Supplemental Information
Molecular Pharmacology
Endomorphin-2: a biased agonist at the μ-opioid receptor

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Table S1. Parameters from fitting GIRK data from LC as shown in Fig. 1 of main text. Data were fitted in GraphPad Prism to sigmoid curves with variable slope. The agonist minimum response in each case was constrained to zero. Where shown, slices were pretreated for 30 min with 30 nM β-FNA. Values are fitted values ± SEM of the fitting process. Values in brackets denote either the % reduction in fitted maximum caused by βFNA pretreatment, or the fold-shift of the fitted EC_{50} caused by βFNA pretreatment.

	DAMGO		**Etorphine**		**Endomorphin-2**	
	-β**FNA**	+β**FNA**	-β**FNA**	+β**FNA**	-β**FNA**	+β**FNA**
Maximum response, expressed as % of maximum response to 100 μM NA	134.9±1.8	125.1±1.5 (7.3%)	135.5±1.8	114.7±1.5 (15.4%)	128.7±0.2	99.8±2.2 (22.5%)
Log EC_{50} (log_{10} of molar concentration of agonist)	-7.1±0.02	-6.0±0.02 (11.5-fold)	-8.1±0.03	-7.4±0.03 (5.6-fold)	-6.9±0.01	-6.5±0.03 (2.6-fold)
Hill slope	1.03±0.05	0.92±0.03	0.88±0.05	1.01±0.05	1.23±0.01	1.41±0.03

Table S2. Parameters from fitting GIRK data from Fig. 1 of main text to the operational model of pharmacological agonism. Values are fitted values ± SEM of the fitting process. The Em and N values are shared values for presence and absence of βFNA pretreatment.

	DAMGO		**Etorphine**		**Endomorphin-2**	
	-βFNA	**+βFNA**	**-βFNA**	**+βFNA**	**-βFNA**	**+βFNA**
Operational efficacy (τ)	100.5±44.4	8.5±1.6	43.4±20.0	5.9±1.2	7.5±1.1	2.4±0.6
Em	137.0±3.6		138.6±5.2		129.9±7.0	
N	1.00±0.08		0.91±0.10		1.47±0.22	

Table S3. Parameters from fitting Ser^{375} phosphorylation data as shown in Fig 3B of main text. Data were fitted in GraphPad Prism to sigmoid curves with variable slope. The agonist minimum response in each case was constrained to zero. Values are fitted values ± SEM of the fitting process.

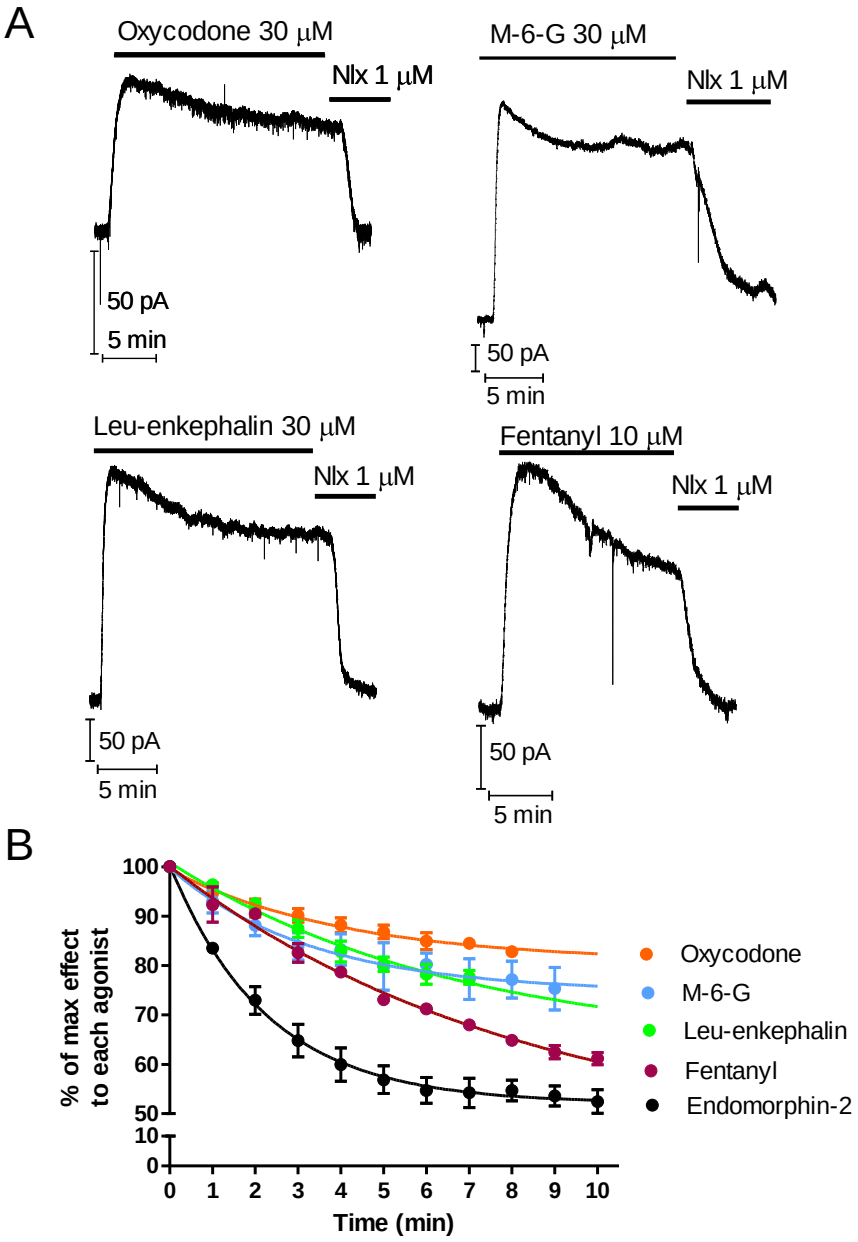
	DAMGO	**Etorphine**	**Endomorphin-2**	**Morphine**
Maximum response, expressed as % of response to 100 μM DAMGO	99.6±9.2	94.4±13.0	103.8±15.1	68.5±46.3
Log EC_{50} (log_{10} of molar concentration of agonist)	-6.14±0.17	-7.66±0.40	-5.91±0.35	-4.60±1.73
Hill slope	1.51±1.12	2.06±2.15	0.96±0.58	0.41±0.28

Table S4. Parameters from fitting Ser^{375} phosphorylation data as shown in Fig 3B of the main text to the operational model of pharmacological agonism, using GraphPad Prism. Values are fitted values ± SEM of the fitting process. Constraints for fitting: Em <101 and shared; N <2.0 and shared; the Ka values (equilibrium dissociation constant) used were those previously determined (McPherson et al., 2010).

	DAMGO	**Etorphine**	**Endomorphin-2**	**Morphine**
Operational efficacy (τ)	1.99±0.66	1.68±0.40	1.69±0.49	0.89±0.12
Em (shared value)	101±40.6			
N (shared value)	2.0±1.0			

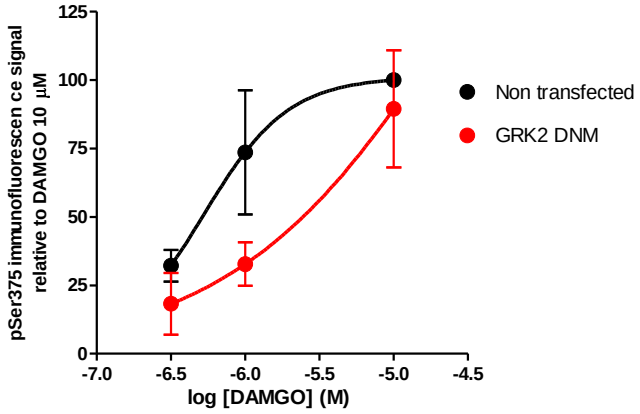
Table S5. Parameters from fitting agonist-induced cell surface loss data as shown in Fig 5A of main text. Data were fitted in GraphPad Prism to sigmoid curves with variable slope. The agonist minimum response in each case was constrained to zero. Values are fitted values ± SEM of the fitting process. †Maximum response to morphine was constrained to 17.0%, the mean maximum response from 4 experiments, as it was not possible to fit the unconstrained data to a sigmoid curve.

	DAMGO	**Etorphine**	**Endomorphin-2**	**Morphine**
Maximum response, expressed as % cell surface loss	47.1±10.3	46.3±5.2	38.1±11.6	17.0†
Log EC_{50} (log_{10} of molar concentration of agonist)	-6.26±0.54	-6.88±0.33	-5.84±0.67	-6.61±0.32
Hill slope	0.48±0.17	0.41±0.09	0.53±0.23	0.43±0.13



Supplemental information Fig. S1. Rate and extent of desensitization of MOPr-evoked GIRK channel currents in rat LC neurones. (A) Outward potassium current recorded from single LC neurons clamped at -60 mV in response to application of saturating concentrations of oxycodone (30 μM), M-6-G (30 μM), leu-enkephalin (30 μM) and fentanyl (10 μM). Agonists, applied for at least 10 min, induced an outward current that was not sustained for the period of drug application (indicated by the solid bar) but declined to a steady state. At the end of each experiment naloxone (Nlx, 1 μM) was perfused. (B) The desensitization phase for each agonist was best fitted to a single phase exponential decay model. Values shown are means ± SEM from 3 to 6 individual recordings. Note that the rate of desensitization to endomorphin-2 is faster than the others, even though the operational efficacy of endomorphin-2 for G protein coupling is similar to or less than the other agonists.

A



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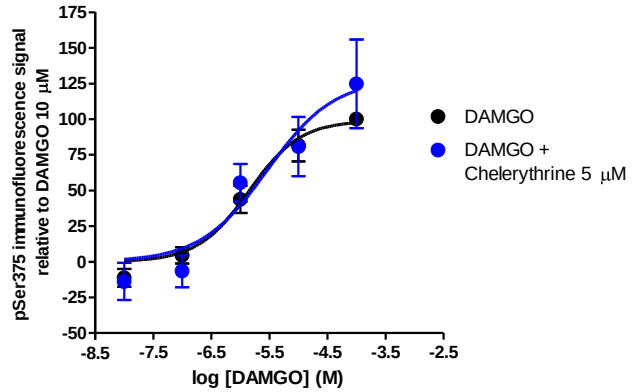
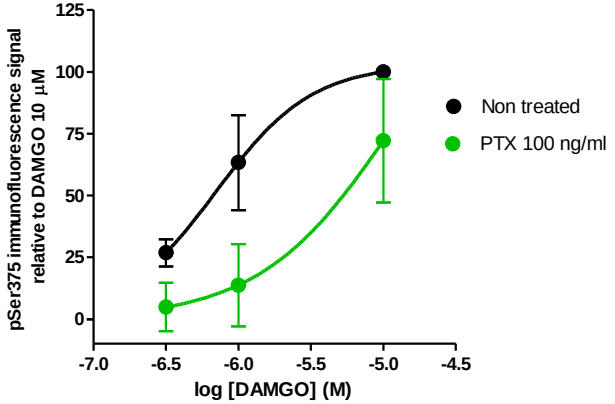
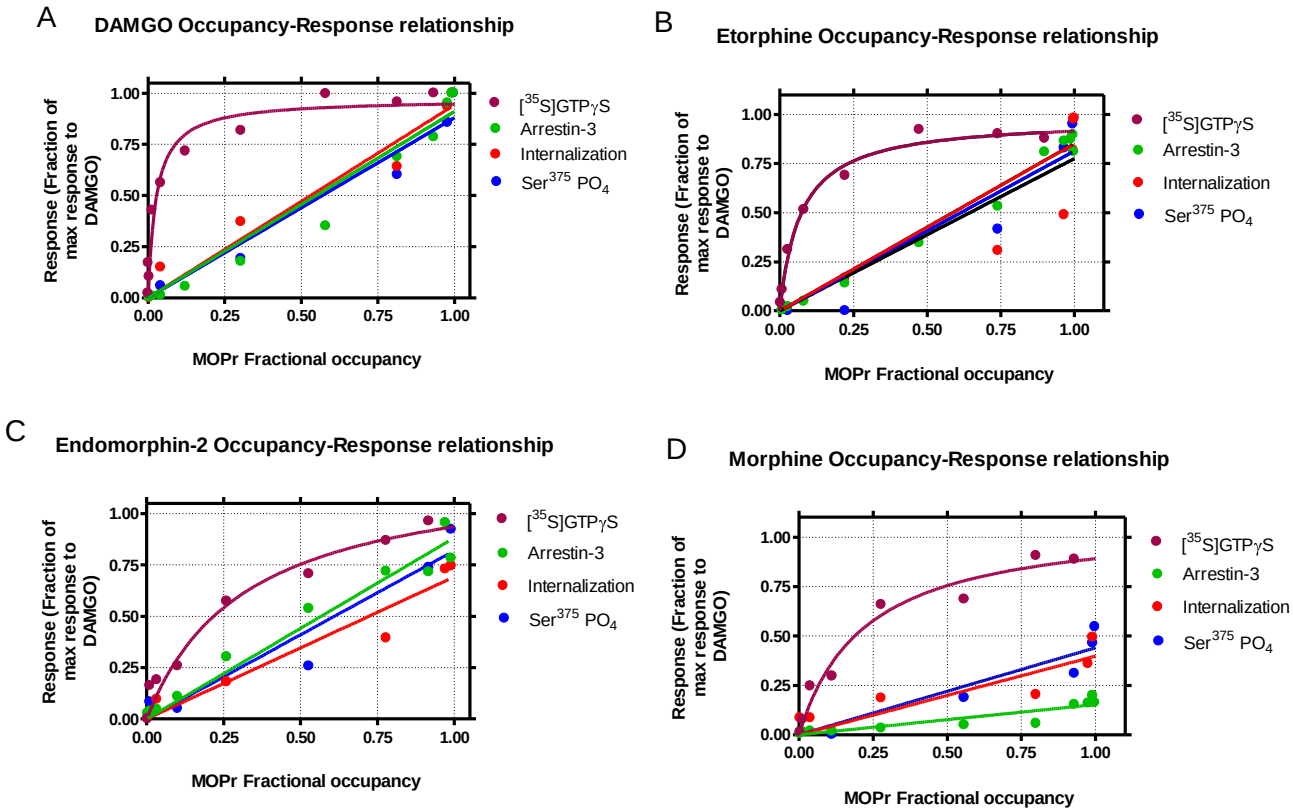


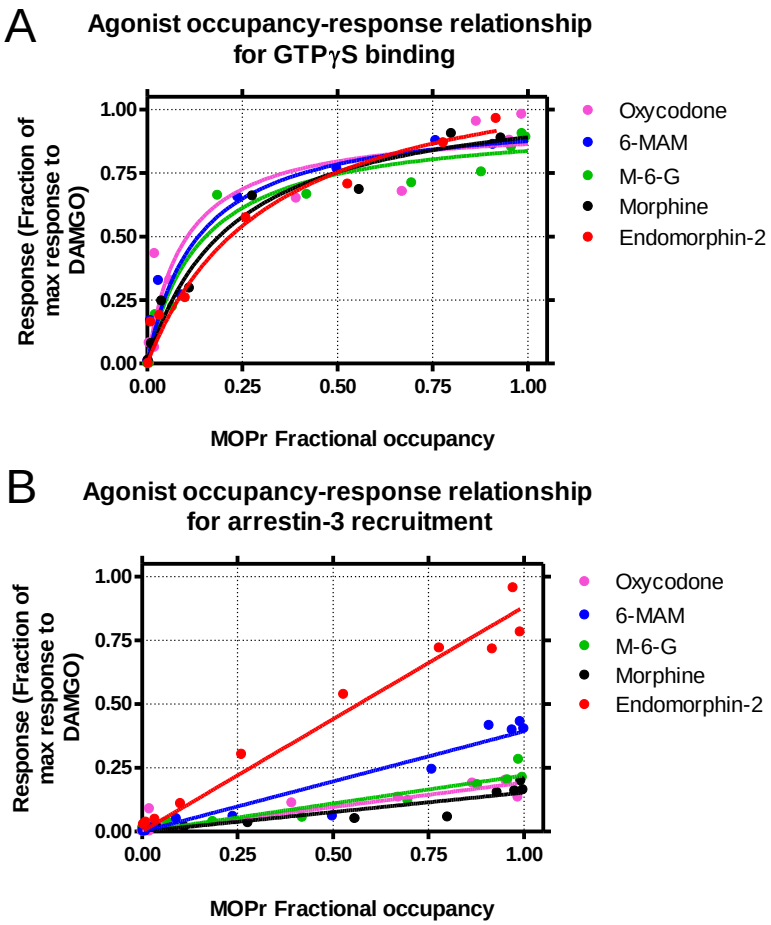
C



Supplemental information Fig. S2. Effect of inhibiton of (A) GRK, (B) PKC or (C) G protein coupling on DAMGO-induced phosphorylation of Ser^{375}. HEK293 cells stably expressing HA-tagged MOPr were plated in 96-well plates and exposed to different concentrations of DAMGO for 10 min. Cells were then fixed and used for immunocytochemical staining with an anti-pSer375 antibody and image analysis undertaken as described in Materials and Methods. For GRK inhibition, cells were transfected with dominant negative mutant GRK2 (GRK2 DNM) 48 h previously; for PKC inhibition, cells were treated with Chelerythrine 5 µM for 20 min prior to and during agonist exposure; for inhibition of G protein coupling cells were incubated with 100 ng/ml pertussis toxin (PTX) overnight. Results shown are means ± SEM of 3-6 independent experiments.



Supplemental information Fig. S3. Fractional receptor occupancy-response relationships for 4 different signalling responses with (A) DAMGO, (B) Etorphine, (C) Endomorphin-2 and (D) Morphine. Previously published (McPherson et al., 2010) concentration-response data was used to determine the occupancy-response relationship for agonist-induced [^{35}S]-GTPγS binding, arrestin-3 recruitment for MOPr agonists, whilst data from the present study was used to determine the occupancy-response relationship for agonist-induced MOPr internalization and Ser^{375} phosphorylation. Fractional receptor occupancy at each concentration of agonist was calculated as described in Materials and Methods. Data for [^{35}S]-GTPγS binding were fitted to a one site binding (hyperbola), whilst data for the other 3 responses were fitted by linear regression. Data points represent the mean response at each level of occupancy.



Supplemental information Fig. S4. Fractional receptor occupancy-response relationships for MOPr agonists. Previously published (McPherson et al., 2010) concentration-response data for agonist-induced [^{35}S]-GTPγS binding and arrestin-3 recruitment was used to determine the occupancy-response relationship for MOPr agonists. Fractional receptor occupancy at each concentration of agonist was calculated as described in Materials and Methods. (A) Relationship between [^{35}S]-GTPγS binding and fractional receptor occupancy. Data were fitted to a one site binding (hyperbola) in Graphpad Prism. Data points represent the mean response at each level of occupancy. (B) Relationship between arrestin-3 recruitment and fractional receptor occupancy. Data were fitted by linear regression as this gave a better fit than to a one site binding (hyperbola) model. Data points represent the mean response at each level of occupancy.