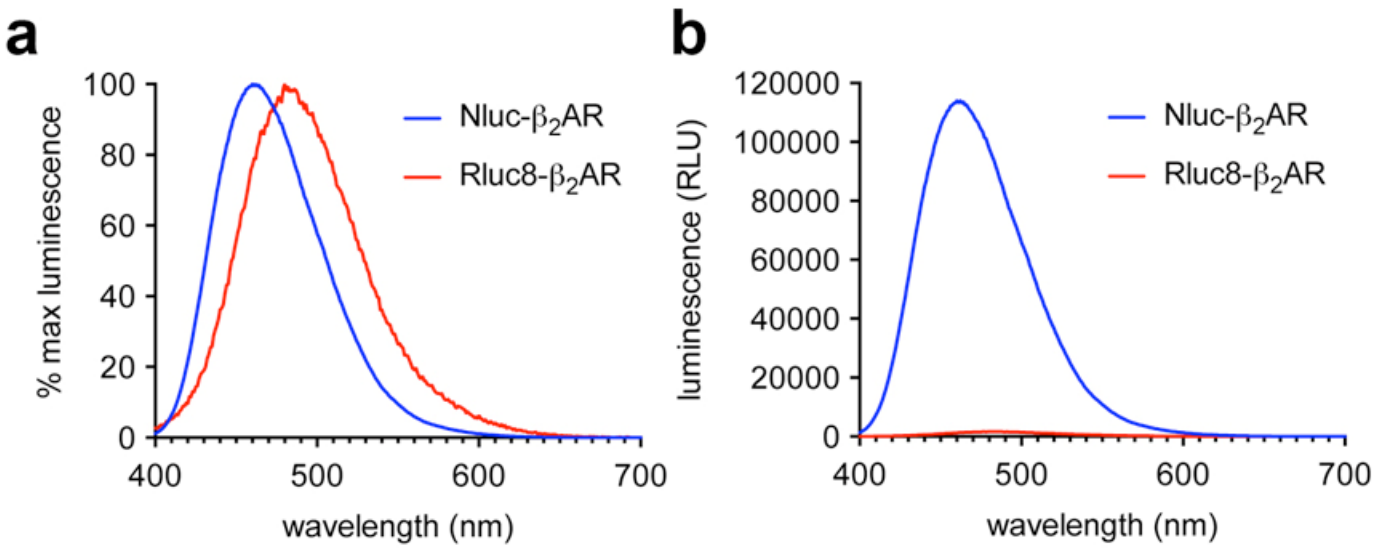
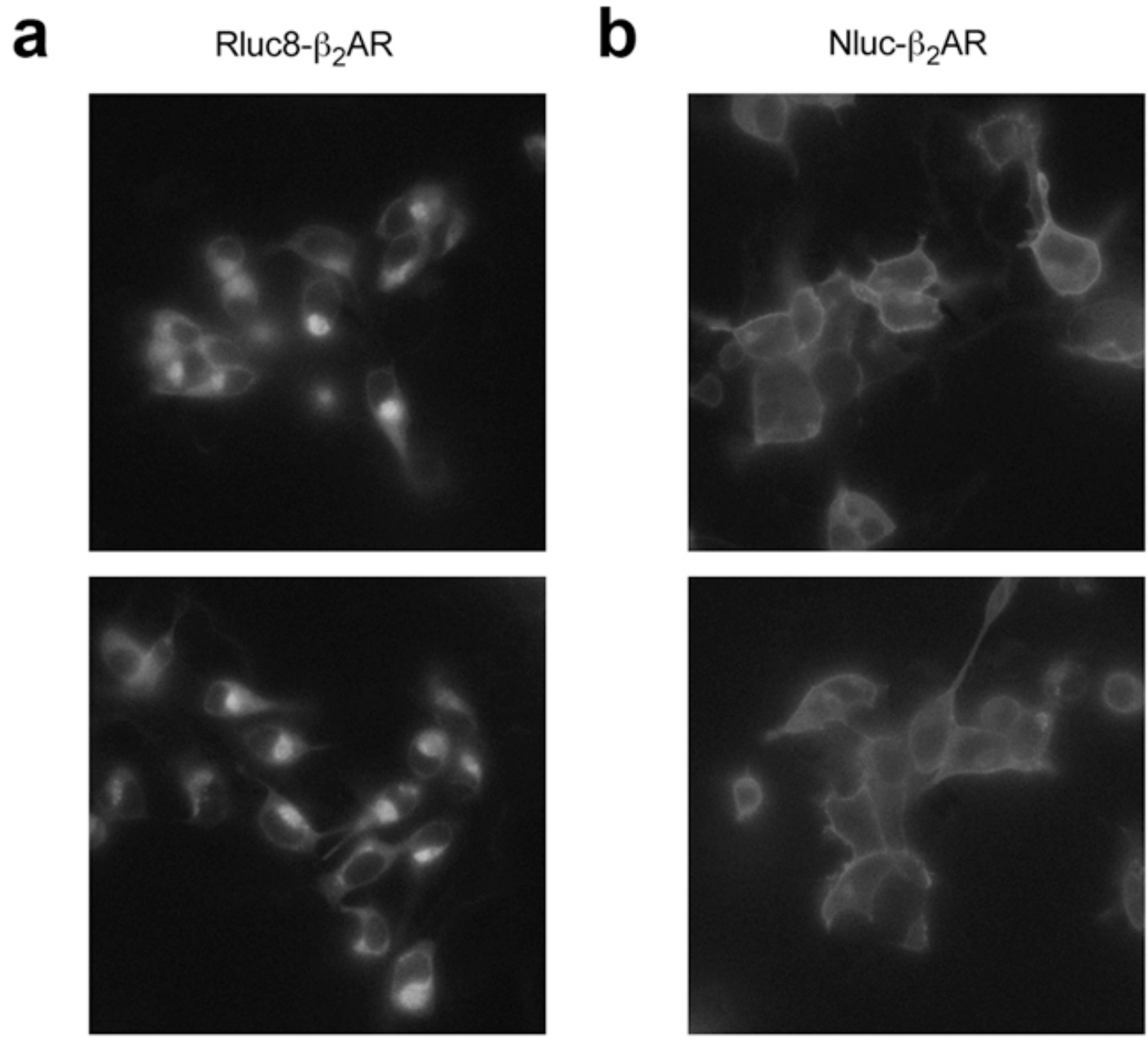


Supplementary Figure 1

Comparison of Rluc8-β_2AR and Nluc-β_2AR luminescence spectra

We generated luminescence spectra with HEK293 cells transiently transfected with Rluc8-β_2AR or Nluc-β_2AR following addition of coelenterazine h or furimazine substrate respectively. (**a**) Presentation as normalized luminescence illustrates that the Nluc emission peak is left-shifted by about 20 nm compared to Rluc8, thus enabling better spectral separation from the acceptor emission. (**b**) Presentation of the same spectra in terms of measured luminescence in relative light units (RLU) without normalization to the peak emission. This illustrates the substantially greater luminescence, and therefore energy transfer potential, of Nluc compared to Rluc8 despite the spectrum being left-shifted (Nluc peak (462 nm): 113,909 RLU; Rluc8 peak (480 nm): 1642 RLU). This becomes more relevant as more red-shifted energy acceptors are utilized, and therefore the spectral overlap of the donor emission spectrum with the acceptor excitation spectrum diminishes. The high luminescence output of Nluc means that this reduced overlap is much less of an issue compared with Rluc8. The data shown here are representative of three independent experiments.

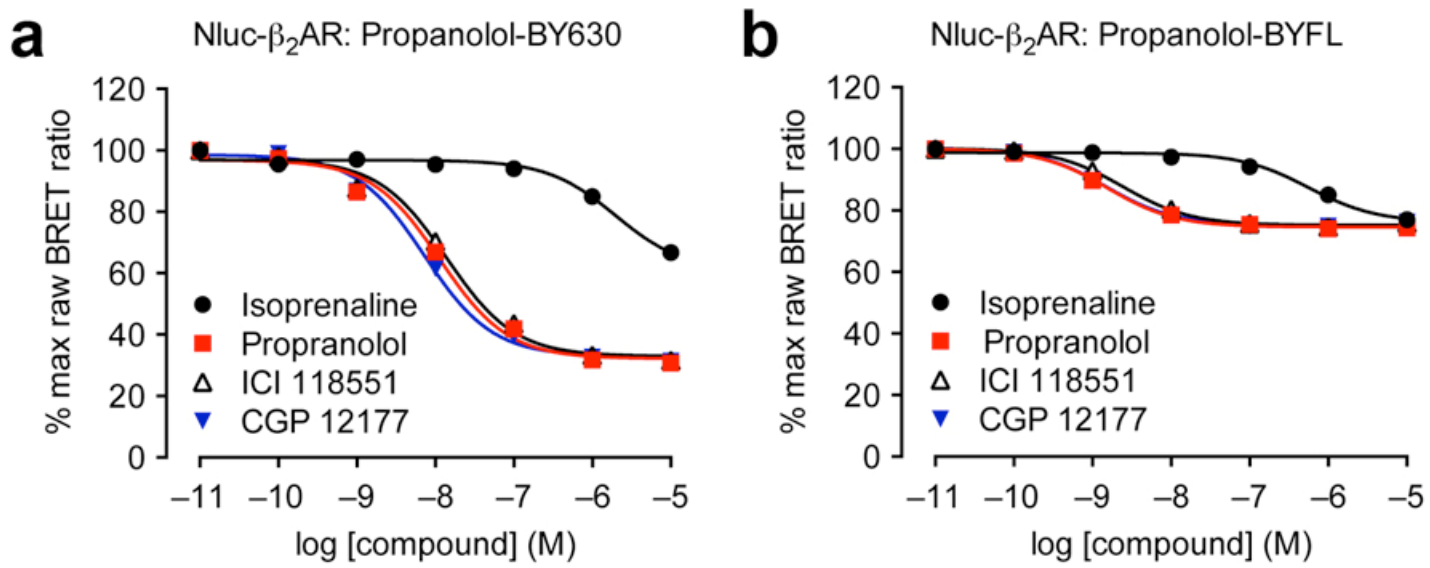


Supplementary Figure 2

Microscopy images comparing the cellular localization of Rluc8-β_2AR and Nluc-β_2AR

(**a**) Images of HEK293 cells transiently transfected with Rluc8-β_2AR indicate that this fusion protein is not appropriately trafficked to the plasma membrane. (**b**) This is in contrast to Nluc-β_2AR that is clearly localized at the plasma membrane. We acquired images of eight different fields of view per sample and two representative fields are shown. Furthermore, the data are representative of three independent experiments. Nluc is derived from the luciferase expressed in deep sea shrimp *Oplophorus gracilirostris*. The native luciferase is secreted by the shrimp in bright luminescent bursts to ward off predators[1]. It has therefore evolved to be secreted and therefore pass through cellular membranes. This is not the case for *Renilla* luciferase. Indeed multiple attempts have been made to generate a secreted *Renilla* luciferase[2]. However, addition of the signal peptide of human interleukin-2 resulted in a secreted form of Rluc with 15 times less activity than cytosolic Rluc in mammalian cells[3]. The reason for this was unclear, but one suggested possibility was that addition of the signal peptide resulted in misfolding in the endoplasmic reticulum.

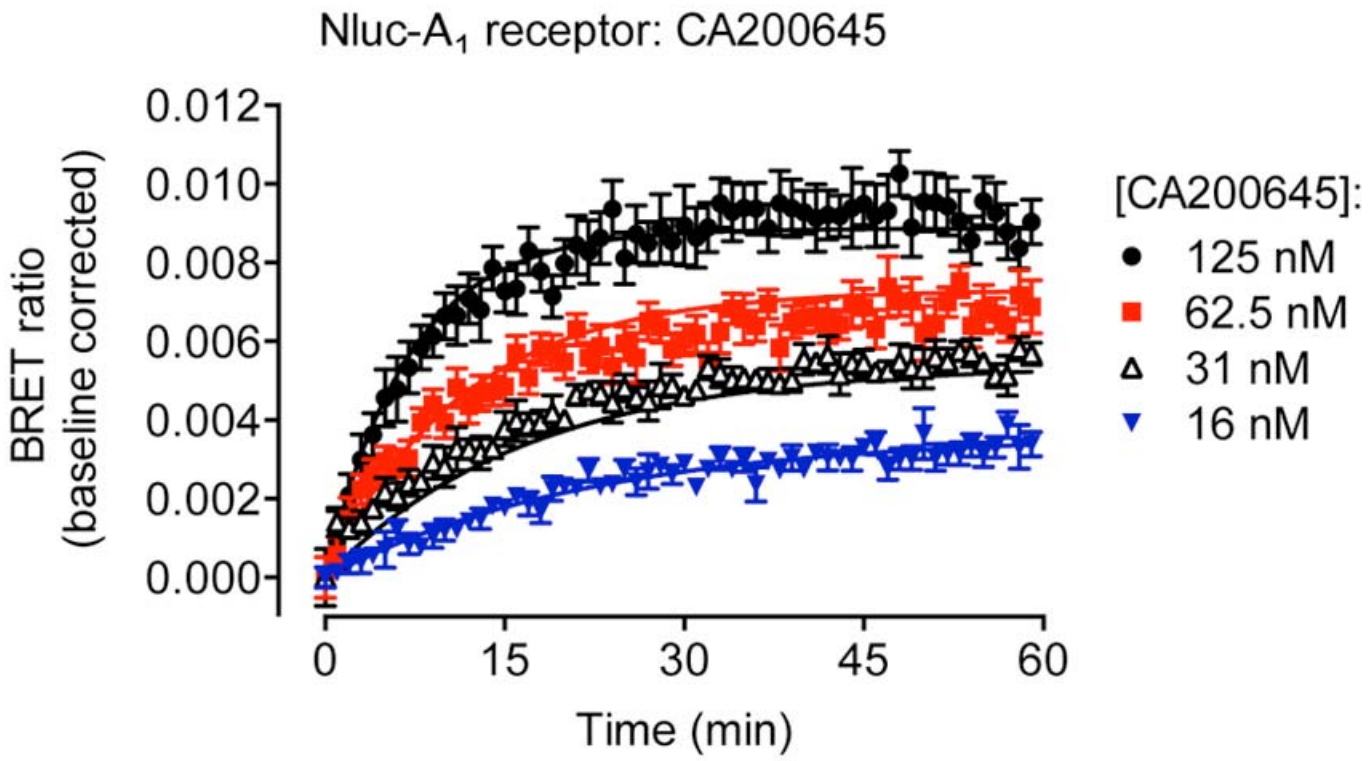
1. Hall, M.P. *et al*. *ACS Chem. Biol*. **7**, 1848–1857 (2012).
2. Tannous, B.A. & Teng, J. *Biotechnol. Adv*. **29**, 997–1003 (2011).
3. Liu, J, O'Kane D.J., Escher, A. *Gene* **203**, 141–148 (1997).



Supplementary Figure 3

BRET binding assessed with propranolol-BY630 and propranolol-BYFL

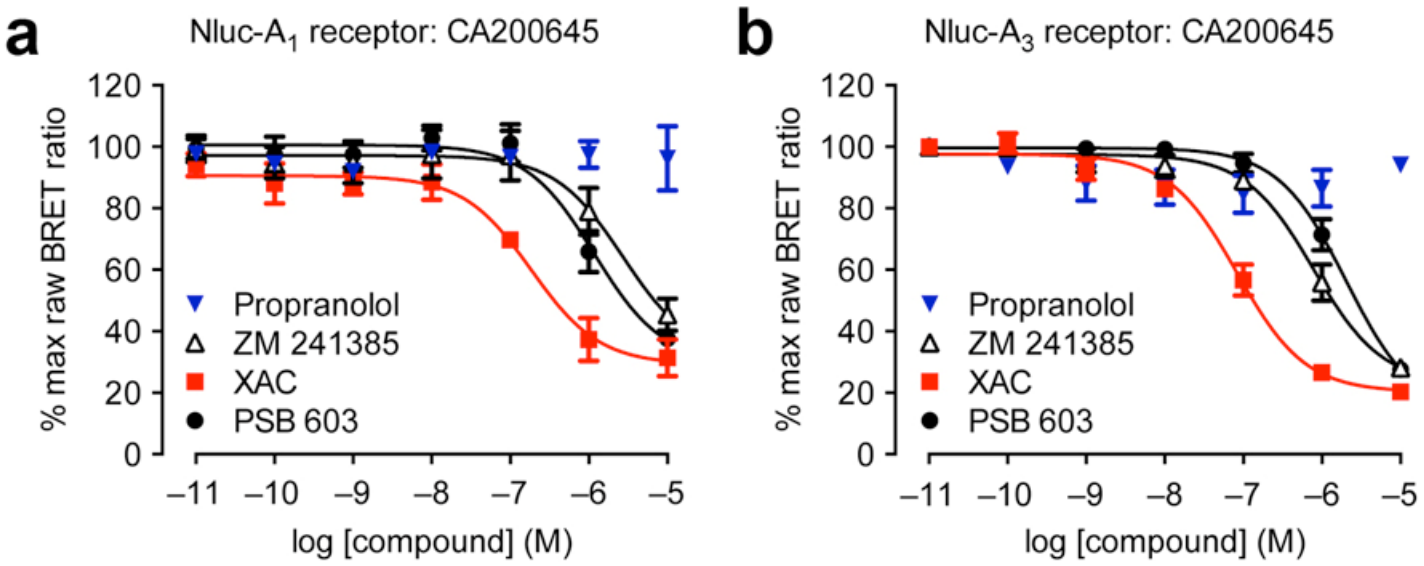
(**a**,**b**) We treated HEK293 cells stably expressing Nluc-β_2AR with 10 nM propranolol-BY630 (**a**) or 10 nM propranolol-BYFL (**b**) and increasing concentrations of unlabeled ligands as shown. In the presence of 10 μM propranolol we observed a decrease of 69.2 ± 1.3% in propranolol-BY630 BRET signal, whereas with propranolol-BYFL we only achieved a 25.6 ± 1.2% decrease in signal. Each data point represents mean ± s.e.m. of five (all curves in (**a**) and propranolol in (**b**)) or four (**b**) separate experiments. In each experiment we made triplicate determinations for each data point.



Supplementary Figure 4

Kinetic measurements of binding of CA200645 to Nluc-A_1 receptor

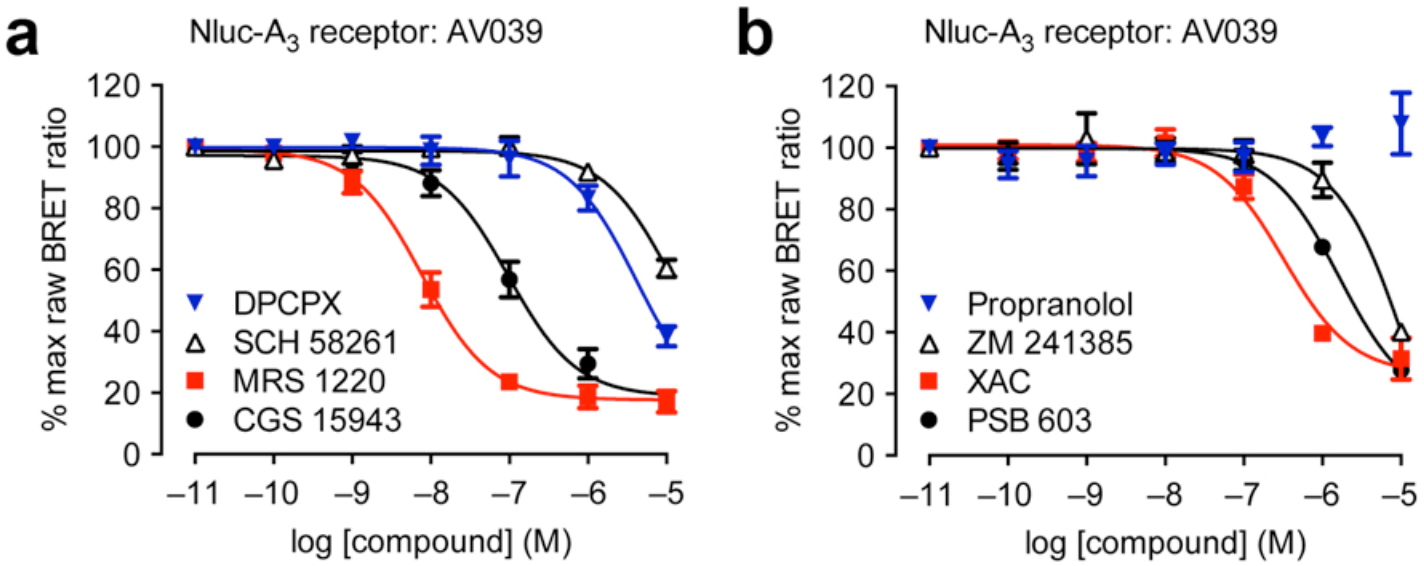
We treated HEK293 cells stably expressing Nluc-A_1 receptor with various concentrations of CA200645. We measured BRET between Nluc and the fluorescent ligand every min for 60 min at room temperature. The data shown are representative of three independent experiments performed in triplicate. From global fitting of the data, the kinetic parameters for CA200645 at Nluc-A_1 receptor are k_{on} = 9.64 ± 0.32 x 10^5 M^{-1} min^{-1} and k_{off} = 0.019 ± 0.005 min^{-1} with a resulting K_D of 20.4 ± 6.9 nM (mean ± s.e.m., n=3).



Supplementary Figure 5

Inhibition of BRET between NanoLuc and CA200645 at the adenosine A_1 and A_3 receptors by four additional compounds

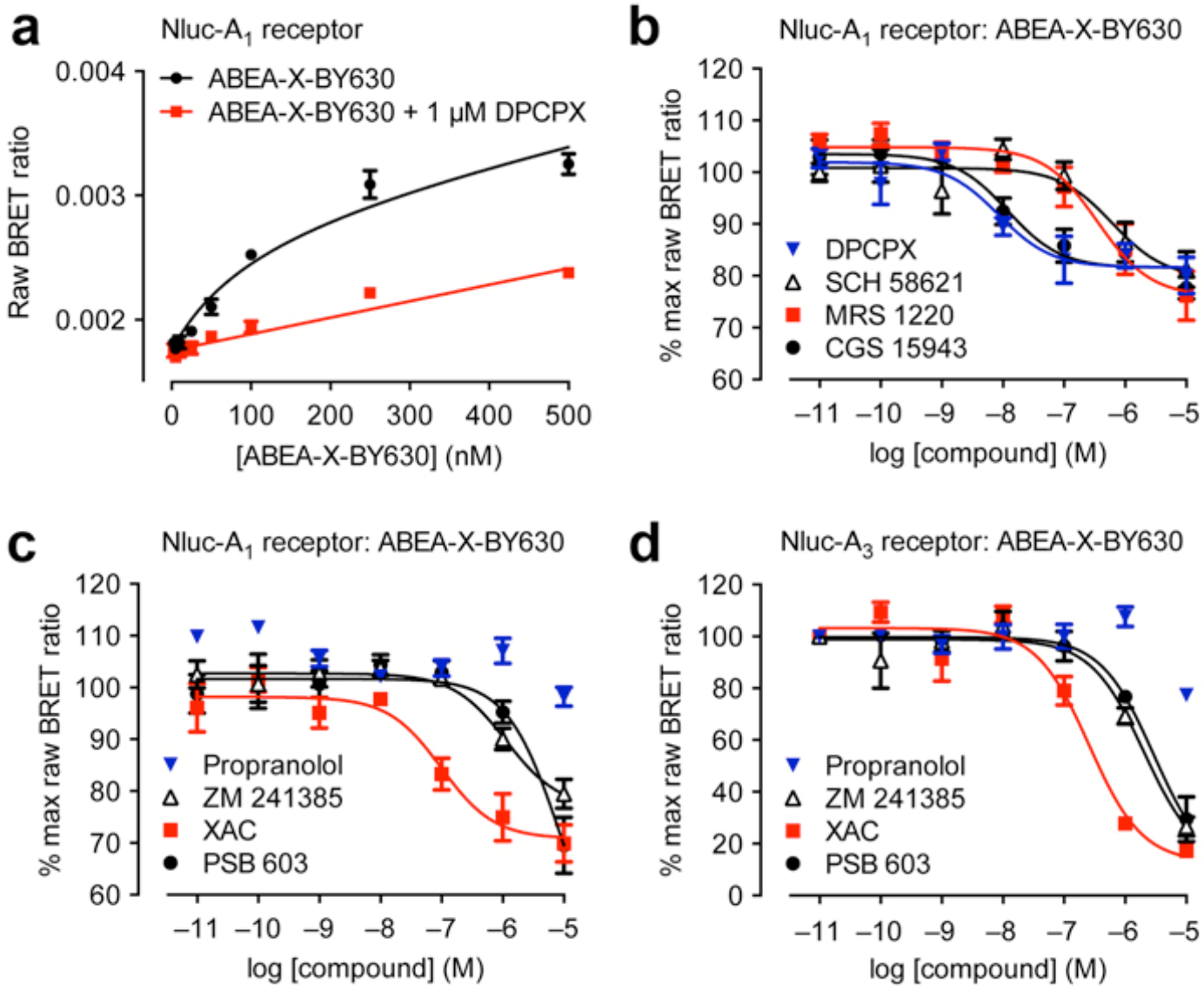
(**a**,**b**) We treated HEK293 cells stably expressing Nluc-A_1 receptor (**a**) or Nluc-A_3 receptor (**b**) with 25 nM CA200645 and increasing concentrations of unlabeled ligands as shown. We monitored the resulting concentration dependent decrease in BRET and each data point represents mean ± s.e.m. of five (**a**: propranolol, ZM241385), four (**a**: XAC, PSB 603; **b**: PSB 603) or three (**b**: propranolol, ZM241385, XAC) experiments performed in triplicate.



Supplementary Figure 6

Inhibition of BRET between NanoLuc and AV039 at the adenosine A_3 receptor by a panel of eight GPCR antagonists

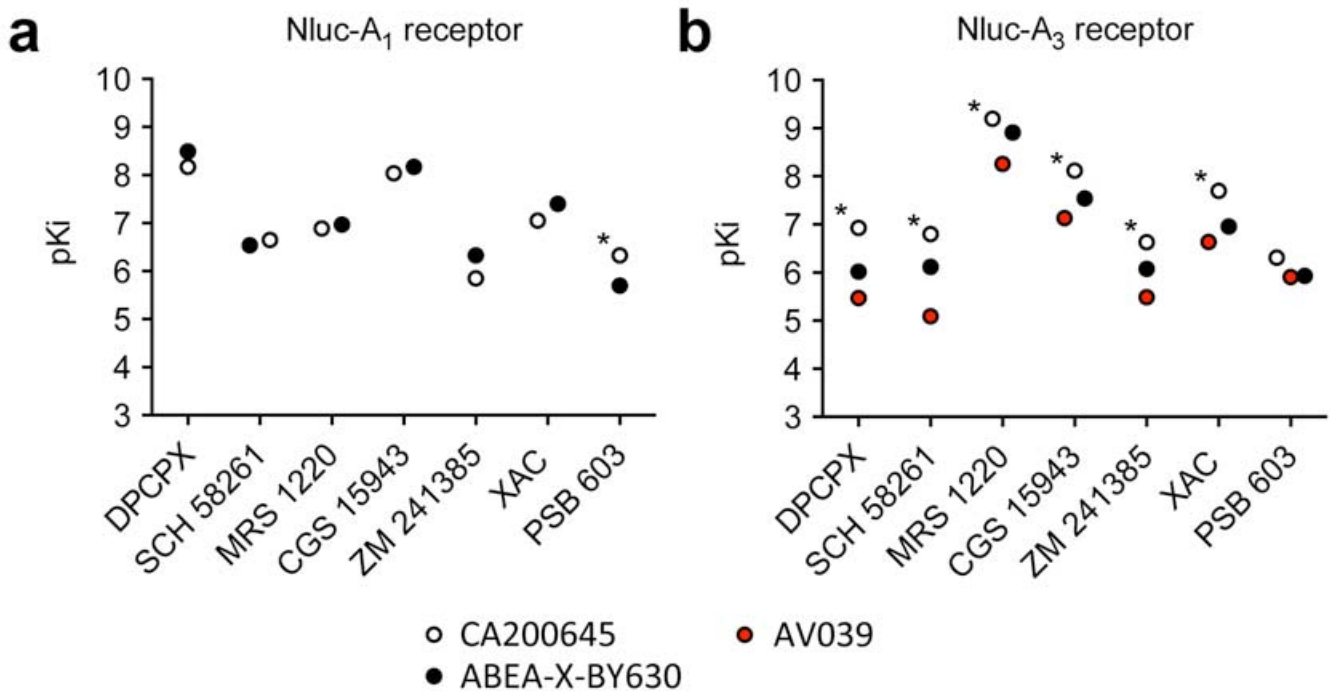
(**a**,**b**) We treated HEK293 cells stably expressing Nluc-A_3 receptor with 10 nM AV039 and increasing concentrations of unlabeled ligands as shown. The resulting concentration dependent decrease in BRET was monitored and each data point represents mean ± s.e.m. of four experiments performed in triplicate.



Supplementary Figure 7

Saturation and competition ligand binding with ABEA-X-BY630 at Nluc-A_1 and Nluc-A_3 receptors

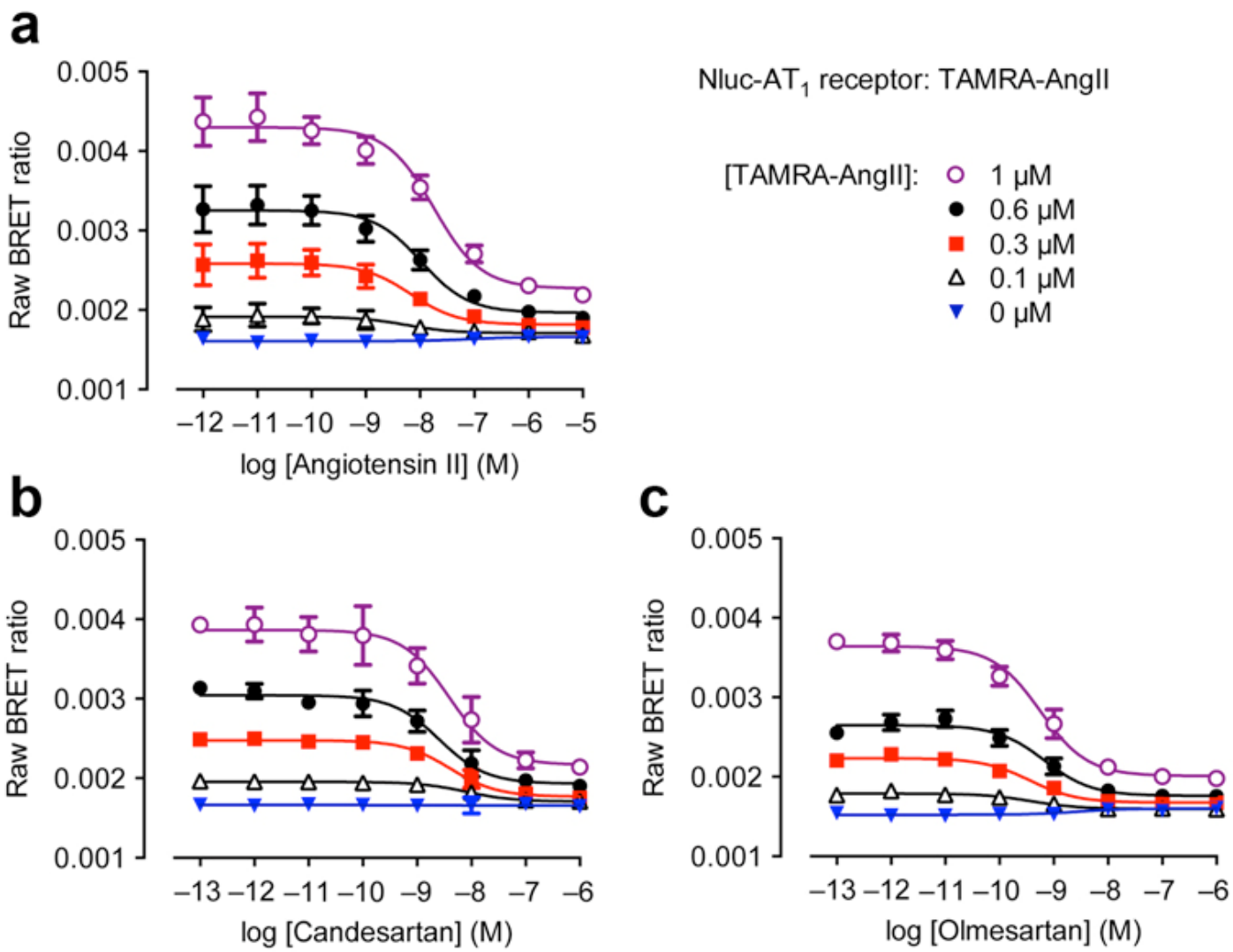
(a) We treated HEK293 cells stably expressing Nluc-A_1 receptor with increasing concentrations of ABEA-X-BY630. We established non-specific binding in the presence of 1 μM DPCPX and measured the resulting BRET ratios after 1 h incubation at 37 °C. (**b–d**) We also performed competition BRET binding assays on Nluc-A_1 (**b, c**) and Nluc-A_3 (**d**) receptor-expressing HEK293 cells treated with 250 nM ABEA-X-BY630 (**b, c**) or 50 nM ABEA-X-BY630 (**d**), along with increasing concentrations of unlabeled ligands as shown. Panel **a** is a representative graph of three experiments performed in triplicate. Data points in **b**, **c** and **d** represent mean ± s.e.m. of four experiments performed in triplicate.



Supplementary Figure 8

A comparison of pK_i values obtained at the adenosine A_1 and A_3 Nluc-tagged receptors using three different fluorescent ligands

(**a**,**b**) We obtained pK_i values for (**a**) the Nluc-A_1 receptor and (**b**) the Nluc-A_3 receptor with the non-selective fluorescent antagonist CA200645, non-selective fluorescent agonist ABEA-X-BY630 and A_3-selective fluorescent antagonist AV039. We have taken pK_i values from Supplementary Tables 1 and 2 (see tables for n numbers). * indicates values which are significantly different ($p<0.05$) using an unpaired t-test (**a**) or one-way ANOVA (**b**). In (**b**) the ANOVA analysis shows that for most competing ligands (with the exception of PSB 603) the data for each competing ligand cannot be described by a single p*K*i value.



Supplementary Figure 9

Competition ligand binding at Nluc-AT$_1$ receptor with varying concentrations of TAMRA-AngII

(**a–c**) We treated cells transiently expressing Nluc-AT$_1$ receptor with 1, 0.6, 0.3, 0.1 or 0 μM TAMRA-AngII and increasing concentrations of (**a**) angiotensin II, (**b**) candesartan and (**c**) olmesartan. We measured BRET between Nluc and TAMRA-AngII. Data points represent mean ± s.e.m. of three experiments performed in duplicate.