers 5 to 15) were maintained in Ham's F12 or DMEM,  
178 respectively, supplemented with 10% FBS, (Invitrogen, Carlsbad, CA), 100 µg/ml  
179 streptomycin, and 100 IU/ml penicillin in 5% CO<sub>2</sub> at 37 °C. For confocal microscopy  
180 experiments, cells were grown on glass coverslips in 6-well plates and transfected with  
181 the indicated constructs using Lipofectamine 2000 in OptiMEM following the  
182 manufacturer's instructions. For BRET and Western blot experiments, cells were grown  
183 on 6-well plates and transfected with the indicated constructs using Lipofectamine 2000  
184 in OptiMEM following the manufacturer's instructions.

185

#### 186 *2.4. Bioluminescence resonance energy transfer (BRET) measurements*

187

188 A detailed description of the BRET measurements applied here can be found in  
189 Supplementary Methods.

190

#### 191 *2.5. Confocal laser-scanning microscopy*

192

193 Cells were grown on glass coverslips and transfected with the appropriate constructs  
194 (using 2 µg/well CB<sub>1</sub>R-mVenus or 0.5 µg/well β-arr2-GFP and 2 µg/well CB<sub>1</sub>R). Cells  
195 were analyzed 22-26 hours later in a modified Krebs-Ringer buffer (see above), using a  
196 Zeiss LSM 710 confocal laser scanning microscope.

197

#### 198 *2.6. Western blot analysis*

199

200 A detailed description of the Western blot measurements applied here can be found in  
201 Supplementary Methods.

202

### 203 *2.7. Data analysis*

204

205 Dose-response curves for G protein,  $\beta$ -arrestin and EPAC BRET measurements were  
206 fitted and statistically compared using built-in algorithms of GraphPad Prism 4.03  
207 (GraphPad Software Inc, San Diego, CA). Equimolar comparison was carried out by  
208 plotting the points of G protein and  $\beta$ -arr2 BRET dose-response curves for vehicle, -8.0  
209 (only by WIN55), -7.5, -7.0, -6.5, -6.0, -5.5 and -5.0 (only by 2-AG)  $\log[\text{WIN55}]$  or  
210  $\log[2\text{-AG}]$  (M) treatments of the same receptor against each other. Equiactive comparison  
211 was carried out by determining the bias factor ( $\beta$ ) using the equation

$$212 \quad \beta = \log \left( \left( \frac{E_{\max,1}}{EC_{50,1}} \frac{EC_{50,2}}{E_{\max,2}} \right)_{mut} \times \left( \frac{E_{\max,2}}{EC_{50,2}} \frac{EC_{50,1}}{E_{\max,1}} \right)_{ref} \right), \text{ (Rajagopal et al. 2011), where } E_{\max,1},$$

213  $EC_{50,1}$ ,  $E_{\max,2}$  and  $EC_{50,2}$  are  $E_{\max}$  and  $EC_{50}$  values from G protein and  $\beta$ -arrestin BRET  
214 dose-response curves, respectively, using  $CB_1R$ -WT as reference receptor. Quantified  
215 Western-blot data were evaluated with two-way ANOVA combined with Holm-Sidak's  
216 post-hoc test, using the software SigmaStat for Windows 3.5 (Systat Software Inc.,  
217 Richmond, CA), and a p value  $<0.05$  was considered significant.

218

## 219 **3. Results**

220

### 221 *3.1. Plasma membrane localization of the $CB_1R$ mutants*

222

223 To investigate whether any of the mutations inserted into the DRY motif of CB<sub>1</sub>R affects  
224 the proper plasma membrane localization of the receptor, CHO cells expressing mVenus-  
225 tagged CB<sub>1</sub>R variants were analyzed using confocal microscopy. In resting cells, CB<sub>1</sub>R-  
226 mVenus is localized both at the plasma membrane and in intracellular vesicles, consistent  
227 with the constitutive internalization of CB<sub>1</sub>R (Fig. 1A). Importantly, D3.49A mutation  
228 strongly impaired plasma membrane localization of CB<sub>1</sub>R, with most of the receptors  
229 being retained in the endoplasmic reticulum of the cells (CB<sub>1</sub>R-D3.49A-mVenus (CB<sub>1</sub>R-  
230 ARY-mVenus) and CB<sub>1</sub>R-D3.49A/Y3.51A-mVenus (CB<sub>1</sub>R-ARA-mVenus), Fig. 1B and  
231 F, respectively). Interestingly, this effect of the D3.49A mutation was reversed by co-  
232 mutation of R3.50, as the double mutant CB<sub>1</sub>R-D3.49A/R3.50A (CB<sub>1</sub>R-AAY) and the  
233 triple mutant CB<sub>1</sub>R-D3.49A/R3.50A/Y3.51A (CB<sub>1</sub>R-AAA) both showed proper plasma  
234 membrane localization (Fig. 1G and H, respectively). The other three mutants, i.e. CB<sub>1</sub>R-  
235 R3.50A (CB<sub>1</sub>R-DAY), CB<sub>1</sub>R-Y3.51A (CB<sub>1</sub>R-DRA) and CB<sub>1</sub>R-R3.50A/Y3.51A (CB<sub>1</sub>R-  
236 DAA) displayed a cellular distribution roughly similar to that of the wild-type receptor  
237 (Fig 1C, D and E, respectively).

238 Since analysis of confocal images is in many cases not sensitive enough to detect fine  
239 changes in receptor distribution, we also applied a more quantifiable approach here, i.e.  
240 we measured the BRET interaction levels between CB<sub>1</sub>R-mVenus and plasma  
241 membrane-targeted Sluc protein. The fraction of the receptors residing on the plasma  
242 membrane of non-stimulated cells (PM/total receptor BRET) was found to be similar in  
243 cells expressing CB<sub>1</sub>R-WT, CB<sub>1</sub>R-AAY or CB<sub>1</sub>R-AAA, whereas CB<sub>1</sub>R-DAY, CB<sub>1</sub>R-  
244 DRA and CB<sub>1</sub>R-DAA showed an ~40% reduction of plasma membrane localization.

245 Furthermore, in accordance with confocal images, the plasma membrane localization of  
246 CB<sub>1</sub>R-ARY and CB<sub>1</sub>R-ARA was shown to be almost completely diminished (Fig. 1I).  
247 Since the plasma membrane localization of the CB<sub>1</sub>R-ARY and CB<sub>1</sub>R-ARA mutants was  
248 severely disrupted, these two mutants were not characterized in the subsequent studies.

249

### 250 *3.2. R3.50A mutation partially affects CB<sub>1</sub>R function*

251

252 R3.50 is the most conserved residue within the DRY motif, therefore we first checked the  
253 functionality of the CB<sub>1</sub>R-DAY mutant. The G protein activation of the receptor was  
254 directly monitored by measuring BRET changes between heterotrimeric G<sub>o</sub> protein  
255 subunits ( $\alpha_o$ -Rluc and YFP- $\beta_1\gamma_{11}$ ) (Turu et al. 2007), co-expressed with wild-type or  
256 mutant CB<sub>1</sub>R. In control experiments measuring BRET donor and acceptor partner  
257 expression directly (i.e. through luminescence and fluorescence counts, respectively) no  
258 significant changes were detected between these values when tested with the different  
259 CB<sub>1</sub>R mutants, suggesting that the observed changes in BRET were not due to alterations  
260 in BRET partner stoichiometry. This applies for all of the G<sub>o</sub> BRET and  $\beta$ -arrestin BRET  
261 experiments presented in this study (data not shown). Dose-response curves performed  
262 with the synthetic CB<sub>1</sub>R agonist WIN55,212-2 (WIN55) or with the endocannabinoid 2-  
263 arachydonoylglycerol (2-AG) showed that the CB<sub>1</sub>R-DAY mutant is impaired, but not  
264 completely disrupted in its ability to activate G<sub>o</sub> proteins. Moreover, CB<sub>1</sub>R-DAY shows a  
265 basal G protein activation similar to that of CB<sub>1</sub>R-WT (Fig. 2A and B). The EC<sub>50</sub> value of  
266 CB<sub>1</sub>R-DAY was also similar to that of CB<sub>1</sub>R-WT, indicating that the G protein binding of  
267 CB<sub>1</sub>R is not affected by the R3.50A mutation (Table 1).

268 Next, the  $\beta$ -arr2 recruitment of CB<sub>1</sub>R-DAY was investigated. GFP-tagged  $\beta$ -arr2 ( $\beta$ -arr2-  
269 GFP) was co-expressed with CB<sub>1</sub>R-DAY in CHO cells, and its distribution was analyzed  
270 under confocal microscopy. Interestingly, we found that in cells co-expressing  $\beta$ -arr2-  
271 GFP and CB<sub>1</sub>R-DAY,  $\beta$ -arr2-GFP was recruited to the plasma membrane in punctuate  
272 structures already in resting cells, indicating an increased basal  $\beta$ -arr2 recruitment of  
273 CB<sub>1</sub>R-DAY (Fig. 2E and G). Such basal recruitment of  $\beta$ -arr2-GFP could not be  
274 observed with CB<sub>1</sub>R-WT (Fig. 2C). This basal recruitment of  $\beta$ -arr2 was the  
275 consequence of a partially active receptor conformation, since treatment with the CB<sub>1</sub>R  
276 inverse agonist AM251 (10  $\mu$ M, 10 min) resulted in the disappearance of most of the  $\beta$ -  
277 arr2 puncta from the plasma membrane (Fig. 2H).

278 After addition of the CB<sub>1</sub>R agonist WIN55 (1  $\mu$ M, 10 min) further translocation of  $\beta$ -  
279 arr2-GFP to the plasma membrane could be observed in case of CB<sub>1</sub>R-DAY, however,  
280 this did not reach the level of  $\beta$ -arr2-GFP recruitment of the CB<sub>1</sub>R-WT (Fig. 2D and F).

281 To evaluate  $\beta$ -arr2 recruitment in a more quantitative manner, translocation of  $\beta$ -arr2 to  
282 the receptors was followed by monitoring BRET changes between  $\beta$ -arr2-Rluc and  
283 plasma membrane targeted mVenus (MP-mVenus). With this assay,  $\beta$ -arr2 recruitment to  
284 the investigated receptor can be monitored without tagging the receptor itself directly,  
285 which is advantageous because the detected BRET changes are not influenced by  
286 possible orientational changes resulting from the introduced receptor mutations.  
287 Furthermore, BRET signal in this assay is only affected via receptors residing on the  
288 plasma membrane, i.e. BRET ratios are not disturbed by intracellular receptor population.  
289 Dose-response curves performed with WIN55 in this  $\beta$ -arr2 BRET assay were in good  
290 accordance with the data obtained by confocal microscopy, i.e. the increased basal  $\beta$ -arr2

291 recruitment of CB<sub>1</sub>R-DAY, as well as a lower  $\beta$ -arr2 recruitment in response to agonist  
292 stimulus were detectable (Fig. 2I). Similar results were obtained with the  
293 endocannabinoid 2-AG (Fig. 2J).

294

### 295 *3.3. Y3.51A mutation increases constitutive activity of CB<sub>1</sub>R*

296

297 Among the three residues of the DRY motif, Y3.51 is the least conserved, and relatively  
298 little is known about its role in 7TMR signaling. To obtain data about its role in CB<sub>1</sub>R  
299 regulation, we tested the CB<sub>1</sub>R-DRA mutant under our experimental settings.  
300 Interestingly, although the maximal G protein activation of this mutant was only  
301 marginally impaired (i.e. a significant change in  $E_{max}$  was only detectable upon 2-AG  
302 stimuli), the G protein BRET dose-response analysis indicated an elevated basal G  
303 protein activation for this mutant (Fig. 3A and B, Table 1). Confocal microscopy analysis  
304 showed that, similarly to the CB<sub>1</sub>R-DAY mutant, basal  $\beta$ -arr2 recruitment of CB<sub>1</sub>R-DRA  
305 occurs (Fig. 3C and E), which could be reversed by inverse agonist treatment (Fig. 3F).  
306 Agonist-induced  $\beta$ -arr2-GFP translocation to the plasma membrane was very weak (Fig.  
307 3D).  $\beta$ -arr2 BRET analysis was in accordance with confocal data, namely, dose-response  
308 curve showed elevated basal  $\beta$ -arr2 recruitment together with a significantly impaired  
309 agonist-induced  $\beta$ -arr2 translocation (Fig. 3G and H).

310

### 311 *3.4. Enhanced $\beta$ -arrestin2 recruitment and reduced G protein activation of the CB<sub>1</sub>R-* 312 *AAV mutant*

313

314 Next, we investigated the signaling properties of the double mutant CB<sub>1</sub>R-AAY. The G  
315 protein activation was monitored by the BRET assay described above. Dose-response  
316 curves carried out with WIN55 or 2-AG showed that the CB<sub>1</sub>R-AAY mutant has  
317 impaired G<sub>o</sub> activation ability (Fig. 4A and B), which is reflected both in the E<sub>max</sub> and the  
318 pEC<sub>50</sub> values of these interactions (Table 1). Moreover, basal G protein activation of this  
319 mutant was significantly lowered ((Fig. 4A and B, Table 1).

320 The β-arr2 recruitment of CB<sub>1</sub>R-AAY was investigated also by β-arr2-GFP co-expression  
321 under confocal microscope. We found that, similarly to CB<sub>1</sub>R-DAY and CB<sub>1</sub>R-DRA,  
322 CB<sub>1</sub>R-AAY recruited β-arr2-GFP to the plasma membrane in non-stimulated cells (Fig.  
323 4C and E). The basal β-arr2 recruitment could be reversed with inverse agonist AM251  
324 treatment (Fig. 4F). Upon addition of WIN55, a very robust translocation of β-arr2-GFP  
325 to the plasma membrane was observed, with practically no β-arr2-GFP remaining in the  
326 cytoplasm (Fig. 4D). We further evaluated the β-arr2 recruitment of CB<sub>1</sub>R-AAY with the  
327 BRET-based method described above. WIN55 and 2-AG dose-response curves showed  
328 that, in addition to the increased basal β-arr2 recruitment of CB<sub>1</sub>R-AAY, this mutant  
329 gained a substantially increased ability to recruit β-arr2 upon agonist stimulus, as shown  
330 by the significant left- and upward shift of the curves (Fig. 4G and H, Table 1). These  
331 results suggest that the signaling of this mutant is shifted from G protein activation  
332 towards β-arr2 recruitment, and therefore CB<sub>1</sub>R-AAY can be considered as a β-arr2-  
333 biased mutant.

334 The characteristics of the triple mutant CB<sub>1</sub>R-AAA) were very similar to that of CB<sub>1</sub>R-  
335 AAY, i.e. a decrease in basal and agonist-induced G protein activation, as well as an  
336 increase in basal and agonist-induced β-arr2 recruitment were observed (data not shown).

337

338 *3.5. The CB<sub>1</sub>R-DAA mutant is G protein-biased*

339

340 In the next set of experiments, the functional characteristics of the CB<sub>1</sub>R-DAA double  
341 mutant receptor were analyzed. Dose-response curves obtained by G<sub>o</sub> protein BRET  
342 assay showed that the CB<sub>1</sub>R-DAA mutant can activate G proteins at a lowered level  
343 (~75% of CB<sub>1</sub>R-WT), although pEC<sub>50</sub> values as well as basal G protein activation  
344 remained unaffected (Fig. 5A and B, Table 1).

345 Confocal microscopy analysis of β-arr2-GFP co-expressed with CB<sub>1</sub>R-DAA showed that  
346 this mutant, similarly to the CB<sub>1</sub>R-DAY, CB<sub>1</sub>R-DRA and CB<sub>1</sub>R-AAY mutants, recruited  
347 β-arr2-GFP to the plasma membrane under control conditions (Fig. 5C and E), and this  
348 was reversed by AM251 treatment (Fig. 5F). Interestingly, no further translocation of β-  
349 arr2-GFP could be detected in these cells upon addition of the CB<sub>1</sub>R agonist WIN55 (Fig.  
350 5C). These results were strengthened by β-arr2 BRET measurements, showing a basal β-  
351 arr2 recruitment for CB<sub>1</sub>R-DAA, which, however, cannot be enhanced by WIN55 or 2-  
352 AG treatment (Fig. 5G and H). These results suggest that, in contrast to CB<sub>1</sub>R-AAY, the  
353 signaling of CB<sub>1</sub>R-DAA is shifted from β-arr2 recruitment towards G protein activation,  
354 and therefore CB<sub>1</sub>R-DAA can be considered as a G protein-biased mutant.

355

356 *3.6. β-arrestin1 recruitment of CB<sub>1</sub>R-AAY mutant is robustly enhanced*

357

358 In our previous study we could not detect significant β-arr1 coupling to the CB<sub>1</sub>R upon  
359 WIN55 stimulus, however, others have suggested that CB<sub>1</sub>R dependent β-arr1



360 recruitment can be present and may regulate ERK1/2 activation of CB<sub>1</sub>R (Laprairie et al.,  
361 2014; Flores-Otero et al., 2014). To test whether DRY mutations of CB<sub>1</sub>R affect the  
362 recruitment of  $\beta$ -arr1, we applied the same BRET based approach as above, i.e. the  
363 plasma membrane translocation of  $\beta$ -arr1-Rluc was monitored, and dose-response curves  
364 were performed using WIN55 and 2-AG as agonists. Our results show that agonist-  
365 induced  $\beta$ -arr1 recruitment is very low in cells expressing CB<sub>1</sub>R-WT, i.e. a significant  
366 increase could only be detected upon 2-AG treatment, whereas the changes obtained with  
367 WIN55 proved to be non-significant. Interestingly, the CB<sub>1</sub>R-AAY mutant displayed a  
368 robustly enhanced ability to recruit  $\beta$ -arr1, both upon WIN55 and 2-AG stimuli. All of  
369 the other three mutants (i.e. CB<sub>1</sub>R-DAY, CB<sub>1</sub>R-DRA and CB<sub>1</sub>R-DAA) produced non-  
370 significant changes in the plasma membrane localization of  $\beta$ -arr1 (Fig. 6A and B).

371

### 372 *3.7. Detailed data analysis strengthens biased signaling of DRY mutant CB<sub>1</sub>Rs*

373

374 The above results suggest that distinct mutations in the conserved DRY motif of the  
375 CB<sub>1</sub>R can differentially affect G protein activation and  $\beta$ -arr2 recruitment of the receptor.  
376 To assess this receptor bias in an exact manner, two different methods, proposed by  
377 Rajagopal et al. (Rajagopal et al. 2011), were applied to analyze data. First, ‘equimolar  
378 comparison’ was carried out, where G protein and  $\beta$ -arr2 responses elicited by the same  
379 ligand concentrations are plotted against each other. In the case of the ‘reference  
380 receptor’, i.e. CB<sub>1</sub>R-WT, this analysis yields a roughly hyperbolic shape with both  
381 WIN55 and 2-AG (Fig. 7A and B, respectively, black circles), reflecting the difference in  
382 the amplification between G protein and  $\beta$ -arr2 assays. Importantly, the points for CB<sub>1</sub>R-

383 AAY are substantially shifted left- and upwards on these graphs, representing bias  
384 towards  $\beta$ -arr2 recruitment (Fig. 7A and B, white triangles). Furthermore, the points for  
385 CB<sub>1</sub>R-DAA are arranged along a horizontal line, demonstrating the bias of this receptor  
386 towards G protein activation (Fig. 7A and B, grey squares). The other method was  
387 ‘equiactive comparison’, where the signaling of each receptor is characterized by a bias  
388 factor ( $\beta$ ), based on the EC<sub>50</sub> and E<sub>max</sub> values from G protein and  $\beta$ -arr2 dose-response  
389 curves (Rajagopal et al. 2011). In case of the reference receptor (CB<sub>1</sub>R-WT), this bias  
390 factor is by definition 0. In the case of CB<sub>1</sub>R-DAA, the  $\beta$  values were 1.42 or 1.61 (for  
391 WIN55 or 2-AG stimuli, respectively), whereas the same values for CB<sub>1</sub>R-AAY were -  
392 1.54 or -1.42, representing more than 10-fold bias of these two mutants towards G protein  
393 activation and  $\beta$ -arr2 recruitment, respectively (Fig. 7C).

394 Taken together, our detailed bias analysis indicated that CB<sub>1</sub>R-AAY and CB<sub>1</sub>R-DAA can  
395 be considered as  $\beta$ -arrestin-biased and G protein-biased mutants, respectively.

396

397 *3.8. Functional assays reflect biased intracellular signaling of CB<sub>1</sub>R-AAY and CB<sub>1</sub>R-*  
398 *DAA*

399

400 Next, we wanted to assess whether the differences seen at the level of receptor-effector  
401 protein coupling are reflected in more distal intracellular signaling events initiated by  
402 CB<sub>1</sub>R activation. First, G<sub>i/o</sub> protein-mediated signaling was assessed by measuring  
403 inhibition of forskolin-induced cAMP accumulation under basal and CB<sub>1</sub>R-stimulated  
404 conditions, using an EPAC-based intramolecular BRET-sensor (Erdelyi et al. 2014). Our  
405 results showed that CB<sub>1</sub>R-WT inhibits cAMP accumulation under non-stimulated

406 conditions, and this is substantially and dose-dependently enhanced upon treatment with  
407 WIN55 (Fig. 8A). Importantly, WIN55-induced cAMP inhibition of the G protein-biased  
408 mutant CB<sub>1</sub>R-DAA was lower but still present, whereas CB<sub>1</sub>R-AAAY, in accordance with  
409 its bias towards  $\beta$ -arr2, failed to induce the inhibition of cAMP accumulation in response  
410 to agonist stimulus (Fig. 8A).

411 Recent data suggest that CB<sub>1</sub>R-induced p42/44 MAP kinase (ERK1/2) activation, which  
412 was formerly suggested to occur via G protein-dependent pathways (Galve-Roperh et al.  
413 2002; Davis et al. 2003; Dalton and Howlett 2012), is also mediated by  $\beta$ -arrestins (Ahn  
414 et al. 2013a; Mahavadi et al. 2014). Therefore, we aimed to study how the ERK1/2  
415 responses correlate with the G protein activation and/or  $\beta$ -arrestin recruitment of the  
416 biased CB<sub>1</sub>R mutants. Western blot experiments carried out with cells expressing CB<sub>1</sub>R-  
417 WT showed a robust increase in the amount of phosphorylated ERK1/2 (pERK1/2) after  
418 5 min treatment with WIN55 (1  $\mu$ M). Moreover, lower but sustained pERK1/2 levels  
419 were also detectable after 20 min WIN55 treatment (Fig. 7B and C). Interestingly, we  
420 found that the  $\beta$ -arr2-biased CB<sub>1</sub>R-AAAY elicited pERK1/2 responses similar to CB<sub>1</sub>R-  
421 WT, both at 5 and 20 min stimulation, whereas the G protein-biased CB<sub>1</sub>R-DAA  
422 produced significantly lower pERK1/2 responses than the wild-type receptor (Fig. 7B and  
423 C). Thus, ERK1/2 activation of the biased DRY mutants correlated well with their  $\beta$ -arr2  
424 recruitment ability, rather than with their G protein activation.

425

#### 426 **4. Discussion**

427

428 In this study, we evaluated the role of the conserved DRY motif in the function of the  
429 CB<sub>1</sub>R. Our goal was to assess its role in mediating basal and agonist-induced G protein  
430 activation and  $\beta$ -arrestin recruitment of CB<sub>1</sub>R, as well as to identify possible differences  
431 caused in these two main effector functions of the receptor. Interestingly, single alanine  
432 mutation of the conserved Arg (R3.50A) resulted only in a ~20% reduction of the G  
433 protein coupling efficiency of CB<sub>1</sub>R, without affecting its basal G protein activation. This  
434 may seem surprising, as crystal structure analysis as well as several mutational data have  
435 suggested a pivotal role for this residue in the G protein coupling of 7TMRs (Zhu et al.  
436 1994; Ballesteros et al. 1998; Rasmussen et al. 2011). However, several other 7TMRs  
437 exist, where similar non-conservative mutations of R3.50 failed to abolish G protein  
438 activation of the receptor (Fanelli et al. 1999; Rovati et al. 2007). Thus, CB<sub>1</sub>R appears to  
439 belong to a subgroup of 7TMRs where this conserved Arg residue plays no absolute role  
440 in the direct receptor-G protein coupling. Furthermore, our results demonstrate a basal  $\beta$ -  
441 arr2 recruitment of the CB<sub>1</sub>R-DAY mutant (or any double or triple mutant carrying the  
442 same mutation), which is in good accordance with previously published data showing  
443 similar characteristics for R3.50H mutants of V<sub>2</sub> vasopressin,  $\alpha_{1B}$  adrenergic and AT<sub>1A</sub>  
444 angiotensin II receptors (Wilbanks et al. 2002). This strengthens the idea that this  
445 conserved Arg somehow prevents arrestin binding in the inactive receptor conformation.  
446 Agonist-induced  $\beta$ -arr2 recruitment of CB<sub>1</sub>R-DAY and CB<sub>1</sub>R-DRA was lowered, which  
447 is most likely to be caused by the lowered plasma membrane localization of these  
448 mutants (Fig. 1I).

449 The most interesting finding of our study is the major difference between the functions of  
450 two double mutants, CB<sub>1</sub>R-DAA and CB<sub>1</sub>R-AAY. Although both mutants contain the

451 R3.50A mutation, and accordingly show increased basal  $\beta$ -arr2 recruitment, their ultimate  
452 characteristics are further determined by the location of the second mutation. Thereby, a  
453 simultaneous lack of D3.49 and R3.50 residues seems to have a dominant-positive effect  
454 on both the  $\beta$ -arr1 and  $\beta$ -arr2 recruitment of CB<sub>1</sub>R (which is also supported by the fact  
455 that the triple mutant CB<sub>1</sub>R-AAA functionally resembles CB<sub>1</sub>R-AAY). Thus, CB<sub>1</sub>R-AAY  
456 is a  $\beta$ -arrestin-biased 7TMR mutant. Interestingly, these characteristics of the CB<sub>1</sub>R-AAY  
457 are similar to those of the formerly described biased mutant angiotensin II receptor AT<sub>1</sub>-  
458 DRY/AAY (AT<sub>1</sub>R-AAY) (Gaborik et al. 2003; Wei et al. 2003). However, an important  
459 difference here is that AT<sub>1</sub>R-AAY is  $\beta$ -arrestin-biased in a way that its G protein  
460 activation is absent while its  $\beta$ -arrestin binding is present but certainly not increased (Wei  
461 et al. 2003, Balla et al., 2012), whereas CB<sub>1</sub>R-AAY is  $\beta$ -arrestin-biased in that its  $\beta$ -  
462 arrestin recruitment is substantially increased, together with a lowered, but not abolished  
463 G protein activating ability. Furthermore, we were able to detect a robustly enhanced  $\beta$ -  
464 arr1 recruitment to CB<sub>1</sub>R-AAY, whereas  $\beta$ -arr1 translocation to CB<sub>1</sub>R-WT was  
465 significant only upon 2-AG stimulus, but not after WIN55 treatment. Thus, it appears that  
466 the recruitment of  $\beta$ -arr1 to CB<sub>1</sub>R-WT is very weak, so that it challenges the limits of  
467 detectability via the (otherwise quite sensitive) BRET approach applied here. However,  
468 our results showing a significant increase of  $\beta$ -arr1 BRET upon 2-AG stimulus are in  
469 accordance with recent results showing higher  $\beta$ -arr1 recruitment by 2-AG compared to  
470 WIN55 (Laprairie et al., 2014). Taken together, recruitment of  $\beta$ -arr1 to CB<sub>1</sub>R-WT is  
471 obviously lower than that of  $\beta$ -arr2, but both are substantially enhanced in the CB<sub>1</sub>R-  
472 AAY mutant. Interestingly, basal G protein activation of CB<sub>1</sub>R-AAY was absent, while  
473 the difference between vehicle-treated and WIN55-stimulated cells remained comparable

474 to that of CB<sub>1</sub>R-WT (Fig. 4A), raising the question whether the reduced E<sub>max</sub> value of  
475 CB<sub>1</sub>R-AAY in this assay reflects a true loss of agonist-induced G protein activation, or it  
476 is caused merely by the absence of basal activity, while WIN55-induced G protein  
477 activation remains unaffected. However, repeating these experiments in HeLa cells,  
478 where basal activity of CB<sub>1</sub>R is minimal (Gyombolai et al. 2013), also showed  
479 substantially impaired WIN55-induced G protein activation of CB<sub>1</sub>R-AAY (Suppl. Fig.  
480 1), suggesting that this mutation reduces not only the basal but also the WIN55-induced  
481 G<sub>o</sub> protein activation of CB<sub>1</sub>R.

482 In contrast, CB<sub>1</sub>R-DAA proved to be G protein-biased, as its β-arrestin recruitment in  
483 response to agonist stimulus was practically absent, but was still able to activate G  
484 proteins, although at a lower level (~70% of the wild type CB<sub>1</sub>R). According to our data,  
485 plasma membrane expression of this mutant is ~40% lower than that of CB<sub>1</sub>R-WT.  
486 However, this extent of decrease is not likely to cause a complete loss of agonist-induced  
487 β-arrestin recruitment, given the ~1:1 stoichiometry of receptor-β-arrestin complex. This  
488 is also supported by the fact that CB<sub>1</sub>R-DAA still binds β-arr2 under basal conditions.  
489 Other 7TMRs described previously as biased mutants include the M<sub>3</sub>-R3.50L designer  
490 muscarinic receptor (Nakajima and Wess 2012) and β<sub>2</sub>-AR-TYY, a triple mutant β<sub>2</sub>-AR  
491 which was rationally designed to be functionally selective (Shenoy et al. 2006).  
492 Interestingly, however, all of these mutants are β-arrestin-biased, i.e. they do not couple  
493 to G proteins but still recruit β-arrestin, albeit at a lowered level. The CB<sub>1</sub>R-DAA mutant  
494 presented here is interesting in this respect, as it is biased towards G protein activation,  
495 whereas its mutations affect a ‘classical’ G protein-coupling region, i.e. the DRY motif.  
496 Intriguingly, although CB<sub>1</sub>R-DAA can hardly recruit β-arrestins in response to agonist

497 stimulus, it still binds  $\beta$ -arr2 to some extent under non-stimulated conditions. This relies  
498 most probably on the presence of the R3.50A mutation, because, as mentioned above, all  
499 of the CB<sub>1</sub>R mutants carrying this mutation recruited  $\beta$ -arr2 constitutively. Thus, it seems  
500 that the absence of the conserved Arg residue can itself determine a receptor  
501 conformation that binds  $\beta$ -arrestin spontaneously. On the other hand, the agonist-induced  
502  $\beta$ -arr2 binding of the receptor can still be strongly influenced in both directions by co-  
503 mutations of the neighboring residues.

504 Taken together, our results obtained with the CB<sub>1</sub>R-AAY and CB<sub>1</sub>R-DAA mutants  
505 strongly support a model where the active G protein-coupled and  $\beta$ -arrestin-bound  
506 conformations of a 7TMR are different. Moreover, receptor states responsible for  
507 constitutive and agonist-induced  $\beta$ -arrestin binding may also show differences.

508 We also demonstrate here that the agonist-induced ERK1/2 phosphorylation shows good  
509 correlation with the  $\beta$ -arr2 recruitment of our biased CB<sub>1</sub>R mutants, rather than their G  
510 protein activation or their ability to inhibit forskolin-induced cAMP accumulation. These  
511 data are consistent with the recently emerging concept of  $\beta$ -arrestin-dependent CB<sub>1</sub>R  
512 signaling, i.e. a  $\beta$ -arrestin-mediated ERK1/2 phosphorylation following CB<sub>1</sub>R activation  
513 (Ahn et al. 2013a; Mahavadi et al. 2014).

514 One of the most interesting questions regarding the DRY mutants presented here is how  
515 (i.e. through which molecular structural rearrangements) the distinct mutations induce  
516 such large differences in the  $\beta$ -arrestin-recruitment of CB<sub>1</sub>R. One simple explanation  
517 would be that mutations of the DRY motif modify primarily the G protein binding of the  
518 receptor, and their effects on the  $\beta$ -arr2 recruitment are merely secondary, resulting from  
519 the assumption that G proteins and  $\beta$ -arrestins compete for the 7TMR binding. However,

520 if this would be the only explanation, one should observe an indirect proportionality  
521 between the G protein-and the  $\beta$ -arrestin binding abilities of the distinct mutants, which is  
522 actually not the case. Thus, mutations of the DRY motif most probably affect  $\beta$ -arr2  
523 binding of CB<sub>1</sub>R independently of its G protein activation. Whether or not the DRY  
524 sequence itself is a part of the docking site for arrestins, can not be answered  
525 unequivocally based on our results. However, previously published data indicating that  
526 the ICL2 loop of 7TMRs, beginning with an intact DRY motif, is part of the  $\beta$ -arrestin  
527 binding site, add interesting aspects to our study (Huttenrauch et al. 2002; Marion et al.  
528 2006). Moreover, two recent studies have provided important insights into the structural  
529 features within the 7TMR- $\beta$ -arrestin complex. Both of these studies point to an important  
530 interaction between the ‘finger loop’ region of  $\beta$ -arrestin and the receptor core, with the  
531 direct involvement of the DRY motif (Shukla et al., 2014; Szczepek et al., 2014).  
532 Combined with these data, our results show good fit with a model where DRY is directly  
533 involved in the  $\beta$ -arrestin binding of CB<sub>1</sub>R. Additionally, mutations of the DRY motif  
534 may also affect  $\beta$ -arrestin binding indirectly, i.e. by inducing structural rearrangements in  
535 the subsequent ICL2, resulting in diverse, sometimes completely opposite  $\beta$ -arrestin  
536 binding phenotypes. However, a more precise understanding of the intramolecular  
537 interactions that mediate these characteristics would require the high resolution crystal  
538 structure data.

539

#### 540 **Declaration of interest**

541 The authors declare no conflict of interest.

542



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547

548 **Author contributions**

549 P.G. designed and carried out most of the experiments and wrote the manuscript. A.D.T.  
550 carried out the  $\beta$ -arr2 BRET experiments, helped with data evaluation and revised the  
551 manuscript. D.T. created the CB<sub>1</sub>R-DAY mutant and carried out important control  
552 experiments. G.T. created the CB<sub>1</sub>R-AAY mutant, helped with data interpretation and  
553 revised the manuscript. L.H. managed the overall project, helped with data interpretation  
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555

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559

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742

743

744

745 **Figure legends**

746

747 **Fig.1 Cellular distribution of wild-type and mutant mVenus-tagged CB<sub>1</sub>R variants**

748 A-H, CHO cells expressing mVenus-tagged CB<sub>1</sub>R variants are visualized using confocal  
749 microscopy. A, CB<sub>1</sub>R-WT-mVenus B, CB<sub>1</sub>R-ARY-mVenus C, CB<sub>1</sub>R-DAY-mVenus, D,  
750 CB<sub>1</sub>R-DRA-mVenus E, CB<sub>1</sub>R-DAA-mVenus F, CB<sub>1</sub>R-ARA-mVenus G, CB<sub>1</sub>R-AAY-  
751 mVenus H, CB<sub>1</sub>R-AAA-mVenus. Images are representative from 3 independent  
752 experiments. Scale bar 10  $\mu$ m. I, PM/total receptor BRET showing the fraction of  
753 mVenus-tagged CB<sub>1</sub>R variants residing on the plasma membrane. 0% reflects no net  
754 BRET interaction and 100% reflects normalized BRET interaction of CB<sub>1</sub>R-WT-  
755 mVenus. Data are mean $\pm$ SEM, n=3, \*p<0.05, ns – non-significant

756

757 **Fig.2 Functional analysis of the CB<sub>1</sub>R-DAY mutant**

758 A-B, Dose-response curves showing G protein activation of CB<sub>1</sub>R-WT (grey curve) and  
759 CB<sub>1</sub>R-DAY (black curve) in CHO cells under basal and different WIN55- (A) or 2-AG-  
760 (B) stimulated conditions, as detected by G<sub>o</sub> protein BRET. 0% reflects total inactivity of  
761 receptors, achieved by inverse agonist treatment (AM251, 10  $\mu$ M), and 100% reflects  
762 maximal WIN55- (A) or 2-AG- (B) induced response ( $E_{max}$ ) of CB<sub>1</sub>R-WT. Data are  
763 mean $\pm$ SEM, n=3-8.

764 C-H, Confocal images showing distribution of  $\beta$ -arr2-GFP in CHO cells co-expressing  
765 CB<sub>1</sub>R-WT (C and D) or CB<sub>1</sub>R-DAY (E-H), under control conditions (C, E and G) and 10  
766 min after WIN55 (1  $\mu$ M, D and F) or AM251 (10  $\mu$ M, H) treatment. Arrows indicate  $\beta$ -

767 arr2-GFP puncta at the plasma membrane. Images are representative from at least 4  
768 independent experiments. Scale bar 10  $\mu$ m.

769 I-J, Dose-response curves showing recruitment of  $\beta$ -arr2 to the plasma membrane by  
770 CB<sub>1</sub>R-WT (grey curve) and CB<sub>1</sub>R-DAY (black curve) in CHO cells under basal and  
771 different WIN55- (I) or 2-AG- (J) stimulated conditions, as detected by BRET between  $\beta$ -  
772 arr2-Rluc and MP-mVenus. 0% reflects total inactivity of receptors, achieved by inverse  
773 agonist treatment (AM251, 10  $\mu$ M), and 100% reflects maximal WIN55- (I) or 2-AG- (J)  
774 induced response ( $E_{max}$ ) of CB<sub>1</sub>R-WT. Data are mean $\pm$ SEM, n=4-7.

775

### 776 **Fig.3 Functional analysis of the CB<sub>1</sub>R-DRA mutant**

777 A-B, Dose-response curves showing G protein activation of CB<sub>1</sub>R-WT (grey curve) and  
778 CB<sub>1</sub>R-DRA (black curve) in CHO cells under basal and different WIN55- (A) or 2-AG-  
779 (B) stimulated conditions, as detected by G<sub>o</sub> protein BRET. 0% reflects total inactivity of  
780 receptors, achieved by inverse agonist treatment (AM251, 10  $\mu$ M), and 100% reflects  
781 maximal WIN55- (A) or 2-AG- (B) induced response ( $E_{max}$ ) of CB<sub>1</sub>R-WT. Data are  
782 mean $\pm$ SEM, n=4-8.

783 C-F, Confocal images showing distribution of  $\beta$ -arr2-GFP in CHO cells co-expressing  
784 CB<sub>1</sub>R-DRA, under control conditions (C and E) and 10 min after WIN55 (1  $\mu$ M, D) or  
785 AM251 (10  $\mu$ M, F) treatment. Arrows indicate  $\beta$ -arr2-GFP puncta at the plasma  
786 membrane. Images are representative from at least 4 independent experiments. Scale bar  
787 10  $\mu$ m.

788 G-H, Dose-response curves showing recruitment of  $\beta$ -arr2 to the plasma membrane by  
789 CB<sub>1</sub>R-WT (grey curve) and CB<sub>1</sub>R-DRA (black curve) in CHO cells under basal and

790 different WIN55- (G) or 2-AG- (H) stimulated conditions, as detected by BRET between  
791  $\beta$ -arr2-Rluc and MP-mVenus. 0% reflects total inactivity of receptors, achieved by  
792 inverse agonist treatment (AM251, 10  $\mu$ M), and 100% reflects maximal WIN55- (G) or  
793 2-AG- (H) induced response ( $E_{max}$ ) of CB<sub>1</sub>R-WT. Data are mean $\pm$ SEM, n=4-7.

794

#### 795 **Fig.4 Functional analysis of the CB<sub>1</sub>R-AAAY mutant**

796 A-B, Dose-response curves showing G protein activation of CB<sub>1</sub>R-WT (grey curve) and  
797 CB<sub>1</sub>R-AAAY (black curve) in CHO cells under basal and different WIN55- (A) or 2-AG-  
798 (B) stimulated conditions, as detected by G<sub>o</sub> protein BRET. 0% reflects total inactivity of  
799 receptors, achieved by inverse agonist treatment (AM251, 10  $\mu$ M), and 100% reflects  
800 maximal WIN55- (A) or 2-AG- (B) induced response ( $E_{max}$ ) of CB<sub>1</sub>R-WT. Data are  
801 mean $\pm$ SEM, n=3-8.

802 C-F, Confocal images showing distribution of  $\beta$ -arr2-GFP in CHO cells co-expressing  
803 CB<sub>1</sub>R-AAAY, under control conditions (C and E) and 10 min after WIN55 (1  $\mu$ M, D) or  
804 AM251 (10  $\mu$ M, F) treatment. Images are representative from at least 3 independent  
805 experiments. Scale bar 10  $\mu$ m.

806 G-H, Dose-response curves showing recruitment of  $\beta$ -arr2 to the plasma membrane by  
807 CB<sub>1</sub>R-WT (grey curve) and CB<sub>1</sub>R-AAAY (black curve) in CHO cells under basal and  
808 different WIN55- (G) or 2-AG- (H) stimulated conditions, as detected by BRET between  
809  $\beta$ -arr2-Rluc and MP-mVenus. 0% reflects total inactivity of receptors, achieved by  
810 inverse agonist treatment (AM251, 10  $\mu$ M), and 100% reflects maximal WIN55- (G) or  
811 2-AG- (H) induced response ( $E_{max}$ ) of CB<sub>1</sub>R-WT. Data are mean $\pm$ SEM, n=4-7.

812

813 **Fig.5 Functional analysis of the CB<sub>1</sub>R-DAA mutant**

814 A-B, Dose-response curves showing G protein activation of CB<sub>1</sub>R-WT (grey curve) and  
815 CB<sub>1</sub>R-DAA (black curve) in CHO cells under basal and different WIN55- (A) or 2-AG-  
816 (B) stimulated conditions, as detected by G<sub>o</sub> protein BRET. 0% reflects total inactivity of  
817 receptors, achieved by inverse agonist treatment (AM251, 10 μM), and 100% reflects  
818 maximal WIN55- (A) or 2-AG- (B) induced response (E<sub>max</sub>) of CB<sub>1</sub>R-WT. Data are  
819 mean±SEM, n=4-8.

820 C-F, Confocal images showing distribution of β-arr2-GFP in CHO cells co-expressing  
821 CB<sub>1</sub>R-DAA, under control conditions (C and E) and 10 min after WIN55 (1 μM, D) or  
822 AM251 (10 μM, F) treatment. Images are representative from at least 3 independent  
823 experiments. Scale bar 10 μm.

824 G-H, Dose-response curves showing recruitment of β-arr2 to the plasma membrane by  
825 CB<sub>1</sub>R-WT (grey curve) and CB<sub>1</sub>R-DAA (black triangles) in CHO cells under basal and  
826 different WIN55- (G) or 2-AG- (H) stimulated conditions, as detected by BRET between  
827 β-arr2-Rluc and MP-mVenus. 0% reflects total inactivity of receptors, achieved by  
828 inverse agonist treatment (AM251, 10 μM), and 100% reflects maximal WIN55- (G) or  
829 2-AG- (H) induced response (E<sub>max</sub>) of CB<sub>1</sub>R-WT. Data are mean±SEM, n=4-7.

830

831 **Fig.6 Dose-response curves showing β-arrestin1 recruitment of CB<sub>1</sub>R mutants**

832 A-B, Dose-response curves showing recruitment of β-arr1 to the plasma membrane by  
833 CB<sub>1</sub>R-WT (black circles), CB<sub>1</sub>R-DAY (white diamonds), CB<sub>1</sub>R-DRA (white circles),  
834 CB<sub>1</sub>R-DAA (white squares) or CB<sub>1</sub>R-AAY (white triangles) in CHO cells under basal  
835 and different WIN55- (A) or 2-AG- (B) stimulated conditions, as detected by BRET

836 between  $\beta$ -arr1-Rluc and MP-mVenus. 0% reflects total inactivity of receptors, achieved  
837 by inverse agonist treatment (AM251, 10  $\mu$ M), and 100% reflects maximal WIN55- (A)  
838 or 2-AG- (B) induced response ( $E_{max}$ ) of CB<sub>1</sub>R-AAV. Data are mean $\pm$ SEM, n=3.  
839 \*p<0.05 vs vehicle treatment.

840

#### 841 **Fig.7 Bias analysis showing functional selectivity of CB<sub>1</sub>R mutants**

842 A-B, Equimolar comparison of CB<sub>1</sub>R-WT (black points), CB<sub>1</sub>R-DAA (grey squares) and  
843 CB<sub>1</sub>R-AAV (white triangles) functions. For each receptor, responses from G protein and  
844  $\beta$ -arr2 BRET dose-response curves, elicited by the same WIN55 (A) or 2-AG (B)  
845 concentration, were plotted against each other. The left- and upward shift of CB<sub>1</sub>R-AAV  
846 points represents bias toward  $\beta$ -arr2 recruitment, whereas the downward shift of CB<sub>1</sub>R-  
847 DAA points indicates G protein bias. Data are mean $\pm$ SEM. C, Equiactive comparison of  
848 CB<sub>1</sub>R-WT, CB<sub>1</sub>R-DAA and CB<sub>1</sub>R-AAV functions. The biased factor ( $\beta$ ) was calculated  
849 for each receptor, based upon  $EC_{50}$  and  $E_{max}$  values of G protein and  $\beta$ -arr2 BRET dose-  
850 response curves obtained with WIN55 (black bars) or 2-AG (grey bars) stimuli, using the  
851 equation described in *Materials and methods*. CB<sub>1</sub>R-WT was used as reference receptor.  
852 Positive values indicate bias towards G protein signaling, whereas negative values reflect  
853  $\beta$ -arrestin bias. Data are mean  $\pm$  SD.

854

#### 855 **Fig.8 Functional assays measuring intracellular signaling of wild-type and mutant** 856 **CB<sub>1</sub>R variants**

857 A, Dose-response curves showing the inhibition of forskolin-induced cAMP  
858 accumulation in CHO cells expressing CB<sub>1</sub>R-WT, CB<sub>1</sub>R-DAA or CB<sub>1</sub>R-AAV under

859 basal and different WIN55-stimulated conditions, measured by the BRET changes of an  
860 EPAC-based intramolecular BRET sensor. BRET was measured 30 min after stimulus.  
861 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251,  
862 10  $\mu$ M), and 100% reflects maximal WIN55-induced response ( $E_{max}$ ) of CB<sub>1</sub>R-WT. Data  
863 are mean $\pm$ SEM, n=6. \*p<0.05 vs basal state, #p<0.05 vs CB<sub>1</sub>R-WT. B, Amounts of  
864 phosphorylated (pERK1/2) and total ERK1/2 proteins detected by Western blot in CHO  
865 cells expressing CB<sub>1</sub>R-WT, CB<sub>1</sub>R-DAA or CB<sub>1</sub>R-AAY, after 0, 5 or 20 min of WIN55 (1  
866  $\mu$ M) treatment. Images are representative from four independent experiments. C,  
867 Quantification of Western blot data. 0% reflects background intensity, and 100% reflects  
868 WIN55-induced pERK1/2 intensity of CB<sub>1</sub>R-WT at 5 min. Data are mean+SEM, n=4,  
869 \*p<0.05 versus CB<sub>1</sub>R-WT at 5 min, #p<0.05 versus CB<sub>1</sub>R-WT at 20 min.

870

### 871 **Supplementary Fig. 1 G protein activation of CB<sub>1</sub>R-AAY mutant in HeLa cells**

872 BRET measurements showing G<sub>o</sub> protein activation of CB<sub>1</sub>R-WT and CB<sub>1</sub>R-AAY in  
873 HeLa cells under basal (white bars) and WIN55-stimulated (1  $\mu$ M, black bars) conditions.  
874 0% reflects total inactivity of receptors, achieved by inverse agonist treatment (AM251,  
875 10  $\mu$ M), and 100% reflects WIN55-induced response of CB<sub>1</sub>R-WT. Data are mean $\pm$ SEM,  
876 n=4. \*p<0.05 vs basal, #p<0.05 vs CB<sub>1</sub>R-WT.

877

### 878 **Table 1. Parameters of G<sub>o</sub> BRET and $\beta$ -arrestin2 BRET dose-response curves for** 879 **the different CB<sub>1</sub>R variants**

880 Bottom and  $E_{max}$  values are expressed as % of  $E_{max}$  of CB<sub>1</sub>R-WT. Data are mean $\pm$ SEM,  
881 n=3-8. \*p<0.05 vs CB<sub>1</sub>R-WT. n.d. – not detectable

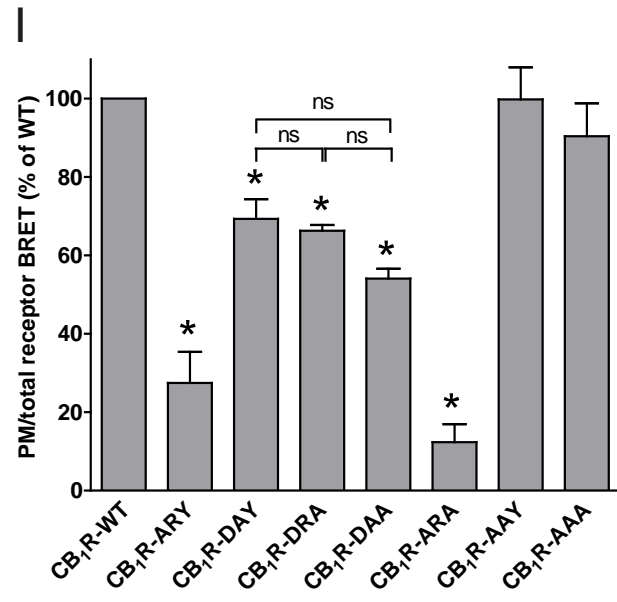
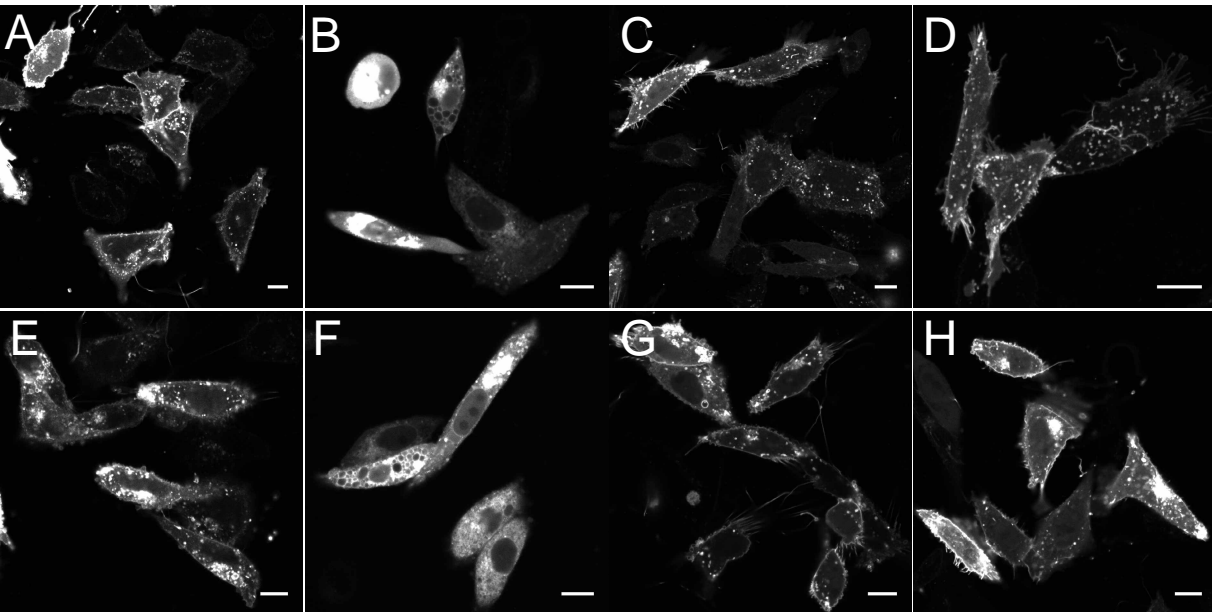


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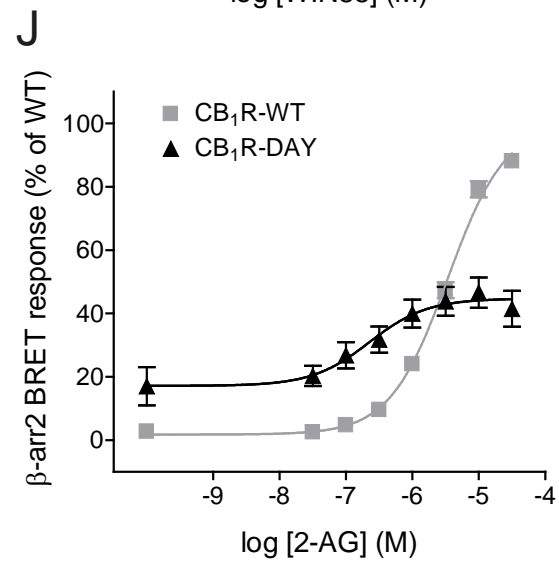
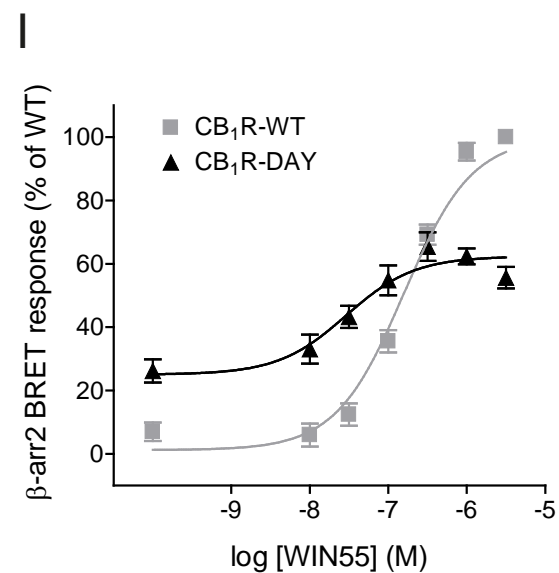
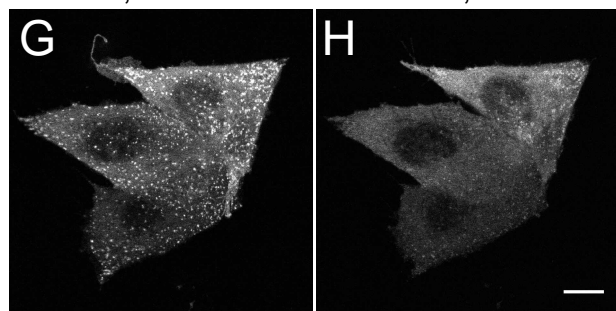
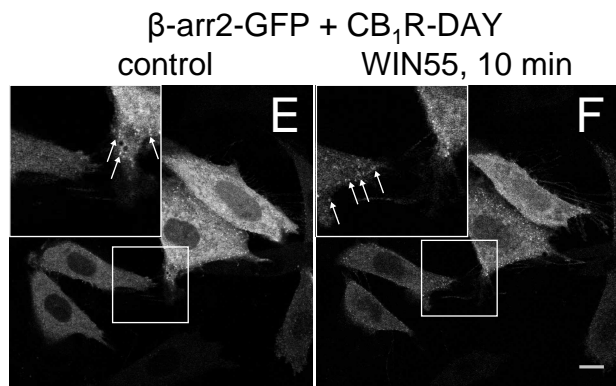
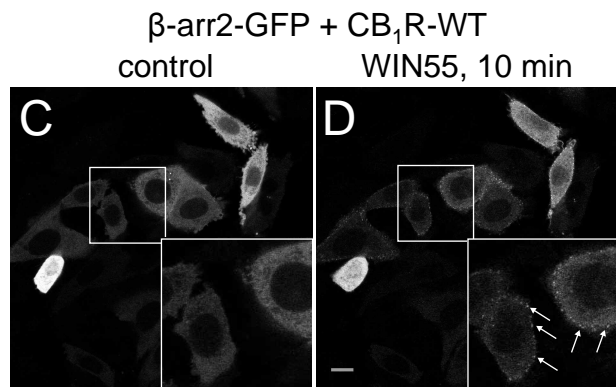
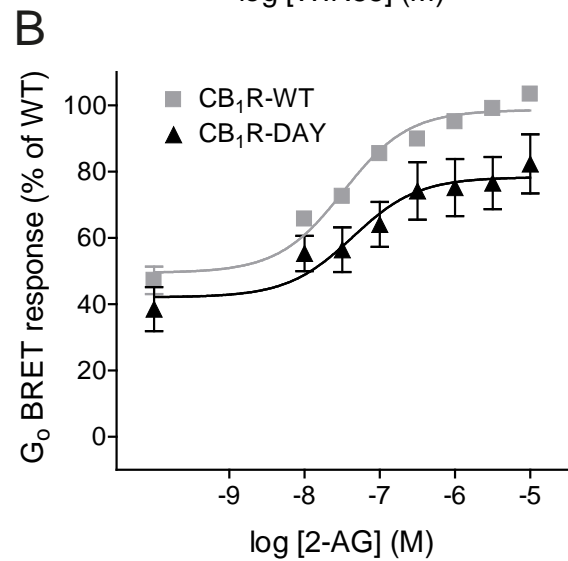
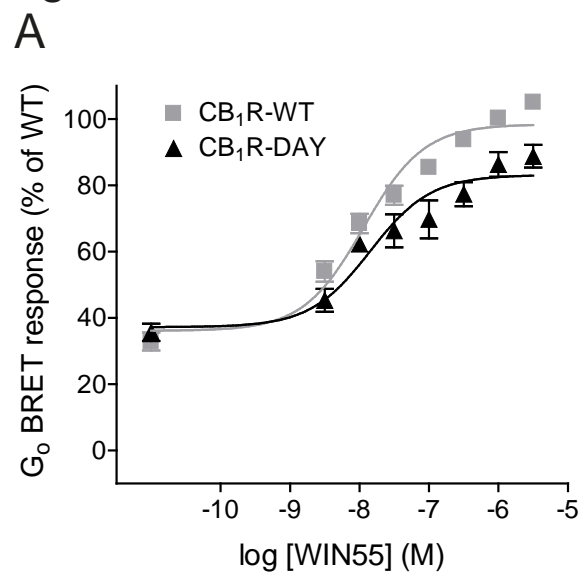
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Figure 1



**Figure 2**



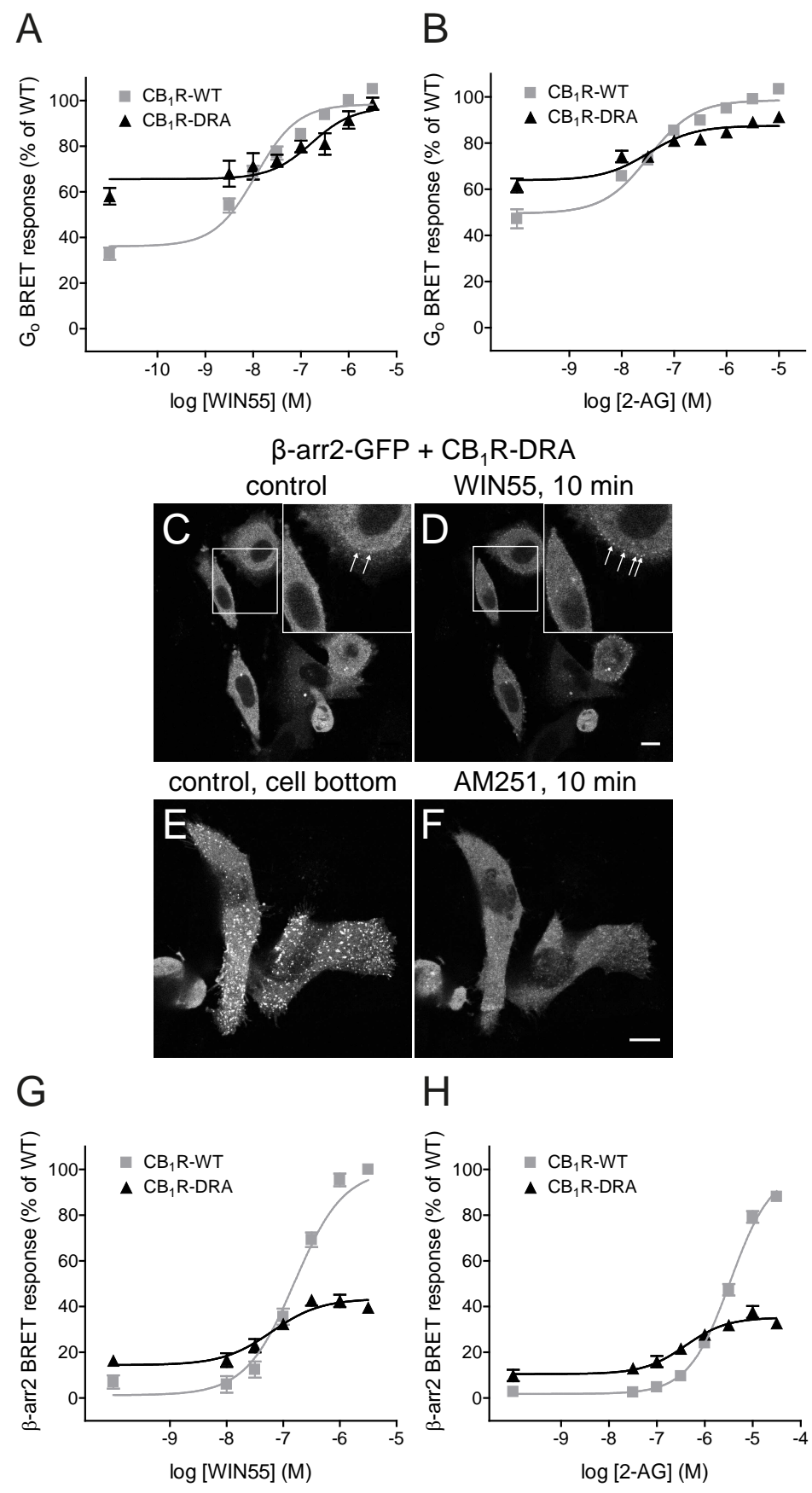
**Figure 3**

Figure 4

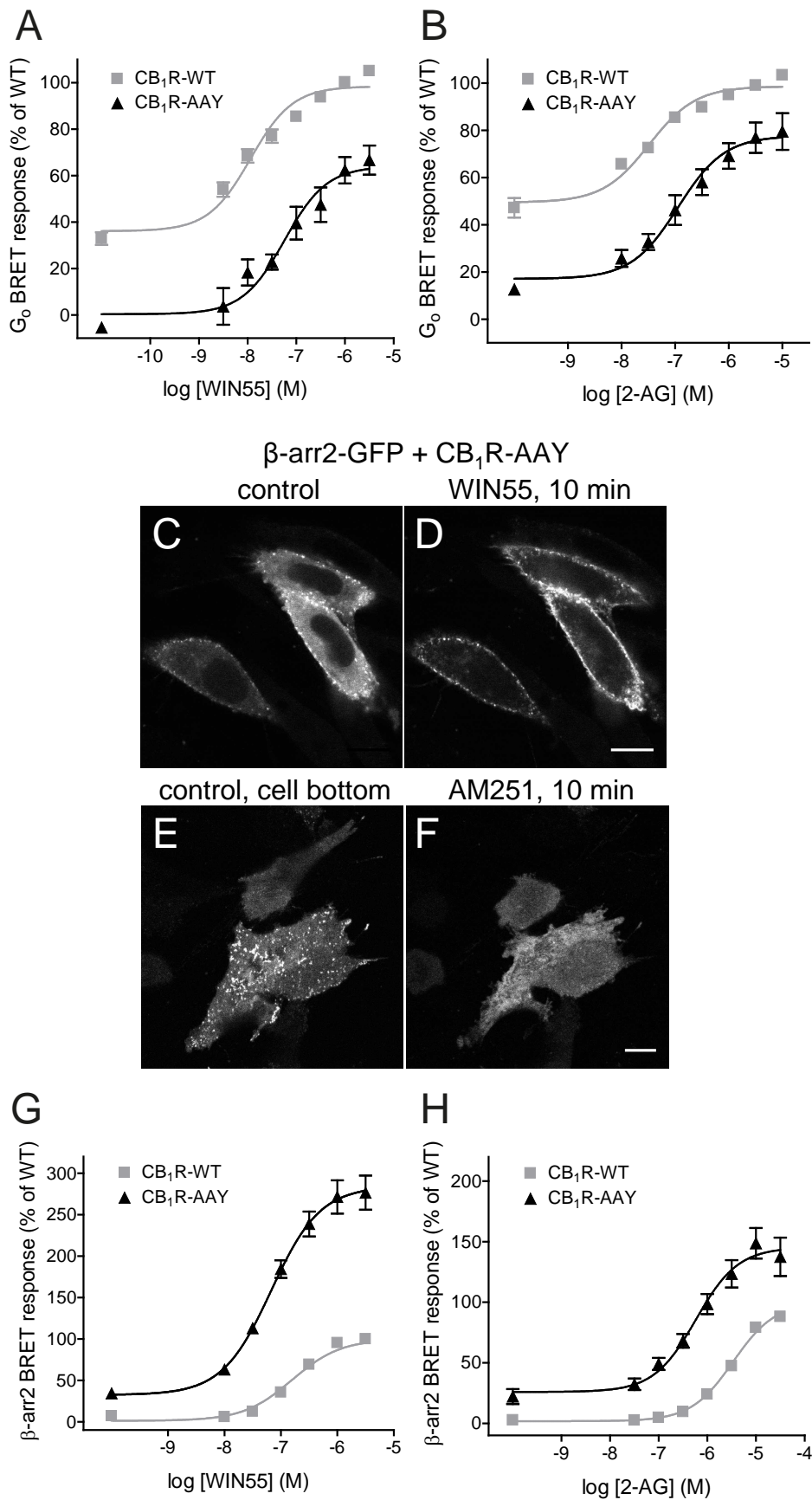
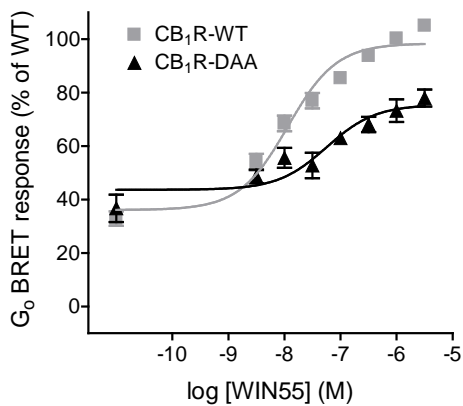
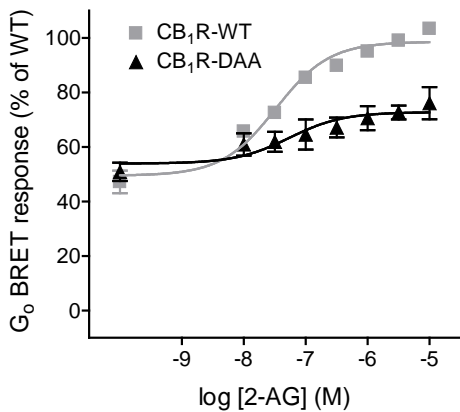


Figure 5

A



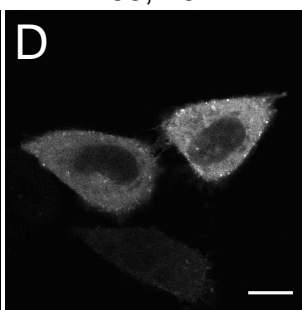
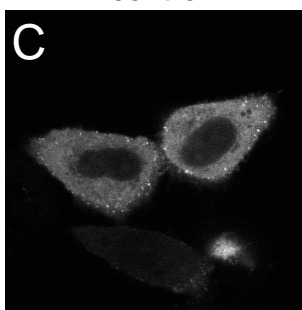
B



$\beta$ -arr2-GFP + CB<sub>1</sub>R-DAA

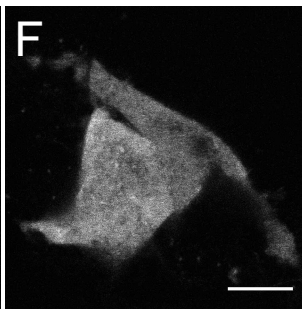
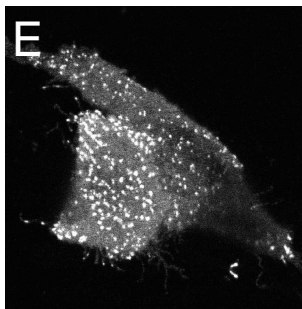
control

WIN55, 10 min

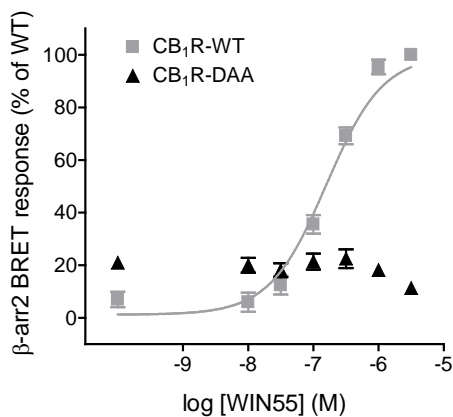


control, cell bottom

AM251, 10 min



G



H

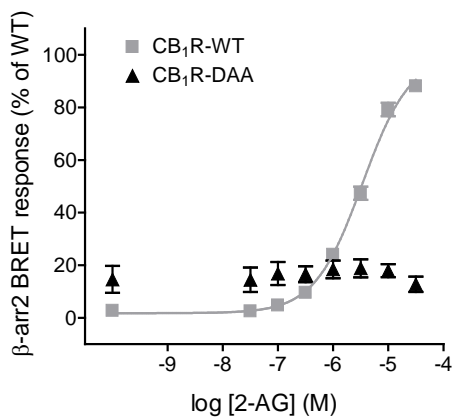
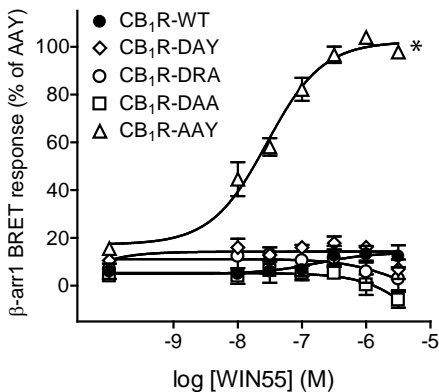
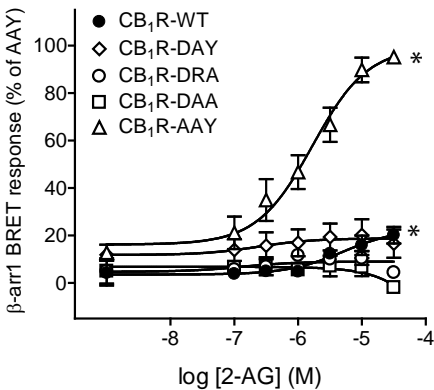


Figure 6

A

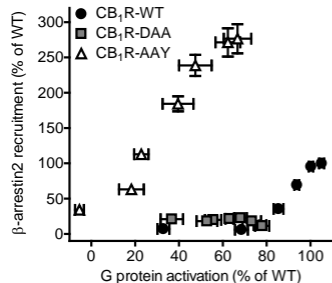


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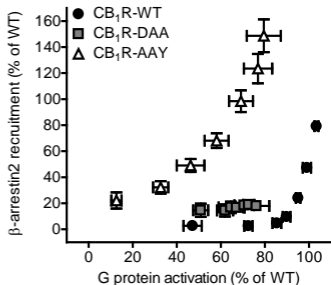


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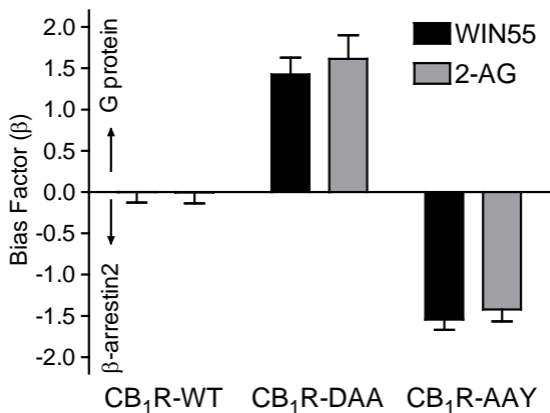
## A



## B



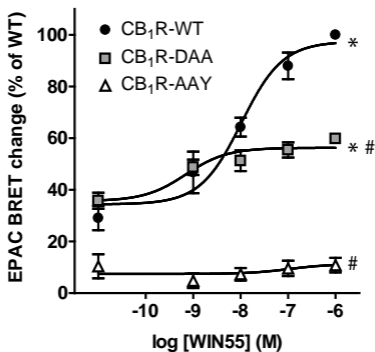
## C



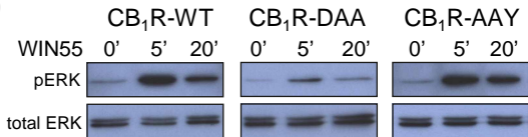


# Figure 8

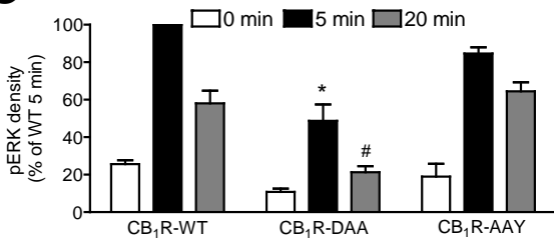
## A



## B



## C



**Table 1**

G <sub>o</sub> BRET						
Receptor	WIN55			2-AG		
	pEC <sub>50</sub>	bottom	E <sub>max</sub>	pEC <sub>50</sub>	bottom	E <sub>max</sub>
CB <sub>1</sub> R-WT	-7.89±0.07	36.85±2.47	100	-7.43±0.07	50.11±2.18	100
CB <sub>1</sub> R-DAY	-7.85±0.17	37.19±3.95	83.12±3.95*	-7.36±0.34	42.11±6.35	78.31±4.10*
CB <sub>1</sub> R-DRA	-6.78±0.27*	65.58±2.62*	97.22±4.31	-7.49±0.18	63.90±2.28*	87.49±1.35*
CB <sub>1</sub> R-DAA	-7.25±0.23	43.62±2.86	75.45±2.92*	-7.26±0.39	53.99±3.56	72.79±2.48*
CB <sub>1</sub> R-AAAY	-7.24±0.17*	0.36±4.24*	64.06±4.43*	-6.95±0.15*	17.12±4.02*	77.69±3.52*

β-arr2 BRET						
Receptor	WIN55			2-AG		
	pEC <sub>50</sub>	bottom	E <sub>max</sub>	pEC <sub>50</sub>	bottom	E <sub>max</sub>
CB <sub>1</sub> R-WT	-6.80±0.051	1.23±2.53	100	-5.47±0.02	1.79±0.87	100
CB <sub>1</sub> R-DAY	-7.53±0.19*	25.13±3.77*	62.36±2.60*	-6.65±0.28*	17.15±3.64*	44.72±2.71*
CB <sub>1</sub> R-DRA	-7.21±0.16*	14.43±2.09*	43.46±1.84*	-6.41±0.16*	10.47±1.69*	35.22±1.52*
CB <sub>1</sub> R-DAA	> -5.0*	20.96±2.35*	n.d.	> -4.5*	14.62±5.10*	n.d.
CB <sub>1</sub> R-AAAY	-7.17±0.10*	32.68±10.90*	284.10±9.93*	-6.24±0.14*	25.91±6.44*	145.30±6.74*

# Supplementary Figure 1

