MATERIAL AND METHODS

Investigation of subcellular localization

HEK293 cells for imaging were cultured on glass coverslips, which were treated with 0.01 mg/ml poly-L-Lysine (BIOCHROM AG, Berlin Germany). For the studies shown here we used YFP- fused GPCR constructs. Cells were overlaid with Hepes-buffered solution (138 nM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM glucose, 2 mg/ml bovine serum albumin, and 10 mM Hepes, pH 7.5) and imaged using a confocal laser-scanning-microscope (LSM 510 META, Carl Zeiss, Jena) with a Plan-Fluar 100x/1.45-object. Microscopy pictures were generated using a 458 nm and a 488 nm laser for excitation and an LP505 emission filter.

Determination of Fluorescence resonance energy transfer

Based on fluorescence resonance energy transfer (FRET) we studied the interaction of MC3R and GHSR as previously described (1,2). Furthermore, we tested homo- and heterodimerization of mutants MC3R I183N, GHSR A204E and GHSR F279L. Therefore, energy transfer-competent forms of auto-fluorescent proteins (cyan-fluorescence protein, CFP and yellow-fluorescence protein, YFP) were attached to the C-terminal tails of the GPCRs. Briefly, 24 h after co-transfection of HEK293 cells with differentially epitope-tagged GPCRs, YFP was photo-bleached at 512 nm and the increase of CFP-emission was measured at excitation at 410 nm. FRET efficiency was calculated as follows: $E = (F_{CFP-max} - F_{CFP-min}) / F_{CFP-max}$ (3). Cells with a molar ratio (YFP:CFP) of less than 1 and over 4 or exhibiting unacceptable signal-to-noise ratios (linear regression analysis giving R^2-values < 0,6) were omitted. The formation of stable dimers is arranged by FRET efficiency between 8 – 25 %.

REFERENCES

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2. Tarnow, P., Rediger, A., Brumm, H., Ambrugger, P., Rettenbacher, E., Widhalm, K., Hinney, A., Kleinau, G., Schaefer, M., Hebebrand, J., Krause, G., Gruters, A., and Biebermann, H. (2008) *Obes Facts* **1**, 155-162
3. Amiri, H., Schultz, G., and Schaefer, M. (2003) *Cell Calcium* **33**, 463-470

FIGURE LEGENDS

Supplementary figure S1: Investigation of dimerization and ligand binding of the MC3R mutant I183N

HEK293 cells were co-transfected with plasmids encoding MC3R-YFP and MC3R-CFP, MC3R I183N-CFP or GHSR-CFP. As positive control the homodimer of MC3R and the MC3R/GHSR heterodimer was used. By FRET analysis we examined the ability of MC3R mutant I183N to form dimers with MC3R **(A)** and with GHSR **(B)**. FRET efficiencies (E) were calculated from the relative increase in CFP emission and the decrease in YFP emission during selective photobleaching at 512 nm. The depicted data represent means ± SEM of 4–6 single cells of one representative measurement of at least three independent transfection experiments performed in triplicates. **(C)** Compared to wild-type MC3R the mutant I183N is still able to bind the ligand but with decreased affinity

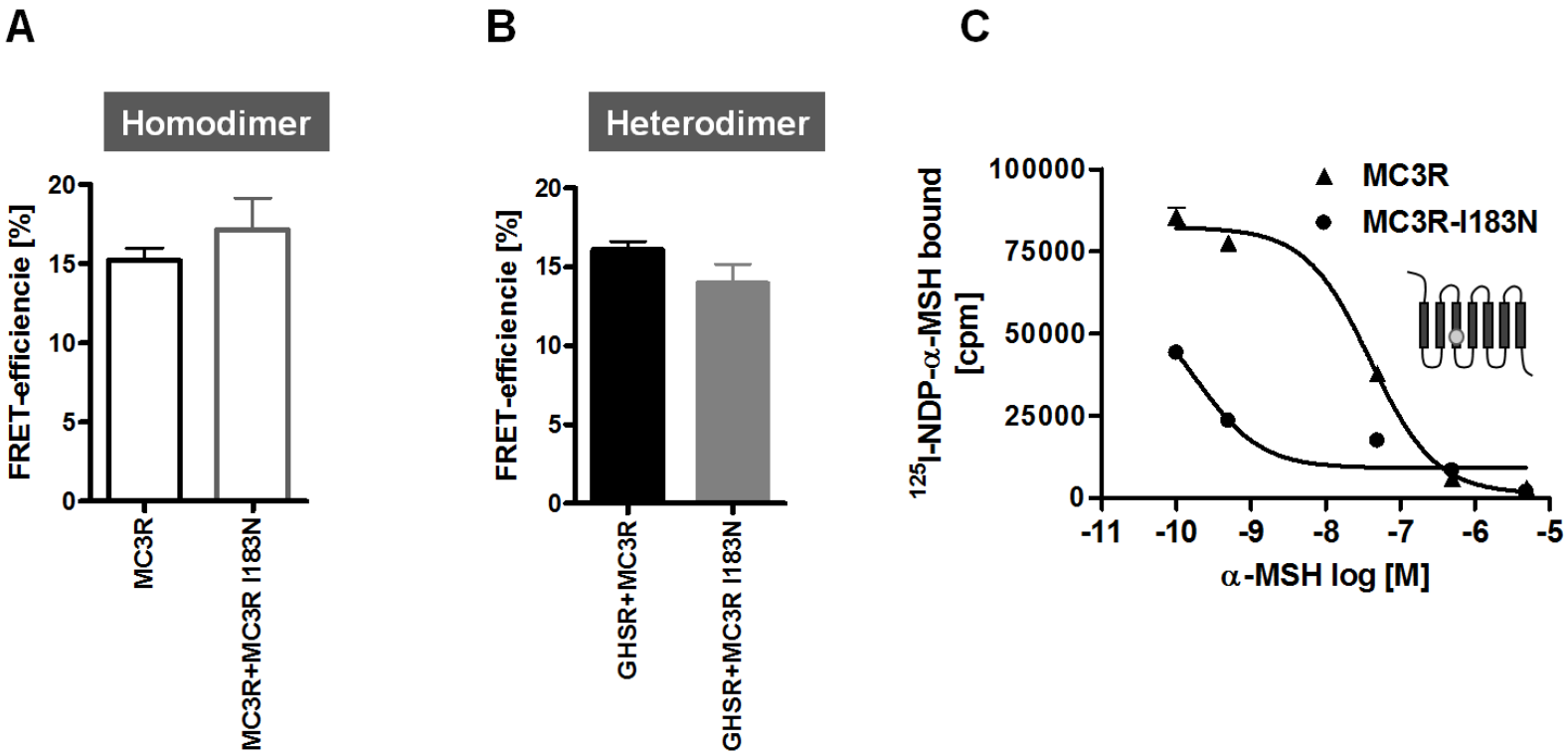
Supplementary figure S2: Signaling properties of natural GHSR mutants

Significant changes of basal and ligand induced activity of GHSR was detected for the mutants A204 and F279L. Twenty-four hours after transfection of HEK293 cells with GHSR mutants and NFAT-luciferase reporter gene constructs activity was quantified in absence or in presence of 1 µM ghrelin. Data represent the mean ± S.E.M. from one representative experiment performed in n=6 out of six independent experiments (see also table 1). Analysis of variance followed by one way ANOVA and Turkey tests. Basal activity of wild-type GHSR compared to the mutants $*p > 0.05$, ligand-induced activity of GHSR wild-type versus mutants **p<0.001.

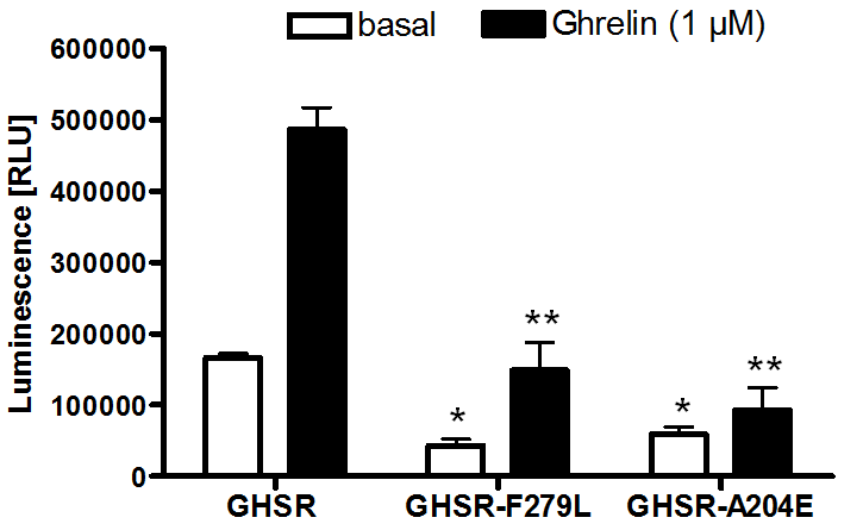
Supplementary figure S3: Investigation of dimerization and intracellular localization of GHSR mutants A204E and F279L

HEK293 cells were co-transfected with plasmids encoding MC3R-YFP and GHSR-CFP, GHSR A204E-CFP or GHSR F279L-CFP. As positive control the homodimer of GHSR and the MC3R/GHSR heterodimer were used. By FRET analysis we examined the competence of GHSR mutants A204E and F279L to form dimers with wt GHSR **(A)** as well as with MC3R **(B)**. FRET efficiencies were calculated from the relative increase in CFP emission and the decrease in YFP emission during selective photobleaching at 512 nm. The depicted data represent means ± SEM of 4-6 single cells of one representative measurement of at least three independent transfection experiments performed in triplicates. **(C)** Fluorophore-tagged GHSR mutants A204E and F279L were used to prove intracellular localization in HEK 293 cells by confocal laser-scanning-microscopy.

Supplementary figure S1



Supplementary figure S2



Supplementary figure S3