Lists of Supplemental Materials

Fig. S7. β-arrestin 1 recruitment levels by G-protein-biased (TAN67), β-arrestin-biased (SNC80), and non-biased (Leu-Enk) δOR agonist in U2OS-δOR-βArr1 cells

Fig. S8. A diagram respresenting the pharmacological competition between two biased agonists and an endnogenous opioid in relations to their ability to modulate ERK1/2 signaling

Supplemental Materials

Table S1. Statistical analysis of anxiety-like behavior upon systemic administration of SNC80 in WT and β-arrestin 2 KO mice

Statistical differences of anxiety-like behaviors in WT or β-arrestin 2 KO mice shown in **Fig. 1**. Significance between groups was calculated by two-way ANOVA followed by a Sidak's multiple comparison (*p<0.05, and ns=not significant).

Subfigure	Behavior test	Genotype	Drug	# of samples	Test	Source of Variation	F-value	p-value	Post hoc analysis	Group Comparison	Mean Diff.	p-value	Significance	-
Figure 1-d	Elevated plus maze test	WT & βarr2 KO	SNC80 (20 mg/kg, s.c.)	WT-Control: 15 WT-SNC80: 15 B2-Control: 21 B2-SNC80: 21	Two-Way ANOVA test	Interaction Genotype factor Drug factor	F(1,68) = 1.429 F(1,68) = 3.15 F(1,68) = 8.781	0.236 0.0804 0.0042	Sidak's Multiple Comparison Test	WT: Con vs. SNC80	-13.0400	0.0164	*	
										B2 KO: Con vs. SNC80	-5.5450	0.3200	ns	
Figure 1-e	Dark-light box test	WT & βarr2 KO	SNC80 (20 mg/kg, s.c.)	WT-Control: 12 WT-SNC80: 11 B2-Control: 20 B2-SNC80: 20	Two-Way ANOVA test	Interaction Genotype factor Drug factor	F(1,59) = 3.677 F(1,59) = 1.039 F(1,59) = 4.978	0.06 0.3122 0.0295	Sidak's Multiple Comparison Test	WT: Con vs. SNC80	-12.5200	0.0232	*	
										B2 KO: Con vs. SNC80	-0.9467	0.9584	ns	
Figure 1-f	Elevated plus maze test (Total movement - min)	WT & βarr2 KO	SNC80 (20 mg/kg, s.c.)	WT-Control: 15 WT-SNC80: 15 B2-Control: 21 B2-SNC80: 21	Two-Way ANOVA test	Interaction Genotype factor Drug factor	F(1,68) = 0.3392 F(1,68) = 20.7 F(1,68) = 0.796	0.5622 <0.0001 0.3754	Sidak's Multiple Comparison Test	WT: Con vs. SNC80	7.9330	0.5615	ns	
										B2 KO: Con vs. SNC80	1.6670	0.9643	ns	
Figure 1-g	Dark-light box test (Total transition)	WT & βarr2 KO	SNC80 (20 mg/kg, s.c.)	WT-Control: 12 WT-SNC80: 11 B2-Control: 20 B2-SNC80: 18	Two-Way ANOVA test	Interaction Genotype factor Drug factor	F(1,57) = 1.754 F(1,57) = 0.1222 F(1,57) = 0.03687	0.1907 0.728 0.8484	Sidak's Multiple Comparison Test	WT: Con vs. SNC80	-2.6140	0.7254	ns	
										B2 KO: Con vs. SNC80	3.5000	0.3949	ns	
Figure S1-a	Dark-light box test (Total distance -cm)	WT & βarr2 KO	SNC80 (20 mg/kg, s.c.)	WT-Control: 12 WT-SNC80: 11 B2-Control: 20 B2-SNC80: 20	Two-Way ANOVA test	Interaction Genotype factor Drug factor	F (1, 59) = 1.949 F (1, 59) = 15.40 F (1, 59) = 19.78	P=0.1679 P=0.0002 P<0.0001	Sidak's Multiple Comparison Test	WT: Con vs. SNC80	-390.8000	0.0011	**	
										B2 KO: Con vs. SNC80	-204.1000	0.0283	*	

Subfigure	Brain region	Genotype	Drug	# of samples	Test	F-value	p-value	Group	Mean	Post hoc analysis	Group Comparison	Mean Diff.	p-value	Significance
Figure S1-b	Dar-light box test (Total distance - cm)	WT	SNC80 (20 mg/kg, i.p) or SL327 (90 mg/kg s.c.)	Con: 19 SNC80: 10 SNC+SL: 12 SL327: 12	One-Way ANOVA test	F (3, 49) = 9.037	P<0.0001	Control SNC80 SNC+SL327 SL327	811.8 1090 663.2 705.8	Tukey's Multiple Comparison Test	Control vs. SNC80	-278.1000	0.0067	**
											Control vs. SNC+SL327	148.6000	0.2258	ns
											Control vs. SL327	106.1000	0.5155	ns
											SNC80 vs. SNC+SL327	426.7000	<0.0001	****
											SNC80 vs. SL327	384.2000	0.0004	***
											SNC+SL327 vs. SL327	-42.5000	0.9585	ns

Table S2. Statistical analysis of ERK1/2 expression levels upon time-series administration of SNC80 in WT and β-arrestin 2 KO mouse brain Statistical differences of ERK1/2 expression levels in WT mice shown in **Fig. 2** and β-arrestin 2 KO mice in **Fig. 3**. Significance between groups was calculated by one-way ANOVA followed by a Tukey's multiple comparison (*p<0.05, **p<0.01, and ns=not significant).

Subfigure	Brain region	Genotype	Drug	# of samples	Test	F-value	p-value	Group	Mean	Post hoc analysis	Group Comparison	Mean Diff.	p-value	Significance
Figure 2-e	Dorsal Striatum	WT	SNC80 (20 mg/kg, ip)	Con: 13 10 min: 13 30 min: 5	One-Way ANOVA test	F (2,28) = 6.776	P=0.0040	Con 10 min 30 min	1 1.472 0.8466	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.4718 0.1534 0.6253	0.0128 0.7398 0.0140	* ns *
Figure 2-f	Nucleus Acumbens	WT	SNC80 (20 mg/kg, ip)	Con: 13 10 min: 13 30 min: 6	One-Way ANOVA test	F (2,29) = 6.645	P=0.0042	Con 10 min 30 min	1 1.363 0.793	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.3632 0.2070 0.5702	0.0317 0.4563 0.0064	* ns **
Figure 2-g	Dorsal Hippocampus	WT	SNC80 (20 mg/kg, ip)	Con: 13 10 min: 12 30 min: 6	One-Way ANOVA test	F (2,28) = 8.252	P=0.0015	Con 10 min 30 min	1 1.819 0.8082	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.8194 0.1918 1.0110	0.0051 0.7923 0.0056	** ns **
Figure 2-h	Amygdala	WT	SNC80 (20 mg/kg, ip)	Con: 13 10 min: 13 30 min: 6	One-Way ANOVA test	F (2,29) = 10.82	P=0.0003	Con 10 min 30 min	1 1.526 0.806	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.5264 0.1940 0.7204	0.0024 0.5284 0.0010	** ns **
Figure 2-i	Ventral Hippocampus	WT	SNC80 (20 mg/kg, ip)	Con: 11 10 min: 10 30 min: 3	One-Way ANOVA test	F (2,21) = 2.82	P=0.0823	Con 10 min 30 min	1 1.138 0.6108	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.1379 0.3892 0.5271	0.6248 0.2039 0.0676	ns ns ns
Figure 3-c	Dorsal Striatum	βarr2 KO	SNC80 (20 mg/kg, ip)	Con: 8 10 min: 7 30 min: 8	One-Way ANOVA test	F (2, 20) = 1.873	P=0.1220	Con 10 min 30 min	1 1.765 1.186	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.7647 -0.1857 0.5790	0.1805 0.8887 0.3608	ns ns ns
Figure 3-d	Nucleus Acumbens	βarr2 KO	SNC80 (20 mg/kg, ip)	Con: 7 10 min: 7 30 min: 7	One-Way ANOVA test	F (2, 18) = 4.903	P=0.0200	Con 10 min 30 min	1 1.891 1.141	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.8913 -0.1407 0.7506	0.0240 0.8907 0.0608	* ns ns
Figure 3-e	Dorsal Hippocampus	βarr2 KO	SNC80 (20 mg/kg, ip)	Con: 8 10 min: 7 30 min: 8	One-Way ANOVA test	F (2, 20) = 0.8178	P=0.4556	Con 10 min 30 min	1 1.165 0.9976	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.1654 0.0024 0.1678	0.5183 0.9998 0.5086	ns ns ns
Figure 3-f	Amygdala	βarr2 KO	SNC80 (20 mg/kg, ip)	Con: 8 10 min: 8 30 min: 8	One-Way ANOVA test	F (2, 21) = 1.472	P=0.2522	Con 10 min 30 min	1 1.236 0.8942	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.2360 0.1058 0.3418	0.4909 0.8531 0.2376	ns ns ns
Figure 3-g	Ventral Hippocampus	βarr2 KO	SNC80 (20 mg/kg, ip)	Con: 8 10 min: 7 30 min: 8	One-Way ANOVA test	F (2, 20) = 2.035	P=0.1569	Con 10 min 30 min	1 1.291 0.9902	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.2905 0.0098 0.3003	0.2167 0.9980 0.1968	ns ns ns

Table S3. Statistical analysis of anxiety-like behavior and ERK1/2 expression levels upon administration of SNC80 in the presence/absence of SL327 in WT mouse brain Statistical differences of anxiety-like behavior and ERK1/2 expression levels in WT mouse brain shown in **Fig. 4** and **Fig. S3**. Significance between groups was calculated by one-way ANOVA followed by a Tukey's multiple comparison (*p<0.05, **p<0.01, ***p<0.001, and ns=not significant).

Subfigure	Behavior test or Brain region	Genotype	Drug	# of samples	Test	F-value	p-value	Group	Mean	Post hoc analysis	Group Comparison	Mean Diff.	p-value	Significance
Figure 4-b	Dar-light box test	WT	SNC80 (20 mg/kg, i.p.) or SL327 (50 mg/kg s.c.)	Con: 7 SNC80: 9 SNC+SL: 11 SL327: 11	One-Way ANOVA test	F (3, 34) = 12.35	P<0.0001	Control SNC80 SNC+SL327 SL327	13.88 30.53 3.418 15.64	Tukey's Multiple Comparison Test	Control vs. SNC80 Control vs. SNC+SL327 Control vs. SL327 SNC80 vs. SNC+SL327 SNC80 vs. SL327 SNC+SL327 vs. SL327	-16.6400 10.4600 -1.7550 27.1100 14.8900 -12.2200	0.0109 0.1496 0.9830 <0.0001 0.0106 0.0325	* ns ns **** * *
Figure 4-c	Dorsal Hippocampus	WT	SNC80 (20 mg/kg, i.p.) or SL327 (50 mg/kg s.c.)	Con: 10 SNC80: 9 SNC+SL: 9 SL327: 9	One-Way ANOVA test	F (3, 33) = 11.68	P<0.0001	Control SNC80 SNC+SL327 SL327	1 1.706 0.9214 0.7122	Tukey's Multiple Comparison Test	Control vs. SNC80 Control vs. SNC+SL327 Control vs. SL327 SNC80 vs. SNC+SL327 SNC80 vs. SL327 SNC+SL327 vs. SL327	-0.6767 0.1076 0.3169 0.7843 0.9936 0.2092	0.0024 0.9247 0.2790 0.0006 <0.0001 0.6459	** ns ns *** **** ns
Figure 4-d	Amygdala	WT	SNC80 (20 mg/kg, i.p.) or SL327 (50 mg/kg s.c.)	Con: 10 SNC80: 10 SNC+SL: 10 SL327: 9	One-Way ANOVA test	F (3, 35) = 10.9	P<0.0001	Control SNC80 SNC+SL327 SL327	1 2.003 1.133 0.5908	Tukey's Multiple Comparison Test	Control vs. SNC80 Control vs. SNC+SL327 Control vs. SL327 SNC80 vs. SNC+SL327 SNC80 vs. SL327 SNC+SL327 vs. SL327	-0.9798 -0.1093 0.4325 0.8705 1.470 0.5419	0.0021 0.9713 0.3442 0.0069 <0.0001 0.1677	** ns ns ** **** ns
Figure S3-a	Dorsal Striatum	WT	SNC80 (20 mg/kg, i.p.) or SL327 (50 mg/kg s.c.)	Con: 9 SNC80: 9 SNC+SL: 9 SL327: 9	One-Way ANOVA test	F (3, 32) = 6.421	P=0.0016	Control SNC80 SNC+SL327 SL327	1 1.543 1.115 0.7734	Tukey's Multiple Comparison Test	Control vs. SNC80 Control vs. SNC+SL327 Control vs. SL327 SNC80 vs. SNC+SL327 SNC80 vs. SL327 SNC+SL327 vs. SL327	-0.5429 -0.1155 0.2266 0.4274 0.7694 0.3420	0.0247 0.9880 0.5960 0.1032 0.0009 0.2490	* ns ns ns *** ns
Figure S3-b	Nucleus Accumbens	WT	SNC80 (20 mg/kg, i.p.) or SL327 (50 mg/kg s.c.)	Con: 10 SNC80: 10 SNC+SL: 10 SL327: 9	One-Way ANOVA test	F (3, 35) = 2.672	P=0.0624	Control SNC80 SNC+SL327 SL327	1 1.319 1.04 0.7877	Tukey's Multiple Comparison Test	Control vs. SNC80 Control vs. SNC+SL327 Control vs. SL327 SNC80 vs. SNC+SL327 SNC80 vs. SL327 SNC+SL327 vs. SL327	-0.3193 -0.0404 0.2123 0.2790 0.5316 0.2526	0.3248 0.9963 0.6809 0.4427 0.0394 0.5501	ns ns ns ns * ns
Figure S3-c	Ventral Hippocampus	WT	SNC80 (20 mg/kg, i.p.) or SL327 (50 mg/kg s.c.)	Con: 10 SNC80: 9 SNC+SL: 10 SL327: 9	One-Way ANOVA test	F (3, 34) = 2.734	P=0.0588	Control SNC80 SNC+SL327 SL327	1 1.164 0.7771 0.8315	Tukey's Multiple Comparison Test	Control vs. SNC80 Control vs. SNC+SL327 Control vs. SL327 SNC80 vs. SNC+SL327 SNC80 vs. SL327 SNC+SL327 vs. SL327	-0.1522 0.2347 0.1803 0.3869 0.3325 -0.0545	0.7419 0.3879 0.6299 0.0655 0.1549 0.9833	ns ns ns ns ns ns

Table S4. Statistical analysis of fear-related behavior upon systemic administration of SNC80 or TAN67 in WT and β-arrestin 2 KO mice Statistical differences of fear-related behaviors in WT or β-arrestin 2 KO mice shown in **Fig. 5**. Significance between groups was calculated by two-way ANOVA followed by a Bonferroni's multiple comparison (*p<0.05, ****p<0.0001, and ns=not significant).

Subfigure	Behavior test	Genotype	Drug	# of samples	Test	Source of Variation	F-value	p-value	Post hoc analysis	Group Comparison	Mean Diff.	p-value	Significance
Figure 5-c	Fear potentiated startle test (Raw startle)	WT	SNC80 (20 mg/kg, i.p.)	Control: 21 SNC80: 21	Two-Way ANOVA test	Interaction Stimulation factor Drug factor	F(2,120) = 20.42 F(2,120) = 92.00 F(1,120) = 63.99	<0.0001 <0.0001 <0.0001	Sidak's Multiple Comparison Test	Blank: Con vs. SNC80 Noise: Con vs. SNC80 Noise+Light: Con vs. SNC80	-0.0126 0.5879 1.0310	0.9994 <0.0001 <0.0001	ns **** ****
Figure 5-d	Fear potentiated startle test (FPS testing)	WT	SNC80 (20 mg/kg, i.p.)	Control: 21 SNC80: 20	Unpaird t test			p=0.0085					
Figure 5-f	Fear potentiated startle test	βarr2 KO	SNC80 (20 mg/kg, i.p.)	Control: 8 SNC80: 8	Two-Way ANOVA test	Interaction Stimulation factor Drug factor	F(2,42) = 20.22 F(2,42) = 51.52 F(1,42) = 40.4	<0.0001 <0.0001 <0.0001	Sidak's Multiple Comparison Test	Blank: Con vs. SNC80 Noise: Con vs. SNC80 Noise+Light: Con vs. SNC80	-0.0355 0.2139 0.5812	>0.9999 0.0103 <0.0001	ns * ****
Figure 5-g	Fear potentiated startle test (FPS testing)	βarr2 KO	SNC80 (20 mg/kg, i.p.)	Control: 8 SNC80: 8	Unpaird t test			p=0.0003					
Figure 5-i	Fear potentiated startle test	WT	TAN67 (25 mg/kg, i.p.)	Control:8 TAN67: 8	Two-Way ANOVA test	Interaction Stimulation factor Drug factor	F(2,42) = 0.7245 F(2,42) = 25.06 F(1,42) = 0.2754	0.4905 <0.0001 0.6025	Sidak's Multiple Comparison Test	Blank: Con vs. SNC80 Noise: Con vs. SNC80 Noise+Light: Con vs. SNC80	-0.0113 0.1123 -0.3664	>0.9999 0.9736 0.5189	ns ns ns
Figure 5-j	Fear potentiated startle test (FPS testing)	WT	TAN67 (25 mg/kg, i.p.)	Control: 7 TAN67: 8	Unpaird t test			p<0.0001					

Table S5. Statistical analysis of ERK1/2 expression levels upon time-series administration of TAN67 in WT mousee and SNC80 in β-arrestin 1 KO mouse brain Statistical differences of ERK1/2 expression levels in WT and β-arrestin 1 KO mouse brain shown in Fig. 6. Significance between groups was calculated by one-way ANOVA followed by a Tukey's multiple comparison (*p<0.05, **p<0.01, and ns=not significant).

Subfigure	Brain region	Genotype	Drug	# of samples	Test	F-value	p-value	Group	Mean	Post hoc analysis	Group Comparison	Mean Diff.	p-value	Significance
Figure 6-a	Dorsal Striatum	WT	TAN67 (25 mg/kg, i.p)	Con: 7 10 min: 7 30 min: 7	One-Way ANOVA test	F (2, 18) = 5.276	P=0.0157	Con 10 min 30 min	1 0.8769 0.5824	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	0.1231 0.4176 0.2945	0.6279 0.0142 0.0931	ns * ns
Figure 6-b	Nucleus Acumbens	WT	TAN67 (25 mg/kg, i.p)	Con: 7 10 min: 7 30 min: 6	One-Way ANOVA test	F (2, 17) = 5.701	P=0.0127	Con 10 min 30 min	1 0.6152 0.5227	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	0.3848 0.4773 0.0925	0.0447 0.0161 0.8190	* * ns
Figure 6-c	Dorsal Hippocampus	WT	TAN67 (25 mg/kg, i.p)	Con: 7 10 min: 7 30 min: 7	One-Way ANOVA test	F (2, 18) = 5.09	P=0.0177	Con 10 min 30 min	1 1.021 0.739	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.0207 0.2610 0.2817	0.9799 0.0410 0.0266	ns * *
Figure 6-d	Amygdala	WT	TAN67 (25 mg/kg, i.p)	Con: 7 10 min: 6 30 min: 6	One-Way ANOVA test	F (2, 16) = 5.455	P=0.0156	Con 10 min 30 min	1 0.851 0.6697	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	0.1490 0.3303 0.1813	0.3216 0.0118 0.2189	ns * ns
Figure 6-e	Ventral Hippocampus	WT	TAN67 (25 mg/kg, i.p)	Con: 6 10 min: 6 30 min: 6	One-Way ANOVA test	F (2, 15) = 1.092	P=0.3607	Con 10 min 30 min	1 1.063 0.8009	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.0628 0.1991 0.2618	0.9388 0.5426 0.3582	ns ns ns
Figure 6-f	Dorsal Striatum	βarr1 KO	SNC80 (20 mg/kg, i.p)	Con: 7 10 min: 6 30 min: 6	One-Way ANOVA test	F (2,16) = 1.62	P=0.2288	Con 10 min 30 min	1 1.191 1.271	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.1907 -0.2710 -0.0803	0.4580 0.2226 0.8743	ns ns ns
Figure 6-g	Nucleus Acumbens	βarr1 KO	SNC80 (20 mg/kg, i.p)	Con: 7 10 min: 6 30 min: 6	One-Way ANOVA test	F (2,16) = 0.5181	P=0.6053	Con 10 min 30 min	1 1.179 1.232	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.1791 -0.2319 -0.0528	0.7426 0.6112 0.9759	ns ns ns
Figure 6-h	Dorsal Hippocampus	βarr1 KO	SNC80 (20 mg/kg, i.p)	Con: 7 10 min: 6 30 min: 6	One-Way ANOVA test	F (2,16) = 11.48	P=0.0008	Con 10 min 30 min	1 2.812 2.874	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-1.8120 -1.8740 -0.0625	0.0027 0.0020 0.9902	** ** ns
Figure 6-i	Amygdala	βarr1 KO	SNC80 (20 mg/kg, i.p)	Con: 7 10 min: 6 30 min: 6	One-Way ANOVA test	F (2,16) = 4.668	P=0.0253	Con 10 min 30 min	1 1.498 1.67	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-0.4981 -0.6695 -0.1714	0.1080 0.0261 0.7564	ns * ns
Figure 6-j	Ventral Hippocampus	βarr1 KO	SNC80 (20 mg/kg, i.p)	Con: 7 10 min: 6 30 min: 6	One-Way ANOVA test	F (2,16) = 5.097	P=0.0194	Con 10 min 30 min	1 2.024 2.377	Tukey's Multiple Comparison Test	Con vs. 10 min Con vs. 30 min 10 min vs. 30 min	-1.0240 -1.3770 -0.3530	0.0907 0.0201 0.7372	ns * ns

Table S6. Antibody information for the Western blot Lists of primary and secondary antibodies that were used in the study were included in the table.

Name of primary antibody	*Company*	*Molecular Weight (kDa)*	*Source*	*Dilution ratio*	*Catalog number*	*Lot number*
p44/42 MAPK (Erk1/2) (L34F12)	Cell Signaling, MA	42, 44	Mouse	1:2,000 for WB; 1:250 for IF	4696S	22
phospho-ERK1/2 (Tyr 204)	Santa Cruz Biotechnology, Dallas, TX	42, 44	Rabbit	1:2,000 for WB	7976-R	C1113
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP®	Cell Signaling, MA	42, 44	Rabbit	1:2,000 for WB; 1:200 for IF	4370S	24
p38 MAPK (D13E1) XP®	Cell Signaling, MA	38	Rabbit	1:2,000	8690S	6
Phospho-p38 MAPK (Thr180/Tyr182)	Cell Signaling, MA	38	Rabbit	1:2,000	9211S	23
JNK (D-2)	Santa Cruz Biotechnology, Dallas, TX	46, 54	Mouse	1:2,000	7345	L3015
p-JNK (G-7)	Santa Cruz Biotechnology, Dallas, TX	46, 54	Mouse	1:2,000	6254	B2117
α-Tubulin	Santa Cruz Biotechnology, Dallas, TX	50	Mouse	1:2,000	5286	G3117
Name of secondary antibody	***Company***	***Molecular Weight (kDa)***	***Source***	***Dilution ratio***	***Catalog number***	***Lot number***
IRDye® 680LT	Li-Cor, Lincoln, NE	-	Mouse	1:5,000	926-68020	60824-02
IRDye® 800CW	Li-Cor, Lincoln, NE	-	Rabbit	1:5,000	926-32211	C61103-06
Alexa fluor 594 Goat Anti-Rabbit IgG (H+L) Antibody	Life Technologies (Thermo Fisher), Waltham, MA	-	Rabbit	1:1,000	A-11012	-
Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) Antibody	Life Technologies (Thermo Fisher), Waltham, MA	-	Mouse	1:1,000	A11001	-

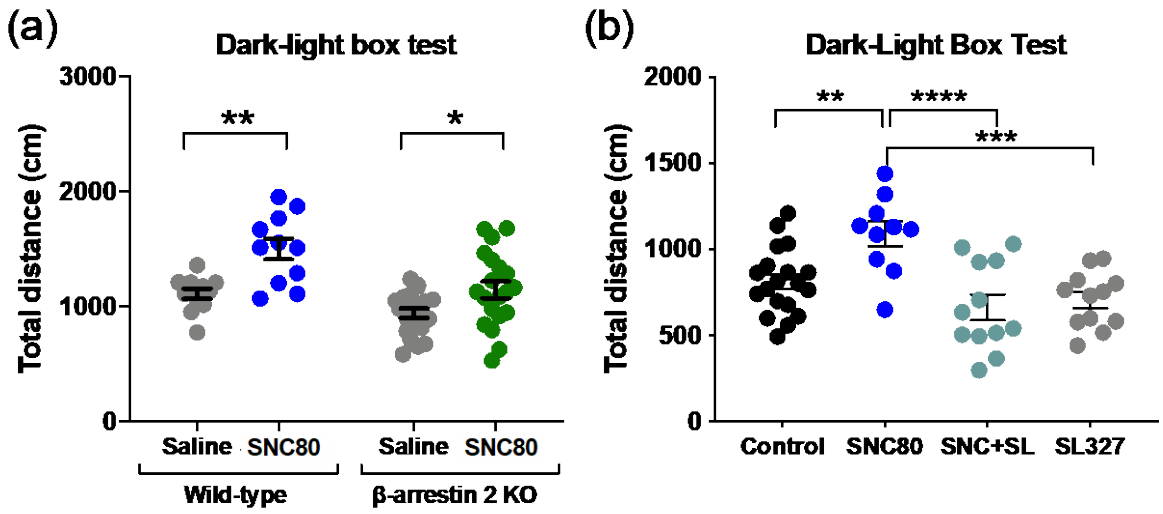

Figure S1. Locomotor effects of drug/vehicle treatment in the WT or β-arrestin 2 KO mice in the dark light upon administration of drugs (a) Traveled distance of WT (control: n=12, SNC80: n=11) and β-arrestin 2 KO mice (control: n=20, SNC80: n=20) upon administration of SNC80 (20 mg/kg, i.p.) the dark light box test shown in **Fig. 1e,g**. **(b)** Traveled distance of WT mice upon administration of SNC80 (20 mg/kg, i.p. / control: n=8, SNC80: n=12, SNC+SL: n=12, SL327: n=12) in presence or absence of 50 mg/kg SL327 in dark light box test shown in **Fig. 4b**. SNC80-induced hyperlocomotion corresponds with a previous report (*11*). (For (a), Significance was calculated by two-way ANOVA $F_{1,59}$=1.949, p=0.1670, WT p=0.0011, β-arrestin 2 KO p=0.0283 after Sidak's multiple comparison; for (b), one-way ANOVA $F_{3,49}$=9.037, p<0.001, control vs. SNC80 p=0.007, SNC80 vs. SNC+SL p<0.0001, SNC+SL vs. SL327 p<0.0004 followed by a Tukey's multiple comparison; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; all values are shown as individual data points ± S.E.M.).

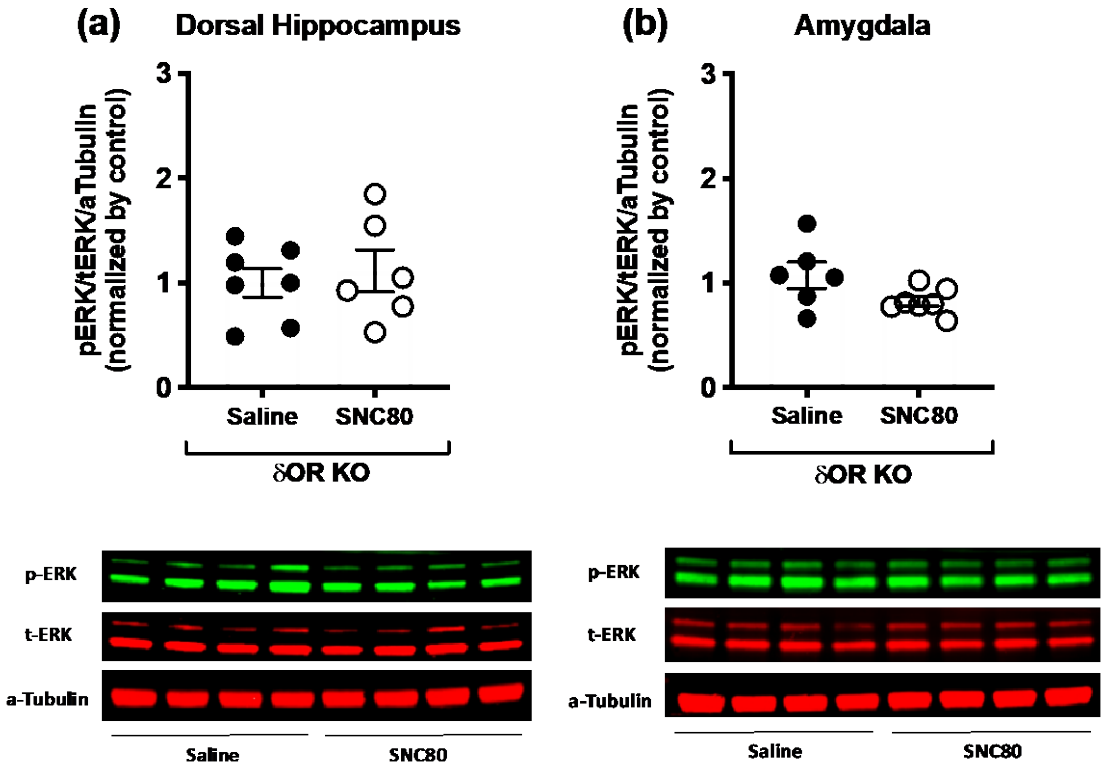

Figure S2. SNC80 does not activate ERK1/2 in the dorsal hippocampus and the amygdala of δOR KO mice (a, b) Unlike **Fig. 2g,h**, systemic administration of SNC80 (20 mg/kg, i.p.) 10 minutes prior to the brain tissue collections did not affect ERK1/2 activation profile in the dorsal hippocampus (Saline: n=8, SNC80: n=7) and the amygdala (Saline: n=7, SNC80: n=8) of δOR KO mice.

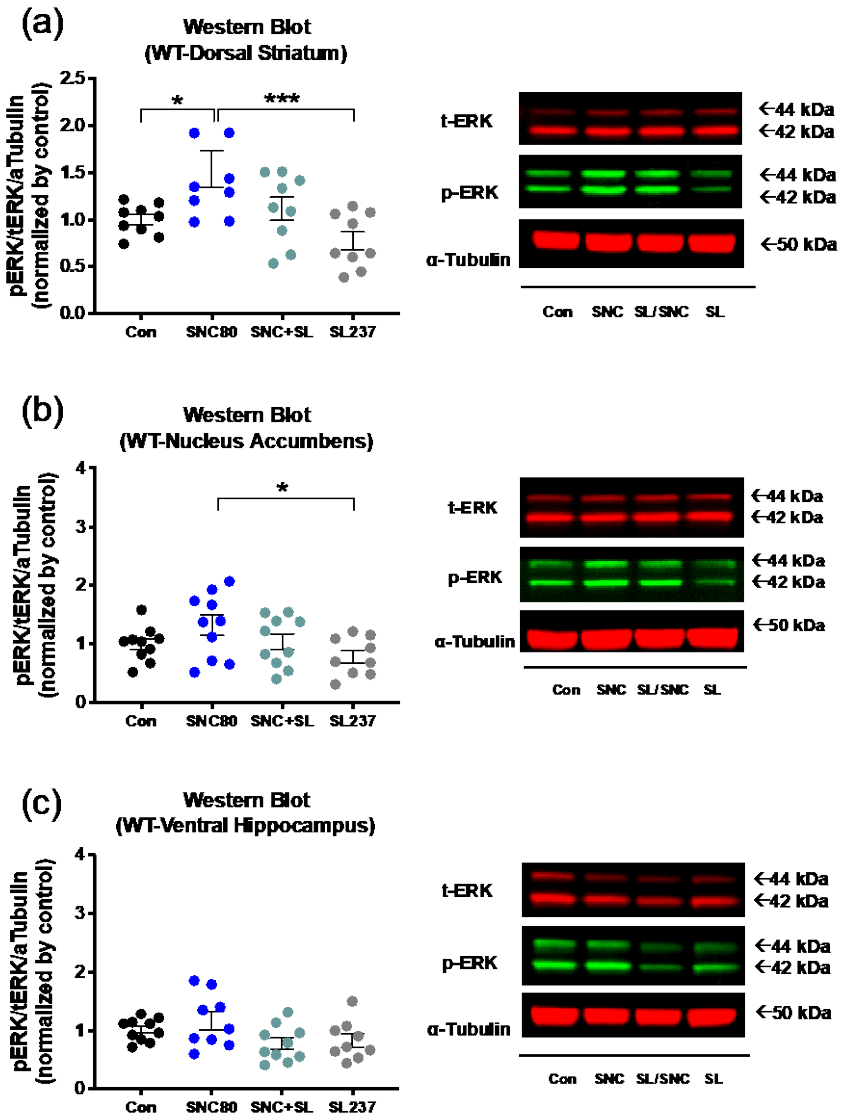

Figure S3. SNC80-induced ERK1/2 activation is partly affected by SL327 in the striatal regions of the brain SL327 (50 mg/kg, s.c.) attenuated SNC80 (20 mg/kg, i.p.)-induced ERK1/2 phosphorylation in the striatum similar to Fig. 4c,d **(a)** and similar trends were observed in the nucleus accumbens **(b)**. **(c)** Yet, no change was observed in the ventral hippocampus similar to Fig. 2i. The number of samples is listed in Table S3. (Significance was calculated by one-way ANOVA followed by a Sidak's or Tukey's multiple comparison; *p<0.05, ***p<0.001; all values

are shown as individual data points ± S.E.M.; SNC+SL means SNC80+SL327 and SL means SL327).

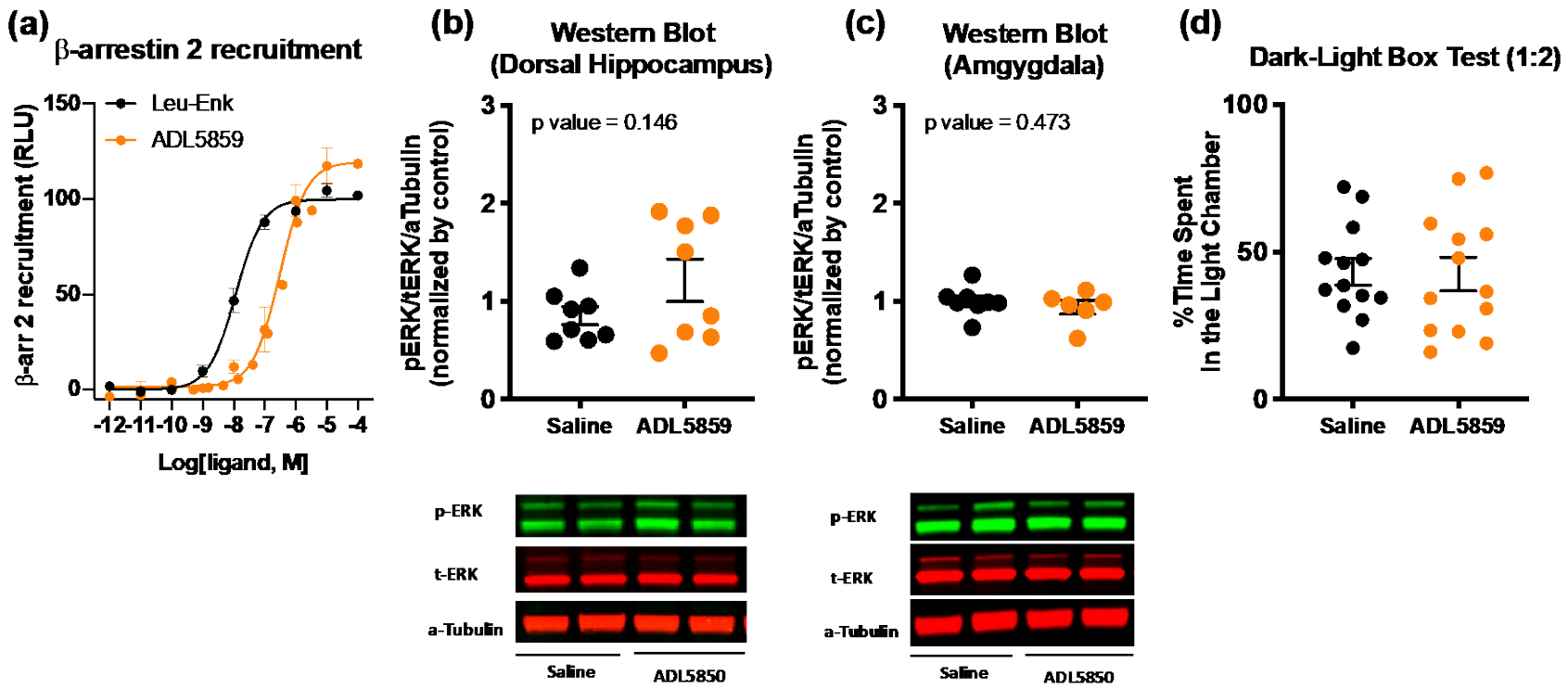

Figure S4. A δOR agonist, ADL5859, does not affect ERK1/2 activity and anxiety-like behaviors of WT mice (a) Dose-dependent β-arrestin 2 recruiment levels by ADL5859 and leucine-enkephalin (leu-enk) were evaluated using cellular assays in CHO-δOR-βarr2 cells (All recruiment levels were normalized by leu-enk and leu-enk was normalized as 100 %). **(b, c)** Systemic administration of ADL5859 (30 mg/kg, p.o.) did not affect ERK1/2 activation profile in the dorsal hippocampus (Saline: n=8, SNC80: n=8) and the amygdala (Saline: n=8, SNC80: n=6) of WT mice. ADL5859 was administered 10 minutes prior to the brain tissue collection. **(d)** No

changes in anxiety-like behaviors in the dark/light box test were observed by systemic administration of ADL5859 (30 mg/kg, p.o.) (Saline: n=13, SNC80: n=13). ADL5859 was administered 30 mintues prior to the behavior testing.

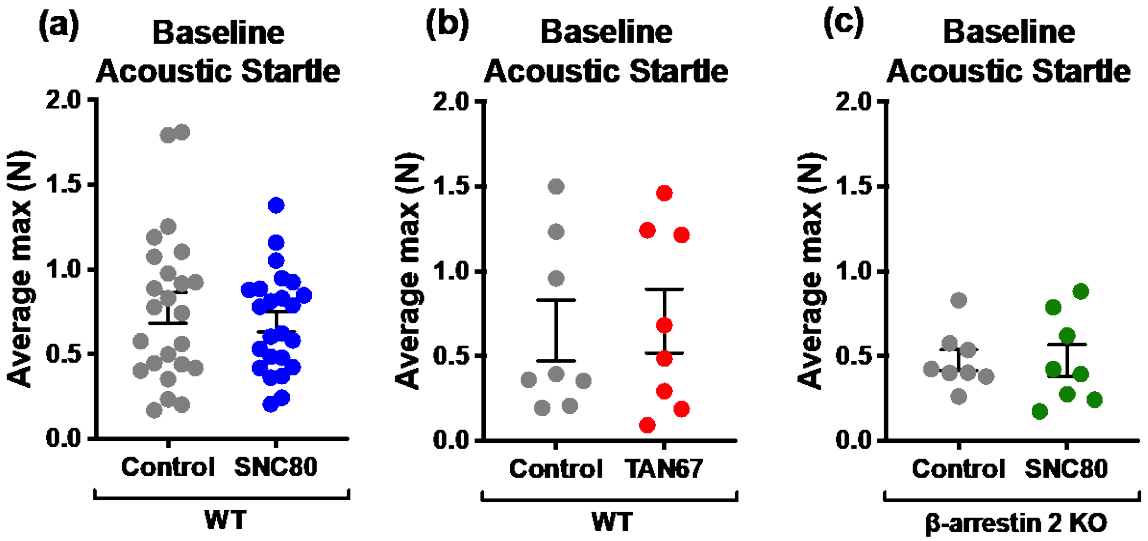

Figure S5. Mice groups for FPS tests were counterbalanced based on baseline acoustic startle response (a) No significance was observed between groups of control *vs.* SNC80 (Control: n=24, SNC80: n=24) **(b)** or control *vs.* TAN67 (Control: n=8, TAN67: n=8) of WT mice. **(b)** Also no significance was observed between control *vs.* SNC80 of β-arrestin 2 KO mice (Control: n=8, SNC80: n=8). All values are shown as individual data points ± S.E.M.

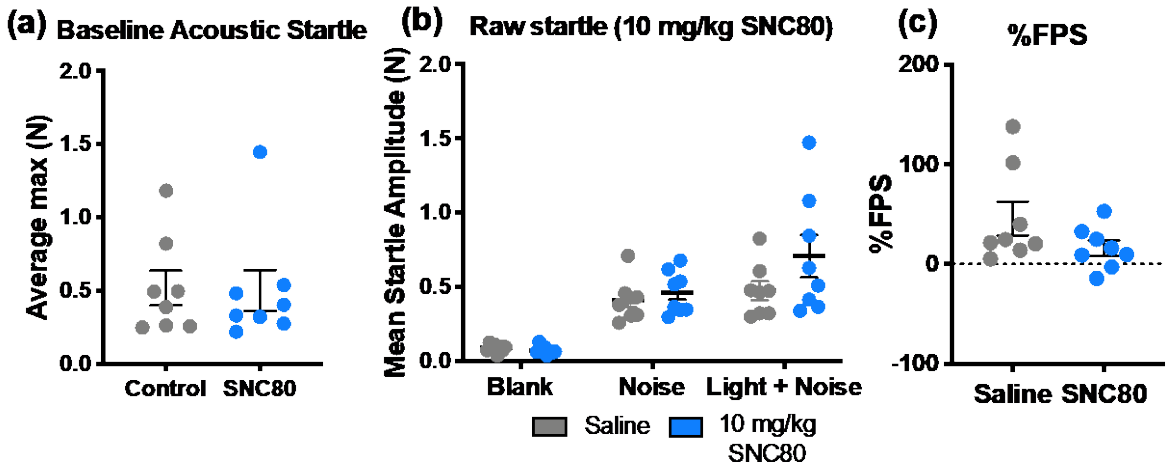

Figure S6. A low dose SNC80 does not affect fear-related behavior of WT mice Fear potentiated startle responses were evaluated upon administration of SNC80 (10 mg/kg, i.p.) in WT mice (Saline: n=8, SNC80: n=8). SNC80 was administered 30 mintues prior to the testing. **(a)** Prior to the testing, mice were measured with baseline acoustic startle and no difference was observed between groups (no drugs were administered for this period). **(b)** 10 mg/kg SNC80 did not affect the raw startle response to either 'noise' alone or 'light+noise' condition. **(c)** 10 mg/kg SNC80 also did not affect %FPS.

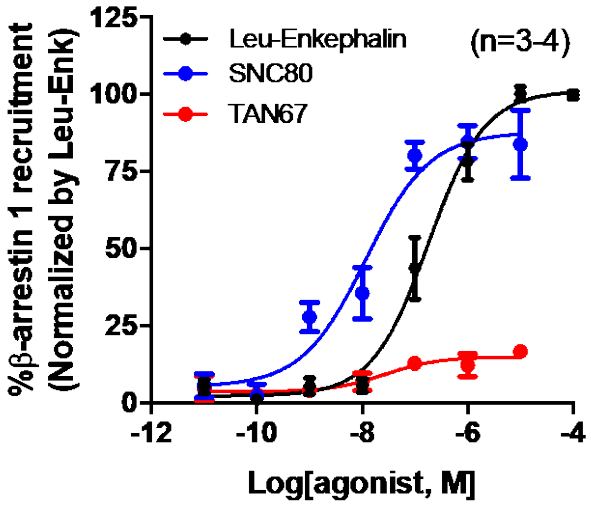

Figure S7. β-arrestin 1 recruitment levels by G-protein-biased (TAN67), β-arrestin-biased (SNC80), and non-biased (Leu-Enk) δOR agonist in U2OS-δOR-βArr1 cells Dose-dependent β-arrestin 1 recruiment levels by TAN67 (n=3), SNC80 (n=3), and Leu-enkephalin (n=4) were evaluated using the cellular assay in U2OS-δOR-βArr1 cells. SNC80 revealed the highest efficacy of recruiment and TAN67 showed the lowest. (All recruiment levels were normalized by leu-enk and leu-enk was normalized as 100 %).

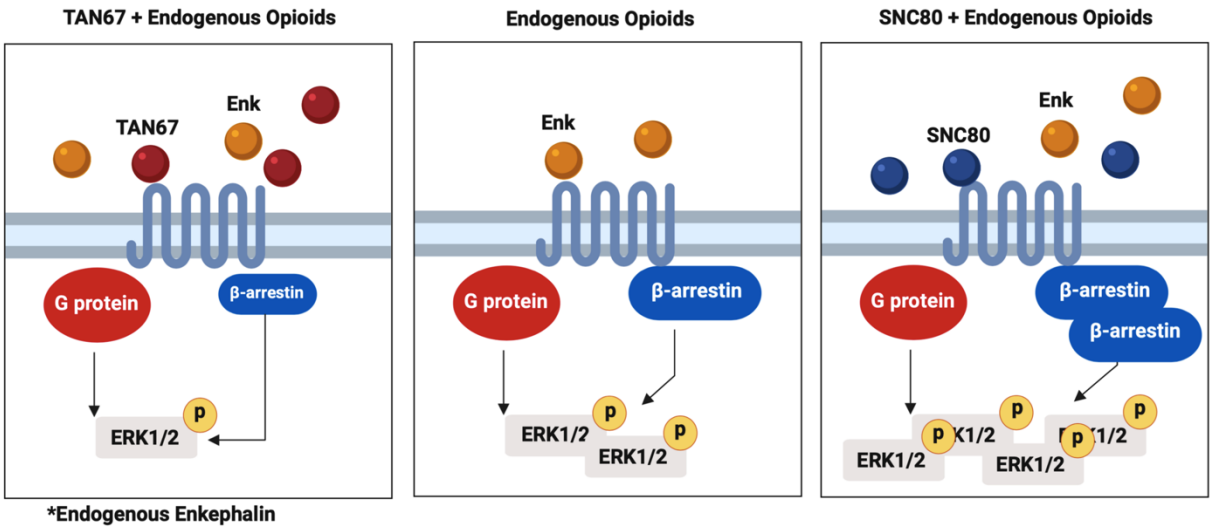

Figure S8. A diagram respresenting the pharmacological competition between two biased agonists and an endnogenous opioid in relations to their ability to modulate ERK1/2 signaling Unlike with cells, the brain has endogenous opioids that bind to δOR. As endogenous opioids such as Leu-Enk, an analog of endogenous opioids, have better ability to recruit β-arrestin proteins than TAN67 as shown in **Fig. S7**, δOR is less likely to recruit β-arrestin and potentially activate less ERK1/2 upon administration of TAN67 in the brain. Likewise, SNC80, which has a better ability to recruits β-arrestin proteins than Leu-Enk, recruits more β-arrestins via δOR and potentially activates more ERK1/2 upon administration of SNC80 in the brain (Right). Yet, it is notworthy that SNC80 and TAN67 have comparable levels of G protein-mediated response (*14*).