

Original Article

The essential role for aromatic cluster in the $\beta 3$ adrenergic receptor

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Aim: To explore the function of the conserved aromatic cluster F213^{5,47}, F308^{6,51}, and F309^{6,52} in human $\beta 3$ adrenergic receptor (h $\beta 3$ AR).

Methods: Point mutation technology was used to produce plasmid mutations of h $\beta 3$ AR. HEK-293 cells were transiently co-transfected with the h $\beta 3$ AR (wild-type or mutant) plasmids and luciferase reporter vector pCRE-luc. The expression levels of h $\beta 3$ AR in the cells were determined by Western blot analysis. The constitutive signalling and the signalling induced by the $\beta 3$ AR selective agonist, **BRL** (**BRL37344**), were then evaluated. To further explore the interaction mechanism between **BRL** and $\beta 3$ AR, a three-dimensional complex model of $\beta 3$ AR and **BRL** was constructed by homology modelling and molecular docking.

Results: For F308^{6,51}, Ala and Leu substitution significantly decreased the constitutive activities of $\beta 3$ AR to approximately 10% of that for the wild-type receptor. However, both the potency and maximal efficacy were unchanged by Ala substitution. In the F308^{6,51}L construct, the EC₅₀ value manifested as a “right shift” of approximately two orders of magnitude with an increased E_{max} . Impressively, the molecular pharmacological phenotype was similar to the wild-type receptor for the introduction of Tyr at position 308^{6,51}, though the EC₅₀ value increased by approximately five-fold for the mutant. For F309^{6,52}, the constitutive signalling for both F309^{6,52}A and F309^{6,52}L constructs were strongly impaired. In the F309^{6,52}A construct, **BRL**-stimulated signalling showed a normal E_{max} but reduced potency. Leu substitution of F309^{6,52} reduced both the E_{max} and potency. When F309^{6,52} was mutated to Tyr, the constitutive activity was decreased approximately three-fold, and **BRL**-stimulated signalling was significantly impaired. Furthermore, the double mutant (F308^{6,51}A_F309^{6,52}A) caused the total loss of $\beta 3$ AR function. The predicted binding mode between $\beta 3$ AR and **BRL** revealed that both F308^{6,51} and F309^{6,52} were in the **BRL** binding pocket of $\beta 3$ AR, while F213^{5,47} and W305^{6,48} were distant from the binding site.

Conclusion: These results revealed that aromatic residues, especially F308^{6,51} and F309^{6,52}, play essential roles in the function of $\beta 3$ AR. Aromatic residues maintained the receptor in a partially activated state and significantly contributed to ligand binding. The results supported the common hypothesis that the aromatic cluster F[Y]5.47/F[Y]6.52/F[Y]6.51 conserved in class A G protein-coupled receptor (GPCR) plays an important role in the structural stability and activation of GPCRs.

Keywords: $\beta 3$ adrenergic receptor; constitutive activity; mutation; aromatic residue; G protein-coupled receptors

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Introduction

G protein-coupled receptors (GPCRs), which are characterised as seven transmembrane (TM) helices, comprise a large superfamily of membrane receptors involved in a wide range of signalling transduction pathways, which are mainly activated by agonists. Their activation mechanisms have been studied for decades, and the results show that they can transit through inactive-state and several active-state conformations with or without ligands^[1, 2]. Although GPCRs are activated by ligands with different chemical natures, they are believed to

share a common molecular activation mechanism^[3, 4]. A number of biochemical and biophysical approaches, including site-directed spin labelling and various fluorescent technologies, have been applied to study the activation-associated conformational changes of GPCRs^[5–8]. These studies indicated that the movement of the intracellular segments of TMs, especially the change of the orientations of TM-III and TM-VI, is likely a key element in the activation of GPCRs. Alternative experimental approaches including metal-ion site or disulphide engineering revealed that the extracellular segments of the TMs appear to move in the opposite direction of the intracellular segments. Thus, a global toggle switch mechanism for GPCR activation was proposed, whereby the extracellular segment of TM-VI was bent towards TM-III while the intracellular part moved away from TM-III^[3, 9].

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At the cytoplasmic surface, the E/DRY motif on TM-III is conserved among all classes of GPCRs. These amino acids form a network of polar interactions that bridge TM-III and TM-VI and that stabilise the inactive-state conformation, which is called the 'ionic lock'^[10]. For β_2 adrenergic receptor (β_2 AR), mutations of these residues, which weakened the strength of the ionic lock, increased constitutive activity^[11]. This interaction network has been observed in crystal structures of the dark-state rhodopsin^[12, 13], and it was broken in several other GPCR crystal structures bound with antagonists, such as β_1 adrenergic receptor (PDB code: 2VT4^[14]) and A2A adenosine receptor (PDB code: 3EML^[15]). Microswitch during the process of GPCR activation has also been explored for decades. The W6.48 of the CWXP motif in GPCR, which is located at the bottom of the main ligand-binding pocket, is generally expected to function as a key microswitch in GPCR activation^[16, 17]. The W6.48 changes position and interaction partners during receptor activation^[17-19]. Moreover, the aromatic cluster around W6.48, F[Y]5.47/F[Y]6.52/F[Y]6.51, is proposed to play a role in initiating the receptor activation^[20]. However, the detailed functional mechanisms of these residues need further investigation.

There are three members of β ARs (β_1 AR, β_2 AR, and β_3 AR) that belong to class A GPCRs. β_3 AR was originally discovered in the 1980s. Early studies identified that human β_3 AR was mainly expressed on the surface of both white and brown adipocytes, mediating metabolic effects such as lipolysis and thermogenesis^[21]. More recent reports showed that β_3 AR is also an attractive target for drugs against overactive bladder^[22, 23], anxiety and depressive disorders^[24, 25]. Understanding the mechanism of β_3 AR activation should facilitate rational drug discovery and the design of new types of β_3 AR agonists. In this study, we employed site-directed mutagenesis and molecular docking to explore the detailed function of the conserved aromatic cluster F213^{5.47}/F309^{6.52}/F308^{6.51} in β_3 AR.

Materials and methods

Reagents

Ham's F12 nutrient medium, G418 and pCDNA3.1 were purchased from Invitrogen (Carlsbad, CA, USA). The human pCDNA3.1- β_3 AR (pCDNA3.1-h β_3 AR) plasmid was obtained from Missouri S&T cDNA Resource Center (Rolla, MO, USA). The pCRE-Luc plasmid containing four copies of a consensus CRE was obtained from Stratagene (La Jolla, CA, USA). CHO-K1 and HEK-293 cells were purchased from ATCC (Rockville, MD, USA). Recombinant cell line CHO-mock was constructed in-house (CHO-K1 cells were stably co-transfected with pCDNA3.1-mock plasmid and pCRE-luc plasmid). Fetal bovine serum was supplied by Hyclone (Logan, UT, USA). FuGENE6 Transfection Reagent was supplied by Roche (Indianapolis, IN, USA). The Steady-Glo Luciferase Assay System was obtained from Promega (Madison, WI, USA). β_3 AR selective agonist BRL37344 (BRL) was obtained from Tocris Bioscience (Bristol, UK). Phosphatase and protease inhibitor cocktail was obtained from Sigma (St Louis, MO, USA). PVDF membranes were obtained from Bio-Rad Laboratories (Rich-

mond, CA, USA). Anti- β_3 AR antibody (c-20) was obtained from Santa Cruz (Santa Cruz, CA, USA). Anti- β -Actin antibody was obtained from Sigma (St Louis, MO, USA). HRP-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). ECL substrate was obtained from Pierce (Rockford, IL, USA).

Mutant construction, cell culture and transfections

Human β_3 AR mutants were constructed by a PCR-based site-directed mutagenesis approach. The PCR products were digested with *DpnI* restriction enzyme and were then transformed into competent cells. All of the mutants were verified by DNA sequencing analysis. HEK-293 cells were plated at a density of approximately 20000 cells/well in 96-well plates with 100 μ L of high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a humidified 5% CO₂/95% air atmosphere at 37°C. After 24 h, the cells were co-transfected with pCDNA3.1-h β_3 AR (wild-type or mutant) and the luciferase reporter plasmid pCRE-Luc using FuGENE6 Transfection Reagent according to the manufacturer's instructions.

CRE-luciferase activity test

After 24 h of transfection, an additional 100 μ L of DMEM containing different concentrations of BRL37344 (BRL) was added. Then, the plate was incubated at 37°C (5% CO₂) for 3 h. The media were removed, and luciferase activities were detected using Steady-Glo Luciferase Assay System on the Flexstation III instrument (Molecular Devices, CA, USA).

Western blotting

Cells were harvested and homogenised in RIPA lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1 mmol/L PMSF) with a phosphatase and protease inhibitor cocktail. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with primary anti- β_3 AR antibody (1:200) and anti- β -Actin antibody (1:1000), followed by HRP-conjugated secondary antibodies (1:5000). The resulting immunoblots were visualised using ECL substrate.

Homology modelling and molecular docking

The construction of the homology model of β_3 AR (32-237, 282-361) was based on the structure of β_2 AR with InsightII^[26]. GPCR has at least two functional states: inactive (R) and active (R*), which have different conformations. To obtain an active conformation of β_3 AR, the structure of β_2 AR binding with an agonist (PDB ID: 3P0G^[27]) was used for our homology modelling, which was retrieved from the Protein Data Bank. On the basis of the sequence alignment result, the coordinates in conserved regions were assigned. The N and C terminal residues and loops were added using Loop_Search module and refined using Discover module. Disulfide bonds were defined between Cys110 and Cys196 and between Cys189 and Cys195. Finally, the whole structure model was minimised by SYB-

YL6.8 (Tripos Inc, St Louis, MO, USA). The stereochemical quality was assessed by Procheck^[28].

Molecular docking was performed using AutoDock4.2^[29]. To prepare both the receptor and **BRL**, all hydrogens were initially added, Gasteiger charges were computed, and the non-polar hydrogens were then merged with the corresponding non-polar atoms. The receptor was considered completely rigid for the docking study. The active site was defined by a grid box as large as 60×60×60 points with a grid spacing of 0.375Å using AutoGrid4. The box was centred on the centre of the Asp117 residue in the receptor. The docking parameters were prepared as follows: *ga_pop_size*, 150; *ga_num_evals*, 2500000; *ga_num_generations*, 27000; *ga_run*, 100 and *rmstol*, 2.0. The Lamarckian genetic algorithm was applied to account for protein-ligand interactions. Finally, the ligand conformation was selected considering both the predicted binding free energy and binding modes in the β3AR pocket.

Statistical analysis

EC₅₀ values were determined by nonlinear regression using GraphPad Prism software (San Diego, CA, USA).

Results

The relationship between the expression level and the pharmacological properties of β3AR

To explore the influence of the protein expression level/transfection efficiency of plasmid DNAs on the detected pharmacological properties of β3AR, we adjusted the percentage of β3AR in DNA mixture (pCDNA3.1-hβ3AR and pCDNA3.1) to mimic the different expression level/transfection efficiency of the receptor. The constitutive activity (basal activity) and response stimulated by **BRL** were determined. The basal activity and maximal efficacy stimulated by **BRL** were both reduced less than 50%, and the EC₅₀ value was reduced less than three-fold as the percentage of β3AR in the DNA mixture changed from 100% to 20% (Table 1). Therefore, in our experimental system, the expression level/transfection efficiency of β3AR or mutants did not significantly influence the pharmacological properties of β3AR.

Table 1. Changes in the potency (EC₅₀) and response (*R*_{max} and *R*_{min}) in relation to the levels of the receptor expression/transfection efficiency.

| β3AR (%) | Basal activity (<i>R</i> _{min}) (%) | <i>R</i> _{max} (%) | lg EC ₅₀ |
|----------|--|-----------------------------|---------------------|
| 100% | 100±12.7 (4) | 265.6±19.9 (2) | -6.9±0.3 |
| 80% | 78.8±10.3 (4) | 194.5±9.1 (2) | -6.9±0.2 |
| 20% | 67.9±9.1 (4) | 220.3±8.1 (2) | -6.7±0.1 |

Expression levels of β3AR were mimicked by transfecting different ratios of pCDNA3.1-hβ3AR plasmid to pCDNA3.1. The pharmacological properties were determined by luciferase activity following stimulation by **BRL**. *R*_{min} represents the basal activity (constitutive activity). The maximal response (*R*_{max}) and *R*_{min} are given as percentages of *R*_{min} value of the one with 100% β3AR plasmid. Values are shown as mean±SEM, values for *n* are shown in parentheses.

Functional analysis of F308^{6.51} in β3AR

To validate the function of F308^{6.51}, this aromatic residue was experimentally substituted with non-aromatic amino acid (Ala or Leu) and aromatic amino acid (Tyr), respectively. The results showed that all the mutants were well expressed in transfected HEK-293 cells as determined by Western blot analysis (the data for mutant F308^{6.51}Y were not obtained) (Figure S1A). The activities of mutants were examined in transfected HEK-293 cells by measuring receptor-mediated luciferase activities. As was observed with substitution of F308^{6.51}, both Ala and Leu substitution significantly decreased the constitutive activities of β3AR to approximately 10% of that for the wild-type receptor. However, both the potency and maximal efficacy were unchanged by Ala substitution. In the F308^{6.51}L construct, the EC₅₀ value manifested as a “right shift” of approximately two orders of magnitude with an increased *E*_{max} (Table 2, Figures 1A and 1B). Impressively, the molecular pharmacological phenotype was similar to the wild-type receptor for the introduction of Tyr at position 308^{6.51}, though the EC₅₀ value increased by approximately five-fold for the mutant (Table 2, Figure 1C). Thus, F308^{6.51} is essential for the constitutive signalling for the β3AR, and it could be involved in the activation process to some extent.

Table 2. Pharmacological properties of the wild-type and mutated β3ARs.

| β3AR (%) | Basal activity (<i>E</i> _{min}) (%) | <i>E</i> _{max} (%) | lg EC ₅₀ |
|----------------|--|-----------------------------|---------------------|
| Wide-type β3AR | 100.0±1.9 (22) | 260.6±5.2 (15) | -8.0±0.1 |
| F308A | 10.7±0.7 (5) | 252.4±14.2 (3) | -7.8±0.2 |
| F308L | 9.5±0.9 (6) | 314.3±6.0 (4) | -6.2±0.1 |
| F308Y | 96.2±7.0 (6) | 260.0±14.1 (4) | -7.3±0.3 |
| F309A | 7.7±0.5 (6) | 235.5±5.6 (4) | -6.6±0.1 |
| F309L | 5.3±0.4 (6) | 119.5±3.4 (4) | -5.1±0.1 |
| F309Y | 29.4±1.2 (6) | - | - |
| F308A_F309A | 5.9±0.2 (6) | 9.8±0.4 (4) | - |
| F213A | 6.7±0.5 (6) | 92.5±4.3 (4) | -5.3±0.1 |
| W305A | 4.8±0.5 (6) | 38.9±1.0 (4) | -6.3±0.1 |

The constructs were expressed in transiently transfected HEK-293 cells. The efficacy data on basal activity (*E*_{min}) and maximal response (*E*_{max}) are expressed as a percentage of the basal activity on the wild-type β3AR. Values are shown as mean±SEM, values for *n* are shown in parentheses.

Functional analysis of F309^{6.52} in β3AR

F309^{6.52} was also substituted with non-aromatic amino acid (Ala or Leu) and aromatic amino acid (Tyr), respectively. According to the Western blot analysis results, Ala substitution reduced the expression of the receptor in transfected HEK-293 cells by approximately 25%, while the expression level of the Leu substituted mutant was increased by approximately 25% compared with the wild-type receptor (Figure S1). However, constitutive signalling for both F309^{6.52}A and F309^{6.52}L constructs were strongly impaired (Table 2, Figures 1D and 1E). In the F309^{6.52}A construct, **BRL**-stimulated signalling showed a normal *E*_{max} but reduced potency (lgEC₅₀

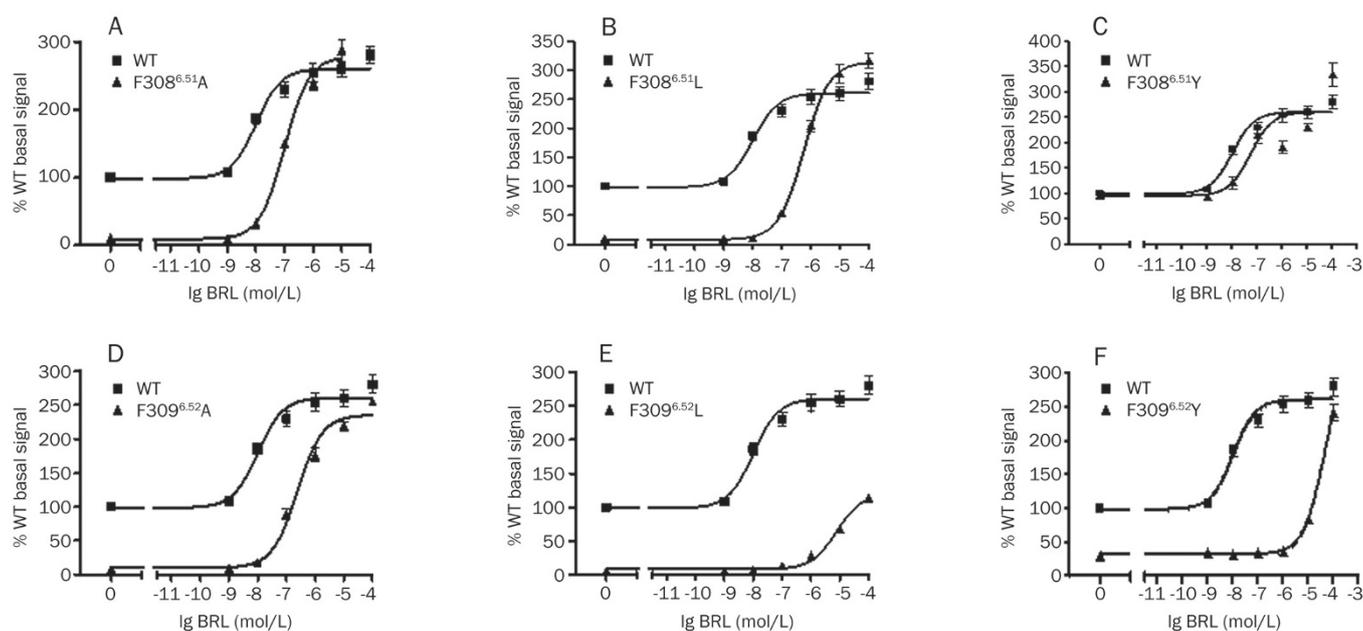


Figure 1. The pharmacological properties of HEK-293 cells transiently transfected with wild-type and mutated β 3ARs (F308^{6.51}A, F308^{6.51}L, F308^{6.51}Y, F309^{6.52}A, F309^{6.52}L, and F309^{6.52}Y). The efficacy is detected as luciferase activity.

value is -6.6). Leu substitution of F309^{6.52} reduced both the E_{max} and potency (Table 2, Figures 1D and 1E). When F309^{6.52} was mutated to Tyr, the constitutive activity was decreased approximately three-fold, and **BRL**-stimulated signalling was significantly impaired (Table 2, Figure 1F). Thus, F309^{6.52} is essential for the constitutive signalling of β 3AR and is important for the agonist-induced signalling.

To further explore the importance of F308^{6.51} and F309^{6.52}, the double mutant F308^{6.51}A_F309^{6.52}A was constructed. The result showed that the protein expression level was reduced to approximately 30%, and both the constitutive and **BRL**-stimulated signalling were almost totally diminished (Table 2, Figures 2A and S1B).

When F213^{5.47} and W305^{6.48} were mutated to Ala, we observed that the constitutive activities and maximum responses (stimulated by **BRL**) were also dramatically impaired for both mutants (Table 2 and Figures 2B and 2C). The result was similar to that for the molecular pharmaco-

logical phenotype for the ghrelin receptor, a member of class A GPCR^[30].

The predicted binding model of β 3AR and **BRL**

With the aim to explore the roles of the aromatic residues F213^{5.47}/F309^{6.52}/F308^{6.51} on the structural level, the interaction mechanism of β 3AR and **BRL** was predicted by molecular docking. The 3D structure of β 3AR was constructed by homology as described in the Materials and methods, and the stereochemical quality of the β 3AR model was finally examined by PROCHECK. The result of PROCHECK showed that 94.4% of the residues were in the most favoured regions and that no residue was in the disallowed regions (Figure S2). **BRL** was then docked (via software AutoDock4.2^[29]) into the binding pocket of β 3AR. As shown in Figure 3, **BRL** formed a hydrogen bond with residues Asp117, Ser208, and Asn312 (Figure 3A), which is consistent with the results reported for β AR family members^[31-35]. F308^{6.51} and F309^{6.52} were in the

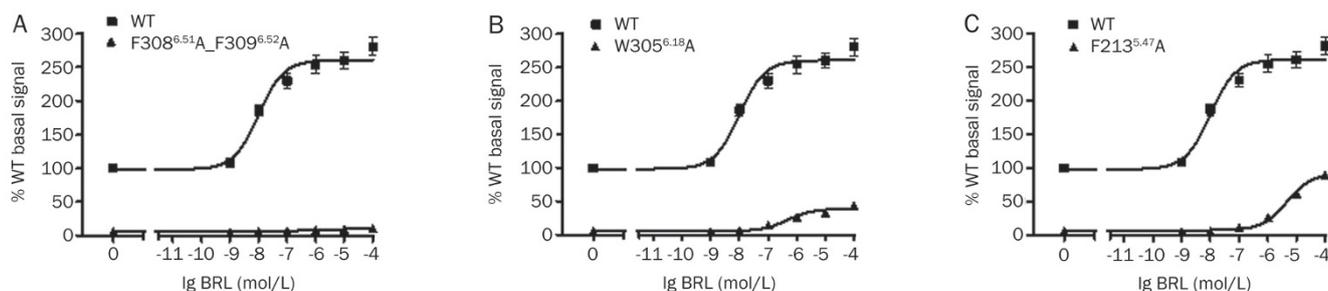


Figure 2. The pharmacological properties of HEK-293 cells transiently transfected with wild-type and mutated β 3ARs (F308^{6.51}A_F309^{6.52}A, W305^{6.48}A, F213^{5.47}A). The efficacy is detected as luciferase activity.

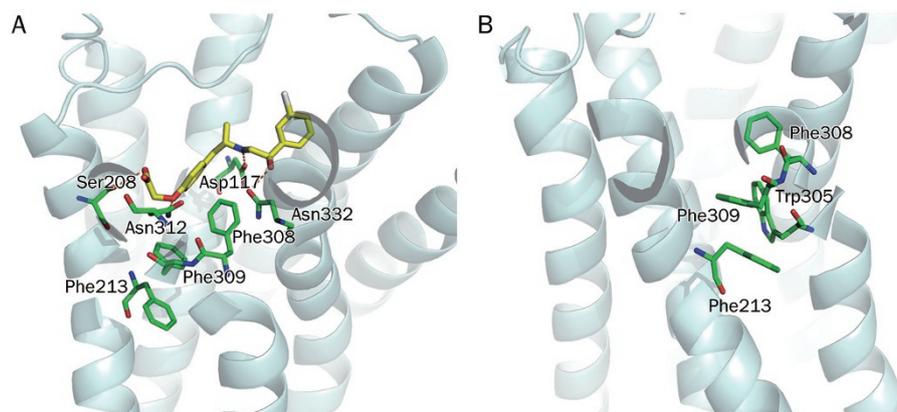


Figure 3. (A) The predicted binding mode of $\beta 3\text{AR}$ and **BRL**. (B) A π - π stacking is formed between F213^{5.47} and F309^{6.52} in the aromatic cluster F213^{5.47}/F309^{6.52}/F308^{6.51} in $\beta 3\text{AR}$. Residues of $\beta 3\text{AR}$ are indicated in green sticks. **BRL** is shown in yellow sticks. The dashed lines in red represent hydrogen bonds.

binding pocket, and they had hydrophobic interactions with **BRL**, while F213^{5.47} was below the binding pocket (Figure 3A). A π - π stacking is formed between F2135.47 and F3096.52 in the aromatic cluster F2135.47/F3096.52/F3086.51 in $\beta 3\text{AR}$ (Figure 3B).

Discussion

The aromatic cluster F[Y]5.47/F[Y]6.52/F[Y]6.51 of GPCR is proposed to play a role in receptor activation^[20]. F5.47 was found to serve as an aromatic lock for the proposed active conformation of the W6.48 “toggle switch” in seven-transmembrane receptor activation^[30]. F6.52 was reported to be highly correlated with W6.48 and may mediate the TM6 Pro-kink^[19]. In $\beta 3\text{AR}$, the conserved aromatic cluster is F213^{5.47}/F309^{6.52}/F308^{6.51}, and we studied the cluster’s function on a cellular and structural level.

For GPCR assays, the most common cells used for generating cell lines are CHO-K1 and HEK-293 cells. With the aim to obtain a sensitive luminescent signal for our luciferase reporter gene system, three cell lines were chosen and compared: CHO-K1, HEK-293 and the in-house cell line CHO-mock. The results indicated that HEK-293 was the most suitable cell line for the transient expression of $\beta 3\text{AR}$ (Figure S3A and S3B). Hence, HEK-293 cells were transfected with wild-type or mutated $\beta 3\text{AR}$ in our study.

To explore the influence of transfection efficiency on the luciferase signal, different ratios of pCDNA3.1-h $\beta 3\text{AR}$ plasmid to pCDNA3.1 plasmid were designed to mimic the different protein expression levels of $\beta 3\text{AR}$. The results demonstrated that the expression level of $\beta 3\text{AR}$ did not greatly influence the luciferase signal in our experimental system (Table 1). In fact, this result was confirmed in our later Western bolt analysis results. For example, on residue F309^{6.52}, Ala substitution reduced the expression of the receptor in transfected HEK-293 cells by approximately 25% and Leu substitution increased the expression approximately 25% compared to the wild-type receptor. In both cases, the constitutive signalling was strongly impaired (Table 2, Figures 1D and 1E).

From the results of the mutation of F308^{6.51} and F309^{6.52}, and the double mutants, we discovered that both residues are essential for the constitutive activation of $\beta 3\text{AR}$. Moreover, we showed that F309^{6.52} was not only essential for the constitutive signalling but also very important for the agonist-induced signalling. However, the results for F308^{6.51} demonstrated that this residue might not be critical for **BRL**-induced signalling efficacy, as both the potency and maximal efficacy were unaltered by Ala substitution (Table 2, Figures 1A). However, F308^{6.51} could be involved in the activation process to some extent.

Homology and molecular docking were carried out to further explore the role of the aromatic cluster on structural level. The predicted binding mode demonstrated that both F308^{6.51} and F309^{6.52} were located in the binding pocket and that they had hydrophobic interactions with **BRL** (Figure 3A), which are consistent with our results and the newly reported observations^[36]. However, through the homology and molecular docking studies, we could not conclude that F309^{6.52} played a more important role than F308^{6.51}, which did not correspond exactly to our experimental results. One possible reason for these results is that during the docking process, the conformation of $\beta 3\text{AR}$ was completely rigid; however, it has been reported that the conserved residue W305^{6.48} (W6.48) changes position during the receptor activation, which might drive the adjacent residues, F308^{6.51} and F309^{6.52} (Figure 3B), to undergo conformational changes^[19]. Thus, rigid protein docking might not be accurate enough to predict the interaction mechanisms between ligands and GPCRs. Interestingly, as observed in Figure 3B, F213^{5.47} could form a π - π stacking with F309^{6.52}. This interaction might have an essential role for both F213^{5.47} and F309^{6.52} in the signal transduction of $\beta 3\text{AR}$.

As the aromatic cluster is highly conserved in GPCR, we carried out a statistic analysis throughout the class A GPCR. We surveyed all the members in the subfamily with the criterion of F[Y]5.47/F[Y]6.52/F[Y]6.51, as phenylalanine and tyrosine are highly homologous. The search results (Table S1) showed that the aromatic cluster resided in 18.5% of GPCR

Class A members, and to be more precise, 72.1% of the amine subfamily, 75.7% of the rhodopsin subfamily, 91.3% of the gonadotropin-releasing hormone subfamily, and 18.4% of the peptide subfamily (the alignment and classification were taken from GPCRDB, <http://www.gpcr.org/>). Hence, the aromatic cluster is highly conserved in amine, rhodopsin, and gonadotropin-releasing hormone subfamilies and is important in a portion of the peptide subfamily members. In the class A GPCR, Phe or Tyr are conserved in the position of 5.47 (41.9%), 6.51 (75.6%), and 6.52 (75.1%, including β -ionone for rhodopsin).

In summary, we explored the importance of the aromatic residues near the binding pocket of β 3AR by applying cell biology technologies and the molecular docking method. We discovered that the aromatic properties of F308^{6.51} and F309^{6.52} were essential in maintaining the receptor in a partially activated state as shown by the constitutive activity. Moreover, both of the residues contributed to hydrophobic interactions with BRL in binding with β 3AR. In addition, the π - π stacking between F213^{5.47} and F309^{6.52} might explain their major roles in the signal transduction of β 3AR. From a survey of the class A GPCR, we found that the aromatic cluster F[Y]5.47/F[Y]6.52/F[Y]6.51 was highly conserved, therefore, the aromatic cluster might have a common mechanism in GPCR activation.

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Author contribution

Prof He-yao WANG and Prof Wei-liang ZHU designed and supervised the research and revised the manuscript; Hai-yan CAI performed the research, analysed data and wrote the manuscript; Prof Kai-xian CHEN helped with part of the research idea and helped revised the manuscript; Zhi-jian XU, Jie TANG, and Ying SUN helped with part of the research.

Supplementary information

Supplementary figures are available at website of Acta Pharmacologica Sinica on NPG.

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