Molecular determinants of the olfactory receptor Olfr544 activation by azelaic acid

Trung Thanh Thacha, Yu-Jung Honga, Sangho Leeb, Sung-Joon Leea, Sung-Lea, Sung-Joon Leea,∗

a Department of Biotechnology, BK21-PLUS, College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Republic of Korea
b Department of Biological Sciences, Sungkyunkwan University, Suwon 16419, Republic of Korea

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The mouse olfactory receptor Olfr544 is expressed in several non-olfactory tissues and has been suggested as a functional receptor regulating different signaling pathways. However, the molecular interaction between Olfr544 and its natural ligand, azelaic acid (AzA), remains poorly characterized, primarily due to difficulties in the heterologous expression of the receptor protein on the cell membrane and lack of entire protein structure. In this report, we describe the molecular determinants of Olfr544 activation by AzA. N-terminal lucy-flag-rho tag ensured the heterologous expression of Olfr544 on the Hana3A cell surface. Molecular modeling and docking combined with mutational analysis identified amino acid residues in the Olfr544 for the interaction with AzA. Our data demonstrated that the Y109 residue in transmembrane helix 3 forms a hydrogen bond with AzA, which is crucial for the receptor-ligand interaction and activation. Y109 is required for the Olfr544 activation by AzA which, in turn, stimulates the Olfr544-dependent CREB-PGC-1α signaling axis and is followed by the induction of mitochondrial biogenesis in Olfr544 wild-type transfected Hana3A cells, but not in mock or Y109A mutant transfected cells. Collectively, these data indicated that a hydrogen bond between Y109 residue and AzA is a major determinant of the Olfr544-AzA interaction and activation.

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1. Introduction

Olfactory receptors (ORs), the largest superfamily of G protein-coupled receptors (GPCRs), are primarily expressed in the olfactory epithelium that detects and discriminates a vast number of odorants [1,2]. Although this family was first identified in 1991 [3], the molecular basis of the interactions between receptor and odorant molecules remain poorly understood, primarily due to the lack of reliable structural information on ORs. ORs belong to the rhodopsin-like class A GPCRs and possess seven transmembrane (TM) helices [4,5]. In contrast to small GPCR families that show over 80% sequence identity among family members, ORs feature a high variability in sequence homology and therefore may adopt different conformations. Such diversity in the protein sequences of ORs is consistent with the fact that each OR is recognized and activated by diverse odor molecules [3,6]. Molecular modeling studies have shown that amino acid residues in TM3, TM5, TM6 and extracellular loop 2 (EL2) are commonly involved in odorant recognition by forming the OR ligand binding pocket [5–7].

ORS are primarily expressed on the olfactory epithelium but also ectopically in various non-olfactory tissues and with unknown functions [8]. Olfr544 is a medium-chain dicarboxylic acid receptor known to bind azelaic acid (AzA) and suberic acid, which are C9 and C8 dicarboxylic acids commonly observed in grains [9]. Olfr544 is ectopically co-expressed with olfactory marker proteins in either bladder interstitial cells or thyroid parafollicular cells, suggesting the presence of Olfr544-mediated downstream signaling in these tissues [8]; Olfr544 is also expressed in pancreatic α-cells of mouse islets, where its response to AzA leads to glucagon secretion [9]. Our primary data also showed that Olfr544 was prominently expressed in several non-olfactory tissues, such as adipose, liver, and intestine (data not shown). These data raised the possibility that Olfr544 could be a functional receptor in non-olfactory tissues. In the comparative modeling combined with mutational studies, Abaffy et al. have previously reported that Olfr544 interacts with AzA. In the study, hydrophobic residues, including I112, V113 (TM3), V202 and V206 (TM5), formed part of the ligand pocket and were involved in determining carbon length preference [6,10]. Likewise, using modeling and mutational analysis, Kawasaki et al. showed that the N-terminal hydrophobic core formed by helix 8 and TM1-2 of Olfr544 is crucial for the rapid response via Golf [11]. However, both models are ambiguous in quality due to a local disruption in
the TM4 or TM5 structure [10,11]. In addition, it remains unclear which residues are specifically involved in the interaction between Olfr544 and AzaA.

The interactions between ORs and selective ligands have been investigated with the combined approaches of molecular modeling and functional biological assays of the receptor [5,12]. For such studies, the heterologous expression of OR is a prerequisite to examine the role of odorants as ligands [12–15]. Importantly, Matsunami and colleagues generated a cell system, HEK293T-derived Hana3A cells, that stably expresses molecular chaperones for OR trafficking to the plasma membrane and Gαolf, an important signaling component of ORs. Thus, the Hana3A cell line provides a functional heterologous expression system for ORs and has been applied to various functional studies of OR, including de-orphanization [15]. In addition, N-terminal tags have shown to improve cell surface targeting of OR. For instance, N-terminal tags derived from either the first 20 amino acids of rhodopsin (rho tag) [16] or the 17 amino acid signal peptide from a single-spanning membrane protein (lucy tag) [14] enhance receptor folding and cell surface expression.

In the present study, we investigated the Olfr544-AzaA interaction with an improved in silico docking model of Olfr544-AzaA and an N-terminal lucy-flag-rho tagged Olfr544 heterologous expression system using Hana3A cells. Our results demonstrated that the Y109 residue in the TM3 of Olfr544 was required for AzaA-driven Olfr544 activation. Expression of lucy-flag-rho-Olfr544 stimulated CRE-luciferase activity in Hana3A cells, but the activity was not observed in cells transfected with Olfr544 mutants (Y109A or Y109F).

2. Materials and methods

2.1. Cell culture and transient transfection

Hana3A cells were obtained as a kind gift from Dr. Hiroaki Matsunami, Duke University. Cells were maintained and grown under standard conditions in Eagles minimal essential medium/Earle’s balanced salt solution (MEM/EBSS, HyClone, USA) supplemented with 10% fetal bovine serum (FBS, HyClone) and 100 units/mL of penicillin together with 100 mg/ml streptomycin (PEST, Welgene Inc., Korea). When the cells reached approximately 80% confluence in 6-well plates, they were transiently transfected with 1 μg of each plasmid for 12 h using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Subsequently, the transfected cells were treated with AzaA (Sigma, USA) for 1 h.

2.2. Total RNA extraction, reverse transcription and quantitative real-time PCR

The total RNA was isolated from cells using the RNAiso Plus reagent (Takara Bio Inc., Japan). Reverse transcription to synthesize cDNA was performed from 1 μg total RNA template using the ReverTra Ace™ RT Master Mix kit (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. Real-time PCR was performed with the Thunderbird™ SYBR® qPCR Mix reagent (Takara Bio Inc., Japan) and the iQ5 Cycler System (Bio-Rad, USA). The expression levels were normalized to the level of the housekeeping gene ribosomal protein L32, as described elsewhere [17].

2.3. Immunoblotting analysis

Cell lysates were prepared using RIPA buffer (10 mM Tris–HCl pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 1 mM EDTA) supplemented with 1× Halt™ protease and phosphatase inhibitor reagent (Thermo, USA) at 4 °C. Soluble proteins were collected from the supernatant fraction after centrifugation at 13,000 rpm for 15 min. Equal protein concentrations, determined by bicinchoninic acid protein assay kit (Pierce Biotechnology, USA), were prepared in sample buffer (5% β-mercaptoethanol) for 5 min, separated by SDS-PAGE, and subsequently transferred onto a nitrocellulose membrane (Daeilab, Korea). The membrane was blocked in PBS containing 5% (w/v) non-fat dried milk and 0.1% Tween 20 (v/v); successively, the membrane was incubated with specific primary antibodies, as previously described [17]. Anti-flag antibody (1:500) was purchased from Sigma; anti-CREB (1:250), anti-p-CREB (Ser133; 1:500), anti-β-actin (1:1000), and anti-PI3K-1α (1:500) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Immunoblotting image was obtained using the ChemiDoc™ touch imaging system and the Image Lab 5.2 software (Bio-Rad, USA).

2.4. Fluorescence immunostaining

Hana3A cells were first seeded on a glass poly-o-lysine-coated coverslips up to a confluency of 70%–80% and transiently transfected with Olfr544 expression vectors for 12 h. The transfected cells were washed with PBS (pH 7.4) and fixed for 10 min using PBS supplemented with 4% paraformaldehyde. Fixed cells were then blocked for 45 min in PBS containing 5% (w/v) bovine serum albumin (BSA), incubated with the anti-flag antibody for 4 h at 4 °C, followed by 1 h incubation with the Alexa Fluor 488-conjugated secondary antibody (dilution 1:1000; Invitrogen, USA). Subsequently, cellular nuclei were stained with DAPI (Invitrogen, USA) for 3 min. The coverslip was subsequently embedded in anti-fade mounting solution (Thermo Fisher Scientific, USA). Image analysis was performed using a Zeiss LSM510 META confocal microscope and the Zeiss LSM700 version 3.2 software (Carl Zeiss, Germany).

2.5. cAMP response element (CRE)-luciferase reporter assay

Hana3A cells were seeded overnight in 24-well plates. Olfr544, CRE-luciferase reporter, and Renilla expression vectors were transiently co-transfected into the cells using Lipofectamine 2000 (Invitrogen, CA, USA) following the manufacturer’s instructions. Eighteen hours after transfection, the cells were stimulated with AzaA for 1 h. Luciferase activity was assessed using a Dual-Glo luciferase assay kit (Promega, WI, USA). The luminescence of the samples was quantified using a Victor X2 multilabel place (PerkinElmer, USA). The firefly luminescence signal was normalized to that of Renilla luciferase. EC50 was determined using GraphPad Prism with steady-state analysis.

2.6. Comparative modeling of Olfr544 and Olfr544-AzaA docking

We derived a structural model of Olfr544 (residues 19–313) using three crystal structures of GPCR-agonist complexes as templates: κ-opioid receptor-JDTic (PDB ID, 4DJH), A2A adenosine receptor-ZM241385 (PDB ID, 3EML) and M2 muscarinic acetylcholine receptor-an antagonist (PDB ID, 3UON). Initially, 5 apo models of Olfr43 were derived based on sequence alignments obtained using the Modeller [18] with the best discrete optimized protein energy (DOPE) score of ~37430, compared to the ~34460 DOPE score of an average template. These models were then refined and submitted to energy minimization using the Galaxy [19] and Molprobity [20], respectively. Olfr544-AzaA complexes were generated using SwissDock [21]. Three-dimensional graphics were prepared using PyMol [22]. Interactions between Olfr544 and AzaA were analyzed using LigPlot [23].
2.7. Cloning and single amino acid substitution of Olfr544

DNA encoding the full-length Olfr544 sequence was amplified from an Olfr544 template (MG215211, Origen, USA). The PCR product was purified and sub-cloned into a pMV18s plasmid (courtesy of Dr. Jeniffer Pluznick, Johns Hopkins University) between EcoRI and XhoI restriction enzyme sites. The final construct contained the N-terminal lucy-flag-rho tag followed by Olfr544. The plasmid was amplified by transforming Escherichia coli DH5α cells. For site-directed mutagenesis to substitute a single amino acid, the Olfr544 construct was mutated by PCR with sets of targeted mutation primers (Table S1) according to the Quick-change kit protocol supplied (Aligent technologies, USA). The identities of all constructs were confirmed by DNA sequencing.

2.8. Mitochondrial DNA content and density determination

Relative human mtDNA was assayed through real-time qPCR from total DNA as described elsewhere [24,25]. Specific sets of primers were used to amplify a 218 bp amplicon on the mitochondrial NADH dehydrogenase subunit 1 (ND1) spanning from 14620 to 14838 in the human mitochondrial genome (Table S1). Mitochondrial density was determined using the Mitotracker Green probe (Molecular Probes) following the manufacturer’s instructions and our previously described protocol[26]. Briefly, after transient transfection with the appropriate expression vectors for 12 h, the cells were stimulated with 50 μM of AzA for 10 h. The cells were then incubated with Green probes (400 nM) for 30 min at 37 °C, followed by washing with PBS. Green fluorescence intensity was measured at excitation and emission wavelength of 490 and 516 nm, respectively, using a SpectraMax microplate reader (Molecular Devices Cor., USA). Images were obtained using a confocal microscope and the Zeiss LSM700 version 3.2 software (Carl Zeiss, Germany). Figures show representative results obtained from at least triplicate experiments.

2.9. Statistical analysis

All data are presented as the mean ± SEM. Student’s t-test or one-way ANOVA followed by Tukey’s HSD test were used for two or multiple group comparisons, respectively, and performed using the GraphPad Prism. A value of \( P < 0.05 \), denoted by *, was considered significant. Values that are denoted by different letters are significantly different (\( P \geq 0.05 \)).

3. Results and discussion

3.1. Functional expression of Olfr544 in Hana3A cells

Heterologous expression of ORs on the cell surface is a prerequisite for the physiological analysis of these receptors. Cleavable lucy tag has been shown to promote OR protein trafficking or endoplasmic reticulum exit that stabilizes the receptor and prevents misfolding [14]. In addition, rho tag enhances OR protein folding and surface expression [16]. Therefore, we constructed an N-terminal lucy-flag-rho-fused Olfr544 sequence cloned in a pMV18s plasmid. As mentioned earlier, the Hana3A cell line stably expresses RTP1L, RTP2, REEP1 and Gaolf, which assist trafficking the OR proteins to the plasma membrane [27]. Hence, N-terminal tagged Olfr544 was transfected into Hana3A cells. Immunoblotting analysis showed that Olfr544 proteins are expressed on the plasma membrane (Fig. 1A) and fluorescence immunostaining with an anti-flag antibody

![Fig. 1. Functional expression of Olfr544 in the Hana3A cell system.](image)
confirmed the expression of Olfr544 on the cell surface (Fig. 1B).

Next, we performed a functional assay for Olfr544 in Hana3A cells. Odorant ligand binding to OR changes the receptor conformation, which activates $G_{olf}$ [28] and increases the intracellular cAMP concentration [29,30], subsequently activates the PKA-CREB signaling axis. For this reason, we performed a CRE-luciferase assay to determine the functional relevance of Olfr544 expressed in Hana3A cells (Fig. 1C). Forskolin, an adenylyl cyclase activator, was used as a positive control. We showed that with the transfection of lucy-flag-rho-Olfr544 into Hana3A cells, AzA trans-activated CREB resulting in an increased CRE-luciferase activity. The EC$_{50}$ value of AzA was 16.8 ± 1.4 μM in Hana3A cells transfected with N-terminal tagged Olfr544; at the same time, AzA did not increase the CRE-luciferase activity in cells that expressed the lucy-flag-rho tag only (mock). The EC$_{50}$ value of forskolin was 88 ± 14 nM (Fig. 1D). These data suggested that the activation of Olfr544 by AzA stimulated the PKA-CREB signaling pathway in Hana3A cells.

3.2. Structural modeling of the Olfr544-AzA complex

To characterize the Olfr544-AzA molecular interaction, we derived a structural model of the Olfr544-AzA complex using the Modeller [18]. The most crucial step in comparative modeling is to find the optimal sequence alignment between a target protein and the template structure. ORs share not more than 25% sequence identity with rhodopsin-like GPCR, thereby the generated models may be suboptimal in quality [31]. To overcome this obstacle, we truncated flexible regions in the N- and C-termini of Olfr544 and used multiple template structures in order to reduce such ambiguity. Overall, the structure of Olfr544 resembles that of a rhodopsin-like GPCR family member and consists of seven transmembrane helices named TM1-7, which are followed by an intracellular C-terminal helix 8 (residues 301–312) that is highly conserved among GPCR family members. There is no local disruption in all seven TM sequences (Fig. 5A). An important feature of our model is a disulfide bond formed between the C101 residue in TM3 and the C183 residue in the extracellular loop 2 (EL2) that is highly conserved among rhodopsin-like receptors [32]. Ramachandran plot showed 290 allowed residues among a total of 293 in the structure (99%), indicating that our model is reliable (Fig. S1B) [33]. The superposition between our Olfr544 model and two other models previously reported by Bavan et al. and Kawasaki et al., showed differences in the mobile EL2 and intracellular loop 3 (IL3) with root mean square distances (RMSD) between C$_{a}$ atoms of 3.6 and 6.3 Å, respectively (Fig. S1C) [11,34]. By contrast, superposition of the 7 TMs showed a high level of conservation with an RMSD value ranging between 1.0 and 1.8 Å (Fig. S1C), demonstrating that our models qualify as a reliable OR structure for further analysis.

To characterize the interaction between Olfr544 and AzA, we docked AzA into the structural model of Olfr544 using the SwissDock [21]. The final model of the Olfr544-AzA complex revealed that AzA sits nicely in the ligand binding pocket of Olfr544 (Fig. 2A).

**Fig. 2.** Olfr544-AzA complex model. (A) Olfr544 is represented by a ribbon diagram. N- and C-termini are labeled. The AzA ligand is depicted as a ball-and-stick model in green. (Insert) The ligand binding pocket is enlarged showing the residues that interact with AzA as stick models. Two hydrogen bonds formed by the S263 and Y109 residues in the Olfr544 and AzA complex are indicated as dotted lines with the relative distances labeled. (B) LigPlot analysis showing the interactions between the Olfr544 residues and AzA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
positioned on TM3, interact with AzA via hydrophobic and hydrogen bonds, respectively (Fig. 2B). A conserved H185 residue in EL2 forms a hydrophobic interaction with AzA. T259 and S263 residues on TM6 bind to AzA via hydrophobic and hydrogen bonds, respectively (Fig. 2A & B). These inter-molecular interactions between residues in Olfr544 and AzA are characteristic of the OR-ligand interaction in which TM3, TM6 and EL2 constitute the odorant-binding sites\[^5,35\].

### 3.3. Mutational analysis of Olfr544-AzA interaction and activation

Using our Olfr544-AzA complex model, we selected 5 residues (M105, Y109, H185, T259, S263) and examined their requirements or AzA binding by mutation analysis. After cloning 5 mutated forms of Olfr544, the relative protein expressions were determined by immunoblotting with an anti-flag antibody to ensure similar expression levels among the mutant proteins and the wild-type form (Fig. 3A). Quantitative analysis of luciferase activity showed that all mutants showed a reduced activation of Olfr544 by AzA (Table 1 & Fig. 3B&C). Among them, H185A and T259A did not affect EC\(_{50}\) values and M105A slightly reduced EC\(_{50}\). However, S263A reduced the Olfr544 activation by 2.5-fold. Importantly, the Y109A mutation showed virtually no activation. These results were consistent with our rigid body complex model indicating that Y109 and S263 residues interact with AzA. Furthermore, to validate whether Y109 binds AzA via a hydrogen bond, we mutated this residue (Y109F mutant) and repeated the luciferase assay. Phenylalanine (F) is identical to tyrosine (Y), except for missing a –OH group for hydrogen-bond formation. Similar to the Y109A mutation, the Y109F variant exhibited a very weak luciferase activity following the Olfr544 activation by AzA (Fig. 3B&C). Taken together, these data demonstrated that the interaction via hydrogen bond between Y109 and AzA is crucial for the Olfr544 activation.

### 3.4. Tyr-109 residue is required for the Olfr544 activation by AzA

Since the Y109 residue in the Olfr544 sequence is critical for the interaction and activation by AzA (Fig. 3), we examined whether the same residue is required for the AzA-activation of Olfr544 in the PKA-CREB cellular signaling axis by introducing an Y109A mutant receptor in the Hana3A cell system. Since the EC\(_{50}\) value of Olfr544 bound to AzA is 16.8 ± 5.1 \(\mu\)M, we stimulated the cells with AzA at a 50 \(\mu\)M concentration. Not surprisingly, we observed that pCREB (Ser133) was significantly induced in Olfr544 (WT)-transfected cells, but its expression was not affected in mock and AzA-stimulated cells transfected with the Olfr544 (Y109A) mutant (Fig. 4A). In accordance with the fact that CREB activity is increased by phosphorylation, we examined whether the peroxisome proliferator-activated receptor \(\gamma\) coactivator-1\(\alpha\) (PGC-1\(\alpha\)) was induced. Since the PGC-1\(\alpha\) promoter possesses a conserved binding site for CREB spanning from \(\text{5'-C0}133\) to \(\text{5'-C0}116\) (human) or \(\text{5'-C0}146\) to \(\text{5'-C0}129\) (mice), CREB activation highly up-regulates PGC-1\(\alpha\) transcription \[^{36,37}\]. Quantitative real-time PCR results showed that the expression of PGC-1\(\alpha\) mRNA level was significantly increased by

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Table 1

<table>
<thead>
<tr>
<th>Olfr544</th>
<th>EC(_{50}) ((\mu)M)</th>
<th>(R^2)</th>
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<tbody>
<tr>
<td>WT</td>
<td>16.8 ± 5.1</td>
<td>0.98</td>
</tr>
<tr>
<td>mock</td>
<td>n.d.(^a)</td>
<td>–</td>
</tr>
<tr>
<td>M105A</td>
<td>34.1 ± 25.1</td>
<td>0.95</td>
</tr>
<tr>
<td>Y109A</td>
<td>v.w.(^b)</td>
<td>–</td>
</tr>
<tr>
<td>Y109F</td>
<td>v.w.(^b)</td>
<td>–</td>
</tr>
<tr>
<td>H185A</td>
<td>20.2 ± 3.4</td>
<td>0.99</td>
</tr>
<tr>
<td>T259A</td>
<td>22.2 ± 7.8</td>
<td>0.93</td>
</tr>
<tr>
<td>S263A</td>
<td>46.1 ± 13.2</td>
<td>0.98</td>
</tr>
</tbody>
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\(^{a}\) n.d., not determined. \(^{b}\) v.w., very weak. \(^{c}\) Goodness of fit \((R^2 \geq 0.90)\), defined as how well a curve fits a set of observations.
2.5-fold in Olfr544 (WT) but not in the Y109A mutant (Fig. 4B). Importantly, immunoblot results confirmed that the PGC-1α protein was induced by 1.9-fold in Olfr544 (WT) compared with mock, whereas the expression of PGC-1α did not change in AzA-stimulated cells transfected with the Y109A mutant (Fig. 4A).

As a major transcriptional regulator of cellular energy mechanisms, PGC-1α directly affects mitochondrial biogenesis and respiratory function [38,39]. Hana3A cells were generated from HEK293T cells, a human embryonic kidney cell line. PGC-1α is highly expressed in kidneys, which contain a high number of cellular mitochondria and levels of oxygen consumption similar to that of the heart [40,41]. Mitochondrial biogenesis has been shown to be a crucial process in renal recovery from injury due to aging, tissue hypoxia, and other acute or chronic kidney diseases [40,42]. Olfr544 activation by AzA induces PGC-1α expression both at the mRNA and protein level, suggesting that it may also induce mitochondrial biogenesis in Hana3A cells. Mitochondrial transcription factor A gene (tfam), a key activator of mitochondrial transcription and a transcriptional target of PGC-1α [43], was increased by 2.3-fold in Olfr544 expressing cells (WT) compared to mock, while there was no virtual difference between the Y109A mutant and the mock group (Fig. 4A). Further quantitative real-time PCR results showed that mitochondrial abundance, assessed as mtDNA content, was induced, approximately by 2.5-fold, in Olfr544 (WT).
compared to mock and the Y109A mutant (Fig. 4C). This result suggested that Olfr544 activation induces mitochondrial DNA synthesis and transcription as well as biogenesis in Hana3A cells. Consistently, our mitotarker-probed mitochondria images demonstrated that Olfr544 (WT) overexpression substantially enhanced mitochondrial density in Hana3A cells, but not in the Y109A mutant (Fig. 4D). Together, these data indicated that the Y109 residue is required for Olfr544 activation by AzaA, which result in the stimulation of the CREB-PGC-1α signaling axis in Hana3A cells.

In summary, using molecular modeling with multiple templates for Olfr544, we proposed an Olfr544 model that was improved compared with those previously reported. Based on Olfr544-AzaA structural information, together with CRE-luciferase reporter assays in Hana3A cells, we demonstrated that the Y109 residue in the Olfr544 amino acid sequence is a key element required for the interaction with AzaA via hydrogen bond interaction. Furthermore, we demonstrated that AzaA-driven Olfr544 activation plays a substantial role in the induction of mitochondrial biogenesis through stimulation of the CREB-PGC-1α signaling axis. These results suggested that the ectopic expression of Olfr544 in metabolic tissues may regulate cellular energy metabolism. Further in vivo studies should be performed to investigate the effect of AzaA-driven Olfr544 activation.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.02.104.

References

