Supporting Information

Human β-Defensin-1 and β-Defensin-3 (Mouse Ortholog mBD14) Function as Full Endogenous Agonists at Select Melanocortin Receptors

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Supplemental Table 1: Analytical Data for the Peptides Synthesized in this Study[a]

Peptide	Structure	HPLC *k'* (system 1)	HPLC *k'* (system 2)	M+1 (calcd)	mass spectral analysis (M+1), (purity, %)
$mBD1_{red}$	H-DQYKCLQHGGFCLRSSCPSNTKLQGTCKPDKPNCCK-NH_2	4.2	8.5	3988.6	3990.2 (>95)
mBD1	H-DQYKCLQHGGFCLRSSCPSNTKLQGTCKPDKPNCCK-NH_2	3.8	7.4	3982.6	3983.4 (>95)
$mBD3*_{red}$	H-FLPKTLRKFFCRIRGGRCAVLNCLGKEEQIGRCSNSGRKCCRKKK-NH_2	5.3	10.0	5185.8	5186.0 (>95)
mBD3*	H-FLPKTLRKFFCRIRGGRCAVLNCLGKEEQIGRCSNSGRKCCRKKK-NH_2	4.3	8.1	5178.0	5179.0 (>95)
$hBD1_{red}$	H-DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK-NH_2	5.1	10.0	3933.6	3933.7 (>95)
hBD1	H-DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK-NH_2	4.3	8.7	3927.0	3928.0 (>95)
$hBD3_{red}$	H-GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK-NH_2	6.6	11.2	5157.0	5158.0 (>95)
hBD3	H-GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK-NH_2	5.8	10.0	5151.0	5152.0 (>95)

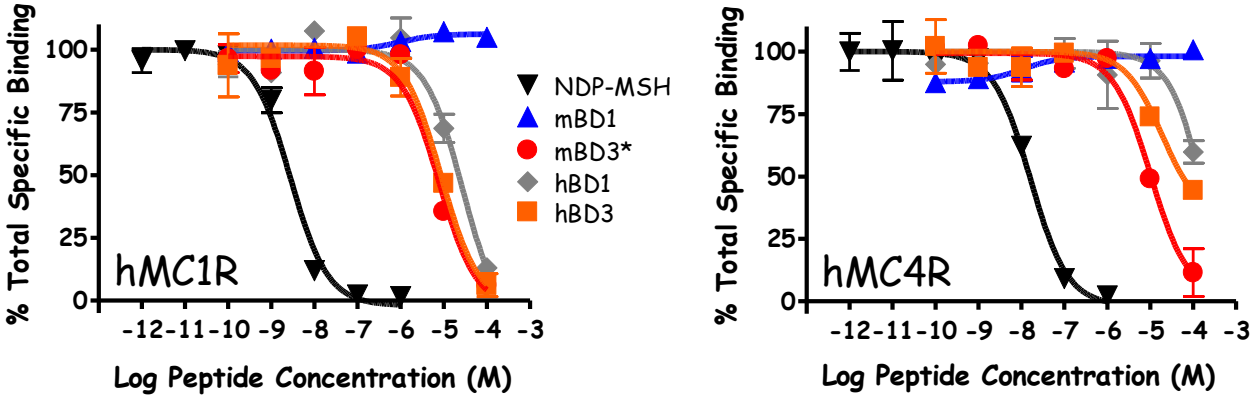
The accepted mouse ortholog nomenclature for hBD3 is mBD14,[1] but will be referred to as mBD3* in this manuscript for simplicity.
[a]HPLC k' = [(peptide retention time – solvent retention time)/solvent retention time] in solvent system 1 (10% acetonitrile in 0.1% trifluoroacetic acid/water and a gradient to 90% acetonitrile over 35 min) or solvent system 2 (10% MeOH in 0.1% trifluoroacetic acid/water and a gradient to 90% MeOH over 35 min). An analytical Vydac C18 column (Vydac 218TP104) was used with a flow rate of 1.5 mL/min. The peptide purity was determined by HPLC at a wavelength of 214 nm.

Supplemental Table 2: Competitive Displacement Binding Results of β-Defensins at the Mouse MC4R and Human MC1R and MC4R Using Radiolabeled [^{125}I]-NDP-MSH.[a]

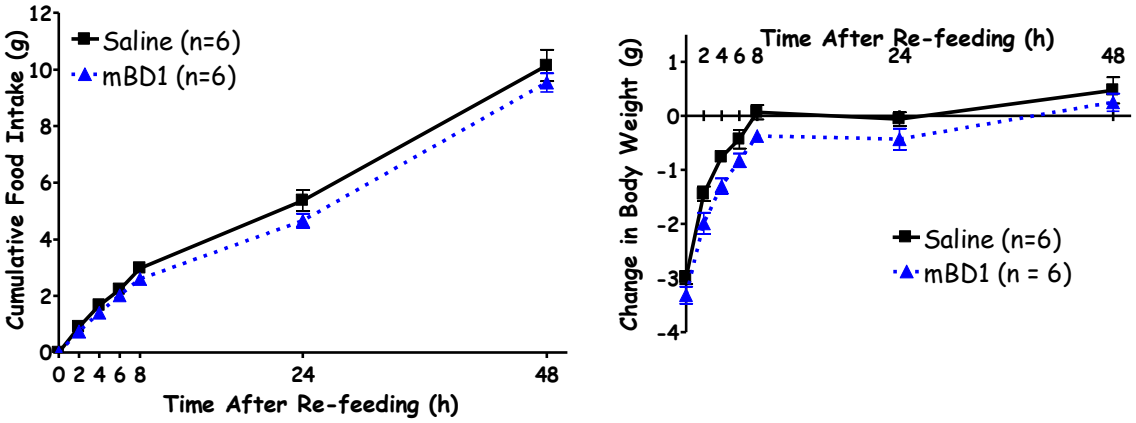
Peptide	mMC4R	hMC1R	hMC4R
	IC_{50} (nM)		
NDP-MSH	4 ± 2	3.0 ± 0.2	11 ± 5
hBD3	17,000 ± 7,000	7,000 ± 1,000	>100,000
hBD1	26,000 ± 1,000	26,000 ± 2,000	>100,000
mBD3*	5,000 ± 1,000	6,000 ± 3,000	18,000 ± 13,000
mBD1	>100,000	>100,000	>100,000

The accepted mouse ortholog nomenclature for hBD3 is mBD14,[1] but will be referred to as mBD3* in this manuscript for simplicity. [a]The indicated errors represent the standard deviation of the mean determined from at least two independent experiments. >100,000 nM indicates that the compound was examined but lacked the ability to competitively displace the radiolabeled compound to 75% maximum response (IC_{50}) at up to 100 μM concentrations.

Supplemental Figure 1: Illustrations of competitive binding studies at the hMC1R and hMC4R. Radiolabeled [^{125}I]-NDP-MSH was used for these studies. The accepted mouse ortholog nomenclature for hBD3 is mBD14,[1] but will be referred to as mBD3* in this manuscript for simplicity.



Supplemental Figure 2: Cumulative food intake and change in body weight of male mice receiving saline vehicle or 100 µg mBD1 (in 3 µL) via ICV administration at the beginning of fast (t = -22 h). Data shown as mean ± SEM. Data analyzed by regular two-way ANOVA followed by a Bonferroni post-test.



Experimental

Reagents and General Methods: The coupling reagents 1-[*bis*(dimethylamino)methylene]-1H-benzotriazolium hexafluorophosphate 3-oxide [HBTU], amino acids, and resins were purchased from Peptide Internationals. Dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), glacial acetic acid, methanol (MeOH), acetonitrile, Tris buffer, and anhydrous ethyl ether were purchased from Fisher (Fair Lawn, NJ). Trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), piperidine, glutathione, guanidine hydrochloride, phenol, and thioanisole, were purchased from Sigma (St. Louis, MO). *N*,*N*-diisopropylethylamine (DIPEA) and triisopropylsilane (TIS) were purchased from Aldrich (Milwaukee, WI). All reagents and chemicals were ACS grade or better and were used without further purification. Radiolabeled [^{125}I]-NDP-MSH was received from Dr. Robert Speth (Nova Southeastern University). Peptide purity (determined to be > 95%) was assessed by analytical RP-HPLC in two different solvent systems before functional characterization in the AlphaScreen bioassay.

***Peptide Synthesis*:** Peptides were synthesized using standard fluorenylmethoxycarbonyl (Fmoc) chemistry.[2-3] Full-length human and mouse β-defensins were individually synthesized (0.3 mmol scale) using a manual microwave synthesizer (CEM Discover SPS) on rink-amide-MBHA resin. Removal of the Fmoc was achieved by treatment with 20% piperidine in DMF (1 x 2 min at room temperature, 1 x 4 min using microwave irradiation using 30W at 75 °C for 4 min). After washing the resin with DMF, the presence of a free amine was confirmed through a ninhydrin Kaiser test.[4] The amino acid coupling step was performed using 3.1 eq Fmoc-amino acid, 3 eq HBTU, and 5 eq DIPEA and irradiating at 75 °C, 30 W for 5 min [5.1 eq Fmoc-Arg(Pbf), 5 eq HBTU, 7 eq DIPEA, and a 10 min irradiation were used for Arg]. Coupling of Cys and His was performed at a lower coupling temperature (50° C). Coupling progress was monitored using the Kaiser test and repeated as necessary.[4] Upon completion, peptides were cleaved from the resin and side chained deprotected with a cleavage cocktail of 90:5:2.5:2.5 TFA:TIS:thioanisole:water for 2 hrs. Peptides were precipitated using cold (4 °C) anhydrous diethyl ether. Crude peptides were purified using a Shimadzu RP-HPLC system with a photodiode array detector and a semipreparative RP-HPLC C18 bonded column (Vydac 218TP1010, 1 cm x 25 cm). Purified linear peptides were at least 95% pure as determined by analytical RP-HPLC (Vydac 218TP104, 4.6 mm x 25 cm) and possessed the correct molecular mass as determined by MALDI-TOF MS (Biflex III, Bruker Daltonics, University of Minnesota's Center of Mass Spectrometry and Proteomics) or ESI-TOF MS (Bruker Bio TOF II, University of Minnesota's Department of Chemistry).

Oxidative folding of the purified linear peptides was achieved by dissolving peptides to a concentration of 0.1 mg/mL in a redox buffer (1.0 M guanidine hydrochloride, 0.1 M Tris buffer, 1.0 mM reduced glutathione, 0.1 mM oxidized glutathione, pH ~8.0) accompanied by stirring for 48 hrs. Monitoring by HPLC indicated a single major peak for all peptides. The folded products were purified by HPLC and characterized as fully oxidized by RP-HPLC and mass spectrometry as described above (SI Table 1). On occasion, the pure cyclic defensin peptides showed broader peak due to possible migrating non-covalent conformers as reported in earlier report by Candille *et al*.[5]

AlphaScreen cAMP Functional Bioassay: Synthesized peptides were pharmacological characterized at the human and mouse MC1R, MC3-5R using the cAMP AlphaScreen assay (PerkinElmer) according to the manufacturer's instructions and as previously reported.[6-8] The hMC1R, hMC3-5, and mMC3-5R were stable expressed in HEK-293 cells. The mMC1R was transiently expressed in HEK-293 cells 48-72 h prior to the assay using the calcium phosphate method and 4 μg DNA.[9] Peptide ligands were dissolved in DMSO or water at a stock concentration of 10^{-2} M.

Briefly, cells 70-90% confluent were dislodged with Versene (Gibco®) at 37 °C and plated 10,000 cells/well in a 384-well plate (Optiplate™) with 5 μL freshly prepared stimulation buffer (1X HBSS, 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA, pH = 7.4) with 1 unit anti-cAMP acceptor beads per well. The cells were stimulated with the addition of 5 μL stimulation buffer containing peptide (10^{-4} to 10^{-10} M; 10^{-6} to 10^{-12} M for NDP-MSH) or forskolin (10^{-4} M) and incubated in the dark at room temperature for 2 hr. Due to the 1:1 dilution of the peptide solution with stimulation buffer containing acceptor beads, the highest concentration of DMSO in the assay was 0.5%, below the recommended limit of 2% from PerkinElmer.

Following stimulation, streptavidin donor beads (1 unit) and biotinylated-cAMP (1 unit) were added to the wells in a subdued light environment with 15 μL lysis buffer (5 mM HEPES, 0.3% Tween-20, 0.1% BSA, pH = 7.4) and the plates were incubated in the dark at room temperature for an additional 2 hr. Plates were read on a Enspire (PerkinElmer) Alpha-plate reader using a pre-normalized assay protocol (set by the manufacturer).

The agonist EC_{50} values represent the mean of duplicate replicates performed in at least three independent experiments. The EC_{50} value estimates, and their associated standard errors of the mean, were determined by fitting the data to a nonlinear least-squares analysis using the PRISM program (v4.0, GraphPad Inc.). The ligands were assayed as TFA salts and not corrected for peptide content.

Receptor Competitive Displacement Binding Studies: HEK-293 cells expressing the mMC4, hMC1R, and hMC4R were maintained as described above. One day preceding the experiment, $0.1\text{-}0.3\times10^{6}$ cells/well were plated into Primera 24 well plates (Falcon). The peptides were used to competitively displace the ^{125}I-radiolabeled [^{125}I]-NDP-MSH (100,000 cpm/well). The ligands were assayed as TFA salts. Dose-response curves (10^{-6} to 10^{-12} M) of [^{125}I]-NDP-MSH and IC_{50} values were generated and analyzed by nonlinear least-squares analysis and the PRISM program (v4.0, Graph-Pad Inc.). The percent total specific binding was determined based upon the nonspecific values obtained using 10^{-6} M [^{125}I]-NDP-MSH and the [^{125}I]-NDP-MSH dose-response curves as controls. Each experiment was performed using duplicate data points and repeated in at least two independent experiments. The standard deviation errors of the mean were derived from the average percent specific binding values from at least two independent experiments and using the PRISM program (v4.0, GraphPad Inc.).

Feeding Studies

Animals: All studies performed were conducted in accord with accepted standards of humane animal care and were approved by the Institutional Animal Care and Use Committee at the University of Minnesota. The mice (mixed genetic background from C57BL/6J and 129/Sv inbred strains) were individually housed in standard polycarbonate conventional cages. Mice were maintained on a 12 h light/dark cycle (lights off was at 11:59 AM) in a temperature-controlled room 23-25 ^{o}C with free access to normal chow and water unless noted.

Cannula surgery and placement validation: Mice were anesthetized with a mixture of ketamine (87 mg/kg) and xylazine (2.6 mg/kg) administered IP and prophylactically dosed with flunixin meglumine (2.5 mg/kg) (FluMegluine, Clipper Distribution Company). Following placement in a stereotaxic apparatus (Cartesian Instruments), a midline anterior to posterior incision was made. A 26-gauge cannula was inserted into the lateral cerebral ventricle, (coordinates: 1.0 mm lateral and 0.46 mm posterior to bregma and 2.3 mm ventral to the surface of the skull) and fixed with n-butyl cyanoacrylate glue (3M Vetbond) and dental cement. After allowing the glue and cement to dry, the skin was sutured. Mice were given a S.Q. injection of 0.5 mL of saline, placed on heating pad, and monitored until recovered. All mice were allowed to rest at least 7 days post-surgery prior to compound injections. Surgeries were performed on mice 7-9 weeks of age for fasting studies, and 12-14 weeks of age for *ad libitum* studies. Cannula placement was confirmed as described in Marsh *et al.*[10] using 2.5 μg of human PYY(3-36) (Bachem).

Feeding Regimens: All studies utilized a Latin Square paradigm. The initial experimental paradigm involved an *ad libitum* feeding paradigm in which mBD1 was administered via ICV injections at the indicated dose 2 h before lights out (t = 0 h). An additional fasting feeding paradigm was also employed, utilizing 100 μg (20.9 nmol) mBD1. mBD1 was administered at the start of a 22 h fast, after which food was reintroduced to the cage 2 h prior to lights out. Peptide was initially dissolved in water and saline was used to adjust the concentration. Cumulative food intake and body weight were measured at t = 0, 2, 4, 6, 8, 24, and 48 h. Data was analyzed using a two-way ANOVA with between-sessions variable of compound and the within subset variable of time, followed by a Bonferroni post-test. Statistical significance was defined as $p < 0.05$.

References

1. Roehrl, J.; Yang, D.; Oppenheim, J. J.; Hehlgans, T. Identification and biological characterization of mouse β-defensin 14, the orthologue of human β-defensin 3. *J. Biol. Chem.* **2008,** *283* (9), 5414-5419.

2. Carpino, L. A.; Han, G. Y. 9-Fluorenylmethoxycarbonyl function, a new base-sensitive amino-protecting group. *J. Am. Chem. Soc.* **1970,** *92* (19), 5748-5749.

3. Carpino, L. A.; Han, G. Y. The 9-fluorenylmethoxycarbonyl amino-protecting group. *J. Org. Chem.* **1972,** *37* (22), 3404-3409.

4. Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **1970,** *34* (2), 595-598.

5. Candille, S. I.; Kaelin, C. B.; Cattanach, B. M.; Bin, Y.; Thompson, D. A.; Nix, M. A.; Kerns, J. A.; Schmutz, S. M.; Millhauser, G. L.; Barsh, G. S. A β-defensin mutation causes black coat color in domestic dogs. *Science* **2007,** *318* (5855), 1418-1423.

6. Singh, A.; Tala, S. R.; Flores, V.; Freeman, K.; Haskell-Luevano, C. Synthesis and pharmacology of α/β^3-peptides based on the melanocortin agonist Ac-His-DPhe-Arg-Trp-NH_2 sequence. *ACS Med. Chem. Lett.* **2015,** *6* (5), 568-572.

7. Lensing, C. J.; Freeman, K. T.; Schnell, S. M.; Adank, D. N.; Speth, R. C.; Haskell-Luevano, C. An in vitro and in vivo investigation of bivalent ligands that display preferential binding and functional activity for different melanocortin receptor homodimers. *J. Med. Chem.* **2016,** *59* (7), 3112-3128.

8. Ericson, M. D.; Schnell, S. M.; Freeman, K. T.; Haskell-Luevano, C. A fragment of the *Escherichia coli* ClpB heat-shock protein is a micromolar melanocortin 1 receptor agonist. *Bioorg. Med. Chem. Lett.* **2015,** *25* (22), 5306-5308.

9. Chen, W. B.; Shields, T. S.; Stork, P. J. S.; Cone, R. D. A colorimetric assay for measuring activation of G_s- and G_q-coupled signaling pathways. *Anal. Biochem.* **1995,** *226* (2), 349-354.

10. Marsh, D. J.; Hollopeter, G.; Huszar, D.; Laufer, R.; Yagaloff, K. A.; Fisher, S. L.; Burn, P.; Palmiter, R. D. Response of melanocortin-4 receptor-deficient mice to anorectic and orexigenic peptides. *Nat. Genet.* **1999,** *21* (1), 119-122.