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A Direct *In Vivo* Comparison of The Melanocortin Monovalent Agonist Ac-His-DPhe-Arg-Trp-NH_2 versus The Bivalent Agonist Ac-His-DPhe-Arg-Trp-PEDG20-His-DPhe-Arg-Trp-NH_2: A Bivalent Advantage

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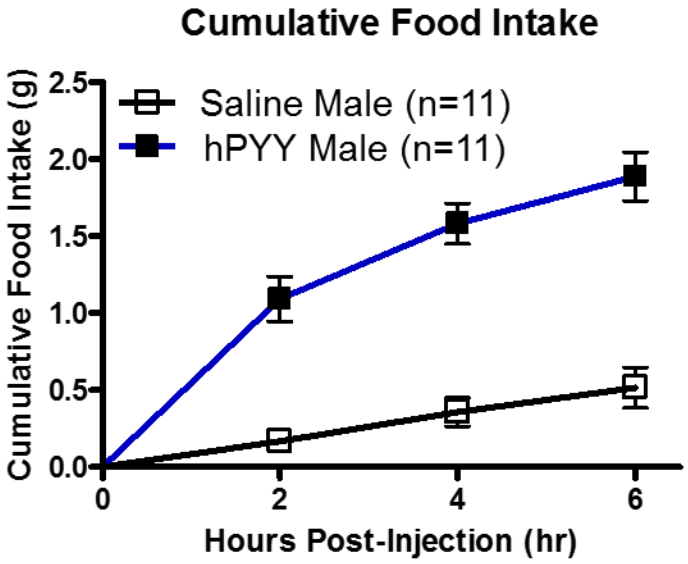
		Amount of Intact Peptide (%)							
Analog	**Sequence**	0.5 h	1.5 h	3 h	6 h	8 h	24 h	48 h	72 h
NDP-MSH	Ac-Ser-Try-Ser-Nle-Glu-**His-DPhe-Arg-Trp**-Gly-Lys-Pro-Val-NH_2	92	77	76	40	31	3	0	0
α-MSH	Ac-Ser-Try-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	71	30	8	1	0	0	0	0
CJL-1-14	Ac-**His-DPhe-Arg-Trp**-NH_2	99	90	87	80	69	42	22	12
CJL-1-87	Ac-**His-DPhe-Arg-Trp**-(PEDG20)-**His-DPhe-Arg-Trp**-NH_2	96	90	72	51	40	3	0	0
CJL-5-35-4	Ac-**His-DPhe-Arg-Trp**-(PEDG20)-NH_2	101	92	85	70	63	33	8	3
CJL-1-116	(PEDG20)-**His-DPhe-Arg-Trp**-NH_2	98	89	81	68	59	20	4	1
CJL-1-31	Ac-**His-DPhe-Arg-Trp**-$(Pro\text{-}Gly)_6$-**His-DPhe-Arg-Trp**-NH_2	0	0	0	0	0	0	0	0
CJL-5-35-1	Ac-**His-DPhe-Arg-Trp**-$(Pro\text{-}Gly)_6$-NH_2	2	0	0	0	0	0	0	0
CJL-1-41	$(Pro\text{-}Gly)_6$-**His-DPhe-Arg-Trp**-NH_2	0	0	0	0	0	0	0	0

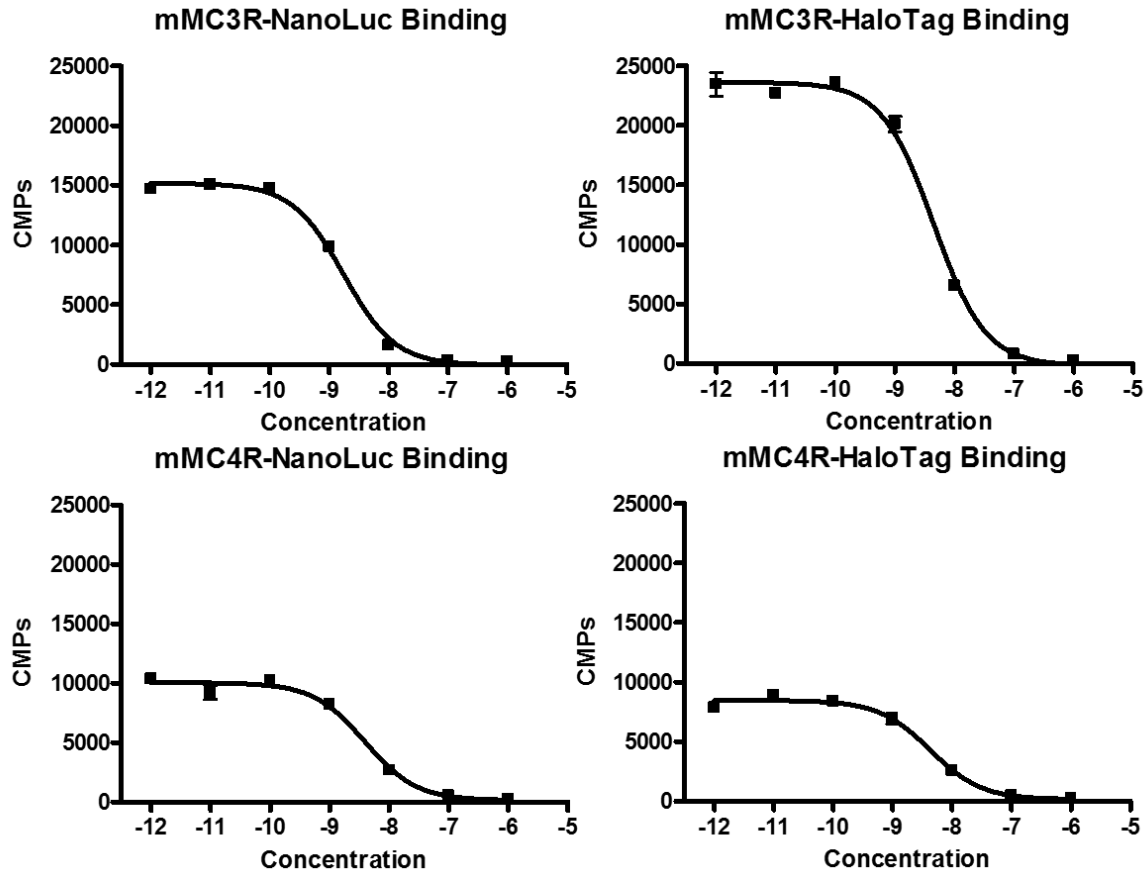
Supplemental Table 1. Amount of intact peptide from *in vitro* serum stability of bivalent ligands and control ligands. Ligands (10 μM) were incubated in mouse serum and monitored for degradation of the parent molecule by LC-ESI^+-MS/MS. The PEDG20 based compounds were relatively metabolically stable, whereas $(Pro\text{-}Gly)_6$ based compounds were rapidly degraded.

	NDP-MSH IC_{50} (nM) Mean±SEM
mMC3R-NanoLuc®	1.1±0.7
mMC3R-HaloTag®	3.1±1.4
mMC4R-NanoLuc®	2.8±1.1
mMC4R-HaloTag®	2.5±1.8

Supplemental Table 2. Competitive radioligand binding assays on the BRET receptor constructs. Unlabeled NDP-MSH was used to displace ^{125}I-NDP-MSH in a dose-response manner to calculate the IC_{50} values. The reported errors are the standard error of the mean (SEM) determined from at least two independent experiments.



Supplemental Figure 1. Average results from hPYY cannulation validation experiments. Mice were administered 2.5 µg of human (h)PYY_{3-36} two hours before lights out. Food intake was measured manually. Validated mice ate at least 0.8 g more food after hPYY administration compared to saline administration at the 4 h time point. On average mice ate 0.4 ± 0.1 g at the 4 h time point after saline administration compared to 1.6 ± 0.1 g after hPYY administration.



Supplemental Figure 2. Representative radioligand binding curves of the BRET receptor constructs. Unlabeled NDP-MSH was used to displace ^{125}I-NDP-MSH in a dose-response manner to calculate the IC_{50} values. The reported errors are the standard error of the mean (SEM) determined from two replicate wells in a single experiment.

Supplemental Discussion

Coexpression of mMC3R and mMC4R Effects on Functional Potency and Discussion of Expression Levels

One noteworthy trend observed in the cAMP functional coexpression experiments is that for all compounds coexpression of the mMC3R transiently in mMC4R stable cells resulted in slightly more potent EC_{50} values than the opposite transfection order in which stable mMC3R cells were transiently transfected with the mMC4R plasmid. This was especially true with dosing of CJL-1-14 and CJL-1-87 that both resulted in about 2-fold differences in EC_{50} values. Although this is considered to be within experimental error of 3-fold, these changes are still worth discussing and may indicate that further study will be necessary.

These differences likely have to do with the ratio of the amount of mMC3R and mMC4R expressed on the cell surface. It is dogma in the field that transient transfection results in a greater expression of receptors on the cell membrane than selected stable receptor populations of cells. Therefore, when the mMC3R is transiently transfected into the stable mMC4R cells, it is anticipated that more mMC3R than mMC4R would be expressed on the cell surface. If heterodimers do exist on the cell surface, the relative concentration of each receptor would be hypothesized to affect the equilibrium of the ratios of mMC3R monomers, mMC4R monomers, mMC3R homodimers, mMC4R homodimers, and mMC3R-mMC4R heterodimers (or how they compose higher-order oligomers). Considering CJL-1-87 has 45- to -350 fold higher binding affinity (depending on the radioligand used) at the mMC4R compared to the mMC3R, these shifts in equilibrium between dimer species would affect how much of the dosed CJL-1-87 is binding mMC4R homodimers, mMC3R homodimers, or mMC3R-mMC4R heterodimers. To our knowledge, there is no technology feasibly capable of distinguishing how much of a ligand is binding to each of the different dimer states at this time. However, the increased potency in the coexpression system suggests that the heterodimerization of the mMC3R-mMC4R could play a role in the altered *in vivo* effects of CJL-1-87 compared to CJL-1-14, and that the mMC3R-mMC4R heterodimer may be a future neuronal molecular drug target.