The Endomorphin-1/2 and Dynorphin-B Peptides Display Biased Agonism at the Mu Opioid Receptor

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Abstract

Background: Opioid agonist activation at the mu-opioid receptor (MOR) can lead to a wide variety of physiological responses. Many opioid agonists share the ability to selectively and preferentially activate specific signaling pathways, a term called biased agonism. Biased opioid ligands can theoretically induce specific physiological responses and might enable the generation of drugs with improved side effect profiles.

Methods: Dynorphins, enkephalins, and endomorphins are endogenous opioid agonist peptides that may possess distinct bias profiles; biased agonism of endogenous peptides could explain the selective roles of these ligands *in vivo*. Our purpose in the present study was to investigate biased signaling and potential underlying molecular mechanisms of bias using ^{35}S-GTPγS and cAMP assays, specifically focusing on the role of adenylyl-cyclases (ACs) and regulators of G-protein signaling proteins (RGSs) in CHO, N2a, and SH-SY5Y cell lines, all expressing the human MOR.

Results: We found that Endomorphin-1/2 preferentially activated cAMP signaling, while Dynorphin-B preferentially activated ^{35}S-GTPγS signaling in most cell lines. Experiments carried out in the presence of an isoform selective RGS-4 inhibitor, and siRNA knock-down of AC6 in N2a cells did not significantly affect the bias properties of endomorphins, suggesting that these proteins may not play a role in endomorphin bias.

Conclusion: We found that Endomorphin-1/2 and Dynorphin-B displayed contrasting bias profiles at the MOR, and ruled out potential AC6 and RGS4 mechanisms in this bias. This identified signaling bias could be involved in specifying endogenous peptide roles *in vivo*, where these peptides have low selectivity between opioid receptor family members.

Introduction

Opioid analgesics such as morphine, oxycodone, and fentanyl are the most widely used clinical analgesics to manage acute and chronic pain [1]. However, despite their effectiveness, their long-term clinical application is limited due to serious unwanted side-effects. It has been hypothesized that structurally different agonist ligands can interact with the mu opioid receptor (MOR) and stabilize it in different active conformations [2]. These conformations then can induce different downstream signaling cascades, such as G-protein coupling vs. β-arrestin recruitment, eventually leading to different cellular responses and physiological outcomes. This phenomenon is referred to as “functional selectivity” or “biased agonism” and can be exploited for drug discovery and drug design [3]. In theory, by developing biased agonist ligands we would be able to enhance the desired physiological effects such as analgesia and to minimize unwanted side-effects such as respiratory depression.

Biased G protein vs. arrestin agonism of opioid agonist ligands at the MOR has been extensively studied, albeit with significant recent controversy [4]; however, biased agonism at endogenous opioid peptides has been poorly studied [5, 6]. One seeming paradox in the study of endogenous opioid function is that the peptides are generally poorly selective between opioid family members [7, 8], even when multiple opioid receptor family members are expressed in the same synapse [9]. Biased agonism could explain how poorly-selective peptides evoke specific roles at specific receptors in the same synapse. Some studies have analyzed signaling bias in endogenous opioid peptides, finding in one study that the endomorphin peptides display bias for cAMP signaling [6]. We thus sought to expand these studies and identify potential molecular mechanisms of action by which opioid peptide bias might be evoked.

There is a plethora of signal transduction regulators that might be involved in the regulation of opioid-mediated pathway bias [10]. Current evidence suggests that the first-line primary regulators might be integral membrane proteins, permanently or transiently associated with the activated receptor-G-protein complex [10]. G-protein coupled receptor tyrosine-kinases (GRKs), guanine nucleotide exchange factors (GEFs), regulators of G-protein signaling (RGSs) and/or different isoforms of adenylyl-cyclases (ACs) might be key players of the regulation of biased signaling [11, 12]. For example, GRKs phosphorylate serine and threonine residues in the intracellular loops of opioid receptors, creating a binding platform for β-arrestins. RGS proteins might be important for the regulation of G-protein mediated signaling bias by effecting G-protein transition states [13]. ACs catalyze the formation of cAMP, a second intracellular messenger, which plays a regulatory role in the activation

of cyclic nucleotide gated ion-channels as well as in the activation of Protein Kinase A. Since RGS proteins were shown to modulate GPCR signaling [14] and ACs were reported to be important switches of GPCR signaling [15, 16] we hypothesized that they might also play a role in the previously described endomorphin pathway bias [6].

For our studies on endogenous opioid signaling bias, we first established CHO, N2a, and SH-SY5Y cell lines, all expressing the human MOR, and determined the signaling properties of these ligands using ^{35}S-GTPγS coupling and cAMP accumulation assays. The CHO and N2a lines overexpress MOR cDNA, while the SH-SY5Y line expresses endogenous MOR, permitting a more endogenously-relevant comparison. We found that Endomorphin-1/2 displayed bias towards cAMP signaling and Dynorphin-B towards G protein signaling at the MOR. Focusing on Endomorphin/cAMP bias, we sought to identify potential molecular mechanisms using a cell permeable RGS4 inhibitor and siRNA knockdown of the AC6 isoform. Neither intervention altered Endomorphin-1/2 cAMP bias at the MOR, suggesting these proteins are not responsible for Endomorphin-1/2 signaling bias. Overall, we have identified and confirmed signaling bias for endogenous Endomorphin and Dynorphin peptides that may impact their selective *in vivo* roles; we also ruled out potential molecular mechanisms for this bias and identified areas for future study to further explore signaling bias of endogenous ligands.

Materials and Methods

Cell Culture

CHO cells stably expressing cloned MOR (HA-hMOR1-CHO cells) were produced by electroporation with the human MOR N-3xHA tag cDNA (GeneCopoeia). Cells were grown on 10cm dishes in DMEM/F-12 50/50 mix w/ L-glutamine & 15mM HEPES (Corning) containing 10% heat inactivated fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 500 µg/mL G418 under 5% CO_2 at 37°C. N2a cells stably expressing the MOR were created with an identical method. SH-SY5Y cells were cultured in DMEM/F-12 media as above except without G418 selection. The CHO and N2a cells were enriched into high expressing populations using flow cytometry, selecting the top ~2% of expressing cells. The resultant cell lines were evaluated by immunocytochemistry, Western blot, and in the molecular pharmacology assays below.

Inhibition of Forskolin Stimulated cAMP Accumulation

At ~80% confluence, cells were plated into 96-well plates (5,000-8,000 cells/well) and grown in the same medium and conditions as described above for 24 hrs. The cells were then serum starved for 1-4 hrs (CHO) or 24 hrs (N2a). After a 20 min incubation at 37°C with 500 μM 3-Isobutyl-1-methylxanthine (IBMX), serum free medium containing 500 μM IBMX, 100 μM Forskolin (Enzo Life Sciences), and the appropriate agonists (all opioid peptides from Tocris) were added and then incubated for 15min at 37°C. The reaction was terminated by removing the medium and adding 60μL of ice-cold assay buffer (50mM Tris-HCl pH 7.4, 100mM NaCl, 5mM ethylenediaminetetraacetic acid [EDTA]). Plates were sealed with boiling mats and then boiled at 95°C for 10 min. Plates were then centrifuged at 3,200 rpm, 4°C, for 15min to remove debris. 50μL of lysate was transferred to a 96-well plate. Lysate was incubated with ~1 pmol ^{3}H-cAMP (PerkinElmer), and 7 μg protein kinase A (Sigma Aldrich) with 0.05% Bovine Serum Albumin (BSA). The assay was incubated at room temperature for 1 hr. The reactions were then harvested onto GF/B filter plates (PerkinElmer) via rapid filtration by a 96-well plate Cell Harvester (Brandel) and washed 3 times with ice-cold water. Filter plates were dried, 40μL of Microscint-PS scintillation cocktail was added to each well, and then counted in a TopCount or Microbeta2 (PerkinElmer) microplate scintillation counter.

For the AC6 knockdown experiments, cells were transfected with siRNA targeting AC6 via electroporation. After recovering for 72 hrs cells were subjected to the cAMP assay described above or qPCR for knockdown evaluation. For RGS inhibition experiments, cells were pre-treated with 10μM CCG50014 during the IBMX treatment, then subjected to the cAMP assay described above.

^{35}S-GTPγS Coupling in Cell Membrane Preparations

Assays were performed as previously described [17]. Briefly, previously frozen cell pellets were homogenized on ice with a teflon-on-glass dounce homogenizer in cold homogenization buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA) and centrifuged at 20,000g for 30min at 4°C. Membranes were re-suspended in cold assay buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 40 μM Guanosine Diphosphate [GDP] [CHO] or 100 μM GDP [N2a, SH-SY5Y]). 10-15 μg of membrane protein was incubated with 0.1nM ^{35}S-GTPγS (Perkin Elmer) and concentration curves of agonist in a total volume of 200 μL for 1 hour at 30°C. Reactions were harvested onto GF/B filter plates (PerkinElmer) via rapid filtration by a 96-well format Brandel cell harvester. Plates were dried, 40 μL of Microscint-PS scintillation cocktail was added to each well,

and then counted in a TopCount or Microbeta2 microplate scintillation counter. Basal counts were subtracted and data normalized to maximum stimulation of DAMGO positive control agonist, expressed as mean ± SEM. Concentration-response curves were fit, and efficacy and potency values calculated, using GraphPad Prism 7.

Whole-Cell ^{35}S-GTPγS Coupling

MOR-CHO cells were plated at 15,000 cells/well in a 96 well plate, and recovered overnight. Cells were serum starved for 1 hr then treated with 100 µL of 100 µg/mL saponin in homogenization buffer described above for 4 min. Cells were then incubated with 100 µL assay buffer, 25µL ^{35}S-GTPγS (~0.1nM) and 25 µL of agonist. The assay was harvested after 1 hr incubation, and the plate was read and data analyzed as described for ^{35}S-GTPγS coupling above.

Biased Signaling Analysis

Biased signaling was quantified utilizing the simplified version that only requires E_{MAX} and EC_{50} values, assuming a Hill coefficient of 1 [18]. Briefly, mean $\Delta Log(E_{MAX}/EC_{50})$ values for each pathway were determined in relation to the reference compound, met-enkephalin. $\Delta\Delta Log(E_{MAX}/EC_{50})$ values were then determined by subtracting cAMP $\Delta Log(E_{MAX}/EC_{50})$ values from GTPyS or whole-cell GTPyS $\Delta Log(E_{MAX}/EC_{50})$ values. 95% confidence intervals (CI) for the respective values were then determined and plotted, with N = 3 independent experiments each.

Results

We characterized four endogenous opioid peptides (Dynorphin-A, Dynorphin-B, Met-Enkephalin, and Leu-Enkephalin) and two putative endogenous opioid peptides (Endomorphin-1 and Endomorphin-2), along with an exogenous MOR-selective opioid peptide positive control, DAMGO. Opioid peptides were evaluated using ^{35}S-GTPyS coupling, whole-cell ^{35}S-GTPyS coupling, and cAMP accumulation in MOR-CHO cells; ^{35}S-GTPyS coupling and cAMP accumulation in MOR-N2a cells; and ^{35}S-GTPyS coupling in SH-SY5Y cells which endogenously express MOR and the delta opioid receptor (DOR).

Endogenous Opioid Peptides Display Different Bias Profiles at the MOR

All molecular pharmacology results using the ^{35}S-GTPγS and cAMP assays are shown in **Figure 1**, with the quantified potency and efficacy values shown in **Table 1**. The opioid peptides all display full efficacy, with varying potency, in ^{35}S-GTPγS coupling at MOR in CHO cells (**Figure 1A**). All compounds similarly show full efficacy agonism in the cAMP assay in CHO cells; the endomorphins, however, appeared to become significantly more potent in the cAMP assay relative to the other compounds (**Figure 1B**).

The ^{35}S-GTPγS coupling assay is performed on membrane preparations, devoid of the soluble intracellular components of the cell. Therefore, to determine whether intracellular factors affect opioid peptide mediated ^{35}S-GTPγS coupling, we ran the assay in whole cells, using saponin to make the cells permeable to ^{35}S-GTPγS. As shown in **Figure 1C**, in the whole-cell assay opioid peptides displayed similar ^{35}S-GTPγS coupling characteristics. The potency rank-orders were mostly maintained with small changes in the potency of some compounds. Of interest, Dynorphin-A now displayed a significant increase in efficacy (144.0% ± 21.4) not seen in the membrane preparation ^{35}S-GTPγS or the cAMP assay.

We repeated this analysis in MOR-N2a cells, to determine if the same peptide molecular pharmacology could be observed in a separate cell type. In general, we found that the results in MOR-expressing N2a cells were similar to the CHO cells, with similar rank-order potencies (**Figure 1D-E**). Interestingly, Dynorphin-A displayed an almost 50% increase in E_{MAX} (146.9 ± 8.3); this finding was the same as for the whole-cell ^{35}S-GTPγS assay in CHO cells (**Figure 1C**).

Lastly, we performed ^{35}S-GTPγS coupling in SH-SY5Y cell membranes, which express native human MOR and DOR. We again found that the results generally match the rank-order potencies found for ^{35}S-GTPγS in CHO and N2a cells; this suggests that the molecular pharmacology and potential bias observed is also present at native receptors that are not overexpressed (**Figure 1F, Table S1**). We again observed an enhanced efficacy for Dynorphin-A in these cells, matching the whole cell CHO and N2a results (**Figure 1F, Table S1**).

Next we quantified ^{35}S-GTPγS vs. cAMP bias [$\Delta\Delta\text{Log}(E_{MAX}/EC_{50})$] using the method described above with Met-Enkephalin as our reference (**Table 1, Figure 2**). As our qualitative observations suggested, the Endomorphins were generally biased towards cAMP inhibition in both cell lines (**Figure 2**). The exceptions were Endomorphin-1 just missed significance in membrane prep CHO cells (**Figure 2A**), and Endomorphin-2 just missed significance in N2a cells (**Figure 2C**). Overall their bias was significant and consistent towards cAMP signaling. Interestingly, Dynorphin-B was biased towards ^{35}S-GTPγS coupling in all three groups compared to

cAMP inhibition (**Figure 2**). Dynorphin-A was biased towards ^{35}S-GTPγS in CHO membranes alone but not whole-cell CHO or in MOR-N2a cells, suggesting that this compound is not consistently biased. Leu-Enkephalin remained unbiased in all groups.

$G\alpha_O$, AC6, and RGS4 Are Not Involved in Endomorphin Signaling Bias

To test potential mechanisms of Endomorphin bias, we examined several candidates: selective recruitment of $G\alpha_O$ proteins, selective inhibition of AC6, and regulation by RGS4. We first examined the role of $G\alpha_O$ signaling on Endomorphin inhibition of cAMP production. Upon knockdown of $G\alpha_O$ we observed no change in the potency of the endomorphins for inhibition of cAMP accumulation (**Figure S1**). Next we took a similar approach and performed a knockdown of AC6 in MOR-N2a cells, and performed the cAMP assay. Knockdown of AC6 resulted in >50% reduction in AC6 mRNA (**Figure S2**). When the cAMP assay was performed on cells after knockdown of AC6, there was no change in the potency of Endomorphin-1/2 (**Figure 3A-B**). Lastly, we sought to determine the role of RGS4 in Endomorphin cAMP bias. After treating MOR-CHO cells with vehicle or an RGS4 inhibitor, CCG50014, the Endomorphins were tested in the cAMP assay. Inhibition of RGS4 in MOR-CHO cells did not change the potency of the Endomorphins (**Figure 3C-D**). Together these results suggest that $G\alpha_O$, AC6, and RGS4 are not involved in Endmorphin cAMP signaling bias.

Discussion

Our screening of opioid peptides at the MOR in ^{35}S-GTPγS coupling in membranes, ^{35}S-GTPγS coupling in whole cells, and cAMP inhibition has revealed differing bias profiles of these endogenous, or putatively endogenous, peptides. Notably, the endomorphins were significantly and consistently biased towards cAMP inhibition over ^{35}S-GTPγS coupling. Dynorphin-B was significantly and consistently biased towards ^{35}S-GTPγS coupling over cAMP inhibition. These results replicate some of the findings in [6] where the Endomorphins showed significant biased towards cAMP inhibition over ^{35}S-GTPγS coupling. However, our results do differ in extending the analysis to whole-cell ^{35}S-GTPγS coupling and to including the N2a and SH-SY5Y cell lines, demonstrating that the Endomorphins display this bias in different cell contexts and assays. In addition, we found that Dynorphin-B was biased towards ^{35}S-GTPγS over cAMP, results not observed in Thompson et al. Also of note, Thompson et al. could not detect $G\alpha_i$ mediated agonism by Dynorphin-A at MOR, a result we have

consistently observed. These observations could be explained by differences in cell backgrounds, differences in bias quantitation methods, or the use of DAMGO for reference standard instead of Met-Enkephalin. Our results could also be confounded by systemic and observational bias, however, this possibility is minimized by using the ΔΔ bias quantitation method and by replicating our studies in multiple cell lines and assay types. Regardless of these differences, the Endomorphin cAMP bias remains clear and consistent, lending confidence to the findings from both labs.

The results in Thompson et al. had already suggested no effect on Endomorphin bias by inhibition of Gβ/γ subunits or A-kinase anchoring protein. Our results further extend this investigation of the mechanism of Endomorphin bias towards cAMP inhibition. Utilizing siRNA to knockdown AC6 or $G\alpha_O$ and pharmacological inhibition of RGS4, we have ruled out these three proteins in mediating this mechanism. Further work will be necessary to identify mechanisms of bias for Endomorphins, as well as mechanisms mediating bias in general. These could include other RGS proteins, of which there are more than 20 members, other AC isoforms, or other signaling proteins entirely that could link G protein activation to cAMP modulation, such as small GTPases (Ras, etc.) or scaffold/anchoring proteins (other A Kinase Anchoring Proteins, etc.).

In general, studies on signaling bias have focused on exogenous drugs in the context of drug discovery and development [19]. In contrast, little attention has been paid to signaling bias of endogenous ligands, with some exceptions [6, 20]. As a consequence, signaling bias as an endogenous means to regulate systems physiology is unstudied. Among the opioid peptides, it's been observed that these ligands in general lack selectivity between the opioid family members [8]; and yet, specific roles have been identified for each ligand despite multiple opioid receptors expressed at the same synapses [9]. Signaling bias could provide a means of "post-receptor" selectivity whereby specific signaling cascades are evoked downstream of specific receptors to alter specific physiology, even though multiple receptors might be bound. Testing this hypothesis could provide insight into the role of signaling bias and specific signaling cascades in systems physiology, and further guide the development of biased agonists as novel therapeutics. Our investigation and those like it [6] are the first necessary step to define endogenous ligand signaling bias before the bias hypothesis can be tested *in vivo*.

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Table 1

CHO-Memb	GTP EC_{50}	GTP E_{MAX}	cAMP EC_{50}	cAMP E_{MAX}	Δ GTP	Δ cAMP	ΔΔ	ΔΔ 95% CI
Met-Enk	41.8 ± 7.3	90.2 ± 1.2	6.40 ± 0.69	65.5 ± 5.8	0	0	0	0
Leu-Enk	155.9 ± 17.7	90.0 ± 2.7	25.4 ± 7.2	67.1 ± 4.2	-0.631	-0.5491	-0.0819	0.549
Endo-1	116.8 ± 28.8	85.6 ± 3.5	4.70 ± 2.20	67.8 ± 7.8	-0.4036	0.2282	-0.06318	0.763
Endo-2	204.9 ± 28.0	82.0 ± 1.5	4.60 ± 2.25	66.0 ± 5.8	-0.7805	0.2345	-1.015	0.705 *
Dyn-A	266.1 ± 31.2	93.2 ± 1.1	235.3 ± 107.7	65.5 ± 9.1	-0.8683	-1.493	0.6247	0.618 *
Dyn-B	294.7 ± 24.1	85.2 ± 0.3	344.4 ± 125.0	59.9 ± 0.8	-1.009	-1.714	0.705	0.359 *
CHO-Whole	**Whole EC_{50}**	**Whole E_{MAX}**	**cAMP EC_{50}**	**cAMP E_{MAX}**	**Δ Whole**	**Δ cAMP**	**ΔΔ**	**ΔΔ 95% CI**
Met-Enk	28.6 ± 2.5	106.0 ± 3.9	6.40 ± 0.69	65.5 ± 5.8	0	0	0	0
Leu-Enk	55.4 ± 15.7	106.3 ± 4.9	25.4 ± 7.2	67.1 ± 4.2	-0.3235	-0.5491	0.2256	0.585
Endo-1	134.2 ± 76.2	109.6 ± 16.8	4.70 ± 2.20	67.8 ± 7.8	-0.6503	0.2282	-0.8785	0.711 *
Endo-2	92.9 ± 3.0	95.6 ± 8.4	4.60 ± 2.25	66.0 ± 5.8	-0.6026	0.2345	-0.8371	0.626 *
Dyn-A	470.4 ± 270.6	144.0 ± 21.4	235.3 ± 107.7	65.5 ± 9.1	-1.101	-1.493	0.392	0.718
Dyn-B	305.5 ± 9.4	107.3 ± 1.0	344.4 ± 125.0	59.9 ± 0.8	-1.013	-1.714	0.701	0.312 *
N2a	**GTP EC_{50}**	**GTP E_{Max}**	**cAMP EC_{50}**	**cAMP E_{MAX}**	**Δ GTP**	**Δ cAMP**	**ΔΔ**	**ΔΔ 95% CI**
Met-Enk	35.4 ± 3.3	107.6 ± 7.6	16.2 ± 4.6	67.6 ± 1.5	0	0	0	0
Leu-Enk	124.4 ± 16.4	99.6 ± 5.0	131.5 ± 73.6	75.4 ± 17.1	-0.5403	-0.7915	0.2512	1.05
Endo-1	107.4 ± 24.1	86.4 ± 3.2	19.4 ± 6.2	63.5 ± 2.6	-0.5744	-0.1283	-0.4461	0.272 *
Endo-2	122.3 ± 14.5	88.6 ± 5.1	31.9 ± 15.2	65.3 ± 7.4	-0.6345	-0.3468	-0.2877	0.35
Dyn-A	665.5 ± 121.9	146.9 ± 8.3	374.5 ± 69.1	62.5 ± 4.3	-1.176	-1.403	0.227	0.585
Dyn-B	829.2 ± 213.5	117.5 ± 7.4	480.2 ± 151.8	68.4 ± 2.1	-1.185	-1.442	0.257	0.195 *

Table 1: Data Summary of Screening and Biased Signaling Quantitation. Data values for ^{35}S-GTPγS coupling in MOR-CHO membranes and cAMP inhibition in MOR-CHO cells (CHO-Memb), whole MOR-CHO cells (CHO-Whole), and MOR-N2a cells (N2a) along with calculated bias values and associated error (SEM for potency/efficacy, 95% CI for bias). See Methods for bias calculation procedures. * = significantly different from Met-Enk reference by 95% CI range that does not overlap with 0.

Figure Legends

Figure 1: **Screening of Endogenous Opioids at the Mu Opioid Receptor. A)** ^{35}S-GTPγS coupling in MOR-CHO membranes. **B)** Inhibition of forskolin-stimulated cAMP accumulation in MOR-CHO cells. **C)** ^{35}S-GTPγS coupling in whole MOR-CHO cells. **D)** ^{35}S-GTPγS coupling in MOR-N2a membranes. **E)** Inhibition of forskolin-stimulated cAMP accumulation in MOR-N2a cells. **F)** ^{35}S-GTPγS coupling in SH-SY5Y membranes. Data presented as the mean ± SEM of the % of maximum DAMGO stimulation (^{35}S-GTPγS coupling) or % of forskolin-stimulated cAMP accumulation (cAMP assay). N = 3 independent assays, performed in duplicate.

Figure 2: Graphical Depiction of Biased Signaling Quantitation. **A)** Graphical pathway bias analysis for ^{35}S-GTPγS coupling in MOR-CHO membranes vs. cAMP inhibition. **B)** Graphical pathway bias analysis for ^{35}S-GTPγS coupling in whole MOR-CHO cells vs. cAMP inhibition. **C)** Graphical pathway bias analysis for ^{35}S-GTPγS coupling in MOR-N2a membranes vs. cAMP inhibition. Graphs depict calculated $\Delta\Delta\text{Log}(E_{MAX}/EC_{50})$ values with associated 95% CIs. Bias factors were considered significant when the 95% CI did not overlap with 0 (marked with *).

Figure 3: Endomorphin Bias is Not Due to RGS4 or AC6. **A)** MOR-N2a cells were transfected with siRNA against a non-coding control or **B)** AC6. Leu-Enkephalin and Endomorphin-1/2 were then tested in the cAMP assay. **C)** MOR-CHO cells were pre-treated with vehicle or **D)** 10 μM CCG50014 for 20 min, then subjected to the cAMP assay as previously described. Data represents the mean ± SEM of % of forskolin-stimulated cAMP accumulation. N = 3-4 independent experiments, performed in duplicate.

Figure 1

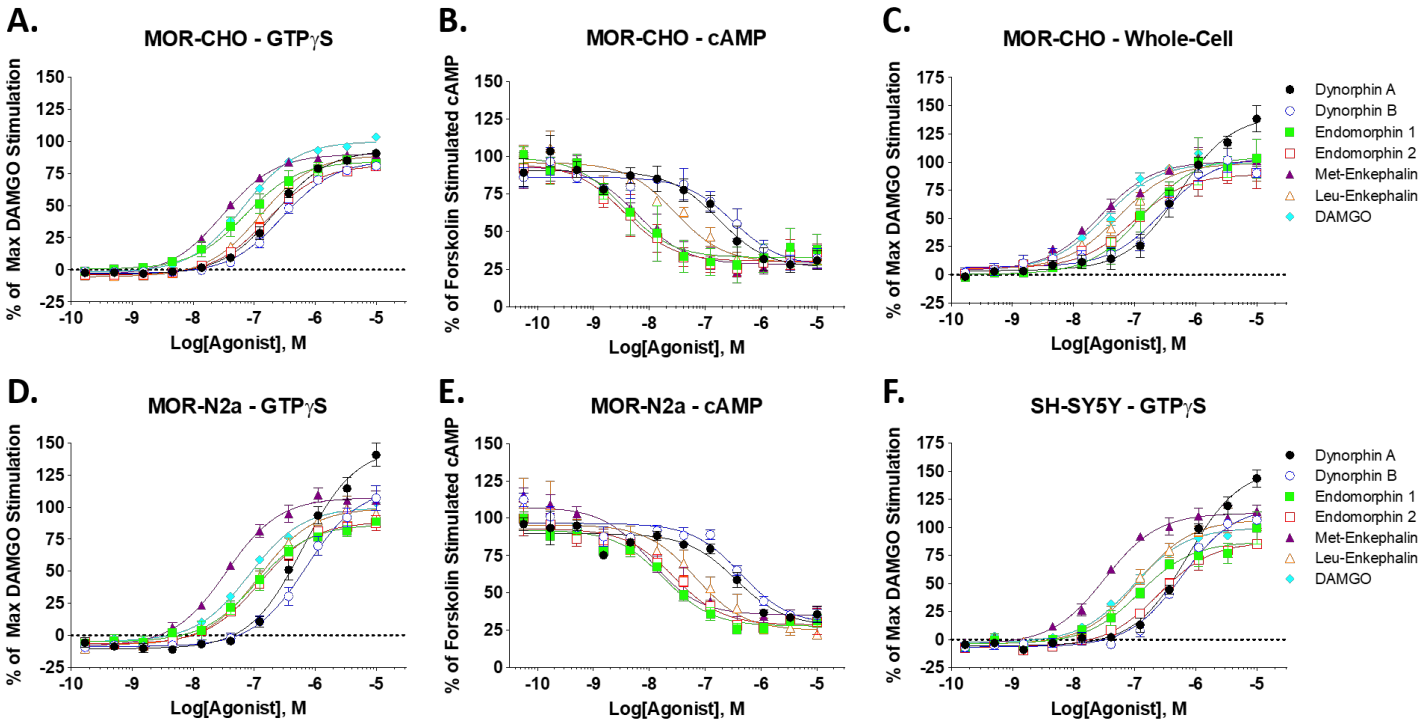

A.
MOR-CHO - GTPγS
% of Max DAMGO Stimulation
Log[Agonist], M
B.
MOR-CHO - cAMP
% of Forskolin Stimulated cAMP
Log[Agonist], M
C.
MOR-CHO - Whole-Cell
% of Max DAMGO Stimulation
Log[Agonist], M
Dynorphin A
Dynorphin B
Endomorphin 1
Endomorphin 2
Met-Enkephalin
Leu-Enkephalin
DAMGO
D.
MOR-N2a - GTPγS
% of Max DAMGO Stimulation
Log[Agonist], M
E.
MOR-N2a - cAMP
% of Forskolin Stimulated cAMP
Log[Agonist], M
F.
SH-SY5Y - GTPγS
% of Max DAMGO Stimulation
Log[Agonist], M
Dynorphin A
Dynorphin B
Endomorphin 1
Endomorphin 2
Met-Enkephalin
Leu-Enkephalin
DAMGO

Figure 2

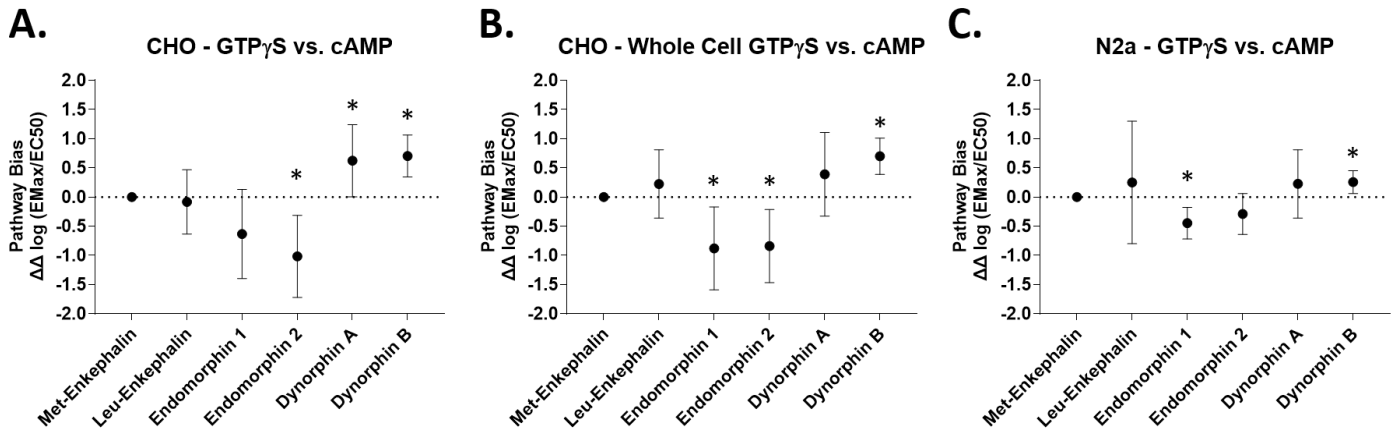
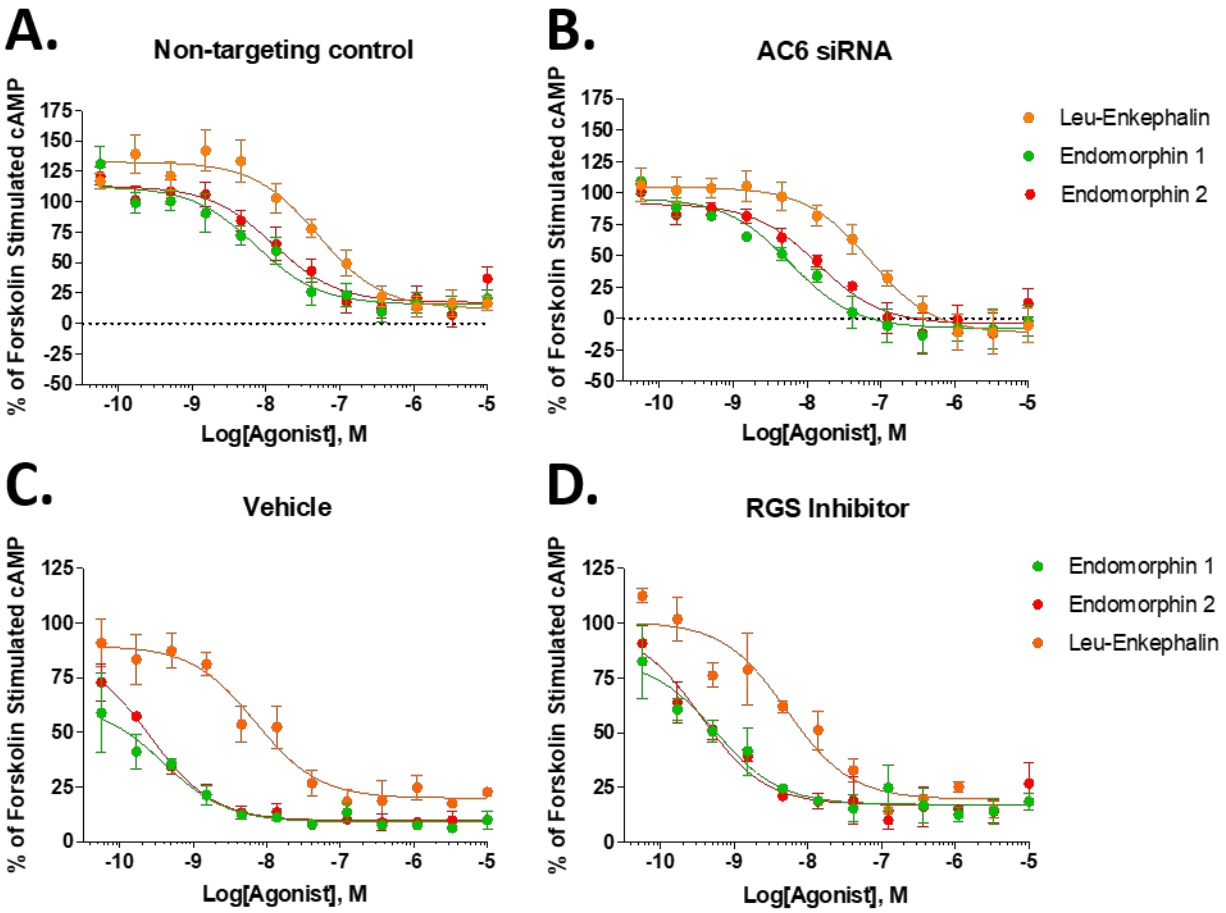

A.
CHO - GTPγS vs. cAMP
B.
CHO - Whole Cell GTPγS vs. cAMP
C.
N2a - GTPγS vs. cAMP
Pathway Bias
ΔΔ log (EMax/EC50)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
*
Met-Enkephalin
Leu-Enkephalin
Endomorphin 1
Endomorphin 2
Dynorphin A
Dynorphin B

Figure 3



E.

EC_{50} (nM)	NT Control	AC6 siRNA	Vehicle	RGS Inhib.
Leu-Enk	56.6 ± 11.08	63.2 ± 17.4	8.07 ± 2.68	3.33 ± 1.92
Endo 1	9.78 ± 2.11	6.2 ± 1.2	0.26 ± 0.04	0.42 ± 0.24
Endo 2	15.0 ± 3.4	14 ± 3.2	0.54 ± 0.25	1.20 ± 0.69

The Endomorphin-1/2 and Dynorphin-B Peptides Display Biased Agonism at the Mu Opioid Receptor

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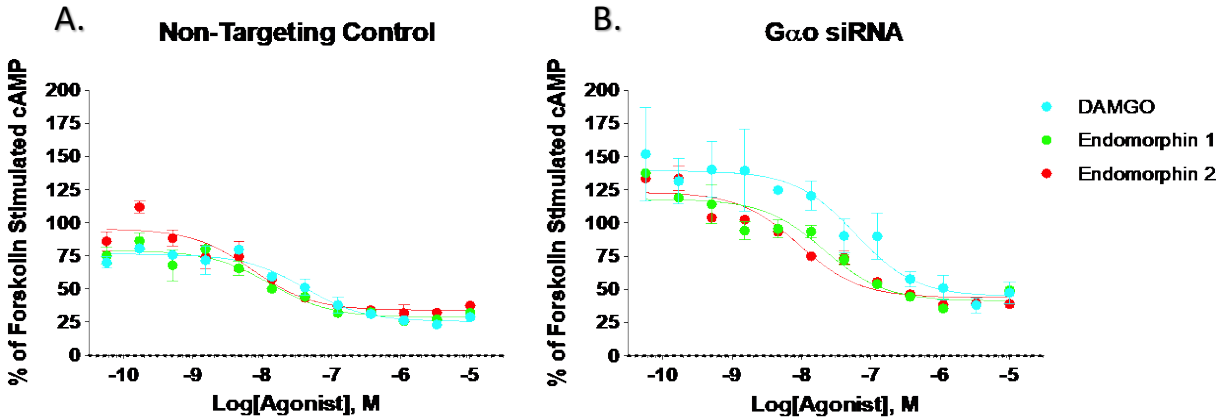
Supplemental Data

Figure S1.



C.

EC50 (nM)	**DAMGO**	**Endo 1**	**Endo 2**
NT	40.9	13.2	7.1
siRNA	59.6	21.3	9.3

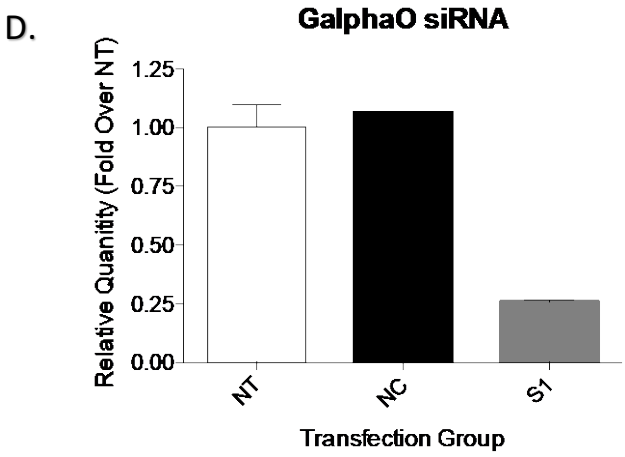


Figure S1: Endomorphin Bias is Not Due to Selective Gαo Recruitment. A) MOR-N2a cells transfected with a non-targeting control siRNA and then treated with varying concentrations of agonist. **B)** MOR-N2a cells transfected with siRNA targeting Gαo and then treated with varying concentrations of agonist. **C)** Table of potency and efficacy values from **A** and **B**, suggesting that Gαo knockdown does not reverse endomorphin cAMP bias. **D)** qPCR results of relative Gαo mRNA quantities after transfecting with negative control siRNA (NC), Gαo siRNA (S1), or non-transfected cells (NT). Results suggest ~75% knockdown. All data represented as mean values of N=1 independent experiment performed at least in duplicate.

Figure S2.

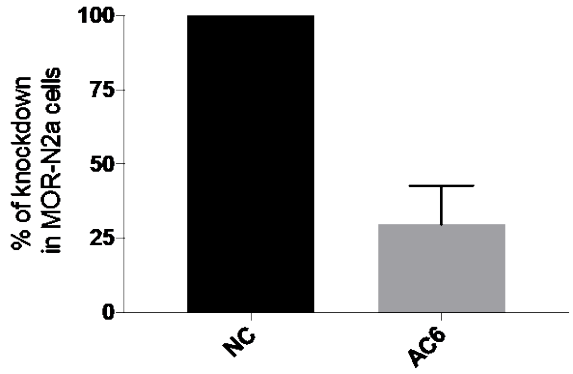


Figure S2: Quantification of AC6 Knockdown in MOR-N2a Cells. qPCR quantification of relative levels of AC6 mRNA after siRNA knockdown in MOR-N2a cells. Data represented as the mean ± SEM of N=3 independent experiments. ~75% knockdown achieved.

Table S1

	EC50 (nM)	Fold EC50 v Met	EMax (%)
Met-Enkephalin	32.5 ± 5.2	1.00 ± 0.16	112.9 ± 8.6
Leu-Enkephalin	118.4 ± 20.6	3.64 ± 0.63	107.3 ± 4.3
Endomorphin 1	122.1 ± 39.2	3.75 ± 1.20	86.9 ± 10.6
Endomorphin 2	247.7 ± 5.9	7.62 ± 0.18	87.2 ± 6.2
Dynorphin A	733.9 ± 13.7	22.6 ± 0.42	153.4 ± 5.3
Dynorphin B	596.8 ± 73.1	18.3 ± 2.2	118.9 ± 0.38
DAMGO	109.5 ± 15.8	3.37 ± 0.48	100

Table S1: **Potency and efficacy of various opioid peptides using [^{35}S]-GTPγS coupling in SH-SY5Y cells.** Data taken from the curves reported in **Figure 1F**. Data represents the mean ± SEM of the EC50, Fold EC50, and Emax (% of Max DAMGO) of each ligand from N=3 independent experiments performed in duplicate.

Supplemental Methods

Quantitative Real-Time PCR

Quantitative RT-PCR was performed using an OMEGA MicroElute Total RNA kit for RNA isolation and Qiagen RT^2 SYBR green qPCR kit and mastermix for the reverse transcription reaction. Both were performed according to the manufacturer's instructions. Briefly, HA-MOR-N2a cells previously transfected with the AC6 or Gαo siRNA constructs (Gao siRNA: CGCCAAAGACGUGAAAUUACUCCTG; AC6 siRNA: GCAUUGAUGAUUCUAGCAAAGACAA) were harvested 72 hrs after the transfection and were subjected to lysis, RNA extraction and reverse transcription reaction. For the qPCR assay, cDNA was used with a Qiagen RT^2 SYBR green qPCR kit with the following primers: AC6-F: GGTTTGACAAGCTGGCTGC; AC6-R: CTCCATGTGGTTAGCCAGGG; Gαo-F: CTTTGGGCGTGGAGTATGGT; Gαo-R: ATCGGTTGAAGCACTCCTGG. RT reaction conditions were set as: 10 min. (25°C), 120 min. (37°C), 5 min. (85°C) and hold at (4°C). Cycling conditions were 15s at 95°C, 45s at 60°C for 40 cycles. Data were normalized to GAPDH control, and converted into a percentage by normalization to negative control siRNA.