#### **Lists of Supplemental Materials**

- 2 Table S1. Statistical analysis of anxiety-like behavior upon systemic administration of SNC80 in
- 3 WT and  $\beta$ -arrestin 2 KO mice
- 4 **Table S2.** Statistical analysis of anxiety-like behabior and ERK1/2 expression levels upon time-
- 5 series administration of SNC80 in WT and  $\beta$ -arrestin 2 KO mouse brain
- 6 Table S3. Statistical analysis of ERK1/2 expression levels upon administration of SNC80 in the
- 7 presence/absence of SL327 in WT mouse brain
- 8 **Table S4.** Statistical analysis of fear-related behavior upon systemic administration of SNC80 or
- 9 TAN67 in WT and  $\beta$ -arrestin 2 KO mice
- 10 Table S5. Statistical analysis of ERK1/2 expression levels upon time-series administration of
- 11 TAN67 in WT mousee and SNC80 in  $\beta$ -arrestin 1 KO mouse brain
- 12 **Table S6.** Antibody information for the Western blot
- 13 Fig. S1. Locomotor effects of drug/vehicle treatment in the WT or  $\beta$ -arrestin 2 KO mice in the
- 14 dark light upon administration of drugs
- **Fig. S2.** SNC80 does not activate ERK1/2 in the dorsal hippocampus and the amygdala of  $\delta OR$
- 16 KO mice
- 17 Fig. S3. SNC80-induced ERK1/2 activation is partly affected by SL327 in the striatal regions of
- 18 the brain
- **Fig. S4.** A δOR agonist, ADL5859, does not affect ERK1/2 activity and anxiety-like behaviors of
- 20 WT mice
- Fig. S5. Mice groups for FPS tests were counterbalanced based on baseline acoustic startle
  response
- 23 Fig. S6. A low dose SNC80 does not affect fear-related behavior of WT mice

- **Fig. S7.** β-arrestin 1 recruitment levels by G-protein-biased (TAN67), β-arrestin-biased (SNC80),
- 2 and non-biased (Leu-Enk) δOR agonist in U2OS-δOR-βArr1 cells
- 3 Fig. S8. A diagram respresenting the pharmacological competition between two biased agonists
- 4 and an endnogenous opioid in relations to their ability to modulate ERK1/2 signaling

### **Supplemental Materials**

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

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

Subfigure	Beh avior 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	WF& Barr2KO	SNC80 (20	WT-Control: 15 WT-SNC80: 15	Two Way	Interaction Genolype factor	F(1,68) = 1.429 F(1,68) = 3.15	0.236 0.0904	Sidak's Mulliple	WT: Can vs. SNC80	-13.0400	0.0164	*	/			
	maze test Print pair 2 107	mg/kg, s.c.)	B2-Control: 21 B2-SNC80: 21	ANOVA test	Drug factor	F (1.68) = 8.781	0.0042	Comparison Test	B2 KO: Con vs. SNC80	-5.5450	0.3200	ns	] /				
Figure 1-e	Dark-light box wr.s. Rer	WE& Barr2KO	SNC80 (20	WI-Control: 12 WI-SNC80: 11	Two Way	Interaction Genolype factor	F(1,59) = 3.677 F(1,59) = 1.039	0.06 0.3122	Sidak's Mulliple	WT: Can vs. SNC80	-12.5200	0.0232	*	/			
	test	wie polizku	mg/kg, s.c.)	B2-Control: 20 B2-SNC80: 20	ANOVA lest	Drug factor	F (1.59) = 4.978	0.0295	Comparison Test	B2 KO: Con vs. SNC80	-0.9467	0.9584	ns	1 /			
Figure 1-f	e 1-f Elevated plus maze test (Total WF& Barr2 KC		SNC80 (20	WT-Control: 15 WT-SNC80: 15	Two-Way	Interaction Genolype factor	F(1,68) = 0.3392 F(1,68) = 20.7	0.5622 <0.0001	1 Sidak's Mulliple	WT: Can vs. SNC80	7.9330	0.5615	ns	1 /			
	movement - min)	wie polizku	mg/kg, s.c.)	B2-Control: 21 B2-SNC80: 21	ANOVA lest	Drug factor	F(1.68) = 0.796	0.3754		B2 KO: Con vs. SNC80	1.6670	0.9643	ns	1 /			
Figure 1-g	Figure 1-g Dark-light box test (Total W					SNC80(20	WT-Control: 12 WT-SNC80: 11	Two-Wiley	Interaction Genotype factor	F(1,57) = 1.754 F(1,57) = 0.1222	0.1907 0.728	Sidak's Mulliple	WT: Can vs. SNC80	-2.6140	0.7254	ns	1 /
	transition)	ni a panzio	mg/kg, s.c.)	B2-Control: 20 B2-SNC80: 18	ANOVA test	Drug factor	F(1.57) = 0.03687	0.8484	Comparison Test	B2 KO: Con vs. SNC80	3,5000	0.3949	ns	1/			
Figure S1-a	Dark-light box test (Total	WF& Barr2KO	SNC80 (20	WT-Control: 12 WT-SNC80: 11	Two-Willy	interaction Genotype factor	F (1, 59) = 1.949 F (1, 59) = 15.40	P=0.1679 P=0.0002	Sidak's Mulliple	WT: Can vs. SNC80	-390.8000	0.0011		]/			
	distance cm)	-	mg/kg, s.c.)	B2-Control: 20 B2-SNC80: 20	ANOVA test	Drug factor	F (1, 59)= 19.78	P≤0.0001	Comparison Test	B2 KO: Con vs. SNC80	-204.1000	0.0283		7			
Subfigu re	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 (50 mg/kg s.c.)	Con: 19 SNC80: 10 SNC+SL: 12 SL327: 12	<b>One-Way</b> ANOVA test	F (3, 49)= 9.037	P≪0.0001	Control SNC80 SNC SL327 SL327	811.8 1090 663.2 705.8	Tukey's Mulliple Camparison Test	Control vs. SNC80 Control vs. SNC+SL327 Control vs. SL327 SNC80 vs. SNC+SL327 SNC80 vs. SL327	-278,1000 148,6000 106,1000 426,7000 384,2000	0.0067 0.2258 0.5155 <0.0001 0.0004	# NS NS			
						aL)	31.427.12				31.321	7058		SNC+SL327 vs. SL327	-42,5000	0.9585	ns

5

- 1 Table S2. Statistical analysis of ERK1/2 expression levels upon time-series administration of SNC80 in WT and β-arrestin 2 KO
- 2 mouse brain Statistical differences of ERK1/2 expression levels in WT mice shown in Fig. 2 and  $\beta$ -arrestin 2 KO mice in Fig. 3.
- 3 Significance between groups was calculated by one-way ANOVA followed by a Tukey's multiple comparison (\*p<0.05, \*\*p<0.01, and
- 4 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		
			SNC 80 (20	Corc 13	One-War			Can	1	Tukey's Mulliple -	Convs. 10 min	-0.4718	0.0128			
Figure 2-e	Dorsal Striatum	WT	mg/kg,ip)	10 min: 13	ANOVA test	F (2,28) = 6.776	P=0.0040	10 min	1472	Comparison Test -	Convs. 30 min	0.1534	0.7398	ns		
			ngng, (p)	30 min: 5	ANOTAUS	AUTA @SL		30 min	0.8466	Companson rest	10 min vs. 30 min	0.6253	0.0140			
	Nucleus		SNC 80 (20	Core 13	One-War			Can	1	Tukey's Mulliple -	Convs. 10 min	-0.3632	0.0317	*		
Figure 24	Acumbens	WE	mg/kg, ip)	10 min: 13	ANOVA test	F (2,29) = 6.645	P=0.0042	10 min	1.363	Comparison Test	Convs. 30 min	0.2070	0.4563	ns		
	Acambaia		mging, spj	30 min: 6	ANOTALS			30 min	0.793	Campanaan reat	10 min vs. 30 min	0.5702	0.0064	#		
	Dorsal		SNC 80 (20	Con: 13	One-War			Can	1	Tukey's Mulliple	Convs. 10 min	-0.8194	0.0051			
Figure 2-g	Hippocampus	WT	ma/ka, ip)	10 min: 12	ANOVA test	F (2,28) = 8.252	P=0.0015	10 min	1.819	Comparison Test -	Convs. 30 min	0.1918	0.7923	ns		
	mppocampus		inging, (p)	30 min: 6	ANOTACA			30 min	0.8082	Companaul real -	10 min vs. 30 min	1.0110	0.0056	**		
			SNC 80 (20	Con: 13	One-War			Can	1	Tukey's Mulliple	Convs. 10 min	-0.5264	0.0024	**		
Figure 2-h	Amygdala	WT	WT mg/kg, ip)	10 min: 13	ANOVA test	F (2,29) = 10.82	P=0.0003	10 min	1.526	Comparison Test	Convs. 30 min	0.1940	0.5284	ns		
			(110 mg, 14)	30 min: 6	Anovates			30 min	0.806	Companadi reat	10 min vs. 30 min	0.7204	0.0010			
	Ventral		SNC 80 (20	Con: 11 One Wa	One War		P=0.0823	Can	1	Tukey's Mulliple Comparison Test	Convs. 10 min	-0.1379	0.6248	ns		
Figure 2-i	Figure 2-i Venual W Hippocampus W		mg/kg, ip)	10 min: 10	ANOVA test			10 min	1.138		Convs. 30 min	0.3892	0.2039	ns		
			inging, cp)	30 min: 3	ANOTALS			30 min	0.6108		10 min vs. 30 min	0.5271	0.0676	ns		
		n βarr2KO	2KO SNC80(20 ma/ka, ip)	Con: 8	<b>One-Way</b> ANOVA test	F (2, 20) = 1.873	P=0.1220	Can	1	Tukey's Mulliple Comparison Test	Convs. 10 min	-0.7647	0.1805	ns		
Figure 3-c	Dorsal Striatum			10 min: 7				10 min	1.765		Convs. 30 min	-0.1857	0.8887	ns		
			inging, (p)	30 min: 8	ANOTACA			30 min	1,196	Companaul real -	10 min vs. 30 min	0.5790	0.3608	ns		
	Nucleus		SNC 80 (20	Com 7	One-Way		P=0.0200	Can	1	Tukey's Mulliple Comparison Test	Convs. 10 min	-0.8913	0.0240	*		
Figure 3-d	Acumbens		maller in)	10 min: 7	ANOVA test	F (2, 18) = 4.903		10 min	1.891		Convs. 30 min	-0.1407	0.8907	ns		
	Acuindens		-	-		inging, ip)	30 min: 7	Anovaus			30 min	1.141	Companson rest	10 min vs. 30 min	0.7506	0.0608
	Dorsal		SNC 80 (20	Con: 8	One-War			Can	1	Tukey's Mulliple -	Convs. 10 min	-0.1654	0.5183	ns		
Figure 3-e	Hippocampus	βал2КО	mg/kg, ip)	10 min: 7	ANOVA test	F (2, 20) = 0.8178	P=0.4556	10 min	1.165	Comparison Test	Convs. 30 min	0.0024	0.9998	ns		
	mppocampus		inging, cp)	30 min: 8	ANOTAUS			30 min	0.9976	Campansan rea	10 min vs. 30 min	0.1678	0.5086	ns		
			SNC 80 (20	Con: 8	One-War			Can	1	Tukey's Mulliple -	Convs. 10 min	-0.2360	0.4909	ns		
Figure 3-f	Amygdala	βarr2KO	ma/ka, ip)	10 min: 8	ANOVA test	F (2, 21) = 1.472	P=0.2522	10 min	1236	Comparison Test	Convs. 30 min	0.1058	0.8631	ns		
			110 mg, (p)	30 min: 8	ANOTACA			30 min	0.8942	Gumparistin rest	10 min vs. 30 min	0.3418	0.2376	ns		
	Ventral		SNC 80 (20	Con: 8	One-Way	F (2, 20) = 2.035		Can	1	Tukey's Mulliple - Comparison Test -	Convs. 10 min	-0.2905	0.2167	ns		
Figure 3-g	Hippocampus	βал 2КО	mg/kg, ip)		ANOVA test		P=0.1569	10 min	1.291		Convs. 30 min	0.0098	0.9980	ns		
	ingep octainget as			30 min: 8	JING TA COL			30 min	0.9902	Comparadit real	10 min vs. 30 min	0.3003	0.1968	ns		

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

Subfigure	Behavior test or Brain region	Genotype	Drug	#ofsamples	Test	F-value	p-value	Group	Mean	Post hoc analysis	Group Comparison	Mean Diff.	p-value	Significance
											Canitol vs. SNC80	-16.6400	0.0109	•
			SNC 80 (20	Con: 7				Caniral	13.88		Control vs. SNC+SL327	10,4600	0.1496	ns
Figure 4-b	Dar-light box test	WE	mg/kg,ip)or	SNC 80: 9	One-Way	F (3, 34) = 12.35	P⊴0.0001	SNC80	30.53	Tukey s Mulliple	Cantral vs. SL327	-1.7550	0.9830	ns
	Del-Igik DOA (CS.				ANOVA test	1 (3, 34) - 12 35	1 -0.0001	SNC+SL327	3.418	Comparison Test	SNC80 vs. SNC+SL327	27.1100	<0.0001	***
			S.C.)	SL327: 11				SL327	15.64		SNC80 vs. SL327	14.8900	0.0106	•
											SNC+SL327 vs. SL327	-12.2200	0.0325	*
											Canitrol vs. SNC80	-0.6767	0.0024	
			SNC 80 (20	Con: 10				Caniral	1		Control vs. SNC+SL327	0.1076	0.9247	ns
Figure 4-c	Dorsal	W	mg/kg,ip)or	SNC 80: 9	One-Way	F (3, 33)= 11.68	P≤0.0001	SINCRO	1.706	Tukey's Mulliple	Cantral vs. SL327	0.3169	0.2790	ns
	Hippocampus		SL327 (50 mg/kg		ANOVA test	F (3, 33) = 11.08	PS0.0001	SNC+SL327 SL327	0.9214 0.7122	Comparison Test	SNC80 vs. SNC+SL327	0.7843	0.0006	
			s.c.)	SL327: 9							SNC80 vs. SL327	0.9936	<0.0001	
											SNC+SL327 vs. SL327	0.2092	0.6459	ns
											Control vs. SNC80	-0.9798	0.0021	**
			SNC 80 (20	Con: 10		F (3, 35) = 10.9	P≪0.0001	Caniral SNC80 SNC+SL327 SL327	1		Control vs. SNC+SL327	-0.1093	0.9713	ns
Figure 4-d	Amygdala	WT		SNC 80: 10	One-Way				2.003	Tukey s Mulliple	Cantral vs. SL327	0.4325	0.3442	ns
- Marcara	Ang game		SL327 (50 mg/kg s.c.)		ANOVA test				1.133	Comparison Test	SNC80 vs. SNC+SL327	0.8705	0.0069	
				SL327:9					0.5908		SNC80 vs. SL327	1.4120	<0.0001	***
											SNC+SL327 vs. SL327	0.5419	0.1677	ns
		Strington MIT	male inter		One-Way ANOVA test	F (3, 32)= 6.421	P=0.0016	Caniral SNC80 SNC+SL327 SL327			Canirol vs. SNC80	-0.5429	0.0247	*
				r SNC80:9					1		Control vs. SNC+SL327	-0.1155	0.9180	ns
Figure \$3.a	Dorsal Striatum								1.543 1.115	Tukey's Mulliple Comparison Test	Caniral vs. SL327	0.2266	0.5960	ns
											SNC80 vs. SNC+SL327	0.4274	0.1032	ns
									0.7734		SNC80 vs. SL327 SNC+SL327 vs. SL327	0.7694 0.3420	0.0009	
											Canini vs. SIL327			ns
			SNC 80 (20	Con: 10				Caniral	1		Canital vs. SNC+SL327	-0.3193 -0.0404	0.3248	ns
	Nucleus			SNE 80: 10	One-War			SNC80	1.319	Tukey's Mulliple	Caniral vs. SH3-3L327	0.2123	0.6909	IIS IIS
Figure S3-b	Accumbens	WT	SL327 (50 mg/kg		ANOVA test	F (3, 35) = 2.672	P=0.0624	SNC+SL327	104	Comparison Test	SNCR0 vs SNC+SL327	02790	04427	ns
	Heedinacata		SCJ2 (SO Mgrag SCJ	SL 327: 9	Antonn dat			SL327	0.7877	Companyan real	SNC80 vs. SL327	0.5316	0.0394	1
								CALA .	0.1011		SNC+SI 327 vs SI 327	0.2526	0.5501	ns
											Control vs. SNC80	-0.1522	0.7419	ns
			SNC 80 (20	Con: 10				Caniral	1		Caninal vs. SNC+SL327	0.2347	0.3879	ns
Cimera 62 a	Ventral	WE	mg/kg,ip)or	SNC 80: 9	One Way	F (3, 34) = 2.734	P=0.0588	SNC80	1.164	Tukey's Mulliple Comparison Test	Caniral vs. SL327	0.1903	0.6299	ns
Figure \$3-c	Hippocampus	-	SL327 (50 mg/kg		ANOVA test			SNC+SL327 SL327	0.7771 0.8315		SNC80 vs. SNC+SL327	0.3869	0.0655	ns
				SL 327: 9							SNC80 vs. SL327	0.3325	0.1549	ns
			•								SNC+SL327 vs. SL327	-0.0545	0.9833	ns

- 1 Table S4. Statistical analysis of fear-related behavior upon systemic administration of SNC80 or TAN67 in WT and β-arrestin
- 2 **2 KO mice** Statistical differences of fear-related behaviors in WT or β-arrestin 2 KO mice shown in **Fig. 5**. Significance between groups
- 3 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	G roup C omparison	Mean Diff.	p-value	Significance
Figure 5-c	Fear potentiated startle test iR aw Wi	wr	SNC 80 (20	Control: 21	Two Way	Interaction Stimulation factor	F(2,120) = 20.42 F(2,120) = 92.80	<0.0001 <0.0001	Sidakî's Mulliple	Blank: Con vs. SNC80 Noise: Con vs. SNC80	-0.0126 0.5879	0.9994 <0.0001	ns 
	startie)		mg/kg, i.p.)	SN C 80: 21	ANOVA test	Drug factor	F (1,120) = 63.99	<0.0001	Comparison Test	Noise+Light Con vs. SNC80	1.0310	<0.0001	****
Figure 5-d	Fear potentiated		SNC 80 (20	Control: 21								/	
	startle test (FPS testing)	WT	mg/kg, i.p.)	SN C 80: 20	Unpaird ttest			p=0.0085					
		β <b>ап</b> 2К0		Control: 8	Two-Way ANOVA test	Interaction	F(2,42) = 20.22	<0.0001		Blank: Con vs. SNC80	-0.0355	>0.9999	ns
Figure 5-f	Fear potentiated startle test		SNC 80 (20 mg/kg, i.p.)			Stimutation factor Drug factor	F(2,42) = 51.52 F(1,42) = 40,4	<0.0001 <0.0001	Sidak's Mulliple	Noise: Con vs. SNC80	0.2139 0.5812	0.0103	-
	SHOLOG OF SHO			SN C 80: 8	ANOTA US	Ding racion	F(1,42) = 40.4	<0.0001	Companison rest	Noise+Light Con vs. SNC80	0.58 12	<0.000 I	
Figure 5-g	Fear potentiated		SNC 80 (20	Control: 8									
i igure 3-g	startle test (FPS testing)	ßarr2 KO	mg/kg, i.p.)	SN C 80: 8	Unpaird ttest			p=0.0003			Martin Martin Martin Martin Martin		
				Controt8		Interaction	F(2,42) = 0.7245	0.4905		Blank: Con vs. SNC80	-0.0113	>0.9999	ns
Figure 5-i	Fear potentiated	WT	TAN67 (25	CONTOLO	Two Way	Stimulation factor	F(2,42) = 25.06	<0.0001	Sidaki's Mulliple	Noise: Con vs. SNC80	0.1123	0.9736	ns
	startle test		mg/kg, i.p.)	TA N67: 8	ANOVA test	Drug factor	F(1,42) = 0.2754	0.6025	Companson Test	Noise+Light Con vs. SNC80	-0.3664	0.5189	ns
Figure 5-j	Fear potentiated	WT	TAN67 (25	Control: 7	I and the set			p≪0.0001					
	test (FPS testing)	WI	mg/kg, i.p.)	TA N67: 8	Unpaird ttest			p≤0.0001			Martin Contraction and Contraction of the Contracti		

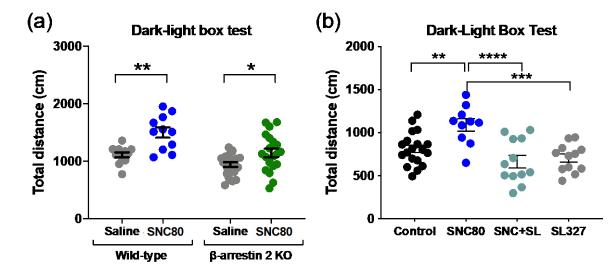
- 1 Table S5. Statistical analysis of ERK1/2 expression levels upon time-series administration of TAN67 in WT mousee and SNC80
- 2 in β-arrestin 1 KO mouse brain Statistical differences of ERK1/2 expression levels in WT and β-arrestin 1 KO mouse brain shown in
- 3 Fig. 6. Significance between groups was calculated by one-way ANOVA followed by a Tukey's multiple comparison (\*p<0.05,
- 4 \*\*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	
	Drain region	Generate		Con: 7		1-ruide	p-ruide	Con			Con vs. 10 min	0 1231	0.6279	IIS	
Figure 6-a	Dorsal Striatum	W	TAN67 (25	10 min: 7	One-Way	F (2, 18) = 5.276	P=0.0157	10 min	0.8769	Tukey's Mulliple	Con vs. 30 min	0.4176	0.0142	*	
			mg/kg,ip)	30 min: 7	ANOVA test	. (2, 13, 22.12		30 min	0.5824	Comparison Test	10 min vs. 30 min	0.2945	0.0931	ns	
Eigene 6 h	igure 6-b Nucleus Acumbens		TAN67 (25	Con: 7	One-Way	F (2, 17) = 5.701		Con	1	Tukey's Mulliple	Con vs. 10 min	0.3848	0.0447	*	
riguieo-u		WE	mg/kg,ip)	10 min: 7	ANOVA test		P=0.0127	10 min	0.6152	Comparison Test	Con vs. 30 min	0.4773	0.0161		
			ngag, cp)	30 min: 6	ANUTA USL			30 min	0.5227		10 min vs. 30 min	0.0925	0.8190	ns	
	Dorsal	WT	TAN67 (25	Con: 7	One-Way	F (2, 18) = 5.09		Con	1	Tukey's Mulliple Comparison Test	Con vs. 10 min	-0.0207	0.9759	ns	
Figure 6-c	Hippocampus		mg/kg,ip)	10 min: 7	ANOVA test		P=0.0177	10 min	1.021		Con vs. 30 min	0.2610	0.04 10	*	
	пфросанраз		mgmg, cp)	30 min: 7	HIGH CA			30 min	0.739		10 min vs. 30 min	0.2817	0.0266	•	
				TAN67 (25	Con: 7	One-Way			Con	1	Tukey's Mulliple	Con vs. 10 min	0.1490	0.3216	ns
Figure 6-d	Amygdala	W	mg/kg,ip)	10 min: 6	ANOVA test	F (2, 16) = 5.455	P=0.0156	10 min	0.851	Comparison Test	Con vs. 30 min	0.3303	0.0118	-	
				30 min: 6	1110111-000			30 min	0.6697	Contraction 1 and	10 min vs. 30 min	0.1813	0.2189	ns	
Figure 6-e	Ventral	WT	TAN67 (25	Con: 6	One-Way ANOVA test	F (2, 15) = 1.092	P=0.3607	Con	1	Tukey's Mulliple Comparison Test	Con vs. 10 min	-0.0628	0.9388	ns	
i igui cu-c	Hippocampus		mg/kg,ip)	30 mm; 6				10 min	1.063		Con vs. 30 min	0.1991	0.5426	ns	
	nippocanipus		ngag, cp)		ANUTA USL			30 min	0.8009	Companyon rest	10 min vs. 30 min	0.2618	0.3582	ns	
Figure 6-f		ßarr1 KO	arr1 KO SNC80 (20 mg/kg, i.p)	Con: 7	) min: 6 Une-way	F (2,16)= 1.62	P=0.2288	Con	1	Tukey's Mulliple Comparison Test	Con vs. 10 min	-0.1907	0.4590	ns	
riguicoa	Dorsal Striatum			10 min: 6				10 min	1.191		Con vs. 30 min	-0.2710	0.2226	ns	
				30 min: 6				30 min	1271		10 min vs. 30 min	-0.0903	0.8743	ns	
Figure 6-g	Nucleus	1	SNC80(20	Con: 7 One-Way	1		Con	1	Tukey's Mulliple	Con vs. 10 min	-0.1791	0.7426	ns		
riguieo-g	Acumbens	βarri KO		10 min: 6	ANOVA test	F (2,16) = 0.5181	P=0.6053	10 min	1,179	Comparison Test	Con vs. 30 min	-0.2319	0.6112	ns	
	Acuments		mg/kg, i.p)	30 min: 6	ANUVA USL			30 min	1232	Companyanties	10 min vs. 30 min	-0.0528	0.9759	ns	
Cimero C b	Dorsal		SNC80 (20	Con: 7	One Way			Con	1	Tukey's Multiple	Con vs. 10 min	-1.8120	0.0027	**	
Figure 6-h		farri KO		10 min: 6	ANOVA test	F (2,16) = 11.48	P=0.0008	10 min	2812		Con vs. 30 min	-18740	0.0020	**	
	Hippocampus		mg/kg, i.p)	30 min: 6	ANUVA USI			30 min	2874	Comparison Test	10 min vs. 30 min	-0.0625	0.9902	ns	
C: C :			CHC00 (70	Con: 7	0 W		1	Con	1	Tedan da Malificata	Con vs. 10 min	-0.4981	0.1080	ns	
Figure 6-i	Amygdala	Barri KO	r1 KO SNC80 (20 mg/kg, i.p)	10 min: 6	One-Way	F (2,16) = 4.668	P=0.0253	10 min	1.498	Tukey's Mulliple Comparison Test	Con vs. 30 min	-0.6695	0.0261	•	
				30 min: 6	ANOVA test	. (2,12)		30 min	1.67		10 min vs. 30 min	-0.1714	0.7564	ns	
ri e i	Manatari		ENC00/00	Con: 7	() Wf	F (2,16) = 5.097	P=0.0194	Con	1	Tukey's Mulliple	Con vs. 10 min	-10240	0.0907	ns	
Figure 6-j	Ventral	Barri KO	SNC80 (20	en	One Way			10 min	2024		Con vs. 30 min	-13770	0.0201	1	
	Hippocampus		mg/kg, i.p)	30 min: 6	ANOVA test	.,,,		30 min	2377	Comparison Test	10 minys 30 min	-0.3530	0.7372	ns	

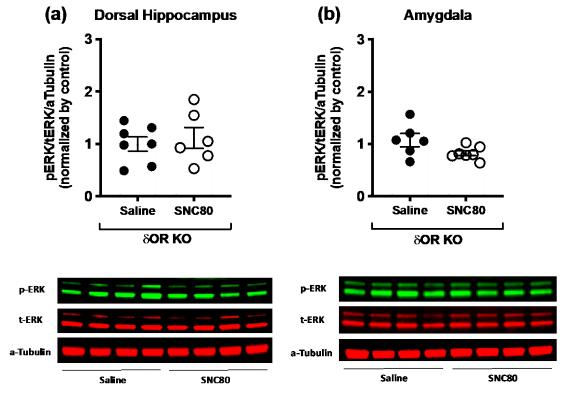
# **1 Table S6. Antibody information for the Western blot** Lists of primary and secondary antibodies

# 2 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	-	



2 Figure S1. Locomotor effects of drug/vehicle treatment in the WT or β-arrestin 2 KO mice 3 in the dark light upon administration of drugs (a) Traveled distance of WT (control: n=12, 4 SNC80: n=11) and  $\beta$ -arrestin 2 KO mice (control: n=20, SNC80: n=20) upon administration of 5 SNC80 (20 mg/kg, i.p.) the dark light box test shown in Fig. 1e,g. (b) Traveled distance of WT 6 mice upon administration of SNC80 (20 mg/kg, i.p. / control: n=8, SNC80: n=12, SNC+SL: n=12, 7 SL327: n=12) in presence or absence of 50 mg/kg SL327 in dark light box test shown in Fig. 4b. 8 SNC80-induced hyperlocomotion corresponds with a previous report (11). (For (a), Significance 9 was calculated by two-way ANOVA  $F_{1,59}=1.949$ , p=0.1670, WT p=0.0011,  $\beta$ -arrestin 2 KO 10 p=0.0283 after Sidak's multiple comparison; for (b), one-way ANOVA F<sub>3.49</sub>=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 11 followed by a Tukey's multiple comparison; p<0.05, p<0.01, p<0.01, p<0.001, p<0.001; all 12 13 values are shown as individual data points  $\pm$  S.E.M.).



2 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.

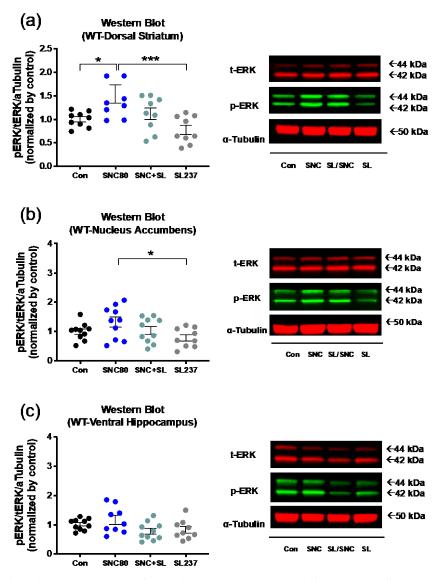
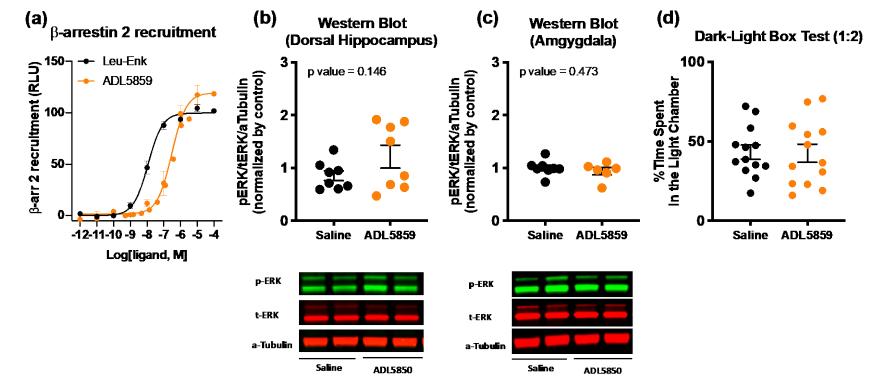


Figure S3. SNC80-induced ERK1/2 activation is partly affected by SL327 in the striatal
regions of the brain SL327 (50 mg/kg, s.c.) attenuated SNC80 (20 mg/kg, i.p.)-induced ERK1/2
phosphorylation in the striatum similar to Fig. 4c,d (a) and similar trends were observed in the
nucleus accumbens (b). (c) 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</li>

- 1 are shown as individual data points  $\pm$  S.E.M.; SNC+SL means SNC80+SL327 and SL means
- 2 SL327).



2 Figure S4. A δOR agonist, ADL5859, does not affect ERK1/2 activity and anxiety-like behaviors of WT mice (a) Dose-dependent

3 β-arrestin 2 recruiment levels by ADL5859 and leucine-enkephalin (leu-enk) were evaluated using cellular assays in CHO-δOR-βarr2

- 4 cells (All recruiment levels were normalized by leu-enk and leu-enk was normalized as 100 %). (b, c) Systemic administration of
- 5 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
- 6 amygdala (Saline: n=8, SNC80: n=6) of WT mice. ADL5859 was administered 10 minutes prior to the brain tissue collection. (d) No

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

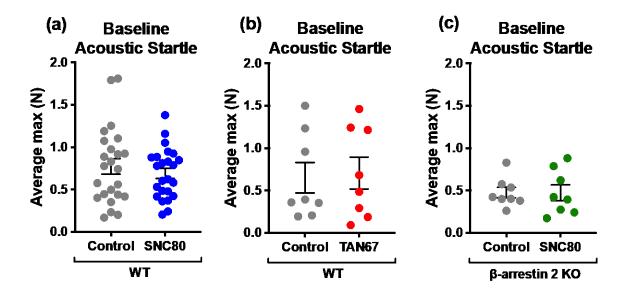
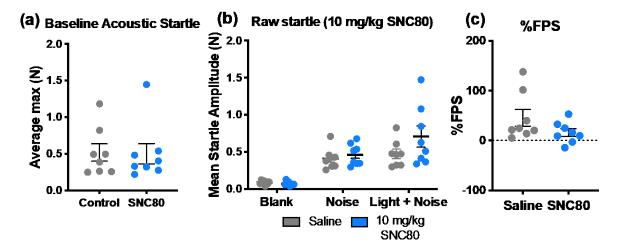


Figure S5. Mice groups for FPS tests were counterbalanced based on baseline acoustic startle response (a) No significance was observed between groups of control *vs*. SNC80 (Control: n=24, SNC80: n=24) (b) or control *vs*. TAN67 (Control: n=8, TAN67: n=8) of WT mice. (b) Also no significance was observed between control *vs*. SNC80 of  $\beta$ -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.

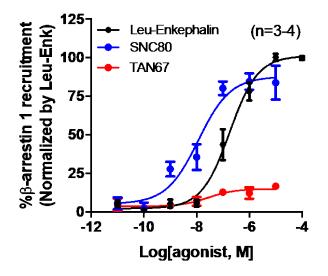


Figure S7. β-arrestin 1 recruitment levels by G-protein-biased (TAN67), β-arrestin-biased (SNC80), and non-biased (Leu-Enk) δOR agonist in U2OS-δOR-βArr1 cells Dose-dependent β-arrestin 1 recruiment levels by TAN67 (n=3), SNC80 (n=3), and Leu-enkephalin (n=4) were evaluated using the cellular assay in 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 %).

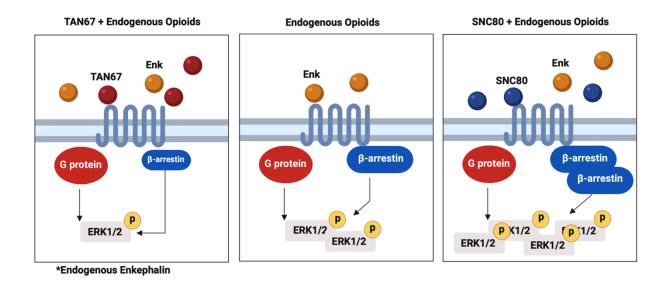


Figure S8. A diagram respresenting the pharmacological competition between two biased agonists and an endnogenous opioid in relations to their ability to modulate ERK1/2 signaling Unlike with cells, the brain has endogenous opioids that bind to  $\delta$ OR. As endogenous opioids such as Leu-Enk, an analog of endogenous opioids, have better ability to recruit  $\beta$ -arrestin proteins than TAN67 as shown in Fig. S7,  $\delta$ OR is less likely to recruit  $\beta$ -arrestin and potentially activate less ERK1/2 upon administration of TAN67 in the brain. Likewise, SNC80, which has a better ability to recruits  $\beta$ -arrestin proteins than Leu-Enk, recruits more  $\beta$ -arrestins via  $\delta$ OR 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*).