Supplementary Materials

Data analysis

Competition-binding curves between [^{3}H]NMS and ACh in the absence or presence of BQCA were fitted to a two-site allosteric ternary complex model (equation S1)

$$Y = \frac{[A]Frac_{Hi}}{[A] + \left(\frac{K_A K_B}{\alpha'[B] + K_B}\right)\left(1 + \frac{[I]}{K_H} + \frac{[B]}{K_B} + \frac{\alpha[I][B]}{K_H K_B}\right)} + \frac{[A](1 - Frac_{Hi})}{[A] + \left(\frac{K_A K_B}{\alpha'[B] + K_B}\right)\left(1 + \frac{[I]}{K_L} + \frac{[B]}{K_B} + \frac{\alpha[I][B]}{K_L K_B}\right)} \quad \text{(S1)}$$

where Y is percentage (vehicle control) binding, [A], [B], and [I] are the concentrations of [^{3}H]NMS, BQCA and ACh respectively, K_A and K_B are the equilibrium dissociation constants of [^{3}H]NMS and BQCA, respectively, K_H and K_L are the equilibrium dissociation constants of ACh for the high- and low-affinity receptor state, respectively, $Frac_{Hi}$ is the proportion of receptors in the high-affinity receptor state, and α′ and α are the cooperativities between BQCA and [^{3}H]NMS or ACh respectively.

Table S1: Allosteric ternary complex model (Equation S1) binding parameters for the interaction between BQCA, ACh and [^{3}H]NMS at the hM_1 mAChR. Estimated parameter values represent the mean ± SEM of 3 experiments performed in duplicate.

Parameter	Value
$\mathbf{pK_B}$ [a]	3.82 ± 0.11
$\mathbf{pK_H}$ [b]	5.15 ± 0.23
$\mathbf{pK_L}$ [c]	4.08 ± 0.24
Log$\boldsymbol{\alpha}$ [d]	2.35 ± 0.30
Log$\boldsymbol{\alpha'}$ [e]	-100

[a] Negative logarithm of the equilibrium dissociation constant of BQCA.

[b] Negative logarithm of the high affinity equilibrium dissociation constant of ACh.

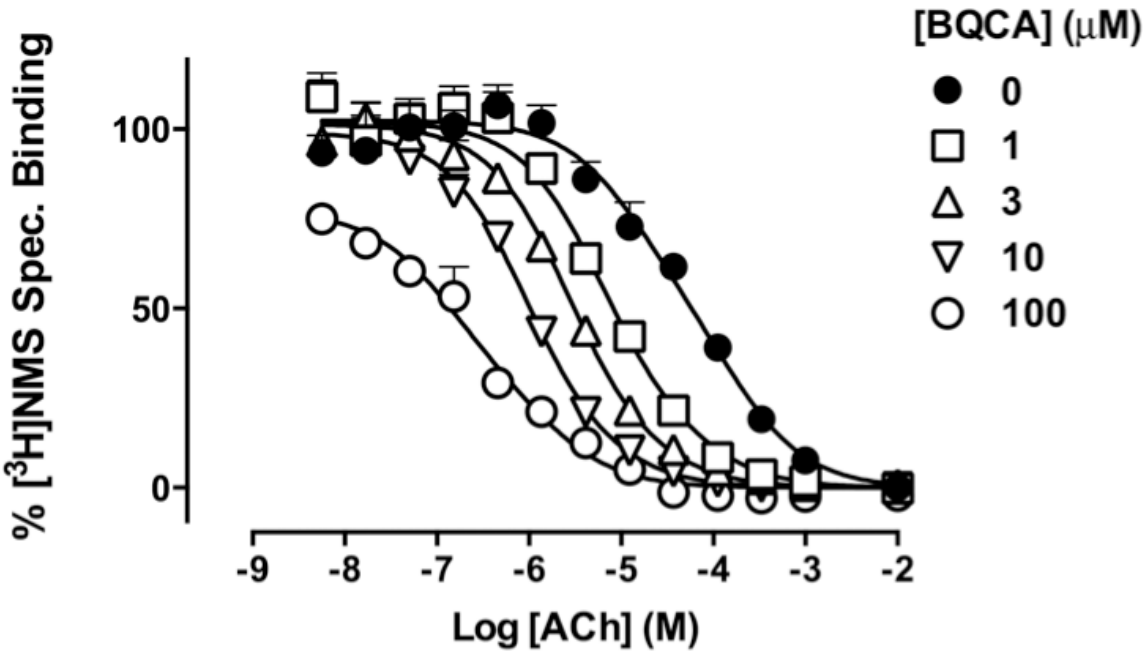
[c] Negative logarithm of the low affinity equilibrium dissociation constant of ACh.

[d] Logarithm of the binding cooperativity factor between BQCA and ACh .

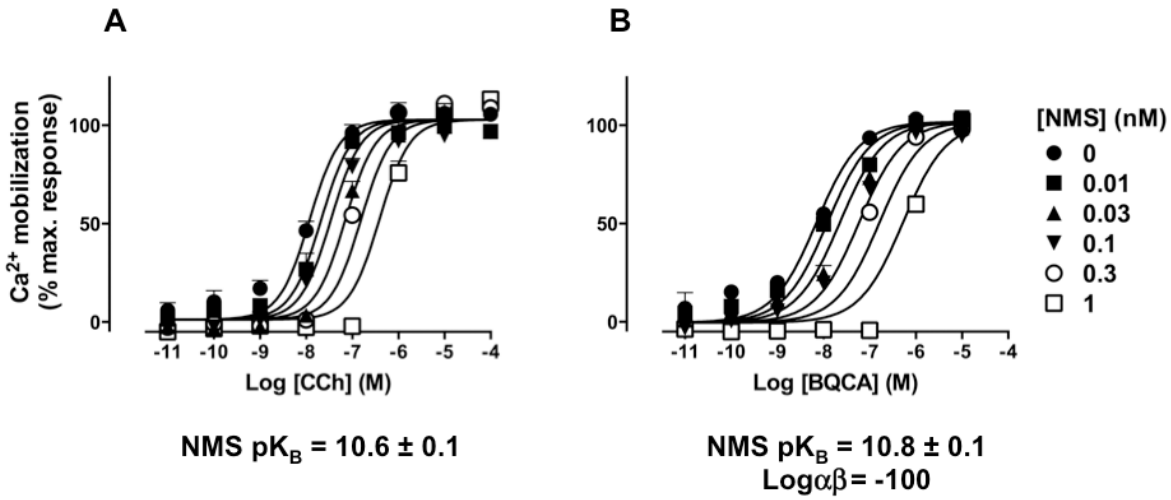
[e] Logarithm of the binding cooperativity factor between BQCA and [^{3}H]NMS; this parameter was constrained to an arbitrarily low value, consistent with very high negative cooperativity between the modulator and radioligand.

Reference

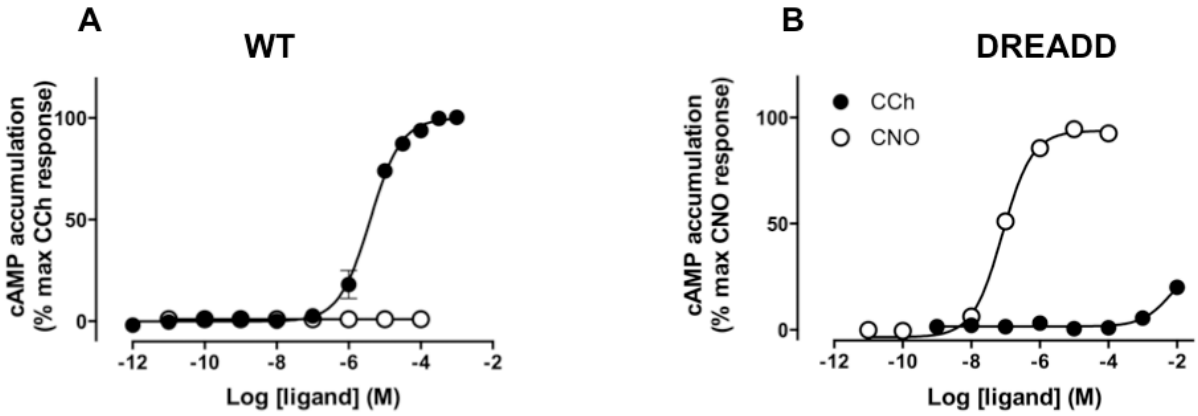
(1) ARUNLAKSHANA, O., and SCHILD, H. O. (1959) *Br J Pharmacol* **14**, 48–58



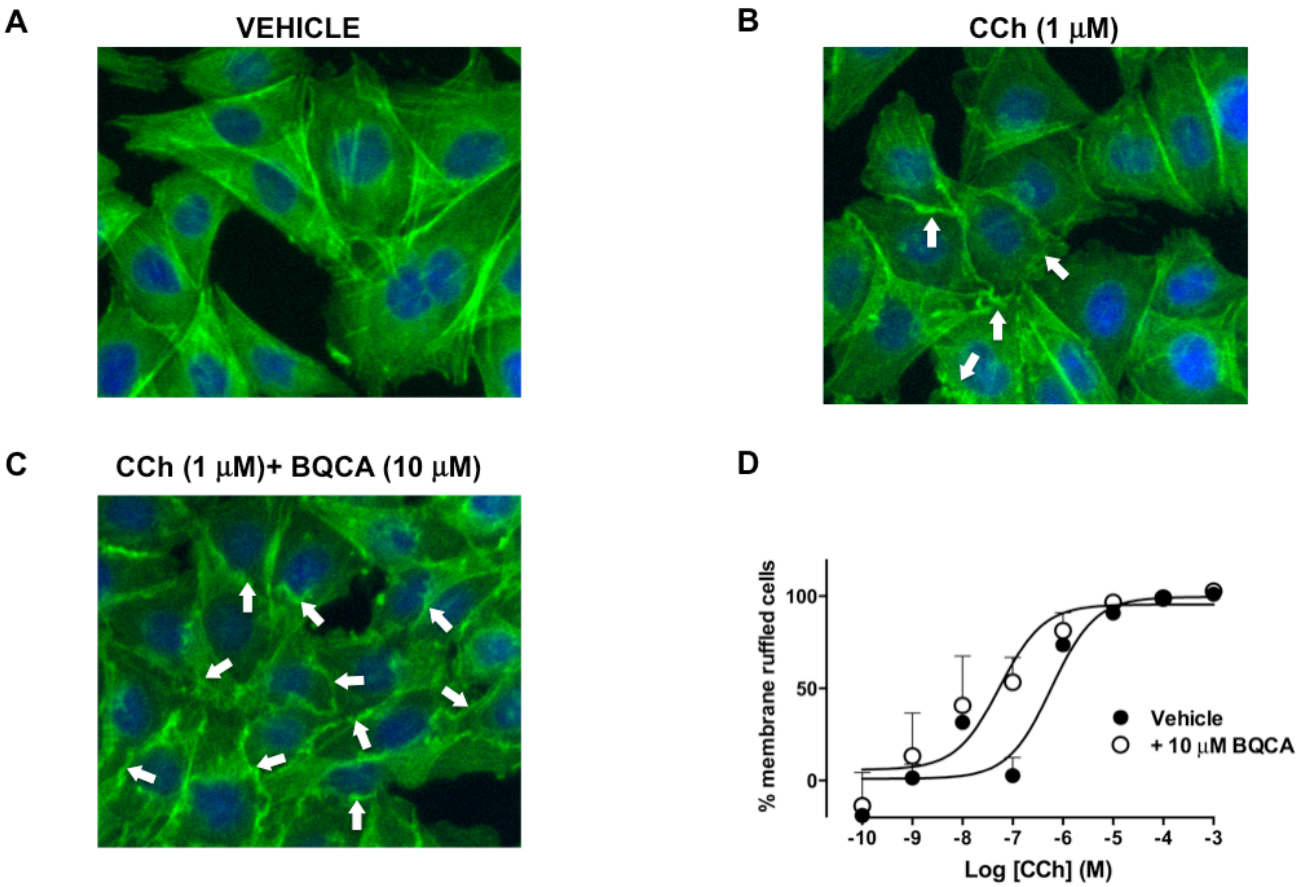
Supplementary Figure 1. BQCA is a positive allosteric modulator of ACh binding affinity. ACh-mediated inhibition of the equilibrium binding of [^{3}H]NMS in the absence or presence of BQCA. Data points represent the mean + SEM of four independent experiments performed in triplicate. Curves drawn through the data points represent the best fit of a two-state allosteric ternary complex model (equation S1; Table S1).



Supplementary Figure 2. BQCA exhibits very high negative cooperativity with an inverse agonist. Interaction between the inverse agonist, NMS, and **(A)** the orthosteric agonist, CCh or **(B)** the allosteric agonist/modulator, BQCA, in an assay of intracellular Ca^{2+}mobilization. Data represent the mean + SEM of three independent experiments. Curves through the data represent the best fit of **(A)** a competitive model of interaction (1) or **(B)** an operational allosteric ternary complex model. For the latter, the global cooperativity parameter ($\log\alpha\beta$) was not significantly different from -100, and was constrained as such.



Supplementary Figure 3. The DREADD M_1 mAChR shows a loss of responsiveness to CCh, but a gain in responsiveness to clozapine-N-oxide (CNO). Comparison of ligand signaling in a cAMP accumulation assay at the **(A)** wild type (WT) or **(B)** the DREADD ($Y^{106}C/A^{196}G$) receptor. Data points represent the mean + SEM of three experiments.



Supplementary Figure 4. BQCA potentiates CCh-mediated membrane ruffling via the M_1 mAChR. **(A-C)** Epifluorescence micrographs of FlpIn CHO hM_1 mAChR cells treated with ligand, fixed, stained with Hoechst 33342 nuclear dye (blue) and Alexa-568 phalloidin (green) and imaged using a 20X objective on an IN Cell 1000 analyzer. **(A)** Cells treated with serum-free media, which displayed smooth edges with an even distribution of actin at the membrane. **(B)** Cells treated with 1μM CCh, where some cells display a ruffled membrane consisting of convoluted actin structures at the membrane (white arrows). **(C)** Cells treated with 1μM CCh and 10 μM BQCA, which mostly display a ruffled morphology (white arrows), indicating potentiation of the CCh response. **(D)** Concentration-response to CCh in the absence or presence of 10 μM BQCA. Data are represented as mean percentage of cells exhibiting membrane ruffling in the absence of ligand + SEM collected from three experiments.