Editor's comments:

1. Materials and methods: Please provide the product numbers (catalog numbers) of the main chemicals and reagents.

Response: we corrected them in line 141, 146, 148, 174, 179.

2. Results: A full statistical report is required for each dataset. Please provide the relevant test statistics and exact p-values supporting every statement regarding statistical significance. For detailed statistical reporting requirements, please refer to the Submission guidelines and the following article: https://link.springer.com/article/10.1007/s43440-020-00110-5#Sec6

Response: We have provided full statistical reporting, including the name of the statistical test, exact p values for every statement of statistical significance throughout the Results section, figure legends, and Table 1,

3. Figure and table captions:

- Please define all abbreviations, symbols, and acronyms used in each figure and table;

- Please include the names of the statistical tests used for the analyses, where applicable;

- Please ensure that the information is presented in the same order and style in all figure and table captions.

Response: We have provided them.

4. Please provide the ORCID IDs of all authors.

Response: We have provided them.

5. Keywords should not be capitalized.

Response: we corrected them in line 75.

6. Please include an overall list of abbreviations used in the manuscript, arranged alphabetically.

Response: we have provided it in line 518.

7. For SEM, please do not use periods.

Response: we corrected them.

8. For p-values, use lowercase.

Response: we corrected them.

9. Please number the lines throughout the document.

Response: we corrected them.

10. Please delete the last data availability statement, as it is duplicated.

Response: we have deleted it.

11. The reviewers' comments are available via the link below. Please address all reviewer comments.

Response: we have addressed each of the reviewers' comments point by point, as detailed in our responses and have revised the manuscript accordingly.

12. Please mark all changes made in response to the reviewers' and editor's comments and submit the marked-up manuscript as a Related file.

Response: we have marked them.

Reviewer 1

The authors submitted an article entitled " Luteolin inhibits human 5-HT3A receptor function through a noncompetitive and voltage-independent mechanism" to Pharmacological Reports.

This functional study explores the effect of luteolin, a natural flavonoid used in different pharmacological applications, on the ionotropic human serotonin 5-HT3A receptor. Using heterologous expression in xenopus oocytes and TEVC recordings, the authors demonstrate an inhibitory effect on serotonin(5-HT)-evoked currents with an IC50 = 6.9 +/- 0.8 µM. Concentration-response curves show a reduce maximal response to 5-HT and I/V curves do not display voltage sensitivity of the inhibition. In silico docking with AutoDock suggested a luteolin binding site at the interface between extracellular ligand binding domains, and site directed mutagenesis identifies I66 (Chain B) and W178 (Chain E) as involved in the inhibition potency of luteolin on h5-HT3AR.

Overall, the article is well-written and the effect of luteolin is clearly observed. However, some major and minor comments are raised as detailed below

Response: First of all, thank you for your thorough review and precise observations. We hope that this revised manuscript meets your expectations.

Major comments:

- The molecular docking results identifies the same binding site as the one identified in previous structural studies with other antagonists (i.e. https://doi.org/10.1038/s41594-024-01282-x). The presence of I66 and W178 surrounding the ligands were already reported. Functionally, W178 in the human 5-HT3AR (https://doi.org/10.1006/mcne.2002.1160) and the equivalent W183 in the mouse receptor (https://www.jbc.org/article/S0021-9258(18)30590-8/fulltext) have already been characterized by substitution with Serine or Tyrosine. It showed no response to 5-HT or antagonists (mCPBG) for W183S and a lower potency for 5-HT (~2 order of magnitude) for W183Y. While no substitution in Alanine is reported, it is not possible to compare the effect, however, these previous studies would suggest that W183A mutant would decrease the EC50 for 5-HT and a complete loss of luteolin inhibition. The characterization of luteolin-induced inhibition on W178S in h5-HT3AR would be a convincing control.

Response: We made the W178S mutant as requested by the reviewer. This mutant showed no response to 5-HT at all, so we could not test luteolin on it — this fits with the mouse W183S data and confirms W178 is important for receptor function (lines 322–334, 408–414). We also note that the luteolin binding site from our docking overlaps with the vortioxetine binding pocket the reviewer mentioned (lines 415–418). These new results are shown in Figure 5E (lines 712–728) and Table 1 (line 731).

- In the Discussion section, a comparative analysis is necessary with previous structural and functional studies that included h5-HT3AR antagonists. Moreover, a discussion should be added to establish a correlation between the inhibitory effect of luteolin discovered on h5-HT3A receptor and the phenotypic effects already described.

Response: We compared our findings with previous h5-HT3AR antagonist studies: the docking-identified luteolin site overlaps the vestibule pocket described for vortioxetine, granisetron, and palonosetron (lines 415–418), and our W178S result (lines 322–334, 408–414).

Minor comments:

- Page 2/background: luteolin has also negative side effects that should be mentioned, which also justify a study to minimize these effects.

Response: We have added negative side effects in line 90-93.

- Page 5/Materials:

o Indicate the vehicle used for dilution of luteolin

Response: We provided it in line 142

.

o Indicate the plasmid in which the OriGene's gene was subcloned

Response: We did not perform additional subcloning; the OriGene cDNA clone (catalog no. RG228377) was used as provided, in the pCMV6-AC-GFP vector."

- Page 5/ Preparation of Xenopus laevis oocytes and microinjection:

o change "cultures" to "batches"

Response: We have corrected it in line 157

o Indicate how the cRNA is purified and what is the quantity of cRNA injected per oocyte.

Response: We have provideded it in line 165 and 157-164.

- Page 6/ Molecular docking analysis: the "PDB ID: VHH15 " is not correct and shoulbbe corrected (certainly in "4PIR").

Response: We have corrected it in line 187

- Page 7/ Two-electrode voltage-clamp recording: Indicate the type of amplifier and digidata that were used.

Response: We have corrected it in line 203 and 204

- Page 7/ Data analysis and statistics: define “k” in the equation.

Response: We have corrected it in line 225-226

- Page 8/ Results/ Luteolin-mediated inhibition of human 5-HT3A receptors:

o Show the data of luteolin application alone, since it has been shown with the other antagonist Vortioxetine, a partial agonist effect on h5-HT3AR and an antagonist effect on rodent 5-HT3AR (https://doi-org.insb.bib.cnrs.fr/10.1038/s41594-024-01282-x).

Response: We have added them in new figure 1B and C.

o The following assumption “ luteolin does not preferentially bind to the closed state, but instead may interact more effectively with receptors after activation” is too speculative since luteolin could bind equally to closed and open states with similar observed effects.

Response: We removed them.

- Page 10: Fig. 4A and B panels are not cited.

Response: We have added them in line 306.

- Page 11

o Indicate in which amino acids, the indicated residues were substituted.

Response: We have corrected it in line 316

o Before "Discussion": change “Vmax” in “Emax”.

Response: We have used Vmax consistently throughout the manuscript to denote the maximal current amplitude derived from the Hill equation fit. To maintain internal consistency, we have retained it throughout the revised manuscript.

o Before "Discussion": indicate that I66A, W178A and the double mutant changed the IC50 and comment it.

Response: We provided it in line 387-390

- Page 13: change “nonspecific suppressor of channel activity” in “pore blocker”.

Response: We have corrected it in line 408

Reviewer 2

The article « Luteolin inhibits human 5-HT3A receptor function through a noncompetitive and voltage-independent mechanism" sought to characterise the effect of a plant molecule, luteolin, on 5-HT3A serotonin receptors using a conventional pharmacological approach. Firstly, through electrophysiological studies involving heterologous expression of the 5-HT3A receptor in Xenopus oocytes and measurement of current, and through modelling of the molecular interaction between the ligand (luteolin) and its receptor. The authors also tested mutants of the 5-HT3A receptor to identify the residues involved in luteolin's antagonistic activity. This is a relatively straightforward pharmacological study. There are several elements in this article that must be revised in order to significantly enhance its value and its suitability for publication.

In the abstract, the rationale for testing the pharmacological effect of luteolin on 5-HT3A receptors is not explained. This should be clarified, specifically mentioning the value of identifying 5-HT3A receptor antagonists, particularly their potential therapeutic benefits (irritable bowel syndrome, vomiting, etc.), and why luteolin was chosen for testing on 5-HT3A receptors.

Response: We thank the reviewer for their thorough and constructive evaluation of our manuscript, and for the thoughtful comments that have helped us substantially improve its clarity and rigor. We address each point below.

We have revised the Abstract Background to explain the therapeutic rationale for identifying 5-HT3A receptor antagonists (lines 39–41) and to clarify why luteolin was selected for testing, based on its identification through a primary screening of flavonoid compounds (lines 52–55)."

Luteolin inhibits the current induced by serotonin, but its action on 5-HT3A receptors is antagonistic. The title of this article should be amended to 'Luteolin antagonises human 5-HT3A receptor function through a non-competitive and voltage-independent mechanism'. The concept of 'inhibition' needs to be revised within the article.

Response: We have corrected it in line 1-2

Intro : "luteolin may interact directly with ion channel proteins"

Specify the ion channels known to be targeted by luteolin: Kir, Kv, BKCa and Ca^{2+} channels… (doi: 10.1016/j.lfs.2019.02.028; 10.3390/antiox11101892; 10.1111/j.1755-5922.2010.00212.x). This section needs to be better documented, particularly to highlight luteolin's low selectivity for receptor channels.

Response: We have introduced it in line 98-101

Results

« with partial outward rectification ». Given the I/V curve, the current is outwardly rectifier, not inward.

Figure 2B: This is an inhibitory effect, so this figure needs to be amended to show not the % of inhibition but the % of current induced by serotonin as a function of [luteolin] - in other words, a decreasing sigmoid curve rather than an increasing one. The same applies to Figure 5D.

Response: We have corrected it in Figure 2B and Figure 5D

IC50 = 6.9 +- 0.8 µM (unit to be added on the figure)

Response: We have corrected it in Figure 2B.

Figure 2D : add the EC50 values in the text or in the figure caption so that they can be compared. Equivalent EC50 values for different concentrations of an antagonist mean that it can be classified as a non-competitive antagonist. This point must be discussed in the Results section (« Noncompetitive modulation of human 5-HT3A receptors by luteolin »).

Response: We provided it in line 291-294.

« Figure 3. Structural analysis of the luteolin-binding site in wild-type and mutant 5-HT3A receptors. » Is this a wt receptor model? Where is the mutant? Where are the mutations? Is the title of this figure correct? I understood that this was a model of molecular interaction between luteolin and the wild-type 5-HT3A receptor: is that correct?

Response: We have corrected figure legend in line 694.

In this model, the serotonin binding site (Fig 3AB) must be clearly indicated to assess the non-competitive antagonistic nature of luteolin. This figure must therefore be completed.

Response: We provided it in Figure 3.

Figure 5D: the figure must be amended so that the inhibition of the current can be seen as a decreasing, rather than an increasing, sigmoid curve.

Response: We have corrected it in Figure 5D

The discussion is based on a very small number of references, which raises questions about its validity. The discussion therefore needs to be revisited, restructured, expanded and rewritten, drawing on evidence from the literature. At present, it is far too weak: there are only six references cited!

Response: We have substantially expanded and restructured the Discussion, increasing the number of references.

Minor :

Intro: Our findings identify luteolin as a potent antagonist of human 5-HT3A receptor

Response: We have corrected it in line 135.

Results : « In contrast, application of luteolin alone did not elicit any measurable current in oocytes injected with 5-HT3A cRNA at a holding potential of −80 mV (data not shown) ». please add these data, at least as a suppl figure.

Response: We have added them in Fig 1B and C.

Discussion: “Such binding would disrupt”

Response: We have corrected it in line 380.

Reviewer 3

The manuscript authored by Yun and colleagues describes pharmacological experiments involving 5-HT3A ionotropic serotonin receptors and a proposed novel naturally occurring antagonist, luteolin. On examination, the experimental work appears sound and accurately executed and analyzed. However, the work is not suitable for publication in its present form, and revision is required, together with consideration of the following points.

1. The abstract states that luteolin was identified through primary screening, but there is no mention of this in the main body of the work. Details of how the antagonist was identified would be a useful addition to the manuscript.

Response: First of all, we would like to sincerely thank the reviewer for the thorough and insightful evaluation of our manuscript. We greatly appreciate the time and expertise devoted to this review, and we hope that the revised manuscript adequately addresses the concerns raised. We have added a description of the primary screening approach that led to the identification of luteolin in the revised Materials and Methods section (lines 52–56).

2. The Methods state that Student's t-test was used in analyzing differences "between control and treatment groups", but there is no mention of where this has been used in the Results section or in Table 1. If using this test, the authors should ensure that any repeated measures involving multiple comparisons with WT receptors should be considered with the statistical analysis.

Response: We have clarified that Student's paired t-test was used specifically for two-group comparisons within the same oocyte (lines 227–229), and this is now explicitly reported in the Results (line 245) and Figure 1 legend (lines 670–671).

3. The text mentions significant changes in Vmax values with certain mutants vs WT, though an increase in IC50 would support the mutated residues contributing to the potency of luteolin. This is not described in the text in the Results section.

Response: We agree that the increase in IC50 observed for several mutants provides important complementary evidence for the involvement of these residues in luteolin potency, in addition to their effect on Vmax.

We have revised the Results section to explicitly describe and statistically evaluate the IC50 changes observed for I66A, W178A, and W178Y.

4. Table 1 requires attention. It lacks units for the Vmax and IC50; I66A is included twice with widely different results and R87A is missing. The Table includes data for a double mutant I66A + W178A, but these data are not described or discussed. The double mutation appears to "correct" the loss of luteolin potency found with the single mutants, which does not seem logical, whilst there is an additive effect on the reduction in Vmax.

Response: We have corrected Table 1 by adding units to the Vmax (%) and IC50 (μM) columns (line 736), removing the erroneous duplicate entry and confirming that all nine single-point mutants, including R87A, are now correctly and uniquely represented (Table 1, lines 731–732). We have also added a description and discussion of the I66A+W178A double-mutant data, which were previously omitted from the text (Discussion, lines 418–430).

5. The key data supporting the idea that luteolin is not a competitive antagonist appears to be in Figure 2D where luteolin suppresses the 5-HT concentration-response curves rather than a parallel shift. This is not described in the Results or considered in the Discussion.

Response: We note that this concentration-response comparison is shown in Figure 2E. We have revised the Results (lines 284–298). We have also expanded the discussion to explain that this suppression of the maximal response (lines 364–376).

6. On the voltage-dependence of the antagonism, this requires quantification of the percent inhibition at positive and negative voltages. The ramp traces provided in Figure 2C suggest that the inhibition at negative voltages is stronger than with positive voltages: about 70% inhibition at -80 mV vs about 40% inhibition at 50 mV. Quantitative data are required to support any claim regarding voltage dependance of inhibition by luteolin.

Response: Quantitative data addressing the voltage dependence of luteolin's inhibitory effect are provided in New Figure 2D.

7. A useful addition to the structures presented in Figure 4 would be the location of the 5-HT binding site for comparison with the location of the docked luteolin.

Response: We provided it in Figure 3.

8. Unfortunately, whilst the legend appeared in the compiled manuscript, although after Figure 2, Figure 5 itself was missing.

Response: We have corrected them.

Minor points:

9. Please state the solvent used to make 100 mM luteolin stock solution.

Response: We have provideded it in line 142

10. There is some reptation in the Methods section on oocyte preparation, where the Xenopus

anesthetization on ice is stated twice.

Response: We have corrected them in line 157

11. Please state how much cRNA was injected into each oocyte.

Response: We have corrected them in line 165

12. The PDB code for the receptor structure used in docking seems incorrect. The publication associated with the structure should be cited.

Response: We have corrected them in line 187

Luteolin antagonises human 5-HT3A receptor function through a non-competitive and voltage-independent mechanism

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Abstract

Background

The 5-hydroxytryptamine type 3A (5-HT3A) receptor is a ligand-gated ion channel that mediates fast excitatory serotonergic signaling and plays a key role in emesis and gastrointestinal sensory transmission. 5-HT3A receptor antagonists are of substantial therapeutic interest for the management of chemotherapy-induced nausea and vomiting and irritable bowel syndrome, yet the interaction of naturally occurring flavonoids with this receptor remains largely unexplored. Luteolin is a naturally occurring flavonoid widely distributed in vegetables, fruits, and medicinal herbs. It exhibits diverse pharmacological activities, including antioxidant, anti-inflammatory, neuroprotective, and anticancer effects. Although luteolin has been shown to modulate multiple intracellular signaling pathways, its direct action on serotonin-gated ion channels remains poorly understood. In this study, we investigated the effect of luteolin on human 5-HT3A receptors and found that it inhibits receptor activity, thereby identifying a new molecular target of this flavonoid and a potential candidate scaffold for 5-HT3A receptor antagonist development

Methods

Luteolin was identified through screening of flavonoid compounds for activity at human neuroreceptors and neuronal ion channels using two-electrode voltage-clamp recordings and

was selected for further study based on its potent inhibition of 5-HT3A receptor-mediated currents. Primary screening identified luteolin as a potent inhibitor of the human 5-HT3A receptor, prompting further examination of its mode of action. Human 5-HT3A receptors were heterologously expressed in Xenopus laevis oocytes, and serotonin-induced currents were recorded using the two-electrode voltage-clamp method. To investigate the molecular determinants of inhibition, computational docking and structural modeling were combined with site-directed mutagenesis.

Results

Luteolin caused a marked and reversible suppression of 5-HT-evoked currents in a concentration-dependent manner. The inhibitory effect displayed noncompetitive behavior relative to serotonin and was not influenced by membrane potential, indicating a voltage-independent mechanism. Concentration-response analysis yielded an IC_{50} of 6.9 ± 0.8 μM. Structure-guided mutational analysis further identified I66 and W178 as important determinants of luteolin sensitivity.

Conclusions

Collectively, these findings demonstrate that luteolin potently inhibits human 5-HT3A receptor function and provide mechanistic insight into its action at both functional and residue-specific levels.

Keywords: serotonin receptor, ligand-gated ion channel, luteolin, flavonoid, site-directed mutagenesis, molecular docking.

Introduction

Luteolin is a naturally occurring flavonoid widely present in vegetables, fruits, and medicinal herbs. It has attracted considerable attention because of its broad spectrum of biological and

pharmacological activities, including antioxidant, anti-inflammatory, neuroprotective, and anticancer effects[1-4]. Previous studies have shown that luteolin modulates multiple intracellular signaling pathways involved in cell survival[5], apoptosis[6, 7], oxidative stress[8, 9], and inflammatory responses[5, 10, 11]. In particular, luteolin has been reported to suppress tumor cell proliferation[12, 13], attenuate epithelial–mesenchymal transition[14], and regulate reactive oxygen species- and endoplasmic reticulum stress-related signaling cascades[12, 15] in several experimental models. Despite these diverse actions, the direct effects of luteolin on membrane proteins, especially ligand-gated ion channels, remain insufficiently characterized. However, in vitro and animal studies have reported that luteolin's biological activity is accompanied by dose-dependent cytotoxicity and, at high doses, adverse effects such as weight loss and organ dysfunction, compounded by its limited oral bioavailability and promiscuous target engagement[16]. Defining luteolin's precise molecular targets, such as membrane receptors and ion channels, is therefore an important step toward structure-based strategies that minimize such adverse effects while preserving its beneficial activities. Natural flavonoids have been reported to influence several classes of ion channels and receptors, suggesting that, in addition to their established intracellular effects, they may directly modulate membrane excitability and synaptic signaling. Luteolin in particular has been shown to modulate several classes of ion channels, including voltage-gated (Kv) and inward-rectifier (Kir) potassium channels in vascular smooth muscle, large-conductance Ca^{2+}-activated potassium (BKCa) channels, and voltage-gated Ca^{2+} channels[17-19]. This broad, largely non-selective engagement across structurally unrelated channel families is consistent with luteolin's promiscuous target profile noted above, and underscores the need to define its molecular targets, including ligand-gated ion channels, in a receptor-specific manner. Given its polyphenolic scaffold and demonstrated ability to modulate membrane proteins across multiple channel classes, luteolin may similarly interact directly with ligand-gated ion channel proteins such as the 5-HT3A receptor. Nevertheless, whether luteolin alters the activity of human 5-HT3A receptors, and if so by what mechanism, has not been clearly established. Elucidation of such an interaction would broaden our understanding of luteolin pharmacology and may also provide

new insight into the modulation of serotonin-gated ion channels by plant-derived small molecules.

Among serotonin receptors, the 5-hydroxytryptamine type 3 (5-HT3) receptor is unique in that it is a ligand-gated ion channel rather than a G protein-coupled receptor[20, 21]. The 5-HT3 receptor belongs to the Cys-loop receptor superfamily and mediates fast excitatory neurotransmission in response to serotonin. The 5-HT3 receptor is assembled from five subunits, of which the 5-HT3A subunit is essential for the formation of functional homomeric channels [22, 23]. Homomeric 5-HT3A receptors have been widely used as an experimental model for elucidating the structural and functional mechanisms of receptor activation, ion permeation, and pharmacological modulation. Because the receptor contains a well-defined extracellular ligand-binding domain and a transmembrane ion-conducting pore, it provides an attractive system for examining how small molecules regulate ligand-gated ion channel activity[24, 25]. Functionally, 5-HT3 receptors are expressed in both central and peripheral nervous systems, where they participate in nociceptive transmission, autonomic regulation, emesis, and gastrointestinal sensory signaling[26, 27]. These receptors are therefore of considerable clinical importance, particularly as therapeutic targets for the prevention of chemotherapy-induced nausea and vomiting and for modulation of visceral sensory pathways[28-31]. A growing body of evidence indicates that naturally occurring compounds can modulate Cys-loop receptors through orthosteric or allosteric mechanisms; however, comparatively little is known about how flavonoids interact with 5-HT3 receptors, particularly at the human 5-HT3A subtype.

In the present study, we investigated the effect of luteolin on human 5-HT3A receptor function using two-electrode voltage-clamp recordings in Xenopus laevis oocytes expressing recombinant receptors. To further define the mechanism underlying luteolin-mediated inhibition, we combined electrophysiological analysis with computational docking, structural modeling, and site-directed mutagenesis. Our findings identify luteolin as a potent antagonist of human 5-HT3A receptor activity and reveal key molecular determinants contributing to its

action.

Materials and Methods

Materials

Luteolin (purity >98%; catalog no. 440025) was obtained from Merck KGaA (Darmstadt, Germany). A 100 mM stock solution of luteolin was prepared in DMSO prior to use. The final DMSO concentration in the recording solution did not exceed 0.01%, a concentration confirmed not to affect $I_{5\text{-}HT}$ in control experiments. The cDNA encoding the human 5-HT3A receptor subunit (GenBank accession no. BC004453) was purchased from OriGene Technologies, Inc. (catalog no. RG228377, Rockville, MD, USA). MDL-72222 (3-tropanyl-3,5-dichlorobenzoate), a selective 5-HT3 receptor antagonist, was purchased from Abcam (catalog no. ab146635, Cambridge, UK). Unless otherwise specified, all additional reagents were supplied by Merck KGaA (Darmstadt, Germany).

Preparation of Xenopus laevis oocytes and microinjection

The experimental procedure is based on the approach presented in the previous study[32]. Xenopus laevis frogs were maintained under approved institutional animal care conditions (CNU IACUC-YB-2016-07) in a controlled environment with a 12 h light/dark cycle and an ambient temperature of approximately 20 °C. All animal handling and husbandry procedures followed the guidelines of the Korean Xenopus Resource Center for Research (KXRCR000001). Each frog contributed up to four batches. For oocyte extraction, frogs were anesthetized on ice for around 2 h, and a small incision was made to access the abdominal region without damaging internal organs. The isolated tissue was treated with collagenase (2 mg/mL) in OR2 solution containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2.5 mM sodium pyruvate, and 5 mM HEPES (pH 7.45) for 1.5 h with gentle shaking to remove surrounding follicular layers. Defolliculated oocytes were then washed thoroughly and maintained at 18 °C

in ND96 solution consisting of 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES (pH 7.45). The incubation medium was replaced twice each day. For heterologous expression, cRNA encoding the human 5-HT3A receptor (10 ng) was injected into oocytes using a microinjector fitted with pulled glass capillaries with tip diameters of approximately 15–20 µm. Electrophysiological recordings were performed 48 h after injection. Serotonin and luteolin were freshly diluted in ND96 solution immediately before application, and their effects on 5-HT3A receptor-mediated currents were assessed during voltage-clamp experiments.

Generation of 5-HT3A receptor mutants and cRNA synthesis

Point mutations in the human 5-HT3A receptor were introduced using the QuikChange XL site-directed mutagenesis method with Pfu DNA polymerase and mutation-specific primer pairs (catalog no. 200516, Agilent Technologies). PCR products were transformed into Escherichia coli DH5α competent cells, and transformants were selected on ampicillin-containing agar plates, as described previously. Plasmid DNA from positive colonies was purified, and the desired nucleotide substitutions were confirmed by DNA sequencing. After sequence verification, plasmids were linearized with SphI and used as templates for capped cRNA synthesis with the mMESSAGE mMACHINE transcription kit (catalog no. AM1344, Thermo Fisher Scientific). The synthesized cRNA was dissolved in RNase-free DEPC-treated water and stored at −80 °C at a final concentration of 1,000 µg/mL.

Molecular docking analysis

Docking simulations were carried out on a 64-bit Windows 10 computer equipped with an Intel Core i7 processor (2.20 GHz) and 16 GB RAM using AutoDock Tools version 1.5.6 (The Scripps Research Institute, La Jolla, CA, USA). The structural model of the human 5-HT3A receptor was obtained from the Protein Data Bank (PDB ID: 8AXD), and the three-dimensional structure of luteolin (PubChem CID: 5280445) was retrieved from PubChem. Receptor and

ligand preparation, grid definition, and docking parameter setup were completed in AutoDock Tools. Docking poses were ranked according to binding energy, predicted inhibition constant (Ki), and intermolecular interaction energy. To further analyze receptor–ligand contacts, the docked complexes were examined using LigPlot version 4.5.3 (EMBL-EBI) and PyMOL version 1.8.4.2 (Schrödinger). LigPlot was used to visualize hydrogen bonds and hydrophobic interactions, whereas PyMOL was used to inspect residue proximity and estimate intermolecular distances. These analyses were subsequently used to guide the selection of candidate residues for mutational testing.

Two-electrode voltage-clamp recording

The experimental procedure is based on the approach presented in the previous study[33]. Electrophysiological measurements were conducted 2 days after cRNA injection using the two-electrode voltage-clamp technique at room temperature. Oocytes were impaled with two borosilicate glass electrodes filled with 3 M KCl and connected to an oocyte clamp amplifier (OC-725C, Warner Instruments) coupled to a Digidata data acquisition system (Digidata 1440A, Molecular Devices). Signals were filtered, digitized, and recorded using pClamp 8 software. Unless otherwise stated, oocytes were voltage-clamped at a holding potential of −80 mV. Immediately before recording, luteolin stock solution was diluted in ND96 recording buffer to the desired concentrations. Oocytes were continuously superfused with ND96 throughout the experiment. Agonist-induced inward currents were measured under control conditions, during luteolin exposure, and after washout to determine the magnitude and reversibility of inhibition. For concentration-inhibition experiments, luteolin was applied cumulatively or stepwise at multiple concentrations with sufficient wash intervals between applications to permit current recovery and reduce receptor desensitization. In separate experiments, current-voltage relationships were analyzed using voltage-step or voltage-ramp protocols in the absence or presence of luteolin.

Data analysis and statistics

Peak inward currents induced by serotonin ($I_{5\text{-HT}}$) were measured at multiple 5-HT concentrations to establish concentration-response curves and to assess the modulatory influence of luteolin on 5-HT3A receptor-mediated currents. The concentration-dependent inhibition produced by luteolin was analyzed by fitting normalized current responses to the Hill equation.:

$$y/V_{max} = (A)^n/(A^n + k^n),$$

In this equation, Y represents the peak current observed at each luteolin concentration, Vmax indicates the maximum current amplitude, and [A] refers to the concentration of luteolin. IC_{50} corresponds to the concentration required to inhibit the response by 50%, where k is the Hill coefficient, reflecting the slope of the concentration-response curve. All values are expressed as mean ± SEM. Differences between two groups ($I_{5\text{-HT}}$ in the absence vs presence of luteolin) were evaluated using Student's paired t-test, as measurements were obtained from the same oocyte before and after luteolin application. Comparisons of Vmax and Hill coefficient values among wild-type and the nine mutant 5-HT3A receptors (Table 1) were performed using one-way ANOVA followed by Dunnett's post hoc test, with wild-type as the reference group. Statistical analyses were performed using Origin (version 8.5, OriginLab Corporation, Northampton, MA, USA). A value of $p < 0.05$ was considered statistically significant; exact P values are reported throughout the Results section.

Results

Luteolin-mediated inhibition of human 5-HT3A receptors

Figure 1A shows the molecular structure of luteolin. To examine its effect on human 5-HT3A receptor activity, two-electrode voltage-clamp recordings were performed in Xenopus laevis oocytes expressing the recombinant receptor. When applied together with 5-HT, luteolin substantially reduced the inward current evoked by 5-HT ($I_{5\text{-HT}}$), demonstrating an inhibitory

action on 5-HT3A receptor-mediated responses. In contrast, application of luteolin alone did not elicit any measurable current in oocytes injected with H_2O (Fig. 1B) or 5-HT3A cRNA (Fig. 1C) at a holding potential of −80 mV. Co-treatment with 100 μM 5-HT and 30 μM luteolin significantly decreased the peak amplitude of $I_{5\text{-HT}}$ compared with application of 5-HT alone ($p = 0.0011$, $n = 5–7$ oocytes from four frogs), and this inhibition was readily reversed after washout. At 30 μM, luteolin suppressed $I_{5\text{-HT}}$ by 73.4 ± 8.9% (Fig. 1D). As a positive control, 0.5 μM of the selective 5-HT3A receptor antagonist MDL-72222 (3-tropanyl-3,5-dichlorobenzoate) also inhibited $I_{5\text{-HT}}$, confirming functional receptor expression in the oocyte system. The extent of inhibition produced by luteolin was similar under co-application and pre-application protocols, indicating that prior exposure did not enhance its inhibitory efficacy. Since pre-incubation generally allows more time for ligand association than simultaneous application, compounds with strong affinity for the resting receptor state often display greater inhibition after pre-application [34-36]. All recordings were obtained at −80 mV, and the data are presented as mean ± SEM (n = 5–7 from four independent frogs).

Concentration-dependent inhibition of $I_{5\text{-HT}}$ by luteolin in 5-HT3A receptor

Co-application of luteolin with 100 μM 5-HT produced a concentration-dependent suppression of peak 5-HT-evoked currents in oocytes expressing human 5-HT3A receptors (Fig. 2A and B). Peak current amplitudes were defined as the maximal responses to 5-HT, and inhibition was calculated relative to the control response measured in the same oocyte. Fitting the concentration-inhibition data with the Hill equation yielded an IC_{50} of 6.9 ± 0.8 μM and a Hill coefficient of 1.2 ± 0.2 (7–9 oocytes per concentration from five independent frogs), indicating an inhibition profile with an approximately unity slope. The inhibitory effect was reversible upon washout, and the extent of inhibition increased progressively with luteolin concentration. These results demonstrate that luteolin inhibits human 5-HT3A receptor-mediated currents in a reversible, concentration-dependent manner.

Voltage-independent inhibition of $I_{5\text{-HT}}$ by luteolin in human 5-HT3A receptors

Luteolin suppressed $I_{5\text{-HT}}$ across the entire voltage range tested (−80 to +50 mV) in Xenopus oocytes expressing human 5-HT3A receptors, with the membrane potential returned to −80 mV between applications. As shown in Fig. 2C and D, 5-HT produced a characteristic current–voltage relationship with partial inward rectification and a reversal potential near 0 mV, consistent with the properties of a nonselective cation conductance. Co-application of 3 or 30 µM luteolin reduced the current amplitude by a comparable proportion throughout the I–V curve without altering the reversal potential. A two-way repeated-measures ANOVA revealed a significant main effect of luteolin treatment ($p < 0.003$) and of membrane potential ($p < 0.001$) on $I_{5\text{-HT}}$ amplitude, but no significant interaction between treatment and voltage ($p = 0.2653$), indicating that the inhibitory effect of luteolin did not depend on membrane potential, and the inhibitory effect was readily reversible upon washout. These results support the conclusion that luteolin acts as a voltage-independent inhibitor of 5-HT3A receptor-mediated currents rather than affecting ion permeation properties.

Noncompetitive modulation of human 5-HT3A receptors by luteolin

To further clarify the basis of this voltage-independent inhibition, we examined the effect of luteolin on 5-HT concentration-response relationships. Oocytes expressing 5-HT3A receptors were challenged with increasing concentrations of 5-HT in the absence or presence of luteolin (3 or 30 µM, Fig. 2E). Luteolin reduced 5-HT-evoked responses across the concentration range tested, with a greater effect at 30 µM than at 3 µM (two-way repeated-measures ANOVA; luteolin concentration: $p = 0.0004$; treatment-by-concentration interaction: $p = 0.0003$; 30 µM vs 3 µM, all $p < 0.01$). The EC_{50} for 5-HT was 9.3 ± 0.3 µM under control conditions and was significantly increased to 19.1 ± 2.9 µM in the presence of 3 µM luteolin and to 28.3 ± 9.1 µM in the presence of 30 µM luteolin, indicating a rightward shift of the 5-HT concentration-response curve without a corresponding reduction in maximal response. These findings indicate that luteolin suppresses receptor activation without evidence of voltage dependence,

consistent with an inhibitory mechanism mediated through allosteric modulation rather than altered ion permeation. Thus, luteolin appears to attenuate 5-HT3A receptor function by interfering with receptor activation efficiency.

Docking analysis of luteolin binding to the 5-HT3A receptor

To define structural elements associated with luteolin sensitivity, we performed comparative docking analyses using wild-type and mutant 5-HT3A receptor models. AutoDock 4.0 predicted that luteolin occupies a binding pocket at the subunit interface near the extracellular vestibule pore entrance (Fig. 3). In the highest-scoring wild-type conformation, luteolin showed a more favorable interaction pattern than in the mutant models, indicating that mutation weakens ligand accommodation within this region (Fig. 4A and B). Distance measurements from representative docking poses identified I66 and W178 as the closest contacting residues in the wild-type receptor, with values of 3.4 and 3.5 Å for I66 and 3.4 and 3.9 Å for W178 (Fig. 4C). In the corresponding mutant models, these separations increased to 4.7 and 5.8 Å for I66A and 3.9 and 4.5 Å for W178A (Fig. 4D), suggesting reduced hydrophobic interactions and altered pocket architecture. These in silico findings support I66 and W178 as key structural determinants of the predicted luteolin-binding site.

Effects of point mutations on luteolin inhibition of 5-HT3A receptors

To test the functional importance of these residues, we examined luteolin sensitivity in a panel of 5-HT3A point mutants. Receptors containing alanine substitutions at I66, W85, R87, N123, T176, S177, W178, M223, and Y229 all produced robust 5-HT-evoked inward currents comparable to those of the wild-type receptor, allowing direct functional comparison across constructs. Luteolin markedly inhibited $I_{5\text{-HT}}$ in wild-type receptors, and inhibition was also observed in the I66A and W178A mutants (Fig. 5), although the magnitude of suppression differed from that in wild type. The inhibitory effects of luteolin across all nine mutants are

summarized in Table 1. To further validate the structural importance of W178, we generated the W178S mutant, corresponding to the loss-of-function tryptophan substitution previously characterized at the homologous position (W183) in the mouse 5-HT3A receptor[37, 38]. n that study, the antagonist mCPBG showed roughly a two-fold difference in potency between W183S and W183Y, whereas in our study luteolin exhibited a similar inhibitory potency at both W178A and W178S (Fig. 5E and Table 1). Interestingly, in the W178S mutant, luteolin produced only a slight inhibition of the 5-HT-evoked current, together with an additional inward current following the peak, a pattern not seen with W178A or W178Y. (Fig. 5E). Consistent with this prior report, W178S receptors failed to generate detectable inward currents in response to 100 μM 5-HT (n = 8 oocytes from 4 frogs), precluding pharmacological evaluation of luteolin sensitivity at this construct. This loss-of-function phenotype confirms that W178 is essential for normal receptor gating and further supports its identification as a critical structural determinant within the luteolin-binding pocket. Concentration-inhibition curves generated from normalized peak-current data were fitted with the Hill equation to estimate Vmax and Hill coefficient values. One-way ANOVA followed by Dunnett's post hoc test showed that I66A and W178A exhibited significantly reduced Vmax relative to wild type ($p < 0.0001$; I66A: $p = 0.00012$, W178A: $p = 0.0004$), whereas the remaining mutants did not differ from wild type (all $p > 0.05$). In parallel, luteolin potency was also significantly reduced at these positions, as reflected by markedly increased IC_{50} values for I66A (64.3 ± 4.6 μM), W178A (24.1 ± 3.2 μM), and W178Y (21.5 ± 4.8 μM) compared with wild type (5.8 ± 0.4 μM) (one-way ANOVA, $p < 0.0001$; Dunnett's post hoc test, I66A: $p = 0.00012$, W178A: $p = 0.0004$, IW178Y: $p = 0.0004$). Notably, the I66A+W178A double mutant showed a near-complete loss of Vmax but only a modest change in IC_{50} (6.7 ± 2.8 μM), suggesting that the combined substitution primarily abolished the efficacy of luteolin inhibition rather than its binding affinity. Hill coefficients did not differ significantly among receptors ($p = 0.3621$; Table 1). Together, the concurrent changes in Vmax and IC_{50} indicate that I66 and W178 contribute both to the potency and the maximal inhibitory efficacy of luteolin at the human 5-HT3A

receptor. The remaining mutations produced little or no effect on these parameters. Together, these results identify I66 and W178 as key residues governing the maximal inhibitory efficacy of luteolin at the human 5-HT3A receptor.

Discussion

In the present study, luteolin was identified as a previously unrecognized inhibitor of the human 5-HT3A receptor. Using two-electrode voltage-clamp recordings in Xenopus laevis oocytes expressing the human 5-HT3A receptor, we observed that luteolin suppressed 5-HT-induced inward currents in a concentration-dependent and reversible manner. Electrophysiological characterization further demonstrated that the inhibitory action of luteolin was both noncompetitive and voltage-independent, with an IC_{50} value of 6.9 ± 0.8 μM. In parallel, structure-based docking and mutational analysis revealed that I66 and W178 are important determinants of luteolin sensitivity. Taken together, these findings indicate that luteolin acts as a noncompetitive inhibitor of the human 5-HT3A receptor and provide a structural basis for understanding its modulatory action on receptor signaling.

Several observations support the conclusion that luteolin inhibits the human 5-HT3A receptor through a noncompetitive mechanism. The absence of voltage dependence suggests that its action is unlikely to involve direct occlusion of the ion-conducting pore, since pore blockers commonly exhibit sensitivity to membrane potential[39-41]. The suppression of the maximal 5-HT-evoked response by luteolin, rather than a simple parallel shift of the concentration-response curve, provides the strongest evidence against a purely competitive mechanism of action. Because increasing the concentration of 5-HT could not restore the control level of receptor activation, luteolin behaves as an insurmountable antagonist, a hallmark of noncompetitive or allosteric inhibition. The accompanying increase in EC_{50} indicates that luteolin also reduces the apparent potency of 5-HT, a pattern consistent with negative allosteric modulation, in which binding at a site distinct from the orthosteric pocket alters both the efficiency of channel gating and the apparent affinity of the agonist, without directly occluding

the 5-HT binding site itself. In addition, the inhibitory effect was readily reversible, consistent with a non-covalent interaction between luteolin and the receptor. Combined with the mutational identification of I66 and W178, these data support the interpretation that luteolin interacts with the extracellular region of the receptor rather than with the transmembrane channel lumen. Such binding would disrupt the conformational coupling process through which agonist binding is translated into channel opening[42, 43]. This interpretation is in line with established models of Cys-loop receptor function, in which ligand-induced perturbations in the extracellular domain can allosterically alter gating transitions without directly competing at the orthosteric agonist-binding site[44, 45].

Our structural modeling together with site-directed mutagenesis indicates that I66 and W178 are important molecular determinants of luteolin-mediated inhibition of the human 5-HT3A receptor. Notably, the I66A and W178A single mutants, as well as the I66A/W178A double mutant, exhibited significant shifts in IC_{50} values for luteolin compared with the wild-type receptor (IC_{50} = 5.8 ± 0.4 μM for wild-type vs. 64.3 ± 4.6 μM for I66A, 24.1 ± 3.2 μM for W178A, and 6.7 ± 2.8 μM for the double mutant; Table 1). These residues are positioned within the extracellular domain, a region that plays a central role in coupling ligand recognition to channel gating in Cys-loop receptors. In such a framework, I66 and W178 may act by shaping a binding-competent microenvironment that supports productive receptor–ligand interaction and stabilizes a receptor conformation less favorable for activation. The implication of W178 is particularly noteworthy because aromatic residues in the extracellular domain of Cys-loop receptors often play critical roles in transducing ligand-induced conformational changes. Although luteolin does not appear to behave as a simple orthosteric competitor, perturbation at W178 could nonetheless interfere with the structural transitions that normally link agonist binding to channel opening. Likewise, the contribution of I66 suggests that the inhibitory site may extend beyond a single focal contact and instead involve a broader extracellular surface or cleft in which multiple residues collectively determine ligand accommodation. From this perspective, luteolin may inhibit receptor function not by directly blocking ion permeation, but by allosterically uncoupling agonist engagement from the gating machinery. This view is

consistent with the electrophysiological profile observed here, particularly the voltage independence and reversibility of inhibition, both of which argue against a classical pore-blocking mechanism[46, 47]. The residue dependence identified in the present study therefore provides mechanistic specificity that strengthens the interpretation of luteolin as an extracellularly acting noncompetitive inhibitor rather than pore blocker. This result is also supported by the W178S mutation, which is similar to the loss-of-function W183S mutation that was already reported in the mouse 5-HT3A receptor and completely abolished 5-HT-evoked currents, making pharmacological evaluation impossible. This confirms that W178 has an essential structural role in receptor gating, and it shows that alanine substitution, not serine, had to be used in order to keep enough receptor function to isolate the specific contribution of W178 to luteolin sensitivity.

The luteolin-binding site identified here overlaps with the extracellular vestibule pocket described for structurally characterized 5-HT3 receptor antagonists, including vortioxetine [48], granisetron [49], and palonosetron [50], whose binding involves residues corresponding to I66 and W178 . The I66A+W178A double mutant exhibited a near-complete loss of luteolin-mediated inhibition (Vmax = 2.4 ± 1.2%, compared with 33.4 ± 2.1% for I66A and 24.5 ± 3.5% for W178A alone), a reduction disproportionately greater than predicted from the individual single-mutant effects, suggesting that I66 and W178 act cooperatively rather than independently in mediating luteolin's inhibitory efficacy. In contrast, the IC_{50} value for the double mutant (6.7 ± 2.8 μM) was similar to that of wild type, in apparent contrast to the increased IC_{50} values observed for each single mutation. However, because the maximal response of the double mutant was reduced to near-baseline levels, the concentration-inhibition curve was poorly constrained, and the resulting IC_{50} estimate should be interpreted with caution rather than as evidence of restored luteolin potency. Taken together, these data suggest that I66 and W178 jointly contribute to luteolin's inhibitory efficacy at the human 5-HT3A receptor, while the apparent IC_{50} value in the double mutant likely reflects the limited reliability of curve-fitting under conditions of minimal residual response rather than a genuine recovery of potency. This convergence suggests that the extracellular vestibule constitutes a common, druggable

hotspot for chemically diverse ligands. However, luteolin's noncompetitive, voltage-independent profile and distinct polyphenolic scaffold suggest a mode of engagement that may differ mechanistically from classical competitive antagonists, warranting further structural comparison.

These findings also have implications for the broader pharmacology of 5-HT3 receptors. Clinically established 5-HT3 receptor blockers have demonstrated that this receptor family is a highly tractable therapeutic target, particularly in disorders involving emesis and visceral sensory signaling[51-53]. In that context, the identification of luteolin as a chemically distinct inhibitor is of interest not because it surpasses existing drugs in potency, but because it expands the structural space of ligands capable of modulating 5-HT3A receptor function. Molecules with alternative scaffolds can reveal receptor microenvironments that are not readily apparent from studies of classical antagonists alone and may ultimately help define new allosteric pharmacophores. Such diversity is particularly relevant for Cys-loop receptors, where subtle differences in extracellular architecture can translate into marked differences in ligand sensitivity, subtype selectivity, and gating behavior[54-56]. Accordingly, luteolin may serve as a useful chemical probe for dissecting extracellular allosteric mechanisms in 5-HT3A receptors and related ligand-gated ion channels. An additional point of interest is that luteolin possesses a relatively compact and synthetically tractable flavonoid framework. Although its potency is modest compared with that of optimized therapeutic antagonists, this level of activity is sufficient to justify its consideration as a lead-like scaffold for structure-guided optimization. The present residue-mapping results offer a practical starting point for such efforts. Derivatization designed to improve steric complementarity around the I66 and W178-associated pocket, or to strengthen local hydrogen-bonding interactions with neighboring residues, may enhance affinity while preserving the reversible and voltage-independent inhibitory profile observed here. At the same time, any medicinal chemistry campaign would need to balance potency with factors such as receptor selectivity, metabolic stability, membrane permeability, and the tendency of polyphenolic compounds to engage multiple cellular targets. Thus, while luteolin itself may be better viewed as a mechanistic lead than as a direct

therapeutic candidate, its interaction with 5-HT3A receptors highlights a pharmacologically exploitable binding region within the extracellular domain. The physiological relevance of this inhibitory action also merits consideration. 5-HT3A-containing receptors are widely involved in fast excitatory serotonergic transmission in both peripheral and central pathways, including circuits associated with nausea, emesis, visceral sensation, and gastrointestinal reflex control. Because dysregulated serotonergic signaling has been implicated in functional gastrointestinal disorders and abnormal sensory processing, inhibition of 5-HT3A receptors remains a plausible mechanism for modulating these pathophysiological processes. In this regard, the ability of luteolin to suppress human 5-HT3A receptor currents suggests that flavonoid-based chemotypes may have utility in exploring how extracellular allosteric inhibition influences gut–brain signaling and sensory excitability. The inhibitory effect of luteolin on human 5-HT3A receptors may help explain some of its previously reported biological effects. Luteolin has antidepressant-like effects, and part of this is thought to involve serotonin signaling, including blocking 5-HT reuptake. Direct 5-HT3 receptor antagonism could be another way luteolin affects serotonin activity, since 5-HT3 receptors are also linked to mood regulation. Also, 5-HT3 receptor antagonists are well known to reduce nausea and vomiting by blocking signals from vagal afferents and the chemoreceptor trigger zone. Since luteolin is a flavonoid commonly found in vegetables and fruits, its ability to inhibit 5-HT3A receptor currents suggests it might also affect gut-brain serotonin signaling related to nausea, gut sensation, and motility. Although the present study does not address in vivo efficacy, tissue exposure, or system-level physiological outcomes, it establishes a molecular basis that can support future translational investigation in more complex experimental settings.

In summary, the present study identifies luteolin as a reversible, concentration-dependent inhibitor of the human 5-HT3A receptor that acts through a noncompetitive and voltage-independent mechanism. Functional and structural analyses converge in supporting a key role for I66 and W178 in shaping luteolin sensitivity, thereby implicating the extracellular domain as a critical site of action. Beyond defining the pharmacological profile of a single flavonoid, these findings provide new insight into extracellular determinants of 5-HT3A receptor

488 modulation and suggest that flavonoid-like chemotypes may represent useful starting points
489 for the development of new probes targeting serotonergic ligand-gated ion channel signaling.

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CRediT authorship contribution statement

Jeongyeon Yun: Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Methodology, Data curation. Jaehui Yang: Investigation, Conceptualization. Methodology, Data curation. Yejin Kim: Visualization, Validation. Myungmi Moon: Formal analysis, Data curation. Hye Duck Yeom: Methodology, Visualization. Virginia Njoki Kamau: review & editing. Mee-Hyun Lee: review & editing. Gihyun Lee: Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. Junho H Lee: Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Ethics approval and consent to participate

The animal experiments were approved by the Animal Ethical and Welfare Committee (Approval No. CNU IACUC-YB-2016-07).

Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

List of Abbreviations

Abbreviation	Full term
5-HT	5-hydroxytryptamine (serotonin)
5-HT3A	5-hydroxytryptamine type 3A receptor subtype
cRNA	complementary RNA
DEPC	diethyl pyrocarbonate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
$I_{5\text{-HT}}$	5-HT-evoked inward current
IACUC	Institutional Animal Care and Use Committee
IC_{50}	half-maximal inhibitory concentration
Ki	inhibition constant
MDL-72222	3-tropanyl-3,5-dichlorobenzoate
ND96	standard Xenopus oocyte bath/recording solution
OR2	oocyte Ringer solution 2
PCR	polymerase chain reaction
PDB	Protein Data Bank
SEM	standard error of the mean
Vmax	maximal current amplitude

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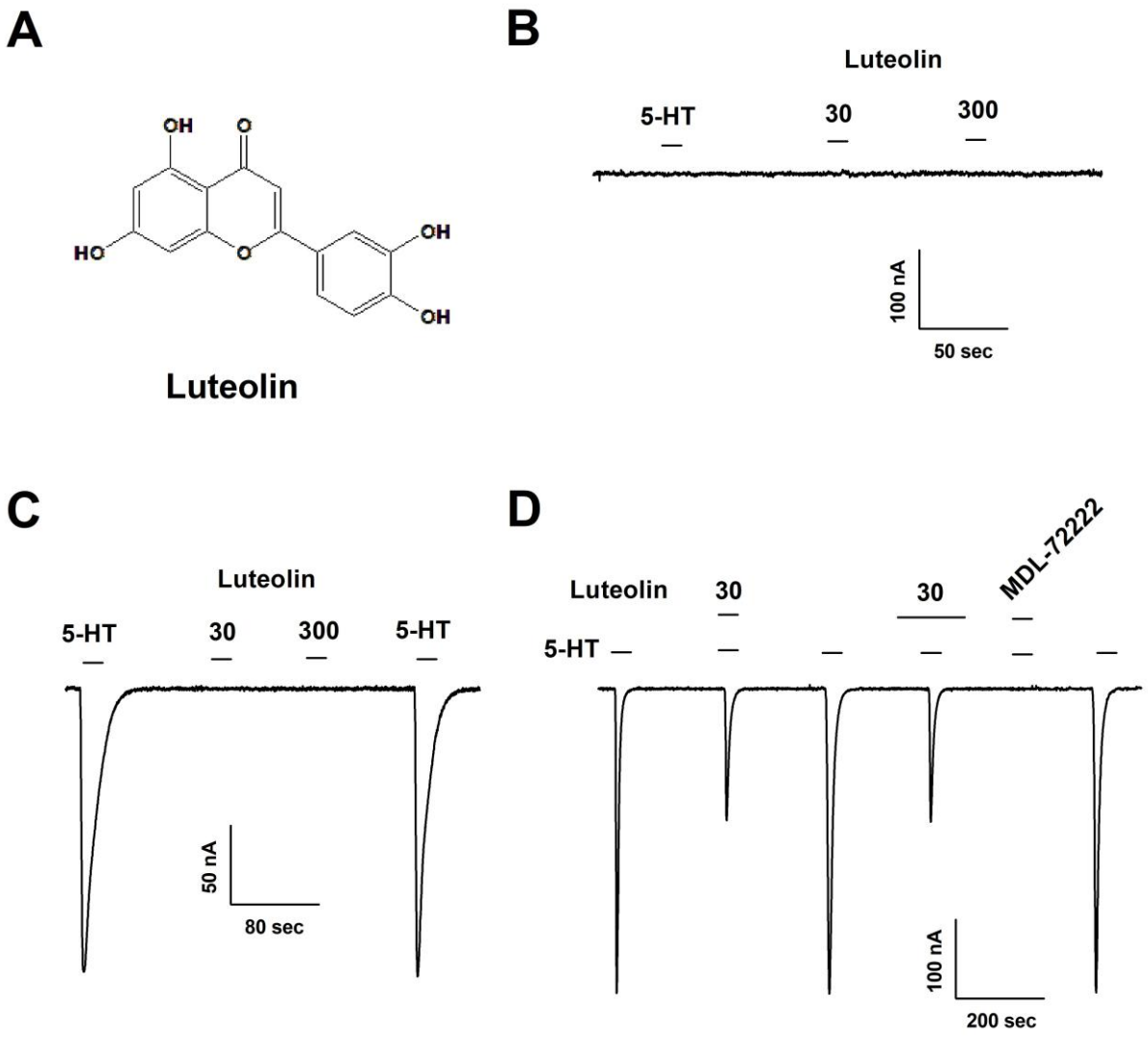

Figure 1. Chemical structure of luteolin and its inhibitory action on human 5-HT3A receptors. (A) Chemical structure of luteolin. (B) Representative current trace recorded from a water (H_2O)-injected oocyte exposed sequentially to 100 μM 5-HT (5-hydroxytryptamine; serotonin), 30 μM luteolin, and 300 μM luteolin, showing no measurable current in the absence of 5-HT3A receptor expression. (C) Representative current trace recorded from an oocyte expressing human 5-HT3A receptors, using the same application protocol as in (B). 5-HT (100 μM) evoked a robust inward current ($I_{5\text{-HT}}$, 5-HT-evoked inward current), whereas luteolin alone (30 or 300 μM) elicited no current; a second application of 5-HT evoked a response of similar amplitude, confirming that luteolin does not act as an agonist at the 5-HT3A receptor and that the receptor response remained stable throughout the recording. (D) Representative current traces recorded from oocytes exposed to 100 μM 5-HT alone or together with 30 μM luteolin,

667 with luteolin administered either simultaneously with 5-HT or as a preincubation treatment.
668 The selective 5-HT3A receptor antagonist 0.5 µM MDL-72222 (3-tropanyl-3,5-
669 dichlorobenzoate) inhibited $I_{5\text{-}HT}$. Data are expressed as mean ± SEM (n = 8–10 oocytes from
670 four frogs, n = number of oocytes). Statistical comparison between 5-HT alone and 5-HT +
671 luteolin was performed using a paired Student's t-test ($p = 0.0011$).

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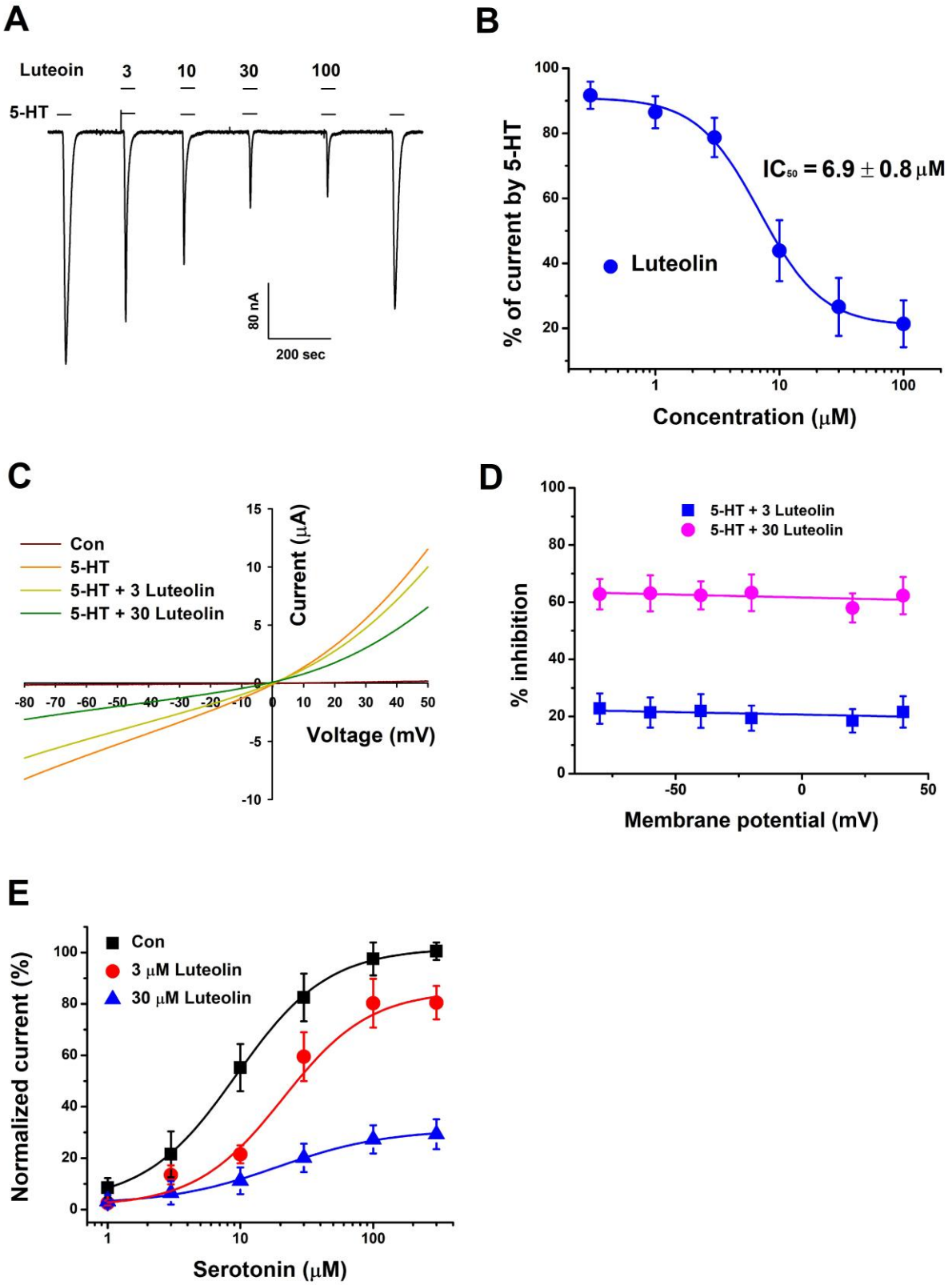

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674 Figure 2. Characterization of luteolin-mediated inhibition of the 5-HT3A receptor.

675 (A) Concentration–inhibition relationships were obtained from oocytes expressing human 5-

HT3A receptors by measuring peak inward currents at a holding potential of −80 mV. Responses to 100 μM 5-HT were first recorded alone and then during co-application with increasing concentrations of luteolin. (B) The inhibitory action of luteolin on 5-HT-induced currents was evaluated by comparing the mean peak amplitudes evoked by 5-HT alone with those recorded in the presence of luteolin. (C) Current-voltage (I–V) relationships obtained using voltage ramps from −80 to +50 mV (holding potential, −80 mV) under control conditions (Con), during application of 100 μM 5-HT alone, and during co-application of 5-HT with 3 or 30 μM luteolin. (D) Percentage inhibition of $I_{5\text{-}HT}$ by 3 μM (blue squares) or 30 μM (magenta circles) luteolin plotted as a function of membrane potential, derived from the I–V data in (C). The lack of voltage dependence across the range tested (−80 to +40 mV) indicates that luteolin inhibits $I_{5\text{-}HT}$ independently of membrane potential. (E) Concentration–response curves for 5-HT were constructed at −80 mV in the absence or presence of 3 or 30 μM luteolin. Currents evoked by different concentrations of serotonin during co-application with luteolin showed that luteolin reduces 5-HT3A receptor function through a noncompetitive inhibitory mechanism (n = 9–12 from four independent frogs).

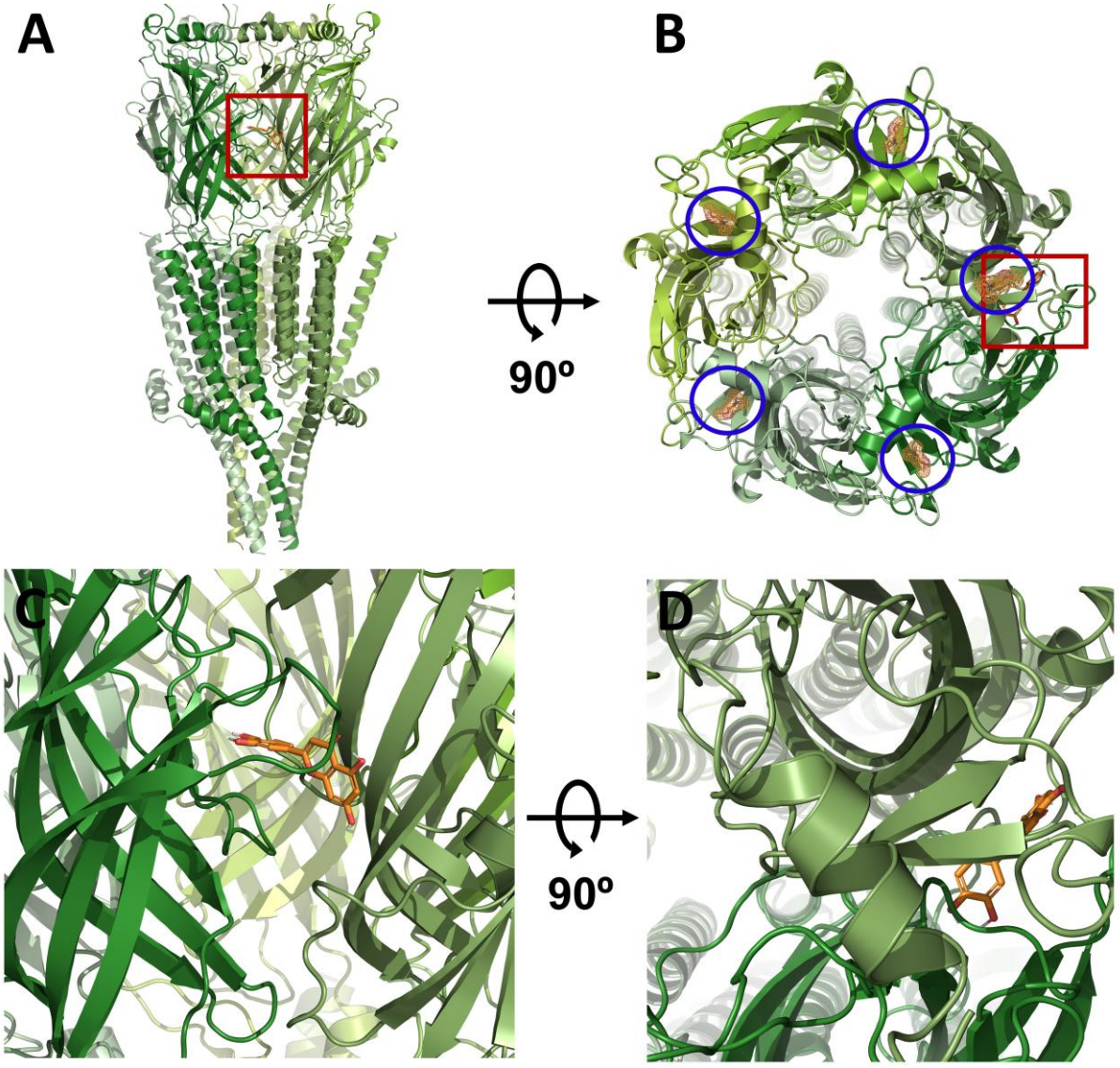


Figure 3. Structural analysis of the luteolin-binding site in wild-type 5-HT3A receptors.

(A) Lateral view of the homopentameric 5-HT3A receptor. The red box indicates the luteolin-binding site identified by docking analysis, located within the extracellular domain at the interface between adjacent subunits. (B) Top view of the same receptor model, rotated 90° relative to (A). Blue circles indicate the five orthosteric 5-HT-binding sites, each located at a subunit interface, the red box indicates the luteolin-binding site shown in (A). (C) Close-up lateral view of the boxed region in (A), showing the predicted docking pose of luteolin (orange sticks) within the binding pocket. (D) Close-up top view of the boxed region in (B), rotated 90° relative to (C), showing the predicted orientation of luteolin within the same pocket.

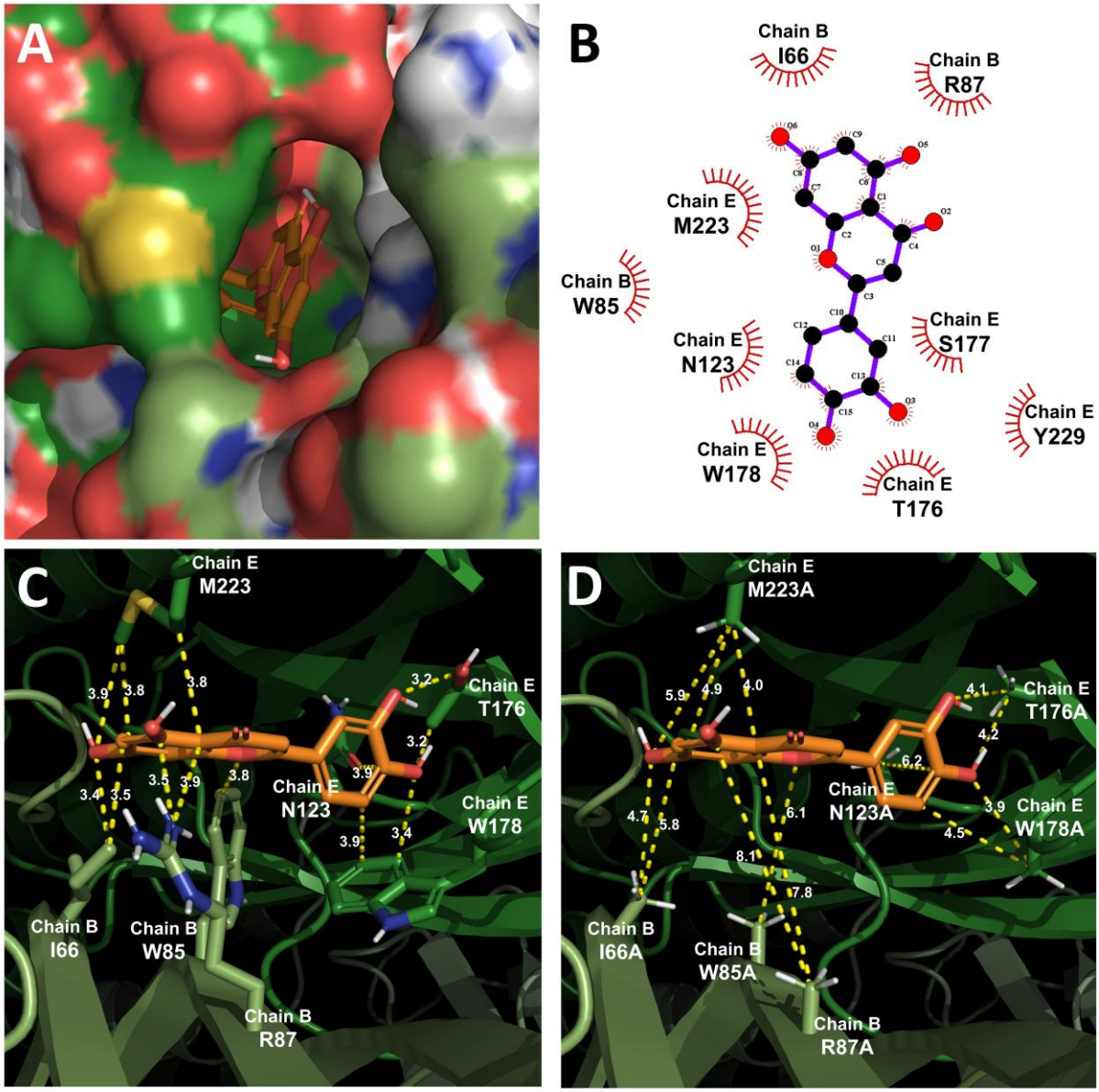
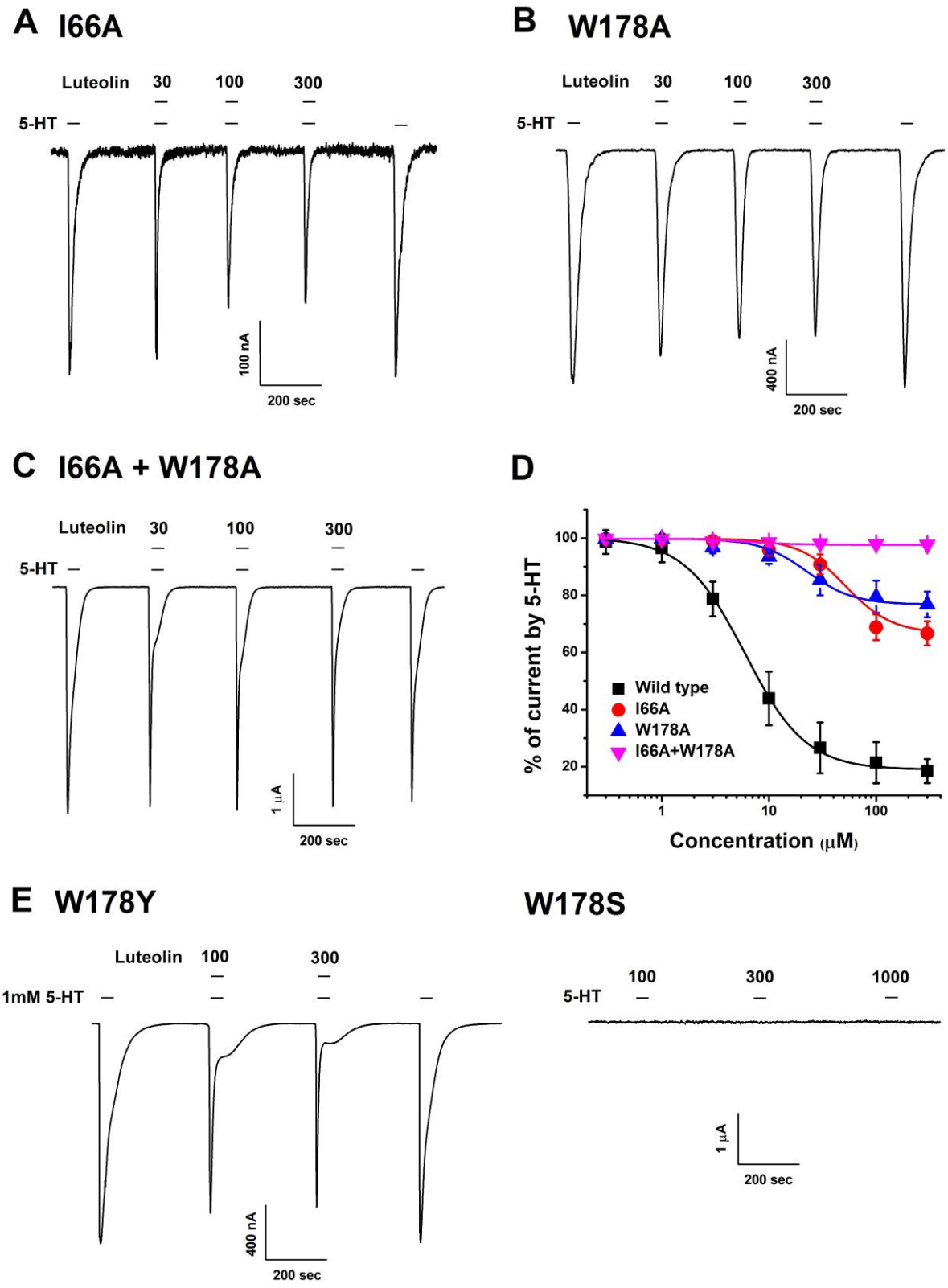


Figure 4. Structural characterization of luteolin binding to the 5-HT3A receptor.

(A) Docking analysis positioned luteolin within a pocket formed at the interface between the extracellular and transmembrane domains of the 5-HT3A receptor. (B) A two-dimensional interaction map depicts the predicted orientation of luteolin in the binding site and the residues involved in ligand recognition. (C, D) Comparison of docking interactions in the wild-type (C) and mutant receptors (D) indicates that individual substitutions differentially alter the pattern of luteolin–receptor contacts.



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712 Figure 5. Effects of luteolin on I66 and W178 mutant 5-HT3A receptors.

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(A–C) Representative current traces of 5-HT-evoked inward current recorded from oocytes expressing I66A (A), W178A (B), or I66A+W178A double-mutant (C) 5-HT3A receptors at a holding potential of −80 mV. Currents evoked by 100 μM 5-HT alone were compared with those evoked in the presence of 30, 100, or 300 μM luteolin. (D) Concentration-inhibition relationships for luteolin at wild-type and mutant 5-HT3A receptors, expressed as the percentage of $I_{5\text{-}HT}$ remaining relative to the response to 5-HT alone (100%). Symbols denote wild-type (■), I66A (●), W178A (▲), and I66A+W178A (▼). Corresponding maximal inhibition (Vmax), IC_{50}, and Hill coefficient values are listed in Table 1. (E) Representative current traces recorded from oocytes expressing the W178Y or W178S mutant receptor at a holding potential of −80 mV, evoked by 1 mM 5-HT (a higher concentration than used in A–C, reflecting the reduced 5-HT sensitivity of these substitutions) alone or in the presence of luteolin (100 or 300 μM for W178Y; 100, 300, or 1000 μM for W178S). The W178S mutant failed to generate a detectable inward current in response to 5-HT at any concentration tested, precluding evaluation of luteolin sensitivity at this construct (see Table 1). Values are shown as mean ± SEM (n = 7–11 oocytes from four individual frogs).

Table 1 Inhibitory effect of luteolin on wild-type and mutant 5-HT3A receptors

	V_{max}	IC_{50}	nH
Wild	81.1 ± 0.9	5.8 ± 0.4	1.4 ± 0.3
I66A	33.4 ± 2.1**	64.3 ± 4.6	1.3 ± 0.5
W178A	24.5 ± 3.5**	24.1 ± 3.2	1.5 ± 0.3
I66A + W178A	2.4 ± 1.2**	6.7 ± 2.8	1.4 ± 0.3
W85A	84.5 ± 2.4	5.5 ± 2.3	1.2 ± 0.5
R87A	72.1 ± 2.5	6.2 ± 5.2	1.1 ± 0.5
N123A	81.4 ± 2.7	5.3 ± 3.1	1.5 ± 0.6
T176A	76.1 ± 2.8	7.4 ± 2.3	1.3 ± 0.5
S177A	77.1 ± 2.7	5.5 ± 2.2	1.4 ± 0.5
M223A	84.1 ± 2.4	7.4 ± 4.2	1.1 ± 0.5
Y229A	79.0 ± 6.4	5.5 ± 2.6	1.2 ± 0.6
W178S	ND	ND	ND
W178Y	28.5 ± 6.7**	21.5 ± 4.8	1.2 ± 0.4

Statistical comparisons of Vmax and Hill coefficient between wild-type and each mutant receptor were performed using one-way ANOVA. **p < 0.001 vs wild-type. The data are expressed as mean ± SEM (n = 6–8) obtained from four individual frogs. Membrane currents were recorded at a holding potential of −80 mV. The IC_{50} values (μM), Hill coefficient (nH), and Vmax (%) were calculated as described in the Materials and Methods section. ND (not determined; no functional response to 5-HT)