SUPPLEMENTAL FIGURES

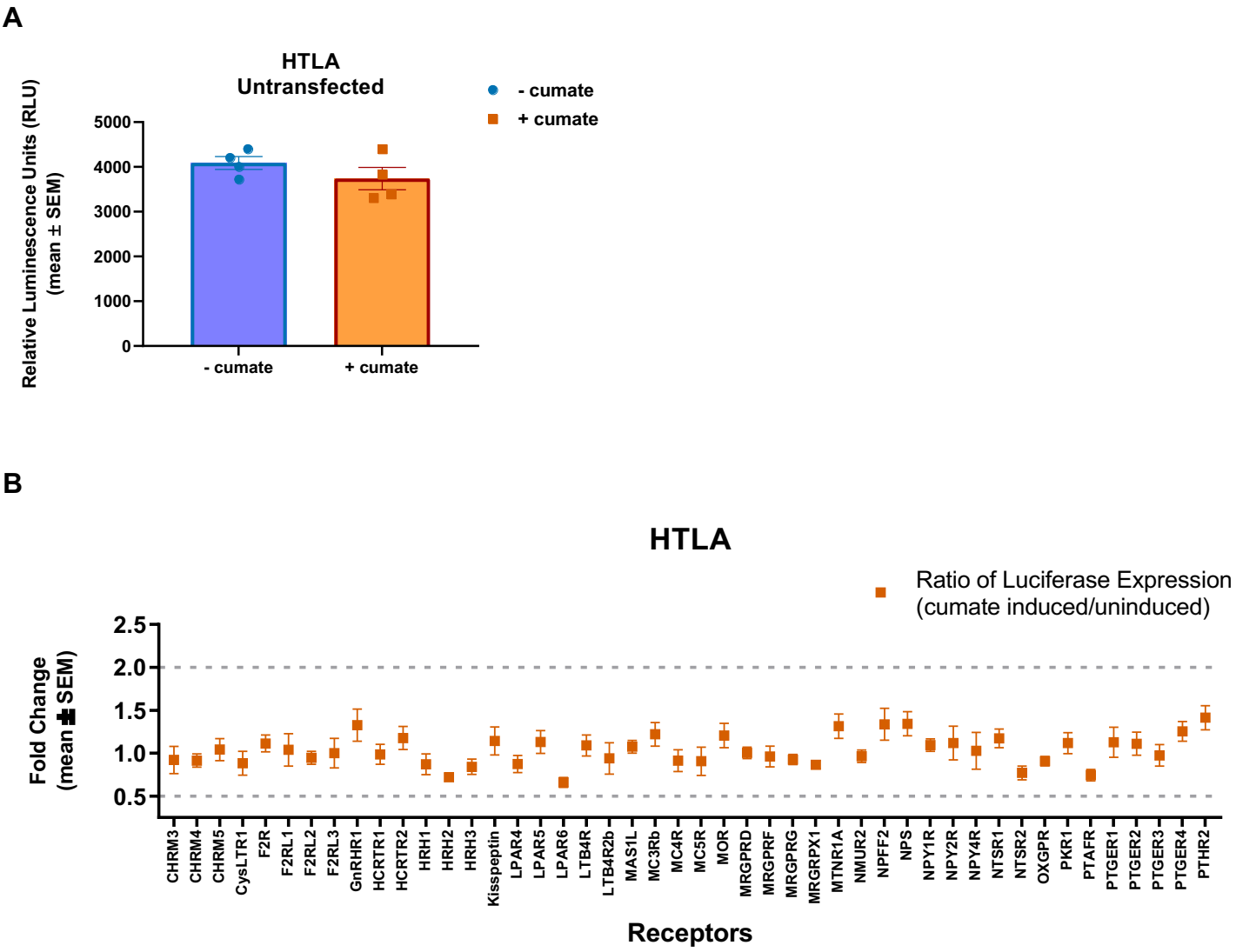


Figure S1. Assessment of the effect of cumate in the PRESTO-Tango (cumate-independent system). To confirm that cumate itself does not possess any agonistic or antagonistic properties in a cumate-independent system, HTLA cells from the PRESTO-Tango platform were plated in the presence and absence of cumate (30 µg/mL) (A) and were transfected with a panel of diverse GPCR Tango constructs (B). 96 hours following its initial addition, fold changes in basal arrestin recruitment were calculated between the wells in the absence or presence of cumate. All error bars represent SEM (Fig S1A: n = 12, with 3 technical replicates from 4 biological samples; Fig S1B: n = 4, with 4 technical replicates from one biological sample). Generic receptor codes refer to the GPCR-Tango constructs.

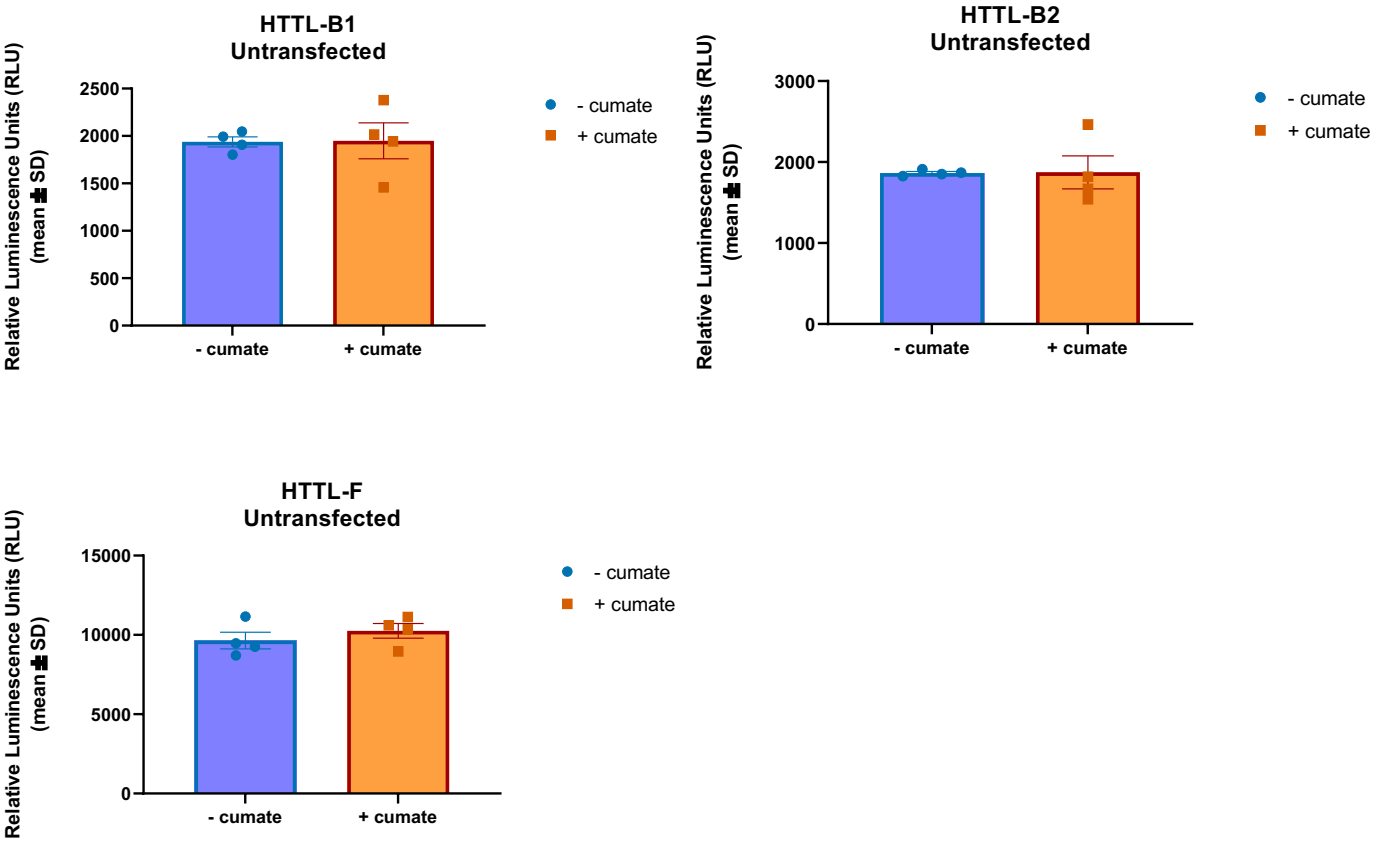
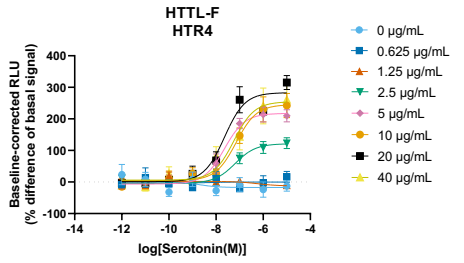
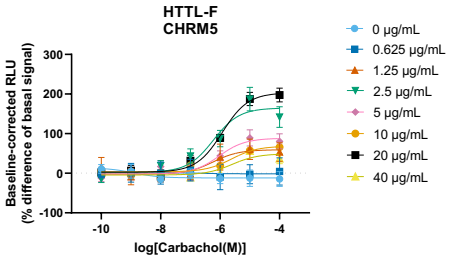


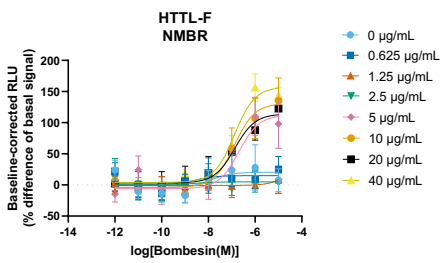
Figure S2. Baseline signal of Tango-Trio cell lines. HTTL-B1, HTTL-B2, and HTTL-F cells were plated in the presence or absence of cumate (30 µg/mL), which was maintained throughout (totalling approximately 72 hours). As per standard protocol, cells were serum starved for the last 24 hours of the experiment, and luminescence was subsequently read to compare the differences in baseline signal of untransfected cells. All error bars represent SD (n = 12, with 3 technical replicates from 4 biological samples).



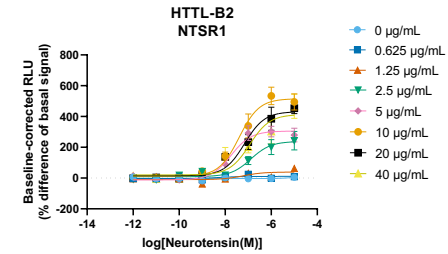
	Bottom	Top	LogEC50	EC50	Fold
0 µg/mL	110821	88503	-8.818	1.522e-009	0.7986
0.625 µg/mL	140317	144855	-8.189	6.476e-009	1.032
1.25 µg/mL	148336	127534	-6.247	5.666e-007	0.8598
2.5 µg/mL	138637	319190	-7.188	6.493e-008	2.302
5 µg/mL	166049	563879	-7.643	2.277e-008	3.396
10 µg/mL	222037	733880	-7.148	7.106e-008	3.305
20 µg/mL	199343	756945	-7.642	2.281e-008	3.797
40 µg/mL	206147	690066	-7.245	5.685e-008	3.347



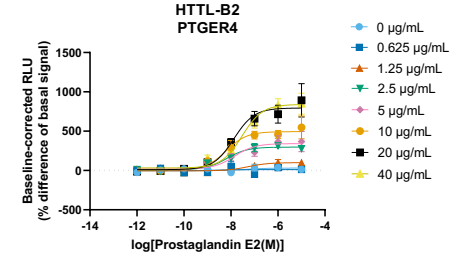
	Bottom	Top	LogEC50	EC50	Fold
0 µg/mL	169971	132039	-8.899	1.261e-009	0.7768
0.625 µg/mL	154668	150573	-6.582	2.616e-007	0.9735
1.25 µg/mL	154068	251206	-6.233	5.848e-007	1.630
2.5 µg/mL	136045	351622	-6.226	5.948e-007	2.585
5 µg/mL	332946	640221	-6.037	9.187e-007	1.923
10 µg/mL	539380	877098	-5.615	2.424e-006	1.626
20 µg/mL	272263	803835	-5.926	1.187e-006	2.952
40 µg/mL	303321	473188	-5.593	2.552e-006	1.560



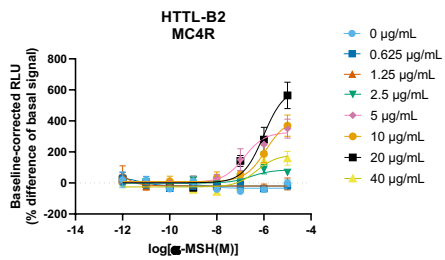
	Bottom	Top	LogEC50	EC50	Fold
0 µg/mL	9439	11845	-7.875	1.333e-008	1.255
0.625 µg/mL	12768	14631	-8.568	2.705e-009	1.146
1.25 µg/mL	12528	Unstable	-0.7912	0.1617	Unstable
2.5 µg/mL	7726	8136	-8.686	2.063e-009	1.053
5 µg/mL	10237	23096	-6.698	2.005e-007	2.256
10 µg/mL	7740	18599	-6.888	1.295e-007	2.403
20 µg/mL	13587	28217	-6.870	1.348e-007	2.077
40 µg/mL	11441	28312	-6.881	1.316e-007	2.475



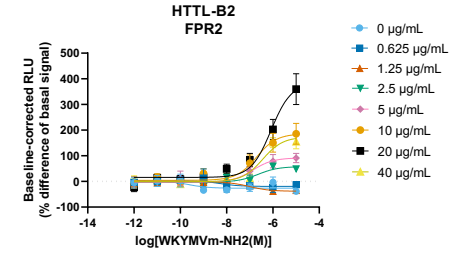
	Bottom	Top	LogEC50	EC50	Fold
0 µg/mL	23502	Unstable	-1.505	0.03127	Unstable
0.625 µg/mL	25021	28514	-8.233	5.844e-009	1.140
1.25 µg/mL	18001	27817	-7.219	6.036e-008	1.545
2.5 µg/mL	27249	84454	-6.886	1.300e-007	3.099
5 µg/mL	17525	80781	-7.750	1.779e-008	4.610
10 µg/mL	35343	194540	-7.395	4.025e-008	5.504
20 µg/mL	35045	161795	-7.161	6.903e-008	4.617
40 µg/mL	33364	141844	-6.938	1.153e-007	4.251



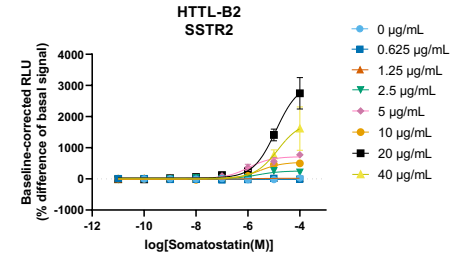
	Bottom	Top	LogEC50	EC50	Fold
0 µg/mL	11321	14823	-7.299	5.024e-008	1.309
0.625 µg/mL	9098	10396	-8.591	2.567e-009	1.143
1.25 µg/mL	7864	16507	-7.281	5.236e-008	2.099
2.5 µg/mL	9957	36207	-8.174	6.696e-009	3.636
5 µg/mL	11869	44285	-7.858	1.387e-008	3.731
10 µg/mL	5764	31765	-8.198	6.337e-009	5.511
20 µg/mL	7670	62150	-7.877	1.327e-008	8.103
40 µg/mL	8771	62554	-7.533	2.933e-008	7.132



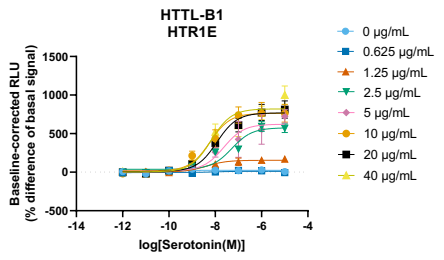
	Bottom	Top	LogEC50	EC50	Fold
0 µg/mL	7932	4999	-8.654	2.220e-009	0.6302
0.625 µg/mL	14308	8113	-11.32	4.753e-012	0.5670
1.25 µg/mL	Unstable	8667	-14.70	2.018e-015	Unstable
2.5 µg/mL	3805	7091	-6.549	2.823e-007	1.864
5 µg/mL	7902	32342	-6.836	1.459e-007	4.093
10 µg/mL	41916	196455	-5.919	1.204e-006	4.687
20 µg/mL	34368	240977	-6.011	9.759e-007	7.012
40 µg/mL	13889	52423	-6.314	4.858e-007	3.775



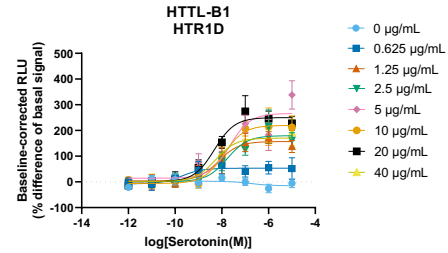
	Bottom	Top	LogEC50	EC50	Fold
0 µg/mL	10526	7651	-9.625	2.372e-010	0.7269
0.625 µg/mL	27685	21659	-8.288	5.153e-009	0.7824
1.25 µg/mL	39278	24763	-6.994	1.013e-007	0.6304
2.5 µg/mL	47610	77195	-6.639	2.296e-007	1.621
5 µg/mL	43509	85730	-6.861	1.377e-007	1.970
10 µg/mL	73621	201615	-6.703	1.981e-007	2.739
20 µg/mL	184987	772697	-6.080	8.317e-007	4.177
40 µg/mL	84915	234172	-6.442	3.618e-007	2.758



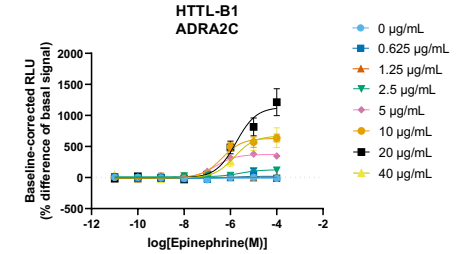
	Bottom	Top	LogEC50	EC50	Fold
0 µg/mL	3751	3589	-6.884	1.306e-007	0.9568
0.625 µg/mL	4719	3600	-9.819	1.517e-010	0.7628
1.25 µg/mL	6308	7905	-5.687	2.058e-006	1.253
2.5 µg/mL	7173	24970	-5.620	2.399e-006	3.481
5 µg/mL	5025	35898	-5.964	1.086e-006	7.144
10 µg/mL	7606	56052	-5.551	2.812e-006	7.369
20 µg/mL	18598	454358	-4.920	1.202e-005	24.43
40 µg/mL	16150	266805	-4.853	1.403e-005	16.52



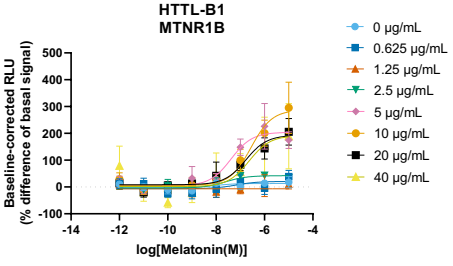
	Bottom	Top	LogEC50	EC50	Fold
0 µg/mL	7406	9973	-10.18	6.643e-011	1.347
0.625 µg/mL	7844	9049	-8.025	9.436e-009	1.154
1.25 µg/mL	10081	25205	-8.388	4.096e-009	2.500
2.5 µg/mL	13011	64133	-7.287	5.166e-008	4.929
5 µg/mL	8895	54261	-7.638	2.304e-008	6.100
10 µg/mL	8506	63935	-8.231	5.882e-009	7.516
20 µg/mL	7509	59656	-7.929	1.179e-008	7.945
40 µg/mL	7305	64479	-8.175	6.688e-009	8.827



	Bottom	Top	LogEC50	EC50	Fold
0 µg/mL	14469	11953	-6.767	1.711e-007	0.8261
0.625 µg/mL	6138	10188	-9.711	1.945e-010	1.660
1.25 µg/mL	9874	24466	-8.138	7.274e-009	2.478
2.5 µg/mL	10954	30320	-7.769	1.703e-008	2.768
5 µg/mL	7250	23236	-7.659	2.194e-008	3.205
10 µg/mL	6493	22094	-7.921	1.199e-008	3.403
20 µg/mL	6953	24274	-8.319	4.799e-009	3.491
40 µg/mL	5664	15120	-8.269	5.383e-009	2.669



	Bottom	Top	LogEC50	EC50	Fold
0 µg/mL	15171	12949	-9.163	6.870e-010	0.8535
0.625 µg/mL	13018	17343	-5.935	1.161e-006	1.332
1.25 µg/mL	Unstable	10624	-15.19	6.461e-016	Unstable
2.5 µg/mL	14894	29975	-5.573	2.674e-006	2.013
5 µg/mL	8398	41840	-6.635	2.320e-007	4.982
10 µg/mL	11114	88819	-6.363	4.337e-007	7.992
20 µg/mL	20020	258605	-5.754	1.761e-006	12.92
40 µg/mL	13450	110965	-5.826	1.491e-006	8.250



	Bottom	Top	LogEC50	EC50	Fold
0 µg/mL	16586	19320	-8.094	8.045e-009	1.165
0.625 µg/mL	18421	23707	-7.185	6.530e-008	1.287
1.25 µg/mL	28214	Unstable	7.958	90711713	Unstable
2.5 µg/mL	17685	26145	-7.610	2.453e-008	1.478
5 µg/mL	14218	41319	-7.384	4.129e-008	2.906
10 µg/mL	25759	94106	-6.560	2.752e-007	3.653
20 µg/mL	26864	72574	-6.773	1.686e-007	2.702
40 µg/mL	20240	58094	-6.641	2.283e-007	2.870

Figure S3. Changes in baseline signals and fold windows due to cumate induction. HTTL-B1, HTTL-B2 and HTTL-F cells were plated in increasing concentrations of cumate (0, 0.625, 1.25, 2.5, 5, 10, 20, and 40 µg/mL), which was maintained throughout the entire experiment, and were transfected with select GPCRs. Transfected cells were stimulated with the receptor specific agonist and dose-response curves were built using XY analysis for non-linear regression curve and the 3-parameters dose-response stimulation function, followed by baseline correction. All error bars represent SEM (n = 3, with three technical replicates from one biological sample). Generic receptor codes refer to the GPCR-Tango constructs.

		pEC50				Emax (in % of vehicle response for EMTA & % difference from baseline for Tango-Trio)			
		βArrestin1 /GRK2 EMTA	βArrestin1 Tango-Trio	βArrestin2 /GRK2 EMTA	β-Arrestin2 Tango-Trio	βArrestin1 /GRK2 EMTA	βArrestin1 Tango-Trio	βArrestin2 /GRK2 EMTA	β-Arrestin2 Tango-Trio
Receptor (Gene Name)	**Ligand**								
HTR1B	Serotonin	7.6	6.96	7.685	6.49	36.35	490	57.78	393.4
HTR1D	Serotonin		7.66		6.89		-14.78		347.9
HTR2A	Serotonin	6.709	8.22	7.028	5.76	518.1	24.81	778.6	29.24
HTR2B	Serotonin	7.809	6.5	8.168	11.31	171.6	80.54	247.2	-26.67
HTR2C	Serotonin	7.995	8.76	8.456	9.33	1564	-28.38	705	14.12
AGTR1	Angiotensin II	8.64	6.62	8.84	6.41	207.8	289.2	220.6	7802
CCKAR	Cholecystokinin	8.329	unstable	8.721	7.11	1374	unstable	1520	332.9
PTGER1	PGE2		6.95		unstable		71.02		unstable
PTGER2	PGE2	6.515	6.38	6.925	5.95	107.8	400.8	100.5	363
PTGER3	PGE2		8.53		6.324		-24.13		19.44
PTGER4	PGE2	8.921	8.62	9.25	8.35	251.5	326.9	146.8	1192
EDNRA	Endothelin-1	8.232	8.9	8.381	8.7	588.1	-8.86	1581	-2.98
GHSR	Ghrelin	8.249	6.81	8.515	6.14	68.41	217.8	169.6	396.8
GNRHR	GnRH		10.32		7.43		-31.52		70.49
GPBAR1	Litocholic acid		unstable		10.41		Unstable		-34.66
LPAR1	O-LPA	7.528	11.93	7.464	11.21	289.4	-90.04	232	-32.09
LPAR2	O-LPA	6.773	6.13	7.078	11.33	715.2	-41.64	938.9	-64.29
MC4R	α-MSH		7.01	7.449	7.28		346.3	76.44	1351
OPRM1	DAMGO	7.099	9.19	7.424	6.7	1008	-35.21	1198	142.9
MTNR1A	Melatonin	8.937	8.03	8.75	9.11	78.64	754.1	157.5	425.5
MTNR1B	Melatonin		7.12		8.38		290		168
OPRL1	Nociceptin	8.249	6.15	8.716	6.17	147.5	73.78	238.1	-50.64
OXTR	Oxytocin	8.091	8.78	8.6	8.67	128.3	-13.08	229.8	82.31
HCRTR2	Orexin-A	8.471	6.9	8.558	6.58	852.9	315.7	863.9	3850
P2RY2	UTP	5.235	unstable	5.36	7.29	184.7	unstable	94.68	24.6
F2R	TFLLR-NH2	5.082	unstable	5.177	-2.55	445.5	unstable	469.5	unstable
S1PR1	Sphingosine 1-phosphate	7.269	6.47	7.459	6.5	477	233.6	557.1	182.8
SSTR2	Somatostatin	8.815	5.66	8.918	6.83	2867	866	1820	1714
AVPR1A	AVP	7.743	10.25	7.824	9.08	890.1	40.7	934.7	40.67
AVP2R	AVP	7.603	7.52	7.846	7.03	353.3	258.6	334.2	1804
VIPR1	VIP	9.109	6.92	9.254	unstable	1367	28.97	1060	unstable
NPY1R	NPY		8.2	8.815	6.28		53.76	42.09	106.1
NPY5R	NPY		7.92		7.07		210.3		1046

Table S1. Comparison of the pharmacological parameters extracted from EMTA and Tango-Trio.
Absolute pEC50 and Emax values of β-arrestin-1/2 activity at GPCRs stimulated with common ligands were extracted from EMTA[1] and Tango-Trio studies. Tango-Trio pEC50 data was extracted from the non-linear least-squares regression analysis using the sigmoidal dose-response function (3-parameters modeled using Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X))) and Emax values from the baseline correction as percentage difference using 100* (Value-Baseline)/Baseline), both provided in GraphPad Prism 9.0. (n = 3, with three technical replicates from one biological sample). Generic receptor codes refer to the GPCR-Tango constructs.

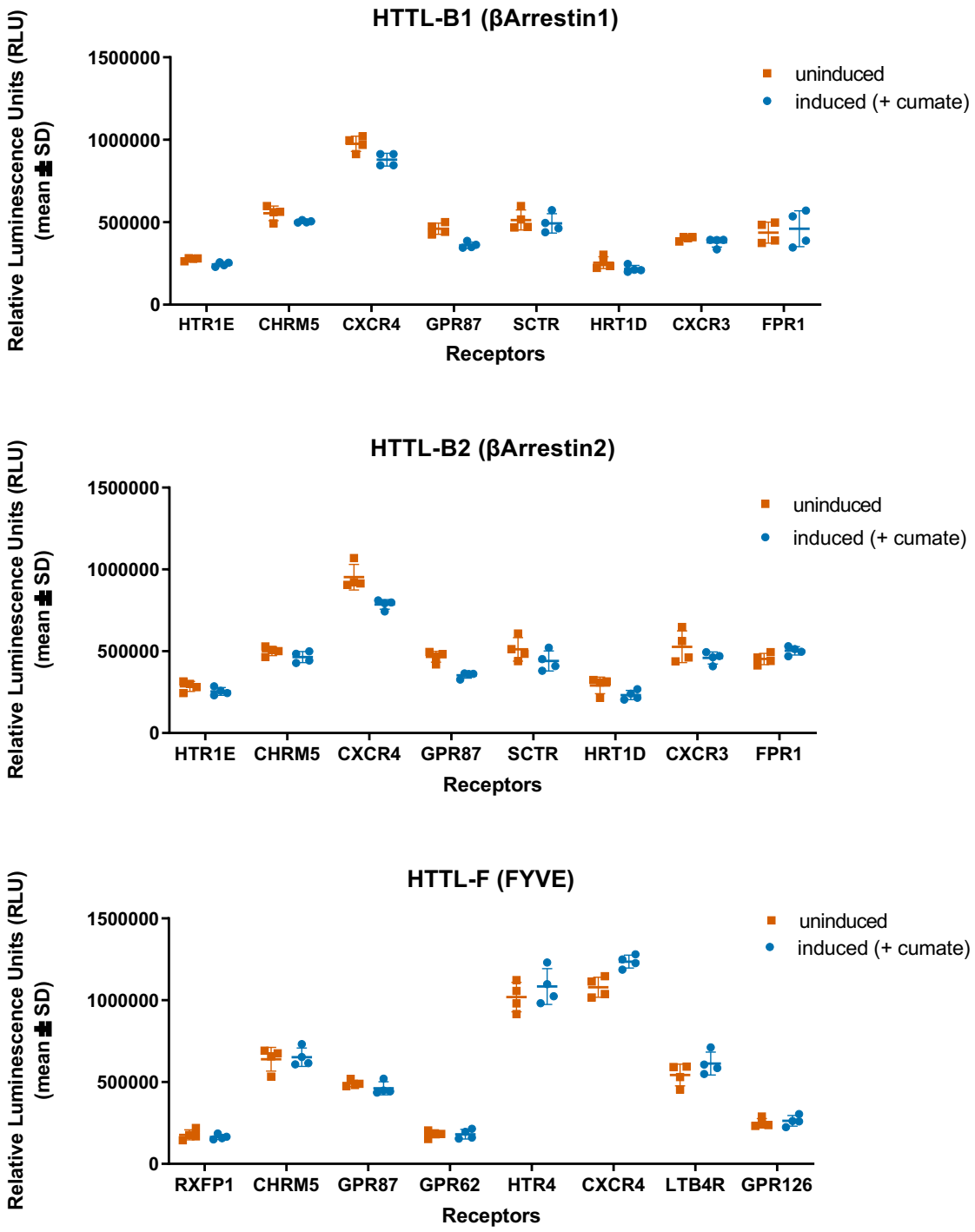
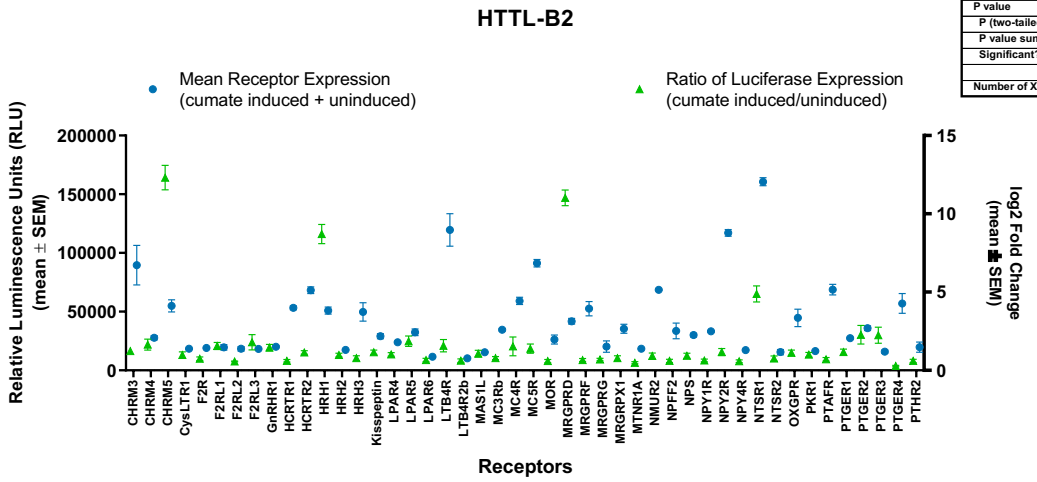


Figure S4. Receptor surface expression following cumate induction of fusion protein expression HTTL-B1, HTTL-B2 and HTTL-F were plated in the presence or absence of cumate (30 µg/mL) and were transfected with a select number of positive GPCR hits from the constitutive HTS. ELISA experiments were subsequently carried out on transfected cells to determine receptor surface expression. All error bars represent SD (n = 4, with four technical replicates from one biological sample). Generic receptor codes refer to the GPCR-Tango constructs.

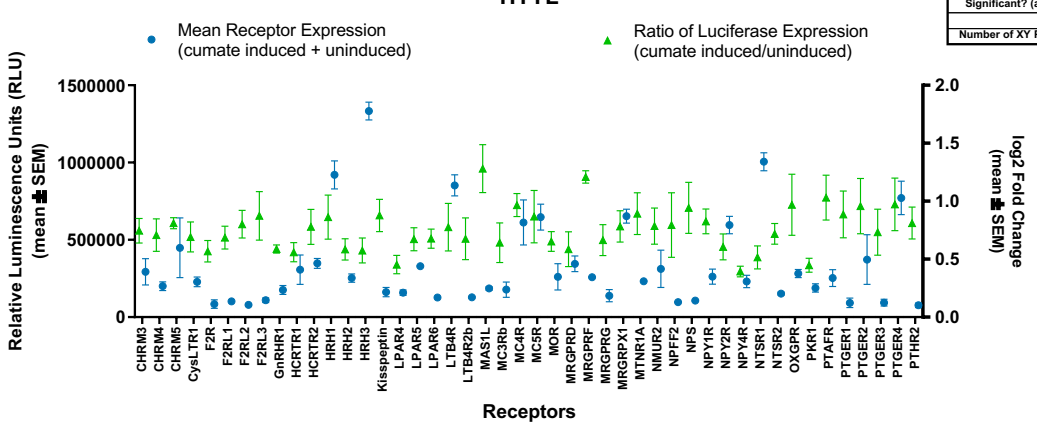
A

Pearson r	
r	0.2084
95% confidence interval	-0.08714 to 0.4703
R squared	0.04344
P value	
P (two-tailed)	0.1645
P value summary	ns
Significant? (alpha = 0.05)	No
Number of XY Pairs	46

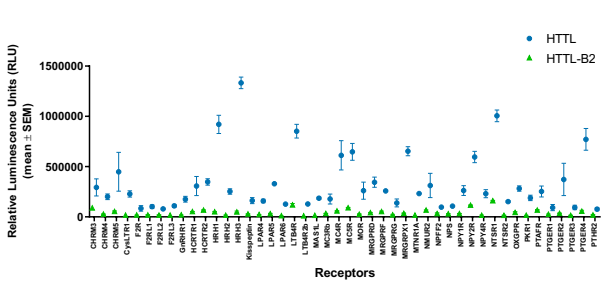


B

Pearson r	
r	-0.03125
95% confidence interval	-0.3187 to 0.2614
R squared	0.0009767
P value	
P (two-tailed)	0.8366
P value summary	ns
Significant? (alpha = 0.05)	No
Number of XY Pairs	46

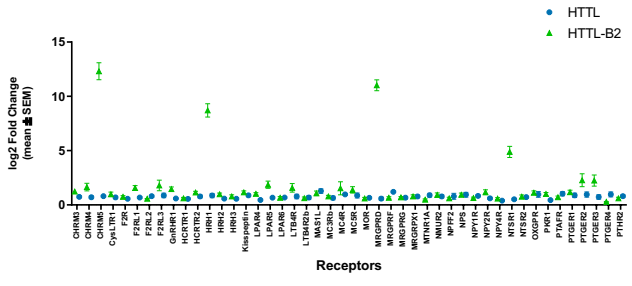


C



Pearson r	
r	0.6659
95% confidence interval	0.4656 to 0.8013
R squared	0.4434
P value	
P (two-tailed)	<0.0001
P value summary	****
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	46

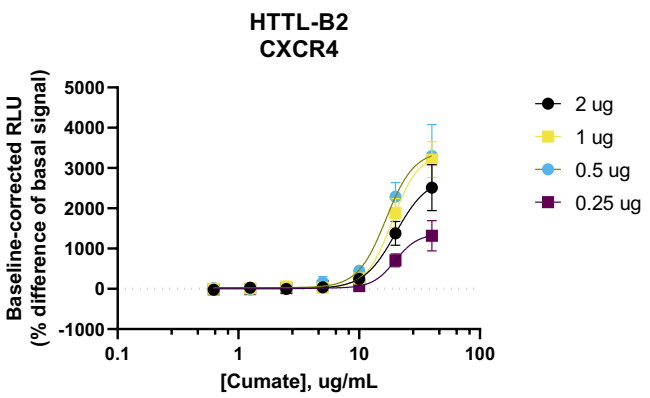
D



Pearson r	
r	-0.04866
95% confidence interval	-0.3342 to 0.2451
R squared	0.002368
P value	
P (two-tailed)	0.7481
P value summary	ns
Significant? (alpha = 0.05)	No
Number of XY Pairs	46

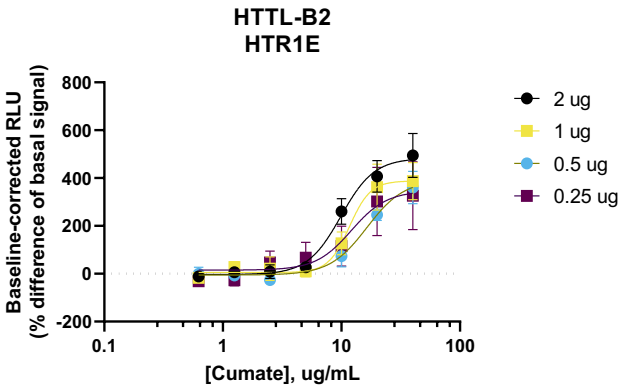
Figure S5. Comparison of receptor expression and constitutive activity across panel of GPCRs. HTTL-B2 (A) and HTTL (B) were plated in the presence or absence of cumate (30 μg/mL) and were transfected with a panel of GPCRs with a varying range of constitutive activities. ELISA experiments were subsequently carried out on transfected cells to determine receptor surface expression, and log2 fold changes in constitutive arrestin recruitment were calculated between the wells in the absence or presence of cumate. Mean receptor expression (C) and log2 fold changes in constitutive arrestin recruitment (D) were also compared between the HTTL and HTTL-B2 cell lines. Pearson correlation coefficients (r) and corresponding p values were computed between data sets using GraphPad Prism. All error bars represent SEM (n = 4, with four technical replicates from one biological sample). Generic receptor codes refer to the GPCR-Tango constructs.

A



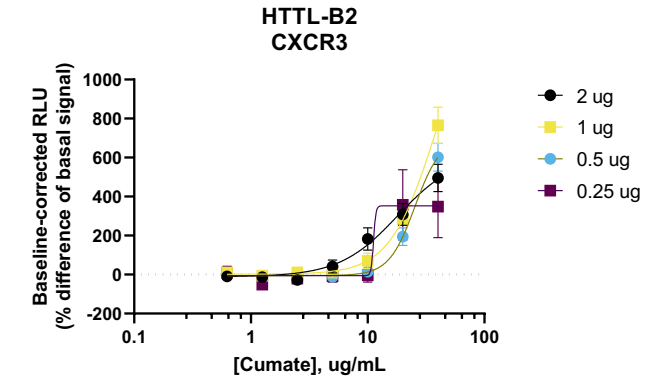
	EC50	logEC50	Span	Fold
2 ug	20.05	1.302	137867	28.29
1 ug	18.88	1.276	816291	30.91
0.5 ug	16.67	1.222	360203	27.49
0.25 ug	19.82	1.297	50063	12.56

B



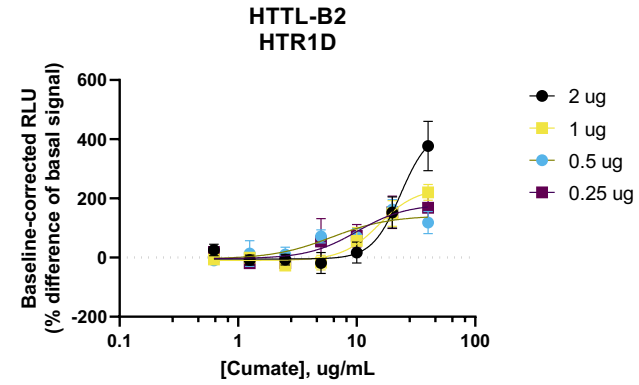
	EC50	logEC50	Span	Fold
2 ug	9.896	0.9955	22407	6.026
1 ug	11.68	1.067	51472	4.646
0.5 ug	16.41	1.215	43555	5.200
0.25 ug	12.12	1.084	18167	3.877

C



	EC50	logEC50	Span	Fold
2 ug	18.09	1.257	28108	8.086
1 ug	37.73	1.577	669417	15.09
0.5 ug	25.45	1.406	376911	8.477
0.25 ug	11.23	1.051	25814	4.811

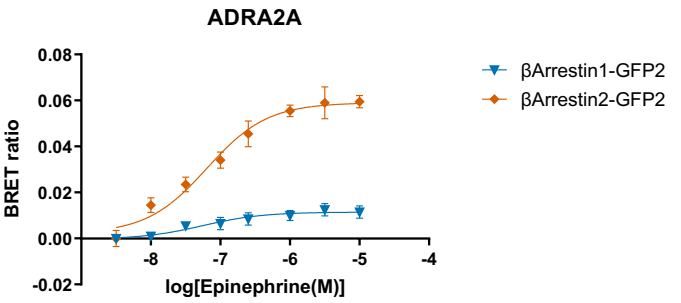
D



	EC50	logEC50	Span	Fold
2 ug	23.47	1.370	259717	5.594
1 ug	15.51	1.190	47183	3.706
0.5 ug	6.125	0.7871	24546	2.548
0.25 ug	10.10	1.004	31572	2.920

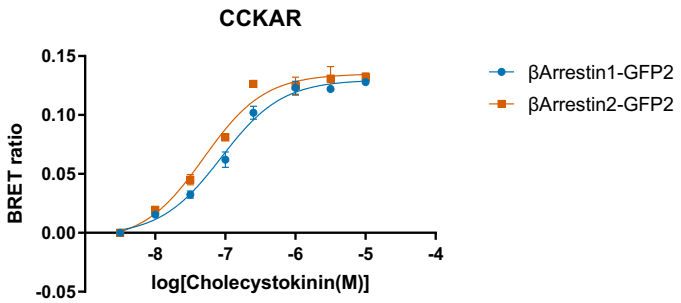
Figure S6. Titration of GPCR Tango DNA and consequent effects on constitutive activity. HTTL-B2 cells were seeded in 6-well plates and transfected with various amount of CXCR4 (A), HTR1E (B), CXCR3 (C), and HTR1D (D)Tango DNA, ranging from 0.25 ug – 2 ug total per well. Transfected cells were stimulated with cumate, and dose- response curves were built using XY analysis for non-linear regression curve and the 4-parameters dose-response stimulation function, followed by baseline correction. All error bars represent SEM (n = 3, with three technical replicates from one biological sample). Generic receptor codes refer to the GPCR-Tango constructs.

A



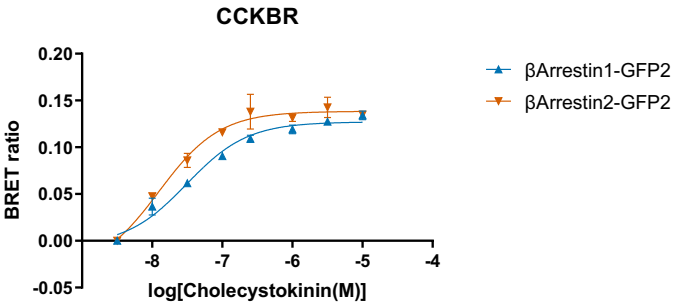
	EC50
βArrestin1-GFP2	5.536e-008
βArrestin2-GFP2	6.425e-008

B



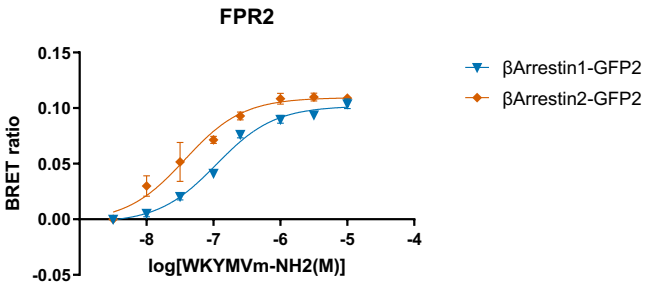
	EC50
βArrestin1-GFP2	8.723e-008
βArrestin2-GFP2	4.926e-008

C



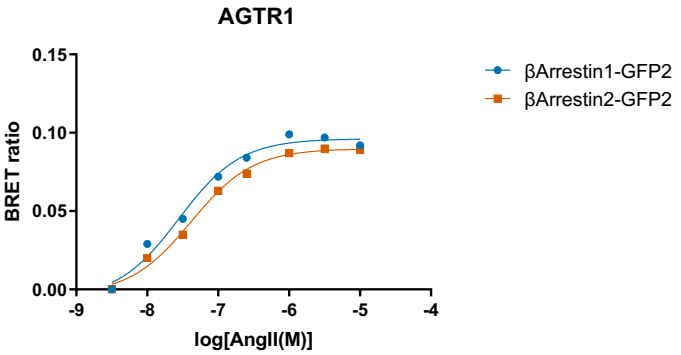
	EC50
βArrestin1-GFP2	3.112e-008
βArrestin2-GFP2	1.248e-008

D



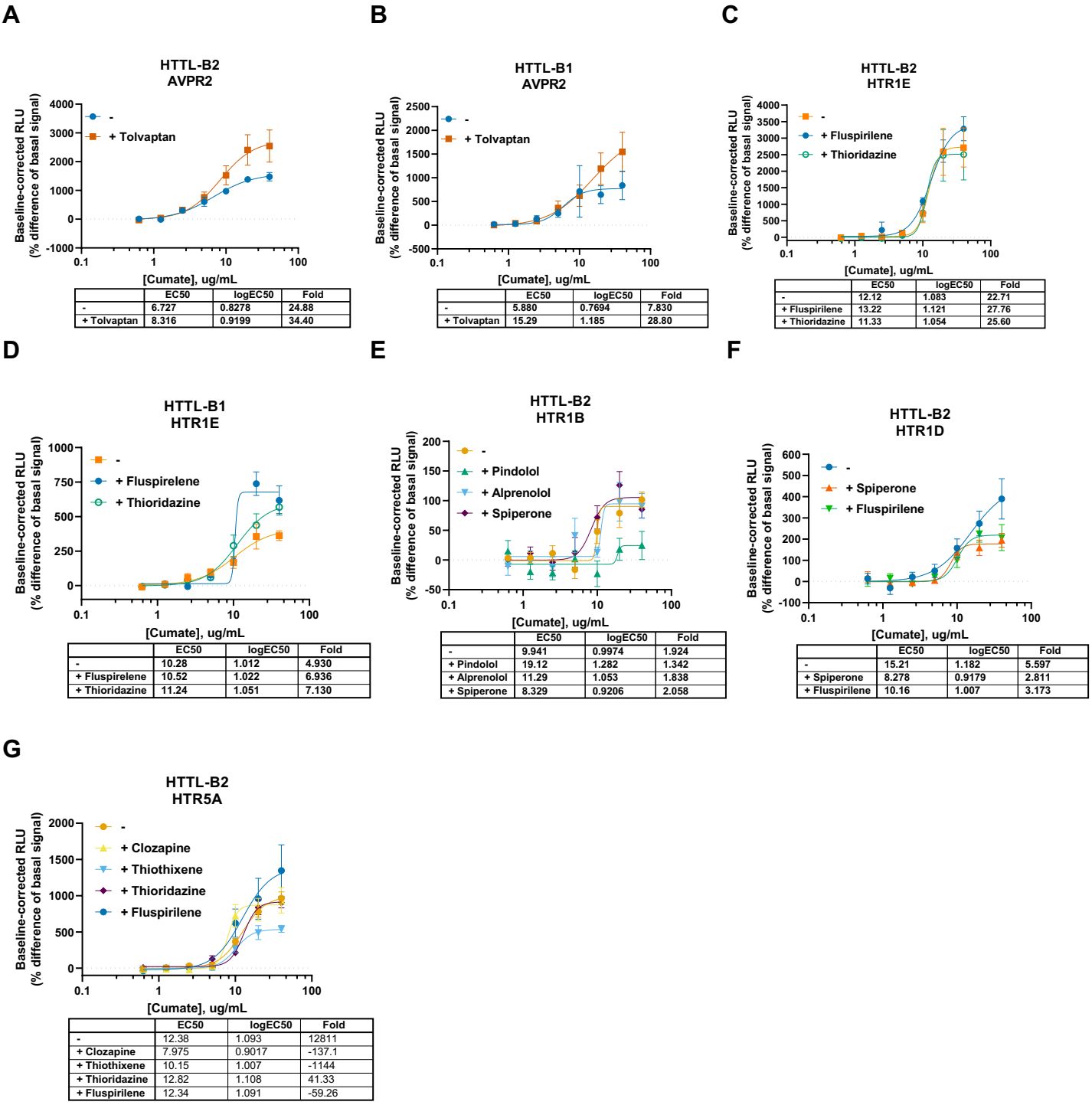
	EC50
βArrestin1-GFP2	1.104e-007
βArrestin2-GFP2	3.734e-008

E



	EC50
βArrestin1-GFP2	2.787e-008
βArrestin2-GFP2	4.163e-008

Figure S7. Orthogonal determination of arrestin isoform selectivity using BRET2. HEK293T cells were transfected with β-arrestin1/2-GFP2, as well as the following GPCRs: ADRA2A (A), CCKAR (B), CCKBR (C), FPR2 (D), AGTR1 (E). Following transfection, cells were stimulated with serial dilutions of selective agonist, and read with 405 nm (RLuc8-Coelenterazine 400a) and 500 nm (GFP2) emission filters. Dose-response curves were built using XY analysis for non-linear regression curve and the 3-parameters dose-response stimulation function, followed by baseline correction. All error bars represent SEM (n = 2, with two technical replicates from one biological sample). Generic receptor codes refer to the GPCR-Rluc8 constructs.



	EC50	logEC50	Fold
-	6.727	0.8278	24.88
+ Tolvaptan	8.316	0.9199	34.40

	EC50	logEC50	Fold
-	5.880	0.7694	7.830
+ Tolvaptan	15.29	1.185	28.80

	EC50	logEC50	Fold
-	12.12	1.083	22.71
+ Fluspirilene	13.22	1.121	27.76
+ Thioridazine	11.33	1.054	25.60

	EC50	logEC50	Fold
-	10.28	1.012	4.930
+ Fluspirelene	10.52	1.022	6.936
+ Thioridazine	11.24	1.051	7.130

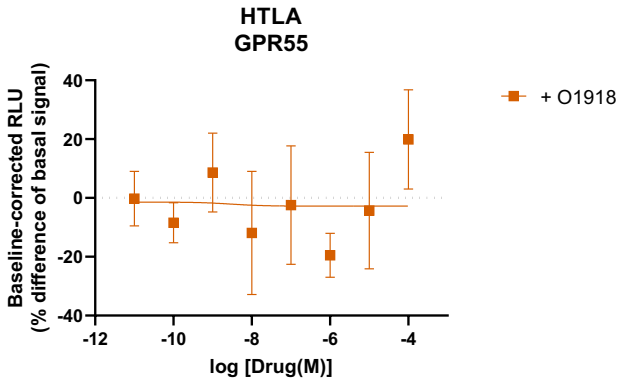
	EC50	logEC50	Fold
-	9.941	0.9974	1.924
+ Pindolol	19.12	1.282	1.342
+ Alprenolol	11.29	1.053	1.838
+ Spiperone	8.329	0.9206	2.058

	EC50	logEC50	Fold
-	15.21	1.182	5.597
+ Spiperone	8.278	0.9179	2.811
+ Fluspirilene	10.16	1.007	3.173

	EC50	logEC50	Fold
-	12.38	1.093	12811
+ Clozapine	7.975	0.9017	-137.1
+ Thiothixene	10.15	1.007	-1144
+ Thioridazine	12.82	1.108	41.33
+ Fluspirilene	12.34	1.091	-59.26

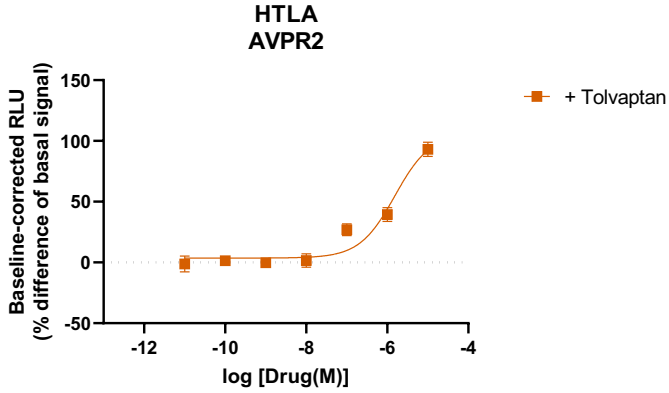
Fig S8. Applications and further investigations into basal activities revealed by Tango-Trio. HTTL-B1 and HTTL-B2 were transfected with GPCRs exhibiting strong basal arrestin recruitment. Transfected cells were stimulated as cumate dose-response in the presence or absence of the following inverse agonists/antagonists at saturating (EC80) concentrations: Tolvaptan at AVPR2 (A-B), Fluspirelene and Thioridazine at HTR1E (C-D), Pindolol, Alprenolol and Spiperone at HTR1B (E), Spiperone and Fluspirelene at HTR1D (F), and Clozapine, Thiothexene, Thioridazine and Fluspirelene at HTR5A (G). Transfected cells were stimulated as a cumate dose-response, and stimulation curves were built using XY analysis for non-linear regression curve and the 4-parameters dose-response stimulation function, followed by baseline correction. All error bars represent SEM. Data are representative of 2 biological replicates, with 3 technical replicates each. Generic receptor codes refer to the GPCR-Tango constructs.

A



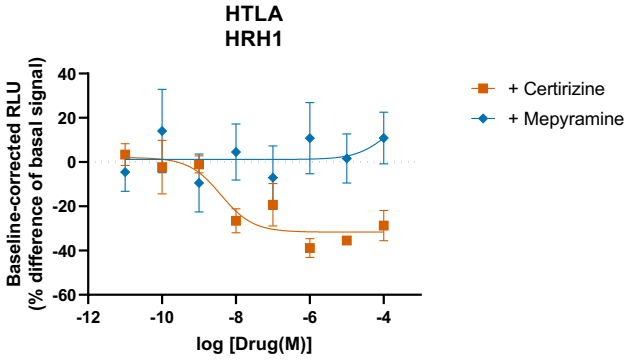
	+ O1918
LogEC50	-8.518
EC50	3.037e-009
Fold	0.9859

B



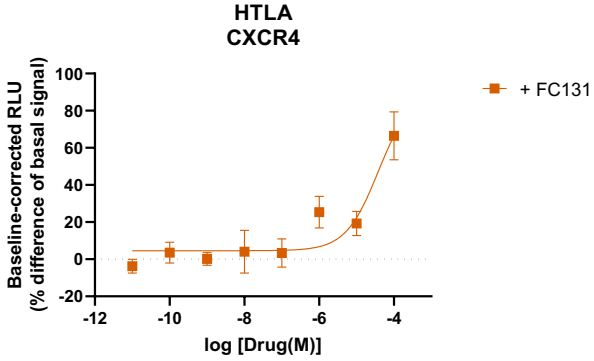
	+ Tolvaptan
LogEC50	-5.814
EC50	1.534e-006
Fold	1.987

C



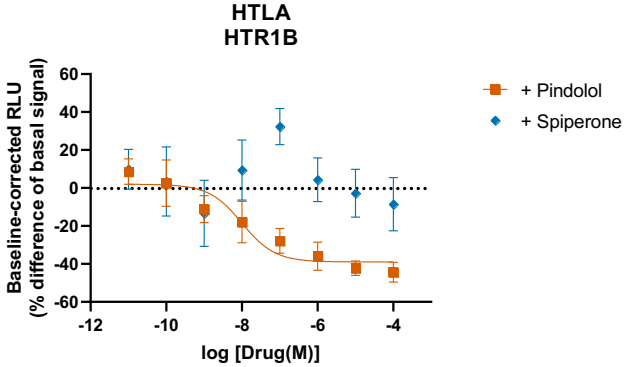
	+ Certirizine	+ Mepyramine
LogEC50	-8.407	-3.866
EC50	3.913e-009	0.0001361
Fold	0.6694	1.226

D



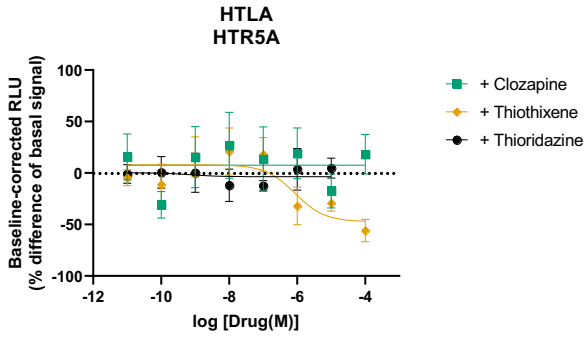
	+ FC131
LogEC50	-4.424
EC50	3.768e-005
Fold	1.812

E



	+ Pindolol	+ Spiperone
LogEC50	-7.998	-8.318
EC50	1.004e-008	4.805e-009
Fold	0.5985	1.047

F



	+ Clozapine	+ Thiothixene	+ Thioridazine
LogEC50	Unstable	-6.067	-9.133
EC50	Unstable	8.580e-007	7.369e-010
Fold	Unstable	0.4876	0.9616

Figure S9. Orthogonal characterization of select inverse agonists and antagonists using PRESTO-Tango. HTLA cells were transfected with GPCRs exhibiting strong constitutive arrestin recruitment from the primary Tango-Trio screen, serving to validate the findings shown in Figure 7. Transfected cells were stimulated with a dose-response curve using the following inverse agonists/antagonists: O-1918 at GPR55 (A), Tolvaptan at AVPR2 (B), Cetirizine and Mepyramine at HRH1 (C), FC131 at CXCR4 (D), Pindolol and Spiperone at HTR1B (E), and Clozapine, Thiothexene, and Thioridazine at HTR5A (F). Stimulation curves were built using XY analysis for non-linear regression curve and the 3-parameters dose-response stimulation function, followed by baseline correction. All error bars represent SEM (Figs S8A, C-F: n = 3, with three technical replicates from one biological sample; Fig S8B: n = 6, with three technical replicates from two biological samples). Generic receptor codes refer to the GPCR-Tango constructs.

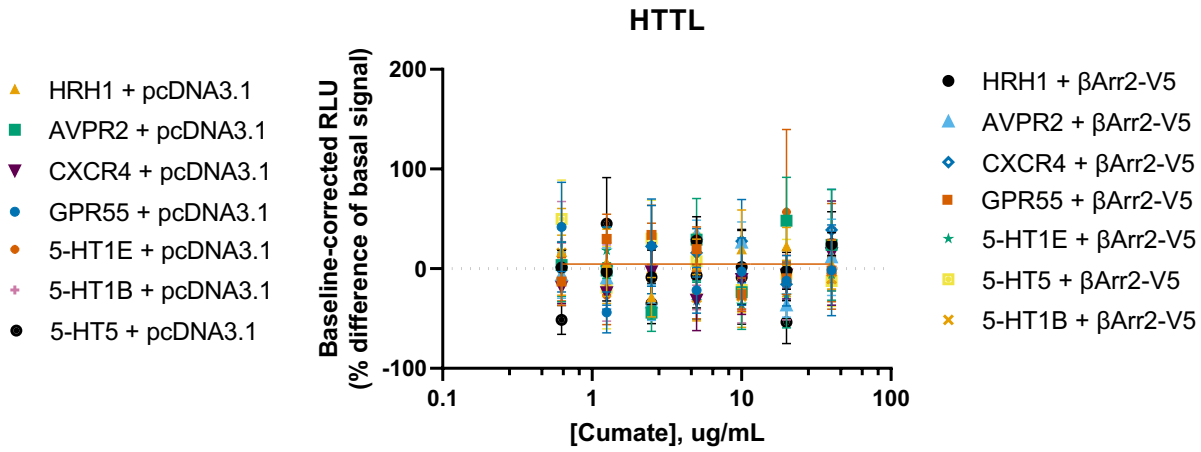
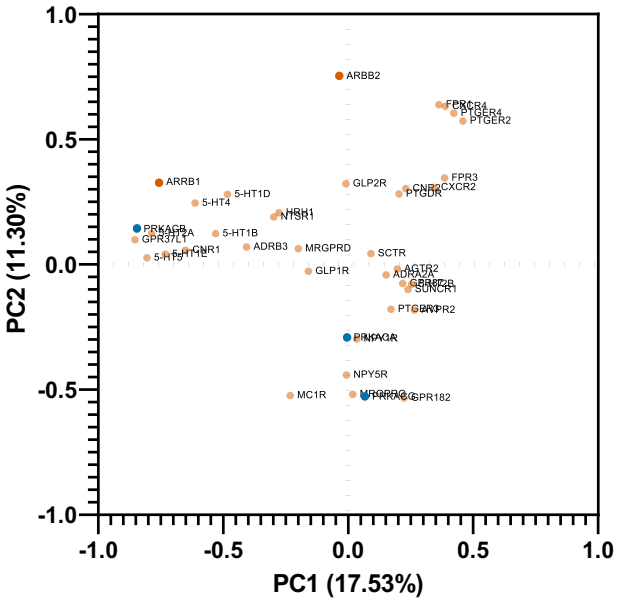
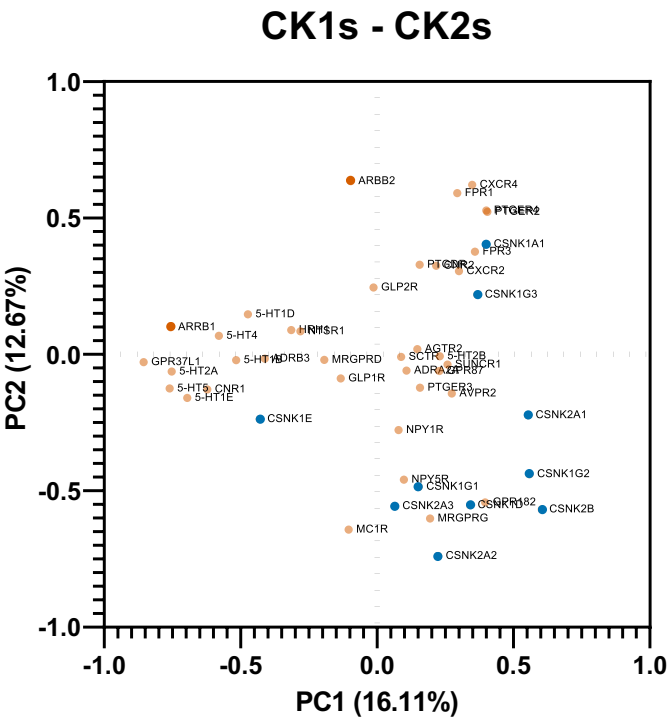


Figure S10. Expression of GPCR-Tango constructs in the absence of TEV protease. HTTL cells were co-transfected with select GPCR constructs (tested with antagonists/inverse agonists in Fig 7 and Fig S8), and either βArr2 (not tagged to TEV protease) or pcDNA3.1 as a control. Transfected cells were stimulated as a cumate dose-response, and stimulation curves were built using XY analysis for non-linear regression curve and the 4-parameters dose-response stimulation function, followed by baseline correction. All error bars represent SEM (n = 3, with three technical replicates from one biological sample), and generic receptor codes refer to the GPCR-Tango constructs.

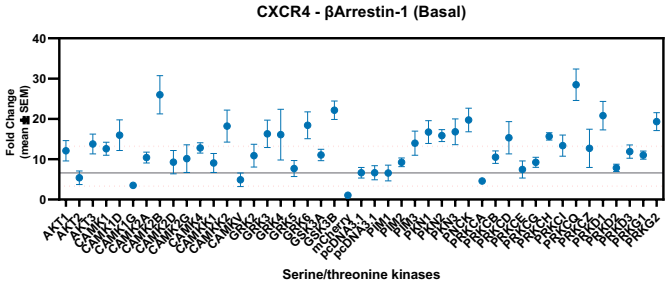
A


PKAs
PC2 (11.30%)
PC1 (17.53%)
1.0
0.5
0.0
-0.5
-1.0
ARBB2
ARRB1
5-HT4
5-HT1D
GLP2R
FPR3
PTGER2
CXCR2
PTGDR
MRGPRD
SCTR
GLP1R
AGTR2
NPY5R
MC1R

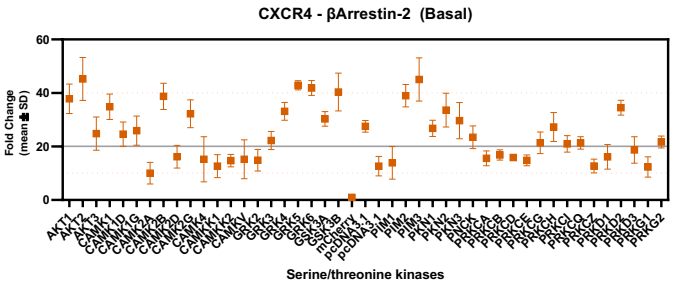
B


CK1s - CK2s
PC2 (12.67%)
PC1 (16.11%)
1.0
0.5
0.0
-0.5
-1.0
ARBB2
CXCR4
CSNK1A1
FPR3
CXCR2
GLP2R
CSNK1G3
ARRB1
5-HT1D
5-HT4
GPR37L1
5-HT2A
MRGPRD
SCTR
GLP1R
PTGER3
AVPR2
CSNK1E
CSNK2A1
NPY1R
CSNK1G2
CSNK1G1
CSNK2A3
CSNK2B
MRGPRG
MC1R
CSNK2A2

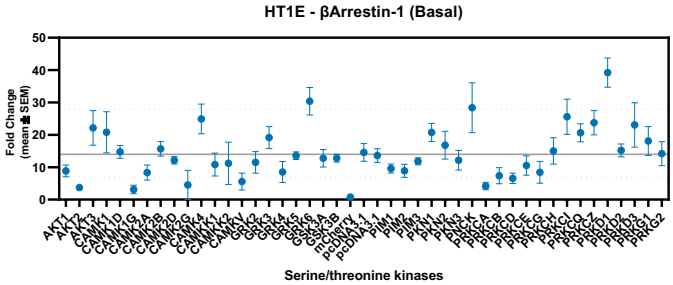
C


CXCR4 - βArrestin-1 (Basal)
Fold Change (mean ± SEM)
Serine/threonine kinases

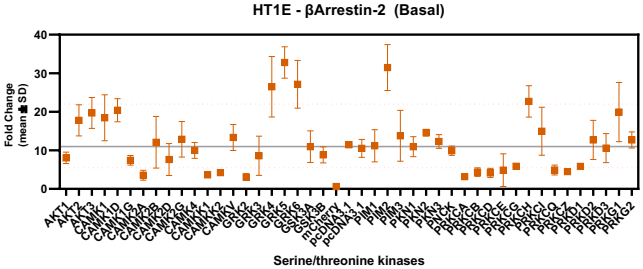
D


CXCR4 - βArrestin-2 (Basal)
Fold Change (mean ± SD)
Serine/threonine kinases

E


HT1E - βArrestin-1 (Basal)
Fold Change (mean ± SEM)
Serine/threonine kinases

F


HT1E - βArrestin-2 (Basal)
Fold Change (mean ± SD)
Serine/threonine kinases

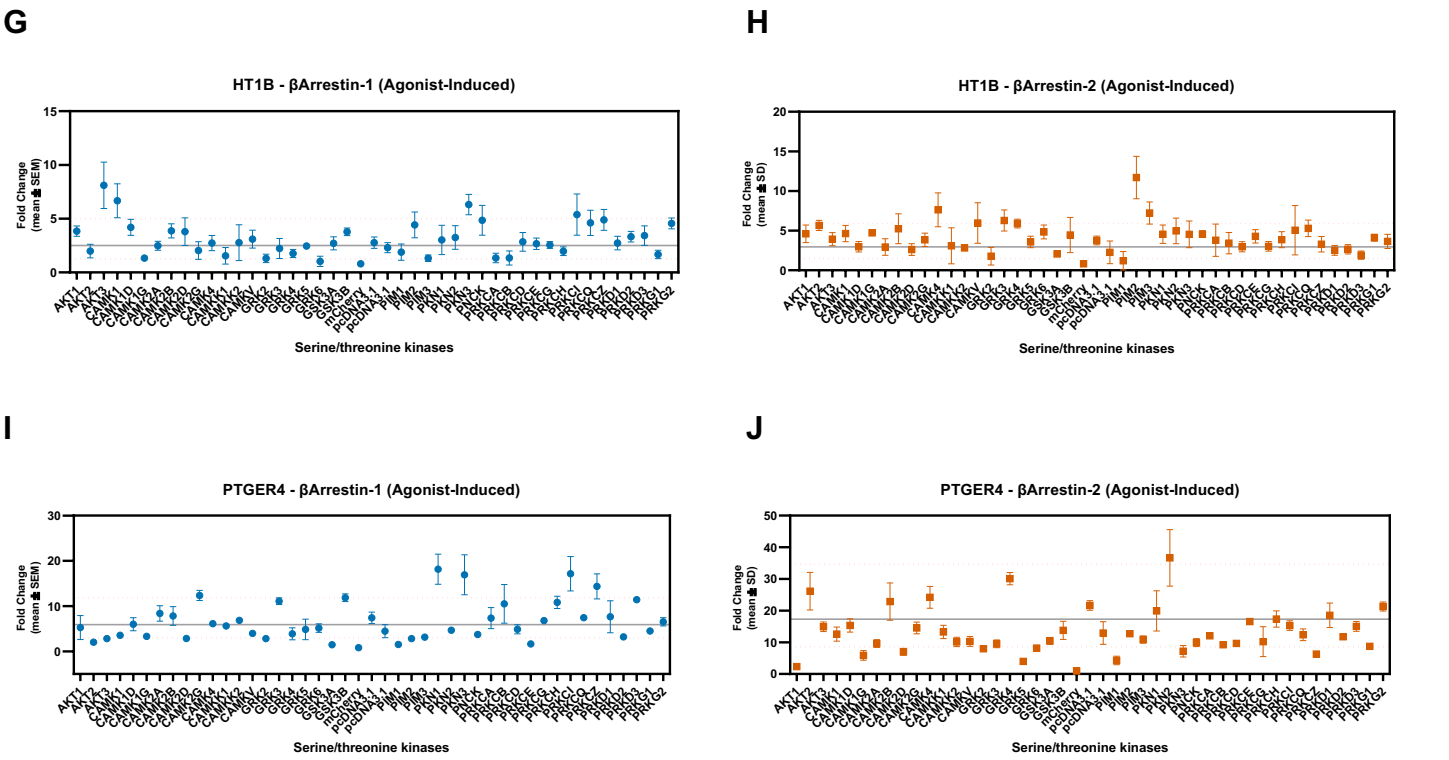


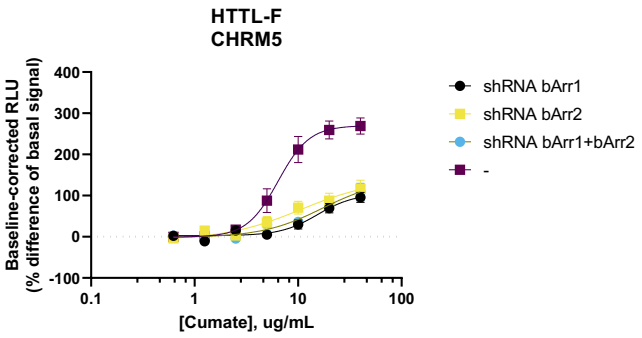
Figure S11. Investigations into the interplay between serine/threonine (ST) kinases and β-arrestin-1/-2 activity. Human Protein Atlas (HPA) RNA consensus tissue gene data (version 21.0 and Ensembl version 103.38.) summarizing the expression levels in 55 tissues was extracted for β-arrestin-1 and -2 (ARRB1 and ARRB2), select receptors with high constitutive selectivity for one arrestin isoform over the other (GPR182, AGTR2, ADRA2A, GPR37L1, SCTR, ADRB3, PTGER4, SUNCR1, PTGER3, MRGPRG, NPY5R, NPY1R, GLP1R, FPR1, MC1R, FPR3, 5-HT5, MRGPRD, GPR87, CXCR4, HRH1, AVPR2, 5-HT4, 5-HT2A, NTSR1, GLP2R, 5-HT1D, CXCR2, 5-HT1B, 5-HT1E, PTGER2, 5-HT2B, PTGDR), and either PKA (A) or CK1s and CK2s (B). The data was analyzed using principal component analysis; β-arrestin-1 and -2 are denoted with red, ST kinases with blue, and GPCRs with orange symbols.

Besides the *in silico* approach, the effect of ST kinases on basal β-arrestin activity were assessed by plating HTTL-B1 and HTTL-B2 cells in alternating rows with or without cumate (30 µg/mL). Three select GPCRs, including CXCR4 (C, D) and HT1E (E, F) were co-transfected with a panel of approximately 50 ST kinases, and pcDNA3.1 as positive controls. Similarly, the effect on agonist-induced activity was determined by co-transfecting the ST kinase panel with three select GPCRs, including HT1B (G, H) and PTGER4 (I, J), followed by receptor-specific agonist stimulation of serotonin and prostaglandin E2, respectively. Fold changes in arrestin activity were calculated between the wells in the absence/presence of cumate or agonist, and hit cut-offs were set as the double/half of the mean arrestin recruitment from the pcDNA3.1 controls. Fold changes are the means calculated from quadruplicate conditions, generated from one screen (n = 4, 4 technical replicates from one biological sample). Generic receptor codes refer to the GPCR-Tango constructs.

Position		Residue	Kinase	Peptide	Score	Cutoff
TM3	143	S	AGC/GRK/GRK/GRK4	IVHLCAISLDRYWSI	14.576	12.794
TM3/ICL2	149	S	AGC	ISLDRYWSITQAIEY	1.983	1.925
TM3/ICL2	149	S	CAMK/CAMK2	ISLDRYWSITQAIEY	6.965	6.8
TM3/ICL2	149	S	AGC/Akt/AKT2	ISLDRYWSITQAIEY	27.652	24.834
TM3/ICL2	149	S	CAMK/PIM/PIM1	ISLDRYWSITQAIEY	0.001	0.001
TM3/ICL2	149	S	CAMK/PKD/PRKD1	ISLDRYWSITQAIEY	6.896	6.813
TM3/ICL2	149	S	AGC/PKC/PKCi/PRKCZ	ISLDRYWSITQAIEY	43.877	41.793
TM3/ICL2	151	T	CAMK/PIM/PIM1	LDRYWSITQAIEYNL	0.001	0.001
ICL3	261	T	AGC/PKC/PKCa/PRKCG	VAAPPGGTERRPNGL	139.391	116.631
ICL3	273	S	AGC/GRK/GRK	NGLGPERSAGPGGAE	15.317	13.902
ICL3	273	S	AGC/GRK/BARK/GRK3	NGLGPERSAGPGGAE	14.32	13.381
ICL3	303	T	CAMK/PIM	APAGPRDTDALDLEE	15.858	15.765
ICL3	311	S	Other/CAMKK/CAMKK-Meta/CAMKK1	DALDLEESSSSDHAE	1.798	1.714
ICL3	312	S	AGC/GRK/GRK/GRK4	ALDLEESSSSDHAER	13.396	12.794
ICL3	313	S	AGC/GRK/BARK	LDLEESSSSDHAERP	168.686	165.626
ICL3	313	S	AGC/PKC/PKCi/PRKCZ	LDLEESSSSDHAERP	42.001	41.793
ICL3	314	S	AGC/GRK/BARK	DLEESSSSDHAERPP	182.515	165.626
ICL3	314	S	AGC/PKC/PKCi	DLEESSSSDHAERPP	3.754	3.394
ICL3	314	S	CAMK/PIM/PIM2	DLEESSSSDHAERPP	6.428	6.373
ICL3	314	S	AGC/GRK/BARK/GRK2	DLEESSSSDHAERPP	159.313	147.674
ICL3	339	S	AGC/PKG	GKGKARASQVKPGDS	11.051	9.739
ICL3	339	S	AGC/PKG/PRKG1	GKGKARASQVKPGDS	12.154	10.046
ICL3	339	S	AGC/GRK/GRK/GRK4	GKGKARASQVKPGDS	12.836	12.794
ICL3	346	S	AGC/PKN	SQVKPGDSLPRRGPG	120.087	109.869
ICL3	346	S	AGC/PKN/PKN1	SQVKPGDSLPRRGPG	84.393	75.607
ICL3	355	T	AGC/GRK/GRK	PRRGPGATGIGTPAA	14.575	13.902
ICL3	355	T	CAMK/PIM/PIM1	PRRGPGATGIGTPAA	0.001	0.001
ICL3	359	T	Other/CAMKK/CAMKK-Meta/CAMKK2	PGATGIGTPAAGPGE	21.838	20.956
ICL3	375	S	CAMK/CAMK1/CAMK1	RVGAAKASRWRGRQN	41.256	40.948

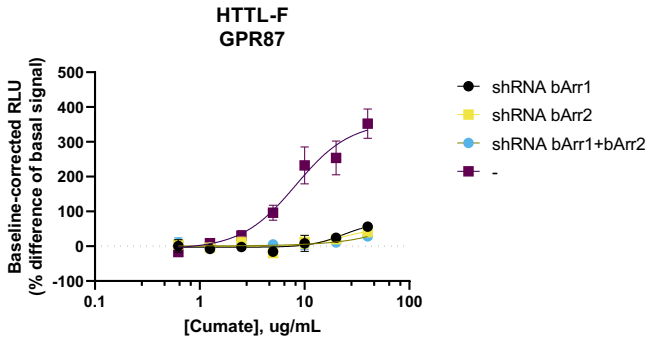
Table S2. Prediction of kinase-specific phosphorylation sites in human ADRA2A receptor using GPS5.0 algorithm. Multiple ST kinases, including AKT, GSK3s, PIMs, PKGs, PKCs, PKNs, PKDs, CAMKs, and GRKs were selected in the GPS5.0 software, and the human ADRA2A sequence was inputted for identification of potential phosphorylation sites. The results are presented in a tabular format, including the position and residue, implicated kinase, predicted scores and pre-defined cut-off values under a high prediction threshold.

A



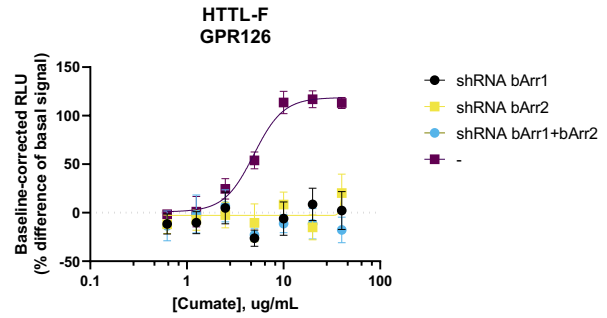
	EC50	logEC50	Span	Fold
shRNA bArr1	15.38	1.187	459303	1.993
shRNA bArr2	10.60	1.025	669083	2.622
shRNA bArr1+bArr2	17.20	1.236	564353	2.461
-	6.405	0.8065	139806	3.725

B



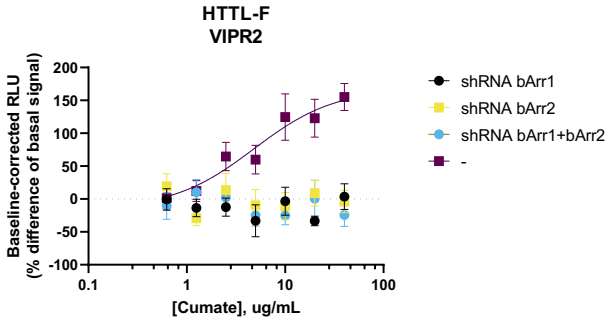
	EC50	logEC50	Span	Fold
shRNA bArr1	23.39	1.369	30849	1.744
shRNA bArr2	21.66	1.336	29759	1.513
shRNA bArr1+bArr2	557592	5.746	Unstable	Unstable
-	8.221	0.9149	48577	4.983

C



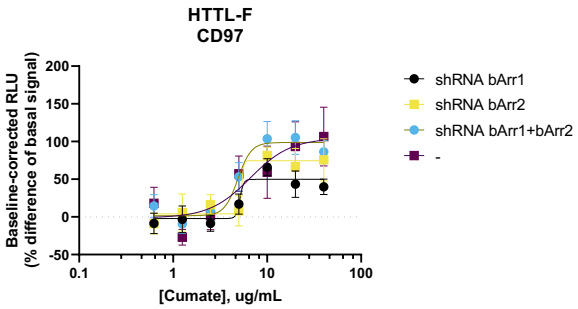
	EC50	logEC50	Span	Fold
shRNA bArr1	3.069	0.4871	30035	1.651
shRNA bArr2	42.20	1.625	Unstable	Unstable
shRNA bArr1+bArr2	3.419	0.5339	21766	1.486
-	4.921	0.6920	38098	2.169

D



	EC50	logEC50	Span	Fold
shRNA bArr1	8.957	0.9522	63180	1.713
shRNA bArr2	4.508	0.6540	46960	1.685
shRNA bArr1+bArr2	3.386	0.5297	43671	1.472
-	4.821	0.6831	57025	3.172

E



	EC50	logEC50	Span	Fold
shRNA bArr1	5.126	0.7098	59654	1.532
shRNA bArr2	5.352	0.7285	71885	1.678
shRNA bArr1+bArr2	4.878	0.6883	94061	1.956
-	6.606	0.8200	67253	2.072

F

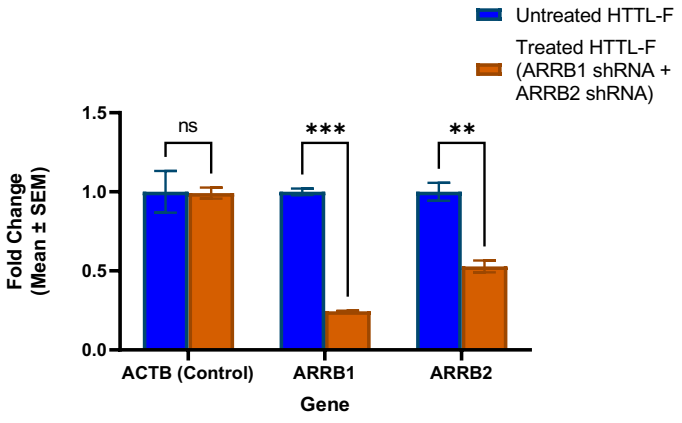


Figure S12. Internalization of GPCRs in HTTL-F following β-arrestin-1 and β-arrestin-2 knockdown. Lentiviral β-arrestin-1 and -2 shRNA plasmids were transfected in HEK293T cells, along with psPAX2 and VSV-G vectors. The medium was replaced the following day with complete fresh medium, and lentiviral shRNA medium was collected following 48 hours transfection. For the knockdown experiment, HTTL-F cells were seeded in either complete medium or in the previously prepared lentiviral β-arrestin-1 and -2 shRNA medium (combined at a 1:1 ratio), with infection of cells facilitated with polybrene at 8 μg/mL. HTTL-F cells were transfected with GPCRs demonstrating high constitutive internalization: CHRM5 (A), GPR87 (B), GPR126 (C), VIPR2 (D), and CD97 (E). Transfected cells were stimulated as a cumate dose-response, and stimulation curves were built using XY analysis for non-linear regression curve and the 4-parameters dose-response stimulation function, followed by baseline correction. All error bars represent SEM (n = 3, with three technical replicates from one biological sample), and generic receptor codes refer to the GPCR-Tango constructs. qPCR was performed on untreated and infected HTTL-F cells to confirm sufficient knockdown of β-arrestin-1 and -2 (F). Fold change of gene expression was assessed with multiple unpaired t test using the FDR method of Benjamini & Yekutieli (n = 2, with two technical replicates from one biological sample).