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Letter

Serotonin 2A Receptor (5-HT$_{2A}$R) Activation by 25H-NBOMe Positional Isomers: *In Vitro* Functional Evaluation and Molecular Docking

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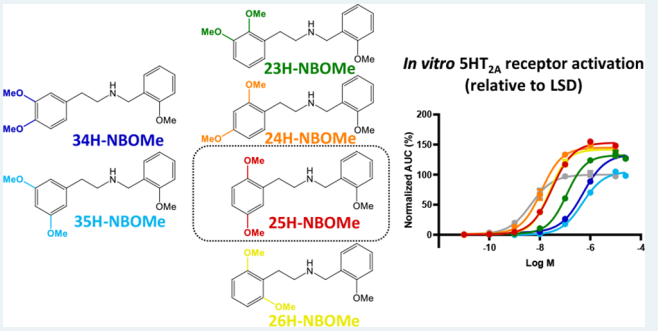
ABSTRACT: Serotonergic psychedelics are defined as compounds having serotonin 2A receptor (5-HT$_{2A}$R) activation as an important pharmacological mechanism. These compounds include the phenylalkylamine class, containing substances with e.g. 2C-X structures (phenethylamines) or their *N*-methoxybenzyl analogues (NBOMes). Besides their abuse potential, psychedelics are increasingly recognized for having therapeutic benefits. However, many psychedelics remain incompletely characterized, even concerning their structure–activity relationships. Here, five positional isomers of 25H-NBOMe, with two methoxy groups on the different positions of the phenyl ring of the phenethylamine moiety, were subjected to split-nanoluciferase assays assessing the *in vitro* recruitment of cytosolic proteins to the 5-HT$_{2A}$R. Furthermore, molecular docking at the 5-HT$_{2A}$R allowed estimation of which residues interact with the specific isomers' methoxy groups. Although the optimal substitution pattern of *N*-unsubstituted phenylalkylamines has been extensively studied, this is the first comparative evaluation of the functional effects of the positioning of the methoxy groups in the phenethylamine moiety of NBOMes.



KEYWORDS: *bioassay, structure–activity relationship, serotonin receptor, molecular docking, psychedelic, new psychoactive substances*

Serotonergic psychedelics are defined as substances that have activation of the serotonin 2A receptor (5-HT$_{2A}$R) as a main pharmacological mechanism.[1] Within the group of serotonergic psychedelics, substances displaying a broad structural variety can be retrieved, both traditionally known substances and new psychoactive substances (NPS), belonging to three different subgroups: ergolines (such as the prototypical psychedelic substance LSD, lysergic acid diethylamide), tryptamines (such as psilocybin), and phenylalkylamines (such as the naturally occurring mescaline).[2] Within the latter group, compounds can be categorized into subgroups, among which are 2C-X (phenethylamines), DOx (phenylisopropylamines), and 25X-NBOMes (*N*-benzyl derivatives of the 2C-X substances).[3] Serotonergic psychedelics comprise a substantial portion of the 950 individual NPS that had been cumulatively reported by the beginning of 2020.[4]

The complex mechanism of action of psychedelics has caused this group of substances to be incompletely characterized. On the one hand, psychedelic substances are consumed for the induction of mystical experiences, empathic feelings, and alterations in consciousness, potentially resulting in severe side effects such as agitation, hyperthermia, rhabdomyolysis, and even death.[1,5,6] On the other hand, psychedelics are increasingly recognized for their potential therapeutic effects. This translates into clinical trials for the treatment of addictions, mood and anxiety disorders, and for the relief of distress concerning death, mainly with LSD and psilocybin.[7]

Substantial efforts have been invested in the characterization of psychedelic substances, their structure–activity relationships, and their receptor interaction(s). Very recently, structural data became available for the 5-HT$_{2A}$R interacting with different ligands.[8,9] Interestingly, these structures hint at differential binding modes for the prototypical psychedelic substance LSD and the *N*-benzyl substituted phenethylamine 25CN-NBOH, a substance highly similar to those in the recently emerging NBOMe class.[8] Despite extensive data on several aspects of the structure–activity relationship of phenylalkylamine psychedelics, an in-depth examination of the influence of the position of the methoxy groups on the

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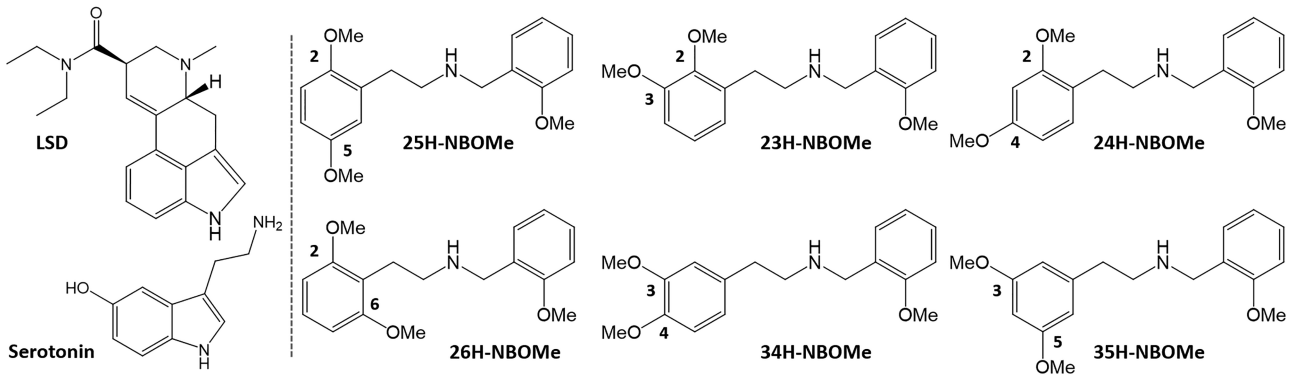

Figure 1. Structures of the substances tested in the 5-HT_{2A}R bioassays: the reference substances LSD and serotonin and the "conventionally substituted" 25H-NBOMe, together with the five tested positional isomers. The numbers in the name of the positional isomer correspond to the methoxy group positions in the phenethylamine fragment, e.g. 25H-NBOMe has one methoxy group at position 2 and one methoxy group at position 5.

phenethylamine moiety in NBOMes, while leaving the *N*-methoxybenzyl group intact, has remained unexplored.[2] In this study, we compared the potential of a set of five isomers of 25H-NBOMe (of which the structures are given in Figure 1) to induce similar effects to the "conventional" 25X-NBOMes at a molecular level. In the name of the positional isomers, the number (i.e., 25, 23, 24, 26, 34, and 35) indicates the positions at which methoxy groups were introduced, e.g. 25H-NBOMe has methoxy groups at positions 2 and 5 of the phenyl ring of the phenethylamine moiety of the molecule. Recently, the synthesis and spectral characterization of these five positional isomers of 25H-NBOMe has been described.[10] Functional characterization was performed by assessing the potential of these isomers to induce recruitment of cytosolic proteins to the activated 5-HT_{2A}R. To this end, we employed previously established bioassays, expressing either β-arrestin 2 (βarr2) or miniGα_q with the 5-HT_{2A}R in the NanoBiT system, in which a split nanoluciferase is functionally complemented following recruitment of the cytosolic protein to the activated 5-HT_{2A}R.[11,12] Additionally, a molecular model was used, based on adaptations of the above-mentioned published cryo-EM structure,[8] for the molecular docking of each of the tested substances into the 5-HT_{2A}R, suggesting interactions between the methoxy groups on different positions and specific receptor residues.

PHARMACOLOGICAL CHARACTERIZATION

To assess the functionality of the NBOMe positional isomers, the Nanoluciferase Binary Technology (NanoBiT) was employed. This technique was specifically developed for the real-time monitoring of protein−protein interactions in live cells. To this end, two nonfunctional parts of the nanoluciferase are each fused to one of the potentially interacting proteins, in this case the 5-HT_{2A}R and a cytosolic protein. When an agonist activates the receptor, the cytosolic protein is recruited, leading to association of the split parts, which can be monitored via a luminescent readout in the presence of the enzyme's substrate.[13] We previously established a bioassay to monitor the recruitment of βarr2, later complemented with an analogous miniGα_q recruitment assay, allowing us to generate concentration−response curves.[11,12] These can be used to derive the potency and efficacy of the test compounds, as compared to a reference agonist, of which the efficacy is arbitrarily set at 100%. For the functional characterization of the described positional isomers, several conditions were taken into consideration to exclude the possibility of making presumptuous conclusions based on the assay chosen, the method of analysis (with kinetic implications), or the included reference agonist. Therefore, besides performing the analyses with our standard βarr2 recruitment assay with LSD as a reference agonist, we also conducted a miniGα_q recruitment assay. In both assays, both serotonin and LSD were included in all experiments as reference agonists. Additionally, data analysis was conducted using the area under the curve (AUC) of either the first 30 min of the real-time activation profile or the full (standard) 2 h activation profile. The EC_{50} (as a measure of potency) and E_{max} (as a measure of efficacy) values for all assessed conditions are summarized in Tables 1 (βarr2) and 2 (miniGα_q). Figure 2 provides concentration−response curves for all substances, calculated for the 2 h activation profile, with either LSD (A and B) or serotonin (C and D) as the reference agonist.

As a "standard" setup, we routinely apply the βarr2 recruitment assay with LSD as a reference agonist and using the 2 h activation profiles for the calculation of the AUC. Table 1 and the corresponding Figure 2A readily show that there are substantial differences in the potency and efficacy of the compounds that are diversely substituted. The "conventionally substituted" 25H-NBOMe serves as the point of comparison for the diversely substituted isomers, yielding a low nanomolar potency (an EC_{50} value of 11.4 nM) in the bioassay and a high efficacy relative to LSD (an E_{max} of 164%). Only one of the isomers is slightly more potent than 25H-NBOMe: 24H-NBOMe yielded an EC_{50} value of 3.88 nM in the βarr2 recruitment assay. The potency of 26H-NBOMe (8.70 nM) is similar to that of 25H-NBOMe. The highest EC_{50} value (33.6 nM) of all isomers with a 2-methoxy substituent, and hence the lowest potency, was observed for 23H-NBOMe. The least potent substances in the βarr2 recruitment assay appeared to be 34H-NBOMe and 35H-NBOMe, both lacking the 2-methoxy substituent. This omission resulted in a markedly reduced *in vitro* potency, with EC_{50} values in the higher nanomolar range (248 and 343 nM, respectively). Concerning the efficacies of the compounds, a more narrow range is obtained. 25H-NBOMe appears to have the highest efficacy (164%) of all tested substances, although its 95% confidence interval is overlapping with those of 24H-, 26H-, and 34H-NBOMe, with efficacies of 145, 156, and 147%, respectively. Although the efficacies obtained for 23H-NBOMe (123%) and 35H-NBOMe (118%) were lower than that of 25H-NBOMe,

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Table 1. Summary of the Potency (EC_{50}) and Efficacy (E_{max}, Where the E_{max} of Either Serotonin or LSD is Set to 100%) Values of All the Tested Compounds in the βarr2 Assay, Calculated Using Either the Full 120 Min or the First 30 Min Activation Profiles[a]

	120 min activation profiles			
	reference agonist LSD		reference agonist serotonin	
compound	EC_{50} (nM, CI)	E_{max} (%, CI)	EC_{50} (nM, CI)	E_{max} (%, CI)
LSD	7.43 (3.54–12.9)	100 (91–109)	7.29 (3.50–12.7)	87.1 (79.4–95.4)
serotonin	7.62 (4.43–13.2)	116 (107–126)	7.24 (4.43–11.9)	100 (93.9–108)
23H-NBOMe	33.6 (19.8–57.3)	123 (115–131)	33.7 (19.7–57.9)	115 (107–123)
24H-NBOMe	3.88 (2.33–6.37)	145 (136–154)	3.89 (2.30–6.45)	136 (127–145)
25H-NBOMe	11.4 (6.36–20.4)	164 (151–180)	11.0 (5.60–21.5)	134 (121–148)
26H-NBOMe	8.70 (5.81–12.5)	156 (147–165)	8.74 (5.91–12.4)	146 (138–154)
34H-NBOMe	248 (111–646)	147 (129–182)	238 (101–691)	120 (104–152)
35H-NBOMe	343 (187–645)	118 (107–135)	310 (165–593)	94.1 (83.8–108)
	30 min activation profiles			
	reference agonist LSD		reference agonist serotonin	
compound	EC_{50} (nM, CI)	E_{max} (%, CI)	EC_{50} (nM, CI)	E_{max} (%, CI)
LSD	24.3 (ND)	100 (91–110)	23.8 (ND)	79.4 (72.0–87.3)
serotonin	11.5 (7.13–18.9)	128 (119–139)	10.9 (7.60–15.8)	100 (94.8–106)
23H-NBOMe	74.4 (55.8–95.9)	133 (128–139)	74.5 (53.9–98.8)	114 (109–119)
24H-NBOMe	8.34 (6.11–11.1)	154 (147–161)	8.32 (5.85–11.5)	132 (125–139)
25H-NBOMe	24.3 (13.4–45.0)	179 (163–199)	22.9 (12.8–41.8)	130 (118–143)
26H-NBOMe	16.3 (13.0–20.8)	165 (159–172)	16.4 (12.4–22.0)	142 (135–149)
34H-NBOMe	488 (226–1707)	162 (142–218)	458 (191–2287)	118 (101–171)
35H-NBOMe	678 (385–1295)	137 (123–162)	625 (353–1148)	98.5 (88.8–115)

[a]The structures of the compounds are provided in Figure 1. Data are from three independent experiments. CI: 95% confidence interval. ND: confidence interval not determined.

Table 2. Summary of the Potency (EC_{50}) and Efficacy (E_{max}, Where the E_{max} of Either Serotonin or LSD is Set to 100%) Values of All the Tested Compounds in the miniGα_q Recruitment Assay, Calculated Using Either the Full 120 Min or the First 30 Min Activation Profiles[a]

	120 min activation profiles			
	reference agonist LSD		reference agonist serotonin	
compound	EC_{50} (nM, CI)	E_{max} (%, CI)	EC_{50} (nM, CI)	E_{max} (%, CI)
LSD	7.40 (1.82–17.7)	100 (84–115)	7.23 (1.73–17.5)	39.6 (33.7–46.0)
serotonin	66.4 (35.5–151)	256 (230–301)	63.5 (33.9–143)	103 (92.5–121)
23H-NBOMe	147 (71.8–436)	81.2 (70–102)	146 (73.8–408)	31.9 (27.5–39.6)
24H-NBOMe	22.1 (12.1–42.1)	144 (129–161)	22.2 (12.4–41.6)	56.9 (51.2–63.4)
25H-NBOMe	49.6 (27.2–84.8)	141 (128–155)	49.2 (26.6–85.4)	58.8 (53.3–64.9)
26H-NBOMe	40.9 (23.7–68.2)	136 (124–148)	40.9 (23.9–67.7)	53.3 (48.8–58.2)
34H-NBOMe	974 (ND)	134 (ND)	947 (ND)	55.3 (45.6–297)
35H-NBOMe	1097 (ND)	89.1 (74–219)	1087 (ND)	37.1 (30.5–112)
	30 min activation profiles			
	reference agonist LSD		reference agonist serotonin	
compound	EC_{50} (nM, CI)	E_{max} (%, CI)	EC_{50} (nM, CI)	E_{max} (%, CI)
LSD	17.7 (10.1–38.6)	98.2 (85–114)	16.3 (8.59–38.7)	31.9 (27.4–37.2)
serotonin	88.5 (46.1–213)	305 (272–365)	62.2 (22.5–269)	87.9 (74.4–121)
23H-NBOMe	236 (ND)	89.6 (75–112)	191 (ND)	27.1 (22.6–33.3)
24H-NBOMe	37.4 (20.6–66.4)	171 (155–189)	35.1 (17.5–68.7)	54.3 (48.5–61.2)
25H-NBOMe	86.2 (57.0–124)	181 (167–196)	129 (69.2–263)	74.8 (65.6–88.8)
26H-NBOMe	68.0 (38.6–115)	162 (147–181)	68.3 (34.0–129)	51.9 (46.0–59.4)
34H-NBOMe	1321 (629–33 780)	156 (130–340)	1334 (ND)	51.8 (ND)
35H-NBOMe	1393 (ND)	107 (94–163)	1296 (683–10 760)	48.3 (41.0–83.6)

[a]Data are from three independent experiments. CI: 95% confidence interval. ND: confidence interval not determined.

it is remarkable that all substances could still be categorized as (at least) equally efficacious as LSD and serotonin.

The potency for the reference psychedelic substance LSD, yielding an EC_{50} value of 7.43 nM, closely corresponds with our previously reported values (5.95 nM, 5.96 nM, 6.41 nM and 5.95 nM), obtained by measuring βarr2 recruitment to the 5-HT_{2A}R in the NanoBiT system.[11,12,14]

Also when using serotonin as a reference and/or when using a 30 min rather than a 2 h read-out for the βarr2 recruitment assay (Table 1), the derived potency values for the different substances yielded the same conclusions as those from our "standard" setup. Despite an upward shift in the absolute EC_{50} values when using a shorter read-out time, the ranking order of potencies of the assessed isomers remained unaltered, irrespective of whether serotonin or LSD was used as the reference agonist, and whether the 30 min or 2 h AUC was employed for calculation. A change in reference agonist or analysis time also did not yield different conclusions when considering the efficacies of the different isomers. With a single exception (35H-NBOMe; E_{max} 94.1 and 98.5 %, with serotonin as a reference), the efficacies of all different isomers superseded those of both LSD and serotonin. All methods of analysis classified 24H-, 25H-, 26H-, and 34H-NBOMe as the more efficacious substances and 23H- and 35H-NBOMe as the less efficacious substances.

As different assays could potentially lead to different outcomes, additionally, a different but highly analogous bioassay was taken, monitoring the recruitment of miniGα_q (of which the engineering was described by Nehmé et al.[15]) to the 5-HT_{2A}R in the NanoBiT system, as previously described.[12] A first prominent observation here is that in this assay setup, the efficacy of serotonin exceeds that of LSD 2.5- to 3-fold. This finding matches the description of LSD as a less efficacious 5-HT_{2A}R agonist than serotonin.[1,16−18] When comparing the results in Table 2 with those in Table 1, and

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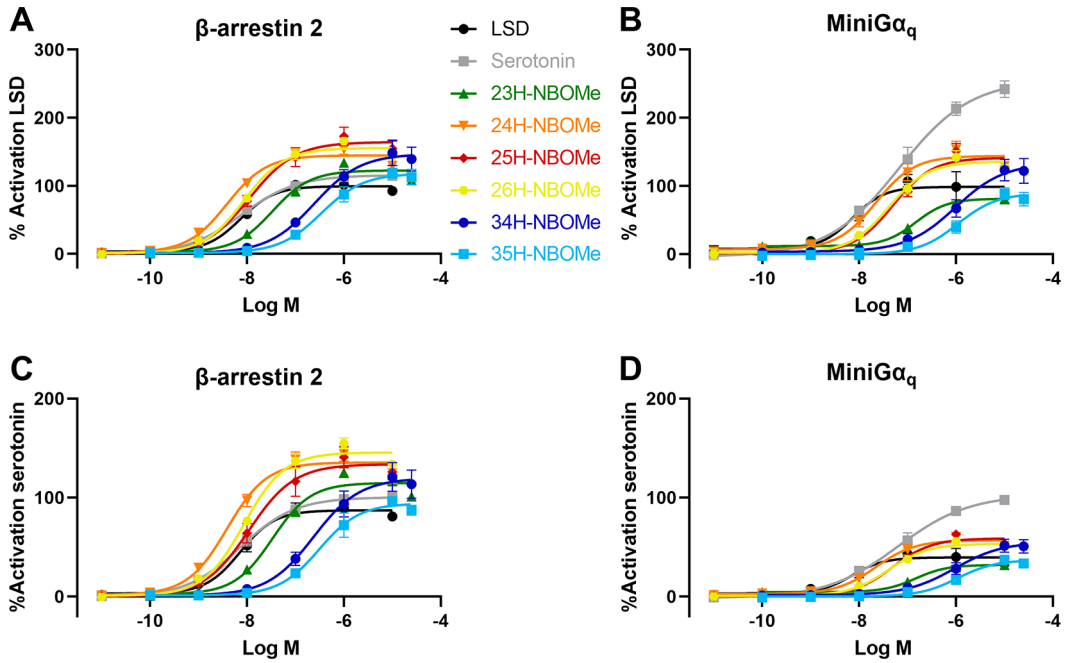

Figure 2. Concentration−response curves obtained by stimulation of the 5-HT$_{2A}$R followed by recruitment of (A and C) βarr2 or (B and D) miniGα_q constructs in the NanoBiT system. Data points are given as the mean of three independent experiments (each performed in duplicate) ± SEM (standard error of the mean). The AUC is normalized in each independent experiment for the maximum response (100%) of the reference agonist LSD (A and B) or serotonin (C and D).

with each other, an upward shift in EC_{50} values can be observed, and when considering serotonin as a reference, a downward shift in the E_{max} of all NBOMes is observed, with the efficacy of none of the NBOMes exceeding that of serotonin in this assay format. Yet, despite these global differences between both assays, comparative analysis of the different NBOMe isomers revealed that, also here, the rank order of potencies remained unaltered, as did the classification of the isomers into more and less efficacious substances. Interestingly, the differences in efficacies were somewhat more pronounced in this assay format; the efficacy of 24H-, 25H-, 26H-, and 34H-NBOMe consistently exceeded that of LSD, while that of 23H-NBOMe was consistently lower. 35H-NBOMe also showed a trend toward efficacy values near to or lower than that of LSD, although its low potency did not allow the setup of ideal concentration−response curves.

Overall, this extensive set of experiments led us to conclude that neither the functional assay used, nor the reference agonist, nor the time point of analysis influenced the conclusions drawn for this set of NBOMe positional isomers, i.e. that 24H-NBOMe is the most potent positional isomer, followed by 26H- and 25H-NBOMe, with a lower potency for 23H-NBOMe, and even lower potencies for 34H and 35H-NBOMe. Smaller differences were observed between the isomers in terms of efficacy, classifying 25H-, 26H-, 24H-, and 34H-NBOMe as the more efficacious substances and 23H- and 35H-NBOMe as the less efficacious substances in the set.

Little to no pharmacological information is available on the tested positional isomers, with four of these (23H-, 26H-, 34H-, and 35H-NBOMe) never having been functionally evaluated before.[10] Rickli et al. reported a potency value of 490 nM for 25H-NBOMe, employing a FLIPR assay, while Eshleman et al. obtained an EC_{50} value of 40 nM by measuring inositol-phosphate 1 formation.[16,17] Braden et al. found a slightly increased potency for 24H-NBOMe (4.0 nM) as compared to 25H-NBOMe (15.3 nM), employing an inositol phosphate accumulation assay, with similar efficacies for the two compounds.[18] From this limited amount of pharmacological data, and as also confirmed by our experiments, it is clear that the use of different signaling events as a readout method can severely impact the obtained numbers, thereby hampering straightforward interpretation and comparability of results obtained in different assays. However, apart from the findings obtained with the FLIPR assay, our findings and the order of magnitude of the obtained potency values correspond with the literature.

The optimal substitution pattern of the phenyl group of the *N*-unsubstituted phenylalkylamine psychedelics has been studied extensively, both *in vitro* and *in vivo*. In PiHKAL, the most-explored patterns are 2,4,5-substitution of the phenyl ring and 3,4,5-substitution, with the former being concluded to be the most effective. It has been suggested that the 2,5-dimethoxy pattern is to be kept intact for optimal psychedelic activity as well as receptor activity and affinity, while the substituent at position 4 can be modified. Furthermore, it is hypothesized that the 2,4,6-substitution pattern could involve potentially active substances. On the other hand, receptor mutation studies have suggested that the 5-substituent is of lesser importance for phenethylamines than for phenylisopropylamines. This emphasizes the dramatic impact of the position of the methoxy groups on the psychedelic activity and receptor binding/activation of an individual substance.[2,19,20] In a recent study, the 2- and 5-desmethoxy analogues of 2C−B and DOB were individually tested both in an *in vitro* Ca^{2+} release assay and *in vivo* through the head twitch response in mice. This study reported modestly reduced binding affinities and functional potencies at the 5-HT$_{2A}$R and 5-HT$_{2C}$R, with the removal of the 2-methoxy group having a more severe impact than that of the 5-methoxy group. Removal of either, however, appears to have a more dramatic impact on the *in*

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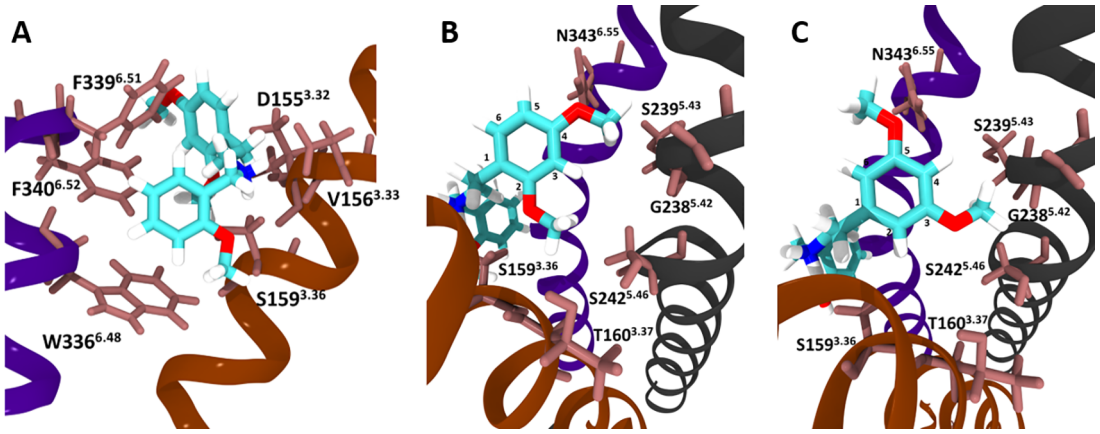

Figure 3. Visual representations of the NBOMe isomers docked into the binding pocket of the 5-HT_{2A}R (based on PDB: 6WHA).[8] (A) 24H-NBOMe, as seen from the perspective of the *N*-benzyl moiety; (B) 24H-NBOMe and (C) 35H-NBOMe bound to the 5-HT_{2A}R looking from the perspective of the phenyl group, with specific mentioning of the residues proposed to interact with the methoxy groups on the phenethylamine moiety.

Table 3. Summary of the Interaction Energies (kcal/mol) between Compounds and the 5-HT_{2A}R

compound	$S159^{3.36}$	$D155^{3.32}$	$V156^{3.33}$	$W336^{6.48}$	$F339^{6.51}$	$F340^{6.52}$
LSD	−3.50	−18.5	−13.5	−2.30	−10.49	−6.39
25CN-NBOH	−9.34	−18.3	−10.7	−7.30	−8.76	−7.73
23H-NBOMe	−11.5	−19.8	−11.3	−9.06	−9.41	−8.72
24H-NBOMe	−9.86	−19.5	−11.2	−8.66	−9.35	−8.36
25H-NBOMe	−9.90	−19.3	−11.0	−8.58	−9.01	−7.54
26H-NBOMe	−9.77	−20.7	−12.9	−8.70	−9.16	−7.89
34H-NBOMe	−8.59	−20.7	−10.6	−8.62	−9.30	−8.39
35H-NBOMe	−8.72	−20.49	−10.1	−8.54	−10.27	−7.25

Table 4. Summary of the Interaction Energies (kcal/mol) Involved in the Proposed Interaction between the Methoxy Groups on the Phenyl Ring and Nearby Amino Acid Residues on the 5-HT_{2A}R

compound	$T160^{3.37}$	$G238^{5.42}$	$S242^{5.46}$	$S239^{5.43}$	$V235^{5.39}$	$N343^{6.55}$	$L229^{ECL2}$
LSD	−1.25	−1.54	−6.46	−4.01	−2.28	−5.02	−9.91
25CN-NBOH	−2.38	−3.07	−1.90	−2.13	−0.68	−1.69	−2.98
23H-NBOMe	−2.77	−2.04	−4.86	−1.29	−0.57	−0.99	−0.56
24H-NBOMe	−2.14	−1.07	−1.74	−2.18	−3.38	−2.25	−1.13
25H-NBOMe	−2.43	−0.82	−1.81	−0.59	−0.93	−1.61	−2.25
26H-NBOMe	−2.51	−0.93	−1.70	−0.60	−0.58	−0.73	−1.76
34H-NBOMe	−0.47	−2.69	−3.23	−2.29	−3.35	−0.95	−1.09
35H-NBOMe	−0.21	−2.26	−2.57	−1.46	−1.60	−3.18	−2.46

vivo head twitch response.[21] Similarly, the employed *in vitro* recruitment assays also hint at the importance of the 2-methoxy group for strong activation of the 5-HT_{2A}R in the group of NBOMes. Not only do we find markedly decreased potencies for the 34H- and 35H-NBOMe isomers, we also find that 24H- and 26H-NBOMe are (at least) equally as potent as 25H-NBOMe with, in comparison, a decreased potency of 23H-NBOMe. This underscores the importance of the 2-methoxy group and, at the same time, suggests that a substituent at position 3 on the phenyl group of the phenethylamine moiety reduces *in vitro* receptor activation, or that the introduction of a methoxy group at the position next to position 2 may negatively impact the positive effect of that methoxy group. Distinct interactions of the distinctly substituted NBOMes at the 5-HT_{2A}R are indeed supported by molecular docking data, as discussed further.

While multiple reports are available on the substitution pattern of the phenyl group of *N*-unsubstituted phenylalkylamine psychedelics (e.g., 2C-X and DOx), this effect is largely unstudied in their *N*-methoxybenzyl counterparts. The introduction of a methoxybenzyl group at the *N*-position of the phenethylamine results in the NBOMe group of substances, with higher affinities and potencies reported for 25H-NBOMe than for the unsubstituted counterpart 2C-H.[2,14,17,18] Literature indicates that the 4-substitution, albeit very important for the activity of 2C-X and DOx substances, is not as essential for the receptor activation by NBOMes.[3,14] Furthermore, in an attempt to reduce the high first-pass metabolism of NBOMes, several substitutions of the methoxy group at position 5, reportedly the metabolic "soft spot" of NBOMes, have been explored. When assessing the functionality of these molecules, it was concluded that the 2,5-dimethoxy motif is not necessarily as imperative for the *in vitro* functionality as it was in the *N*-unsubstituted psychedelics, finding reduced affinity but comparable potency and efficacy in a Ca^{2+}/Fluo-4 assay upon omission of the 5-methoxy group of

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25B-NBOMe.[22] Our findings are consistent with the aforementioned literature, as changing the position of the 5-methoxy group in additionally 2-methoxy substituted NBOMes does not necessarily result in severely altered potencies and efficacies, at least *in vitro*. In addition, in this study, we find the 2,4-dimethoxy pattern to result in a higher potency and similar efficacy as the 2,5-dimethoxy pattern in NBOMes with no further phenethylamine moiety substituents.

MOLECULAR DOCKING

Attempting to provide an explanation for the differences in potencies/efficacies observed for the isomers, molecular docking was performed, with a model based on adaptations of the recently published cryo-EM structure of the 5-HT_{2A}R in complex with 25CN-NBOH (PDB: 6WHA).[8] Figure 3A shows the position of 24H-NBOMe in the orthosteric binding pocket of the receptor, in which all of the isomers bind. Figure 3B and 3C focus on the proposed interactions of the methoxy groups on the phenethylamine moiety of 24H-NBOMe and 35H-NBOMe with the amino acid residues of the receptor, providing insight into the modeled interaction of specific residues with the methoxy groups. The analogous figures for the other isomers and 25CN-NBOH are provided in Supplementary Data. Tables 3 and 4 show the calculated interaction energies between the molecules and specific 5-HT_{2A}R residues, with the latter specifically focusing on the interactions of the methoxy groups on the phenethylamine moiety of the isomers. The values in these Tables reflect the model-derived strength of the interactions between a certain amino acid residue of the binding pocket and the respective ligand. The more negative the given energy, the stronger the proposed interaction would be. These values are obtained through Prime MM-GBSA (Molecular Mechanics-Generalized-Born Surface Area), a tool for which the calculated values have previously been shown to provide a good estimate for the relative binding affinities of a set of ligands.[23,24] The model showed that all NBOMe isomers can be docked in the same binding pocket as 25CN-NBOH, despite the change of the hydroxyl group on the *N*-benzyl moiety into a methoxy group. This latter group specifically stabilizes the molecule through an H-bond with $S159^{3.36}$ (as also reflected by the interaction energies, lying between −8.5 and −11.5 kcal/mol), and through hydrophobic interaction with $S159^{3.36}$, $W336^{6.48}$ and $S373^{7.46}$. The nitrogen atom is observed to form a salt bridge with $D155^{3.32}$, which has been previously defined as critical for receptor interaction.[8] The strongly negative values in Table 3 (between −18 and −20 kcal/mol) are indeed consistent with a strong salt bridge-type interaction with $D155^{3.32}$. The *N*-benzyl moiety is stabilized in the binding pocket by hydrophobic interactions with $W336^{6.48}$ and $F339^{6.51}$, and both the *N*-benzyl moiety and the phenyl group of the phenethylamine function are stabilized by interaction with $F340^{6.52}$. Additionally, the strongly negative values obtained for $F339^{6.51}$ and $F340^{6.52}$ confirm the strength of this interaction, consistent with pi stacking interactions. The reference compound LSD interacts with $S242^{5.46}$ stronger than NBOMe compounds due to an H-bond between the indole on LSD and $S242^{5.46}$, in which NBOMes cannot participate (Table 4).

Because of the ability of the utilized model to predict to a reasonable extent the interactions of LSD and 25CN-NBOH with the 5-HT_{2A}R, as described by Kim et al.,[8] it was additionally used to assess the interactions with the methoxy groups of the phenethylamine moiety of the panel of NBOMe positional isomers. In contrast to the energies presented in Table 3, the interaction energies in Table 4, representing the interactions of the respective methoxy groups with residues $T160^{3.37}$, $G238^{5.42}$, $S239^{5.43}$, $S242^{5.46}$, $V235^{5.39}$, $N343^{6.55}$, and $L229^{ECL2}$, indicate relatively weak interactions with the isomers. This Table, however, shows strong interaction energies between LSD and receptor residues $S242^{5.46}$ and $L229^{ECL2}$, consistent with observations by Kim et al.[8] While it is not straightforward to speculate on the significance of the interaction energies between the receptor residues and the different isomers, as depicted in Table 4, some patterns do seem to emerge. When all substances carrying a methoxy group at a certain position have a stronger interaction energy (more negative value), this suggests an interaction between the methoxy group at that position and the concerned receptor residue. Important here is to additionally consider the steric effects that are relevant when two methoxy groups are placed on adjacent positions (e.g., 23H-NBOMe and 34H-NBOMe). Overall, this approach allows to propose an explanation for certain trends in interaction energies. A hydrophobic interaction between a methoxy group on position 2 of the phenyl ring of the phenethylamine moiety (as is the case in 23H-, 24H-, 25H-, and 26H-NBOMe) with $T160^{3.37}$, $S159^{3.36}$, and $V156^{3.33}$ is proposed. The model also indicates hydrophobic interactions between $G238^{5.42}$ and $S242^{5.46}$ and a methoxy group at position 3, as reflected by a weaker interaction of these residues with substances lacking a 3-methoxy group. For 25CN-NBOH, $G238^{5.42}$ would interact with the cyano group rather than with a methoxy group through a weak electrostatic interaction. The occurrence of a methoxy group at position 4 appears to be linked to a stronger hydrophobic interaction (lower interaction energies) with residues $S239^{5.43}$ and $V235^{5.39}$. Overall, substances containing a 5-methoxy group show a relatively stronger hydrophobic interaction with residues $N343^{6.55}$ and $L229^{ECL2}$ than substances lacking that group, with the exception of 24H-NBOMe, where the 4-methoxy group may also interact with $N343^{6.55}$. However, the obtained values for these latter two residues indicate substantially weaker interactions with these residues than those observed for LSD. Lastly, a methoxy group at position 6 appears to contribute to the already strong interaction with $V156^{3.33}$ and $D155^{3.32}$ and weakly interacts with $L229^{ECL2}$. Based on the interactions proposed here, it is clear that there is no trivial or "single-residue" explanation for the observed lower potencies and efficacies of 23H-, 34H-, and 35H-NBOMe, suggesting a concerted impact of the interaction with several residues. It must also be taken into consideration that this model was adapted from a mini$G\alpha_q$ bound 5-HT_{2A}R structure and that the presence of βarr2 could differentially impact ligand interaction. Within the context of this study, it was not possible to generate binding data; however, such data may help to explain some of the observed differences in receptor activation by different positional isomers.

Even though *in vitro* data on receptor activation provide valuable information on newly synthesized substances, the extrapolation to the *in vivo* effects in humans remains difficult. On the one hand, the actual mechanism inducing psychedelic effects (on a molecular level) remains elusive. Contributing factors could involve biased agonism, receptor dimerization, and activation of receptors other than the 5-HT_{2A}R.[25,26] Several additional factors besides receptor activation need to be taken into account. An example of such a factor is the potential (first pass) metabolism of these substances, as

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NBOMes are prone to a high intrinsic clearance. The 5-methoxy group of NBOMes has been defined to be the metabolic soft spot of the molecule, and the omission of this group was found to decrease the intrinsic clearance. The effect of the reintroduction of this methoxy group at a different position has not been assessed.[22,27] Additionally, the potencies and efficacies reported here only reflect the ability of the compounds to recruit certain cytosolic proteins to the receptor. From these data, it is not possible to derive receptor binding affinities of the substance, because efficacy and potency do not necessarily correlate with the affinity. It is therefore theoretically possible that certain isomers would have higher receptor affinities than others, but would yield a "less effective" receptor configuration, and thus a lower potency and/or efficacy for a given pathway. In addition, it remains elusive how the methoxy groups on different positions of the phenyl group of the phenethylamine moiety of NBOMes will impact the pharmacokinetic and pharmacodynamic properties of the molecules *in vivo*. Besides that, the use of artificial systems such as cell lines expressing assay components, as used here, may cause another layer of difficulty in the interpretation of obtained *in vitro* results.[28]

In conclusion, we report on the functional characterization of a set of positional isomers of 25H-NBOMe, in which the methoxy groups are placed in different positions of the phenethylamine moiety. More specifically, the impact of the structural changes on the ability of the activated 5-HT_{2A}R to recruit cytosolic proteins was assessed. To this end, bioassays monitoring the recruitment of either βarr2 or miniGα_q to the 5-HT_{2A}R were employed, yielding a luminescent readout upon functional complementation of the assay components. The results show clear differences in the EC_{50} (as a measure of potency) and E_{max} (as a measure of efficacy) values of the differentially substituted isomers. Overall, the isomers with a methoxy group on position 2 were more potent than those that did not have this substituent, with 24H-NBOMe being the most potent substance tested, and 23H-NBOMe being slightly less potent than 25- and 26H-NBOMe. The two isomers lacking the 2-methoxy group, 34- and 35H-NBOMe, were substantially less potent than the others. In terms of efficacy, the differences are less evident, with 23H- and 35H-NBOMe being markedly less efficacious than all other isomers, although they remained approximately as efficacious as LSD. The results showed that nor the reference agonist, nor the assay employed, nor the method of analysis influenced these findings. Data obtained from molecular docking of these substances into a 5-HT_{2A}R model suggest specific residues that interact with the specific methoxy groups on the phenyl moiety of the phenethylamine part of the molecule which, in a concerted manner, result in the observed differential receptor activation potential of the differently substituted isomers. While the methoxy pattern on the phenylalkylamine moiety has been described extensively for *N*-unsubstituted psychedelics, this is the first report comparatively assessing the functional effects of this isomerization in their NBOMe counterparts.

MATERIALS AND METHODS

Chemicals and Reagents. Dulbecco's modified Eagle's medium (DMEM, supplemented with GlutaMAX), Hank's Balanced Salt Solution (HBSS), and penicillin/streptomycin (10 000 IU/mL and 10 000 μg/mL) were purchased from Thermo Fisher Scientific (Pittsburgh, PA, United States). The FuGENE HD Transfection Reagent, Nano-Glo Live Cell reagent, and the Nano-Glo LCS Dilution buffer were procured from Promega (Madison, WI, United States). Fetal bovine serum (FBS), poly-D-lysine hydrobromide, methanol, and the analytical standards of LSD (lysergic acid diethylamide) and serotonin were bought from Sigma-Aldrich (Overijse, Belgium). The analytical standard of 25H-NBOMe hydrochloride 2-(2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)-ethanamine was from Chiron AS (Trondheim, Norway). The positional isomers of 25H-NBOMe, depicted in Figure 1, were synthesized as described previously and dissolved in methanol.[10]

Cell Culture and the 5-HT_{2A}R Activation Assays. Human Embryonic Kidney (HEK) 293T cells were routinely cultured in DMEM (GlutaMAX, supplemented with 10% heat-inactivated FBS, 100 IU/mL of penicillin, 0.25 μg/mL amphotericin B and 100 μg/mL streptomycin) in a humidified atmosphere of 37 °C and 5% CO_2. For the 5-HT_{2A}R activation assays, the cells were seeded in 6-well plates at a density of 500 000 cells per well, following the protocol as described before.[11,12] Following overnight incubation, the cells were transfected with 1.65 μg of both the 5-HT_{2A}R and the cytosolic protein (either βarr2 or the miniGα_q protein) in the NanoBiT system (NanoLuc Binary Technology), employing FuGENE in a 3:1 FuGENE:DNA ratio, according to the manufacturer's protocol. After 24 h, the cells were seeded in poly-D-lysine coated 96-well plates at a density of 50 000 cells per well and incubated overnight. The cells were then rinsed twice with HBSS, and 100 μL of HBSS was added to each of the wells. To this, 25 μL of Nano-Glo Live Cell Substrate was added (diluted 1/20 in the Nano-Glo LCS Dilution buffer, according to the manufacturer's protocol), and the plate was transferred to the Tristar^2 LB 942 multimode microplate reader (Berthold Technologies GmbH & Co, Germany). After the equilibration phase, 10 μL of the 13.5× concentrated agonist solution was added, and the luminescent signal was monitored for 2 h. Each experiment was performed in duplicate with at least three independent experiments per compound. For the purpose of normalization of the data, on each 96-well plate, a concentration curve was run of the reference agonists LSD and serotonin. Appropriate solvent controls were included per condition.

Data Processing and Analysis. The obtained time-luminescence profiles were corrected for interwell variability and used for the calculation of the AUC, using either the full activation profiles of 2 h or only the first 30 min, as described previously in more detail.[29] After subtraction of the AUC of the solvent control, the data were used for the fitting of a sigmoidal concentration−response curve through the four-parametric nonlinear regression model in GraphPad Prism software (San Diego, CA, United States). For each separate experiment, the data were normalized to the maximal response of the reference agonist, either LSD or serotonin, alternately set at 100% for a comparison between the obtained results. The data of all individual experiments were then pooled to determine the total EC_{50} and E_{max} values per substance.

Molecular Docking. The recently published cryo-EM structure (PDW: 6WHA) of the 5-HT_{2A}R in complex with miniGα_q and the psychedelic substance 25CN-NBOH was used as a starting template.[8] Each of the structures of the -NBOMe isomers and LSD and 25CN-NBOH (as controls) was built and optimized in Spartan '18 Parallel Suite (Wave function, Irvine, CA, United States) and molecularly docked, employing induced fit docking (Schrödinger, NY, United

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States). To reduce atom clashing, ligand−receptor complexes were minimized using the OPLS3 force field in Prime (Schrödinger). Prime MM-GBSA (Schrödinger) was used for the calculation of the amino acid interaction energy with the individual residues.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.0c00189.

Figure S1: visual representation of the docking of 25CN-NBOH and 23H-, 25H-, 26H-, and 34H-NBOMe in the binding pocket of the 5-HT_{2A}R (PDF)

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Notes

The authors declare no competing financial interest.

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