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Delineation of the functional properties and the mechanism of action of TMPPAA, an allosteric agonist and positive allosteric modulator of 5-HT$_3$ receptors

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ABSTRACT

We have previously identified a novel class of 5-hydroxytryptamine type 3 receptor (5-HT$_3$R) agonists sharing little structural similarity with orthosteric 5-HT$_3$R ligands (Jørgensen et al., 2011). In the present study we have elucidated the functional characteristics and the mechanism of action of one of these compounds, *trans*-3-(4-methoxyphenyl)-*N*-(pentan-3-yl)acrylamide (TMPPAA). In electrophysiological recordings TMPPAA was found to be a highly-efficacious partial agonist equipotent with 5-HT at the 5-HT$_3$A receptor (5-HT$_3$AR) expressed in COS-7 cells and somewhat less potent at the receptor expressed in *Xenopus* oocytes. The desensitization kinetics of TMPPAA-evoked currents were very different from those mediated by 5-HT. Moreover, repeated TMPPAA applications resulted in progressive current run-down and persistent non-responsiveness of the receptor to TMPPAA, but not to 5-HT. In addition to its direct activation, TMPPAA potentiated 5-HT-mediated 5-HT$_3$AR signalling, and the allosteric link between the two binding sites was corroborated by the analogous ability of 5-HT to potentiate TMPPAA-evoked responses. The agonism and potentiation exerted by TMPPAA at a chimeric α7-nACh/5-HT$_3$A receptor suggested that the ligand acts through the transmembrane domain of 5-HT$_3$AR, a notion further substantiated by its functional properties at chimeric and mutant human/murine 5-HT$_3$ARs. A residue in the transmembrane helix 4 of 5-HT3A was identified as an important molecular determinant for the different agonist potencies exhibited by TMPPAA at human and murine 5-HT$_3$ARs. In conclusion, TMPPAA is a novel allosteric agonist and positive allosteric modulator of 5-HT$_3$Rs, and its aberrant signalling characteristics compared to 5-HT at the 5-HT$_3$AR underline the potential in Cys-loop receptor modulation and activation through allosteric sites.

1. Introduction

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) mediates its physiological effects through a plethora of G-protein coupled receptors and a family of ligand-gated cation-selective ion channels, the 5-HT type 3 receptors (5-HT$_3$Rs) [1]. The 5-HT$_3$Rs belong to the Cys-loop receptor superfamily, which also comprises nicotinic acetylcholine receptors (nAChRs), γ-aminobutyric acid type A receptors (GABA$_A$Rs), glycine receptors (GlyRs) and a zinc-activated channel [2–7]. The receptors are membrane-bound homomeric or heteromeric complexes assembled from five subunits. In the case of human 5-HT$_3$Rs, five subunits (5-HT3A-E) assemble into homomeric 5-HT$_3$A and heteromeric 5-HT$_3$AB, 5-HT$_3$AC, 5-HT$_3$AD and 5-HT$_3$AE receptor complexes (5-HT$_3$A and 5-HT$_3$AB being the major physiological receptor subtypes), whereas other species only express 5-HT3A and 5-HT3B subunits [2,3,8,9]. The functions mediated by 5-HT$_3$Rs in serotonergic transmission and as heteroreceptors regulating the activity in other neurotransmitter systems make the receptors interesting therapeutic targets. Competitive 5-HT$_3$R antagonists are clinically administered drugs in the treatment of nausea and emesis arising after operations or from radiation and chemotherapy. Further,

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5-HT_3R ligands are investigated as putative therapeutics in gastrointestinal disorders, various psychiatric and cognitive disorders, and pain [8,10,11].

As other Cys-loop receptors, the pentameric 5-HT_3R complex consists of an extracellular domain (ECD) made up by the N-termini of the subunits, a transmembrane domain (TMD) consisting of the four transmembrane α-helices TM1-4 in the subunits, and an intracellular domain (ICD) composed by the intracellular loops in the subunits [3,4,12]. Signal transduction is initiated by agonist binding to the orthosteric sites located at subunit interfaces in the ECD of the receptor, which subsequently elicits the opening of the ion channel in the TMD hereby enabling the flux of ions through the channel [3,4,13,14]. Following this *activation*, the receptor will either return to its resting unbound/closed-channel conformation upon release of the agonist from its binding site (*deactivation*) or transition into an agonist-bound/closed-channel conformation (*desensitization*). The desensitized receptor can subsequently convert back to the active state or return to its resting state (*reactivation* and *recovery from desensitization*, respectively) [4,14–16].

The numerous allosteric transitions underlying Cys-loop receptor function means that the signalling events elicited by the orthosteric agonist can be modulated by concomitant binding of ligands to numerous allosteric sites topographically distinct from the orthosteric site [4,14,15]. Positive allosteric modulators (PAMs) enhance the affinity/potency and/or the efficacy of the orthosteric agonist at the receptor, whereas negative allosteric modulators (NAMs) do the opposite. Allosteric agonists capable of directly activating the receptor have also been identified, and some of these exhibit PAM activity in addition to their intrinsic activity and are referred to as "ago-PAMs". The receptor modulation exerted by any allosteric ligand arises from its effects on one or several of the multiple energy barriers associated with the allosteric transitions between different receptor conformations. As a result hereof, the modulatory characteristics displayed by a range of allosteric ligands can differ dramatically [4,14–16].

In contrast to the abundance of potent and selective allosteric modulators of nAChRs and $GABA_A$Rs published over the years [15–17], allosteric modulators of 5-HT_3Rs reported to date have typically emerged from discoveries of 5-HT_3R activity in drugs with already identified pharmacological activities at other targets (see [18,19] and references herein). The fact that most of these modulators display significantly lower potencies at the 5-HT_3Rs than at their original targets has limited their potential as pharmacological tools, and thus the development of novel allosteric modulators could potentially be valuable for future explorations of the receptors.

In 2011 we reported the discovery of a series of benzamide analogues and homologues as a novel class of 5-HT_3R agonists [20]. The structures of these compounds were substantially different from those of orthosteric 5-HT_3R ligands, not least in their lack of a positive charge at physiological pH, an essential pharmacophore element for orthosteric ligand binding to 5-HT_3Rs and other Cys-loop receptors [3,13,19]. In agreement with this, the compounds did not compete with the orthosteric radioligand [^{3}H]GR65530 for binding to the 5-HT_3R. Further, analogues from the series were shown to potentiate 5-HT-mediated 5-HT_3R signalling in a fluorescence-based functional assay [20]. Hence, we proposed that the compounds could act as ago-PAMs at 5-HT_3Rs while stressing that additional investigations were needed in order to fully draw this conclusion [20]. In the present study we have characterized the functional properties of one of these compounds, *trans*-3-(4-methoxyphenyl)-*N*-(pentan-3-yl)acrylamide (TMPPAA, analogue **3d** in the original paper [20], Fig. 1), at the 5-HT_3A receptor (5-HT_3AR) in elaborate electrophysiology studies and elucidated the molecular basis for its activity at the receptor.

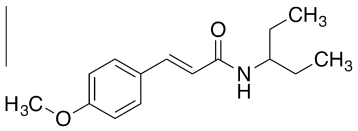

Fig. 1. Chemical structure of *trans*-3-(4-methoxyphenyl)-*N*-(pentan-3-yl)acrylamide (TMPPAA).

2. Materials and methods

2.1. Materials

Culture media, serum, antibiotics, buffers for cell culture and the LipofectAMINE™ Plus regent transfection kit were obtained from Invitrogen (Paisley, UK). The Polyfect transfection reagent was obtained from Qiagen (Hilden, Germany). ACh, 5-HT, metoclopramide, probenecid, poly-D-lysine and the 3,3′,5,5′-tetramethyl benzidine liquid substrate system were purchased from Sigma (St. Louis, MO). PU02 was obtained from Chembridge Corporation (San Diego, CA) and ondansetron, (±)-zacopride, clozapine, picrotoxin and propofol from Tocris Cookson (Bristol, UK). TMPPAA was synthesized in-house essentially as described previously [20]. The FLIPR™ Membrane Potential Blue (FMP) assay and the Fluo-4/AM dyes were purchased from Molecular Devices (Crawley, UK) and Molecular Probes (Eugene, OR), respectively. Defolliculated stage V–VI *Xenopus laevis* oocytes were obtained from Lohmann Research Equipment (Castrop-Rauxel, Germany). The cDNAs encoding for the rat α7 nAChR subunit (rα7), human 5-HT3A (h5-HT3A) and mouse 5-HT3A (m5-HT3A) subunits were kind gifts from Drs. J.W. Patrick, J. Egebjerg and D. Julius, respectively, and the stable h5-HT_3AR-HEK293 cell line was a kind gift from Dr. C. Rojas [21].

2.2. Molecular biology

The cDNAs used in the study were in pCIneo (h5-HT3A, m5-HT3A and rα7/m5-HT3A) and pGEMHE (human α7 (hα7) and h5-HT3A used for the oocyte experiments) vectors. The numbering of amino acid residues in the receptor subunits in this study [h5-HT3A (BC002354.2), m5-HT3A (NM_013561.2) and rα7 (NM_012832.2)] is based on the non-mature proteins (i.e., including the signal peptides). The chimeric and myc-tagged subunits were generated by splicing by overlap extension PCR [22]. The fusion points in the chimeric constructs were as follows (m5-HT3A sequences underlined): h5-HT3A/m5-HT3A (...F234-T235-V236-/-I242-I243-R244...), m5-HT3A/h5-HT3A (...F239-T240-V241-/-V237-I238-R238...) and h5-$HT3A^{m5\text{-}HT3A\text{-}ICL2}$ (...V327-R328-L329-/-V335-H336-K337... and ...R426-V427-G428-/-S447-V448-L449...). The myc-m5-HT3A construct was made by insertion of the myc epitope sequence (n-EQKLISEEDL-c) immediately after the m5-HT3A signal peptide sequence between residues G23 and S24 (predicted by SignalP 4.1 [23]). The construction of the rα7/m5-HT3A (...T223-V224-/-I242-I243-R244...) and myc-h5-HT3A constructs has been described previously [24,25]. Point mutations were introduced into the cDNAs for h5-HT3A, m5-HT3A and myc-tagged versions of these using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). The absence of unwanted mutations in all cDNAs created by PCR was verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany).

2.3. Cell culture and transfections

All cell lines were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The stable h5-HT_3AR-HEK293 cell line