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Functional characterization in vitro of twelve naturally occurring variants of the human pancreatic polypeptide receptor *NPY4R*

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ABSTRACT

Obesity has become a global health problem and therefore understanding of the mechanisms regulating hunger and satiety is of utmost importance for the development of new treatment strategies. The Y4 receptor, encoded by the *NPY4R* gene, and its ligand pancreatic polypeptide (PP) have been reported to mediate a satiety signal. Multiple genetic studies have reported an association between *NPY4R* copy number and body weight. The gene also displays several SNP variants, many of which lead to amino acid differences, making it interesting to study. We have investigated the functional properties of 12 naturally occurring amino acid sequence variants of the Y4 and interpret the results in relation to sequence conservation and our structural model of the human Y4 receptor protein. Three receptor variants, Cys201^{ECL2}Tyr, Val271$^{6.41}$Leu and Asn318$^{7.49}$Asp, were found to completely lose functional response, measured as inositol phosphate turnover, while retaining membrane expression. They display high sequence conservation and have important roles in the receptor structure. For two receptor variants the potency of PP was significantly decreased, Cys34^{NT}Ser (EC50 = 2.9 nM, $p < .001$) and Val135$^{3.46}$Met (EC50 = 3.0 nM, $p < .01$), compared to wild-type Y4 (EC50 = 0.68 nM). Cys34 forms a disulphide bond with Cys298, linking the N-terminal part to ECL3. The Val135$^{3.46}$Met variant has an amino acid replacement located in the TM3 helix, one helix turn above the highly conserved ERH motif. This position has influence on the network of residues involved in receptor activation and subsequent inactivation. Sequence conservation and the structural model are consistent with these results. The remaining seven positions had no significant effect on the receptor's functional response compared to wild-type Y4. These positions display more variation during evolution. Understanding of the interactions between the Y4 receptor and its native PP agonist and the effects of amino acid variation on its functional response will hopefully lead to future therapeutic possibilities.

1. Introduction

Obesity is a highly heritable disease (Elks et al., 2012) that has become one of the major health problems across the globe during the past few decades (World Health Organization, 2014). It is a complex polygenic and multifactorial disease which makes it difficult to study specific genetic factors influencing body weight.

Multiple single nucleotide polymorphisms (SNPs) and gene copy number variation (CNV) regions are associated with obesity (Loos, 2009; Willer et al., 2009; Sha et al., 2009; Lindgren et al., 2009; Bochukova et al., 2010; Jarick et al., 2011; Wang et al., 2010). One such CNV region is located at 10q11.22 (Wang et al., 2010; Sebat et al., 2004; Park et al., 2010; Sudmant et al., 2010) and it encompasses the three genes *NPY4R*, *GPRIN2* and *SYT15*. Out of the three, *NPY4R* is the most likely candidate to play a role in energy metabolism and obesity development.

The *NPY4R* gene encodes the Y4 receptor which belongs to the neuropeptide Y (NPY) family of rhodopsin-like (class A) G protein-coupled receptors. Like all GPCRs, they are comprised by seven transmembrane helices (TM) connected with intracellular (ICL) and extracellular loops (ECL). Humans have four functional NPY-family receptor subtypes: Y1, Y2, Y4 and Y5 (Larhammar and Salaneck, 2004) and a pseudogenised Y6 receptor.

In mammals, the NPY family of peptides consists of three members: NPY, peptide YY (PYY) and pancreatic polypeptide (PP). All three peptides are 36 amino acids long, have an amidated carboxyterminal tyrosine and share considerable sequence identity (Sundström et al., 2008). The NPY family peptides regulate energy metabolism, hunger

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and satiety (Herzog, 2003), lipid metabolism and insulin secretion (Renshaw and Batterham, 2005; Huda et al., 2006), as well as many other physiological parameters. NPY stimulates appetite (Edwards et al., 1999) through the Y1 and Y5 receptors (Lecklin et al., 2002). In contrast, PYY and PP inhibit appetite (Batterham et al., 2002; Batterham et al., 2003; Kojima et al., 2007; Ueno et al., 1999; Akerberg et al., 2010), PYY acting primarily on Y2 (Batterham et al., 2002) and PP on Y4 (Kojima et al., 2007).

A structural model for the human Y4 receptor has been proposed based on crystal structures for other class A receptors followed by mutagenesis and measurements of functional response to PP stimulation (Pedragosa-Badia et al., 2014). Several residues in the outer parts of the transmembrane regions were identified to be important for receptor activation by PP. The Y4 receptor like the other NPYR family subtypes can couple via Gi and Go as well as Gq (Misra et al., 2004).

Among the NPY family receptors, Y4 is the fastest evolving functional member (Wraith et al., 2000). *NPY4R* displays multiple SNPs and was duplicated in the common ancestor of Denisovans, Neanderthals and the modern human (Shebanits et al., submitted). We have recently reported that the CNV ranges from 2 to 8 copies in one study sample (Shebanits et al., 2018) and from 3 to 11 in a different study sample (Shebanits et al., in preparation). The extensive CNV and the substantial SNP variation make the *NPY4R* gene a challenge to study, but due to its role in appetite inhibition the Y4 receptor is an attractive target for anti-obesity drug development. Understanding of the interactions between Y4 and PP, the consequences of the genetic variation, both CNV and SNP, will hopefully facilitate studies of therapeutic possibilities.

In our previous investigations of the copy number and sequence variation of the NPY4R gene we identified 12 SNP variants resulting in amino acid changes. We report here studies of the functional response of all of these naturally occuring sequence variants of the Y4 receptor protein. We interpret the results in the light of extensive evolutionary sequence comparisons of Y4 across vertebrates as well as with other NPY-family receptor subtypes and more distantly related peptide receptors. Furthermore, we have generated a structural model of the Y4 receptor that provides the structural basis for interpretation of the functional results.

2. Methods

2.1. Study Samples

We have screened the coding part of the NPY4R for SNPs in 24 individuals from 1000 Genomes Project (NA10847, NA10851, NA12155, NA12717, NA12878, NA18504, NA18510, NA18517, NA18519, NA18524, NA18529, NA18536, NA18542, NA18603, NA18627, NA18745, NA18760, NA18795, NA18940, NA18948, NA18949, NA18959, NA18961, NA19238) (Abecasis et al., 2010) and used the data from the Northern Sweden Population Health Study (NSPHS) in order to find non-sense and miss-sense mutations. We have studied 12 mutated versions of Y4 receptor.

2.2. Mutant analysis of 24 samples from 1000 Genomes Project

A number of PCR primes that cover the whole gene in a step-wise fashion was developed to analyse the SNPs in the coding part of the *NPY4R* (Supplementary Table 1).

2.3. Numbering and nomenclature used for receptor residues

Mutants are named using three-letter abbreviations for the amino acid residue, followed by a sequence correlative number and the introduced amino acid. Superscripts following receptor residue numbers denote *N*-terminus (NT), extracellular loop 2 (ECL2) and GPCR numbering according to Ballesteros and Weinstein. The most conserved residue in a TM is designated *x*.50, where *x* is the TM number. All other residues on the same TM region are numbered relative to this reference residue.

2.4. Site-directed mutagenesis

A pcDNA3.1-C-eGFP vector containing wild-type human Y4 (WT hY4) receptor coding region was ordered from GenScript and used as a parental template. Tha AccuPrime *Pfx* DNA Polymerase kit (Thermo Fisher Scientific) was used to generate $Cys34^{NT}Ser$, $Ala81^{2.44}Thr$ and $Arg240^{5.64}Cys$ mutants, QuikChange II XL (Stratagene) was used to generate $Ala99^{2.62}Ser$. Both kits were used according to the manufacturers' instructions. Briefly, each mutation was introduced via PCR using specifically designed primers (Supplementary Table 2) and the parental template. Coding regions of the cloned mutant plasmids were sequenced to confirm the introduced mutation. After the sequence confirmation, the plasmids were purified using PureLink HiPure Plasmid purification kit (Thermo Fisher Scientific) for transfection. Mutants $Thr126^{3.37}Met$, $Val135^{3.46}Met$, $Cys201^{ECL2}Tyr$, $Arg239^{5.63}Trp$, $Arg239^{5.63}Gln$, $Val271^{6.41}Leu$, $Val276^{6.46}Met$ and $Asn318^{7.49}Asp$ have been purchased from GenScript.

2.5. Functional assay

The inositol phosphate (IP) assay was performed by cotransfection of each receptor plasmid with a chimeric G-protein plasmid. The chimeric Gαq-protein (curtesy of E. Kostenis) has last four amino acids replaced by the corresponding amino acids from Gαi and can change a Gαi signal transduction pathway to the Gαq pathway, leading to IP generation(Kostenis, 2002).

Human embryonic kidney (HEK) 293 cells at 90–95% confluence were cotransfected with the chimeric Gαi4 and either a WT hY4 plasmid or the mutant hY4 plasmids, using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) and Opti-Mem cell culture medium (Thermo Fisher Scientific) according to the manufacturer's protocol. Myo[2 − ^{3}H]inositol (PerkinElmer) at 3 μCi/mL was added the next day. On the following day, the cells were detached with PBS/EDTA mixture (0.2 g/L) and resuspended in the IP assay buffer (10 mM LiCl, 20 mM Hepes, 137 mM NaCl, 5 m M KCl, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM glucose). The cells were preincubated for 10 min and then stimulated with serial dilution of hPP for 30 min at 37 °C. An equal volume of ice-cold 0.8 M perchloric acid was added and incubated on ice for 30 min to lyse the cells. The reaction was terminated by using a neutralising buffer ($KOH/KHCO_3$). Ion exchange chromatography on AG 1-X8 resin (Bio-Rad) was used to isolate the generated [^{3}H]inositol phosphates. The resin was washed with 5 nM Na_2B_2 and 60 mM NH_4-formate, and the [^{3}H]inositol phosphates were eluted with 1 M NH_4-formate and 0.1 M formic acid (method adapted from Johansson et al. 2007 (Johansson et al., 2009)). Following elution, the samples were mixed with OptiPhase HiSafe (PerklinElmer) and the ^{3}H radioactivity was measured with a liquid scintillation counter (PerklinElmer). Each sample was run in duplicates for each concentration and each assay was performed in triplicates. WT hY4 was used as a reference in each run.

2.6. Detection of receptor expression

Coverslips were coated with 0.1 mg/mL poly-D-lysine and were seeded with HEK 293 cells transiently expressing WT hY4 or mutant receptors. Cells, transfected without a plasmid, were used as a negative control. Cells were cultured for 48 h at 37 °C in 5% CO_2. The coverslips were washed twice with PBS and the cells were fixed with 70% ethanol for 10 min. The coverslips were mounted upside down on glass slides using DAPI-containing mounting medium. Acquired fluorescence was detected with an inverted confocal microscope (Zeiss LSM 510 Meta, Carl Zeiss Inc., Thornwood, NY) and a 63× oil immersion objective (NA = 1.4) and visualized via the accompanying LSM software (Carl

Table 1
Potencies of PP for wild-type Y4 and 12 naturally occurring Y4 variants.

Peptide	WT-hY4		Fold change K_i / PP K_i	n
	EC50(nM)	95% CI		
WT Y4	0.68	0.46; 1.0	1.0	13
Cys34^{NT}Ser	2.9***	0.86; 9.9	4.3	4
Ala81$^{2.44}$Thr	0.26	0.11; 0.62	0.4	3
Ala99$^{2.62}$Ser	1.2	0.71; 1.9	1.8	3
Thr126$^{3.37}$Met	0.39	0.25; 0.62	0.6	4
Val135$^{3.46}$Met	3.0**	0.34; 26	4.4	3
Cys201^{ECL2}Tyr	–	–	–	3
Arg239$^{5.63}$Trp	0.61	0.26; 1.5	0.9	3
Arg239$^{5.63}$Gln	1.6	1.1; 2.3	2.4	3
Arg240$^{5.64}$Cys	0.49	0.19; 1.3	0.7	4
Val271$^{6.41}$Leu	–	–	–	3
Val276$^{6.46}$Met	0.69	0.43; 1.1	1.0	3
Asn318$^{7.49}$Asp	–	–	–	3

Zeiss Inc.). The colours during microscopy were distinctly green (hY4-eGFP) or blue (cell nuclei). However, due to a technical problem with the camera filters during photography, some green fluorescence leaked into the blue channel in the photographs leading to cyan instead of green (Supplementary Fig. 1). This artifact was absent in negative control (cells that were not transfected with plasmid containing eGFP) (Supplementary Fig. 1).

2.7. Data analysis

To calculate the EC50 values of PP for Y4 and mutants, the functional assay data was analysed with the GraphPad Prism 5.0 software using non-linear regression curve fitting function. As the EC50 value is relative to the maximum response in each experiment, it will not vary with the exact expression level of the receptor. For each receptor, the assays were performed at least three times. The results were presented as geometric mean with 95% confidence interval (Table 1). The statistical analyses of pEC50 were performed using one-way ANOVA with Dunnett post hoc tests (Fig. 3). $P < .01$ is used to define statistically significant difference.

2.8. Homology modelling

A model of the Y4 receptor was generated with the GPCR-ModSim web server (http://open.gpcr-modsim.org) (Esguerra et al., 2016) following the protocol for homology modelling reported in (Vasile et al., 2018). Briefly, after obtaining a multiple sequence alignment of the Y4 sequence (from position 22 to position 340) against the curated crystallized GPCRs in fully active conformation available in the server, three templates were chosen to model the Y4 receptor in its active state: the human M2 muscarinic acetylcholine receptor (PDB code 4mqs), metarhodopsin II (PDB code 3pqr) and the beta2 adrenergic receptor (PDB code 3sn6). The server provides a pairwise sequence identity for each region of the GPCR in order to select the best template(s) for each segment. The alignment of the sequences was manually modified to account for distorsions of the transmembrane helices and 50 models were generated, following the protocol of Modeller (Webb and Sali, 2014) (embedded in the webserver), and complying with the set of spatial restraints and the sequence alignment previously created. The models were ranked based on the value of scoring function and their stereochemical quality was evaluated with Molprobity (Chen et al., 2010): the best model was chosen for the representation of the Y4

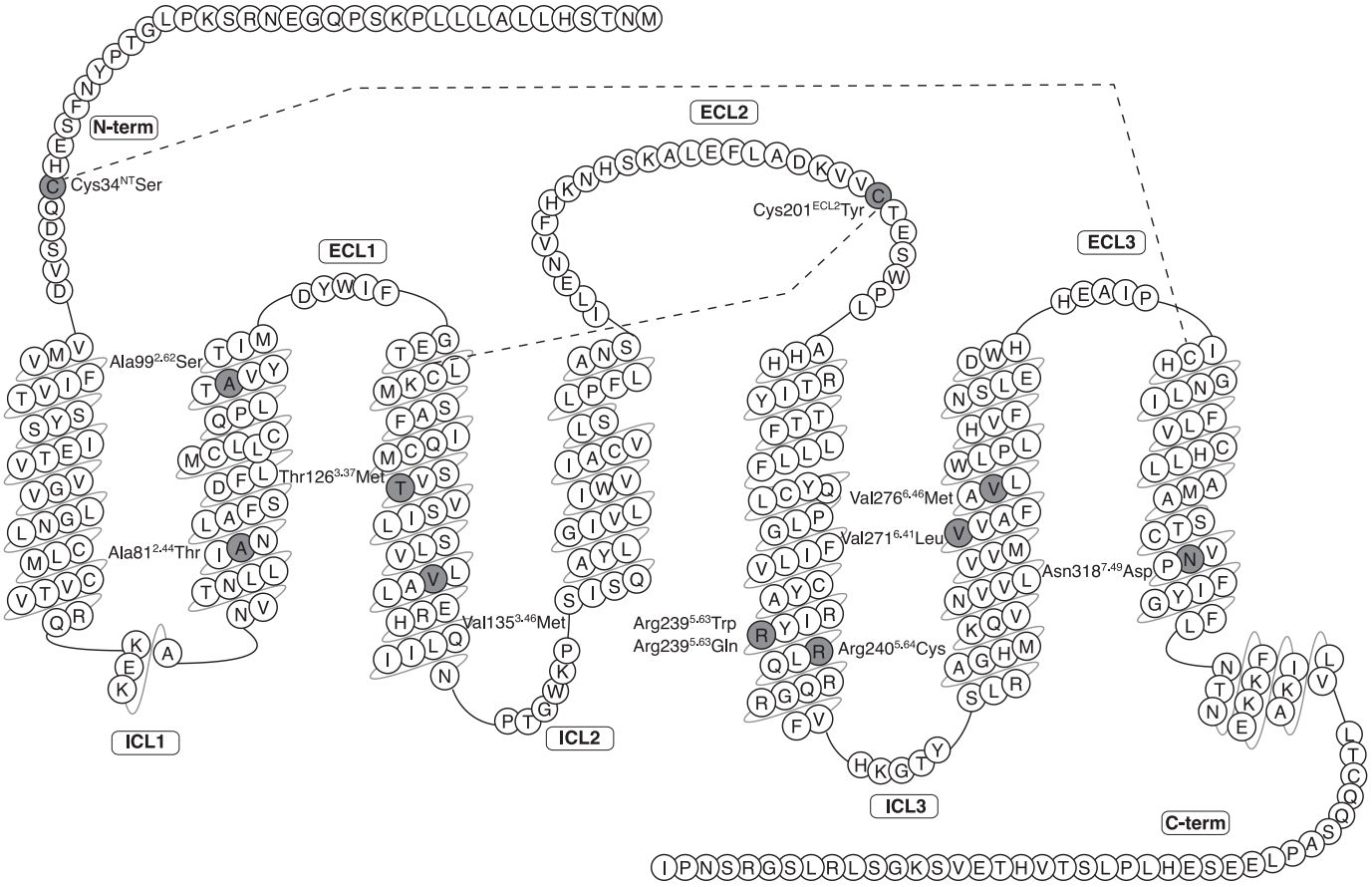

Fig. 1. Schematic representation of the Y4 receptor. Nonsynonymous mutations identified in this study are marked with grey colour. The figure is adopted from GPCRdb with modifications.

receptor.

3. Results

3.1. Analysis of receptor variants

We found seven nonsynonymous variants (Cys34^{NT}Ser, Ala81$^{2.44}$Thr, Ala99$^{2.62}$Ser, Cys201^{ECL2}Tyr, Arg240$^{5.64}$Cys, Val276$^{6.46}$Met and Asn318$^{7.49}$Asp) among 24 samples sequenced in the 1000 Genomes Project. A total of eight nonsynonymous SNPs was found in the data set of 1009 individuals from the Northern Sweden Population Health Study (Ala99$^{2.62}$Ser, Thr126$^{3.37}$Met, Val135$^{3.46}$Met, Arg239$^{5.63}$Trp, Arg239$^{5.63}$Gln, Arg240$^{5.64}$Cys, Val271$^{6.41}$Leu, Val276$^{6.46}$Met). Thus, three of the variants were represented in both study samples, namely Ala99$^{2.62}$Ser, Arg240$^{5.64}$Cys and Val276$^{6.46}$Met. All the variants are summarised in Fig. 1.

3.2. Functional assay of PP for WT Y4 and Y4 mutants

All of the receptor variants were generated by mutagenesis of a wild-type Y4 receptor construct in the pcDNA3.1-C-eGFP vector and functionally expressed after transfection of HEK 293 cells. We used a functional assay measuring inositol phosphate turnover in order to study the in vivo signaling of the mutated receptors in comparison with the WT Y4 receptor in response to the natural agonist human PP. Y4 is naturally coupled to the Gαi protein, but in order to study the production of the inositol phosphates, the chimeric Gαq-protein with the last four C-terminal residues from Gαi was co-transfected with the Y4 plasmid. All experiments were run in duplicate and repeated at least three times and always with the WT construct in parallel as reference.

PP had a statistically significant decrease in potency on two of the variants, Cys34^{NT}Ser and Val135$^{3.46}$Met (Table 1, Fig. 2, Fig. 3). Both of these displayed a rather modest difference to the wild-type, but nevertheless significant with $p < .001$ and $p < .01$, respectively.

The response was completely absent in three of the variants, namely Cys201^{ECL2}Tyr, Val271$^{6.41}$Leu and Asn318$^{7.49}$Asp. All three were expressed on the cell surface as shown by fluorescence of the eGFP tag,

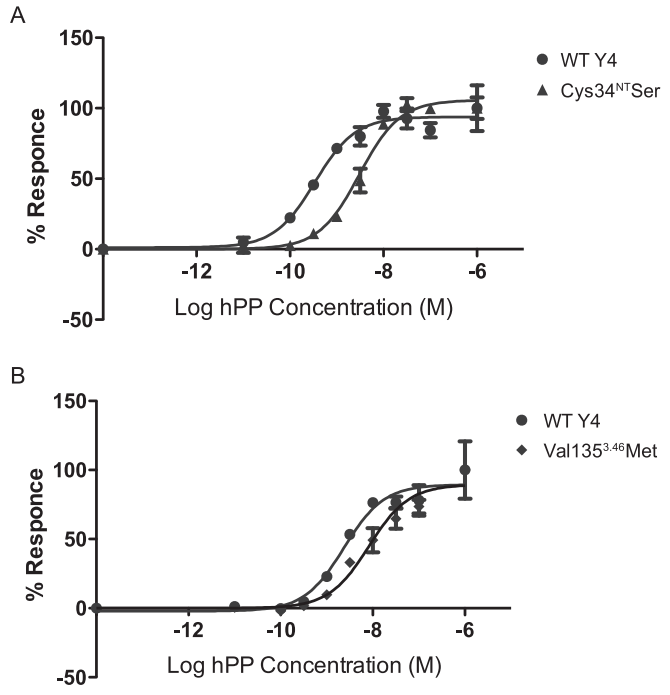

Fig. 2. Examples of typical dose-response curves that were used to calculate the EC50 values (for WT hY4 and Cys34^{NT}Ser and Val135$^{3.46}$Met mutants).

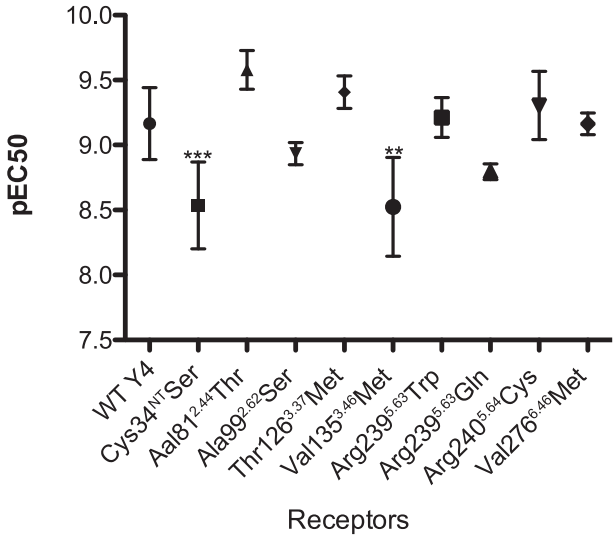

Fig. 3. A comparison of the pEC50 values of PP in the IP assay for WT Y4 and mutant receptors. Data are presented as mean ± SD (**p < .01; *** p < .001).

although Val271$^{6.41}$Leu had considerable fluorescence in intracellular vesicles and Asn318$^{7.49}$Asp had low albeit clear cell surface expression (not shown).

The remaining seven variants showed no difference to the WT receptor in their functional response (Fig. 3).

The EC_{50} values are presented as the geometric mean of the n independent experiments. Three receptor variants showed no detectable response. One-way ANOVA with Dunnett post hoc analysis was performed for the pEC_{50} values. ***: p < .001, **: $p < .01$.

3.3. Homology modelling

A model of the human Y4 receptor was generated as described in the methods section. The structural information obtained with homology modelling of Y4 helps with the qualitative interpretation of the mutagenesis data as described below.

The loss of receptor response in the case of Cys201Tyr is explained by the disruption of the disulphide bridge with Cys114$^{3.25}$, the function of which is to bring ECL2 and TM3 close to each other. Regarding the Asn318$^{7.49}$Asp variant, the presence of Asp87$^{2.50}$ in the vicinity of Asn318$^{7.49}$ makes hard to accept a second negative charge, consistent with loss of receptor response. The last mutant for which no response was detected, Val271$^{6.41}$Leu, is found in a very apolar environment composed of Leu227$^{5.51}$, Leu231$^{5.55}$ and Leu267$^{6.37}$. The introduction of a longer sidechain can be structurally deleterious.

Two variants had significantly, albeit modestly, reduced response. Cys34 in the N-terminus forms a disulphide bridge with Cys298$^{7.29}$ on the outer tip of TM7. The Cys34Ser variant has lost this interaction. As in the case of the Cys201Tyr mutant, the perturbation of a second disulphide bridge between Cys34 and Cys298$^{7.29}$ would be consistent with the observed 4.3-fold reduction of potency compared to the wild type. The Val135$^{3.46}$Met variant had 4.4-fold reduction in potency. The Val sidechain points towards TM2 and TM7 and it is surrounded by Leu79$^{2.42}$, Arg139$^{3.50}$ and Tyr322$^{7.53}$, hence the Met mutation is unfavourable for sterical reasons.

The other seven amino acid variants that we have found did not result in a significantly different response compared to the wt Y4 receptor. The sidechain of Ala81$^{2.44}$ faces the membrane and this position is deep in the bilayer, thus the mutation into Thr does not have a detrimental effect on PP potency. The sidechain of Ala99$^{2.62}$, instead, faces TM1 and it is found in a very hydrophobic environment, with the exception of Tyr48$^{1.39}$, whose hydroxyl group points away from Ala99$^{2.62}$.

Its mutation into Ser is not easily accepted in such surroundings, although no sterical clash arises. The Thr126$^{3.37}$Met variant is a big change in the physical characteristics of the sidechain, but the potency appears unaltered, probably due to the absence of polar interactions involving the threonine sidechain and the possible accommodation of the bulky sidechain of methionine between TM3 and TM5.

According to our model, Arg239$^{5.63}$ and Arg240$^{5.64}$ are positioned in the lower part of TM6 with their sidechains exposed to the solvent. Moreover, they are surrounded by other arginines. The change of Arg239$^{5.63}$ into Trp or Gln and the change of Arg240$^{5.64}$ to Cys do not affect the receptor response to its agonist PP most probably because they do not cause major changes in the part of the receptor involved in the ligand or G protein binding.

The final variant Val276$^{6.46}$Met involves a valine sidechain that is completely surrounded by the lipid membrane why its change into methionine would not be expected to have any effect on the potency.

4. Discussion

Due to the complex genetics of the *NPY4R* gene, displaying both extensive CNV and SNP variation, it is not possible to discuss the amino acid variants in terms of allelic products. Rather, we cautiously call them receptor variants, as they may arise either from distinct copies of the *NPY4R* gene on the same chromosome or from alleles for either of the duplicates.

We identified twelve naturally occurring Y4 variants in the two study samples that we have analysed. Three of these variants were found in both of the cohorts: Val271$^{6.41}$Leu (Aerts et al., 2016), Arg240$^{5.64}$Cys and Val276$^{6.46}$Met (Sjödin, 2005). These are the only ones that have been investigated pharmacologically before, to our knowledge. The remaining nine variants were found either in one or the other of the two cohorts. One of these, Ala81$^{2.44}$Thr was analysed in one of the previous studies, but only in silico, where it was predicted to have no effect (Aerts et al., 2016).

Three of the variants produced no functional response at all, namely Cys201^{ECL2}Tyr Val271$^{6.41}$Leu and Asn318$^{7.49}$Asp. Cells transfected with Cys201^{ECL2}Tyr exhibited membrane expression but had a high number of vesicles containing the receptor (Supplementary Fig. 1). The loss of response for Cys201Tyr was expected because this cysteine forms a disulphide bond with Cys114 in ECL1, holding together this region with ECL2, in a large number of class A GPCRs including all of the NPYR subtypes and species. Presumably this disulphide is required for the NPY receptors to form a functional binding site for the native peptide agonists.

Cells transfected with Val271$^{6.41}$Leu displayed membrane expression and, as well as Cys201^{ECL2}Tyr, had a high number of vesicles containing the receptor (Supplementary Fig. 1). Our structural model shows that this side chain is surrounded by three Leu side chains why a Leu also at position 271 may make this space too crowded, resulting in a distorted receptor structure. This Val at this position is highly conserved in all NPY-family receptors except two Y4 sequences that have Ile (a shark and a pufferfish) and most of the Y5 sequences which likewise have an Ile. Thus, only Val and Ile seem to be generally acceptable at this position in the NPYR family. The related receptors for NPFF, PRLP and QRFP have either Val or Ala at this position (see Fig. 5 (Xu et al., 2018)).

The complete loss of response for the change Asn318$^{7.49}$Asp was expected because it affects a functionally crucial residue with very high conservation not only across species for Y4 and across NPY-family receptor subtype, but also in many other class A GPCRs. Asn318$^{7.49}$ interacts with Asp105$^{2.50}$ to regulate receptor activation-inactivation (see (Hulme, 2013)). A change of Asn318$^{7.49}$ to Asp may interfere with the nearby Asp87$^{2.50}$ according to our structural model.

Two receptor variants displayed significantly reduced potency, requiring > 4-fold higher PP concentration at EC50. Regarding the Cys34^{NT}Ser variant, Cys34 forms a disulphide bond with Cys298 at the outer end of ECL3. This cysteine pair is found in all Y1-subfamily receptors, i.e., Y1, Y4, Y6 and Y8 (lost in mammals). However, it does not exist in Y2, Y5, or Y7 suggesting that it is not absolutely essential for NPYR function. Therefore, it is not unexpected that the Cys34Ser variant is expressed on the cell surface (Supplementary Fig. 1).

The Val135$^{3.46}$Met variant involves an amino acid replacement rather deep in the TM3 helix, just one helix turn above the highly conserved ERH motif. This position may have some interaction with the network of residues involved in receptor activation and subsequent inactivation. Indeed, it is highly conserved across NPY receptor subtypes, having and Ile in all subtypes including Y4 in most species except mammals, all of which have a Val. Also the closest relatives of the NPYR family have and Ile, namely the receptors for NPFF, PRLP and QRFP (see Fig. 5 in (Xu et al., 2018)). The structural model shows that a Met at this position will have sterical problems with the surrounding side chains in TM2, TM3 and TM7, namely Leu79$^{2.42}$, Arg139$^{3.50}$ and Tyr322$^{7.53}$.

The remaining seven Y4 variants did not differ significantly from the WT in functional response (Fig. 3). All of these display more variability among species and NPY receptor subtypes and are likely to represent more or less neutral variants. Detailed analyses of the structural model Fig. 4 shows that these positions seem able to accommodate that variant amino acid side chains without obvious disturbance of either receptor structure, ligand binding or G protein interaction.

Thus, in conclusion, our analyses of the functional responses of twelve naturally occurring Y4 receptor variants are in good agreement with the degree of variation at each of these positions across species and NPYR subtypes, and also other class A GPCRs. The functional consequences of the genetic variation of NPY4R is likely to be a combination of gene copy number variation, amino acid changes and differences in regulatory regions of the genes. Hence, it is not immediately obvious how much each variant gene may contribute to the phenotype of the carrier of that variant, neither the three variants that have lost functional response nor the two that have a reduced functional

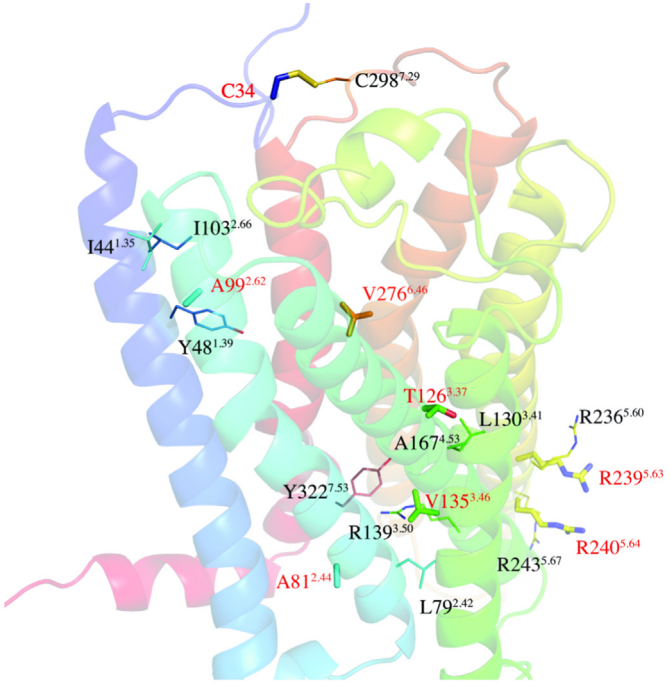

Fig. 4. Homology model of the human Y4 receptor and the locations of the variant amino acid residues investigated in this study. The three variants that completely lost receptor response are not shown. The mutated residues are depicted with thicker sticks and red label, while their surroundings are indicated with black labels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

response. If these variants would occur in individuals with only two or three rather than several more copies of the *NPY4R* gene, it is of course more likely that they will cause a reduction in the physiological response to PP.

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Conflict of interests

Authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.npep.2019.05.004.

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