Two Naturally Occurring Mutations in the Type 1 Melanin-Concentrating Hormone Receptor Abolish Agonist-Induced Signaling

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ABSTRACT

The melanin-concentrating hormone (MCH) receptor type 1 (MCHR1) is a seven-transmembrane domain protein that modulates orexigenic activity of MCH, the corresponding endogenous peptide agonist. MCHR1 antagonists are being explored as a potential treatment for obesity. In the current study, we examined the pharmacological impact of 11 naturally occurring mutations in the human MCHR1. Wild-type and mutant receptors were transiently expressed in human embryonic kidney 293 cells. MCHR1-mediated, G[i]-dependent signaling was monitored by using luciferase reporter gene assays. Two mutants, R210H and P377S, failed to respond to MCH. Five other variants showed significant alterations in MCH efficacy, ranging from 44 to 142% of the wild-type value. For each of the MCH-responsive mutants, agonist potency and inhibition by (S)-methyl 3-((3-(4-(3-acetamidophenyl)piperidin-1-yl)propyl)carbamoyl)-4-(3,4-difluorophenyl)-6-(methoxymethyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (SNAP-7941), an established MCHR1 small-molecule antagonist, were similar to wild type. To explore the basis for inactivity of the R210H and P377S mutants, we examined expression levels of these receptors. Assessment by enzyme-linked immunosorbent assay revealed that cell surface expression of both nonfunctional receptors was comparable with wild type. Overnight treatment with SNAP-7941, followed by washout of antagonist, enhanced MCH induced signaling by the wild-type receptor and restored MCH responsiveness of the P377S but not the R210H variant. It is of note that the two loss-of-function mutants were identified in markedly underweight individuals, raising the possibility that a lean phenotype may be linked to deficient MCHR1 signaling. Formal association studies with larger cohorts are needed to explore the extent to which signaling-deficient MCHR1 variants influence the maintenance of body weight.

Introduction

The melanin-concentrating hormone (MCH) receptor type 1 (MCHR1) is a G protein-coupled receptor (GPCR) that is expressed in both the mammalian brain and the periphery (Chung et al., 2009). Within the central nervous system, receptor expression is most abundant in the cortex, hippocampus, amygdala, and nucleus accumbens (Saito et al., 2001; Chung et al., 2010). Stimulation with MCH, the receptor’s endogenous ligand, triggers Gαi-mediated inhibition of cAMP formation and consequent downstream signaling (Saito et al., 1999; Shimomura et al., 1999). In contrast to the wide tissue distribution of the receptor, expression of MCH in the brain is restricted to the hypothalamus and zona incerta (Pissios and Maratos-Flier, 2003; Antal-Zimanyi and Khawaja, 2009). Extensive genetic and pharmacological studies in rodents have implicated the MCH system in the control of feeding and metabolism as well as in anxiety and depression. Central injection or infusion of MCH (Qu et al., 1996; Gomori et al.,...
2003) or overexpression of the corresponding gene (Ludwig et al., 2001) leads to increased food intake, whereas genetic deletion of MCH expression results in lean mice (Shimada et al., 1998; Kokkotou et al., 2005; Alon and Friedman, 2006). Consistent with these observations, animals with targeted disruption of the MCHR1 gene have reduced body weight (Chen et al., 2002; Marsh et al., 2002). The role of this receptor in modulating body weight is also supported by pharmacological studies using drugs that block MCHR1 function. Acute administration of a small-molecule antagonist, (S)-methyl 3-((3-(4-(3-acetamidophenyl)piperidin-1-yl)propyl)carbamoyl)4-(3, 4-difluorophenyl)-6-(methoxymethyl)-2-oxo-1,2,3,4-tetrahydroprymidine-5-carboxylate (SNAP-7941), to rodents was shown to decrease MCH-induced food intake. When the same drug was chronically given to rats with diet-induced obesity, a decrease in body weight resulted (Borowsky et al., 2002). Since these initial observations, an increasing number of small-molecule MCHR1 antagonists have been studied in rodents and shown to reduce body weight. In addition, several of these antagonists, including SNAP-7941, show anxiolytic and/or antidepressant properties when assessed in murine models (Antal-Zimanyi and Khawaja, 2009; Chung et al., 2010). The development of MCHR1 antagonists is therefore being actively pursued as a potential therapeutic for obesity and/or mood disorders in humans.

To explore whether naturally occurring genetic alterations in the MCHR1 predispose to obesity, there has been ongoing interest in identifying human variants of this receptor. Two of these naturally occurring mutants, D32N and R317Q, have been pharmacologically characterized in vitro. With both of these variants, function and expression were indistinguishable from corresponding wild-type values (Gibson et al., 2004; Wermter et al., 2005).

To determine the potential impact of 11 additional MCHR1 missense variants that have been reported in the literature and genomic databases (Gasteiger et al., 2003; Wermter et al., 2005; Wheeler et al., 2008), we pharmacologically characterized each of these receptor isoforms. We found that several mutations cause a significant alteration in MCH efficacy with little, if any, impact on agonist potency or receptor expression compared with wild type. In addition, we discovered that two MCHR1 variants (R210H and S377P) virtually abolish receptor-mediated signaling. Consistent with the anticipated metabolic phenotype resulting from loss-of-function isoforms (i.e., reduced body weight), it is intriguing that the two inactivating MCHR1 mutations were identified in underweight subjects but not in a parallel cohort of obese individuals (Wermter et al., 2005). This observation raises the possibility that selected MCHR1 variants protect against obesity. Our findings also suggest that certain effects of SNAP-7941 extend well beyond antagonist activity. This small molecule increases cell surface expression of the wild-type receptor and rescues MCH responsiveness of one of the functionally deficient receptor mutants (P377S). The potential of MCHR1 antagonists to paradoxically agonist stimulation, and the possible impact of pharmacogenomic variability, will need to be considered in the development of such compounds as therapeutics.

**Materials and Methods**

**Materials.** Cell culture media, fetal bovine serum, and Lipofectamine reagents were obtained from Invitrogen (Carlsbad, CA). Peroxidase-conjugated, anti-hemagglutinin (HA) monoclonal antibody (3F10) and BM-blue (3.3′-5′-tetramethylbenzidine), a peroxidase substrate, were purchased from Roche Applied Science (Indianapolis, IN). The reporter gene plasmids encoding luciferase under the control of either a serum-response element (SRE5x) or cAMP-response element (CRE5x) and the plasmids encoding the chimeric G protein, GoβγS, and β-galactosidase have been described previously (Hearn et al., 2002; Beinborn et al., 2005, 2010). MCH was purchased from Bachem California (Torrance, CA). Synthesis of the nonpeptide MCHR1 antagonist SNAP-7941 has been reported previously (Goss and Schaus, 2008).

**Cell Culture.** Human embryonic kidney (HEK) 293 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were maintained at 37°C in a humidified environment containing 5% CO2.

**Receptor Plasmids.** The MCHR1 encoding cDNA used in this study was obtained from the Missouri S&T cDNA Resource Center (Rolla, MO). The corresponding 422-amino acid receptor sequence is identical to that of the MCHR1 construct reported by Wermter et al. (2005) (National Center for Biotechnology Information reference sequence NM_005297.2). This protein-coding cDNA was amplified by polymerase chain reaction and subcloned into pcDNA1.1 (Invitrogen), providing the wild-type template for site-directed mutagenesis. Receptor constructs including missense polymorphisms/mutations (listed below) were generated by oligonucleotide-directed, site-specific mutagenesis as described previously (Blaker et al., 1998). A parallel set of corresponding wild-type and mutant constructs was also made in which a hemagglutinin epitope tag (YPYDVPDYA) was inserted between the first and second receptor amino acids (methionine and serine, respectively), thus enabling detection by ELISA. The coding region of each construct was sequenced to confirm introduction of only the designated amino acid substitutions.

Eight of the mutants that were pharmacologically characterized (shown in Fig. 1) correspond to single-nucleotide polymorphisms listed in the ExPasy Proteomics server (http://www.expasy.ch)

![Fig. 1. A diagram of MCHR1 illustrating the position of missense mutations within the receptor protein. Residues in the wild-type receptor are indicated by single-letter codes. The positions of the 11 amino acids that are the focus of this study are indicated by numbering relative to the initiator methionine M1. In addition, a previously characterized polymorphism affecting residue 32D is included as a control. Boxed positions indicate two mutations that were found in this study to abolish agonist-induced receptor function. N, amino terminus; C, carboxyl terminus.](image-url)
Luciferase Reporter Gene Assays. Receptor-mediated signal-
ning via inhibitory G\(\alpha\)o proteins was assessed by using a luciferase
reporter gene assay as described previously (Hearn et al., 2002;
Al-Falaj, et al., 2008). In brief, HEK293 cells were plated at a density of
2000 cells per well onto clear-bottom, white 96-well plates (Costar;
Corning Glassworks, Corning, NY) and grown for 2 days to ~80%
confluence. Cells were then transiently transfected in serum-free
medium using Lipofectamine reagent (Invitrogen) with cDNAs encod-
ing 1) wild-type or mutant MCHR1 (or the empty expression
vector pcDNA1.1), 2) an SRE-luciferase reporter gene (SRE\(_{\text{bgal}}\)-lucif-
erase), 3) a chimeric G\(\alpha\)q protein (Gq(5i)), and 4) a control cDNA
encoding \(\beta\)-galactosidase linked to a constitutively active cyto-
megalovirus promoter. Introduction of the five C-terminal residues of
G\(\alpha\)o into the Gq protein (Gq(5i)) directs G\(\alpha\)o-coupled receptor-
mediated signaling to activation of the SRE-luciferase reporter gene
(Conklin et al., 1993). Forty-eight hours after transfection, the cells
were incubated for 4 to 6 h with or without ligand (MCH and/or
SNAP-7941). The medium was then gently aspirated and luciferase
activity was measured by using Steadylite reagent (PerkinElmer Life
and Analytical Sciences, Waltham, MA). The \(\beta\)-galactosidase
substrate 2-nitrophenyl \(\beta\)-D-galactopyranoside A was subsequently
added, followed by further incubation at 37°C for 30 to 60 min.
Substrate cleavage was quantified by measurement of optical den-
sity at 420 nm using a SpectraMax microplate reader (Molecular
Devices, Sunnyvale, CA). The level of \(\beta\)-galactosidase activity was
used to normalize the luciferase readings in corresponding wells.

As an alternative approach for measuring G\(\alpha\)o-mediated signal-
ing, cells were transfected with cDNAs encoding 1) receptor, 2) a
cAMP-responsive element-luciferase reporter gene (CRE\(_{\text{bgal}}\)-lucif-
erase), and 3) \(\beta\)-galactosidase. Forty-eight hours after transfection,
the cells were incubated for 4 to 6 h in medium containing MCH
together with 5 \times 10\(^{-7}\) M forskolin, a receptor-independent stimulus
of cAMP formation and consequent CRE\(_{\text{bgal}}\)-luciferase activity. The
ability of MCH to trigger receptor-mediated inhibition of forskolin-
induced luciferase activity was monitored as described above.

Additional control experiments compared ligand efficacy with
transfection of 20 ng/well of receptor cDNA and/or an interval of 24 h
between cell transfection and functional assessment. Using these
modified conditions, MCH efficacies at the mutant versus wild-type
receptors were similar to those observed with the standard protocol
(4 ng of receptor cDNA/48 h incubation).

In experiments to assess the effect of overnight exposure to SNAP-
7941, 10\(^{-6}\) M of this compound was added 24 h after transfection.
The next day, cells were washed five times with serum-free media,
incubated for 4 to 6 h with MCH (3 \times 10\(^{-6}\) M), and then lysed for
assessment of luciferase activity.

ELISA of MCHR1 Expression. Receptor expression levels were
determined using a procedure described by Al-Falaj et al. (2008).
In brief, HEK293 cells grown in 96-well plates (Primaria; BD Bio-
sciences, Franklin Lakes, NJ) were transiently transfected with ei-
ther pcDNA1.1 or a plasmid encoding an HA-tagged wild-type or an
HA-tagged mutant MCHR1. Forty-eight hours after transfection,
the cells were washed once with phosphate-buffered saline (PBS), pH
7.4, and fixed with 4% paraformaldehyde in PBS (USB, Cleveland,
OH) for 10 min at room temperature. In a subset of wells, cells were
permeabilized with 0.1% Triton X-100 in PBS for 2 min to enable
detection of total receptor expression levels. Cell surface expression
was measured without the permeabilization step. After washing with
PBS/100 mM glycine, cells were incubated for 30 min in block-
ing solution (PBS/20% bovine serum). A horseradish peroxidase-
conjugated monoclonal antibody (clone 3F10; Roche Applied Science)
directed against the HA epitope was then added to the cells (1:500
dilution in blocking solution). After 1 h, the cells were washed five
times with PBS, and BM-blue solution (Roche Applied Science) (50 \(\mu\)l
per well) was added. After incubation for 30 min at room tempera-
ture, conversion of this substrate by antibody-linked horseradish
peroxidase was terminated by adding 2 M sulfuric acid (50 \(\mu\)l per
well). Converted substrate (which correlates with the amount of
receptor) was assessed by measuring light absorbance at 450 nm with
a SpectraMax microplate reader (Molecular Devices).

In addition to examining receptor expression in the absence of ligand,
in designated experiments SNAP-7941 (10\(^{-6}\) M) was added 24 h after
transfection. ELISA was carried out the next day as described above.

Data Analysis. The half-maximal effective or inhibitory concentra-
tions (EC\(_{50}\) or IC\(_{50}\) values) of MCH and SNAP-7941 were de-
termined by nonlinear curve fitting using Prism 5.0 software (Graph-
Pad Software Inc., San Diego, CA). The negative logarithms of these
dilutions (pEC\(_{50}\) or pIC\(_{50}\)) as well as basal receptor activities and
MCH-stimulated maxima, were compared between wild-type and
mutant receptors using analysis of variance with Dunnnett’s post test.
Analysis of variance was also used to assess the significance of
SNAP-7941 effects on total and surface receptor expression and on
basal and MCH-induced function (Student-Newman-Keuls post
test).

Results

We studied the effect of 11 naturally occurring missense mutations in the MCHR1 that have been reported in the literature and/or selected databases (see Materials and Methods) but have not yet been pharmacologically charac-
terized. In addition, the D32N polymorphism, which was described previously as functionally normal, was included as a control (Wermter et al., 2005). A diagram illustrating the location of individual amino changes within the recep-
tor molecule is shown in Fig. 1.

After expression of MCHR1s in HEK293 cells, basal and
agonist-induced signaling of each mutant was assessed relative to
wild type (summarized in Table 1).

In the absence of MCH, all of the receptors lacked basal
activity (i.e., signaling did not differ from vector-transfected
control cells (no receptor in Table 1). Five variants (T25M, D28V,
D32N, G34R, and Y250H) showed MCH induced max-
imal activity comparable with that at the wild-type receptor.
In contrast, the maximal level of MCH-stimulated function
was significantly increased (by 30–40%) in cells expressing
the G401R or T411M receptor variants. Conversely, activity
of MCH was reduced (by 50% compared with wild type)
with expression of either the G103R, Y210H, or T305M iso-
forms. The most pronounced defects were observed with ex-
pression of the R210H and P377S variants. Each of these
isoforms showed minimal, if any, activation when stimulated with
MCH (Fig. 2, top; Table 1).

To explore whether the altered signaling observed with selected receptor mutants correlates with a change in
MCHR1 expression, ELISAs were carried out using either
permeabilized or nonpermeabilized HEK293 cells (to mea-
sure total versus cell surface expression, respectively). For
the wild-type MCHR1, surface expression was 45.8 ± 3.3% of
total expression (mean ± S.E.M. of eight independent exper-
iments; not shown), suggesting that this receptor is in part
**TABLE 1**

Activity and expression of the wild type and mutant MCHR1 receptors

<table>
<thead>
<tr>
<th>Variant</th>
<th>Basal Activity</th>
<th>Significance vs. No Receptor</th>
<th>MCH Stimulated</th>
<th>Significance vs. WT</th>
<th>Total Expression (% WT)</th>
<th>Significance vs. WT</th>
<th>Surface Expression (% WT)</th>
<th>Significance vs. WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>No receptor</td>
<td>10 ± 2</td>
<td>N/A</td>
<td>11 ± 2</td>
<td>**</td>
<td>0</td>
<td>N/A</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>MCHR1 WT</td>
<td>10 ± 2</td>
<td>N.S.</td>
<td>100</td>
<td>N.A</td>
<td>100</td>
<td>N.S.</td>
<td>100</td>
<td>N.S.</td>
</tr>
<tr>
<td>T25M</td>
<td>10 ± 1</td>
<td>N.S.</td>
<td>116 ± 10</td>
<td>N.S.</td>
<td>112 ± 11</td>
<td>N.S.</td>
<td>114 ± 12</td>
<td>N.S.</td>
</tr>
<tr>
<td>D28V</td>
<td>8 ± 1</td>
<td>N.S.</td>
<td>103 ± 7</td>
<td>N.S.</td>
<td>78 ± 12</td>
<td>N.S.</td>
<td>105 ± 11</td>
<td>N.S.</td>
</tr>
<tr>
<td>D32N</td>
<td>9 ± 1</td>
<td>N.S.</td>
<td>102 ± 8</td>
<td>N.S.</td>
<td>88 ± 5</td>
<td>N.S.</td>
<td>95 ± 7</td>
<td>N.S.</td>
</tr>
<tr>
<td>G34R</td>
<td>10 ± 1</td>
<td>N.S.</td>
<td>93 ± 8</td>
<td>N.S.</td>
<td>78 ± 8</td>
<td>N.S.</td>
<td>99 ± 9</td>
<td>N.S.</td>
</tr>
<tr>
<td>G103R</td>
<td>8 ± 1</td>
<td>N.S.</td>
<td>44 ± 3</td>
<td>**</td>
<td>75 ± 9</td>
<td>N.S.</td>
<td>73 ± 6</td>
<td>**</td>
</tr>
<tr>
<td>Y107H</td>
<td>9 ± 1</td>
<td>N.S.</td>
<td>53 ± 4</td>
<td>**</td>
<td>86 ± 3</td>
<td>N.S.</td>
<td>89 ± 8</td>
<td>N.S.</td>
</tr>
<tr>
<td>R210H</td>
<td>9 ± 1</td>
<td>N.S.</td>
<td>10 ± 1</td>
<td>**</td>
<td>115 ± 12</td>
<td>N.S.</td>
<td>90 ± 8</td>
<td>N.S.</td>
</tr>
<tr>
<td>Y250H</td>
<td>8 ± 1</td>
<td>N.S.</td>
<td>88 ± 13</td>
<td>N.S.</td>
<td>100 ± 16</td>
<td>N.S.</td>
<td>82 ± 7</td>
<td>N.S.</td>
</tr>
<tr>
<td>T305M</td>
<td>8 ± 1</td>
<td>N.S.</td>
<td>46 ± 3</td>
<td>**</td>
<td>92 ± 8</td>
<td>N.S.</td>
<td>94 ± 8</td>
<td>N.S.</td>
</tr>
<tr>
<td>P377S</td>
<td>8 ± 1</td>
<td>N.S.</td>
<td>11 ± 1</td>
<td>**</td>
<td>119 ± 11</td>
<td>N.S.</td>
<td>82 ± 6</td>
<td>N.S.</td>
</tr>
<tr>
<td>G401R</td>
<td>9 ± 1</td>
<td>N.S.</td>
<td>142 ± 6</td>
<td>**</td>
<td>78 ± 9</td>
<td>N.S.</td>
<td>97 ± 8</td>
<td>N.S.</td>
</tr>
<tr>
<td>T411M</td>
<td>10 ± 1</td>
<td>N.S.</td>
<td>132 ± 7</td>
<td>**</td>
<td>99 ± 5</td>
<td>N.S.</td>
<td>94 ± 10</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

N/A, not applicable; N.S., not significant.

*Expressed relative to MCH-stimulated maximal activity at the wild-type receptor = 100%.

**Surface expression, defined as 100% for the wild-type receptor, is 45.8 ± 3.3% of the corresponding total expression (which includes intracellular receptors).

***, P < 0.01.

Intracellularly trapped. For comparison with variant receptors, wild-type total and surface expression were each defined as 100%, whereas background in vector-transfected cells (no receptors) was defined as 0%. This analysis revealed that, with one exception, both total and surface expression of all receptor variants was comparable with wild type (Table 1). The only significant difference was found with the G103R mutant where surface expression was 73% of the control value.

To confirm the major loss of function observed with the R210H and P377S mutations, signaling was examined by using a second reporter gene assay (shown in Fig. 2, bottom). When assessed on a background of prestimulation with forskolin, agonist-induced signaling of the wild-type MCHR1 triggers a concentration-dependent decrease in cAMP-mediated CRE6x-luciferase activity. In contrast, little, if any, MCH effect was observed in cells expressing either the R210H or the P377S variant (Fig. 2, bottom). These findings further support the conclusion that the R210H and P377S receptor isoforms are essentially nonfunctional.

In addition to basal and maximal activity, an important parameter of MCHR1 function is MCH potency (reflected by EC50 values; summarized in Table 2). Corresponding values were determined by using two complementary assays as illustrated above (based on either receptor-mediated, GoS-dependent activation of SREluciferase or receptor-mediated inhibition of forskolin-activated CREluciferase; see Materials and Methods). Excluding the R210H and P377S receptor variants that showed little or no signaling, MCH potencies at all other receptor mutants fell within a 3.5-fold range of the corresponding wild-type value (Table 2). A significantly different EC50 value (versus wild type) was found only for the Y107H mutant when assessed with the GoS-mediated signaling assay. When the forskolin-activated CREluciferase assay was used, MCH potency at this mutant was also decreased versus wild type; however, the change did not quite reach significance.

We next investigated whether naturally occurring MCHR1 mutations influence the ability of a prototype nonpeptide antagonist, SNAP-7941 (Borowsky et al., 2002), to inhibit MCH-induced function. At the wild-type MCHR1, this compound results in a concentration-dependent reduction in MCH-induced signaling with an IC50 of 84 nM (Fig. 3; Table 3).
As discussed above, MCH was unable to stimulate mutants R210H or P377S, which precluded investigation of SNAP-7941 as an antagonist. On all other MCHR1 variants, SNAP-7941 inhibited MCH induced activity with IC₅₀ values comparable with wild type (Table 3). Given some rare precedents for mutation-induced conversion of antagonists to agonists (Strader et al., 1989; Blaker et al., 1998; Kopin et al., 2003), we also examined SNAP-7941 activity on the R210H and P377S variants. Experiments revealed that this compound did not induce signaling at either of the mutants (not shown).

There is precedent in the literature that long-term exposure to nonpeptide antagonists can increase GPCR cell surface expression and/or function (induced by subsequent stimulation with agonist) (Conn and Ulloa-Aguirre, 2010). In principle, this paradoxical response to antagonists may convert loss-of-function variants to agonist-responsive receptors. The possibility of an antagonist-induced increase in cell surface expression was explored with the signaling-deficient MCHR1 isoforms R210H and P377S by using ELISA. Overnight incubation with the nonpeptide compound SNAP-7941 had no significant impact on total expression of either the mutants or the wild-type MCHR1 (Fig. 4). However, SNAP-7941 induced a significant increase in surface expression of the wild-type receptor, overnight incubation and subsequent washout of SNAP-7941 led to increased MCH-induced signaling (Fig. 5) in addition to enhanced receptor expression, we investigated whether this compound affects MCH-induced signaling at the wild-type receptor or at either of the inactive receptor variants. With cells expressing the signaling-deficient MCH-Induced Gq/11-mediated inhibition of forskolin-induced CRE6× luciferase activity, HEK293 cells expressing the wild-type receptor together with Gq/11 were incubated for 4 to 6 h with 10⁻¹⁰ M MCH and increasing concentrations of SNAP-7941. Cells were then lysed and luciferase activity was determined. Results were normalized to the value observed after stimulating the wild-type receptor with MCH in the absence of SNAP-7941 (100%). Data represent the mean ± S.E.M. of five independent experiments, each performed in triplicate.

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**TABLE 2**

MCH potencies at the wild-type and mutant MCHR1 receptors

<table>
<thead>
<tr>
<th>Variant</th>
<th>MCH EC50 (nM)</th>
<th>pEC50 ± S.E.M.</th>
<th>Significance vs. WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCHR1 WT</td>
<td>103</td>
<td>6.99 ± 0.06</td>
<td>N.S.</td>
</tr>
<tr>
<td>T25M</td>
<td>85</td>
<td>7.07 ± 0.11</td>
<td>N.S.</td>
</tr>
<tr>
<td>D28V</td>
<td>85</td>
<td>7.19 ± 0.22</td>
<td>N.S.</td>
</tr>
<tr>
<td>D32N</td>
<td>76</td>
<td>7.12 ± 0.19</td>
<td>N.S.</td>
</tr>
<tr>
<td>G34R</td>
<td>79</td>
<td>7.10 ± 0.14</td>
<td>N.S.</td>
</tr>
<tr>
<td>G103R</td>
<td>142</td>
<td>6.85 ± 0.20</td>
<td>N.S.</td>
</tr>
<tr>
<td>Y107H</td>
<td>351</td>
<td>6.45 ± 0.12</td>
<td>**</td>
</tr>
<tr>
<td>R210H</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Y250H</td>
<td>91</td>
<td>7.04 ± 0.18</td>
<td>N.S.</td>
</tr>
<tr>
<td>T305M</td>
<td>173</td>
<td>6.76 ± 0.11</td>
<td>N.S.</td>
</tr>
<tr>
<td>P377S</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>G401R</td>
<td>49</td>
<td>7.31 ± 0.16</td>
<td>N.S.</td>
</tr>
<tr>
<td>T411M</td>
<td>109</td>
<td>6.96 ± 0.17</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

**TABLE 3**

SNAP-7941 induced inhibition of MCH-mediated signaling at the MCHR1

<table>
<thead>
<tr>
<th>Variant</th>
<th>SNAP IC50 (nM)</th>
<th>SNAP pIC50 ± S.E.M.</th>
<th>Significance vs. WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCHR1 WT</td>
<td>84</td>
<td>7.07 ± 0.08</td>
<td>N.S.</td>
</tr>
<tr>
<td>T25M</td>
<td>112</td>
<td>6.95 ± 0.26</td>
<td>N.S.</td>
</tr>
<tr>
<td>D28V</td>
<td>89</td>
<td>7.05 ± 0.13</td>
<td>N.S.</td>
</tr>
<tr>
<td>D32N</td>
<td>105</td>
<td>6.98 ± 0.26</td>
<td>N.S.</td>
</tr>
<tr>
<td>G34R</td>
<td>73</td>
<td>7.14 ± 0.21</td>
<td>N.S.</td>
</tr>
<tr>
<td>G103R</td>
<td>55</td>
<td>7.26 ± 0.35</td>
<td>N.S.</td>
</tr>
<tr>
<td>Y107H</td>
<td>39</td>
<td>7.41 ± 0.27</td>
<td>N.S.</td>
</tr>
<tr>
<td>R210H</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Y250H</td>
<td>182</td>
<td>6.74 ± 0.42</td>
<td>N.S.</td>
</tr>
<tr>
<td>T305M</td>
<td>113</td>
<td>6.95 ± 0.52</td>
<td>N.S.</td>
</tr>
<tr>
<td>P377S</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>G401R</td>
<td>141</td>
<td>6.85 ± 0.21</td>
<td>N.S.</td>
</tr>
<tr>
<td>T411M</td>
<td>100</td>
<td>7.00 ± 0.35</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

N.S., not significant; N.D., not determined.

**"** P < 0.01.

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Fig. 3. SNAP-7941 inhibits MCH-induced signaling at the wild-type receptor. Top, structure of the nonpeptide ligand, SNAP-7941. Bottom, functional assessment of SNAP-7941. Forty-eight hours after transfection, HEK293 cells expressing the wild-type receptor together with Gq5i and a SRE5× luciferase reporter gene were incubated for 4 to 6 h with 10⁻¹⁰ M MCH and increasing concentrations of SNAP-7941. Cells were then lysed and luciferase activity was determined. Results were normalized to the value observed after stimulating the wild-type receptor with MCH in the absence of SNAP-7941 (100%). Data represent the mean ± S.E.M. of at least three independent experiments, each performed in triplicate.
parallel alterations in MCHR1 cell surface expression with the possible exception of G103R, which shows a concomitant decrease in receptor density.

Given that changes in expression levels do not account for the observed alterations in MCH activity, other mechanisms may be postulated. The location of affected residues within the receptor (Fig. 1) suggest corresponding mechanisms (Chung et al., 2009). The Y107H and G103R substitutions project into the extracellular space and therefore may influence MCH/MCHR1 interactions. In contrast, it is more likely that residue changes within the intracellular domains, i.e., T305M (third intracellular loop) and G401R/T411M (C terminus), affect the receptor's coupling to components of the signaling cascade (e.g., G proteins).

Compared with the relatively modest changes in receptor pharmacology outlined above, a more pronounced phenotype was observed with the R210H and P377S variants. Each of these receptor isoforms was essentially unable to trigger MCH-induced signaling (Figs. 1 and 2; Table 1) despite normal cell surface expression. The observed loss of function in each case may be attributed to disruption of a highly conserved GPCR signature motif that has been linked to receptor activation.

The R210H substitution is localized within the “DRY” motif of the MCHR1 (underlined R corresponds to variant R210). This amino acid sequence was shown in other GPCRs to play an essential role in determining the equilibrium between inactive and active receptor conformations (Rovati et al., 2007). Consistent with this function, a study showed that substitution of the corresponding arginine in the rat MCHR1 isoform with an alanine (R141A) resulted in a significant loss in MCH potency (Aizaki et al., 2009). It seems that the naturally occurring R210H substitution in the human MCHR1, as described here, has an even more pronounced impact by essentially abolishing MCH-induced stimulation.

The P377S substitution, which also leads to a loss of MCH-induced function, is located within a different highly conserved GPCR signature motif, NPXXY (where positions X can be occupied by any amino acids). Again, there is precedent with several receptors that modification of this important motif can compromise ligand induced activity and/or receptor expression (Wess et al., 1993; Galès et al., 2000; Mazna et al., 2008).

Additional characterization of the MCHR1 variants that are unresponsive to MCH revealed that under basal conditions cell surface expression is comparable with wild type. Follow-up studies were done examining the effect of SNAP-7941, a well established antagonist (Borowsky et al., 2002; Goss and Schaus, 2008), on the two inactive mutants and the wild-type receptor. Overnight incubation with this well established antagonist enhances MCH efficacy of the wild-type receptor by ~50%. Parallel experiments with the mutants revealed that preincubation with SNAP-7941 restores MCH-induced signaling at the P377S but not the R210H variant.

Our findings suggest that SNAP-7941 be included in a growing list of small-molecule GPCR antagonists and inverse agonists that are pharmacological chaperones. Such ligands penetrate the cell, bind to trapped receptors, and facilitate processing to the cell surface (Conn and Ulloa-Aguirre, 2010; Nakamura et al., 2010). It seems that SNAP-7941 has chaperone activity at both the wild-type MCHR1 and the P377S mutant. At the wild-type receptor, this ligand triggers partial

Discussion

In this study, we have established that selected naturally occurring mutations in the human MCHR1 can significantly alter the receptor’s ability to signal. Whereas 5 of the 12 receptor isoforms investigated (including the previously reported D22N variant) show a normal phenotype, two of the mutations (G401R and T411M) result in a significant increase in MCH efficacy (by ~30–40%) versus the corresponding wild-type value (Table 1). Conversely, MCH activity was significantly reduced (by ~50%) in cells expressing three other receptor isoforms (G103R, T107H, and T305M). The observed changes in MCH efficacy are not explained by parallel alterations in MCHR1 cell surface expression with the possible exception of G103R, which shows a concomitant decrease in receptor density.
redistribution of the intracellularly trapped pool of MCHR1s to the cell surface, thereby enhancing MCH induced function. At the P377S mutant, not only does SNAP-7941 enhance surface expression but this compound rescues the receptor’s ability to signal (reflected by the fact that MCH activity at this variant is contingent on preincubation with the small molecule). In contrast, at the R210H mutant, SNAP-7941 treatment has no appreciable effect on either receptor expression or function.

The ability of SNAP-7941 to restore agonist-induced signaling of the P377S mutant may reflect an ability of this ligand to stabilize the receptor in one or more conformations that are a prerequisite for agonist activity. It is now increasingly appreciated that GPCRs, like many other proteins, spontaneously undergo conformational changes with varying levels of structural stability (Kobilka and Deupi, 2007). Biophysical studies revealed that interaction with a ligand (agonist, antagonist, or inverse agonist) may stabilize/induce one or several distinct receptor conformations, which in the case of an agonist ligand triggers downstream signaling (Bokoch et al., 2010). GPCR mutations may result in a loss of agonist-induced function by preventing the formation of active receptor conformations and/or increasing the degree of structural instability. To interpret our findings, it is plausible to speculate that expression of the P377S mutant in the presence of SNAP-7941 stabilizes a receptor conformation that can be activated by MCH.

SNAP-7941 can not only modulate the expression and activity of a mutant receptor, but also has the potential to either enhance or block agonist-stimulated function at the wild-type MCHR1 isoform. This observation may have important implications for the future therapeutic use of other antagonists with similar properties (e.g., as drugs for treating obesity and/or mood disorders). Depending on the dosing and timing of antagonist administration, the net effect may either be receptor inhibition (if sufficient drug is present to block endogenous agonist induced MCHR1 signaling) or paradoxical stimulation (caused by enhanced MCH-induced activation resulting from the SNAP-7941 mediated increase in receptor expression).

In addition to dosing and timing, SNAP-7941-induced effects may be further complicated by the differential impact of naturally occurring MCHR1 mutations. Given our observations, a large increase in MCH-mediated function may occur in carriers of the P377S variant that are treated with SNAP-7941, whereas this effect is predicted to be absent in individuals harboring the R210H mutation. The potential for such pharmacogenomic variability should be considered as MCHR1 antagonists are being developed for clinical use.

Based on rodent studies, it is anticipated that either loss or gain of MCHR1 function will predispose to selected phenotypes in humans (e.g., a lean or obese phenotype, respectively) (Antal-Zimanyi and Khawaja, 2009; Pissios, 2009; Chung et al., 2010). As a candidate obesity susceptibility gene, the MCHR1 locus has therefore been screened for mutations (Gibson et al., 2004; Wermter et al., 2005). The variants that have been identified to date are not associated with standard indices of obesity (including body mass index, waist circumference, and glucose tolerance) (Antal-Zimanyi and Khawaja, 2009). However, it is of note that the two major loss-of-function variants described in our work (H210R and P377S) were, respectively, identified in two markedly underweight students, but not in an obese cohort that was studied in parallel (Wermter et al., 2005). Although these are single observations and as such do not prove an association, it is worth considering that the observed phenotype (reduced body weight) is consistent with loss of function of an orexigenic receptor (as demonstrated in this work). Given this possible link, it may be informative to explore the frequency of MCHR1 variants (in particular ones that abolish agonist-induced signaling) in large cohorts of underweight subjects. It is intriguing to speculate that just as there are human GPCR mutations that are linked to obesity there may be other receptor (e.g., MCHR1) variants that predispose to a lean phenotype.

Taken together, our observations establish that naturally occurring MCHR1 missense mutations in the human population have the potential to markedly alter the pharmacological response to MCH and/or to receptor selective nonpeptide antagonists (e.g., SNAP-7941). Furthermore, our data in combination with a published clinical report (Wermter et al., 2005) lead us to speculate that selected MCHR1 variants (i.e., P377S and R210H) may predispose to a lean phenotype. As efforts move forward to develop MCHR1-targeted drugs (Luthin, 2007; Antal-Zimanyi and Khawaja, 2009; Chung et al., 2010), it will be important to consider the potential impact of pharmacogenomic variability and/or chaperone-like effects on the response to therapeutics.

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