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Overexpression of Orphan Receptor GPR61 Increases cAMP Levels upon Forskolin Stimulation in HEK293 Cells: *in vitro* and *in silico* Validation of 5-(Nonyloxy) Tryptamine as a Low-Affinity Inverse Agonist

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Keywords

GPR61 · Constitutive activity · Inverse agonist · Receptor · Molecular modeling · Proteins

Abstract

GPR61 is an orphan receptor that belongs to Class A of G-protein-coupled receptors. It has been reported that GPR61 has a constitutive activity and couples to $G\alpha_s$. In the present study, we characterized GPR61 function and ligand binding by experimental and molecular docking studies. We demonstrated that heterologous expression of GPR61 in HEK293 cells enhanced the cAMP synthesis response to forskolin, whereas the basal cAMP synthesis was unaffected. 5-(Nonyloxy)tryptamine inhibited forskolin-stimulated cAMP production in GPR61-expressing HEK293 cells. These studies highlight that the intrinsic activity of this receptor is only measurable following its synergy with $G\alpha_s$. © 2019 S. Karger AG, Basel

Introduction

G-protein-coupled receptors (GPCRs) are the largest family of cell surface receptors. They are also the most common targets (ca. 34%) for FDA-approved drugs [1]. However, despite the arising interest, >150 GPCRs remain “orphan”; that is with no endogenous ligand discovered to date, and 81 of these orphan receptors belong to the Class A GPCRs [2]. GPR61 (other names: GPCR3, Biogenic amine receptor-like G-protein-coupled receptor, BALGR) is an orphan receptor, which sequence was first identified in 2001 [3]. GPR61 is expressed in various tissues including adrenal gland, hippocampus, and leukocytes including proinflammatory Th17 cells [4–6]. Pharmacological targeting of GPR61 should be explored in greater detail since, for example, this receptor has been reported to be relevant in metabolism as GPR61-deficient mice developed obesity [7].

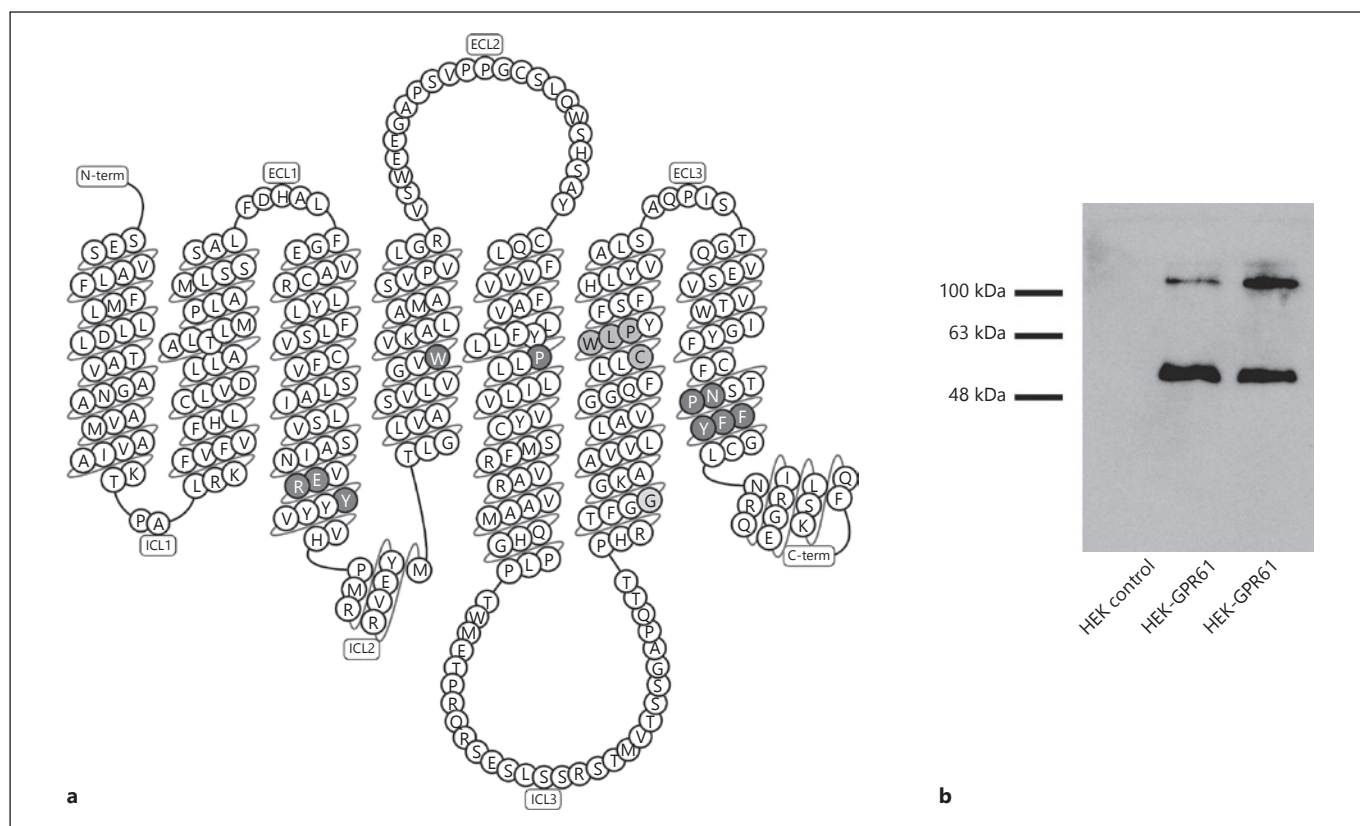


Fig. 1. a GPR61 structure with conserved and important residues/motifs shown highlighted: D(E)RY in TM3; W^{3.50}, P^{4.50}, CWxP in TM6; and NPxxY in TM7. GPR61 has G282^{6.30} in place of D^{6.30}. **b** Myc-immunoreactivity in HEK293 cells stably expressing His-

myc-tagged GPR61. GPR61 is expressed both as a monomer and a dimer, Lanes: 1 = HEK293 control cells, 2 and 3 = HEK293 stably overexpressing His-myc-tagged GPR61.

At a structural level, GPR61 has the conserved tyrosine toggle switch NPxxY and CWxP motifs, which are involved in the receptor activation in the Class A GPCRs [8]. However, the receptor lacks the conserved aspartic acid in the transmembrane domain 6 (D^{6.30} in Ballesteros-Weinstein nomenclature system [9]) that forms a classical “ionic lock” with R^{3.50} keeping GPCRs in an inactive state (Fig. 1a) [10]. The lack of this motif suggests a potential constitutive activity of the receptor and in support of this basal activity of a GPR61-Gα_s construct was increased in comparison to other receptors [11]. Furthermore, 5-(non-yloxy)tryptamine (5-[nonyloxy]-1H-indole-3-ethanamine, 5-NOT) has been described as a low-affinity inverse agonist of the GPR61 receptor [10]. A subsequent paper from the same group demonstrated that a minor truncation in the N-terminus of GPR61 and a V19A mutation reduced the constitutive activity in the same GPR61-Gα_s (³⁵S)GTPφS functional model [12]. It was concluded that the N-terminal region of the receptor might auto-activate

it. The latest study partly supports this finding [13], yet a different one demonstrates that GPR61 is not a constitutively active receptor [14]. Another study has been conducted that described that some brain-expressed GPCRs, including GPR61, may be targets for plasmalogens [15].

Since the intrinsic activity of GPR61 is debatable and no further proofs have been shown that would confirm that 5-NOT is indeed an inverse agonist, in our article, we aimed to investigate 5-NOT-dependent and potential intrinsic activity of GPR61 in cAMP accumulation assay. This study was accompanied by in silico study on a GPR61-binding site.

Experimental

Cell Culture and Cloning

HEK293 stably expressing His-myc-GPR61 and control cells have been generated and characterized previously [6]. The cells were maintained in complete Dulbecco’s Modified Eagle’s Medi-

um (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich) and 1% (v/v) penicillin/streptomycin (Sigma Aldrich, 100 units/mL penicillin and 0.1 mg/mL streptomycin).

SDS/PAGE and Western Blotting

Cells were lysed in 25 mmol/L Tris (pH = 7.4) and resuspended in an equal volume of 2× radioimmunoprecipitation assay buffer and appropriate volume of 2× urea sample buffer. No >30 µg of protein was loaded and run on 4–20% Tris/glycine gels. Proteins were transferred onto PVDF membranes, which were then blocked in 10% nonfat milk for 2 h at room temperature. Membranes were incubated in 1:3,000 anti-myc (Cell Signaling Technology) or 1:3,000 anti-GAPDH (Abcam, Cambridge, UK) in 5% nonfat milk overnight at 4°C. Membranes were incubated subsequently in anti-mouse or anti-rabbit HRP-linked secondary antibody (Cell Signaling Technology) in 5% nonfat milk in wash buffer at room temperature for 2 h. The membrane was subsequently exposed to film and developed. Alternatively, the membrane was developed using ChemiDoc MP system imager (Bio-Rad).

cAMP Accumulation Assay

cAMP accumulation in the cells was measured using AlphaScreen kit (Perkin Elmer) according to the manufacturer's instructions. Briefly, HEK293 cells stably overexpressing His-*myc*-tagged GPR61 were seeded at 10⁵ cells/well in a flat-bottom 96-well plate and incubated overnight at 37°C. On the next day, the medium was removed, and the cells were carefully washed twice in 200 µL of stimulation buffer. Cells were incubated in the stimulation buffer for 20 min at 37°C. In the next step, drugs were added, and the cells were incubated for 20 min at 37°C. Adenylyl cyclase activator forskolin (1.0 µmol/L, Sigma-Aldrich) was then added, and the cells were stimulated for 10 min at 37°C. Subsequently, the supernatants were removed, and the cells were lysed for 20 min in 1× immunassay buffer. Subsequently, 5.0 µL of cell lysate was transferred to appropriate wells of OptiPlate 384-well plate (PerkinElmer). Acceptor beads were prepared at 1:25 dilution in the stimulation buffer and 5.0 µL of this beads suspension was added to lysate and left to incubate for 30 min in the dark at room temperature. In a separate tube mix of biotin-cAMP 1:240 and streptavidin-donor beads 1:150 was prepared and left to incubate for 30 min in the dark. After 30 min, 15 µL of biotin-cAMP and streptavidin-donor beads suspension was added. The plate was left for 4 h in the dark at room temperature before being read on PHERAstar FS HTS microplate reader (BMG LABTECH) using AlphaScreen module.

Homology Modeling

The homology model of inactive GPR61 was generated using the high-resolution structure of dopamine D4 receptor as a template (PDB ID: 5WIU, structural identity ~28%) [16]. The sequence of GPR61 (UniProt ID: Q9BZJ8) was aligned with that of D4R (UniProt ID: P21917) with ClustalX2 [17]. The N- and C-termini were excluded, and the alignment was manually edited to ensure the proper alignment of transmembrane domains. One hundred homology models of the GPR61 receptor were generated with MODELLER 9.19 [18], and the representative one was selected based on DOPE score.

Molecular Docking

Docking of 5-NOT was performed in AutoDock Vina [19] using default settings except for num_modes (20) and exhaustive-

ness (100). Protein and ligands protonation states were set for pH 7.4 with PROPKA [20] and Avogadro [21], respectively. The size of the binding box was set to 30 × 30 × 30 Å³. The docking results were analyzed using LigPlot + [22] and PyMOL (The PyMOL Molecular Graphics System, version 2.0 Schrödinger, LLC).

Statistics and Data Analysis

The statistical analysis and curve fitting were done using Prism 6 (GraphPad). Throughout the manuscript, data are expressed as a mean ± SEM of *n* independent experiments (biological replicates). The Mann-Whitney test was used to test for significant differences between 2 groups, and Kruskal-Wallis test with Dunn's post hoc analysis was used to test for significant differences between >2 groups when *n* ≥ 3, **,### *p* < 0.01, *,# *p* < 0.05.

Results and Discussion

In the present study, we use the HEK293 cells stably expressing His-*myc*-GPR61 (Fig. 1b) that have been previously characterized by us [6]. The data presented in Figure 2a reveal that the control HEK293 cells and HEK293 cells stably expressing the GPR61 receptor (HEK-GPR61) display similar basal cAMP levels. Differences in cAMP levels between the 2 cell lines, however, were only evident following stimulation with forskolin (Fig. 2a) arguing for receptor's synergistic action with gas subunit.

The potential of 5-NOT (Fig. 2b) to act as a low-affinity GPR61 ligand was confirmed. Thus, 5-NOT significantly inhibited the forskolin-stimulated cAMP synthesis within HEK-GPR61 cells (Fig. 2c), consistent with previous pharmacological studies and predictions where 5-NOT acts as an inverse agonist of GPR61 and that GPR61 couples to Gα_s as a cognate G protein. What is more, 5-NOT did not reduce forskolin-induced cAMP levels in the control cells, arguing for the specificity of this compound as a GPR61 ligand (Fig. 2d). Forskolin used at a lower concentration of 1 µmol/L did not elicit measurable differences in cAMP levels in control HEK cells, therefore, it was used at 10 µmol/L, concentration which is consistent with previously published reports on similar applications [23]. In our assay, 5-NOT did not significantly reduce forskolin-induced cAMP in the control cells. The application of forskolin at higher concentrations (e.g., 100 µmol/L) was not possible because of a high final DMSO concentration (1%).

In our model, as mentioned before, we have not been able to confirm the higher basal activity of the receptor, as opposed to the most recent study that has reported such a phenomenon [13]. However, our data, to some extent, support the results of the older paper [14]. The reasons for those differences are debatable, but in both cited

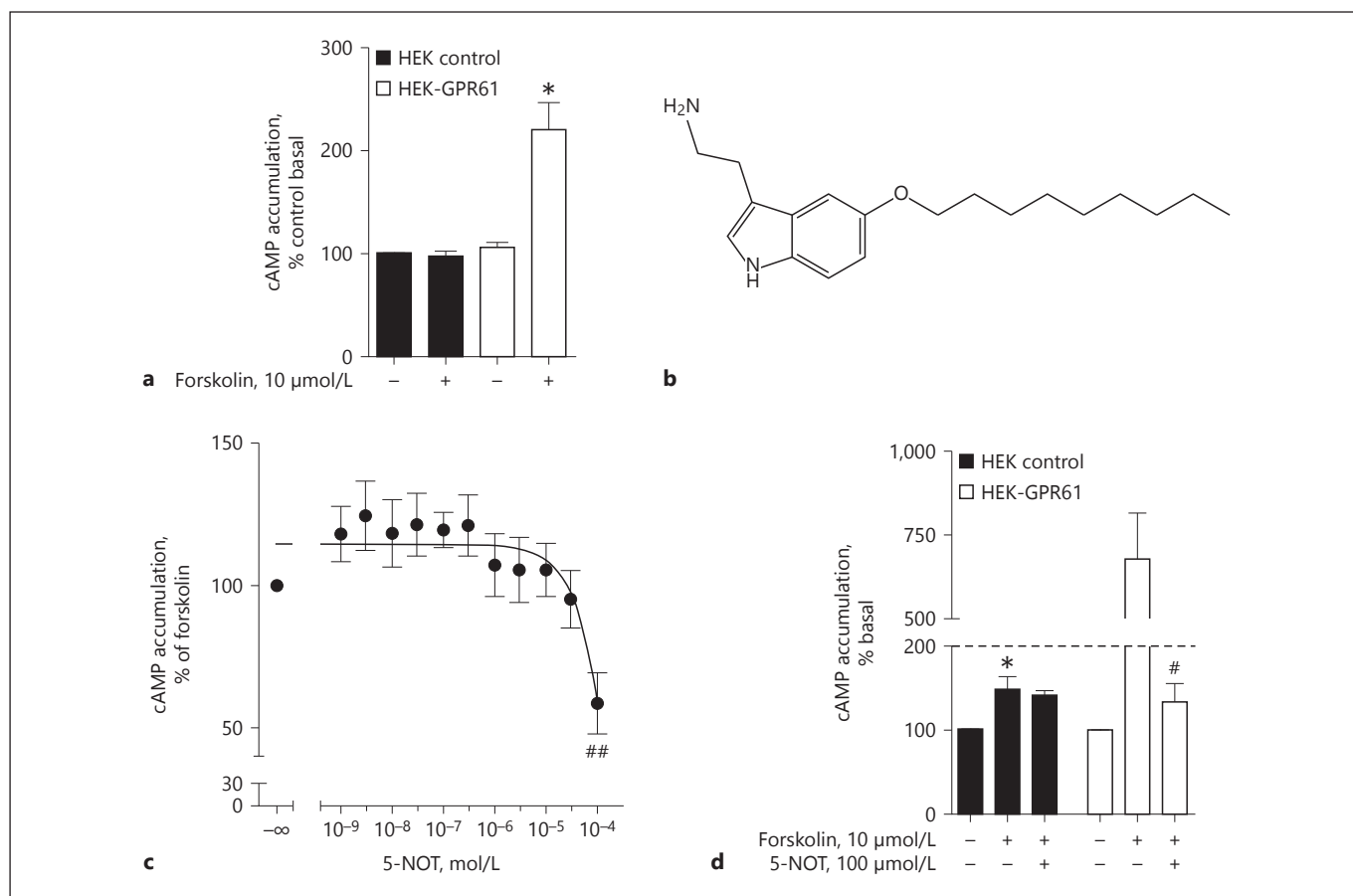


Fig. 2. a cAMP accumulation in HEK293 cells. Forskolin (1 $\mu\text{mol/L}$) induces a significant increase in cAMP production in the cells stably overexpressing GPR61, $n = 4$. **b** Structure of 5-NOT; a putative ligand of GPR61. **c** 5-NOT is a low-affinity inverse agonist of GPR61, $n = 6$. **d** Higher concentration of forskolin (10 $\mu\text{mol/L}$) is required to induce cAMP production in the control cells. 5-NOT

(100 $\mu\text{mol/L}$) does not have an impact on cAMP levels in these cells; HEK-GPR61 cells were used for comparison to demonstrate a visible difference in response to 10 $\mu\text{mol/L}$ forskolin, $n = 3$; * differences between forskolin- and vehicle-treated samples, # differences between forskolin only and forskolin + 5-NOT-treated samples.

papers, different assays were performed, and transiently transfected cells were used to measure the intrinsic activity of GPR61. It has to be noted that receptor levels have great impact on its basal activity and ligand-induced signaling. What is more, it is well-known that stably transfected cell system will behave differently to a transiently transfected one, especially in the signaling readouts. Lastly, we cannot exclude the GPR61 heterodimerization effect on the presented ligand-bound GPR61 mediated cellular responses [6].

We have also attempted to evaluate the binding of 5-NOT to GPR61 by molecular docking (GPR61 model was based on the dopamine receptor D4 structure, Fig. 3a). To analyze the docking results, we used the lowest-energy binding pose of 5-NOT in the predicted receptor binding

pocket (AutoDock Vina calculated energy: -8.0 kcal/mol, $K_d = 1.35$ $\mu\text{mol/L}$; Fig. 3b). The long nonpolar tail of 5-NOT can directly interact with side chains of amino acids of TM5 via hydrophobic interactions. Also, the pose of 5-NOT in the binding pocket supports hydrogen bond formation with neighboring L93^{2,57}, C195 in ECL2 and Y303^{6,51} (Fig. 3b).

Conclusion

In conclusion, the results presented here reveal that GPR61 does not have an increased basal activity, but its overexpression promotes a higher cellular response – cAMP accumulation – to forskolin. We also confirm that

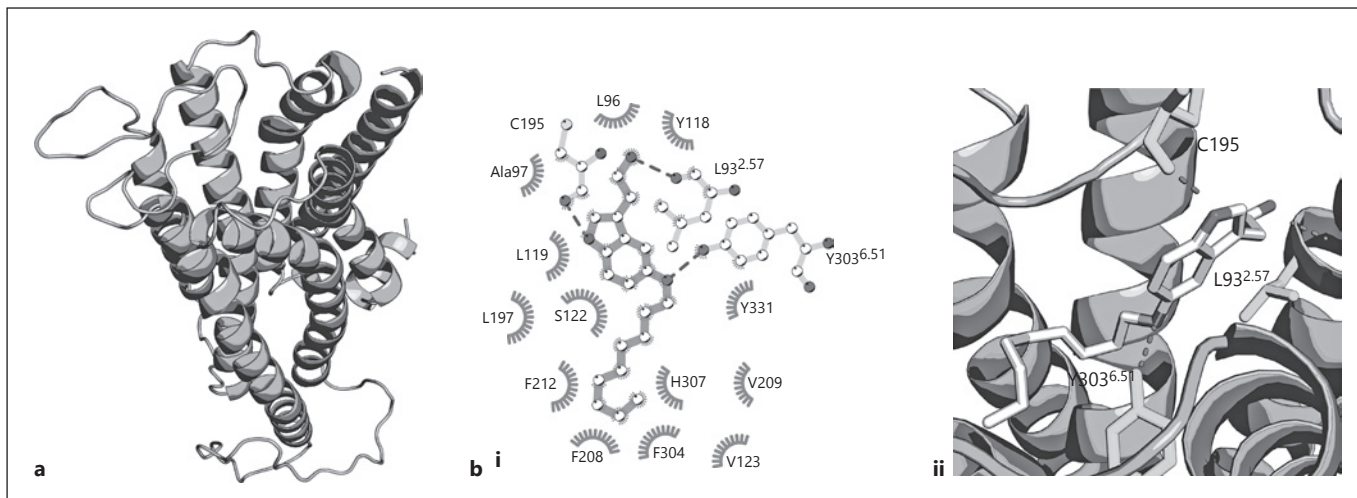


Fig. 3. a GPR61 receptor model. Binding poses of 5-NOT (b) with all the interacting neighboring residues (i) and hydrogen bond interacting residues (ii).

5-NOT is a low-affinity inverse agonist. Additionally, we demonstrate that 5-NOT could bind in the intra-TM orthosteric binding site of GPR61. We have previously shown that GPR61 is highly expressed in Th17 cells, and given that, under certain conditions, it can have a high intrinsic activity – the development of inverse agonist targeting this GPCR is desirable [6]. We consider that our findings may contribute to the process of GPR61 deorphanization.

Disclosure Statement

The authors declare that they have no conflict of interest.

Author Contributions

P.K., G.G., and N.M.B. conceived the project and designed the experiments. P.K. performed all the experiments. P.K. and J.J.S.-K. analyzed the data. P.K. and N.M.B. wrote the paper. N.M.B. supervised the study.

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