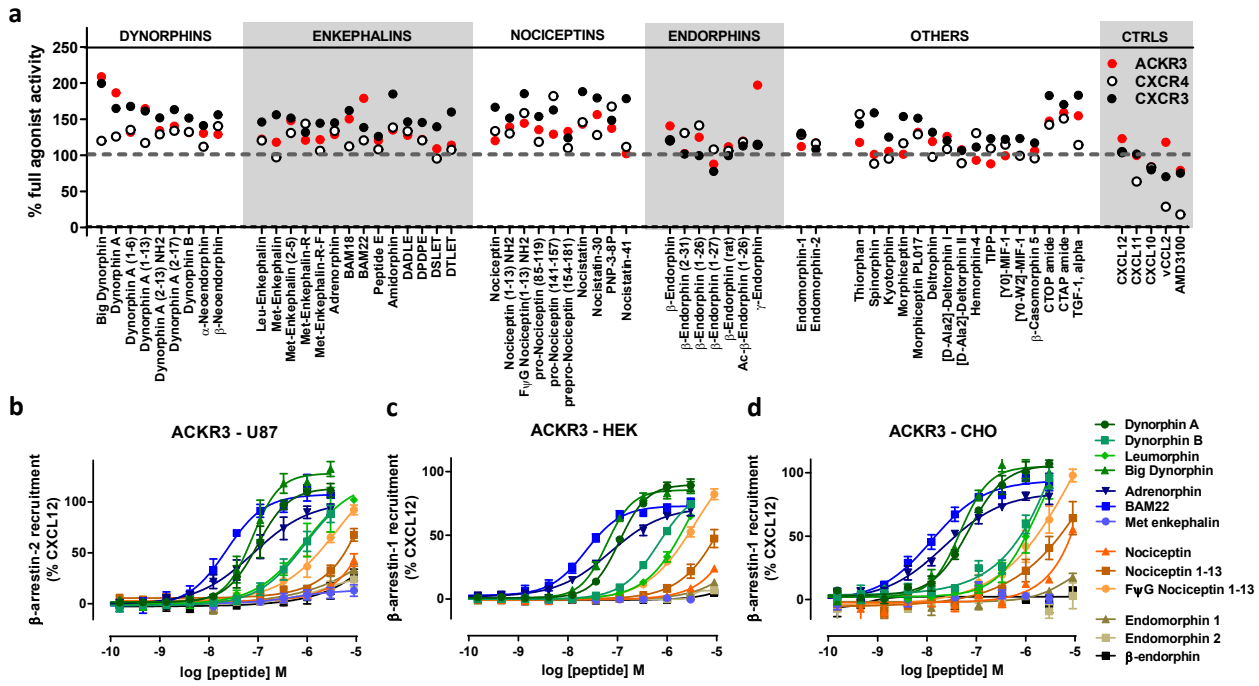
The atypical chemokine receptor ACKR3/CXCR7 is a broad-spectrum scavenger for opioid peptides

Supplementary information

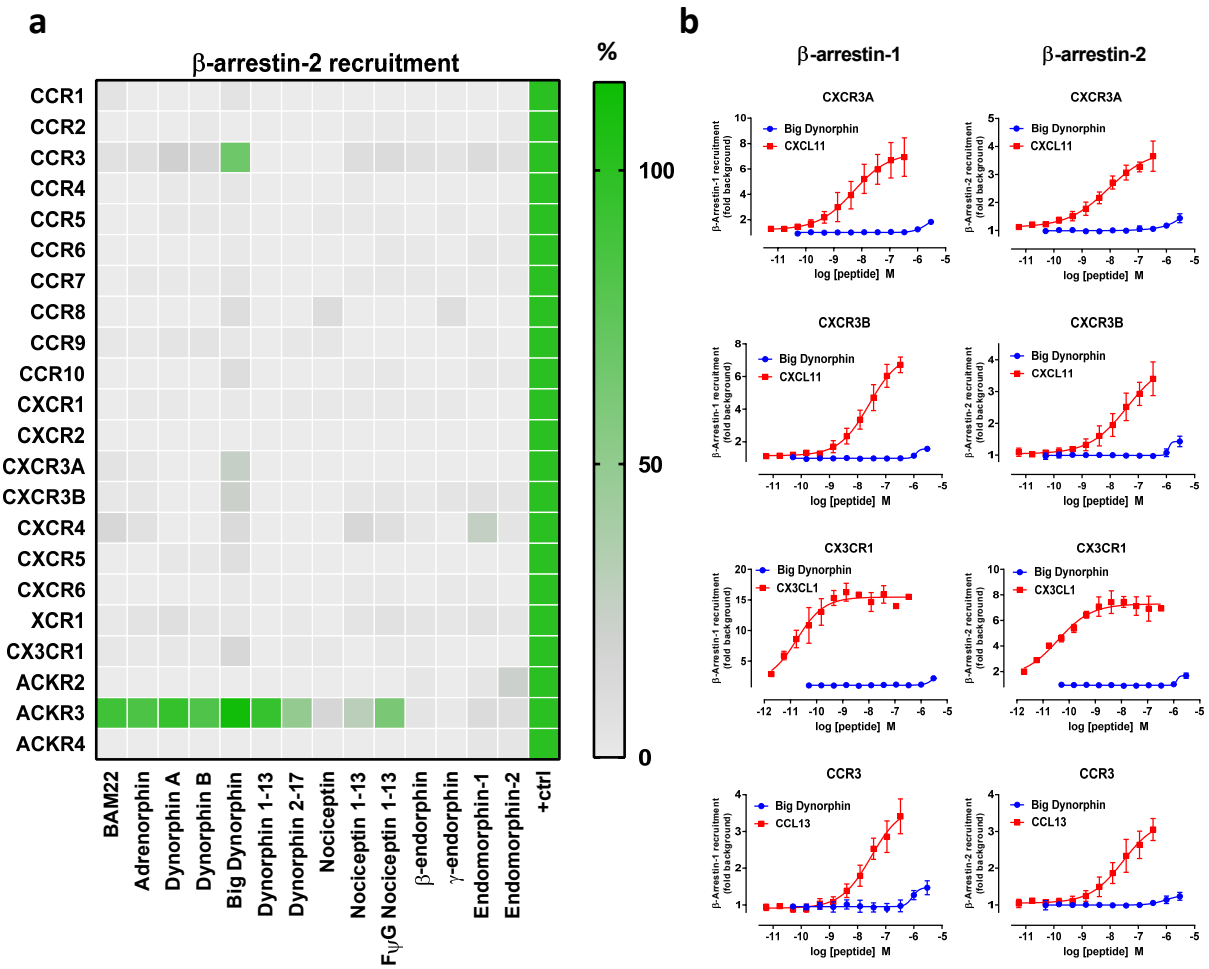
Meyrath, Szpakowska *et al*.

Supplementary Figures 1-8

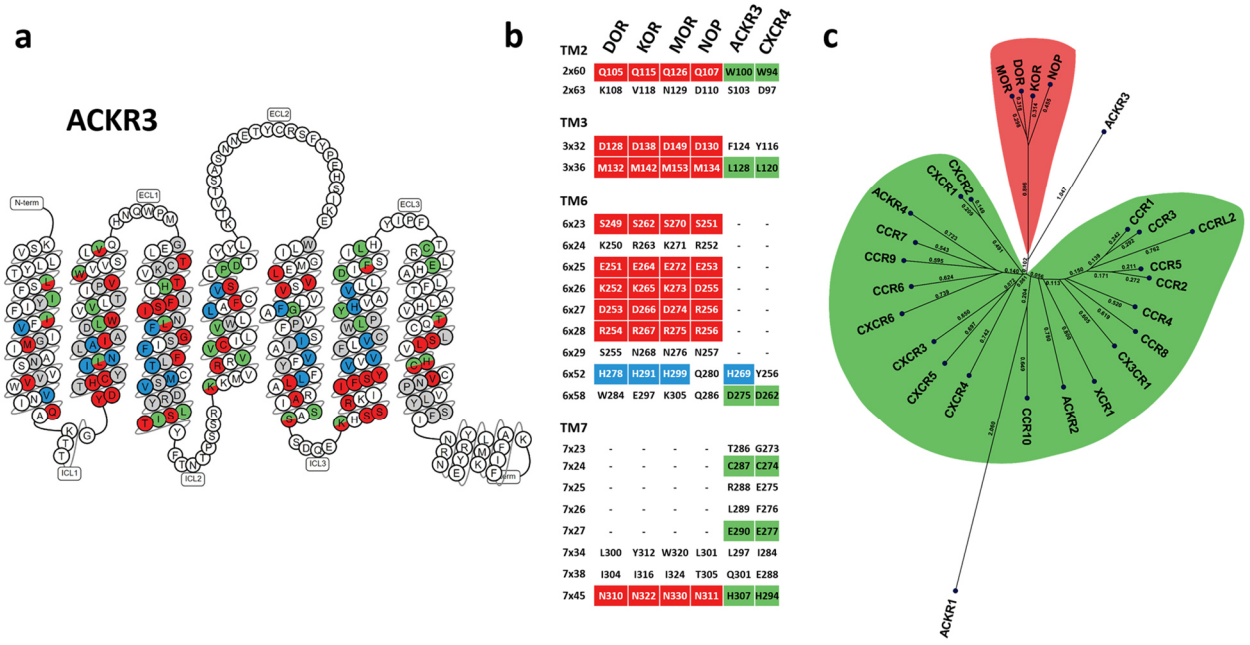
Supplementary Tables 1-10



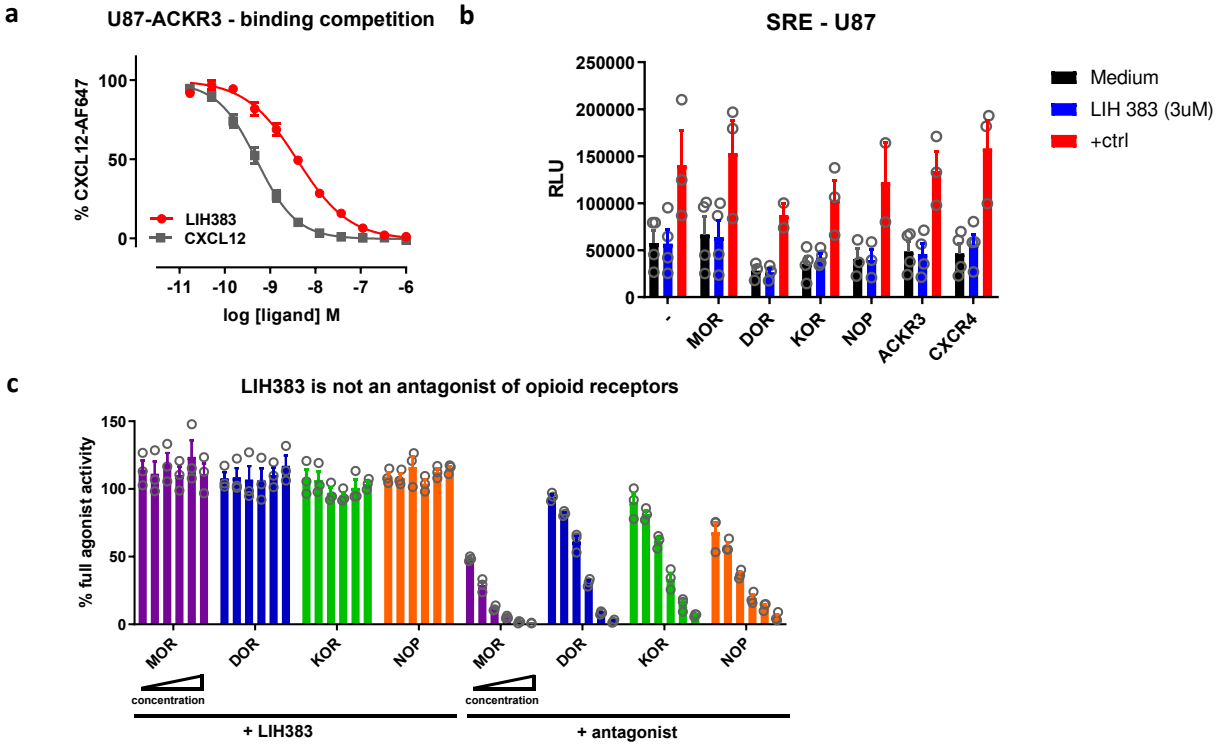
Supplementary Fig 1. Opioid peptide library screening in antagonist mode and confirmation of ACKR3 activation in different cell lines. (**a**) Antagonistic activity of 58 compounds including natural opioid peptides from the four opioid families, variants thereof and small molecule opioid receptor modulators (5 µM) towards ACKR3, CXCR4 and CXCR3 evaluated using β-arrestin-2 recruitment assay in U87 cells. For full peptide names and sequences, see supplementary Table 1. Antagonist activity was measured in the presence of CXCL12 (4 nM for ACKR3, 35 nM for CXCR4) or CXCL11 (50 nM for CXCR3) and results are presented as mean of two technical replicates for ACKR3 and CXCR4. A single measurement was performed for CXCR3. (**b-d**) Comparison of potency and efficacy of different opioid peptides in inducing β-arrestin-2 recruitment to ACKR3 in U87 cells (**b**) or β-arrestin-1 recruitment to ACKR3 in HEK cells (**c**) or in CHO cells (**d**). Results are presented as mean ± S.E.M of independent experiments (n = 3-5). The corresponding EC_{50} and Emax values are summarized in supplementary Table 9. Source data are provided as a Source Data file.



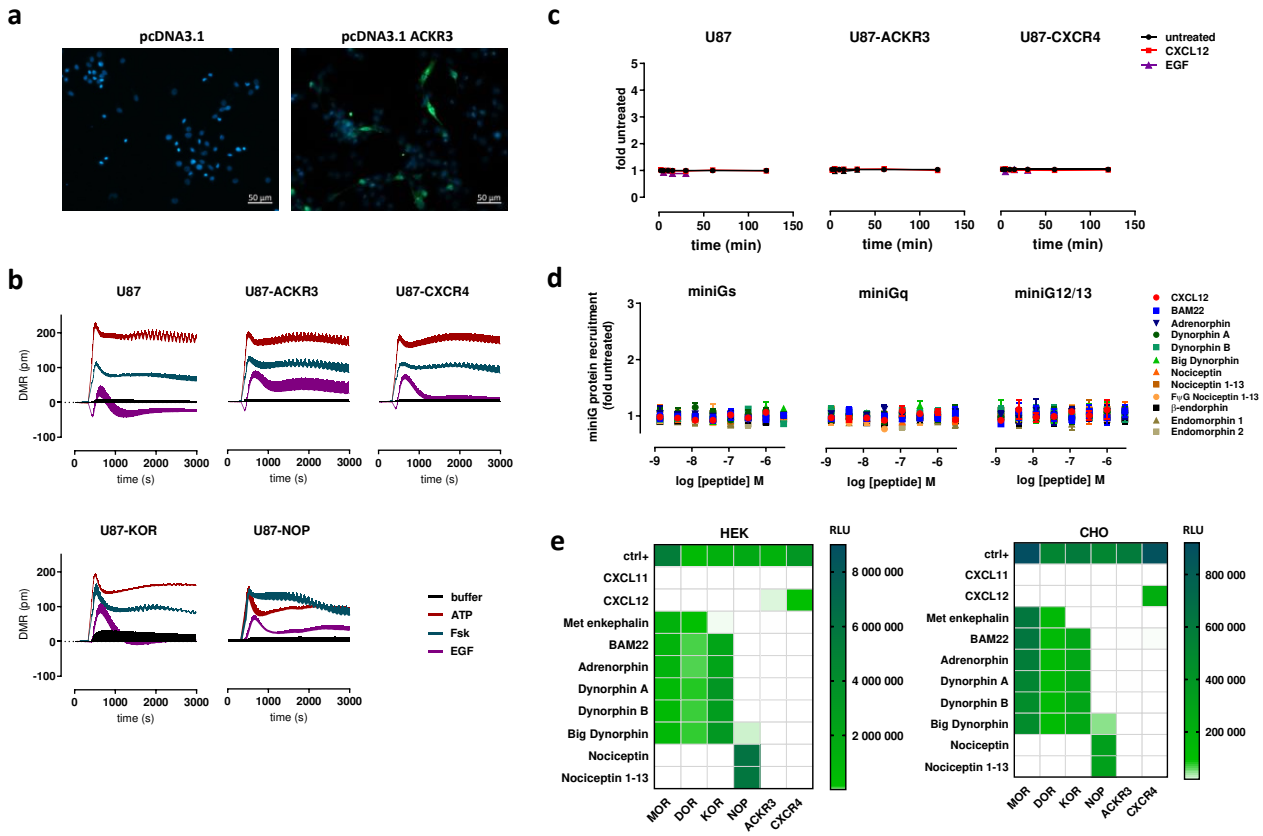
Supplementary Fig 2. Specific activation of ACKR3 by opioid peptides. (a) Agonist activity of opioid peptides (3 µM) representative of the four opioid families towards 18 classical and 3 atypical chemokine receptors evaluated in β-arrestin-2 recruitment assay. Results are expressed as the percentage of signal recorded with agonist chemokines used as positive controls (supplementary Table 3) at a concentration of 100 nM and presented as mean of independent experiments (n=3) (**b**) Comparison of potency and efficacy of big dynorphin with a full agonist chemokine inducing the recruitment of β-arrestin-1 (left) or β-arrestin-2 (right) to the chemokine receptors CXCR3A, CXCR3B, CX3CR1 or CCR3 in U87 cells. Results are expressed as fold change over untreated and presented as mean ± S.E.M of independent experiments (n =3). The corresponding EC_{50} and Emax values are summarized in supplementary Table 10. Source data are provided as a Source Data file.



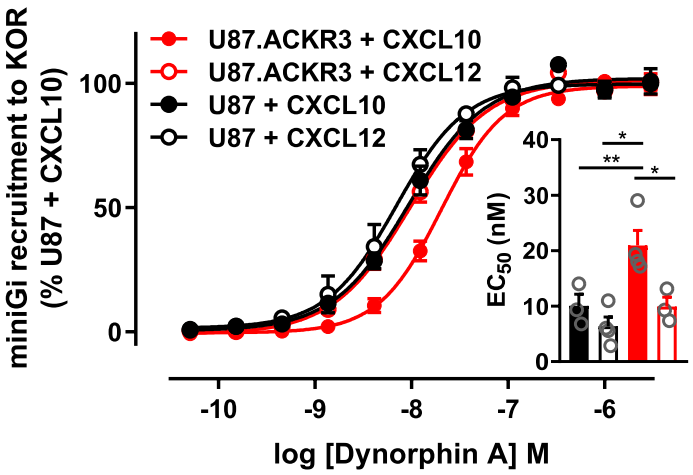
Supplementary Fig 3. Phylogenetic analysis and comparison of residues conserved in ACKR3, opioid and chemokine receptors. (**a**) Snake diagram depicting ACKR3 topology and identifying the positions occupied by residues conserved only in ACKR3 and CXCR4 (green), conserved in opioid receptors but not in ACKR3 (red), conserved separately in chemokine receptors and opioid receptors (green /red), conserved in at least three of the four opioid receptors and ACKR3 (blue) or in opioid receptors as well as ACKR3 and CXCR4 (grey) (GPCRdb). (**b**) Comparison of particular positions such as positions delineating the orthosteric binding pocket of opioid receptors (2x60, 3x32, 3x36, 6x52, 7x34, 7x38 and 7x45) or CXCR4 (2x63, 6x58 and 7x38), the bottom of TM6 (6x23 to 6x29), the top of TM7 (7x23 to 7x27) or key conserved position in chemokine receptors (7x38). (**c**) Phylogenetic tree of the human chemokine and opioid receptors showing that ACKR3 stands on a separate branch in between the chemokine and the opioid receptors (GPCRdb).



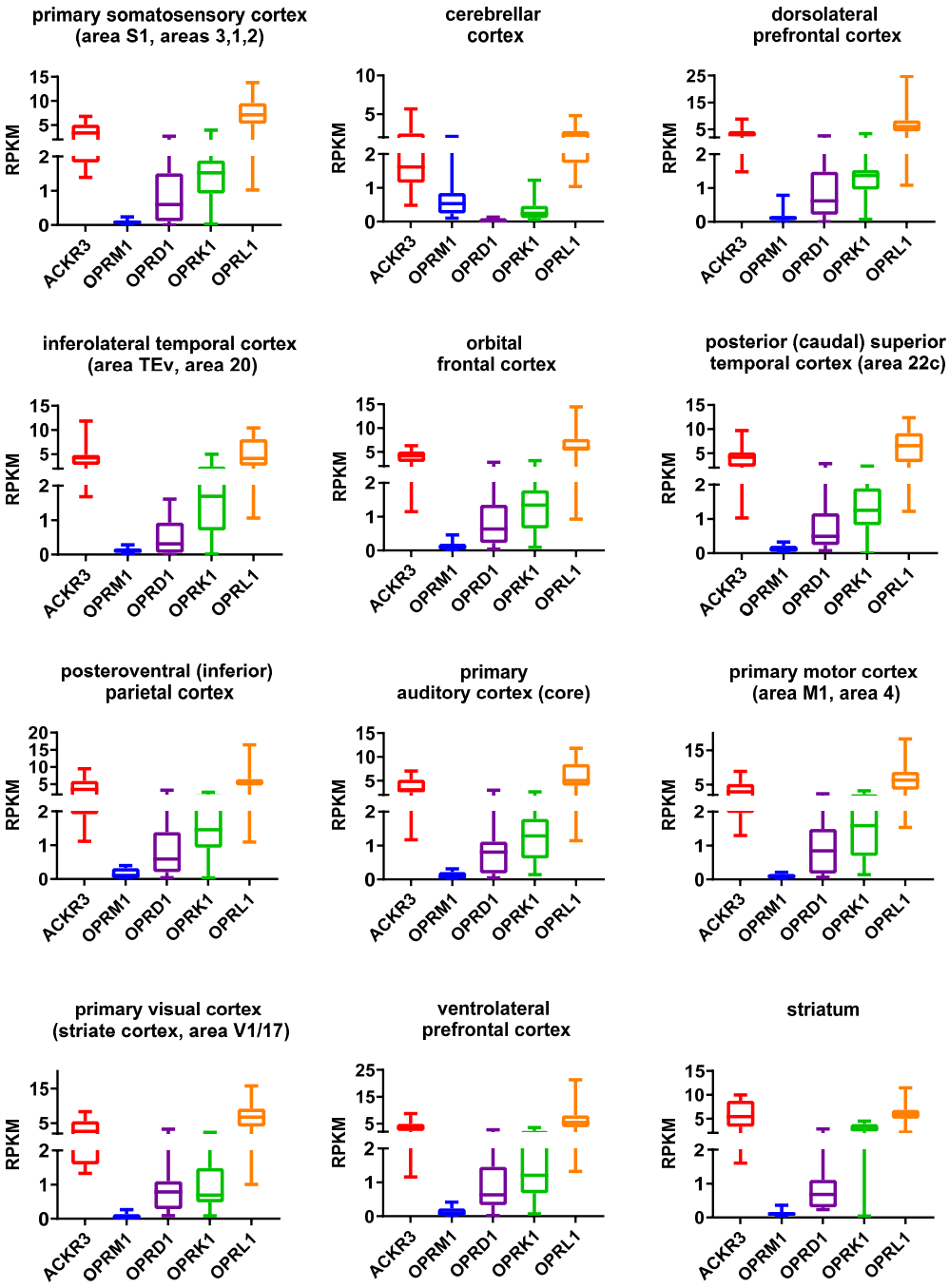
Supplementary Fig 4. Absence of activity and signaling by LIH383 towards classical opioid receptors and ACKR3-binding potency of LIH383. (**a**) Binding competition of increasing concentrations of ACKR3-activating CXCL12 or LIH383 with Alexa Fluor 647-labelled CXCL12 (5 nM) on U87-ACKR3 cells determined by flow cytometry. (**b**) Activation of SRE (ERK1/2) signaling cascade in U87 cells (-) or U87 expressing ACKR3, CXCR4 or classical opioid receptors (MOR, DOR, KOR or NOP) in response to 3 µM LIH383 or positive controls (+ctrl, 30 nM PMA, 10 % FBS). Medium only was used as reference. (**c**) Dose dependent antagonist activity of LIH383 (12.3-3000 nM) towards classical opioid receptors MOR, DOR, KOR and NOP determined by β-arrestin-1 recruitment. Naloxone (4.1-1000 nM) for MOR, DOR and KOR or buprenorphine (111-27000 nM) for NOP were used as positive control antagonists. Antagonist activity was measured following addition of BAM22 (50 nM), met-enkephalin (70 nM), dynorphin A (50 nM), nociceptin (70 nM) on MOR, DOR, KOR and NOP, respectively. Results are presented as mean ± S.E.M of independent experiments (n = 3-5). Source data are provided as a Source Data file.



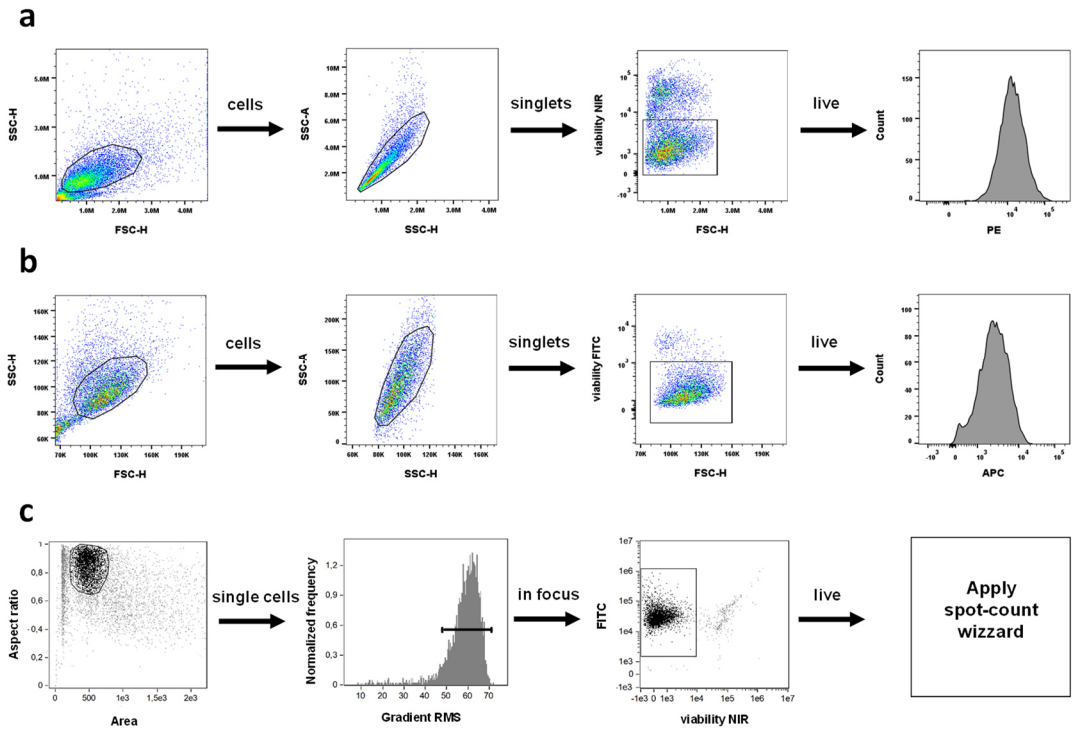
Supplementary Fig 5. Absence of biased/alternative ACKR3 signaling in response to opioid peptides in different cell lines. (**a**) Verification of effective ACKR3 transfection by fluorescence microscopy for DMR experiments. Adherent U87 cells transfected with pcDNA3.1-ACKR3 or an empty pcDNA3.1 vector were fixed and stained with an ACKR3-specific primary mouse antibody (clone 11G8) and a secondary Cy2-conjugated anti-mouse antibody (green). Nuclei were stained with DAPI (blue). Pictures are representative of three independent transfections used for DMR experiments. Scale bar: 50µm. (**b**) DMR profiles of U87 cells expressing (or not) ACKR3, CXCR4 or classical opioid receptors (KOR and NOP) stimulated with 100 µM of adenosine triphosphate (ATP), forskolin (Fsk) or epidermal growth factor (EGF) determined over 3000 seconds. Representative profiles of three to five independent experiments are shown. (**c**) Kinetic analysis of total ERK in U87 cells stably expressing ACKR3 or CXCR4 stimulated by CXCL12 or EGF. Results represent the mean of independent experiments (n=2). **(d)** mini Gs, mini Gq and miniG12/13 recruitment to ACKR3 in response to CXCL12 and opioid peptides monitored in U87 cells. Results represent the mean ± S.E.M of independent experiments (n = 3-5). (**e**) Activation of SRE (ERK1/2) signaling cascade in HEK (left panel) or CHO (right panel) cells expressing or not ACKR3, CXCR4 or classical opioid receptors MOR DOR KOR or NOP in response to chemokines CXCL12 and CXCL11, opioid peptides or positive controls (30 nM PMA, 10 % FBS). Results represent the mean of independent experiments (n = 3). Source data are provided as a Source Data file.



Supplementary Fig 6. Regulation by ACKR3 of opioid peptide availability for classical receptors. Dynorphin A-induced concentration-dependent recruitment of mini Gi to KOR in the presence of tenfold excess of U87 or U87-ACKR3 cells (1.5 x 10^5) pre-treated for 15 minutes with CXCL12 (200nM) to block ACKR3, or the irrelevant chemokine CXCL10 (200nM). The determined EC_{50} values are represented as bars in the inset. Results are presented as mean ± S.E.M of independent experiments (n=3 for U87 + CXCL10 and U87 ACKR3 + CXCL12 and n=4 for both other conditions). * $p< 0.05$, ** $p< 0.01$ by Two-way ANOVA: interaction between cell line and chemokine treatment with Tukey's post hoc test. Source data and statistical analysis parameters are provided as a Source Data file.



Supplementary Fig 7. Relative gene expression of ACKR3 and classical opioid receptors in different brain regions. RNA-Seq RPKM (reads per kilobase per million) values from the open-source brainspan.org database containing data of different brain regions from 14–22 donors (n=14 for striatum, n=15 for dorsolateral prefrontal cortex, n=16 for primary somatosensory cortex and for primary motor cortex, n=17 for orbital frontal cortex and for primary auditory cortex, n=18 for primary visual cortex, n=19 for ventrolateral prefrontal cortex and for posteroventral parietal cortex, n=21 for cerebellar cortex and for inferolateral temporal cortex, n=22 for posterior superior temporal cortex). Box plots encompass 25th to 75th percentile with median as central mark and whiskers extending to most extreme data points.



Supplementary Fig 8. Gating strategy used in experiments to determine receptor surface expression, LIH383-Cy5 and CXCL12-AF647 binding and opioid peptide internalization. (a) Gating strategy for two-color flow cytometry analysis of receptor surface expression reported in figure 6g. After ligand treatment followed by an acid wash, cells were incubated with receptor-specific mAbs (clones 11G8 for ACKR3 and 387301 for KOR) and a secondary PE-conjugated $F(ab')_2$ fragment anti-mouse IgG. Dead cells were excluded using a Zombie NIR viability dye (near infrared). **(b)** Gating strategy for two-color flow cytometry analysis to determine CXCL12-AF647 or LIH-Cy5 binding presented in figure 1g, figure 4f and supplementary figure 4a. Cells were treated with either LIH383-Cy5 or a mixture of CXCL12-AF647 and unlabeled ligand. Dead cells were excluded with Zombie Green viability dye (FITC) and signal was recorded in APC channel. **(c)** Gating strategy for two-color imaging flow cytometry to evaluate opioid peptide uptake and presence of individual intracellular fluorescent spots represented in figure 6a, figure 6b and figure 7e. Gates were set to include only single, in-focus cells. Dead cells were excluded with the Zombie NIR viability dye for FAM-labeled peptides or with Zombie Green viability dye for Cy5-labeled peptides. Geometrical MFI from FITC channel for FAM-labeled peptides, or from APC channel for Cy-5 labeled peptides was determined. The number of spots per cell was determined using a mask-based software wizard.

Supplementary Table 1. Sequences, full names and family of endogenous opioid peptides and variants thereof used in experiments reported in Figure 1A and supplementary Figure 1A.

	Name/Abbreviation used in Fig 1A	Full name and species (as indicated by phoenixpeptide.com)	sequence (three letter code)
Dynorphins	Big Dynorphin	Dynorphin, big (Human, Rat, Mouse, Porcine)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
	Dynorphin A	Dynorphin A (Human, Rat, Mouse, Porcine)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
	Dynorphin A (1-6)	Dynorphin A (1-6) / Endorphin (1-6), alpha-Neo / Leu-Enkephalin-Arg (Human, Rat, Mouse, Porcine)	Tyr-Gly-Gly-Phe-Leu-Arg
	Dynorphin A (1-13)	Dynorphin A (1-13) (Human, Rat, Mouse, Porcine)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys
	Dynorphin A (2-13) NH2	Dynorphin A (2-13) amide (Human, Rat, Mouse, Porcine)	Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-NH2
	Dynorphin A (2-17)	Dynorphin A (2-17) (Human, Rat, Mouse, Porcine)	Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
	Dynorphin B	Dynorphin B / Rimorphin (Human, Rat, Mouse, Porcine)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
	α-Neoendorphin	Endorphin, alpha-Neo (Porcine)	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys
	β-Neoendorphin	Endorphin, beta-neo	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro
Enkephalins	Leu-Enkephalin	Leu-Enkephalin	Tyr-Gly-Gly-Phe-Leu
	Met-Enkephalin	Met-Enkephalin	Tyr-Gly-Gly-Phe-Met
	Met-Enkephalin (2-5)	[Des-Tyr1]-Met-Enkephalin	Gly-Gly-Phe-Met
	Met-Enkephalin-R	Met-Enkephalin-Arg	Tyr-Gly-Gly-Phe-Met-Arg
	Met-Enkephalin-R-F	Met-Enkephalin-Arg-Phe	Tyr-Gly-Gly-Phe-Met-Arg-Phe
	Adrenorphin	Metorphinamide / Adrenorphin	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-NH2
	BAM18	BAM-18P (Bovine)	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln
	BAM22	BAM-22P (Bovine)	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly
	Peptide E	Peptide E / 3200-Dalton Adrenal Enkephalin-Containing Peptide (Bovine)	Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu
	Amidorphin	pro-Enkephalin A (104-129) amide / Amidorphin (Bovine)	Tyr-Gly-Gly-Phe-Met-Lys-Lys-Met-Asp-Glu-Leu-Tyr-Pro-Leu-Glu-Val-Glu-Glu-Glu-Ala-Asn-Gly-Gly-Glu-Val-Leu-NH2
	DADLE	[D-Ala2, D-Leu5]-Enkephalin	Tyr-D-Ala-Gly-Phe-D-Leu
	DPDPE	[D-Pen2, D-Pen5]-Enkephalin	Tyr-D-Pen-Gly-Phe-D-Pen Disulfide bridge: Pen2-Pen5
	DSLET	[D-Ser2]-Leu-Enkephalin-Thr / Delta-Receptor Peptide / DSLET	Tyr-D-Ser-Gly-Phe-Leu-Thr
	DTLET	[D-Thr2]-Leu-Enkephalin-Thr / DTLET	Tyr-D-Thr-Gly-Phe-Leu-Thr
Nociceptins	Nociceptin	Orphanin FQ / Nociceptin (Human, Rat, Mouse, Ox)	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln
	Nociceptin (1-13) NH2	Orphanin FQ (1-13) amide / Nociceptin (1-13) amide (Human, Rat, Mouse, Ox)	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH2
	[F-ψ-G]Nociceptin(1-13) NH2	[Phe-psi-Gly]-Orphanin FQ (1-13) amide / [Phe-psi-Gly]-Nociceptin (1-13) amide (Human, Rat, Mouse, Ox)	[Phe1ψ(CH2-NH)-Gly2]-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH2
	pro-Nociceptin (85-119)	pro-Orphanin FQ (85-119) / pro-Nociceptin (85-119) / Nocistatin-35 (Rat)	Met-Pro-Arg-Val-Arg-Ser-Val-Val-Gln-Ala-Arg-Asp-Ala-Glu-Pro-Glu-Ala-Asp-Ala-Glu-Pro-Val-Ala-Asp-Glu-Ala-Asp-Glu-Val-Glu-Gln-Lys-Gln-Leu-Gln
	pro-Nociceptin (141-157)	pro-Orphanin FQ (141-157) / pro-Nociceptin (141-157)	Phe-Ser-Glu-Phe-Met-Arg-Gln-Tyr-Leu-Val-Leu-Ser-Met-Gln-Ser-Ser-Gln
	prepro-Nociceptin (154-181)	prepro-Orphanin FQ (154-181), free acid (Rat) / prepro-Orphanin FQ (160-187) free acid (Mouse)	Phe-Ser-Glu-Phe-Met-Arg-Gln-Tyr-Leu-Val-Leu-Ser-Met-Gln-Ser-Ser-Gln-Arg-Arg-Arg-Thr-Leu-His-Gln-Asn-Gly-Asn-Val
	Nocistatin	prepro-Orphanin FQ (111-127) / Nocistatin / PNP-3 (Bovine)	Thr-Glu-Pro-Gly-Leu-Glu-Glu-Val-Gly-Glu-Ile-Glu-Gln-Lys-Gln-Leu-Gln
	Nocistatin-30	prepro-Orphanin FQ (98-127) / Nocistatin-30 (Human)	Met-Pro-Arg-Val-Arg-Ser-Leu-Phe-Gln-Glu-Gln-Glu-Glu-Pro-Glu-Pro-Gly-Met-Glu-Glu-Ala-Gly-Glu-Met-Glu-Gln-Lys-Gln-Leu-Gln-
	PNP-3-8P	PNP-3-8P (Bovine)	Glu-Ile-Glu-Gln-Lys-Gln-Leu-Gln
	Nocistatin-41	PNP-2/3 / Nocistatin-41 (Mouse)	Met-Pro-Arg-Val-Arg-Ser-Leu-Val-Gln-Val-Arg-Asp-Ala-Glu-Pro-Gly-Ala-Asp-Ala-Glu-Pro-Gly-Ala-Asp-Ala-Glu-Pro-Gly-Ala-Asp-Asp-Ala-Glu-Glu-Val-Glu-Gln-Lys-Gln-Leu-Gln
Endorphins	β-Endorphin	Endorphin, beta (Human)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
	β-Endorphin (2-31)	Endorphin, beta (2-31) (Human)	Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-
	β-Endorphin (1-26)	Endorphin, beta (1-26) (Human)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala
	β-Endorphin (1-27)	Endorphin, beta (1-27) (Human)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr
	β-Endorphin (rat)	Endorphin, beta (Rat)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Val-His-Lys-Lys-Gly-Gln
	Ac - β-Endorphin (1-26)	acetyl-Endorphin, beta (1-26) (Human)	Ac-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala
	γ-Endorphin	Endorphin, gamma / Lipotropin, beta (61-77) (Human, Bovine, Ovine, Camel)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu
Endomorphins	Endomorphin-1	Endomorphin-1	Tyr-Pro-Trp-Phe-NH2
	Endomorphin-2	Endomorphin-2	Tyr-Pro-Phe-Phe-NH2
Others	Thiorphan	Thiorphan / D,L-3-mercapto-2-benzylpropanoyl-Glycine	DL3-Mercapto-2-Benzylpropanoyl-Glycine
	Spinorphin	Spinorphin (Bovine)	Leu-Val-Val-Tyr-Pro-Trp-Thr
	Kyotorphin	Kyotorphin	Tyr-Arg
	Morphiceptin	Casomorphin (1-4), amide beta / Morphiceptin	Tyr-Pro-Phe-Pro-NH2
	Morphiceptin PL017	[N-MePhe, D-Pro4]-Morphiceptin / PL017	Tyr-Pro-MePhe-D-Pro-NH2
	Deltrophin	Dermenkephalin / Deltrophin (Phyllomedusa sauvageii)	Tyr-D-Met-Phe-His-Leu-Met-Asp-NH2
	[D-Ala2]-Deltorphin I	[D-Ala2]-Deltorphin I	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH2
	[D-Ala2]-Deltorphin II	[D-Ala2]-Deltorphin II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH2
	Hemorphin-4	Hemorphin-4 (H-4) (Human, Bovine)	Tyr-Pro-Trp-Thr
	TIPP	TIPP / delta-Opioid Antagonist	Tyr-Tic-Phe-Phe
	[Y0-W2]-MIF-1	[Tyr0, Trp2]-Melanocyte Inhibiting Factor 1 (MIF-1)	Tyr-Pro-Trp-Gly-NH2
	[Y0]-MIF-1	[Tyr0]-Melanocyte Inhibiting Factor 1 (MIF-1)	Tyr-Pro-Leu-Gly-NH2
	β-Casomorphin 5	Casomorphin (1-5), beta	Tyr-Pro-Phe-Pro-Gly
	CTOP amide	CTOP amide (Cys2, Tyr3, Orn5, Pen7) / Somatostatin Analog	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2 Disulfide bridge: Cys2-Pen7
	CTAP amide	CTAP amide (Cys2, Tyr3, Arg5, Pen7)	D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 Disulfide bridge: Cys2-Pen7
	TGF-1, alpha	Transforming Growth Factor 1 (TGF-1), alpha (Rat)	Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol

Supplementary Table 2. Sequences of selected opioid peptides and their binding competition with CXCL12-AF647 on ACKR3 reported in Figure 1g and supplementary Figure 4a

Name	Sequence	Binding	
		ACKR3	
		IC_{50} (CI) nM	Min %
Dynorphin A	**YGGF**LRRIRPKLKWDNQ	85.3 (64.2-118)	6
Dynorphin A 2-17	-**GGF**LRRIRPKLKWDNQ	~10,000	55*
Dynorphin A 2-13-NH2	-**GGF**LRRIRPKLK-NH2	> 10,000	75*
Dynorphin A 1-13	**YGGF**LRRIRPKLK	122 (96.3 - 162)	9
Dynorphin B	**YGGF**LRRQFKVVT	~1000	33
Leumorphin	**YGGF**LRRQFKVVTRSQEDPNAYSGELFDA	~6000	41*
Big Dynorphin	**YGGF**LRRIRPKLKWDNQKR**YGGF**LRRQFKVVT	32.8 (25.4 - 41.9)	4
Adrenorphin	**YGGF**MRRV-NH2	41.2 (28.2 - 60.1)	7
BAM22	**YGGF**MRRVGRPEWWMDYQKRYG	31.3 (27.6 - 35.5)	3
Met enkephalin	**YGGF**M	NB	110*
Nociceptin	F**GGF**TGARKSARKLANQ	NB	106*
Nociceptin 1-13-NH2	F**GGF**TGARKSARK-NH2	> 10,000	78*
[Phe1Ψ(CH2-NH)-gly2]nociceptin-(1-13)-NH2	[Fψ(CH2-NH)G]GFTGARKSARK-NH2	> 10,000	71*
Endomorphin-1	YPWF-NH2	> 10,000	81*
Endomorphin-2	YPFF-NH2	NB	102*
β-endorphin	**YGGF**MTSEKSQTPLVTLFKNAIIKNAYKKGE	NB	125*
LIH383	F**GGF**MRRK-NH2	4.0 (3.1 - 5.1)	1

IC_{50} values are indicated in nanomolar (nM) with 95 % confidence interval (CI)

NB: No binding competition with 5 nM CXCL12-AF647

Min = minimal remaining AF647 signal measured at 3 µM non-labeled competitor and expressed as % of signal measured for 5 nM CXCL12-AF647 without competitor.

*measured at 9 µM

YGGF motif conserved in most of the opioid peptides is shown in bold. Positively charged residues are underlined

Supplementary Table 3. Activity of positive controls used in Figure 2 and Supplementary Figure 2a

Receptor	Positive ctrl	β-arrestin-1	β-arrestin-2
		Fold untreated ± SEM	Fold untreated ± SEM
CCR1	CCL3	2.01 ± 0.35	1.64 ± 0.14
CCR2B	CCL2	9.49 ± 1.69	7.15 ± 1.05
CCR3	CCL13	2.13 ± 0.19	1.88 ± 0.15
CCR4	CCL22	8.20 ± 0.64	5.49 ± 0.14
CCR5	CCL5	17.00 ± 0.77	9.07 ± 0.24
CCR6	CCL20	13.26 ± 4.37	6.86 ± 0.52
CCR7	CCL19	7.45 ± 1.27	5.94 ± 0.77
CCR8	CCL1	2.91 ± 0.26	1.56 ± 0.28
CCR9	CCL25	5.71 ± 1.38	5.69 ± 1.63
CCR10	CCL27	4.97 ± 1.61	3.05 ± 0.52
CXCR1	CXCL8	30.77 ± 5.68	14.54 ± 1.08
CXCR2	CXCL8	2.46 ± 0.44	2.20 ± 0.24
CXCR3A	CXCL11	8.16 ± 0.62	3.30 ± 0.17
CXCR3B	CXCL11	6.05 ± 0.93	3.49 ± 0.36
CXCR4	CXCL12	1.69 ± 0.04	1.37 ± 0.09
CXCR5	CXCL13	4.85 ± 0.28	3.78 ± 0.10
CXCR6	CXCL16	29.50 ± 5.45	16.52 ± 1.85
XCR1	XCL2	4.75 ± 1.52	3.26 ± 1.07
CX3CR1	CX3CL1	10.24 ± 1.72	8.66 ± 0.86
ACKR2	CCL5	1.42 ± 0.09	1.33 ± 0.11
ACKR3	CXCL12	3.03 ± 0.23	2.79 ± 0.36
ACKR4	CCL19	6.02 ± 0.77	4.39 ± 0.54

Values correspond to fold increase over untreated and represent the mean of three independent experiments ± standard error of the mean.

Supplementary Table 4. Sequences of adrenophin-derived peptides and activity in β-arrestin-1 recruitment towards human opioid receptors reported in Figure 3.

Sequence	Position	Receptor			
		MOR	DOR	KOR	ACKR3
		EC_{50} (CI) nM	EC_{50} (CI) nM	EC_{50} (CI) nM	EC_{50} (CI) nM
YGGFMRRV	Ref.	101 (83.0 - 125)	323 (258 - 409)	65.6 (56.5 – 75.9)	109 (70.7 – 182)
KGGFMRRV	1	NA	NA	NA	NA
AGGFMRRV	1	NA	NA	NA	>10,000
FGGFMRRV	1	NA	NA	NA	9.33 (5.12 – 16.0)
NGGFMRRV	1	NA	NA	NA	NA
LGGFMRRV	1	NA	NA	NA	261 (nc – 573)
Y**A**GFMRRV	2	1094 (776 – 4207)	2317 (nc – 75020)	NA	611 (397 – 1052)
Y**S**GFMRRV	2	NA	NA	NA	~10,000
Y**R**GFMRRV	2	NA	NA	NA	~5,000
YG**A**FMRRV	3	>10,000	1581 (nc – 4986)	315 (253 – 392)	263 (163 – 425)
YG**S**FMRRV	3	>10,000	>10,000	>10,000	668 (465 – 1049)
YGG**Y**MRRV	4	NA	NA	NA	~5,000
YGG**W**MRRV	4	522 (361 – 725)	1018 (844 – 1436)	>10,000	70.3 (38.3 – 136)
YGG**L**MRRV	4	NA	NA	NA	~10,000
YGG**A**MRRV	4	NA	NA	NA	~10,000
YGGFLRRV	5	880 (624 – 1572)	513 (403 – 639)	31.9 (28.4 – 25.8)	684 (368 – 1682)
YGGF**A**RRV	5	>10,000	>10,000	656 (555 -775)	~5,000
YGGF**K**RRV	5	NA	NA	NA	~10,000
YGGF**Q**RRV	5	~5000	>10,000	283 (221 – 366)	~5,000
YGGF**F**RRV	5	363 (225 – nc)	1313 (nc – 5420)	194 (81.3 – 900)	1535 (nc – 4196)
YGGF**N**RRV	5	NA	NA	508 (441 – 586)	~10,000
YGGFM**A**RV	6	35.4 (27.7 – 45.2)	147 (98.5 – 238)	2238 (nc – 12290)	~5,000
YGGFM**K**RV	6	63.8 (50.6 – 79.3)	360 (248 – 533)	416 (355 – 485)	~5,000
YGGFMR**A**V	7	682 (227 – nc)	308 (214 – 461)	1250 (1001 – 2131)	NA
YGGFMR**K**V	7	786 (567 – 1250)	378 (260 – 565)	230 (212 – 251)	~10,000
YGGFMRR**L**	8	294 (235 – 366)	254 (184 – 354)	71.2 (59.6 – 84.5)	153 (104 – 242)
YGGFMRR**I**	8	188 (nc – 260)	320 (215 – 470)	76.9 (55.9 – 105)	115 (81.1 – 177)
YGGFMRR**A**	8	265 (nc – 448)	260 (nc – 427)	132 (59.8 – 343)	201 (109 – 478)
YGGFMRR**F**	8	198 (177 – 220)	212 (171 – 261)	80.9 (66.9 – 96.5)	73.5 (45.2 – 119)
YGGFMRR**K**	8	252 (187 – 342)	527 (383 – 713)	19.2 (14.9 – 24.8)	37.0 (19.1 – 75.0)
YGGFMRR**Q**	8	183 (143 – 240)	259 (180 – 381)	63.1 (53.0 – 74.6)	110 (43.6 – 499)
- GGFMRRV	0	NA	NA	NA	>10,000
AYGGFMRRV	0	308 (238 – 396)	637 (240 – 19890)	205 (157 – 270)	330 (nc – 1394)
YGGFMRRV**A**	9	174 (nc – 270)	272 (186 – 403)	57.5 (49.6 – 66.2	72.3 (50.7 – 101)
YGGFMRRV**R**	9	58.7 (46.1 – 73.5)	193 (136 – 283)	21.9 (20.3 – 23.7)	12.6 (7.47 – 21.2)
yggfmrrv	Daa	NA	NA	NA	>10.000
(YGGFMRRVC)2	dimer	271 (188 – 389)	253 (154 – 337)	79.4 (44.7 – 138)	52.9 (17.9 – 173)

EC_{50} values are indicated in nanomolar (nM) with 95 % confidence interval (CI)

NA: No activity or activity below 10 % of positive control in the concentration range tested

nc; not calculable

Note that all peptides showed no activity towards NOP

Peptides presented in Figure 3 b-e are marked in red

Supplementary Table 5. Potencies of peptide variants combining mutation Y1F with other modifications in β-arrestin-1 recruitment to ACKR3.

2nd generation peptides	ACKR3	
Sequence	**EC50 (nM)**	**pEC50 ± SEM**
FGGFMRR**K** (LIH383)	0.61	9.21 ± 0.17
FGGFMRR**KR**	1.12	8.96 ± 0.20
FGGFMRRV**R**	3.53	8.45 ± 0.10
FGG**W**MRR**K**	4.39	8.36 ± 0.12
FGG**W**MRRV**R**	6.16	8.21 ± 0.14
FGG**W**MRR**KR**	7.34	8.14 ± 0.13
FGG**W**MRRV	10.52	7.98 ± 0.62
FGGFMRR**F**	10.96	7.96 ± 0.29
FGGFMRR**FR**	16.34	7.79 ± 0.24
FGG**W**MRR**FR**	19.07	7.72 ± 0.22
FGG**W**MRR**F**	19.10	7.72 ± 0.20
FGGFMRRV (Y1F adrenorphin)	9.33	8.03 ± 0.11
YGGFMRRV (adrenorphin)	108.7	6.96 ± 0.09
MRRKFGGF (LIH383 ctrl)	inactive	

Modified amino acids with respect to peptide Y1F-adrenorphin (FGGFMRRV) are underlined and in bold

Supplementary Table 6. Activity of LIH383, adrenorphin and chemokines in β-arrestin-1 recruitment to human and mouse ACKR3 and classical human opioid receptors reported in Figure 4 b-f

Name	β-arrestin-1											
	hACKR3		MOR		DOR		KOR		NOP		mACKR3	
	EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %
LIH383	0.61 (0.19 – 1.17)	99	NA	1	NA	1	NA	9	NA	1	2.31 (0.008 – 7.56)	95
LIH383 Ctrl	NA	4	-	-	-	-	-	-	-	-	ND	17
CXCL12	1.21 (0.83 – 1.74)	100	-	-	-	-	-	-	-	-	-	-
CXCL11	2.20 (0.78 – 4.81)	75	-	-	-	-	-	-	-	-	-	-
Adrenorphin	56.5 (36.6 – 95.0)	96	41.6 (30.7 – 57.7)	96	157 (104 – 310)	96	43.5 (33.7 – 57.9)	98	NA	3	-	-
mCXCL12	-	-	-	-	-	-	-	-	-	-	3.32 (1.17 – 33.6)	100
LIH383-Cy5	6.65 (5.52 – 8.29)	100	-	-	-	-	-	-	-	-	-	-

EC_{50} values are indicated in nanomolar (nM) with 95 % confidence interval (CI)

ND: Not determinable since saturation was not reached

NA: No activity or activity below 10 % of positive control in the concentration range tested

Max = maximum signal measured at 3 µM expressed as % of the reference peptide

- = not analyzed

Supplementary Table 7. Activity of selected opioid peptides in mini Gi recruitment to ACKR3, MOR, KOR, DOR and NOP reported in Figure 5e.

Name	Sequence	mini Gi									
		ACKR3		MOR		DOR		KOR		NOP	
		EC50 (CI) nM	Max %	EC50 (CI) nM	Max %	EC50 (CI) nM	Max %	EC50 (CI) nM	Max %	EC50 (CI) nM	Max %
CXCL12		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
BAM22	**YGGF**MRRVGRPEWWMDYQKRYG	NA	NA	17.8 (12.2 – 26.5)	100	1324 (547 - nc)	63	-	-	-	-
Adrenorphin	**YGGF**MRRV-NH2	NA	NA	38.0 (14.23 – 43840)	110	315 (92.6 - nc)	89	-	-	-	-
Met enkephalin	**YGGF**M	NA	NA	170 (97.7 – 453)	89	39.4 (11.7 - nc)	100	-	-	-	-
Dynorphin A	**YGGF**LRRIRPKLKWDNQ	NA	NA	-	-	-	-	11.8 (6.49 – 22.0)	100	-	-
Dynorphin B	**YGGF**LRRQFKVVT	NA	NA	-	-	-	-	6.06 (1.99 – 14.8)	95	-	-
Big Dynorphin	**YGGF**LRRIRPKLKWDNQKR**YGGF**LRRQFKVVT	NA	NA	-	-	-	-	16.1 (8.98 – 28.7)	94	-	-
Nociceptin	F**GGF**TGARKSARKLANQ	NA	NA	-	-	-	-	-	-	5.97 (2.45 – 12.3)	100
Nociceptin 1-13-NH2	F**GGF**TGARKSARK-NH2	NA	NA	-	-	-	-	-	-	1.06 (0.008 – 2.97)	108
[Phe1Ψ(CH2-NH)-gly2]nociceptin-(1-13)-NH2	[Fψ(CH2-NH)G]GFTGARKSARK-NH2	NA	NA	-	-	-	-	-	-	32.2 (21.6 – 53.8)	33
β-endorphin	**YGGF**MTSEKSQTPLVTLFKNAIIKNAYKKGE	NA	NA	1021 (342 - nc)	64	931 (208 - nc)	70	-	-	-	-
Endomorphin-1	YPWF-NH2	NA	NA	162 (37.9 - nc)	66	-	-	-	-	-	-
Endomorphin-2	YPFF-NH2	NA	NA	175 (53.4 - nc)	63	-	-	-	-	-	-

EC_{50} values are indicated in nanomolar (nM) with 95 % confidence interval (CI)

NA: No activity or activity below 10 % of positive control in the concentration range tested

Max = maximum signal measured at 3 µM expressed as % of the reference peptide

nc: not calculable

- = not analyzed

YGGF motif conserved in most of the opioid peptides is shown in bold. Positively charged residues are underlined

Supplementary Table 8. Sequences and annealing temperatures of qPCR primers used for receptor mRNA quantification in human brain samples and smNPC represented in Figure 7b and 7c.

Gene	T_a	Primer Sequence	Reference
ACKR3	57°C	F: 5'-TCGGCAGCATTTTCTTCCTC-3' R: 5'-GCAGTAGGTCTCATTGTTGGAC-3'	*Ikeda et al, 2013*[1]
OPRD	60°C	F: 5'-TCGGGCTCCAAGGAGAAG-3' R: 5'-TCGTCGAGGAAAGCGTAGA-3'	*Ikeda et al, 2013*[1]
OPRK	61°C	F: 5'-CTGCATCTGGCTGCTGTCGT-3' R: 5'-GACGCAGGATCATCAGGGTGTAG-3'	*Ikeda et al, 2013*[1]
OPRM	58°C	F: 5'-CCGTGTGCTATGGACTGA-3' R: 5'-GAAATGCATAAAGGACTGGGTT-3'	*Ikeda et al, 2013*[1]
OPRL	62°C	F: 5'-CTCGGCTGGTGCTGGTGGTA-3' R: 5'-CGTGCAGAAGCGCAGAATGG-3'	*Li et al, 2013*[2]
PPIA	60°C	F: 5'-TCCTGGCATCTTGTCCATG-3' R: 5'-CCATCCAACCACTCAGTCTTG-3'	*Schote et al, 2007*[3]
GAPDH	60°C	F: 5'-GAAGGTGAAGGTCGGAGTC-3' R: 5'-GAAGATGGTGATGGGATTTC-3'	*Schote et al, 2007*[3]

All primer pairs have been validated and used in previous studies (see references[1, 2, 3])

Supplementary Table 9. Sequences of selected opioid peptides and their activity in β-arrestin-1 or β-arrestin-2 recruitment to ACKR3 in different cell lines reported in supplementary Figure 1 b-d.

Name	Sequence	β-arrestin-2		β-arrestin-1			
		ACKR3-U87		ACKR3-HEK		ACKR3-CHO	
		EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %	EC_{50} (CI) nM	Max %
Dynorphin A	**YGGF**LRRIRPKLKWDNQ	97.6 (70.5 – 138)	111	110 (97.2 – 125)	89	70.5 (54.5 – 93.9)	107
Dynorphin B	**YGGF**LRRQFKVVT	908 (373 - nc)	88	673 (572 – 831)	74	~1000	96
Leumorphin	**YGGF**LRRQFKVVTRSQEDPNAYSGELFDA	902 (342 - nc)	92*	2708 (1302 – 92760)	65*	1723 (700 - nc)	88*
Big Dynorphin	**YGGF**LRRIRPKLKWDNQKR**YGGF**LRRQFKVVT	76.6 (54.9 – 109.2)	132	60.7 (52.9 – 69.8)	87	47.5 (36.1 – 61.5)	100
Adrenorphin	**YGGF**MRRV-NH2	81.5 (40.1 – 342)	93	72.6 (51.1 – 114)	69	22.5 (15.0 – 34.8)	81
BAM22	**YGGF**MRRVGRPEWWMDYQKRYG	22.9 (15.4 – 35.6)	106	23.2 (17.8 – 30.6)	75	12.5 (9.10 – 19.6)	93
Met enkephalin	**YGGF**M	ND	13*	NA	0*	NA	1*
Nociceptin	F**GGF**TGARKSARKLANQ	>10,000	43*	ND	24*	~10,000	55*
Nociceptin 1-13-NH2	F**GGF**TGARKSARK-NH2	~10,000	68*	~10,000	47*	~10,000	65*
[Phe1Ψ(CH2-NH)-gly2]nociceptin-(1-13)-NH2	[Fψ(CH2-NH)G]GFTGARKSARK-NH2	4344 (1429 - nc)	92*	3761 (2339 – 9706)	82*	~5000	97*
Endomorphin-1	YPWF-NH2	ND	34*	ND	13*	ND	17*
Endomorphin-2	YPFF-NH2	ND	24*	ND	7*	ND	3*
β-endorphin	**YGGF**MTSEKSQTPLVTLFKNAIIKNAYKKGE	ND	28*	ND	5*	ND	7*

EC_{50} values are indicated in nanomolar (nM) with 95 % confidence interval (CI)

ND: Not determinable since saturation was not reached

NA: No activity or activity below 10 % of positive control in the concentration range tested

nc: not calculable

Max = maximum signal measured at 3 µM expressed as % of signal measured for CXCL12

*measured at 9 µM

YGGF motif conserved in most of the opioid peptides is shown in bold. Positively charged residues are underlined

Supplementary Table 10. Off-target activity of selected opioid peptides in β-arrestin-1 and β-arrestin-2 recruitment towards human chemokine receptors reported in supplementary Figure 2b

Receptor	β-arrestin-1				β-arrestin-2			
	Positive Ctrl		Big Dynorphin		Positive Ctrl		Big Dynorphin	
	chemokine – EC_{50} (CI) nM	Max	EC_{50} (CI) nM	Max	chemokine – EC_{50} (CI) nM	Max	EC_{50} (CI) nM	Max
CXCR3A	CXCL11 – 5.14 (2.03 – 72.7)	6.93	ND	1.83	CXCL11 – 8.65 (4.26 – 57.1)	3.65	ND	1.43
CXCR3B	CXCL11 – 25.6 (16.1 – 61.8)	6.71	ND	1.57	CXCL11 – 40.2 (14.1 – nc)	3.40	ND	1.42
CX3CR1	CX3CL1 – 0.02 (0.000005 – 0.06)	14.00	ND	2.20	CX3CL1 – 0.04 (0.0002 – 0.12)	7.09	ND	1.68
CCR3	CCL13 – 30.1 (15.5 – 195)	3.41	ND	1.47	CCL13 – 28.7 (11.2 – 13220)	3.05	ND	1.23

EC_{50} values are indicated in nanomolar (nM) with 95 % confidence interval (CI)

ND: Not determinable since saturation was not reached

Max = signal measured at 300 nM for chemokines or 3 μM for big dynorphin expressed as fold untreated

nc: not calculable

Supplementary References

1. Ikeda Y, Kumagai H, Skach A, Sato M, Yanagisawa M. Modulation of circadian glucocorticoid oscillation via adrenal opioid-CXCR7 signaling alters emotional behavior. *Cell* **155**, 1323-1336 (2013).

2. Li L, Dong L, Wang S. Expression of the nociceptin/orphanin FQ receptor in the intestinal mucosa of IBS patients. *Exp Ther Med* **6**, 679-683 (2013).

3. Schote AB, Turner JD, Schiltz J, Muller CP. Nuclear receptors in human immune cells: expression and correlations. *Mol Immunol* **44**, 1436-1445 (2007).