IDENTIFICATION OF AN EFFICACY SWITCH REGION IN THE GHRELIN RECEPTOR RESPONSIBLE FOR INTERCHANGE BETWEEN AGONISM AND INVERSE AGONISM

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ABSTRACT:
The carboxy-amidated wFwLL peptide was used as a core-ligand to probe the structural basis for agonism versus inverse agonism in the constitutively active ghrelin receptor. In the ligand, an efficacy switch could be built at the N-terminus as, for example, AwFwLL functioned as a high potency agonist whereas KwFwLL was an equally high potency inverse agonist. The wFw-containing peptides – agonists as well as inverse agonists - were affected by receptor mutations covering the whole main ligand-binding pocket with key interaction sites being an aromatic cluster in TM-VI and VII and residues on the opposing face of TM-III. Gain-of-function in respect of either increased agonist or inverse agonist potency or swap between high potency versions of these properties was obtained by substitutions at a number of positions covering a broad area of the binding pocket on TM-III, -IV and –V. However, in particular space generating substitutions at position III:04 shifted the efficacy of the ligands from inverse agonism towards agonism, whereas similar substitutions at position III:08 one helical turn below shifted the efficacy from agonism toward inverse agonism. It is suggested that the relative position of the ligand in the binding pocket between this “efficacy shift region” on TM-III and the opposing aromatic cluster in TM-VI and TM-VII leads either to agonism – i.e. in a superficial binding mode - or it leads to inverse agonism – i.e. in a more profound binding mode. This relationship between different binding modes and opposite efficacy is in accordance with the Global Toggle Switch Model for 7TM receptor activation.

INTRODUCTION:
7TM receptors (G-protein coupled receptors) constitute one of the largest super-families of proteins, which also serve as targets for a large proportion of current medical drugs. Especially members of the family A or rhodopsin like 7TM receptors, which also is the largest family, are considered to be rather easy drug targets. Most of these receptors are antagonist-prone, i.e. if they are screened with libraries of small organic, drug-like molecules, most if not all of the hits will be antagonists, inhibiting agonist-induced signal transduction, when they are tested in functional assays (1). However, a small proportion of the 7TM receptors including, for example the complement C5a receptor, the melanocortin MC4 receptor and the ghrelin receptor are instead agonist-prone, i.e. most of the screening hits are agonists in functional assays (2). Part of the reason for this is probably that these receptors are characterized by a rather high degree of constitutive, ligand-independent signalling activity, i.e. in the conformational equilibrium these receptors are at least partly biased for active conformation(s) (1). The ghrelin receptor, for example, is among the most constitutively active receptors as it signals with approximately 50 % - depending on the signal transduction pathway - of its maximal signalling capacity without the presence of any hormone (3;4). High constitutive activity has been demonstrated for...
many 7TM receptors in various in vitro settings, in particular when the receptors are over-expressed in heterologous expression systems (5). For the individual receptor it is generally unclear to what degree such ligand-independent receptor signalling is present in the in vivo setting and consequently whether the constitutive signalling is of physiological relevance. This point was recently clarified for the ghrelin receptor with the identification of naturally occurring human mutations, which selectively eliminate its constitutive activity without affecting the affinity, potency or efficacy of the ghrelin hormone (6). Importantly, this mutation which selectively eliminated the constitutive signalling segregated with the development of short stature and the development of obesity (6;7). Similar polymorphisms which selectively eliminate the constitutive activity without affecting the affinity of the endogenous agonist and are associated with morbid obesity have been described for the MC4 receptor (8). This strongly indicates that the constitutive activity of at least the MC4 and the ghrelin receptor and probably many more receptors such as the CB1 receptor is of physiological importance.

Although the Ghrelin receptor clearly is agonist-prone, we have previously found that a low potency antagonist, [DArg¹, DPhe³, DTrp⁵,⁷, Leu¹¹]-substance P, surprisingly acted as a high potency inverse agonist by inhibiting the spontaneous, ligand-independent signalling of the receptor (3). Recently we have, through the synthesis of multiple analogs of this peptide, dissected the molecular determinant for the inverse agonist properties of [DArg¹, DPhe³, DTrp⁵,⁷, Leu¹¹]-substance P and found that the most C-terminal hepta-peptide was sufficient to obtain high potency, full inverse agonism (9). Furthermore, the carboxy-amidated penta-peptide - DTrp-Phe-DTrp-Leu-Leu (wFwLL) appeared to represent the active core which, however, affected the ghrelin receptor with a characteristic shallow biphasic dose-response curve. That is, at low concentrations around 10 nM, wFwLL functioned as a partial agonist, whereas at around 100 nM it acted as a partial inverse agonist (9). The aromatic wFw tripeptide was suggested to represent the essential binding unit in these inverse agonists for the ghrelin receptor (9). In the present study we describe how minor structural modifications of the core wFwLL peptide can convert this dual acting compound into either a pure, high potency agonist or an equally high potency, pure inverse agonist. Similarly, during mutational mapping of the common binding site for these wFw-containing peptides a number of positions were identified in the receptor where mutations improved either their agonist potency or their inverse agonist potency. Importantly, a few mutations could in a systematic manner swap their efficacy from agonism to inverse agonism or the other way around. In particular, two classical ligand interaction positions - III:04 and III:08 - located one helical turn apart on the inner face of TM-III were identified to function as a molecular switch region for the efficacy of the wFw-containing peptides.

**EXPERIMENTAL PROCEDURES**

**Material:** ghrelin peptide was purchased from Bachem (Bubendorf, Switzerland). The N⁴-Fmoc-protected amino acids, 1-hydroxy-benzotriazole (HOBt) and the 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink Amide) resin were purchased from Novabiochem (Germany, Schwalbach). The side chain protecting groups were: tert-butyl (tBu) for Tyr; trityl (Trt) for Gln and His; 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf) for Arg; tert-butyl ester (OtBu) for Asp and t-butoxycarbonyl (Boc) for Lys and diaminopropionic acid (Dap). N,N'-diisopropylcarbodiimide (DIC) was obtained from Sigma-Aldrich (Germany, Taufkirchen). Trifluoroacetic acid (TFA), 1-methyl-2-pyrrolidone, N-ethyldiisopropylamine (DIEA), t-butoanol, thioanisole, piperidine, propionic acid anhydride, O-(7-azobenzotriazol-1-yl)-N,N,N,N-tetramethyluronium hexafluorophosphate (HATU), p-thiocresol, 1,2-ethanediol, and trimethylsilylbromide were purchased from Fluka (Germany, Taufkirchen). Acetonitril (ACN, for HPLC) was obtained from Merck (Germany, Darmstadt). Diethyl ether, dichloromethane (DCM) and dimethylformamide (DMF, peptide synthesis grade) were obtained from Biosolve (Netherlands, Valkenswaard).

**Peptide synthesis** - The inverse agonist peptides were synthesized by solid-phase technique on an automated multiple peptide synthesizer (Syro, MultiSynTech,
Bochum, Germany) by using the Rink amide resin (30 mg, resin loading 0.6 mmol/g) as described recently (10). All peptides were cleaved from the resin in one step by using TFA, precipitated from ice-cold diethyl ether, washed and finally lyophilized. Partially oxidized Met were reduced after lyophilization by applying a mixture of TFA/ ethanedithiol/trimethylbromosilane (97.2:1.6:1.2, v/v/v) for at least 20 min and subsequently recovered from ice-cold diethyl ether, washed and finally lyophilized.

Purification of the peptides was achieved by preparative HPLC on a RP C-18 column (Vydac, 250x25 mm, 10 µm) with a gradient of 20-60 % B in A (A = 0.1 % TFA in water; B = 0.08 % TFA in acetonitrile) over 60 min and a flow of 10 mL/min (λ = 220 nm). The peptides were analyzed by MALDI mass spectrometry on a Voyager-DE RP workstation (Applied Biosystems, Darmstadt, Germany) and by analytical reversed-phase HPLC on a Vydac RP18-column (4.6 x 250 mm; 5 µm, 300 Å) using linear gradients of 10-60 % B in A over 30 min and a flow rate of 0.6 mL/min (λ = 220 nm). The observed masses were in full agreement with the calculated masses and the purity of all peptides was >95 % according to analytical HPLC.

Molecular biology - The human ghrelin/ Growth Hormone Secretagogue receptor cDNA was cloned by PCR from a human brain cDNA library. The cDNA was cloned into the eukaryotic expression vector pCMV-Tag(2B) (Stratagene, La Jolla, CA) for epitope tagging of proteins with a FLAG epitope. Mutations were constructed by PCR using the overlap extension method (11). The PCR products were digested with appropriate restriction endonucleases (BamHI and EcoRI), purified and cloned into the vector pCMV-Tag (2B). All PCR experiments were performed using Pfu polymerase (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. All mutations were verified by restriction endonuclease mapping and subsequent DNA sequence analysis using an ABI 310 automated sequencer.

Transfections and tissue culture - COS-7 cells were grown in Dulbecco’s modified Eagle’s medium 1885 supplemented with 10 % fetal calf serum, 2 mM glutamine and 0.01 mg/ml gentamicin. Cells were transfected with Lipofectamine 2000 (Life Technologies). Phosphatidylinositol turnover - One day after transfection COS-7 cells were incubated for 24 hours with 5 µCi of [3H]-myo-inositol (Amersham, PT6-271) in 1 ml medium supplemented with 10 % fetal calf serum, 2 mM glutamine and 0.01 mg/ml gentamicin per well. Cells were washed twice in buffer, 20 mM HEPES, pH 7.4, supplemented with 140 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM CaCl2, 10 mM glucose, 0.05 % (w/v) bovine serum; and were incubated in 0.5 ml buffer supplemented with 10 mM LiCl at 37°C for 30 min. After stimulation with various concentrations of peptide for 45 min at 37°C, cells were extracted with 10mM formic acid followed by incubation on ice for 30 min. The resulting supernatant was purified on Bio-Rad (Hercules, CA) AG 1-X8 anion-exchange resin. Determinations were made in duplicates.

SRE reporter assay. HEK293 cells (30 000 cells/well) seeded in 96-well plates were transiently transfected. The cells were transfected with SRE-Luc (PathDetect SRE Cis-Reporting System, Stratagene) and the indicated amounts of receptor DNA. The experiment was performed as previously described (13).

Competition binding assays - Transfected COS-7 cells were transferred to culture plates one day after transfection at a density of approximately 5.000 cells per well aiming at 5 - 8 % binding of the radioactive ligand. Two days after transfection competition binding experiments were performed for 3 hours at 4°C using approximately 25 pM of 35S-MK-677 (provided by Andrew Howard, Merck Research Laboratories, NJ). Binding assays were performed in 0.1 ml of a 50 mM Hepes buffer, pH 7.4, supplemented with 1 mM CaCl2, 5 mM MgCl2, and 0.1 % (w/v) bovine serum albumin, 40 µg/ml bacitracin. Non-specific binding was determined as the binding in the presence of 1 µM of unlabeled ghrelin. Cells were washed twice in 0.1 ml of ice-cold buffer and 50µL of lysis buffer/scientillation fluid (Ethoxylated alkylphenol 30% and Diisopropyl naphthalene isomers 70%) was added and the bound radioactivity was counted. Determinations were made in triplicate. Initial experiments showed that steady
state binding was reached with the radioactive ligand under these conditions.

Calculations - IC\textsubscript{50} and EC\textsubscript{50} values were determined by nonlinear regression using the Prism 3.0 software (GraphPad Software, San Diego). \( B_{max} \) value was calculated from the homologous competition binding curve using the equation \( B_{max} = B_0 \times (IC_{50}/[L]) \), where \( B_0 \) indicates the specific bound radioligand and \([L]\) indicates the concentration of free radioligand.
The basal constitutive activity is expressed as percent of the ghrelin induced activation for each mutant construct of the ghrelin receptor. \( F_{mut} \) indicates the fold shift in potency or affinity induced by the structural change in the ligand as compared to the \([D-Arg^1,D-Phe^5,D-Trp^7,9,Leu^{11}]\)-substance P peptide in table I. In table II \( F_{mut} - EC_{50}(mut/WT) \) - indicates the fold shift in potency induced by the mutated receptor as compared to the wild type receptor.

Cell surface expression measurement. Cells were transfected and seeded in parallel with those used for IP accumulation assay. After two wash cycles, cells were fixed and incubated in blocking solution (PBS/0.2 %BSA) for 30 min at room temperature. The cells were kept at room temperature for all subsequent steps. Cells were incubated with anti-FLAG (M2) antibody (Sigma Chemical Co, St.Louise, MO) in 1:300 dilution. After three washes cells were incubated with anti mouse horseradish peroxidase (HRP) (Amersham Bioscience, New Jersey, US) conjugated antibody in dilution 1:4000. After extensive washing the immunoreactivity was revealed by the addition of HRP substrate according to manufacture’s instruction.

Conformation analysis of the wFwLL peptide by molecular dynamics - Molecular dynamic was performed by CHARM using the CHARM22 force field as described in the supplementary materials (14). Briefly, the system was minimized and heated to 310 K followed by equilibration and simulated using Langevin dynamics for 0.1 \( \mu \)s. The molecular dynamic trajectory was analyzed and structures were clustered based on the backbone dihedral angles \( \phi \), psi and the side chain \( \chi_1 \) dihedral angle for wFwLL were used to calculate differences between conformations and define the cluster distances. The average cluster energy (kcal/mol), number of cluster members and estimated probabilities were calculated (see supplementary materials for details)

RESULTS

Modification of the C-terminal end of the wFwLL peptide – The synthetic analogs of the wFwLL peptide were analyzed in COS-7 cells transiently transfected with the ghrelin receptor both in respect of affinity, as measured in competition binding experiments against the radioactively labeled non-peptide agonist \(^{35}\text{S}-\text{MK}-677\), as well as in respect of stimulation of signal transduction, as determined in inositol phosphate turnover assays. Exchange of the C-terminal Leu-Leu motif of wFwLL with Ala-Ala completely eliminated the activity of the peptide as neither agonism nor inverse agonism was observed at any concentration tested (Fig.2A) (Table 1). In accordance with this, no displacement of the radiolabeled \(^{35}\text{S}-\text{MK}677\) was observed for the wFwAA peptide even at a concentration up to 10 \( \mu \)M (Fig.2B). In contrast, exchange of the Leu-Leu motif with a simple Gly-Gly sequence, i.e. corresponding to a wFw peptide being extended with just a peptide backbone spacer to the important C-terminal carboxyamide group, surprisingly enhanced the agonist property, as wFwGG behaved as a 50% partial agonist stimulating IP turnover with a potency of 159 nM and having a binding affinity of 540 nM (Fig.2B) (Table 1). This observation supports the notion that it is the aromatic tri-peptide wFw (DTrp-Phe-DTrp) which – besides the C-terminal carboxy-amide - is the key recognition motif for the wFwLL peptide in the ghrelin receptor (9).

Modification of the N-terminal end of the wFwLL peptide – As previously reported, attachment of a positively charged Lys residue at the N-terminal end of wFwLL results in a hexapeptide, which is an equally potent inverse agonist as the original \([D-Arg^1,D-Phe^5,D-Trp^7,9,Leu^{11}]\)-substance P peptide (9). In the present study we systematically attached positive, negative and neutral amino acid residues at the N-terminal end of wFwLL (Table 1). It was found that Arg was equally efficient as Lys in converting the dual-active wFwLL into a pure, high potency inverse agonist (Table 1). In contrast, extension of wFwLL with the small non-natural, positively charged amino acid diaminopropionic acid resulted in an inverse agonist with a six fold lower potency than
KwFwLL and an affinity of 210 nM (66 fold less) indicating that the positive charge of the side-chain has to be spaced from the free amino terminal by more than two carbons in order to achieve an optimal interaction with the receptor (Table I). In contrast, N-terminal extension of wFwLL with a negatively charged Asp did not improve its potency and efficacy as an inverse agonist, but did abolish the high potency partial agonism of the wFwLL peptide (Fig. 3A).

Surprisingly, N-terminal extension of wFwLL with the small, apolar, aliphatic residue, Ala, completely eliminated the inverse property of the core pentapeptide as AwFwLL instead behaved as a high potency, pure – albeit partial - agonist (EC₅₀ = 15 nM) (Fig. 3). Thus, AwFwLL stimulated inositol phosphate turnover with a potency being even slightly higher than the potency of the structurally similar KwFwLL and the original, mother peptide [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]-substance P, which however both act as inverse agonists (Fig. 3). Interestingly, the binding affinity of AwFwLL as measured in competition against ³⁵S-MK677 was decreased 17 fold compared to the affinity observed for, for example [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]-substance P, which however both act as inverse agonists (Fig. 3). Thus, the wFw tri-peptide appears to constitute a versatile ghrelin receptor binding scaffold, which in a carboxy-amidated penta- or hexa-peptide setting provides either high potency agonism or high potency inverse agonism - depending upon the size and chemical nature of the terminal residues. Thus, wFwLL which by itself display a characteristic, relatively shallow, biphasic dose-response curve is converted into a pure, high potency inverse agonist in the KwFwLL setting and into an equally high potency, pure agonist in the AwFwLL peptide.

Mutational mapping of receptor interactions of KwFwLL and AwFwLL - The two closely related hexa-peptides having opposite efficacies were characterized in a library of receptor mutants with substitutions located at seventeen key positions in the main ligand-binding pocket of the ghrelin receptor (Fig 1). The mutants were selected from a large library of ghrelin receptor mutants to have significant structural changes at the various positions but still to display a reasonably high constitutive activity, which is required in order to be able to study the inverse agonist properties of the ligands. All mutations were expressed at the cell surface to a similar degree as observed for the wild type receptor as determined by cell surface ELISA, i.e. except for the AspII:20Lys and the GlnIII:05Ala constructs, for which the expression was less than half of that observed for the wild type receptor (Table II). The absolute receptor expression was estimated from competition binding experiments to be 55fmol/10⁵ cells. Ghrelin receptor expression levels have previously been estimated in human hypothalamic membranes and dependent on the radioligand the expression levels have been reported to be either at the same level as we find (15) or approximately 20 fold lower (16). However it is difficult to compare Bmax value obtained from tissues samples with Bmax value calculated from transfected cells and especially when different radioligands are used. Concerning expression levels, the important issue is that the constitutive activity of the ghrelin receptor has been shown to be a physiologically important phenomenon in the in vivo setting as carriers of a genetic variant of the receptor, which selectively eliminate its constitutive activity, display a clear phenotype (6).

Most of the mutations of the present study did not affect the constitutive activity, whereas four of the mutations displayed a moderately reduced degree of constitutive activity (i.e. between 15 and 20 % of maximal signalling capacity as compared to the 42 % observed in the wild-type receptor) (Table II). One mutation, [PheVI:16Ala], was included despite a highly reduced constitutive activity (i.e. only 2 %), because it is centrally located in the binding pocket and because it has been shown to be a mutational hit for ghrelin (9). The selected mutations have previously been described in terms of effect on the basic inverse agonist, [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]-substance P and on ghrelin (9). These data are included in Table II for comparison in the form of Fmut values, i.e. the fold-differences of the potency induced by the mutant as compared to the wild-type receptor.

In contrast to the endogenous agonist ghrelin, which only is affected by a few mutations located at key positions in the middle of the binding pocket between TM-III, -VI and -VII (9) (Table II), both the Lys and the Ala modified penta-peptides were affected by a large number of mutations located throughout the...
main ligand binding pocket. The mutational map for the inverse agonist (KwFwLL) and the partial agonist (AwFwLL) were rather similar, as they both were crucially dependent on AspII:20, GlnIII:05, GluIII:09, ArgVI:20 and PheVII:09 (Table II and Fig.4). However, at a few positions the effect of the mutations differed considerably. Thus, substitution of PheVII:06 eliminated the activity of the inverse agonist, KwFwLL, whereas the partial agonist, AwFwLL, was only decreased 7.3 fold in potency. The opposite pattern was observed for two substitutions in TM-V, ValV:08 and PheV:12, which both completely abolished the agonist activity of AwFwLL without having any effect on the inverse agonism of KwFwLL (Table II). Notably, the [PheVI:16Ala], which due to lack of constitutive activity could not be used to map the inverse agonist KwFwLL, was a major hit for the AwFwLL agonist peptide as it is for the endogenous agonist ghrelin (Table II).

Molecular dynamic analysis of the wFwLL core peptide revealed that it preferentially adopts an L-shaped low energy conformation in which the side-chains of the characteristic, aromatic wFw sequence all radiates away from the convex side of the molecule whereas the polar groups of the backbone point inward on the concave side (Fig 4A). As shown in Fig. 4B it is proposed that the wFw-containing peptides bind in the ghrelin receptor in a manner in which the aromatic side-chains of the ligand interdigitate with the aromatic cluster on TM-VI and VII while the polar backbone interact with the polar residues on the opposing face of TM-III.

**Mutations increasing the potency of the peptides** - In mutational analysis of ligand binding and function it is usually loss-of-function effects that are observed. However, as previously reported for the inverse agonist, [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-substance P (9), a number of mutations in the ghrelin receptor in fact increased the potency of either one or the other of the hexa-peptides (Table II). Thus, a large increase in inverse agonist potency (from 32 to 2.6 nM) was observed for KwFwLL in the [SerIV:16Ala] mutant located at the extracellular end of TM-IV (Fig. 5 and 6A). Previously, we found an almost 20-fold increase in inverse agonist potency also for the mother peptide, [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-substance P, in this mutant (9) (Table II). In fact, for KwFwLL a clear two-component dose-response curve was found in the [SerIV:16Ala] mutant consisting of a very high potency component of 0.3 nM (64 %) and a low potency component of 120 nM (36 %) (Fig. 6A). Thus, it could be argued that the space-generating SerIV:16 to Ala mutant was a major mutational “hit” for KwFwLL as it induced an almost 100-fold increase in its inverse agonist potency. In contrast, the agonist AwFwLL was only affected to a minor degree (i.e. an approx. 2-fold increase in potency) by the SerIV:16 mutation in TM-IV, and the natural agonist ghrelin was not affected at all (Table II). In TM-V, however, another space-generating substitution, MetV:05 to Ala, had the opposite profile as it increased the potency of the AwFwLL agonist 50 fold without affecting the potency of the inverse agonist KwFwLL (Table II and Fig. 6B). In conclusion, a substantial, selective increase in potency is observed for the inverse agonist KwFwLL by a space-generating mutation at the extracellular end of TM-IV, [SerIV:16Ala], and for the structurally similar agonist AwFwLL at the extracellular end of TM-V, [MetV:05Ala] (Fig. 5), which indicates that these two structurally related peptides with opposite pharmacological property have significantly different interaction modes at the interface between the extracellular ends of TM-IV and TM-V.

**Mutations swapping between inverse agonism and agonism** - three mutations had a surprising effect as they did not influence the actual potency of the wFw-containing peptides but instead totally swapped their pharmacological property. In TM-IV, Ala substitution of IleIV:20 converted the inverse agonist KwFwLL into an almost equally potent agonist (EC50 = 43 nM as agonist on the mutant versus 32 nM as inverse agonist in the wild-type receptor) having an efficacy of approx. 50 % as compared to the natural agonist ghrelin (Fig. 7E). The efficacy of AwFwLL, which was a partial agonist on the wild-type receptor, was increased to almost full agonism by the IleIV:20 to Ala substitution, while its potency was somewhat decreased (11 fold) (Fig 7C). As previously reported, the inverse agonist potency of [D-Arg1,D-Phe5,D-
Trp<sup>7,9</sup>,Leu<sup>11</sup>]-substance P was decreased 20-fold by the IleIV:20 to Ala substitution (Table II). In TM-III where mutations of GlnIII:05 and GluIII:09 basically eliminate the effects of both AwFwLL and KwFwLL (Table II), substitution of the “neighboring” PheIII:04 and SerIII:08 (Fig. 5) both resulted in an total interchange between agonism and inverse agonism for the N-terminally extended wFwLL hexapeptides, but importantly in opposite directions. Thus, substitution of PheIII:04 with Ser converted the inverse agonist KwFwLL into an efficacious agonist showing a 60 nM potency similar to its potency as an inverse agonist on the wild-type ghrelin receptor (32 nM) (Fig. 7D). The [PheII:04Ser] mutant had very limited effect on the structurally similar agonist AwFwLL (Table II). In contrast, Ala-substitution of SerIII:08 located one helical turn deeper in the main ligand-binding pocket of the receptor (Fig. 5) converted the AwFwLL agonist into an inverse agonist with a potency of 28 nM, which is similar to its potency as an agonist on the wild-type ghrelin receptor (15 nM) (Fig. 7B, Table II). The potency of the KwFwLL inverse agonist was slightly improved by the SerIII:08 to Ala mutation, which previously has been reported to increase the inverse agonist potency of [D-Arg<sup>1</sup>,D-Phe<sup>5</sup>,D-Trp<sup>7,9</sup>,Leu<sup>11</sup>]-substance P approx. four fold. In conclusion, position III:04 and III:08 located one helical turn apart on the inner face of TM-III together constitute an efficacy switch region in the ghrelin receptor (Fig. 5).

**Characterization of selected mutants in SRE signaling**

The ghrelin receptor has previously been demonstrated to signal through activation of serum responsive element (SRE) controlled transcriptional activity conceivably mediated mainly through G12/13 (4). In the SRE signaling pathway the two hexapeptides, KwFwLL and AwFwLL, were affected by the III:04 and III:08 substitutions in TM-III in a similar way as observed for these mutants when the Gq/11 mediated inositol phosphate turnover was measured. However, the starting point for the peptides – especially KwFwLL – was somewhat different in the SRE assay (Fig. 8). Thus, on the wild-type ghrelin receptor the KwFwLL peptide, which in relation to IP turnover clearly was a potent and efficacious inverse agonist (Fig. 3), was a relatively neutral or very low potency inverse agonist in respect of effect on SRE signalling (Fig. 8A). Nevertheless, the SerIII:08 to Ala mutant restored its properties as a high potency inverse agonist (EC<sub>50</sub> = 12) whereas the PheIII:04 to Ala substitution turned the KwFwLL peptide into a high potency (EC<sub>50</sub> = 26 nM) partial agonist (Fig. 8A). The AwFwLL peptide, which in respect of stimulation of IP turnover was a partial agonist, was a full agonist in respect of stimulation of SRE signalling. In this case, the PheIII:04 to Ala mutation only had a slight increasing effect on the agonist potency of the peptide, whereas the SerIII:08 to Ala mutant swapped AwFwLL from being an agonist to being an inverse agonist (Fig. 8B). In Fig. 8C are shown also the effect of these two mutants on the “mother-peptide”, i.e. the inverse agonist [D-Arg<sup>1</sup>,D-Phe<sup>5</sup>,D-Trp<sup>7,9</sup>,Leu<sup>11</sup>]-substance P, i.e. a decrease in inverse agonist potency for [PheIII:04Ala] and an increase in potency for [SerIII:08Ala], respectively. These two mutants had very little effect on the agonist properties of the endogenous ligand ghrelin (Fig. 8C).

**DISCUSSION**

Agonism and inverse agonism are a priori opposite properties. In the present study a characteristic highly aromatic, carboxy-amidated penta-peptide, wFwLL, was used as a core-ligand for the constitutively active ghrelin receptor to probe the structural basis - both in the ligand and in the receptor – for agonism versus inverse agonism. Relatively small structural changes at the N-terminal end of the ligand were found to swap between high potency agonism – as observed for the AwFwLL hexa-peptide - and equally high potency inverse agonism - as observed for example for the KwFwLL peptide. Similarly, relatively small changes in the receptor structure could in a comparable manner swap the efficacy of the peptides from agonism to inverse agonism of equally high potency - or the other way around. Thus, an “efficacy switch region” for the wFw-containing peptides was identified on the inner face of TM-III (Fig. 5), where space generating substitutions at position III:04 shifted the efficacy of the ligands from inverse agonism toward agonism. In contrast, similar substitutions at position III:08 one helical turn below shifted their efficacy from agonism toward inverse agonism. Apparently, the relative position of the ligand in the binding pocket between this “efficacy shift region” on TM-III and the opposing aromatic cluster in TM-VI and TM-VII leads either to agonism – i.e. in a superficial binding mode - or it leads to inverse agonism – i.e. in a more
profound binding mode (Fig. 9). This relationship between different binding modes and opposite efficacy is in accordance with the Global Toggle Switch Model for 7TM receptor activation (see below) (1).

Building an efficacy switch into the wFwLL-containing peptide ligand

The core penta-peptide, wFwLL, has in itself a shallow biphasic efficacy profile composed of a high potency agonism component combined with a low potency inverse agonism component (9). Structural modifications at the C-terminal end of the wFwLL peptide improved the agonist properties of the peptide as illustrated by the wFwGG peptide, which was a pure agonist. However, in no case did we observe that C-terminal changes improved the inverse agonist component of the peptide. In contrast, modifications at the N-terminal end of the wFwLL peptide generated both high potency inverse agonists and equally high potency agonists - depending on the physicochemical properties of the modification. Thus, attachment of a positively charged Lys, Arg, or His residue at the N-terminus provided pure, high potency inverse agonism whereas extension with a small, aliphatic Ala residue resulted in pure, high potency agonism. In contrast, N-terminal extension of the wFwLL peptide with an acidic Asp or a polar, non-charged Gln residue basically just eliminated the high potency, low efficacy agonistic component of wFwLL as these two hexapeptides displayed relatively similar, low potency simple inverse agonism. Thus, in an X-wFwLL hexapeptide setting the N-terminal residue clearly constitute an efficacy switch region.

Mutational mapping of the binding pocket for the peptides – the mutational map for the wFw-containing hexa-peptides is rather complex as it is composed of both loss-of-function hits and significant gain-of-function hits as well as even hits, which convert the efficacy of the ligands – being either agonist or inverse agonist - to the opposite type of efficacy. Nevertheless, just the classical loss-of-function mutations for the AwFwLL agonist peptide are found in all six helices from TM-II to TM-VII, which is in clear contrast to the endogenous agonist ghrelin, which only is affected by very few mutations, which all are located at the interface between TM-III, VI and VII, (Table II). This indicates that the small AwFwLL peptide - in contrast to ghrelin - acts as an agonist by inserting and interacting with residues located across the whole main ligand-binding crevice from TM-II to TM-IV and –V. The overall mutational map for the inverse agonist KwFwLL was rather similar to the map for the AwFwLL agonist peptide; however, with a major difference being that KwFwLL is not affected by the substitutions in TM-V, i.e. mutations at positions V:08 and V:12, which eliminate the function of the AwFwLL peptide (Table II). Furthermore, KwFwLL display improved potency by substitutions in TMIV whereas AwFwLL has improved potency after substitution in TMV. Most likely, the side-chains of the characteristic, aromatic wFw-motif of these peptides interact with the aromatic cluster at the interface of TM-VI and – VII, i.e. PheVI:16, PheVII:06 and PheVII:09 (Fig. 4B). However, this aromatic-aromatic interaction appears to be different for the two peptides as indicated by the fact that the Leu for Phe substitution at position VII:06 is only a minor hit for the agonist AwFwLL (5.4 fold), but is a major hit for the inverse agonist KwFwLL (Table II). The aromatic cluster in the receptor has previously been shown to be essential for the constitutive activity of the ghrelin receptor as demonstrated both by targeted mutagenesis and by the occurrence of a natural mutation of PheVI:16 to Leu, which eliminates the constitutive activity and is associated with short for normal stature and obesity (4;17). In this binding scenario, the polar residues on the opposing, inner face of TM-III, GlnIII:05 and SerIII:08, are likely interaction points for the peptide backbone of the wFw-motif. A schematic binding mode for the KwFwLL peptide is shown in Fig. 4B where it is suggested that the positively charged N-terminal α-amino group of the peptide ligands points toward AspII:20 and the C-terminal carboxy-amide points toward GluIII:09, which both are major hits for the wFw-containing peptides (Table II). In principle the peptides could be oriented in the opposite direction. However, we prefer the orientation indicated in Fig. 4B as it positions the key “efficacy switch residue” of the ligand, i.e. the N-terminal Lys or Ala, in close proximity to the corresponding switch-region in the receptor, i.e. residues III:04 and III:08, which upon mutation makes the corresponding swap in efficacy for the two peptides. The fact that it is the α-amino group and not

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2 This includes a number of unpublished wFw peptides with C-terminal modifications.
the side-chain of the peptide ligands that is pointing toward AspII:20 is further substantiated by introduction of a Lys in this position. As shown in Table II not even the Asp extended wFwLL peptide is improved by introduction of a lysine residue in position II:20.

Molecular efficacy switch in the ghrelin receptor –
It should be noted that the two positions in TM-III, III:04 and III:08, which in the ghrelin receptor constitute the main molecular switch region for the small wFw-containing peptides, are well known positions for ligand interactions in prototype 7TM receptors. In the monoamine receptors, AspIII:08 functions as the classical anchor-point for the positively charged ligands – i.e. agonists as well as inverse agonists or antagonists (18-20). In rhodopsin, GluIII:04 (Glu113) functions as the counter-ion for the LysVII:10 (Lys 256) mediated, covalent Schiff-base attachment of retinal. In relation to the ligands of the present study it is notable that the overall structural difference between the highly efficacious inverse agonist 11-cis retinal and the all-trans agonist form of retinal in fact is not very big (21;22). Importantly, however, during light activation, which turns the inverse agonist 11-cis into the all-trans agonist, the Schiff-base attachment of the ligand is broken (23). In analogy to this, mutations, which disrupt the interaction between the inverse agonist KwFwLL and the Phe in position III:04 in the ghrelin receptor, turn KwFwLL into an agonist (Fig. 7 and 8). It is unclear what the interaction between the inverse agonist KwFwLL and PheIII:04 is in the wild-type receptor. However, the fact that different positively side-chains at the amino-terminal end of the X-wFwLL peptide all provide inverse agonism, would suggest that for example a cation-π interaction could be involved.3 It is suggested that for the wFw-containing peptides a major part of the ligand-receptor interaction - both in the agonist and in the inverse agonist binding mode - involves an aromatic-aromatic interaction between the wFw motif of the ligand and the aromatic cluster on the opposing, inner face of TM-VI and TM-VII across from the molecular switch region in TM-III (shown schematically in Fig. 4B for KwFwLL).

Changing potency and swapping efficacy on a “global efficacy scale” – The wFw-containing peptides constitute a unique collection of structurally similar ligands or chemotype, which covers a broad range of efficacy “starting points” on the wild-type ghrelin receptor - from agonists over neutral ligands to inverse agonists. Interestingly, these peptides are affected by particular substitutions in their common binding pocket in a strikingly similar mode, which – independent on their starting point on the global efficacy scale – can be considered to be either “from inverse agonism toward agonism” - or the other way around, depending on the specific substitution (Fig. 5 and Fig. 9A). The results indicate that a decrease in inverse agonist potency, a swap from inverse agonism to agonism, and an increase in agonist potency all can be considered to be a “movement in the same direction” i.e. from inverse agonism toward agonism (green arrows in Fig. 9A). For example, the PheIII:04 to Ala mutant display such a “from inverse agonism toward agonism” effect on the three different wFw-containing peptides: i.e. it decreases the inverse agonist potency of [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-substance P, it swaps KwFwLL from being an inverse agonist / neutral ligand to an agonist, and it slightly increases the agonist potency for AwFwLL. In the same way, the SerIII:08 to Ala mutant shifts these three wFw-ligands in the opposite direction “from agonism toward inverse agonism”: it swaps AwFwLL from being an agonist to being an inverse agonist, it increases the inverse agonist potency of KwFwLL more than 100-fold, and it also increases the inverse agonist potency of [D-Arg1,D-Phe5,D-Trp7,9,Leu11]-substance P. It should be noted, that none of the mutants, which swap the agonist / inverse agonist property of the wFwLL peptides or increase their potencies, had any effect on the surface expression or the constitutive activity of the receptor as such and they did not affect the potency or the agonist property of the endogenous ligand ghrelin (Table II). This is in accordance with the notion that full activation of the receptor involves a multistep process where the efficacy switch region revealed by the X-wFwLL hexapeptides, does not influence the partial active ligand independent conformation.

Efficacy swapping in the Global Toggle Switch Model for 7TM receptor activation

3 Substitution of PheIII:04 with an Ala was unfortunately not tolerated (data not shown) to exclude the possibility that introduction of a Ser could be responsible for the observed agonism rather than lack of Phe.
According to the Global Toggle Switch Model, especially TM-VI and VII will during receptor activation perform vertical see-saw movements around the central, conserved proline bends through which the well-established, outward rigid body movements of the intracellular segments of these helices is coupled to an inward tilting of the extracellular segments of especially TM-VI into the ligand binding pocket located at the extracellular side of the receptor (1). According to this model, small molecule agonists function as “molecular glue”, which holds the extracellular segments of especially TM-VI in the active inwardly-bent conformation. Such an induced-fit binding mode for small molecule agonists - including a narrowing of the main ligand-binding pocket - has recently gained support from the work of several different groups employing very different experimental approaches (20;25-29).

In the present study seven mutations are described which display a “gain-of-function” effect – i.e. they either swap efficacy from one type to the opposite or they increase agonist or inverse agonist potency4. These seven substitutions cluster in a relatively broad area on the inner faces of the extracellular segments of TM-III, -IV, and –V (Fig. 5). Importantly, however, if the mutants are divided based on which direction they shift the efficacy, a very interesting pattern appears. Thus, substitutions which bias the wFw-containing ligands toward inverse agonism - i.e. either increased inverse agonist potency and/or swap efficacy from agonism to inverse agonism - are located relatively deep in the binding pocket: SerIII:08, SerIV:16, ValV:08 and PheV:12 (red symbols in Fig. 5 and 9A). In contrast, substitutions which in a similar - but opposite - way bias the ligands toward agonism, are located more extracellularly: PheIII:04, IleIV:20 and MetV:05 (green symbols in Fig. 5 and 9A). As discussed above we believe that the wFw-containing peptides in both the agonist and inverse agonist binding mode make significant interactions with the aromatic cluster on TM-VI and –VII. It is suggested, that the space-generating mutants in the lower part of the binding pocket, which bias toward inverse agonism, does so by enabling the ligands to adopt a more deep binding mode in the pocket (Fig. 9). In contrast, the space-generating substitutions in the upper part of the binding pocket, which bias toward agonism, will enable the ligands to bind better in a more superficial manner – importantly still making the aromatic-aromatic interactions with TM-VI and TM-VII (Fig. 9). These different binding modes for agonists versus inverse agonists are in good agreement with the Global Toggle Switch model (1). Thus, a deep binding mode of the ligand would be expected to block the inward movement of the extracellular segments of TM-VI and VII toward TM-III, which is required for activation, i.e. the peptide will function as an inverse agonist. In contrast, a more superficial binding mode of the ligand in the pocket between TM-III, -VI and –VII could - with the right interactions - be expected in stead to allow TM-VI and –VII to move into their active, inwardly bent conformation, i.e. the peptide act as an agonist.

According to this, it should in principle be possible to induce similar changes in potencies and similar swaps between agonism and inverse agonism in other 7TM receptors through similar receptor substitutions. However, such gain-of-function effects can probably only be obtained with certain types of ligands or chemotypes. In the present study, for example, the seven gain-of-function substitutions discussed above do not affect the potency or efficacy of the endogenous agonist ghrelin (Table II). In other receptor systems similar interchanges between agonism and antagonism has been observed by substitutions in other positions of the receptor (30;31). Nevertheless, in for example the C5a receptor it has been reported that substitution of IleIII:08 with a smaller Ala residue can swap the efficacy of a particular peptide analog having a large hydrophobic side chain – cyclohexylalanine – from being an antagonist to becoming an agonist (32). Similarly, also in the C5a receptor a series of non-peptide ligands which are covalently coupled to a Cys residue introduced at position VI:20 on the opposing inner face of TM-VI will gain agonist properties and gain potency by the same space-generating IleIII:08 to Ala substitution (33;34). It was previously proposed that the increased space will allow for an enhanced inward movement of TM-VI toward TM-III which according to the Global Toggle switch Model will result in agonism (1). In the CB1 receptor, which also is highly

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4 Mutations which decrease potency of either an agonist or an inverse agonist may in fact also work on the proposed global efficacy scale in a “positive manner” as shown in Fig. 9A, but if they are not observed to either increase the potency or swap the efficacy of a related ligand, such mutations may just be classical mutational hits which affect the ligand binding / affinity in a negative manner.
constitutive active, space generating mutations in position III:12 (Phe²⁰⁰) - i.e. one helical turn deeper in the receptor than III:08 - converted a small molecule agonist into an inverse agonist (35), which very well could occur through a mechanism similar to the one described in the present study for the AwFwLL agonist peptide in the SerIII:08 to Ala mutation.

A signalling pathway biased inverse agonist - It has previously been demonstrated that the ghrelin receptor couples to both Gq/11 - as measured for example in changes in inositol phosphate turnover - and to G12/13 - as measured by SRE mediated transcriptional activity (4). The wFw-containing “mother peptide”, [D-Arg¹,D-Phe⁵,D-Trp⁷⁹,Leu¹¹]-substance P, functions as an efficacious inverse agonist for both of these signalling pathways with a similar, high potency being 18 and 49 nM, respectively. Interestingly, the KwFWLL hexapeptide, which is a high potency inverse agonist in respect of inhibiting inositol phosphate turnover, was almost unable to inhibit the SRE mediated transcriptional activity (Fig 8). Such, inverse agonists which are biased toward a particular signalling pathway may have important clinical applications or implications as it could be relevant to target only the function of the receptor related to coupling to one of the G-protein but not the other. For example, ghrelin is involved in the control of both appetite and growth hormone secretion and it may be relevant to develop drugs that selectively target one of these functions.

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Reference List


**LEGENDS TO FIGURE**

Fig. 1. **Structure of the wFw-containing peptides and the ghrelin receptor target.** At the top right are shown schematic drawings of the amino acid sequence of the “mother peptide”, the inverse agonist [DArg₁, DPhe⁵, DTrp⁷,⁹, Leu¹¹]-substance P, the core peptide wFwLL, the inverse agonist KwFwLL, and the AwFwLL agonist which were used for mutational mapping (aromatic residues in green, aliphatic hydrophobic in grey, hydrophilic non-charged in pink, and positively charged in blue). In the serpentine diagram of the ghrelin receptor the residues which are mutated in the present study are highlighted in red. Baldwin’s generic numbering system for 7TM receptor residues, which is based on the actual location of the residues in each transmembrane helix is used throughout the paper (36;37). For convenience, the numbering of a highly conserved residues in each helix is marked (also highlighted in white on grey): AsnI:18, AspII:10, ArgIII:26, TrpIV:10, ProV:16, ProVI:15 and ProVII:17 and the proposed first residue of each transmembrane helix is indicated by “1”.

Fig. 2. **Effect of C-terminal modification of the core peptide wFwLL on binding and signalling – Panel A** shows effect on Gq coupling as reflected in inositol phosphate turnover; and **Panel B** shows competition binding experiments using ³⁵S labelled MK-677 as a radioligand by [DArg₁, DPhe⁵, DTrp⁷,⁹, Leu¹¹]-substance P (dashed line), ghrelin (dotted line) and three C-terminally modified version of wFw-LL: wFw-LL (square), wFw-AA (triangle) and wFw-GG (circle). Peptides were tested in COS-7 cells transiently transfected with wild type ghrelin receptor, data are mean ± S.E. of three to ten independent experiments performed in triplicates.

Fig. 3. **Effect of N-terminal modification of the core peptide wFwLL on binding and signalling – Panel A** shows effect on Gq coupling as reflected in inositol phosphate turnover; and **Panel B** shows competition binding experiments using ³⁵S labelled MK-677 as a radioligand. The dose response curve for ghrelin is depicted in a dotted line and the [DArg¹, DPhe⁵, DTrp⁷,⁹, Leu¹¹] substance P curve in dashed line. The following N-terminally extended hexa-peptide versions of wFwLL are shown: Ala (empty circle), Asp (full square), Lys (full circle), and Arg (full triangle). Peptides were tested in COS-7 cells transiently transfected with wild type ghrelin receptor, data are mean ± S.E. of three to ten independent experiments performed in triplicates.

Fig. 4. **A cluster of the most preferred conformations of the wFwLL core peptide and schematic model of the mutational hits and a proposed basic binding mode for the wFw-containing peptides in the main ligand-binding crevice of the ghrelin receptor illustrated by the inverse agonist KwFwLL – Panel A** – 10 random conformations from the most populated cluster of conformations of the wFwLL peptide sampled after...
molecular dynamics simulations (see supplementary material for details) aligned according to the backbone of the wFw sequence. Panel B - A molecular model of the ghrelin receptor - built over the X-ray structure of the inactive form of rhodopsin – is viewed from the extracellular side where only the seven helical domains are shown in solid ribbon with the identified mutational hits highlighted. Classical mutational hits (see Table II for details) which just impair the potency of either the agonist AwFwLL and/or the inverse agonist KwFwLL are shown in dark purple (AspII:20, GlnIII:05, GluIII:09; PheVI:16, ArgVI:20, PheVII:06 and PheVII:09). Mutational hits which shift the ligands toward inverse agonism (i.e. improve inverse agonist potency and/or swap ligands from agonism to inverse agonism – see Fig, 5) are shown in red (SerIII:08, SerIV:16, ValV:08 and PheV:12). Mutational hits which shift the ligands toward agonism (i.e. improve agonist potency and/or swap ligands from inverse agonism toward agonism – see Fig. 5) are shown in green. A schematic model of the KwFwLL inverse agonist has been “docked” into the model in a configuration where the N-terminal molecular switch region of the ligand is placed in proximity to the efficacy switch region of the receptor – positions III:04 and III:08. It is proposed that the alpha NH2-group of the N-terminal residue (Lys in this case Ala in the agonist version) makes a charge-charge, anchoring interaction with AspII:20, and that the central aromatic cluster makes key interactions with the aromatic cluster presented at the interface between TM-VI and TM-VII (including a potential with ArgVI:20) and that the C-terminal double Leu sequence is located in the pocket between TM-III, TM-IV and TM-V. It is envisioned that the polar backbone interacts mainly with polar residue in TM-III including a key interaction with GluIII:09. It is suggested that the positively charged epsilon-NH2 group of the Lys residue of the ligand makes a cat-ion π interaction with PheII:04 (see text for details).

Fig. 5. Overview of the mutational hits for the wFw-containing peptides in TM-II through TM-V. The “inner faces” TM-I to TM-V of a molecular model of the ghrelin receptor built over the X-ray structure of the inactive form of rhodopsin are shown in solid ribbons in a side-view from TM-VI / VII, which are not shown. In stick models are highlighted the side-chains of the mutational hits for the wFw-containing peptides of which AspII:20, GlnIII:05, and GluIII:09 are considered to be classical mutational hits which just impairs either agonist or inverse agonist potency (see Table II). Space-generating substitutions of the relatively deeply located residues: SerIII:08, SerIV:16, ValV:08, and PheV:12 (highlighted in red circle) all shift the efficacy of the peptides toward inverse agonism (see text, discussion for details). In contrast, similar space-generating substitutions of the more extracellularly located residues: PheIII:04, IleIV:20, and MetV:05 (highlighted in green) all shift the efficacy of the peptides toward agonism. Note that the generic nomenclature / numbering system for residues in 7TM receptors used in the present study (36;37) provides information about the actual vertical position of residues in the receptor structure e.g. II:20 and IV:20 at the same level and III:08 and V:08 at the same level etc.

Fig. 6. Mutations in TM-IV and TM-V which improve the potency for the wFw-containing peptides - Panel A - dose response curves for the agonist AwFwLL (open circle) and the inverse agonist KwFwLL (closed circle) on [SerIV:16Ala] mutant of the ghrelin receptor. Panel B - dose response curves for the agonist AwFwLL (open square) and the inverse agonist K-wFwLL (closed square) on the [MetV:05Ala] mutant. In both panels the dose response curves on wild type receptor are included for AwFwLL as a dotted curve and for K-wFwLL as dashed curve. The experiments are made in transiently transfected COS7 cell. Data are mean ± S.E. of three to five independent experiments performed in duplicates.

Fig. 7. Switch between inverse agonism and agonism in wFw-containing peptides due to substitution at positions II:04, III:08 and IV:20 in the ghrelin receptor. Panel A - a helical wheel diagram of the most extracellular located residues of the ghrelin receptor where the residues which has been substitute according to Table II are marked in grey and the mutations [PheIII:04Ser] and [IleIV:20Ala] which shift the inverse agonist KwFwLL toward agonism is highlighted in green and the [SerIII:08Ala] mutation which shifts the agonist AwFwLL toward inverse agonism is highlighted in red. Panels B and C - dose response curves for A-wFwLL on
the wild type ghrelin receptor (dashed line) compared with the [PheIII:04Ser] (open square panel B), [SerIII:08Ala] (closed square panel B), and [IleIV:20Ala] mutations (open circle panel C). Panels D and E - dose response curves for KwFwLL on the wild type ghrelin receptor (dashed line) and on the [PheIII:04Ser] (open square panel D), [SerIII:08Ala] (closed square panel D), and the [IleIV:20Ala] mutations (open circle panel E). Inositol phosphate turnover was measured in transiently transfected COS7 cell, data are mean ± S.E. of three to eight independent experiments performed in duplicates.

Fig. 8. Switch toward agonism by the [PheIII:04Ser] mutant and toward inverse agonism by the [SerIII:08Ala] mutation as measured by modulation of serum response element (SRE) dependent transcription by the ghrelin receptor. Panels A and B – dose-response curve for the KwFwLL (which functions as a neutral ligand or weak inverse agonist in SRE signalling) (panel A) and for the agonist AwFwLL (Panel B) on wild-type ghrelin receptor (dashed line) and in the [PheIII:04Ser] (empty square) and the [SerIII:08Ala] mutations (filled square). Panel C – dose-response curve for ghrelin on the wild-type ghrelin receptor (dotted line) and in the [PheIII:04Ser] (empty circle) and [SerIII:08Ala] mutations (filled circle). Inhibition of basal signalling induced by [DArg¹, DPhe⁵, DTrp⁷,⁹, Leu¹¹]-substance P on the wild-type ghrelin receptor (dashed line) and in the [PheIII:04Ser] (empty square) and [SerIII:08Ala] mutations (filled square). The experiments were performed in transiently transfected HEK293 cell, data are mean ± S.E. of three to five independent experiments performed in duplicates.

Fig. 9. Overview of structural and functional aspects of mutational shifts in efficacy for the wFw-containing peptides either toward agonism or toward inverse agonism in the Ghrelin receptor. Panel A – shifts in efficacy presented on a “global efficacy scale”. In solid lines and symbols are indicated the dose-response curves for an agonist (circles), a neutral ligand / weak agonist (squares) and an inverse agonist (triangles). Green arrows indicate that a decrease in inverse agonist potency, a swap from inverse agonism toward agonism and an increase in agonist potency all can be considered to be “shifts toward agonism” as observed in, for example the [PheIII:04Ala] mutations for the different wFw-containing peptides (Table II). Red arrows indicate that a decrease in agonist potency, a swap from agonism to inverse agonism and an increase in inverse agonist potency all can be considered to be “shifts toward inverse agonism” as observed in, for example the [SerIII:08Ala] mutations. Panel B – The molecular model of the seven helical bundle of the ghrelin receptor as viewed from the side with TM-VI in front. The mutational hits for the wFw-containing peptides are highlighted as described in Fig. 5 and in panel A. A proposed “vertical location” of a wFw-containing peptide is indicated by a green symbol and the suggested translocation of this either deeper into the pocket by the space-generating “green” mutations leading to a shift toward inverse agonism or the opposite translocation higher up in the binding pocket by space-generating “green” mutations which shifts the efficacy toward agonism are indicated by arrows.

**Abbreviations used are:** TM: trans membrane; MC: melanocortin; GHS: growth hormone secretagogues; CB: cannabinoid; Dpr: diaminoproprionic acid; SAR: structure activity relationship; GHRP: growth hormone releasing peptide; SRE: serum responsive element.
Table I: Modifications of the active core peptide wFwLL tested in transiently transfected COS7 cells for potency as inverse agonist measured by inositol phosphate accumulation and for affinity in competition binding study using $^{35}$S-MK677 as radioligand. Bmax value is 55±12 fmol/10^5 cells

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a This compound acts as an agonist
Table II. Mutational mapping of the binding site for the agonist Ghrelin and AwFwLL and the inverse agonists KwFwLL using a library of 16 mutant versions of the Ghrelin receptor with substitutions systematically placed through the main ligand-binding crevice. The potency (EC50) of the compounds in respect of either stimulating (agonist) or inhibiting (invers agonist) the constitutive stimulation of inositol phosphate accumulation was determined in COS-7 cells transiently transfected with either the wild-type or the mutants forms of the Ghrelin receptor. Fmut indicates the fold shift in potency induced by the structural change in the receptor as compared to the wild-type receptor. In the first column is shown the constitutive activity of the mutant receptors expressed as percent basal signaling activity compared to the maximal Ghrelin stimulated activity. In second column expression of each mutation is assessed by cell surface ELISA stated as fraction of wild type receptor expression

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<tr>
<td>AspI:20Asn (Asp&lt;sup&gt;99&lt;/sup&gt;)</td>
<td>56 ± 5 4</td>
<td>0.62 ±0.15 3</td>
<td>3.2 47</td>
<td>&gt;1000 3</td>
<td>&gt;31 1000 3</td>
</tr>
<tr>
<td>AspI:20Lysd (Asp 99)</td>
<td>53 ±2 3</td>
<td>0.46 ±0.23 3</td>
<td>68</td>
<td>&gt;1000 3</td>
<td>&gt;31 &gt;1000 3</td>
</tr>
<tr>
<td>AspI:20Asn (Asp&lt;sup&gt;99&lt;/sup&gt;)</td>
<td>38 ±4 6</td>
<td>1.2 ±0.2 3</td>
<td>1.3 8.9</td>
<td>60 ±12&lt;sup&gt;b&lt;/sup&gt; 6</td>
<td>1.9 41 ±6 3</td>
</tr>
<tr>
<td>AspI:20Lysd (Asp 99)</td>
<td>35 ±3 8</td>
<td>1.2 ±0.1 3</td>
<td>0.9 0.26</td>
<td>21 ±1 5</td>
<td>0.66 28 ±7&lt;sup&gt;c&lt;/sup&gt; 4</td>
</tr>
<tr>
<td>AspI:20Asn (Asp&lt;sup&gt;99&lt;/sup&gt;)</td>
<td>34 ±3 4</td>
<td>0.75 ±0.18 3</td>
<td>250 14</td>
<td>&gt;1000 &gt;31 &gt;1000 3</td>
<td>&gt;67</td>
</tr>
<tr>
<td>AspI:20Lysd (Asp 99)</td>
<td>33 ±2 8</td>
<td>0.78 ±0.1 3</td>
<td>1.4 0.06</td>
<td>2.6 ±1.3&lt;sup&gt;c&lt;/sup&gt; 3</td>
<td>0.08 6.1 ±2 3</td>
</tr>
<tr>
<td>SerIV:16Ala (Ser&lt;sup&gt;123&lt;/sup&gt;)</td>
<td>46 ±2 11</td>
<td>1.0 ±0.3 3</td>
<td>2.5 22</td>
<td>43 ±12&lt;sup&gt;b&lt;/sup&gt; 5</td>
<td>1.3 169 ±10 3</td>
</tr>
<tr>
<td>MetI:05Ala (Met&lt;sup&gt;213&lt;/sup&gt;)</td>
<td>43 ±8 5</td>
<td>1.3 ±0.4 3</td>
<td>1.2 0.47</td>
<td>29 ±2 3</td>
<td>0.91 0.35 ±0.1 3</td>
</tr>
<tr>
<td>MetI:05Ala (Met&lt;sup&gt;213&lt;/sup&gt;)</td>
<td>46 ±4 8</td>
<td>0.53 ±0.06 3</td>
<td>2.9 0.15</td>
<td>27 ±7 3</td>
<td>0.84 &gt;1000 &gt;67</td>
</tr>
<tr>
<td>SerIV:09Ala (Ser&lt;sup&gt;119&lt;/sup&gt;)</td>
<td>46 ±4 8</td>
<td>0.73 ±0.12 3</td>
<td>1.1 0.61</td>
<td>83 ±19 4</td>
<td>2.6 14 ±4 3</td>
</tr>
<tr>
<td>PheI:16Ala (Phe&lt;sup&gt;220&lt;/sup&gt;)</td>
<td>20 ±1 12</td>
<td>0.68 ±0.07 3</td>
<td>1.3 0.11</td>
<td>19 ±5 3</td>
<td>0.59 &gt;1000 &gt;67</td>
</tr>
<tr>
<td>PheI:16Ala (Phe&lt;sup&gt;220&lt;/sup&gt;)</td>
<td>2 ±2 10</td>
<td>0.72 ±0.23 3</td>
<td>41</td>
<td>&gt;1000 4</td>
<td>&gt;67</td>
</tr>
<tr>
<td>ArgI:020-Gln (Arg&lt;sup&gt;283&lt;/sup&gt;)</td>
<td>17 ±4 7</td>
<td>0.67 ±0.26 3</td>
<td>38 4.1</td>
<td>&gt;1000 4</td>
<td>&gt;31 &gt;1000 4</td>
</tr>
<tr>
<td>AsnV:02Ala (Asn&lt;sup&gt;305&lt;/sup&gt;)</td>
<td>16 ±2 5</td>
<td>1.1 ±0.18 3</td>
<td>8.5 0.78</td>
<td>61 ±11 4</td>
<td>1.8 15 ±6 4</td>
</tr>
<tr>
<td>PheI:06Leu (Phe&lt;sup&gt;209&lt;/sup&gt;)</td>
<td>42 ±2 6</td>
<td>1.2 ±0.45 3</td>
<td>1.5 6.7</td>
<td>&gt;1000 3</td>
<td>&gt;31 81 ±7 3</td>
</tr>
<tr>
<td>PheI:09Ala (Phe&lt;sup&gt;312&lt;/sup&gt;)</td>
<td>15 ±1 8</td>
<td>0.64 ±0.14 3</td>
<td>3.8 2.6</td>
<td>&gt;1000 3</td>
<td>&gt;31 &gt;1000 3</td>
</tr>
</tbody>
</table>

<sup>a</sup> KwFwLL has turned into an agonist for this mutant
<sup>b</sup> This IC<sub>50</sub> is a average value for the two component curve with IC<sub>50</sub> of 0.28nM (64%) and 130 nM (36%), respectively.
<sup>c</sup> AwFwLL has turned into an inverse agonist for this mutant
<sup>d</sup> DwFwLL was tested on this mutant. The potency was EC50>1000nM
<sup>e</sup> The receptor concentration as measured from competition binding experiments was 55±12 fmol/10<sup>5</sup> cells
Inverse agonist (KwFwLL)

Core-peptide (wFwLL)

Partiel agonist (AwFwLL)

Inverse agonist- [DArg$^1$,DPhe$^6$,DTyr$^7$,Leu$^{11}$]-substance P

Core-peptide (wFwLL)

Partiel agonist (AwFwLL)
Figure 2

A. % of maximal constitutive activity - IP turnover

Log conc. Ligand (M)

B. % of max bound - S-MK677

Log conc. ligand (M)
AspII:20 PheIII:04 GlnIII:05 ValV:08

Shift toward agonism

Shift toward inverse agonism

Figure 5
Figure 6

A. SerIV:16Ala

B. MetV:05Ala

% of max constitutive activity

IP turnover

Log conc.ligand (M)
Figure 7

% constitutive activity

Log conc. KwFwLL (M)

B.  A-wFwLL
C.  A-wFwLL

D.  K-wFwLL
E.  K-wFwLL

% constitutive activity

Log conc. A-wFwLL (M)

Log conc. KwFwLL (M)

PhelIII:04Ser
SerIII:08Ala

PhelIV:20Ala
SerIII:08Ala

PhelIV:20Ala
IleIV:20Ala
Figure 8

A. K-wFwLL

B. A-wFwLL

C. Ghrelin and SP-analog
Figure 9

A. Graph showing the relationship between log concentration of ligand (M) and percent of constitutive activity. The graph includes curves for agonism and inverse agonism.

B. Diagram illustrating the structure of transmembrane (TM) regions with arrows indicating movement toward agonism and inverse agonism.
Identification of an efficacy switch region in the ghrelin receptor responsible for interchange between agonism and inverse agonism

Birgitte Holst, Jacek Mokrosinski, Manja Lang, Erik Brandt, Rie Nygaard, Thomas M. Frimurer, Annette G. Beck-Sickinger and Thue W. Schwartz

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