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Neurochemical pharmacology of psychoactive substituted N-benzylphenethylamines: high potency agonists at 5-HT_{2A} receptors

Amy J Eshleman[a,b], **Katherine M Wolfrum**[a], **John F Reed**[a], **Sunyoung O Kim**[a,1], **Robert A Johnson**[a], and **Aaron Janowsky**[a,b,c]

[a]Research Service, VA Portland Health Care System, [b]Departments of Psychiatry and Behavioral Neuroscience, and [c]The Methamphetamine Abuse Research Center Oregon Health and Science University, Portland, OR 97239, United States

Abstract

The use of new psychoactive substituted 2,5-dimethoxy-N-benzylphenethylamines is associated with abuse and toxicity in the United States and elsewhere and their pharmacology is not well known. This study compares the mechanisms of action of 2(2,5-dimethoxy-4-methylphenyl)-N-(2-methoxybenzyl)ethanamine (25D-NBOMe), 2-(4-ethyl-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25E-NBOMe), 2-(2,5dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25H-NBOMe), 2-(((4-iodo-2,5dimethoxyphenethyl)amino)methyl)phenol (25I-NBOH); and 2-(2,5-dimethoxy-4-nitrophenyl)-N(2-methoxybenzyl)ethanamine) (25N-NBOMe) with hallucinogens and stimulants. Mammalian cells heterologously expressing 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B} or 5-HT_{2C} receptors, or dopamine, serotonin or norepinephrine transporters (DAT, SERT and NET, respectively) were used to assess drug affinities at radioligand binding sites. Potencies and efficacies were determined using [^{35}S]GTPγS binding assays (5-HT_{1A}), inositol-phosphate accumulation assays (5-$HT_{2A,}$ 5-HT_{2B} and 5-HT_{2C}), and uptake and release assays (transporters). The substituted phenethylamines were very low potency and low efficacy agonists at the 5-HT_{1A} receptor. 25D-NBOMe, 25E-

Authorship contributions

Participated in research design: Eshleman, Janowsky

Conducted experiments: Eshleman, Wolfrum, Reed, Kim, Johnson

Performed data analysis: Eshleman, Wolfrum, Reed, Kim, Johnson

Wrote or contributed to the writing of the manuscript: Eshleman, Janowsky

[1]Present address: Uniformed Services University, Bethesda, MD 20814 USA (SOK)

Conflict of interest

All authors declare that they have no conflicts of interest.

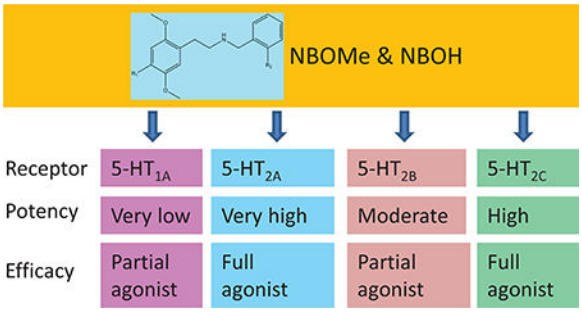
Chemical compounds studied in this article

2-(2,5-dimethoxy-4-methylphenyl)-N-(2-methoxybenzyl)ethanamine (25D-NBOMe); (PubChem CID: 118536027);
2-(4-ethyl-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25E-NBOMe); (PubChem CID: 121230757);
2-(2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (25H-NBOMe); (PubChem CID: 121230760);
2-(((4-iodo-2,5-dimethoxyphenethyl)amino)methyl)phenol (25I-NBOH) (PubChem CID: 10001761);
2-(2,5-dimethoxy-4-nitrophenyl)-N-(2-methoxybenzyl)ethanamine) (25N-NBOMe) (PubChem CID: 118536028)

NBOMe, 25HNBOMe, 25I-NBOH and 25N-NBOMe had very high affinity for, and full efficacy at, $5HT_{2A}$ and $5\text{-}HT_{2C}$ receptors. In the $5\text{-}HT_{2A}$ receptor functional assay, 25D-NBOMe, 25ENBOMe, 25I-NBOH and 25N-NBOMe had subnanomolar to low nanomolar potencies similar to (+)lysergic acid diethylamide (LSD) while 25H-NBOMe had lower potency, similar to serotonin. At the $5\text{-}HT_{2C}$ receptor, four had very high potencies, similar to LSD and serotonin, while 25H-NBOMe had lower potency. At the $5\text{-}HT_{2B}$ receptor, the compounds had lower affinity, potency and efficacy compared to $5\text{-}HT_{2A}$ or $5\text{-}HT_{2C.}$ The phenethylamines had low to mid micromolar affinities and potencies at the transporters.

These results demonstrate that these –NBOMe and –NBOH substituted phenethylamines have a biochemical pharmacology consistent with hallucinogenic activity, with little psychostimulant activity.

Graphical Abstract



Keywords

Substituted phenethylamine; Serotonin receptor; Lysergic acid diethylamide (LSD); NBOMe; Drug abuse

1. Introduction

New psychoactive substances, including psychedelic substituted phenethylamines, are public health and regulatory challenges [1]. Substituted phenethylamines, similar in structure to mescaline, were found in drug seizures in the European Union [2]. The United States Drug Enforcement Administration (DEA) has categorized some hallucinogenic compounds, including the substituted phenethylamines 2,5-dimethoxy-4- methylphenethylamine (2C-D), and 2,5-dimethoxy-4-ethylphenethylamine (2C-E) as Schedule 1 substances, i.e., having no therapeutic use and having high potential for abuse and adverse health effects [3]. Recently developed N-benzylmethoxy (NBOMe) compounds are derivatives of the 2C-X family of phenethylamine hallucinogens (see Figure 1) [4;5]. The drugs are administered orally or sublingually/buccally, available via the internet and known as “n-bomb” [4]. There are several case reports of abuse of 25BNBOMe, 25C-NBOMe, and 25I-NBOMe and their harmful effects include prolonged agitation, hallucinations, seizures, rhabdomyolysis, acute kidney injury and death [6–10]. In animal studies, these three NBOMe compounds substituted for the discriminative stimulus effects of the hallucinogen DOM [11] and were recently categorized as Schedule 1 compounds [12]. There is much less information

available regarding other NBOMe compounds although anectodal evidence indicates that many are psychoactive and hallucinogenic. 25D-NBOMe, 25E-NBOMe and 25H-NBOMe (Fig 1) have been detected on commercially available blotter paper [13;14] and the latter was detected in postmortem blood and urine [15]. 25I-NBOH (Fig 1) has been found on blotter paper [16] and is an N-hydroxybenzyl derivative of the hallucinogen 2C-I, the demethylated analog of 25I-NBOMe.

Agonist activation of 5-HT_{2A} receptors is essential for hallucinogenic activity of serotonergic compounds such as LSD and (-)2,5-dimethoxy-4-methylamphetamine (DOM) [17–19]. Conformational changes of 5-HT_{2A} receptors induced by the binding of LSD are critical for its time course [20]. For the substituted phenethylamine 2,5dimethoxy-4-propylthiophenethylamine (2C-T-7), antagonists of 5-HT_{2A} receptors can decrease drug-induced head twitch behavior in mice and its ability to substitute for the discriminative stimulus properties of LSD in rats [21]. The psychoactive dimethoxyphenethylamine series of compounds, including 2C-D, 2C-E, 2C-I and 2C-T-2 [22] and others, have high affinity and potency at the 5-HT_{2A} receptor and many of these compounds also bind and have high potency agonist activity at additional 5-HT receptors [23;24]. Addition of the NBOMe substituent increases the affinity for the 5HT_{2A} receptor [5;24–26]. The potential interaction of these drugs with the 5-HT_{2B} receptor is of concern, as prolonged activation by agonists at this receptor can cause cardiac valvulopathy [27;28]. The role of the 5-HT2C receptor activation in the psychoactivity of hallucinogens is less well understood (Reviewed in [19]).

The hallucinogenic activity of the NBOMe and NBOH series is indicated by animal studies. A structurally similar compound, 2-([2-(r-cyano-2,5dimethoxyphenyl)ethylamine]methyl)phenyl (25CN-NBOH) is a 5-HT_{2A} receptor agonist, causes head twitches in mice that are inhibited by a 5-HT_{2A} antagonist and substitutes partially for the discriminative stimulus of 2,5-dimethoxy-4-iodoamphetamine (DOI) [5;29]. 25I-NBOMe is more potent at inducing head twitches than the parent compound 2C-I [30]. Derivatives of 25I-NBOMe have high affinity for 5-HT_{2A} and 5-HT_{2c} receptors as determined using [^{125}I]DOI, and 5-HT_{2B} receptor using [^{3}H]LSD and have very low affinity for other 5-HT receptors [31]. 25D-NBOMe, 25E-NBOMe, 25H-NBOMe and 25N-NBOMe have micromolar affinity for 5-HT_{1A} receptors, rank order of affinity of 5-HT_{2A} >5HT_{2C} >5-HT_{2B} receptors, mid-nanomolar potency but low efficacy at the 5-HT_{2A} receptor as measured by Ca^{2+} mobilization assays and have low-mid micromolar affinities for the dopamine, serotonin and norepinephrine transporters (DAT, SERT and NET, respectively)[24]. However, the latter report involved the use of antagonist ligands at the 5-HT_{2A} and 5-HT_{2C} receptors and an agonist ligand at the 5-HT_{1A} receptor.

The goal of this research was to characterize the pharmacological activity of 25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25I-NBOH and 25N-NBOMe at relevant receptors and transporters to aid in the determination of abuse potential and DEA scheduling decisions. We now report results of experiments characterizing the interactions of NBOMe analogues with agonist ligand binding sites and signal transduction across four 5-HT receptors. The specific aims were to 1) determine the affinities of four NBOMe compounds and 25I-NBOH for the 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors using agonist radioligands, as well as affinities for the DAT, SERT and NET, 2) determine the potencies and efficacies of the

compounds as agonists in functional assays for the 5HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors, and 3) determine the potencies and efficacies of the compounds in uptake and release assays with DAT, SERT and NET.

2. Materials and Methods

2.1 Drugs

25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25I-NBOH and 25N-NBOMe were purchased from Cayman Chemicals (Ann Arbor, MI). (+)LSD(-)tartrate, (-)DOM, (-)cocaine, and S(+)METH were provided by the National Institute on Drug Abuse Drug Supply Program (Rockville, MD). [^{3}H]8-OH-DPAT, [^{125}I]2,5-dimethoxy-4-iodoamphetamine (DOI), [^{125}I]RTI-55, [^{3}H]DA, [^{3}H]5-HT, [^{3}H]NE and [^{35}S]GTPγS were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA). The IP-1 Elisa kit was purchased from Cisbio (Bedford, MA). Other reagents were purchased from Sigma (St. Louis, MO).

2.2 5-HT$_{1A}$ receptor: Radioligand binding

Human embryonic kidney cells expressing the human 5-HT$_{1A}$ receptor (HEK-5-HT$_{1A,}$ passage numbers 10, 14, 15 and 20) were used. The methods for transfection of HEK cells, cell membrane preparation, and [^{3}H]8-OH-DPAT agonist binding have been described previously [32]. The density and affinity of [^{3}H]8-OH-DPAT binding sites was 1670 fmol/mg protein and 5.0 nM. Briefly, the binding reaction mixture contained test compound, cell homogenate (0.05 mg of protein) and [^{3}H]8-OH-DPAT (0.5 nM final concentration) in a final volume of 1 ml (assay buffer: 25 mM Tris-HCl, pH 7.4, containing 1 mM ascorbic acid and 10 μM pargyline) and was incubated for 1h. Nonspecific binding was determined with 1 μM dihydroergotamine. The reaction was terminated by filtration through polyethylenimine-soaked “A” filtermats on a Tomtec 96well cell harvester (Tomtec, Hamden, CT) and radioactivity was counted on a Perkin Elmer (Boston, MA) microbeta scintillation counter.

2.3 5-HT$_{1A}$ receptor: [^{35}S]GTPγS binding

The method for [^{35}S]GTPγS binding has been described [32]. In brief, cell membranes (40–75 μg protein) were preincubated (10 min, room temperature) with test compound in duplicate in assay buffer (20 mM HEPES, pH 7.4, 10 mM MgCl2, 100 mM NaCl, and 0.2 mM dithiothreitol). The reaction was initiated by addition of GDP (3 μM) and [^{35}S]GTPγS (~150,000 cpm, 1350 Ci/mmol) in a final volume of 1 ml. The reaction was incubated for 1h at 25ºC and terminated as described above. Agonist efficacy is expressed relative to that of 100 nM 5-HT, which was determined for each experiment.

2.4 5-HT$_{2A}$ and 5-HT$_{2C}$ Receptors: [^{125}I]DOI binding

[^{125}I]DOI binding to 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors was tested in HEK-293 cells expressing either the human 5-HT$_{2A}$ receptor (HEK-5-HT$_{2A}$ cells, passage numbers 1718) or the human 5-HT$_{2C}$ receptor (HEK-5-HT$_{2C}$ cells, passage number 11) adapting methods described earlier [23;32]. The density and affinity of [^{125}I]DOI binding sites were 612 and 900 fmol/mg protein and 3.62 and 4.18 nM for h5-HT$_{2A}$ and h5-HT$_{2C}$ receptors, respectively. Briefly, the binding reaction mixture contained test compound, cell homogenate and [^{125}I]DOI (0.05 nM final concentration) in a final volume of 250 μl (assay buffer: 50

mM Tris-HCl, pH 7.4, containing 5 mM ascorbic acid, 5 mM $CaCl_2$, 10 μM pargyline). The assay was incubated for 1h at 37°C and terminated as described above. Nonspecific binding was determined with 10 μM serotonin.

2.5 5-HT_{2B} Receptor: [^{3}H]5-HT binding

[^{3}H]5-HT binding to 5-HT_{2B} receptors was tested in HEK-293 cells stably expressing the human 5-HT2B receptor (HEK-5-HT2B cells, passage numbers 7–11) adapting methods described earlier for [^{125}I]DOI binding to 5-HT_{2A} and 5-HT_{2C} receptors [23;32]. The cDNA, subcloned into the mammalian expression vector pCMV6-AC, was purchased from Origene (Rockville, MD). The density and affinity of [^{3}H]5-HT binding sites were 1,910 ± 240 fmol/mg protein and 3.56 ± 0.19 nM. Briefly, the binding reaction mixture contained test compound, cell homogenate and [^{3}H]5-HT (3–4 nM final concentration) in a final volume of 250 μl (assay buffer: 50 mM Tris-HCl, pH 7.4, containing 5 mM ascorbic acid, 5 mM $CaCl_2$, 10 μM pargyline). The assay was incubated for 45 min at 37°C and terminated as described above. Nonspecific binding was determined with 10 μM serotonin.

2.6. 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} Receptors: Inositol monophosphate (IP-1) formation

Activation of 5-HT_{2A} (passage numbers 9–17), 5-HT_{2B} (passage numbers 4–8), and 5HT_{2C} receptors (passage numbers 7–10) was tested by measuring the accumulation of inositol monophosphate using the Cisbio IP-1 Elisa kit as described previously [23;32]. Briefly, cells were plated at a density of 400,000 cells per well in 24 well plates. The next day, cells were starved with DMEM for 1 h, medium was removed, and stimulation buffer was added. After 10 min incubation, agonists were added and plates were incubated for 60 min. Cells were lysed, and 50 μl aliquots of the lysates were added to the IP-1 plate. The assay was conducted according to kit instructions. Stimulated IP-1 formation was normalized to the maximal effect of 5-HT, which was determined in each assay.

2.6. Biogenic amine transporters: Inhibition of [^{125}I]RTI-55 binding to, and [^{3}H]neurotransmitter uptake by, hDAT, hSERT or hNET in Clonal Cells

The methods for characterizing radioligand binding and functional uptake assays have been described previously [33]. Human embryonic kidney (HEK-293) cells expressing the recombinant hDAT (HEK-hDAT, passage numbers 7,9,10,17,18,28), hSERT (HEKhSERT, passage numbers 11, 15,16,18,20,26) or hNET (HEK-hNET, generous gift from Dr. Randy Blakely, Florida Atlantic University, passage numbers 9,19–23) were used. The density and affinity of [^{125}I]RTI-55 binding sites was 7.9, 0.85, and 3.6 pmol/mg protein and 1.83, 0.98, and 12.1 nM for DAT, SERT and NET, respectively [33]. Binding assays were conducted with a total particulate membrane preparation. The uptake assay was conducted in duplicate and initiated by the addition of [^{3}H]DA, [^{3}H]5-HT, or [^{3}H]NE (20 nM final concentration) to intact detached cells.

2.7. Biogenic amine transporters: [^{3}H]Neurotransmitter release

The methods for characterizing drug-induced release of pre-loaded [^{3}H]neurotransmitter from HEK-hDAT (passage numbers 24,26,27), HEK-hSERT (passage numbers 13–19) and HEK-hNET (passage numbers 18,19,22–25) cells have been described previously [32]. In

brief, cells were loaded with [^{3}H]neurotransmitter, centrifuged, resuspended in Krebs HEPES buffer (pH 7.4; 122 mM NaCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 μM pargyline, 100 μM tropolone, 0.2% glucose and 0.02% ascorbic acid, buffered with 25 mM HEPES), and added to the superfusion device (Brandel, Gaithersburg, MD). Buffer was perfused for 12–15 min, and the last 6 min (3 fractions) were collected for baseline. Drug was added, and 11 × 2 min fractions of effluent were collected. SDS (1%) was then perfused, and 4 × 2.5 min fractions were collected. Data were normalized to the maximal effects of the positive control METH. Radioactivity in the samples was determined using conventional liquid scintillation spectrometry. Fractional release was the amount of radioactivity in a fraction divided by the total radioactivity remaining in the sample.

2.8. Data analysis

For competition binding assay results, data were normalized to the specific binding in the absence of drug. Three or more independent competition experiments were conducted with duplicate determinations. GraphPAD Prism (La Jolla, CA) was used to analyze the ensuing data, with IC_{50} values converted to K_i values using the Cheng-Prusoff equation [34]. For signal transduction assays, GraphPAD Prism was used to calculate EC_{50} values using data expressed as % 5-HT-stimulation for 5-HT_{1A}-stimulated [^{35}S]GTPγS binding and 5-HT_{2A}-, 5-HT_{2B}- and 5-HT_{2C}-receptor-mediated IP-1 formation and for % total specific [^{3}H]neurotransmitter uptake for transporters. For [^{3}H]neurotransmitter release assays, area under the curve (AUC) for fractional release in the absence or presence of test compound over time was calculated using GraphPad Prism, and EC_{50} values were determined using logarithms of drug concentrations and sigmoidal dose-response nonlinear regression. Differences in affinities, potencies or efficacies were assessed by one way ANOVA using the logarithms of the K_i or EC_{50} values for test compounds and standards. Statistical significance was set at $p<0.05$. Dunnett's multiple comparison test was used to compare NBOMe and NBOH compounds to a drug standard. GraphPad Prism was used to calculate the Spearman correlation coefficient for the affinities and potencies at each 5-HT receptor using the logarithms of the K_i and EC_{50} values.

3. Results

3.1. 5-HT_{1A} receptors

At the recombinant 5-HT_{1A} receptor, 25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25INBOH, 25N-NBOMe and DOM had lower affinities compared to 5-HT (*ps*<0.0001, one way ANOVA followed by Dunnett's multiple comparison test) and LSD had a similar affinity to that of 5-HT (Table 1). The Ki values for the NBOMes and NBOH compounds were all in the micromolar range. Similar results were obtained in the 5-HT_{1A} functional assay, with EC_{50} values for stimulating [^{35}S]GTPγS binding in the micromolar range for 25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25I-NBOH, 25N-NBOMe and DOM, and with significantly lower potencies than 5-HT (ps<0.0001) while LSD had potency similar to that of 5-HT (Table 2, Fig 2A). 25D-NBOMe, 25E-NBOMe, and 25N-NBOMe were partial agonists with lower efficacies for stimulation of [^{35}S]GTPγS binding compared to 5-HT (p<0.05–0.01), while the efficacies of 25H-NBOMe, 25I-NBOH, DOM and LSD were not significantly different from 5-HT (Table 2, Fig 2A). In addition, there was no correlation

between affinities for the [^{3}H]8-OH-DPAT binding site and potencies for stimulating [^{35}S]GTPγS binding, except that both values were low (Fig 3A).

3.2. 5-HT$_{2A}$ receptors

Results were very different with the recombinant 5-HT$_{2A}$ compared to the 5-HT$_{1A}$ receptor. Using the agonist [^{125}I]DOI as the radioligand in the binding assay, affinities of 25D-NBOMe, 25E-NBOMe, 25I-NBOH, 25N-NBOMe and LSD were all higher than that of 5-HT, with subnanomolar Ki values (ps<0.0001, one way ANOVA Dunnett's multiple comparison test, Table 1). 25H-NBOMe and DOM had Ki values that were similar to 5HT Ki values, which were in the low nanomolar range. In the IP-1 functional assay, 25D-NBOMe, 25E-NBOMe, 25I-NBOH, 25N-NBOMe and LSD had higher potencies than 5-HT (ps<0.01–0.0001) at the 5-HT$_{2A}$ receptor, with EC$_{50}$ values ranging from 0.511.5 nM (Table 2, Fig 2B). 25H-NBOMe and DOM had potencies similar to 5-HT, with EC$_{50}$ values of about 40 nM. All compounds except LSD (64.5%) were full agonists at the 5-HT$_{2A}$ receptor, with efficacies ranging from 85.9–95.1% of the maximal stimulation by 5-HT. There was an excellent correlation between affinities for the 5-HT$_{2A}$ [^{125}I]DOI binding site and potencies in the IP-1 signal transduction assay (Fig 3B).

3.3. 5-HT$_{2B}$ receptors

Using the agonist [^{3}H]5-HT as the radioligand in binding assays, 25D-NBOMe, 25E-NBOMe, 25I-NBOH and LSD had higher affinities than 5-HT with Ki values of 2.05, 1.11, 1.91 and 0.57 respectively (p*s*<0.05–0.001). 25N-NBOMe had similar affinity to that of 5-HT. 25H-NBOMe and DOM had lower affinities than 5-HT (p*s*<0.001).

However, in the 5-HT$_{2B}$ receptor IP-1 functional assay, 25D-NBOMe, 25E-NBOMe, 25I-NBOH, 25N-NBOMe and DOM had lower potencies than 5-HT (ps<0.01–0.0001), with EC$_{50}$ values ranging from 23.5–463 nM (Table 2, Fig 2C). LSD and 5-HT had similar, low nanomolar potencies. In addition, only 5-HT and DOM were full agonists at the 5-HT$_{2B}$ receptor. The rest of the compounds had efficacies ranging from 21.3–57.6% of the maximal stimulation by 5-HT (Table 2). There was good correlation between affinities for the 5-HT$_{2B}$ [^{3}H]5-HT binding site and potencies in the IP-1 functional assay (Fig 3C).

3.4. 5-HT$_{2C}$ receptors

At the recombinant 5-HT$_{2C}$ receptor, using the agonist [^{125}I]DOI as the radioligand in the binding assay, the affinities of 25D-NBOMe and 25E-NBOMe were higher than that of 5HT, with subnanomolar Ki values (ps<0.05–0.001, one way ANOVA Dunnett's multiple comparison test, Table 1). 25I-NBOH, 25N-NBOMe and LSD had affinities similar to the affinity of 5-HT, with Ki values in the low nanomolar range. 25H-NBOMe and DOM had lower affinities than 5-HT, with Ki values of 16–19 nM (ps<0.001). In the IP-1 functional assay, 25H-NBOMe and DOM had lower potencies than 5-HT (ps<0.001) with EC$_{50}$ values of 13.8 and 21.9 nM (Table 2). 25D-NBOMe, 25E-NBOMe, 25INBOH, 25N-NBOMe and LSD had similar potencies for stimulation of IP-1 formation compared to 5-HT, with EC$_{50}$ values ranging from 0.95–2.38 nM (Table 2, Fig 2D). All compounds were full agonists at the 5-HT$_{2C}$ receptor. There was good correlation between affinities for the 5-HT$_{2C}$ [^{125}I]DOI binding site and the potencies of drugs in the IP-1 functional assay (Fig 3D).

3.5. DAT, SERT and NET: inhibition of [^{125}I]RTI-55 binding and [^{3}H]neurotransmitter uptake

3.5.1. DAT—25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25I-NBOH, and 25N-NBOMe had very low affinities for the radioligand binding site on the DAT, with values ranging from 8.5–81.4 μM (Table 3). LSD and DOM had no measurable affinity, and the uptake blockers, cocaine and mazindol, had mid- and low-nanomolar Ki values. Similar results were seen for inhibition of [^{3}H]DA uptake, with IC_{50} values for the five compounds being higher than the Ki values. Again there was no measurable effect of LSD and DOM on uptake, while cocaine and mazindol had mid- and low-nanomolar potencies, respectively.

3.5.2. NET—25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25I-NBOH, and 25N-NBOMe had low affinities for the [^{125}I]RTI-55 binding site on the recombinant NET, with values ranging from 4.06–16.3 μM (Table 3). All had higher affinities for NET than for DAT. LSD and DOM had no measurable affinity, and cocaine and mazindol had low micromolar and low nanomolar Ki values, respectively. For all except 25N-NBOMe, IC_{50} values for inhibition of [^{3}H]NE uptake were lower than the Ki values. 25N-NBOMe had similar values in both assays. All five compounds had higher potencies at NET compared to their potencies at DAT. Again there was no measurable effect of LSD and DOM, while cocaine and mazindol had mid- and low-nanomolar potencies, respectively.

3.5.3. SERT—25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25I-NBOH, and 25N-NBOMe had low affinities for the [^{125}I]RTI-55 binding site on the SERT, with values ranging from 1.22 to 5.81 μM (Table 3). All had higher affinities for SERT than for DAT or NET. LSD had mid-micromolar affinity and DOM had no measurable affinity, and cocaine and mazindol had mid-nanomolar Ki values. The –NBOMe compounds had IC_{50} values for inhibition of [^{3}H]5-HT uptake that were similar to their Ki values. 25D-NBOMe, 25E-NBOMe, 25INBOH, and 25N-NBOMe had higher potencies at the recombinant SERT compared to their potencies at DAT or NET. 25H-NBOMe had a rank order of potency of NET > SERT >DAT. There was no measurable effect of LSD, while DOM had mid-micromolar and cocaine and mazindol had mid- and low-nanomolar potencies, respectively.

3.6. [^{3}H]Neurotransmitter Release via DAT, SERT or NET

Assays measuring release of preloaded [^{3}H]neurotransmitter from cells or other tissue preparations have been used to determine if a compound is a transporter substrate [35]. 25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25I-NBOH, 25N-NBOMe, LSD and DOM had little to no efficacy at inducing release of preloaded [^{3}H]neurotransmitter from recombinant DAT, SERT or NET, tested at concentrations from 10 nM – 100 μM (Table 4). Thus, even though the compounds were efficacious at inhibition of uptake at 1–5 μM at SERT (Table 3), they did not induce release, and thus are blockers, not substrates, at SERT. METH was efficacious at all three transporters, with potencies that agree with previous reports [35].

4. Discussion

25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25I-NBOH, and 25N-NBOMe had very high affinities for the 5-HT_{2A} receptor, in the subnanomolar range, except for 25H-NBOMe,

which had low nanomolar affinity. These affinities are higher (3–5 fold) than those reported [24] using an antagonist radioligand. This difference is consistent with [26], in that many substituted phenethylamines in the 25H and 25I families show lower affinity for the 5-HT_{2A} receptor when displacing an antagonist compared to displacement of agonist radioligand. In 5-HT_{2A} functional assays, these compounds had very high potency at inducing the accumulation of inositol phosphates (IP-1 assay). These EC_{50} values were 12–320 times lower (higher potency) than those reported by [24], which were measured using a Ca^{2+} mobilization assay. For 25H-NBOMe and 25I-NBOH, Braden et al. [26] reports EC_{50} values in a radiolabeled inositol phosphate accumulation assay similar to or about three fold lower than the values reported herein. In addition, we observed full functional efficacy for the five compounds, ranging from 86–95% of maximal serotonin effect, in agreement with [26] and much higher efficacy than in the Ca^{2+} mobilization assay [24]. Thus these compounds may be of high concern when considering the possibility of overdose and adverse effects resulting from high potency and full efficacy at the 5-HT_{2A} receptor.

25D-NBOMe, 25E-NBOMe had sub-nanomolar, 25H-NBOMe had low nanomolar, and 25I-NBOH and 25N-NBOMe had about 1 nM affinity for the 5-HT_{2C} receptor. These affinities are higher (4–23 fold) than those reported by [24] who used an antagonist radioligand. This is the first report that, in 5-HT_{2C} functional assays, these compounds had very high potency for inducing the accumulation of inositol phosphates (IP-1 assay). In addition, we observed full functional efficacy for the five compounds, ranging from 91.8–99.4% of maximal 5-HT effect. The role of the 5-HT_{2C} receptor in the psychoactive properties of the substituted phenethylamines is still debatable. A 5-HT_{2C} antagonist does not, but a 5-HT_{2A} antagonist does, modify head twitch behavior by 25CN-NBOH [29]. The affinities of 5-HT_{2A} receptor antagonists correlate with their IC_{50} values for blockade of LSD and DOM as stimuli in drug discrimination studies, while the drugs' affinities for the 5-HT_{2C} receptor do not correlate with the behavioral data [17]. However, activation of 5-HT_{2C} receptors can activate differentially distinct signal transduction pathways, dependent on agonist characteristics, which may contribute to psychoactive properties of these compounds [36]. For example, Canal and Murnane recently hypothesized that the non-addictive nature of many hallucinogens is due to 5-HT_{2C} receptor activation inhibiting potassium Kv1.c channels on nucleus accumbens medium spiny neurons [37].

Hallucinogenic indoleamines depress raphe cell firing by binding with high affinity to somatodendritic 5-HT_{1A} autoreceptors (reviewed in [19]). However, the phenethylamines have very low affinity for 5-HT_{1A} receptors, and thus do not directly mediate this effect. In addition, although these compounds had high affinity for the $5HT_{2B}$ receptor, they had 11–146 times lower potencies at activating 5-HT_{2B} compared to 5-HT_{2A} receptors, and only partial efficacy, which suggests that they could cause cardiac valvulopathy only with chronic and high dose use [28].

Forensic evidence indicates that relevant brain concentrations for 5-HT_{2A} activation are reached following drug ingestion. Human blood concentrations, taken hours after ingestion, of 0.29 ng/ml 25H-NBOMe and 2.80 ng/ml 25C-NBOMe have been measured [15]. The ~0.9 nM 25H-NBOMe blood concentration is lower than its EC_{50} for 5-HT_{2A} IP-1 hydrolysis (40.7 nM) but the ~8 nM 25I-NBOMe concentration is higher than its EC_{50} [26]

and similar concentrations have been reported for 25B-NBOMe [38]. Postmortem analysis of peripheral blood and brain tissue yielded 25I-NBOMe concentrations of 0.405 ng/ml and 2.54ng/g, respectively, suggesting preferential distribution to, and accumulation in, brain tissue [39]. Thus brain concentrations sufficient to activate the $5HT_{2A}$ and $5\text{-}HT_{2C}$ receptors may have been attained. Serum concentrations for other compounds tested herein were not found in the literature.

Consistent with other reports [26;30;40], affinity for some 5-HT receptors increased with the N-benzyl additions to the phenethylamines. For parent compounds of 25D-NBOMe, 25E-NBOMe and 25I-NBOH at $5\text{-}HT_{1A}$, $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors, using the same assay conditions, 2C-D had affinities of 1630, 23.9 and 12.7 nM; 2C-E had affinities of 1190, 4.5, and 5.4 nM; and 2C-I had affinities of 970, 9.3, 10.2 nM, respectively [23]. Comparison of these values with Ki values for the corresponding – NBOMe and –NBOH analogs (Table 1) indicates that affinities for $5\text{-}HT_{1A}$ were decreased while $5\text{-}HT_{2A}$ affinities were increased 35–100 times by addition of –NBOMe and –NBOH moieties, and $5\text{-}HT_{2C}$ affinities were increased 10–18 times, similar to affinity shifts of other –NBOMe [41]. Thus the affinity selectivity for the $5\text{-}HT_{2A}$ receptor was increased with the N-benzyl additions. Modelling of -NBOMe compounds indicates that the increase in $5\text{-}HT_{2A}$ affinities is due to stabilization of the N-benzyl moiety with Phe339 in transmembrane 6 [26]. In Fig 3, the –NBOMe series is grouped to the left with high affinity and potency for both $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors. Selectivity for binding affinity for $5\text{-}HT_{2A}$ over $5\text{-}HT_{1A}$ ranged from 920–20,500 fold across –NBOMe drugs, and between $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ ranged from 2.4–7.4 fold (Table 1). There was also a high selectivity of functional activity for the –NBOMe and –NBOH analogs for $5HT_{2A}$ over $5\text{-}HT_{1A}$ receptors (Table 2), while the potencies at $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ were similar.

The current results indicate that the –NBOMe and –NBOH substituted phenethylamines examined here are full, very high potency agonists at the $5\text{-}HT_{2A}$ and $5\text{-}HT_{2C}$ receptors. The biochemical pharmacology of these compounds is consistent with psychoactive hallucinogenic activity with minimal stimulant activity, as indicated by lower affinities and potencies at neurotransmitter (DA and NE) transporters. At high, toxic doses, these compounds may cause symptoms, including tachycardia and hypertension, that are elicited by activation of additional pathways [6].

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Role of funding source

DEA project officers contributed to study design and had no further role in the collection, analysis and interpretation of data, in the writing of the report, or in the decision to submit the paper for publication.

Abbreviations

(25D-NBOMe)	2-(2,5-dimethoxy-4-methylphenyl)-N-(2-methoxybenzyl)ethanamine
(25E-NBOMe)	2-(4-ethyl-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine
(25H-NBOMe)	2-(2,5dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine
(25I-NBOH)	2-(((4-iodo-2,5dimethoxyphenethyl)amino)methyl)phenol
(25N-NBOMe	2-(2,5-dimethoxy-4-nitrophenyl)-N-(2methoxybenzyl)ethanamine)
(LSD)	(+)Lysergic acid diethylamide
((DOM) -)	2,5dimethoxy-4-methylamphetamine
(DOI)	2,5-dimethoxy-4-iodoamphetamine
(METH)	(S+)methamphetamine
(5-HT)	Serotonin hydrochloride
(DA)	Dopamine hydrochloride
(NE)	Norepinephrine bistartrate
(DAT)	Dopamine transporter
(NET)	Norepinephrine transporter
(SERT)	Serotonin transporter
(2C-B)	2,5-dimethoxy-4-bromophenethylamine
(2C-C)	2,5dimethoxy-4-chlorophenethylamine
(2C-E)	2,5-dimethoxy-4-ethylphenethylamine
(2C-I)	2,5dimethoxy-4-iodophenethylamine
(2C-T-2)	2,5-dimethoxy-4-ethylthiophenethylamine
(DOC)	2,5-dimethoxy-4-chloroamphetamine
([^{125}I]RTI-55)	[^{125}I]methyl (1*R*,2*S*,3*S*)-3-(4-iodophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate
([^{3}H]8-OH-DPAT)	[^{3}H]8-hydroxy-*N*,*N*-dipropyl-2aminotetralin
([^{35}S]GTPγS)	[^{35}S]guanosine triphosphate

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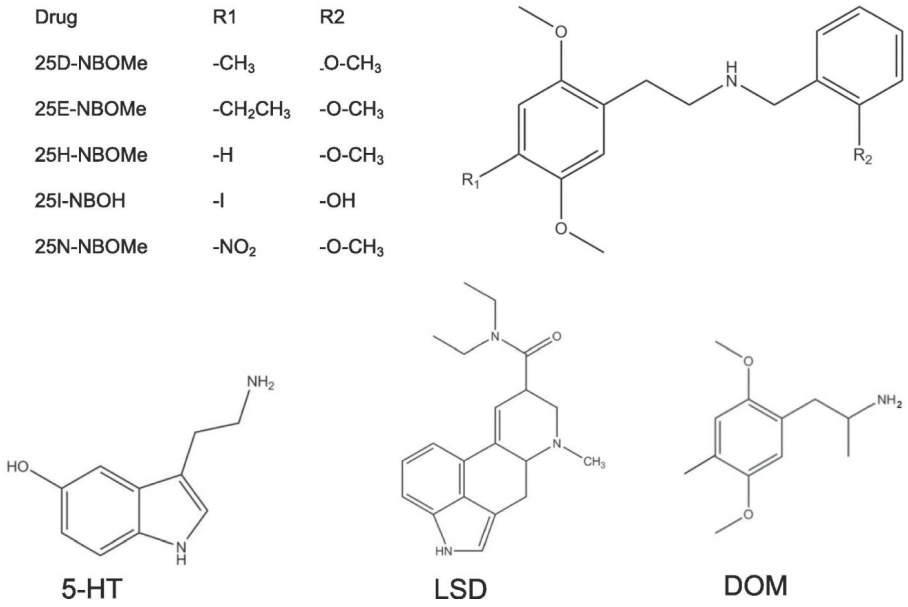

Figure 1.

Chemical structures of 25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25I-NBOH, 25N-NBOMe, 5-HT, LSD and DOM.

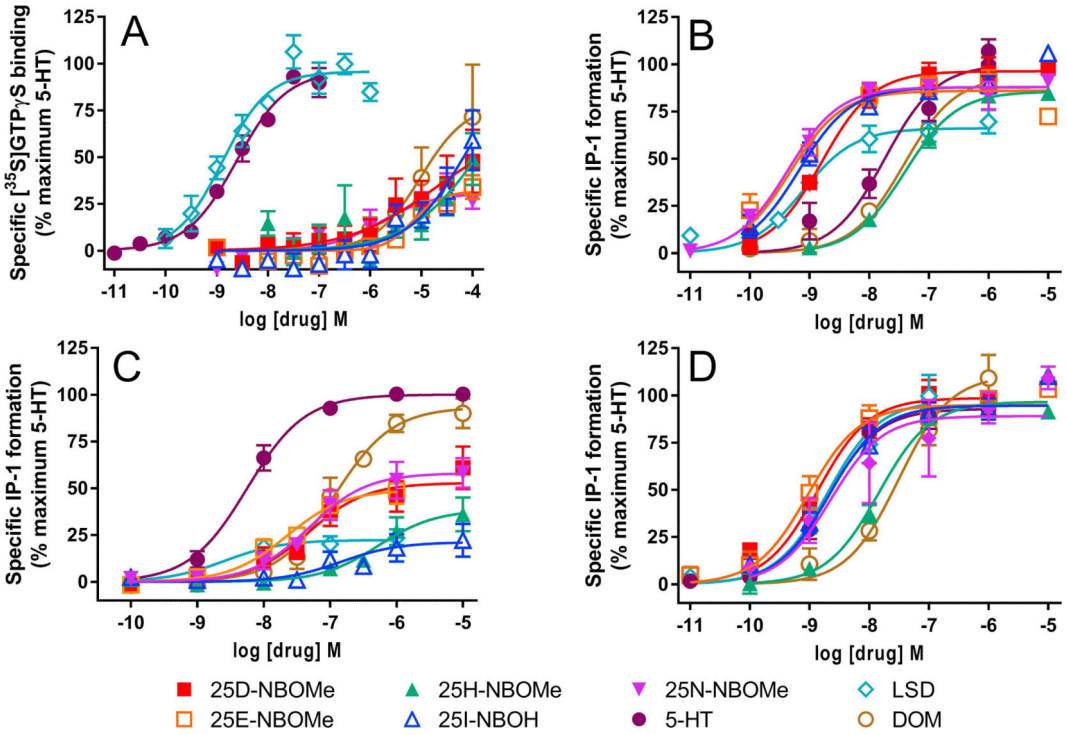

Figure 2.

Agonist activity of -NBOMe phenethylamines at recombinant 5-HT_{1A}, 5-$HT_{2A,}$ 5-HT_{2B} and 5-HT_{2C} receptors. All data were normalized to the maximal effect of 5-HT, which was measured on each experimental day. A. 5-HT_{1A} [^{35}S]GTPγS binding. N=3–7 independent experiments conducted with duplicate determinations. B. 5-HT_{2A} agonist IP-1 assay. N=3–4 independent experiments conducted with duplicate determinations. C. 5-HT_{2B} IP-1 assay. N=4–6 independent experiments conducted with duplicate determinations. D. 5-HT_{2C} IP-1 assay. N=3–4 independent experiments conducted with duplicate determinations. Data shown are mean ± sem.

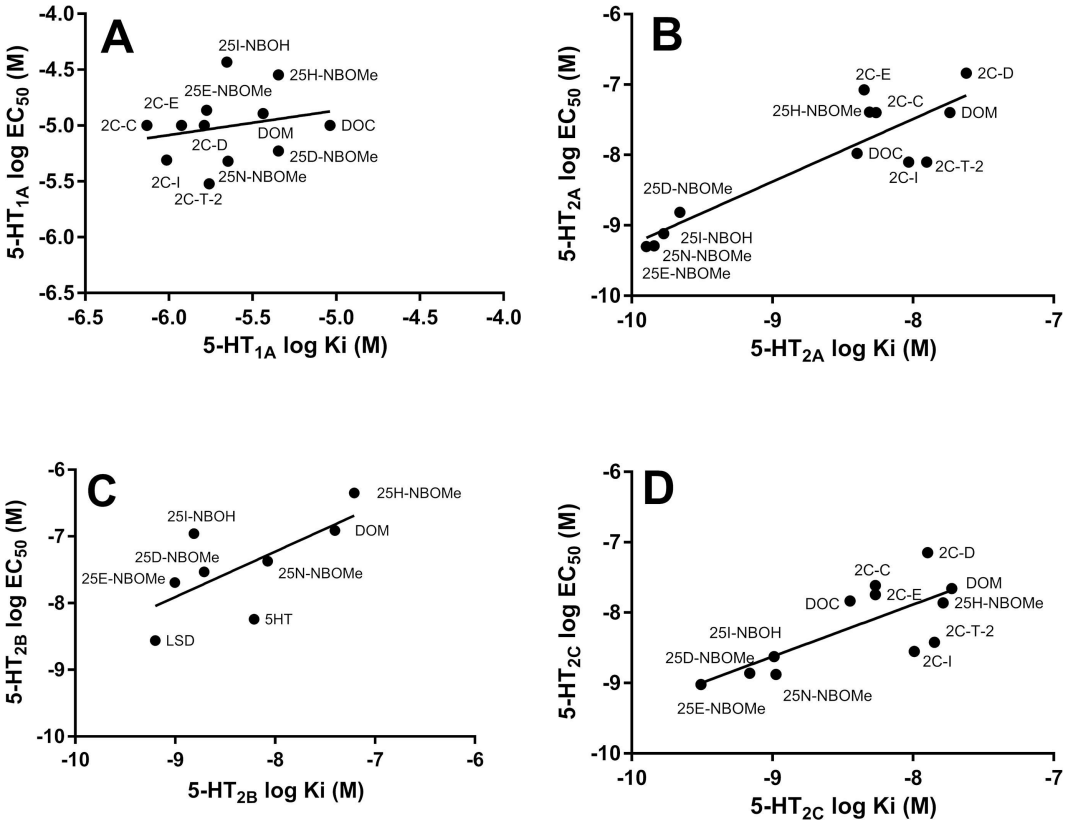

Figure 3.

Correlation of affinities and agonist potencies of substituted phenethylamines at 5-HT_{1A}, 5-$HT_{2A,}$ 5-HT_{2B} and 5-HT_{2C} receptors. The linear regression for the data in each graph is shown. A. 5-HT_{1A} affinities as measured with [^{3}H]8-OH-DPAT binding vs 5-HT_{1A} potencies as measured using [^{35}S]GTPγS binding. Spearman r=0.19, p>0.05. B. 5-HT_{2A} affinities as measured with [^{125}I]DOI binding vs 5-HT_{2A} potencies as measured using the IP-1 assay. Spearman r=0.72, p=0.01. C. 5-HT_{2B} affinities as measured with [^{3}H]5-HT binding vs 5-HT_{2B} potencies as measured using the IP-1 assay. Spearman r=0.76, p<0.05. D. 5-HT_{2C} affinities as measured with [^{125}I]DOI binding vs 5-HT_{2C} potencies as measured with IP-1 assay. Spearman r=0.67, p<0.05. Values for 2C-C, 2C-D, 2C-E, 2C-I, 2C-T-2 and DOC are from [23]

Table 1.

Affinity of substituted phenethylamines and other compounds for recombinant 5-HT_{1A}, 5-$HT_{2A,}$ 5-HT_{2B} and 5-HT_{2C} receptors.

	Inhibition of radioligand binding Ki mean ± sem (nM) (n)				Selectivity (binding ratios)		
Drug	**5-HT_{1A} [^{3}H]8OH-DPAT**	**5-HT_{2A} [^{125}I]DOI**	**5-HT_{2B} [^{3}H]5-HT**	**5-HT_{2C} [^{125}I]DOI**	**5-HT_{2A} : 5-HT_{1A}**	**5-HT_{2A} : 5-HT_{2B}**	**5-HT_{2A} : 5-HT_{2C}**
25D-NBOMe	4510 ± 730 (3)	0.22 ± 0.026 (5)	2.05 ± 0.49 (3)	0.69 ± 0.02 (4)	20500	9.3	3.1
25E-NBOMe	1680 ± 350 (3)	0.127 ± 0.019 (5)	1.11 ± 0.32 (3)	0.311 ± 0.071 (4)	13200	8.7	2.4
25H-NBOMe	4520 ± 3+0 (3)	4.9 ± 1.3 (5)	62.9 ± 7.5 (3)	16.4 ± 5.0 (6)	920	13	3.3
25I-NBOH	2220 ± 590 (3)	0.169 ± 0.013 (5)	1.91 ± 0.48 (6)	1.03 ± 0.24 (4)	13100	11	6.1
25N-NBOMe	2260 ± 230 (3)	0.144 ± 0.042 (5)	8.7 ± 1.6 (3)	1.06 ± 0.35 (4)	15700	60	7.4
5-HT	1.71 ± 0.24 (4)	16.8 ± 2.2 (3)	6.31 ± 0.94 (4)	2.52 ± 0.45 (7)	0.10	0.38	0.15
LSD	1.22 ± 0.23 (4)	0.091 ± 0.044 (3)	0.57 ± 0.14 (3)	1.60 ± 0.27 (4)	13.4	6.3	17.6
DOM	3660 ± 470 (4)	18.3 ± 1.7 (4)	41.0 ± 5.9 (3)	18.9 ± 2.4 (4)	200	2.2	1.0

(n) Number of independent experiments conducted in duplicate.

Binding Hill slopes for h5-HT_{1A} [^{3}H]8-OH-DPAT ranged from -0.58 to -0.90, for h5-HT_{2A} [^{125}I]DOI ranged from -0.32 to -1.78, for h5-HT_{2B} [^{3}H]5-HT ranged from -0.87 to -1.17 and for h5-HT_{2C} [^{125}I]DOI ranged from -0.58 to -0.96.

Table 2.

Potency and efficacy of substituted phenethylamines and other compounds at recombinant 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors.

Drug	EC_{50} mean ± sem (nM) (n) % maximum stimulation ± sem*				Selectivity (functional ratios)		
	5-HT_{1A} Stimulation of [^{35}S]GTPγS binding	5-HT_{2A} Stimulation of IP-1 formation	5-HT_{2B} Stimulation of IP-1 formation	5-HT_{2C} Stimulation of IP-1 formation	5-HT_{2A}: 5-HT_{1A}	5-HT_{2A}: 5-HT_{2B}	5-HT_{2A}: 5-HT_{2C}
25D-NBOMe	5900 ± 1900 $_{(4)}$ 55 ± 20%	1.53 ± 0.10 $_{(3)}$ 95.1 ± 6.8%	32.3 ± 6.8 $_{(4)}$ 47.7 ± 8.1%	1.37 ± 0.36 $_{(3)}$ 96.1 ± 2.5%	3900	21	0.90
25E-NBOMe	13700 ± 5600 $_{(6)}$ 38.0 ± 6.2%	0.50 ± 0.14 $_{(4)}$ 87.1 ± 6.6%	23.5 ± 6.0 $_{(5)}$ 49.4 ± 6.8%	0.95 ± 0.18 $_{(3)}$ 91.8 ± 4.4%	27,000	47	1.9
25H-NBOMe	28400 ± 8600 $_{(4)}$ 52 ± 14%	40.7 ± 8.1 $_{(4)}$ 85.9 ± 1.7%	463 ± 71 $_{(4)}$ 37.7 ± 9.0%	13.8 ± 2.6 $_{(3)}$ 95.5 ± 3.0%	700	11	0.34
25I-NBOH	37000 ± 13000 $_{(3)}$ 74 ± 18%	0.76 ± 0.26 $_{(4)}$ 87.5 ± 4.1%	111 ± 13 $_{(4)}$ 21.3 ± 8.3%	2.38 ± 0.72 $_{(3)}$ 94.0 ± 4.0%	49,000	146	3.1
25N-NBOMe	4800 ± 1800 $_{(5)}$ 35.6 ± 6.6%	0.51 ± 0.19 $_{(3)}$ 87.9 ± 3.4%	47 ± 11 $_{(4)}$ 57.6 ± 8.6%	1.32 ± 0.27 $_{(3)}$ 99.4 ± 0.4%	9400	92	2.6
5-HT	2.88 ± 0.61 $_{(6)}$ 94.7 ± 3.6%	19.4 ± 3.7 $_{(8)}$ 98.7 ± 2.7%	6.3 ± 1.2 $_{(5)}$ 100.2 ± 2.8%	2.8 ± 1.0 $_{(6)}$ 98.9 ± 3.3%	0.15	0.32	0.14
LSD	1.31 ± 0.46 $_{(3)}$ 93.2 ± 6.4%	0.706 ± 0.065 $_{(3)}$ 64.5 ± 6.3%	3.07 ± 0.65 $_{(6)}$ 23.3 ± 4.9%	1.74 ± 0.29 $_{(3)}$ 92.5 ± 4.8%	1.9	4.3	2.5
DOM	12800 ± 4700 $_{(3)}$ 74 ± 25%	39.9 ± 5.4 $_{(3)}$ 94.6 ± 4.1%	145 ± 47 $_{(4)}$ 96.2 ± 3.2%	21.9 ± 5.9 $_{(3)}$ 96.0 ± 4.0%	320	3.6	0.55

(n) Number of independent experiments conducted in duplicate.

*Drug-induced stimulation is normalized to the maximal stimulation by 5-HT.

Table 3.

Affinity and potency of substituted phenethylamines and other compounds at recombinant hDAT, hSERT and hNET.

	Inhibition of [^{125}I]RTI-55 binding Ki mean ± sem (μM) (n)			Inhibition of [^{3}H]neurotransmitter uptake IC_{50} mean ± sem (μM) (n)		
Drug	**hDAT**	**hSERT**	**hNET**	**hDAT [^{3}H]DA**	**hSERT [^{3}H]5-HT**	**hNET [^{3}H]NE**
25D-NBOMe	34.5 ± 4.2 (3)	1.78 ± 0.28 (3)	6.7 ± 1.3 (4)	>85 μM (3)	1.024 ± 0.078 (3)	1.17 ± 0.14 (3)
25E-NBOMe	19.6 ± 1.2 (3)	1.59 ± 0.12 (4)	5.4 ± 1.0 (3)	34 ± 10 (5)	1.44 ± 0.14 (3)	2.31 ± 0.70 (3)
25H-NBOMe	81.4 ± 8.9 (4)	2.22 ± 0.34 (3)	16.3 ± 2.6 (4)	>100 μM (2)	2.08 ± 0.31 (3)	3.65 ± 0.88 (4)
25I-NBOH	8.50 ± 0.38 (3)	1.22 ± 0.21 (3)	4.06 ± 0.65 (3)	30.7 ± 5.0 (3)	1.72 ± 0.25 (3)	0.629 ± 0.093 (4)
25N-NBOMe	37.9 ± 4.6 (3)	5.81 ± 0.90 (3)	11.3 ± 3.5 (3)	>100 μM (2)	5.79 ± 0.53 (3)	15.0 ± 3.8 (4)
5-HT	375 ± 83 (3)	2.33 ± 0.52 (4)	392 ± 10 (4)	3.36 ± 0.32 (4)	0.192 ± 0.049 (3)	7.2 ± 2.5 (6)
LSD	>100 μM (2)	62.9 ± 5.0 (4)	76.9 ± 8.5 (4)	>100 μM (2)	>90 μM (3)	>100 μM (3)
DOM	>100 μM (2)	>100 μM (4)	>100 μM (2)	>100 μM (2)	64 ± 12 (4)	>70 μM (2)
Cocaine	0.595 ± 0.059 (5)	0.468 ± 0.049 (6)	1.88 ± 0.42 (4)	0.37 ± 0.11 (4)	0.361 ± 0.077 (6)	0.212 ± .013 (4)
Mazindol	0.0289 ± 0.0026 (4)	0.127 ± 0.039 (4)	0.0116 ± 0.0035 (5)	0.0154 ± 0.0021 (5)	0.069 ± 0.012 (4)	0.0011 ± 0.0003 (5)

(n) Number of independent experiments conducted in duplicate.

Data are normalized to specific binding or specific uptake in the absence of drugs. Drugs were tested in binding assays at concentrations ranging from 1 nM to either 10 μM or 100 μM. Hill slopes for binding ranged from -0.93 to -2.39.

Table 4.

Potency and efficacy of substituted phenethylamines and other compounds to release preloaded [^{3}H]neurotransmitter from HEK-hDAT, HEK-hSERT and HEK-hNET cells.

Drug	Drug-induced release of [^{3}H]neurotransmitter EC_{50} mean ± sem (μM) (n) % maximum release ± sem*		
	hDAT [^{3}H]DA	hSERT [^{3}H]5-HT	hNET [^{3}H]NE
25D-NBOMe	>100 μM (2)	>100 μM (2)	>33 μM (3)
	0.75 ± 0.48%	0.59 ± 0.24%	14.3 ± 8.5%
25E-NBOMe	>85 μM (3)	>100 μM (2)	>66 μM (3)
	5.8 ± 6.4%	0.21 ± 0.77%	17.1 ± 5.9%
25H-NBOMe	>100 μM (2)	>100 μM (2)	>66 μM (3)
	−0.18 ± 0.26%	−0.27 ± 0.49%	21 ± 12%
25I-NBOH	>100 μM (2)	>100 μM (2)	>100 μM (2)
	−0.56 ± 0.35%	−0.27 ± 0.40%	16 ± 16%
25N-NBOMe	>100 μM (2)	>100 μM (2)	>38 μM (3)
	−0.89 ± 0.91%	−0.25 ± 0.54%	25 ± 15%
LSD	>54 μM (4)	>100 μM (2)	>91 μM (3)
	7.4 ± 4.7%	−0.59 ± 0.55%	35.7 ± 4.2%
DOM	>100 μM (2)	>100 μM (2)	>42 μM (4)
	−0.20 ± 0.16%	2.9 ± 3.4%	25.5 ± 2.0%
METH	0.43 ± 0.15 (5)	27.5 ± 7.0 (4)	0.152 ± 0.056 (3)
	104.1 ± 1.3	104.9 ± 6.8%	106.7 ± 3.3%

(n) Number of independent experiments.

*Maximum release is defined as the maximum release (maximal AUC) induced by METH (1–10 μM, hDAT; 0.3–1 mM, hSERT; 0.3–1 μM, hNET) for each experiment.