UNIVERSITY OF CALIFORNIA SAN DIEGO

If you give a mouse a mushroom: Behavioral effects of hallucinogens in mice

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Neurosciences with a Specialization in Anthropogeny

by

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The Dissertation of Landon Marshall Klein is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2018

DEDICATION

In loving memory of Ira Matthew Klein
(1985-2013)
an inspiration in the ceaseless pursuit of something more

EPIGRAPH

"Mama, mama, many worlds I've come
since I first left home"

- J. Garcia / R. Hunter

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Brandt, S.D., Kavanagh, P. V., Twamley, B., Westphal, F., Elliott, S.P., Wallach, J., Stratford, A., **Klein, L.M.**, McCorvy, J.D., Nichols, D.E., Halberstadt, A.L., 2018. Return of the lysergamides. Part IV: Analytical and pharmacological characterization of lysergic acid morpholide (LSM-775). Drug Test. Anal. 10, 310–322. https://doi.org/10.1002/dta.2222

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ABSTRACT OF THE DISSERTATION

If you give a mouse a mushroom: Behavioral effects of hallucinogens in mice

by

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Doctor of Philosophy in Neurosciences with a Specialization in Anthropogeny

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Classical hallucinogens are a class of psychoactive drugs that reliably alter perception, cognition, and behavior. Recently, a renewed focus on the mechanisms responsible for the effects of hallucinogens has emerged, as a renaissance of recreational availability and therapeutic interest has taken root. Identifying pharmacological substrates of hallucinogen effects allows us to predict potential dangers of novel hallucinogens, improve our understanding of psychotic disorders, and optimize the therapeutic value of hallucinogens while minimizing untoward side effects. The experiments herein use mouse models to address a broad array of questions regarding neural mechanisms of hallucinogen action. First, I

used the head twitch response (HTR), a paroxysmal head rotation induced by hallucinogens in rodents, to probe the relationship between structural characteristics of putative hallucinogens and their potency *in vivo*. These experiments clarified structure-activity relationships of *N*-benzyl-5-methoxytryptamines and revealed that the *in vivo* potency of *N*,*N*-diallyltryptamines can be predicted by affinities at 5-HT_{2A} and 5-HT_{1A} receptors, with positive and negative contributions to potency, respectively. These are the first experiments to quantify the relative contributions of these receptors to the behavioral effects of a class of tryptamine hallucinogens. Next, I demonstrated that mice lacking $mGlu_5$ receptors are hypersensitive to multiple behavioral effects of hallucinogens. This hypersensitivity persists when a 5-HT_{2A} agonist functionally selective for PLC-mediated signaling is administered, indacating a role for the PLC signaling cascade in this phenomenon. These findings suggest that $mGlu_5$ receptors may typically provide tonic inhibition in response to excessive neuronal excitation, mitigating hallucinogen effects. Finally, I used a discrete-trials interval timing task to explore pharmacological mechanisms responsible for hallucinogen effects on interval timing. These experiments first probed the effects of a tryptamine hallucinogen, psilocin, on timing in mice, and revealed effects to depend on activation of 5-HT_{2A} but not 5-HT_{1A} receptors. Then, studies identified a non-monotonic relationship between 5-HT_{2A} activation and timing wherein either increases or decreases from baseline levels of 5-HT_{2A} activation result in similar timing effects, and demonstrated the sufficiency of hallucinogen effects in prefrontal cortex to alter interval timing. These experiments reveal novel insights on the relationship between pharmacological characteristics of hallucinogenic drugs and their behavioral effects.

INTRODUCTION

Classical hallucinogens are a subset of psychoactive drugs that act on the serotonergic system and produce profound changes in perception, cognition, and behavior. Hallucinogens are unique from many other drug classes (e.g. stimulants, opiates, etc.) in their ability to reliably produce qualitatively distinct mental states (Nichols, 2004). Hallucinogens have thus garnered scientific interest for their potential to elucidate the neurophysiology of normal conscious function, as well as their ability to recapitulate certain aspects of psychotic disorders such as schizophrenia (Halberstadt and Geyer, 2013a). Though systematic study of the effects of hallucinogens began in the late 1800s, historical evidence of the ritualistic and recreational consumption of these substances dates back several millennia (Nichols, 2004; Perrine, 1996). Nonetheless, the neural mechanisms by which these drugs produce their characteristic effects are still obscure.

In recent years, the effects of these compounds have taken on additional public health relevance. With the rise of web-based black market drug distribution, the availability of abused hallucinogens has increased dramatically, including several novel, highly potent hallucinogens designed for research that have been co-opted for recreational use (Barratt et al., 2014; Martin, 2013). Though fatalities attributable to abuse of hallucinogens are historically infrequent, abuse of these novel hallucinogens has led to numerous fatalities and cases of acute toxicity (Halberstadt and Geyer, 2013b; Lawn et al., 2014; Nikolaou et al., 2014; Rose et al., 2013; Tang et al., 2014; Walterscheid et al., 2014). Furthermore, hallucinogens have evoked renewed interest by the psychiatric community as promising therapeutic agents for the treatment of symptoms associated with PTSD, end-of-life anxiety, depression, and addiction (Bogenschutz and Johnson, 2016; Carhart-Harris et al., 2015, 2016; Catlow et al., 2013; Dos Santos et al., 2016; Gasser et al., 2015; Mithoefer et al., 2016; Nichols, 2016; Pathania, 2015; Winkelman, 2014). Clarifying the molecular features responsible for the behavioral potency of these drugs could help to predict the potential abuse liability and toxicity of newly emerging hallucinogens and to optimize synthesis of novel hallucinogen-like compounds for therapeutic use. Moreover, improved understanding of the neural circuitry underlying perceptual effects of hallucinogens can unearth new

insights into the pathophysiology of psychotic disorders and reveal novel targets for the treatment of myriad psychiatric illnesses.

Classical hallucinogens include three main structural classes: phenylalkylamines (e.g. mescaline, DOI); tryptamines (e.g. DMT, psilocybin); and ergolines (e.g. LSD). While compounds from all three classes bind to the 5-HT_{2A} receptor, tryptamines and ergolines also possess appreciable affinities for many other 5-HT receptor subtypes (e.g. 5-HT_{1A}) (Halberstadt and Koedood, 2011; Halberstadt and Geyer, 2011; Nichols, 2004). The 5-HT_{2A} receptor is thought to be necessary for most psychoactive effects of classical hallucinogens, as co-administration of a 5-HT_{2A} antagonist blocks the hallucinogenic effects of psilocybin in humans (Kometer et al., 2013; Vollenweider, 1998). Still, hallucinogens vary widely in affinity and efficacy at 5-HT_{2A} and recent evidence indicates that activity at other receptors may modulate their subjective effects (Fiorella et al., 1995; Halberstadt and Koedood, 2011; Nichols, 2016, 2004; Pokorny et al., 2016). For instance, the 5-HT_{2A} receptor is highly homologous to the 5-HT_{2C} receptor, with ~80% sequence homology in the transmembrane domains (Córdova-Sintjago et al., 2012). While both receptors are G_q-coupled and activate similar downstream pathways, distinct cellular and regional localizations result in differential effects of 5-HT_{2A} and 5-HT_{2C} activation on a number of behaviors and neurochemical events (Nichols and Nichols, 2008). All classical hallucinogens also activate the 5-HT_{2C} receptor, and no highly selective agonist for 5-HT_{2A} over 5-HT_{2C} currently exists, complicating investigation of the pharmacological mechanisms for hallucinogen action (Appel et al., 1990; McKenna and Peroutka, 1989). With the recent development of 25CN-NBOH, an agonist with ~23-fold selectivity for 5-HT_{2A} over 5-HT_{2C}, our capacity to explore the sufficiency of 5-HT_{2A} receptors in hallucinogen-associated behaviors has been considerably improved (Fantegrossi et al., 2014; Halberstadt et al., 2016; Hansen et al., 2014). Nonetheless, the necessity and sufficiency of 5-HT_{2A} activation for inducing specific characteristic effects of hallucinogens are still open topics for investigation.

Behavioral effects of hallucinogens are often modeled in rodents using the head twitch response (HTR) assay. HTR is a brief, paroxysmal head rotation in mice and rats that is induced by 5-HT_{2A} agonists and can be ameliorated by highly selective 5-HT_{2A} antagonists and by 5-HT_{2A} gene deletion

(Fantegrossi and Simoneau, 2010; Fantegrossi et al., 2014, 2008, 2006, 2005, González-Maeso et al., 2007, 2003; Halberstadt et al., 2011; Halberstadt and Geyer, 2013a; Schreiber et al., 1995). The propensity for 5-HT_{2A} agonists to induce HTR is highly predictive of hallucinogenic activity in humans, and non-hallucinogenic 5-HT_{2A} agonists such as lisuride fail to induce this response (Egan et al., 1998; González-Maeso et al., 2003; Halberstadt and Koedood, 2011; Halberstadt and Geyer, 2013a). HTR is thus widely accepted as a valid model for assessing hallucinogenic activity in rodents (Halberstadt and Geyer, 2013a, 2013c; Hanks and González-Maeso, 2013). To improve accuracy and throughput of the HTR assay, our lab developed an automated system for HTR assessment that utilizes a small head-mounted magnet and a magnetometer coil. Since voltage deflections in the coil output during the HTR exhibit a characteristic waveform (i.e. 40-160Hz activity, $\geq$ 2 bipolar peaks, amplitude exceeding background noise, duration < 0.15s), head twitches can be easily identified post hoc (Halberstadt and Geyer, 2013c). Automation of HTR detection not only allows higher throughput since multiple subjects can be tested simultaneously, but also improves accuracy, as the behavior is quite rapid and thus susceptible to gross observer error. Our HTR system allows the unprecedented ability to screen large catalogues of compounds for behavioral activity, providing an outstanding opportunity for investigation of structure-activity relationships of classical hallucinogens.

Although the HTR is an excellent tool for assessing structure-activity relationships and pharmacology as they relate to hallucinogen potency, it lacks the face validity to translate to specific behavioral or perceptual effects experienced by humans during hallucinogen intoxication. Whether or not the HTR relates to any specific subjective phenomenon is unclear, as it may simply be a hard-wired, involuntary behavior with no subjective basis. To better assess the mechanisms responsible for the interoceptive cues associated with hallucinogens, many researchers use the drug discrimination paradigm, an operant task in which a mouse or rat is trained to respond for reward on one lever when under the influence of a drug with known subjective qualities (e.g. LSD) and on the other if they are not experiencing the effects of that drug and have instead received vehicle. Once trained, the subject is administered a test drug or vehicle and the proportion of responses made on the drug and the non-drug

levers are quantified (Appel et al., 2004). While this assay permits assessment of the interoceptive similarity of two drugs and reliably indicates similarity between 5-HT_{2A} agonists known to produce similar subjective effects in humans (Benneyworth et al., 2005; Egan et al., 1998; Fantegrossi et al., 2008; Gatch et al., 2011; Glennon, 1986; Glennon et al., 1987, 1979; Glennon and Hauck, 1985; Hirschhorn and Winter, 1971; Smith et al., 2003; Winter et al., 2007), it does not indicate what specific subjective cues are responsible for similarity, which could be entirely independent of the perceptual effects typically associated with hallucinogenesis in humans. This assay also often fails to distinguish hallucinogenic and non-hallucinogenic agonists at the 5-HT_{2A} receptor (Appel et al., 1999; Fiorella et al., 1995; Glennon and Hauck, 1985; White and Appel, 1982) and requires regular administration of hallucinogens for training, which can alter the neuropharmacological characteristics of the subjects and is not consistent with the episodic use patterns of humans (Nichols, 2004).

To permit exploration of the pharmacological and circuit mechanisms underlying the perceptual effects of hallucinogens, we thus developed a mouse discrete-trials interval timing task that can be easily translated to a human design, allowing for similar assessment of psychoactive effects across species (Halberstadt et al., 2016). Hallucinogens have been reported both anecdotally and experimentally to alter the perception of time in humans (Beringer, 1927; Deshon et al., 1952; Hoch et al., 1952; Šerko, 1913; Wackermann et al., 2008; Wittmann et al., 2007). These findings have been corroborated in similar discrete-trials tasks in rats (Asgari et al., 2006; Body et al., 2006; Hampson et al., 2010), and research from our lab has also demonstrated altered interval timing in mice treated with the hallucinogenic amphetamine DOI or the selective 5-HT_{2A} agonist 25CN-NBOH (Halberstadt et al., 2016). We believe this model of a perceptual effect of hallucinogens represents a significant advance in the available experimental toolkit for investigating the mechanisms of hallucinogen effects in rodent models.

The overarching goal of my graduate work has been to explore the relationship between molecular properties of hallucinogens and their perceptual and behavioral effects. Toward this end, I have used mouse models of hallucinogen action to investigate how structural modifications to existing hallucinogens affect their behavioral potency, as well as how interactions with and between specific

neurotransmitter receptors contribute to the behavioral and perceptual effects of these drugs. Finally, I used the aforementioned timing task to better identify the pharmacological mechanisms underlying the effects of hallucinogens on the perception of time. In aggregate, the studies herein provide a broad survey of mechanisms responsible for the behavioral and perceptual effects of hallucinogens in rodents spanning from the relationship between chemical structure and pharmacology to the pharmacological basis of complex perceptual phenomena. These insights can optimize the therapeutic potential of hallucinogens for psychiatric treatment, inform the bases of psychiatric illnesses that produce debilitating psychotic symptoms, and allow more effective public health efforts to combat abuse of emerging novel hallucinogens.

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CHAPTER 1

N-BENZYL-5-METHOXYTRYPTAMINES AS POTENT SEROTONIN 5-HT_2 RECEPTOR FAMILY AGONISTS AND COMPARISON WITH A SERIES OF PHENETHYLAMINE ANALOGUES

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N-Benzyl-5-methoxytryptamines as Potent Serotonin 5-HT$_2$ Receptor Family Agonists and Comparison with a Series of Phenethylamine Analogues

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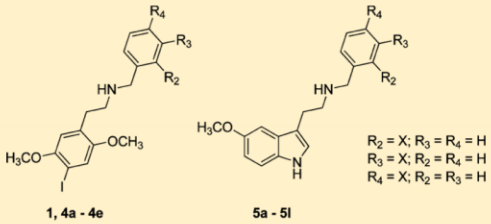
ABSTRACT: A series of *N*-benzylated-5-methoxytryptamine analogues was prepared and investigated, with special emphasis on substituents in the meta position of the benzyl group. A parallel series of several *N*-benzylated analogues of 2,5-dimethoxy-4-iodophenethylamine (2C-I) also was included for comparison of the two major templates (i.e., tryptamine and phenethylamine). A broad affinity screen at serotonin receptors showed that most of the compounds had the highest affinity at the 5-HT2 family receptors. Substitution at the para position of the benzyl group resulted in reduced affinity, whereas substitution in either the ortho or the meta position enhanced affinity. In general, introduction of a large lipophilic group improved affinity, whereas functional activity often followed the opposite trend. Tests of the compounds for functional activity utilized intracellular Ca^{2+} mobilization. Function was measured at the human 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors, as well as at the rat 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors. There was no general correlation between affinity and function. Several of the tryptamine congeners were very potent functionally (EC_{50} values from 7.6 to 63 nM), but most were partial agonists. Tests in the mouse head twitch assay revealed that many of the compounds induced the head twitch and that there was a significant correlation between this behavior and functional potency at the rat 5-HT$_{2A}$ receptor.



KEYWORDS: *Serotonin, 5-HT2 receptors, 5-HT$_{2A}$, agonist, phenethylamine, 5-methoxytryptamine, mouse head twitch*

■ INTRODUCTION

Recently, an extremely potent hallucinogenic phenethylamine, 25I-NBOMe (*N*-(2-methoxybenzyl)-2,5-dimethoxy-4-iodophenethylamine; "smiles") **1** has been available on the illicit drug market.[1] For purposes of enforcement, it is presently considered by the Drug Enforcement Administration (DEA) to be an analogue of 2C-I (**2**), which is currently a Schedule I controlled substance. The procedure to classify **1** as a Schedule I substance has been initiated, and it has been placed temporarily into Schedule I.[2] Unfortunately, several deaths have been associated with the use of **1**,[3−5] but it is not clear whether the deaths resulted from the ingestion of lethal amounts of pure solid drug, or whether the drug has some inherent toxicity that is not normally associated with other hallucinogens.

There has been increasing global interest in **1** and closely related analogues. For example, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) has received a range of notifications from EU Member States about analytically confirmed nonfatal and fatal intoxications associated with **1**. This was then followed by a risk assessment conducted by the Scientific Committee of the EMCDDA in order to assess health and social risks associated with this particular analogue.[6] In addition, the World Health Organization's Expert Committee on Drug Dependence reviewed the status of a range of new substances for its 36th meeting in June 2014, which included **1** and its 4-bromo and 4-chloro analogues.[7] In September 2014, the Council of the European Union decided to subject **1** to control measures and criminal penalties throughout the European Union.[8]

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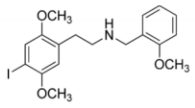

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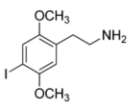

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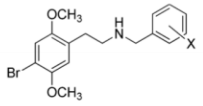
Typically, simple N-alkylation dramatically attenuates or abolishes hallucinogenic activity in phenethylamines.[9,10] The *N*-benzyl moiety, however, confers exceptionally high potency onto the molecule,[11−15] and we have presented evidence that the *N*-benzyl may engage F339 in the human 5-HT_{2A} receptor.[14] We also examined various *N*-arylmethyl substituents and found that a variety of aryl groups were effective in enhancing potency.[16,17] In addition, the presence of a polar substituent at the ortho position of the aryl ring (a possible hydrogen bond acceptor) further enhances activity.[18] Silva et al.[18] also have reported that in an in vitro cylindrical rat tail artery strip **1** had a pEC_{50} of 10.09 and an E_{max} of 30%.

Two decades ago, Glennon et al.[19] reported that the affinities of the *N*-benzyl compound **3a**, as well as the 4-bromo- and 4-iodo-*N*-benzyl compounds, **3b** and **3c**, respectively, were 2−3 times higher than that of the parent primary amine. There have been no further reports on these compounds, and in our own work, we had never examined 3- or 4-substituted benzyl substituents in the phenethylamine series.

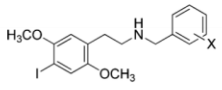


3a X = H, **3b** X = 4-Br, **3c** X = 4-I

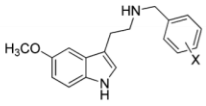
In addition to the phenethylamine type 5-HT_{2A} agonists, certain simple tryptamines possess similar pharmacology, particularly 4- or 5-oxygenated molecules. In the report by Glennon et al., placing an *N*-benzyl moiety on the amine of 5-methoxytryptamine had essentially no effect on affinity. Interestingly, *N*-benzyl-5-methoxytryptamine previously had been reported to be an antagonist of serotonin-induced contraction in the rat stomach fundus, the isolated guinea pig uterus, and the isolated guinea pig taenia cecum.[20] In addition, Leff et al.[21] had shown that *N*-benzyl-5-methoxytryptamine had only weak partial agonist activity at 5-HT_2 type receptors in rabbit aorta and rat jugular vein.

Surprisingly, however, in the Glennon report,[19] a 5-HT_{2A} receptor affinity of 0.1 nM was reported for the *N*-4-bromobenzyl compound (compound **33** in the Glennon report, numbered here as **5f**), with 1000-fold selectivity for 5-HT_{2A} over 5-HT_{2C} receptors. We found these data particularly intriguing. This degree of selectivity was overestimated, however, because affinity at the 5-HT_{2A} receptor was measured by displacement of an agonist ragioligand, whereas affinity at the 5-HT_{2C} receptor was measured by displacement of an antagonist radioligand. Nonetheless, no specific 5-HT_{2A}-selective agonist has been available, although such a compound would be very valuable for serotonin neuroscience research.

Although it was reported[19] that 4-bromo compound **5f** had 0.1 nM affinity at the human 5-HT_{2A} receptor, the 4-fluoro-, 4-chloro-, and 4-iodo-substituted benzyl congeners had reported affinities of 40, 105, and 120 nM, respectively, in that same report. We found this discontinuity in the structure−activity relationship (SAR) puzzling, where the 4-bromo compound would be such an outlier in the family of halogen-substituted benzyls. Further investigation by Jensen, however, revealed that the authentic 4-bromo compound **5f** actually had relatively low affinity for the 5-HT_{2A} receptor, more consistent with the reported affinities of the other halogenated compounds.[22] Although spectroscopic data were not reported by Glennon et al.[19] that might explain the basis for this discrepancy, their publication indicated elemental analysis data to be consistent with the proposed structure. If the elemental analysis data were correct, the mostly likely explanation for the discordant biological data therefore seemed to be that **5f** might have been an isomer other than the 4-substituted compound.



1, X = 2-OCH_3
4a, X = 3-OCH_3
4b, X = 4-OCH_3
4c, X = 2-Br
4d, X = 3-Br
4e, X = 4-Br



5a, X = 2-OCH_3
5b, X = 3-OCH_3
5c, X = 4-OCH_3
5d, X = 2-Br
5e, X = 3-Br
5f, X = 4-Br
5g, X = 3-F
5h, X = 3-Cl
5i, X = 3-I
5j, X = 3-CH_3
5k, X = 3-CF_3
5l, X = 3-SCH_3

On the basis of the hypothesis that the original data were associated with an isomer other than the 4-bromo compound, we subsequently discovered that *N*-3-bromobenzyl compound **5e** did have higher affinity for the 5-HT_{2A} receptor (K_i 1.48 nM), compared to that of the 4-bromo congener **5f** (K_i 11.2 nM). Further, the effect of an ortho-oxygenated *N*-benzyl appeared not to be significant for affinity in the tryptamine series, suggesting perhaps different binding orientations of the *N*-benzyltryptamines versus the *N*-benzylphenethylamines within the receptor. That is, compound **5a** has been reported to have agonist potency (pEC_{50} 7.08) in a rat tail artery assay not significantly different from the compound with an unsubstituted *N*-benzyl moiety (pEC_{50} 7.00), although the E_{max} was slightly higher for the 2′-methoxy compound.[18] These findings prompted us to synthesize a small series of structurally related congeners to determine whether other substitutions might have even greater affinity and/or selectivity for the 5-HT_{2A} receptor.

Thus, in this article we describe the facile synthesis of compounds **1**, **4a−4e**, and **5a−5l**, preliminary screening at a variety of 5HT family receptors, and more detailed testing at human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, including affinity measurements using displacement of the agonist radioligand [^{125}I]-DOI and functional effects in elevating intracellular calcium. We also present behavioral data for the mouse head twitch response (HTR) as a measure of in vivo 5-HT_{2A} receptor activation.[23]

Compound **1** has been previously reported,[24] and the NMR and electron ionization mass spectra of **4a** and **4b** have been reported but without any biological data.[25] We thus decided to compare all of the series members at the same time to elucidate a consistent SAR.

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CHEMISTRY

All of the compounds were most easily prepared using a modification of the facile method first reported by Abdel-Magid et al.[26] The free base of **2** was stirred in 3 mL of MeOH for 30 min with the appropriate aldehyde, followed by reduction of the intermediate enamine with $NaBH_4$. Following appropriate workup, the bases were converted to their HCl or maleate salts and crystallized in good to excellent yields.

PHARMACOLOGY

Affinities at a panel of 5-HT receptors were determined by the NIMH-sponsored PDSP program (http://pdsp.med.unc.edu/kidb.php). Affinities at both the human and rat 5-HT_{2A} and 5-HT_{2C} receptors also were determined, using both agonist and antagonist radioligands. As a measure of functional potency and efficacy, changes in intracellular Ca^{2+} levels were measured using a fluorometric imaging plate reader ($FLIPR^{TETRA}$, Molecular Devices), at the human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, and at the rat 5-HT_{2A} and 5-HT_{2C} receptors. Finally, as a measure of in vivo 5-HT_{2A} receptor activation, we assessed the ability of all compounds to induce the mouse HTR.[23] We hypothesized that functional potency at the rat 5-HT_{2A} receptor might correlate best with the mouse head twitch behavioral data because ligand affinities at the rat 5-HT_{2A} receptor correlate with the mouse 5-HT_{2A} receptor but not with the human 5-HT_{2A} receptor.[27]

RESULTS

Further exploration of a small library of 3-substituted *N*-benzyl tryptamines allowed us to develop a tentative SAR for this series, and it is clear that substituents on the *N*-benzyl 3-position do modulate affinity in the tryptamine series. In the broad screening of 5-HT receptor types, all of the compounds had the highest affinity at the 5-HT2 family of receptors (Tables 1 and 2).

At the 5-HT_{2A} and 5-HT_{2C} receptors, the highest affinity was observed in the competition displacements with [^{125}I]-DOI. Except for **5c** and **5f**, all of the tryptamine compounds had low nanomolar or subnanomolar affinity for the human 5-HT_{2A} receptor. The known phenethylamine **1** had by far the highest affinity at 5-$HT_{2A/2C}$ receptors, with subnanomolar affinity at both subtypes. We have previously reported an affinity for **1** at the human 5-HT_{2A} receptor of 0.04 nM.[14] Of the tryptamines, only the 3-iodobenzyl compound **5i**, had subnanomolar affinity at the 5-HT_{2A} receptor, although all of the tryptamines had high affinity at this receptor. It should be noted that N-methylation of **5e** completely abolished affinity at the 5-HT_{2A} receptor (K_i > 10 μM; data not shown), indicating that tertiary amines are not tolerated in the *N*-benzyltryptamines.

The rank order of affinity of all compounds at the [^{125}I]-DOI-labeled h5-HT_{2C} receptor generally paralleled that measured at the 5-HT_{2A} receptor, although the affinities tended to be somewhat lower. Again, among the tryptamines studied **5i** had the highest affinity at this receptor, as well as at the 5-HT_{2B} receptor. Affinities measured at the [^{125}I]-DOI site tended to be on the order of 5−10 times higher than that at the antagonist labeled sites at both receptors.

Functional potencies at the rat and human 5-HT_{2A} and 5-HT_{2C} receptors and the human 5-HT_{2B} receptor are shown in Table 3. Compound **1** was a nearly full agonist at both receptor types, with a 4.2 nM EC_{50} at the human 5-HT_{2A} receptor and 11 nM EC_{50} at the rat 5-HT_{2A} receptor. The most potent compound was **5a**, with an EC_{50} of 1.9 nM and 85% efficacy at the h5-HT_{2A}. Notably, this compound has the *N*-2-methoxybenzyl substituent, the same as the most potent phenethylamine **1**, suggesting that it may be optimal for activation of the 5-HT_{2A} receptor when placed at the 2-position of the *N*-benzyl moiety. Efficacies of the tryptamines at the rat and human 5-HT_{2A} receptors and human 5-HT_{2C} receptor varied from about 40% to 80%, with a few compounds that were full agonists (e.g., **5a** and **5c**), whereas at the rat 5-HT_{2C} receptor all of the compounds were full agonists.

It is noteworthy that the functional potencies in the rat and human 5-HT_{2A} receptors are essentially identical for phenethylamine compounds **1**, and **4a**−**4e**, yet the potencies for tryptamine compounds **5a**−**5l** are 4−10-fold higher at the human 5-HT_{2A} receptor than at the rat 5-HT_{2A} receptor. This finding may reflect the single amino acid difference in the orthosteric binding site of these two receptors at position 5.46. In the rat or mouse 5-HT_{2A} receptor, residue 5.46 is an alanine, whereas in the human receptor it is a serine. We have previously shown that mutation of this residue in the human receptor from serine to alanine has little effect on affinity or function for phenethylamine 5-HT_{2A} agonists but does have a significant effect for tryptamines.[28] One might infer, therefore, from these potency differences that the indole NH in the

Table 1. Affinities of New Compounds for the Human 5-HT_{2A} and 5-HT_{2C} Receptors Using Both Agonist and Antagonist Radioligands[a]

	h5-HT_{2A} pK_i ± SEM (K_i nM)		h5-HT_{2C} pK_i ± SEM (K_i nM)	
cmpd	[^{3}H]ketanserin	[^{125}I]DOI	[^{3}H]mesulergine	[^{125}I]DOI
1	9.28 ± 0.11 (0.52)	9.80 ± 0.15 (0.16)	9.16 ± 0.09 (0.69)	9.30 ± 0.16 (0.50)
4a	8.81 ± 0.17 (1.5)	9.57 ± 0.09 (0.27)	8.38 ± 0.01 (4.17)	9.90 ± 0.07 (0.13)
4b	7.93 ± 0.13 (11.7)	9.15 ± 0.16 (0.70)	7.85 ± 0.02 (14.1)	8.44 ± 0.14 (3.63)
4c	8.63 ± 0.18 (2.34)	9.42 ± 0.09 (0.38)	8.06 ± 0.07 (8.71)	8.99 ± 0.18 (1.02)
4d	8.40 ± 0.04 (3.98)	9.24 ± 0.12 (0.57)	8.12 ± 0.02 (7.59)	8.79 ± 0.08 (1.62)
4e	7.28 ± 0.14 (52.5)	8.49 ± 0.09 (3.24)	7.34 ± 0.02 (45.7)	8.48 ± 0.25 (3.31)
5a	7.78 ± 0.05 (16.6)	8.82 ± 0.19 (1.51)	7.49 ± 0.14 (32.4)	8.47 ± 0.10 (3.39)
5b	8.11 ± 0.10 (7.76)	8.98 ± 0.14 (1.05)	7.42 ± 0.12 (38.0)	8.23 ± 0.09 (5.89)
5c	7.16 ± 0.16 (69.2)	7.98 ± 0.04 (10.5)	6.90 ± 0.03 (126)	7.85 ± 0.13 (14.1)
5d	7.60 ± 0.12 (25.1)	8.63 ± 0.19 (2.34)	7.00 ± 0.01 (100)	7.85 ± 0.10 (14.1)
5e	8.17 ± 0.11 (6.76)	8.83 ± 0.10 (1.48)	7.58 ± 0.05 (26.3)	8.25 ± 0.11 (5.62)
5f	6.37 ± 0.12 (427)	7.95 ± 0.22 (11.2)	6.60 ± 0.15 (251)	7.54 ± 0.19 (28.8)
5g	7.67 ± 0.04 (21.4)	8.58 ± 0.17 (2.63)	7.32 ± 0.09 (47.9)	8.06 ± 0.14 (8.71)
5h	8.28 ± 0.08 (5.25)	8.98 ± 0.10 (1.05)	7.55 ± 0.06 (28.2)	8.37 ± 0.05 (4.27)
5i	8.46 ± 0.09 (3.47)	9.21 ± 0.16 (0.62)	8.19 ± 0.09 (6.46)	8.98 ± 0.08 (1.05)
5j	8.32 ± 0.17 (4.79)	8.93 ± 0.11 (1.17)	7.65 ± 0.03 (22.4)	8.47 ± 0.08 (3.39)
5k	7.55 ± 0.05 (28.2)	8.53 ± 0.19 (2.95)	6.99 ± 0.06 (102)	7.83 ± 0.26 (14.8)
5l	8.05 ± 0.15 (8.91)	8.51 ± 0.17 (3.09)	7.88 ± 0.23 (13.2)	8.68 ± 0.30 (2.09)

[a] pK_i ± SEM (affinities in nM); n = 3−5 separate displacement curves.

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Table 2. PDSP Screening Affinities for All Compounds at Other Human Serotonin Receptor Types[a]

cmpd	5-HT$_{2B}$	5-HT$_{1A}$	5-HT$_{1B}$	5-HT$_{1D}$	5-ht1e	5-HT$_3$	5-ht5a	5-HT$_6$	5-HT$_7$
1	8.86 ± 0.03 (1.4)	5.99 ± 0.05 (1033)	5.23 ± 0.06 (5886)	6.27 ± 0.05 (533)	>10,000	>10,000	5.55 ± 0.07 (2795)	7.5 ± 0.06 (32)	5.81 ± 0.06 (1542)
4a	8.34 ± 0.03 (4.6)	6.03 ± 0.05 (925)	5.49 ± 0.05 (3232)	6.36 ± 0.05 (439)	5.77 ± 0.05 (1707)	>10,000	7.24 ± 0.06 (57)	7.17 ± 0.06 (67)	6.23 ± 0.06 (583)
4b	7.78 ± 0.03 (17)	5.97 ± 0.05 (1064)	5.8 ± 0.05 (1592)	6.49 ± 0.05 (325)	5.89 ± 0.05 (1285)	>10,000	5.99 ± 0.06 (1020)	7.12 ± 0.03 (75)	5.8 ± 0.06 (1575)
4c	7.7 ± 0.04 (20)	5.94 ± 0.06 (1155)	>10,000	6.37 ± 0.05 (423)	>10,000	>10,000	5.64 ± 0.09 (2290)	6.59 ± 0.06 (257)	5.59 ± 0.05 (2547)
4d	7.89 ± 0.04 (13)	6.17 ± 0.06 (670)	5.80 ± 0.05 (1568)	6.79 ± 0.05 (162)	6.10 ± 0.04 (792)	>10,000	6 ± 0.08 (1009)	6.76 ± 0.06 (175)	6.45 ± 0.05 (355)
4e	7.17 ± 0.04 (68)	6.19 ± 0.06 (649)	5.22 ± 0.05 (5093)	6.51 ± 0.05 (311)	>10,000	5.61 ± 0.05 (2460)	5.73 ± 0.06 (1848)	6.46 ± 0.05 (350)	6.19 ± 0.05 (641)
5a	8.04 ± 0.03 (9)	6.64 ± 0.05 (231)	>10,000	5.89 ± 0.05 (1292)	>10,000	>10,000	>10,000	7.06 ± 0.03 (87)	5.75 ± 0.06 (1770)
5b	8.6 ± 0.03 (2.5)	6.48 ± 0.05 (335)	>10,000	6.48 ± 0.06 (334)	>10,000	>10,000	5.9 ± 0.06 (1261)	7.6 ± 0.03 (25)	6.39 ± 0.05 (406)
5c	7.49 ± 0.03 (33)	7.12 ± 0.06 (76)	5.97 ± 0.04 (1060)	6.79 ± 0.06 (161)	>10,000	>10,000	5.62 ± 0.09 (2388)	6.45 ± 0.03 (353)	7.44 ± 0.05 (37)
5d	7.62 ± 0.03 (24)	6.54 ± 0.05 (286)	>10,000	6.11 ± 0.05 (782)	>10,000	5.21 ± 0.07 (6169)	>10,000	6.69 ± 0.05 (203)	5.96 ± 0.05 (1086)
5e	8.45 ± 0.03 (3.6)	6.81 ± 0.05 (155)	5.19 ± 0.06 (6433)	6.42 ± 0.05 (381)	>10,000	>10,000	6.21 ± 0.06 (612)	7.34 ± 0.03 (45)	6.93 ± 0.06 (116)
5f	6.83 ± 0.03 (150)	7.11 ± 0.05 (78)	5.35 ± 0.05 (4374)	6.57 ± 0.05 (271)	>10,000	>10,000	5.99 ± 0.08 (1034)	6.25 ± 0.03 (566)	6.45 ± 0.06 (358)
5g	7.66 ± 0.03 (22)	6.53 ± 0.04 (295)	5.57 ± 0.06 (2674)	6.50 ± 0.06 (319)	>10,000	>10,000	5.61 ± 0.05 (2450)	7.23 ± 0.03 (59)	6.62 ± 0.05 (242)
5h	8.16 ± 0.02 (6.6)	6.71 ± 0.05 (195)	5.36 ± 0.06 (4392)	6.55 ± 0.06 (282)	>10,000	>10,000	5.64 ± 0.06 (2310)	7.30 ± 0.03 (50)	6.55 ± 0.05 (281)
5i	9.12 ± 0.03 (0.76)	6.91 ± 0.05 (122)	5.53 ± 0.05 2963)	6.70 ± 0.06 (199)	>10,000	>10,000	5.81 ± 0.05 (1536)	7.58 ± 0.03 (27)	7.66 ± 0.05 (22)
5j	8.71 ± 0.03 (1.9)	6.57 ± 0.04 (271)	5.37 ± 0.07 (4241)	6.55 ± 0.06 (283)	5.41 ± 0.05 (3876)	>10,000	5.41 ± 0.06 (3852)	7.21 ± 0.03 (62)	6.67 ± 0.05 (212)
5k	7.56 ± 0.02 (28)	6.62 ± 0.05 (240)	>10,000	6.56 ± 0.06 (278)	>10,000	>10,000	5.51 ± 0.06 (3091)	7.06 ± 0.03 (87)	6.58 ± 0.05 (262)
5l	8.39 ± 0.04 (4.1)	6.90 ± 0.05 (127)	>10,000	6.18 ± 0.05 (659)	>10,000	>10,000	6.08 ± 0.08 (841)	8.01 ± 0.06 (9.7)	6.87 ± 0.05 (136)

[a]pK_i ± SEM, (affinity in nM).

present series also engages this serine in the human receptor but not the alanine in the rat receptor, consistent with mutagenesis studies reported by others.[29,30]

Figure 1 shows an illustrative dose−response curve for compound **5h** in the mouse HTR. HTR data for all compounds are given in Table 4. Although some of the compounds failed to induce the HTR at doses up to 30 mg/kg, most of the "inactive" compounds displayed relatively low potency at 5-HT$_{2A}$ (see Figure 2), so it is possible that they would induce the HTR if tested at higher doses. Importantly, for the subset of compounds that induced the HTR, behavioral potency was significantly correlated with functional potency at the r5-HT$_{2A}$ receptor ($r = 0.69$, $p < 0.03$; Figure 2), but there was no correlation with functional EC$_{50}$ values at the r5-HT$_{2C}$ receptor ($r = 0.17$, $p > 0.1$). Despite the overall correlation between mouse HTR and r5-HT$_{2A}$ potency, the relationship was not always orderly for individual compounds. Compound **1** was by far the most potent compound in that assay, with an ED$_{50}$ of 0.078 mg/kg (data taken from Halberstadt and Geyer[31]). It is not clear why **1** should be so much more potent than any other compound because, for example, **4d** is inactive but appears nearly comparable functionally, with an EC$_{50}$ of 14 nM and efficacy of 69%, compared with an EC$_{50}$ of 11 nM for **1** with an efficacy of 79%. The next most potent compounds in the mouse HTR are **4c** and **5j**, with identical ED$_{50}$s of 2.31 mg/kg, about 300-fold less potent than **1**. Although they have similar functional EC$_{50}$ values (36 and 26 nM), nothing in the functional or binding data can explain their lower potency compared to that of **1**. Further, compounds **5a**, **5b**, and **5g** have virtually identical ED$_{50}$ values in the mouse HTR, yet their functional EC$_{50}$s at the rat 5-HT$_{2A}$ receptor are 21, 34, and 80 nM, respectively.

With the exception of **5k** and **5l**, which had relatively low functional potencies at the r5-HT$_{2A}$ (EC$_{50}$ values of 770 and 120 nM, respectively), all of the meta-substituted *N*-benzyl derivatives of 5-methoxytryptamine induced the HTR. That included the 3-methyl (**5j**; ED$_{50}$ = 2.31 mg/kg), 3-methoxy (**5b**; ED$_{50}$ = 3.28 mg/kg), 3-fluoro (**5g**; ED$_{50}$ = 3.33 mg/kg), 3-chloro (**5h**; ED$_{50}$ = 4.43 mg/kg), 3-bromo (**5e**; ED$_{50}$ = 5.18 mg/kg), and 3-iodo (**5i**; ED$_{50}$ = 7.77 mg/kg) compounds.

The HTR produced by compounds **5b** and **5j** showed a biphasic bell-shaped dose−response function (the response peaked at 10 mg/kg and 30 mg/kg was inactive). Other 5-HT$_{2A}$ agonists, including DOI, DOM, 2C-T-7, and 5-MeO−DIPT, have been shown to produce similar nonmonotonic responses.[32−34] Fantegrossi et al.[34] have argued that the descending arm of the biphasic HTR dose−response is a consequence of 5-HT$_{2C}$ activation, which attenuates the response to 5-HT$_{2A}$ activation. Recently, however, it was reported that *N*-(2-hydroxybenzyl)-2,5-dimethoxy-4-cyanophenethylamine (25CN-NBOH), a 5-HT$_{2A}$ agonist with 100-fold selectivity over 5-HT$_{2C}$, also induces the HTR with a biphasic dose−response.[35] The fact that the descending arm of the response to 25CN-NBOH was not affected by a 5-HT$_{2C}$

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Table 3. Functional Data for New Compounds in Rat and Human 5-HT_{2A} and 5-HT_{2C} and Human 5-HT_{2B} Receptors[a]

	r5-HT_{2A}		h5-HT_{2A}		h5-HT_{2B}		r5-HT_{2C}		h5HT_{2C}	
cmpd	pEC_{50} (EC_{50} nM)	E_{max} %	pEC_{50} (EC_{50} nM)	E_{max} %	pEC_{50} (EC_{50} nM)	E_{max} %	pEC_{50} (EC_{50} nM)	E_{max} %	pEC_{50} (EC_{50} nM)	E_{max} %
5-HT	8.3 ± 0.04 (5.4)	100 ± 1.5	8.7 ± 0.05 (2.0)	100 ± 1.6	9.31 ± 0.04 (0.49)	99.9 ± 1.1	9.70 ± 0.03 (0.20)	99.6 ± 0.73	9.52 ± 0.08 (0.30)	98.5 ± 2.4
1	8.0 ± 0.04 (11)	79.4 ± 1.1	8.4 ± 0.05 (4.2)	86.4 ± 1.4	7.81 ± 0.09 (15)	65 ± 2	7.02 ± 0.05 (95)	104 ± 2	7.38 ± 0.12 (41.7)	92 ± 0
4a	7.6 ± 0.04 (27)	51.7 ± 0.9	7.6 ± 0.03 (28)	71.6 ± 0.9	7.4 ± 0.3 (38)	NA[b]	6.88 ± 0.06 (133)	91 ± 3	7.47 ± 0.36 (33.8)	41 ± 6
4b	7.3 ± 0.04 (50)	53.8 ± 0.9	7.2 ± 0.03 (60)	74.1 ± 0.8	7.1 ± 0.1 (87)	38 ± 2	6.88 ± 0.05 (132)	97 ± 2	7.36 ± 0.31 (43.2)	50 ± 7
4c	7.4 ± 0.06 (36)	65.6 ± 1.6	7.4 ± 0.04 (42)	88.0 ± 1.5	6.82 ± 0.07 (134)	83 ± 3	6.98 ± 0.02 (105)	104 ± 1	7.24 ± 0.13 (57.6)	87 ± 5
4d	7.8 ± 0.03 (14)	68.5 ± 0.9	7.8 ± 0.04 (17)	87.5 ± 1.3	7.05 ± 0.05 (85)	90 ± 2	7.44 ± 0.05 (36)	101 ± 2	7.28 ± 0.17 (57.6)	74 ± 5
4e	6.8 ± 0.03 (150)	67.3 ± 0.9	6.8 ± 0.03 (170)	88.0 ± 1.4	6.21 ± 0.04 (610)	90 ± 2	6.54 ± 0.04 (290)	105 ± 2	6.66 ± 0.14 (200)	77 ± 5
5a	7.7 ± 0.03 (21)	80.9 ± 1.1	8.7 ± 0.05 (1.9)	85.2 ± 1.4	8.2 ± 0.1 (6.7)	52 ± 2	7.79 ± 0.04 (16)	102 ± 2	7.24 ± 0.12 (57.1)	119 ± 6
5b	7.5 ± 0.04 (34)	52.2 ± 0.9	8.2 ± 0.04 (6.2)	70.0 ± 1.0	6.0 ± 0.4 (949)	NA[b]	6.78 ± 0.05 (168)	102 ± 2	6.75 ± 0.15 (178)	65 ± 5
5c	6.7 ± 0.03 (190)	75.0 ± 1.3	7.4 ± 0.04 (42)	84.1 ± 1.3	7.64 ± 0.04 (23)	81 ± 1	7.73 ± 0.04 (19)	102 ± 2	7.12 ± 0.11 (75.1)	112 ± 5
5d	6.3 ± 0.04 (450)	49.7 ± 1.2	7.5 ± 0.05 (30)	74.7 ± 1.5	6.8 ± 0.3 (168)	NA[b]	6.05 ± 0.05 (898)	104 ± 3	6.36 ± 0.09 (439)	94 ± 5
5e	6.9 ± 0.03 (130)	65.5 ± 0.8	7.9 ± 0.04 (13)	73.8 ± 1.1	7.5 ± 0.2 (29)	20 ± 2	6.38 ± 0.04 (422)	112 ± 3	6.49 ± 0.23 (321)	64 ± 8
5f	5.8 ± 0.04 (1500)	77.6 ± 2.4	6.4 ± 0.02 (430)	90.3 ± 1.2	6.54 ± 0.05 (290)	90 ± 2	6.69 ± 0.03 (204)	108 ± 2	6.28 ± 0.14 (529)	83 ± 7
5g	7.1 ± 0.04 (80)	69.1 ± 1.3	8.0 ± 0.1 (10)	89.3 ± 1.1	7.42 ± 0.08 (38)	37 ± 1	7.34 ± 0.07 (46)	100 ± 3	6.72 ± 0.13 (192)	83 ± 5
5h	7.1 ± 0.03 (83)	70.1 ± 1.0	7.9 ± 0.04 (14)	81.2 ± 1.3	7.3 ± 0.2 (50)	NA[b]	6.54 ± 0.04 (286)	105 ± 2	6.50 ± 0.13 (316)	85 ± 6
5i	6.9 ± 0.04 (120)	73.4 ± 1.4	7.8 ± 0.04 (16)	79.0 ± 1.1	7.4 ± 0.2 (43)	31 ± 2	6.51 ± 0.05 (313)	110 ± 3	6.35 ± 0.09 (445)	94 ± 5
5j	7.6 ± 0.04 (26)	56.2 ± 0.9	8.2 ± 0.04 (6.5)	73.3 ± 1.0	NA[b]		6.72 ± 0.04 (192)	104 ± 2	6.54 ± 0.10 (289)	75 ± 4
5k	6.1 ± 0.03 (770)	69.6 ± 1.4	7.1 ± 0.04 (87)	75.5 ± 1.2	6.97 ± 0.07 (107)	51 ± 2	6.79 ± 0.03 (162)	104 ± 2	6.29 ± 0.11 (512)	75 ± 5
5l	6.9 ± 0.05 (120)	32.0 ± 0.7	7.5 ± 0.04 (32)	46.9 ± 0.8	NA[b]		6.69 ± 0.05 (205)	101 ± 2	6.55 ± 0.11 (283)	60 ± 4

[a]Values are pEC_{50} ± SEM, with (EC_{50}) values in nM and E_{max} given in percentage of the maximum response to 5-HT. [b]NA, not active; $E_{max} \leq 15\%$.

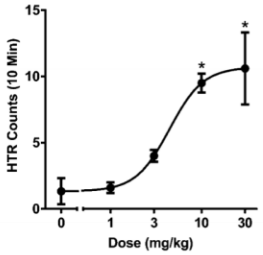

Figure 1. Representative dose–response plot in the mouse head twitch assay for compound **5h**. *$p < 0.05$ versus vehicle (Tukey's test).

antagonist[35] demonstrates that the inhibition of the HTR at high doses does not necessarily result from competing activity at 5-HT_{2C}. One potential alternative explanation for the biphasic HTR is that high levels of 5-HT_{2A} activation may produce competing behaviors that interfere with expression of head shaking. Along those lines, it has been reported that high doses of quipazine, 5-MeO-DMT, and (+)-LSD produce stereotypic behaviors that preclude head shakes and wet dog shakes in rats.[36,37]

■ DISCUSSION

Unfortunately, despite the report by Glennon et al.,[19] compound **5e** was not selective for the h5-HT_{2A} receptor versus the h5-HT_{2C} receptor. Using affinity at the [^{125}I]-DOI-labeled receptors, the selectivity of **5e** was slightly less than 4-fold. Even using affinity at the [^{125}I]-DOI-labeled h5-HT_{2A} receptor and the [^{3}H]-mesulergine-labeled h5-HT_{2C} receptor, "selectivity" was only about 18-fold. The most selective compound in the entire series, with respect to affinity, was **5d**, but with only 6-fold selectivity.

With respect to selectivity in function at the h5-HT_{2A} vs h5-HT_{2C}, the most selective tryptamine was **5j**, with 44-fold selectivity and less than a 3-fold difference in affinity at the agonist-labeled receptors. Indeed, we were disappointed that none of the compounds had high selectivity for the h5-HT_{2A} receptor.

Overall, with the exception of compound **1**, none of the compounds was particularly potent in producing the HTR. This low potency is somewhat surprising, given that many known hallucinogens with high affinity for the 5-HT_{2A} receptor, such

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Table 4. Activity of New Compounds in Producing the Mouse Head Twitch

	ED_{50} mg/kg (95% CI)	test duration (min)	N	dose range	active doses (mg/kg)	max counts	maximally effective dose (mg/kg)	magnitude of peak effect × vehicle
1	0.078 (0.055−0.111)	30	5	0.03−1.0	0.1, 0.3, 1	102.6	1	16.0
4a	4.34 (1.41−13.32)	10	10	0.3−30	3, 10, 30	11.4	30	5.7
4b	inactive		5	0.3−30				
4c	2.31 (1.41−3.77)	20	5	0.3−30	3, 10, 30	23.2	10	3.0
4d	inactive		5−7	0.3−10				
4e	inactive		6	1−30				
5a	3.15 (1.94−5.12)	20	10	0.3−30	10, 30	25.4	10	3.9
5b	3.28 (1.53−7.04)	10	5−6	1−30	10	9.2	10	3.7
5c	inactive		5	30				
5d	inactive		5	0.3−30				
5e	5.18 (2.35−11.38)	10	5−6	1−30	10, 30	14.2	30	4.4
5f	inactive		5	0.3−30				
5g	3.33 (2.25−4.93)	10	6	1−30	10, 30	14.5	10	7.3
5h	4.43 (2.03−9.69)	10	5−6	1−30	10, 30	10.6	30	8.0
5i	7.77 (3.40−17.53)	10	6	1−30	10, 30	20.2	30	3.4
5j	2.31 (0.82−6.51)	10	5	0.3−30	10	14.6	10	3.5
5k	inactive		5	30				
5l	inactive		4−5	30				

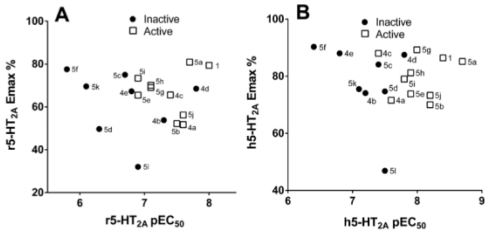

Figure 2. Plots of active and inactive compounds as a function of potency and efficacy at the rat 5-HT_{2A} receptor (panel A) and the human 5-HT_{2A} receptor (panel B).

as 2,5-dimethoxy-4-iodoamphetamine (DOI), *R*-(−)-2,5-dimethoxy-4-methylamphetamine (*R*-DOM), *R*-(−)-2,5-dimethoxy-4-bromoamphetamine (*R*-DOB), 2,5-dimethoxy-4-propylthiophenethylamine (2C-T-7), psilocin, and 5-MeO-*N*,*N*-diisopropyltryptamine (5-MeO−DIPT) produce the head twitch in mice at doses of ≤1 mg/kg.[32,33,38−40] However, certain tryptamine hallucinogens, including 5-MeO-*N*,*N*-dimethyltryptamine (5-MeO-DMT) and α-methyltryptamine, are active within the same dose range (3−30 mg/kg) as the *N*-benzyltryptamines tested herein.[40−42] It is unlikely that the low *in vivo* potencies of the compounds studied here are related to the use of an automated HTR detection system because we have confirmed that the results obtained using this system are consistent with published data based on visual scoring.[23] For example, the potency of LSD measured using the automated system (ED_{50} = 0.13 μmol/kg)[23] is almost exactly the same as the potency assessed using direct observation (ED_{50} = 0.14 μmol/kg).[41] One possible explanation for the low potencies might be rapid first pass metabolism of *N*-benzyl-analogues in general[43] combined with a slow release from subcutaneous tissue due to the highly hydrophobic nature of the compounds.

Substitution on the *N*-benzyl ring has different effects, depending on whether the phenethylamines or the tryptamines are being studied. For example, *ortho*-bromo-substituted tryptamine congener **5d** failed to induce the HTR when tested at doses up to 30 mg/kg (~60 μmol/kg), yet *N*-3-bromobenzyl **5e** is active. By contrast, *N*-2-bromobenzyl phenethylamine **4c** is active, whereas *N*-3-bromobenzyl **4d** is inactive in the HTR assay.

None of the phenethylamines or tryptamines with 4-substituted *N*-benzyl groups, **4b**, **4e**, **5c**, or **5f**, was active in the HTR. All of these compounds were partial agonists with relatively low potency in the r5-HT_{2A} functional assay. Although **5e**, with a 3-substituted *N*-benzyl, has an EC_{50} and E_{max} virtually identical to **4e**, it is active in the HTR assay. It is possible that differences in pharmacokinetics or metabolic lability could explain these data. Nevertheless, if only the compounds active in the mouse HTR assay are compared, one finds a significant correlation between potency in the rat 5-HT_{2A} receptor and potency in the HTR assay, as shown in Figure 3.

Taken together, these data show that for *N*-benzylphenethylamines the highest in vivo potency in mice is associated with an ortho-substituent on the benzyl group, whereas the *N*-benzyltryptamines are more active in vivo when a meta-

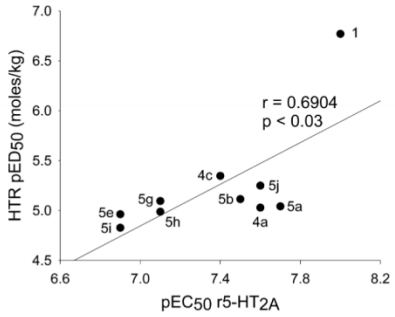

Figure 3. Regression analysis of pED_{50} for the mouse head twitch response on the pEC_{50} for function for active compounds at the rat 5-HT_{2A} receptor; n = 10.

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substituent is present. Hence, there are SAR differences between the *N*-benzyltryptamines and the *N*-benzylphenethylamines for the induction of the HTR, which likely reflect different binding orientations in the 5-HT_{2A} receptor. Obviously, the indole system is larger than a simple phenyl ring, something that would clearly affect the binding modes for the two different series at the orthosteric site. For example, the distance from the indole C(3) atom to the 5-oxygen atom is 4.94 Å, whereas the corresponding distance from the 5-methoxy oxygen to C(1) of the aryl ring is only 3.70 Å. Even the distance of 4.85 Å from C(1) of the aryl ring to the 4-iodo atom of the phenethylamines is less than the 4.94 Å distance measured from C(3) of the indole to the 5-methoxy.

One exception is that for both the *N*-benzyltryptamines and *N*-benzylphenethylamines, oxygenated substituents are tolerated at the ortho- and meta-positions of the benzyl moiety. For example, **1**, **4a**, **5a**, and **5b** are all active in the HTR assay, whereas **4d** and **5d** are inactive over a range of doses. This observation again would be consistent with some structural feature in the 5-HT_{2A} receptor that could engage a polar oxygen atom at the ortho-position of the *N*-benzyl moiety. There has been speculation, based on virtual docking studies with phenethylamines and tryptamines, that an oxygen atom in the ortho-position of the *N*-benzyl moiety may interact with a hydrogen bond donor (possibly the OH of Tyr 370$^{(7.43)}$ in the h5-HT_{2A} receptor.[14,18] It is conceivable that an oxygen atom at the meta-position in *N*-benzyltryptamines also could form a hydrogen bond with Tyr 370, possibly involving a water molecule.

Unfortunately, a 5-HT_{2A} selective agonist did not emerge from this small library of compounds. There are now only two selective 5-HT_{2A} agonists reported,[44,45] but they have not been available for extensive study. Thus, research on 5-HT_{2A} receptor function has been forced to employ either a mixed 5-$HT_{2A/2C}$ agonist such as DOI in combination with a specific 5-HT_{2C} antagonist, or to administer antagonists alone, the latter paradigm really being appropriate to study receptor function only when there are high levels of endogenous receptor activation or constitutive activity of the receptors. Genetic knockout mice have not revealed particular behavioral phenotypes and have served primarily to demonstrate that a particular drug depends on the presence of 5-HT_{2A} or 5-HT_{2C} receptors for its effect. Hence, the psychopharmacology of a "pure" 5-HT_{2A} agonist remains completely unknown. Furthermore, the tremendous present interest in the role of the 5-HT_{2A} receptor in normal brain function makes it imperative that scientists in the field gain access to a 5-HT_{2A} specific agonist so that research into the roles of the 5-HT_{2A} receptor can be more fully elucidated.

METHODS

Chemistry. *General Methods.* Reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) or Alfa Aesar (Ward Hill, MA) and used as delivered, unless otherwise specified. Thin layer chromatography was carried out using J. T. Baker flexible sheets (silica gel IB2-F) with fluorescent indicator, visualizing with UV light at 254 nm or iodine stain. Melting points were determined using a Mel-Temp apparatus and are uncorrected. NMR experiments were carried out using a Bruker Advance 300 MHz instrument, and the chemical shift (δ) values are in parts per million (ppm) relative to tetramethylsilane at 0.00 ppm. The solvent was CD_3OD. NMR samples were dissolved in MeOD. Ph = aromatic protons/carbons of benzyl group; In = aromatic protons/carbons of the indole nucleus; Ar = either phenyl or indole resonances, or phenyl in the case of compounds **1**−**4f**. Coupling constants (J) are presented in Hertz. Abbreviations used in the reporting of NMR spectra include: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, and quint = quintuplet.

Mass spectra were performed by high resolution LC-QTOF-MS on protonated molecules $[M + H]^+$. UHPLC-Q-TOF-MS conditions for UHPLC separation employed a mobile phase consisting of 100% MeCN that included 1% formic acid (organic phase) and an aqueous solution of 1% formic acid (aqueous phase). The column was maintained at 40 °C with a 0.6 mL/min flow rate and 5.5 min acquisition time. The elution was a 5−70% MeCN gradient ramp over 3.5 min, then up to 95% MeCN in 1 min and held for 0.5 min before returning to 5% MeCN in 0.5 min. Q-TOF-MS data were acquired in positive mode scanning from 100 to 1000 m/z with and without auto MS/MS fragmentation. Ionization was achieved with an Agilent JetStream electrospray source and infused internal reference masses. Agilent 6540 Q-TOF-MS parameters: gas temperature, 325 °C; drying gas, 10 L/min; and sheath gas temperature, 400 °C. Internal reference masses of 121.05087 and 922.00979 m/z were used.

For compounds **1** and **4a**−**4e**, 0.5 mmol of the free base of 4-iodo-2,5-dimethoxyphenethylamine[10,46] was stirred for 30 min at room temperature with 0.55 mmol of the appropriate aldehyde in 3 mL of methanol. The reaction was then placed on an ice bath, and 48 mg (1.25 mmol) of $NaBH_4$ was added in three portions over 15 min. The ice bath was removed and the reaction allowed to stir for an additional 15 min. The reaction was then transferred to a separatory funnel with 50 mL of EtOAc. The organic phase was washed three times with saturated NaCl, then dried overnight over Na_2SO_4. The drying agent was removed by suction filtration, and the filtrate was concentrated under reduced pressure. EtOH (1 mL) was added to the amber residue, and the HCl salt was prepared by acidification with 0.5 mL of 1 N HCl/EtOH. Dilution with EtOAc or diethyl ether then led to crystallization of the HCl salts, generally in good yields. In most cases, the supernatant was simply decanted from the crystalline product, followed by resuspension of the crystals in Et_2O and decantation, then air drying to afford the products as white to off-white fine needles. No attempt was made to optimize the yields, but in one case the supernatant was reduced to dryness and the residue crystallized from EtOH/Et_2O to afford an additional 6% of product. This small additional recovery was not deemed sufficient to warrant the extra effort. Thus, all reported yields are those obtained after the first crystallization.

The synthesis of tryptamines **5a**−**5l** followed essentially the same procedure, except that maleate salts were prepared. As an example, 1.0 mmol of 5-methoxytryptamine free base (Aldrich) was stirred for 30 min with 1.10 mmol of the appropriate aldehyde in 5 mL of methanol. The reaction was then placed on an ice bath, and 96 mg (2.5 mmol) of $NaBH_4$ was added in three portions over 15 min. The ice bath was removed and the reaction allowed to stir for an additional 15 min. The reaction was then transferred to a separatory funnel with 50 mL of EtOAc and was washed three times with saturated NaCl. The organic phase was dried overnight over Na_2SO_4, then filtered and concentrated under reduced pressure. Maleic acid (116 mg, 1 mmol) and 1.0 mL of acetone were then added to the residual amber oil, and the solution swirled until all of the maleic acid had dissolved. The reaction was then diluted with 10 mL of EtOAc, and Et_2O was added nearly to the cloud point. In most cases, crystallization occurred rapidly and spontaneously, and the product solution was stored overnight in a cold room. Crystalline products were collected by suction filtration, washed on the filter with EtOAc, and then air-dried to afford white to off-white fine needles.

*N-(2-Methoxybenzyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethan-1-amine Hydrochloride (**1**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 86%; mp 168−170 °C, Lit[24] mp 162−166 °C, 166.[13] ^{1}H NMR (300 MHz, CD_3OD) δ ppm 7.46 (1H, td, J = 8.2, 1.7 Hz, Ar−H), 7.37 (1H, dd, J = 7.6, 1.6 Hz, Ar−H), 7.35 (1H, s, Ar−H), 7.09 (1H, d, J = 8.3 Hz, Ar−H), 7.02 (1H, td, J = 7.5, 1.0 Hz, Ar−H), 6.86 (1H, s, Ar−H), 4.24 (2H, s, NB-CH_2), 3.88 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 3.20−3.25 (2H, m, α-CH_2), 3.03−2.98 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 159.37 (Ar−Cq), 154.44 (Ar−Cq), 153.60 (Ar−Cq), 132.81

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(Ar−CH), 132.73 (Ar−CH), 126.99 (Ar−Cq), 123.19 (Ar−CH), 122.13 (Ar−CH), 120.29 (Ar−Cq), 114.98 (Ar−CH), 112.16 (Ar−CH), 85.04 (Ar−Cq-iodine), 57.59 (OCH_3), 56.71 (OCH_3), 56.24 (OCH_3), 48.1 (NB-CH_2), 48.0 (α-CH_2), 28.49 (β-CH_2). HRMS calculated for $C_{18}H_{23}INO_3$ $[M + H]^+$, 428.07171; observed $[M + H]^+$, 428.07239. The EI mass spectrum also has been reported by Casale and Hays.[25]

*N-(3-Methoxybenzyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethan-1-amine Hydrochloride (**4a**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 85%; mp 171−2 °C. ^1H NMR (300 MHz, CD_3OD) δ ppm 7.38 (1H, t, J = 7.7 Hz, Ar−H), 7.34 (1H, s, Ar−H), 6.98−7.10 (3H, m, Ar−H), 6.86 (1H, s, Ar−H), 4.19 (2H, s, NB-CH_2), 3.83 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 3.22−3.27 (2H, m, α-CH_2), 2.99−3.04 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 161.77 (Ar−Cq), 154.43 (Ar−Cq), 153.63 (Ar−Cq), 133.82 (Ar−Cq), 131.48 (Ar−CH), 127.01 (Ar−Cq), 123.14 (Ar−CH), 122.92 (Ar−CH), 116.53 (Ar−CH), 116.13 (Ar−CH), 114.95 (Ar−CH), 85.00 (Ar−Cq-iodine), 57.59 (OCH_3), 56.68 (OCH_3), 55.93 (OCH_3), 52.23 (NB-CH_2), 48.1 (α-CH_2), 28.65 (β-CH_2). HRMS calculated for $C_{18}H_{23}INO_3$ $[M + H]^+$, 428.07171; observed $[M + H]^+$, 428.07319. The EI mass spectrum has also been reported by Casale and Hays.[25]

*N-(4-Methoxybenzyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethan-1-amine Hydrochloride (**4b**).* This particular compound was extremely difficult to crystallize, providing unfilterable gels upon attempts to crystallize it from EtOH, EtOH/Et_2O, or MeOH/Et_2O. It was finally obtained by dissolving in a minimum amount of boiling acetonitrile and allowing the solution to cool. Upon cooling, the solution also took on a gel-like appearance, but unlike other attempts, this material could be collected by vacuum filtration through a sintered glass filter funnel. The voluminous white solid was washed on the filter with a small amount of cold acetonitrile, then left on the funnel with suction until dry; yield 72%; mp 180−182 °C. ^1H NMR (300 MHz, CD_3OD): δ ppm 7.41 (2H, d, J = 8.7 Hz, 2 x Ar−H), 7.34 (1H, s, Ar−H), 7.00 (2H, d, J = 8.5 Hz, 2 x Ar−H), 6.85 (1H, s, Ar−H), 4.15 (2H, s, NB-CH_2), 3.82 (3H, s, OCH_3), 3.81 (3H, s OCH_3), 3.79 (3H, s OCH_3), 3.18−3.23 (2H, m, α-CH_2), 2.96−3.01 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 162.27 (Ar−Cq), 154.42 (Ar−Cq), 153.64 (Ar−Cq), 132.59 (2 x Ar−CH), 127.05 (Ar−Cq), 124.22 (Ar−Cq), 123.16 (Ar−CH), 115.64 (2 x Ar−CH), 114.94 (Ar−CH), 84.98 (Ar−Cq-iodine), 57.59 (OCH_3), 56.68 (OCH_3), 55.90 (OCH_3), 51.90 (NB-CH_2), 47.8 (α-CH_2), 28.67 (β-CH_2). HRMS calculated for $C_{18}H_{23}INO_3$ $[M + H]^+$, 428.07171; observed $[M + H]^+$, 428.07320. The EI mass spectrum has also been reported by Casale and Hays.[25]

*N-(2-Bromobenzyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethan-1-amine Hydrochloride (**4c**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 79%; mp 170−1 °C. ^1H NMR (300 MHz, CD_3OD): δ ppm 7.74 (1H, dd, J = 7.9, 1.3 Hz, Ar−H), 7.61 (1H, dd, J = 7.7, 1.7 Hz, Ar−H), 7.49 (1H, td, J = 7.5, 1.3 Hz), 7.39 (1H, td, J = 7.9, 1.9 Hz), 7.35 (1H, s, Ar−H), 6.89 (1H, s, Ar−H), 4.42 (2H, s, NB-CH_2), 3.82 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 3.31−3.36 (2H, m, α-CH_2), 3.03−3.08 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 154.46 (Ar−Cq), 153.62 (Ar−Cq), 134.74 (Ar−CH), 133.03 (Ar−CH), 132.81(Ar−CH), 132.32 (Ar−Cq), 129.70 (Ar−CH), 126.87 (Ar−Cq), 125.94 (Ar−Cq), 123.19 (Ar−CH), 114.98 (Ar−CH), 85.08 (Ar−Cq-iodine), 57.60 (OCH_3), 56.73 (OCH_3), 51.99 (NB-CH_2), 48.7 (α-CH_2), 28.62 (β-CH_2). HRMS calculated for $C_{17}H_{20}BrINO_2$ $[M + H]^+$, 475.97166; observed $[M + H]^+$, 475.97212.

*N-(3-Bromobenzyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethan-1-amine Hydrochloride (**4d**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 89%; mp 199−201 °C. ^1H NMR (300 MHz, CD_3OD): δ ppm 7.69−7.74 (1H, m, Ar−H), 7.60−7.66 (1H, m, Ar−H), 7.45−7.51 (1H, m, Ar−H), 7.41 (1H, d, J = 7.7 Hz, Ar−H), 7.35 (1H, s, Ar−H), 6.86 (1H, s, Ar−H), 4.22 (2H, s, NB-CH_2), 3.81 (3H, s, OCH_3), 3.79 (3H, s, OCH_3), 3.22−3.27 (2H, m, α-CH_2), 2.98−3.03 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 154.44 (Ar−Cq), 153.63 (Ar−Cq), 134.97 (Ar−Cq), 134.03 (Ar−CH), 133.87, (Ar−CH), 132.12 (Ar−CH), 129.89 (Ar−CH), 126.93 (Ar−Cq), 124.00 (Ar−Cq), 123.18 (Ar−CH), 114.95 (Ar−CH), 85.06 (Ar−Cq-iodine), 57.59 (OCH_3), 56.70 (OCH_3), 51.51 (NB-CH_2), 48.3 (α-CH_2), 28.68 (β-CH_2). HRMS calculated for $C_{17}H_{20}BrINO_2$ $[M + H]^+$, 475.97166; observed $[M + H]^+$, 475.97281.

*N-(4-Bromobenzyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethan-1-amine Hydrochloride (**4e**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 81%; mp 196−7 °C. ^1H NMR (300 MHz, CD_3OD) δ ppm 7.64 (2H, d, J = 8.7 Hz, 2 x Ar−H), 7.42 (2H, d, J = 8.5 Hz, 2 x Ar−H), 7.34 (1H, s, Ar−H), 6.86 (1H, s, Ar−H), 4.21 (2H, s, NB-CH_2), 3.81 (3H, s, OCH_3), 3.79 (3 H, s, OCH_3), 3.22−3.27 (2H, m, α-CH_2), 2.98−3.03 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 154.43 (Ar−Cq), 153.62 (Ar−Cq), 133.50 (2 x Ar−CH), 133.00 (2 x Ar−CH), 131.71 (Ar−Cq), 126.92 (Ar−Cq), 124.92 (Ar−Cq), 123.16 (Ar−CH), 114.94 (Ar−CH), 85.02 (Ar−Cq-iodine), 57.60 (OCH_3), 56.68 (OCH_3), 51.58 (NB-CH_2), 48.2 (α-CH_2), 28.66 (β-CH_2). HRMS calculated for $C_{17}H_{20}BrINO_2$ Calculated $[M + H]^+$, 475.97166; observed $[M + H]^+$, 475.97268.

*N-(2-Methoxybenzyl)-2-(5-methoxy-1H-indol-3-yl)ethan-1-amine Hydrochloride (**5a**).* Obtained as needles following crystallization from EtOH/EtOAc; yield 91%; mp 232−4 °C. ^1H NMR (300 MHz, CD_3OD): δ ppm 7.42 (1H, td, J = 7.9, 1.7 Hz, Ph-H), 7.33 (1H, dd, J = 7.4, 1.6 Hz, Ph-H), 7.28 (1H, dd, J = 8.9, 0.6 Hz, In−H), 7.16 (1H, s, In−H), 6.97−7.03 (2H, m, Ph-H), 6.95 (1H, d, J = 2.4 Hz, In−H), 6.81 (1H, dd, J = 8.8, 2.4 Hz, In−H), 4.23 (2H, s, NB-CH_2), 3.78 (3H, s, OCH_3), 3.67 (3H, s, OCH_3), 3.28−3.33 (2H, m, α-CH_2, overlapping with solvent), 3.12−3.17 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 159.25 (Ph−Cq), 155.42 (In-Cq), 133.67 (Ar−Cq), 132.77 (Ph−CH), 132.68 (Ph−CH), 128.34 (Ar−Cq), 125.35 (In-CH), 122.12 (In-CH), 120.13 (Ar−Cq), 113.41 (Ph−CH), 113.21 (In-CH), 112.06 (Ph−CH), 109.51 (Ar−Cq), 101.00 (In-CH), 56.35 (OCH_3), 55.93 (OCH_3), 48.90 (α-CH_2), 48.3 (NB-CH_2), 23.21 (β-CH_2). HRMS calculated for $C_{19}H_{23}N_2O_2$ $[M + H]^+$, 311.17540; observed $[M + H]^+$, 311.17548.

*N-(3-Methoxybenzyl)-2-(5-methoxy-1H-indol-3-yl)ethan-1-amine Maleate (**5b**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 84%; mp 124−5 °C. ^1H NMR (300 MHz, CD_3OD): δ ppm 7.33−7.39 (1H, m, Ph-H), 7.26 (1H, dd, J = 8.9, 0.6 Hz, In−H), 7.13 (1H, s, In−H), 6.99−7.01 (4H, m, overlapping 3 x Ph-H, 1 x In−H), 6.80 (1H, dd, J = 8.8, 2.4 Hz, In−H), 6.24 (2H, s, maleate), 4.18 (2H, s, NB-CH_2), 3.81 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 3.28−3.35 (2H, α-CH_2, overlapping with solvent), 3.11−3.16 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 170.89 (maleate), 161.79 (Ph−Cq), 155.39 (In-Cq), 136.79 (maleate), 133.89 (Ar−Cq), 133.60 (Ar−Cq), 131.50 (Ph−CH), 128.44 (Ar−Cq), 125.00 (In-CH), 122.87 (Ph−CH), 116.40 (Ph−CH), 116.15 (Ph−CH), 113.35 (In-CH), 113.07 (In-CH), 109.84 (Ar−Cq), 101.04 (In-CH), 56.39 (OCH_3), 55.88 (OCH_3), 52.16 (NB-CH_2), 49.0 (α-CH_2), 23.36 (β-CH_2). HRMS calculated for $C_{19}H_{23}N_2O_2$ $[M + H]^+$, 311.17540; observed $[M + H]^+$, 311.17572

*N-(4-Methoxybenzyl)-2-(5-methoxy-1H-indol-3-yl)ethan-1-amine Maleate (**5c**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 82%; mp 172−3 °C. ^1H NMR (300 MHz, CD_3OD): δ ppm 7.36 (2H, d, J = 8.0 Hz, 2 x Ph-H), 7.26 (1 H, dd, J = 8.8, 0.5 Hz, In−H), 7.12 (1H, s, In−H), 6.99 (1H, d, J = 2.5 Hz, In−H), 6.97 (2H, d, J = 6.6 Hz, 2 x Ph-H), 6.80 (1H, dd, J = 8.9, 2.4 Hz, In−H), 6.24 (2H, s, maleate), 4.15 (2H, s, NB-CH_2), 3.81 (3H, s, OCH_3), 3.80 (3H, s, OCH_3), 3.27−3.32 (2H, m, α-CH_2, overlapping with solvent), 3.09−3.14 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 170.90 (maleate), 162.24 (Ph−Cq), 155.37 (In-Cq), 136.78 (maleate), 133.60 (Ar−Cq), 132.52 (2 x Ph−CH), 128.45 (Ar−Cq), 124.97 (In-CH), 124.27 (Ar−Cq), 115.64 (2 x Ph−CH), 113.34 (In-CH), 113.06 (In-CH), 109.89 (Ar−Cq), 101.06 (In-CH), 56.39 (OCH_3), 55.89 (OCH_3), 51.78 (NB-CH_2), 48.5 (α-CH_2), 23.38 (β-CH_2). HRMS calculated for $C_{19}H_{23}N_2O_2$ $[M + H]^+$, 311.17540; observed $[M + H]^+$, 311.17632

*N-(2-Bromobenzyl)-2-(5-methoxy-1H-indol-3-yl)ethan-1-amine Maleate (**5d**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 72%; mp 93−5 °C. ^1H NMR (300 MHz, CD_3OD): δ ppm 7.70 (1H, dd, J = 7.9, 1.3 Hz, Ph-H), 7.54 (1H, dd, J = 7.7, 1.9 Hz, Ph-H), 7.45 (1H, td, J = 7.5, 1.4 Hz, Ph-H), 7.36 (1H, td, J = 7.8, 1.8 Hz, Ph-H), 7.26 (1H, dd, J = 8.9, 0.6 Hz, In−H), 7.16

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(1H, s, In−H), 7.02 (1H, d, J = 2.3 Hz, In−H), 6.80 (1H, dd, J = 8.9, 2.4 Hz, In−H), 6.24 (2H, s, maleate), 4.41 (2H, s, NB-CH_2), 3.82 (3H, s, OCH_3), 3.40−3.45 (2H, m, α-CH_2), 3.16−3.21 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 170.89 (maleate), 155.41 (In-Cq), 136.75 (maleate), 134.71 (Ph−CH), 133.64 (Ar−Cq), 133.04 (Ph−CH), 132.76 (Ph−CH), 132.40 (Ar−Cq), 129.65 (Ph−CH),128.44 (Ar−Cq), 125.94 (Ar−Cq), 125.12 (In-CH), 113.38 (In-CH), 113.10 (In-CH), 109.67 (Ar−Cq), 101.06 (In-CH), 56.40 (OCH_3), 51.90 (NB-CH_2), 49.3 (α-CH_2), 23.32 (β-CH_2). HRMS calculated for $C_{18}H_{20}BrN_2O$ $[M + H]^+$, 359.07535; observed $[M + H]^+$, 359.07581.

*N-(3-Bromobenzyl)-2-(5-methoxy-1H-indol-3-yl)ethan-1-amine Maleate (**5e**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 86%; mp 137−8 °C. ^{1}H NMR (300 MHz, CD_3OD): δ ppm 7.67−7.68 (1H, m, Ph-H), 7.61 (1H, dt, J = 7.7, 1.6 Hz, Ph-H), 7.34−7.45 (2H, m, Ph-H), 7.27 (1H, d, J = 8.7 Hz, In−H), 7.14 (1H, s, In−H), 7.01 (1H, d, J = 2.3 Hz, In−H), 6.80 (1H, dd, J = 8.9, 2.4 Hz, In−H), 6.24 (2H, s, maleate), 4.21 (2H, s, NB-CH_2), 3.82 (3H, s, OCH_3), 3.31−3.36 (2H, m, α-CH_2, overlapping with solvent), 3.11−3.16 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 170.92 (maleate), 155.40 (In-Cq), 136.78 (maleate), 135.10 (Ar−Cq), 134.00 (Ph−CH), 133.82 (Ph−CH), 133.61 (Ar−Cq), 132.10 (Ph−CH), 129.81 (Ph−CH), 128.45 (Ar−Cq), 125.01 (In-CH), 124.03 (Ar−Cq), 113.37 (In-CH), 113.08 (In-CH), 109.82 (Ar−Cq), 101.06 (In-CH), 56.42 (OCH_3), 51.52 (NB-CH_2), 49.1 (α-CH_2), 23.40 (β-CH_2). HRMS calculated for $C_{18}H_{20}BrN_2O$ $[M + H]^+$, 359.07535; observed $[M + H]^+$, 359.07547

*N-(4-Bromobenzyl)-2-(5-methoxy-1H-indol-3-yl)ethan-1-amine Maleate (**5f**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 75%; mp 181−3 °C. ^{1}H NMR (CD_3OD): δ ppm 7.60 (2H, d, J = 8.5 Hz, 2 x Ph-H), 7.37 (2H, d, J = 8.5 Hz, 2 x Ph-H), 7.26 (1H, d, J = 8.9 Hz, In−H), 7.13 (1H, s, In−H), 6.99 (1H, d, J = 2.3 Hz, In−H), 6.80 (1H, dd, J = 8.9, 2.4 Hz, In−H), 6.24 (2H, s, maleate), 4.20 (2H, s, NB-CH_2), 3.81 (3H, s, OCH_3), 3.31−3.36 (2H, m, α-CH_2, overlapping with solvent), 3.11−3.16 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 170.89 (maleate), 155.38 (In-Cq), 136.7 5 (maleate), 133.61 (Ar−Cq), 133.51 (2 x Ph−CH), 132.92 (2 x Ph−CH), 131.77 (Ar−Cq), 128.43 (Ar−Cq), 125.02 (In-CH), 124.90 (Ar−Cq), 113.36 (In-CH), 113.06 (In-CH), 109.76 (Ar−Cq), 101.06 (In-CH), 56.41 (OCH_3), 51.50 (NB-CH_2), 48.90 (α-CH_2), 23.40 (β-CH_2). HRMS calculated for $C_{18}H_{20}BrN_2O$ $[M + H]^+$, 359.07535; observed $[M + H]^+$, 359.07597.

*N-(3-Fluorobenzyl)-2-(5-methoxy-1H-indol-3-yl)ethan-1-amine Maleate (**5g**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 78%; mp 150−2 °C. ^{1}H NMR (300 MHz CD_3OD): δ ppm 7.44−7.51 (1H, m, Ph-H), 7.16−7.29 (4H, m, overlapping 3 x Ph-H, 1 x In−H), 7.14 (1H, s, In−H), 7.01 (1H, d, J = 2.4 Hz, In−H), 6.80 (1H, dd, J = 8.9, 2.4 Hz, In−H), 6.24 (2H, s, maleate), 4.24 (2H, s, NB-CH_2), 3.81 (3H, s, OCH_3), 3.31−3.37 (2H, m, α-CH_2, overlapping with solvent), 3.12−3.17 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 170.89 (maleate), 164.38 (Ph−Cq-3′, d, J = 246.2 Hz), 155.40 (In-Cq), 136.74 (maleate), 135.08 (Ph−Cq-1′, d, J = 7.5 Hz), 133.61 (In-Cq), 132.31 (Ph−C-5′, d, J = 8.3 Hz), 128.45 (In-Cq), 126.88 (Ph−C-6′, d, J = 3.0 Hz), 125.00 (In-CH), 117.79 (Ph−C-2′, d, J = 22.5 Hz), 117.60 (Ph−C-4′, d, J = 21.8 Hz), 113.37 (In-CH), 113.08 (In-CH), 109.79 (In-Cq), 101.06, (In-CH), 56.41 (OCH_3), 51.58 (NB-CH_2, J = 1.5 Hz), 49.1 (α-CH_2), 23.38 (β-CH_2). HRMS calculated for $C_{18}H_{20}FN_2O$ $[M + H]^+$, 299.15542; observed $[M + H]^+$, 299.15602.

*N-(3-Chlorobenzyl)-2-(5-methoxy-1H-indol-3-yl)ethan-1-amine Maleate (**5h**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 79%; mp 116−8 °C. ^{1}H NMR (300 MHz, CD_3OD): δ ppm 7.52 (1H, br, s, Ph-H), 7.34−7.49 (3H, m, Ph-H), 7.26 (1H, d, J = 8.9 Hz, In−H), 7.14 (1H, s, In−H), 7.01 (1H, d, J = 2.4 Hz, In−H), 6.80 (1H, dd, J = 8.9, 2.4 Hz, In−H), 6.24 (2H, s, maleate), 4.22 (2H, s, NB-CH_2), 3.82 (3H, s, OCH_3), 3.31−3.37 (2H, m, α-CH_2, overlapping with solvent), 3.12−3.17 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 155.41 (In-Cq), 136.76 (maleate), 136.10 (Ar−Cq), 134.82 (Ar−Cq), 133.60 (Ar−Cq), 131.89 (Ph−CH), 131.04 (Ph−CH), 130.84 (Ph−CH), 129.38 (Ph−CH), 128.45 (Ar−Cq), 125.01 (In-CH), 113.36 (In-CH), 113.08 (In-CH), 109.78 (In-Cq), 101.04 (In-CH), 56.40 (OCH_3), 51.54 (NB-CH_2), 49.1 (α-CH_2), 23.39 (β-CH_2). HRMS calculated for $C_{18}H_{20}ClN_2O$ $[M + H]^+$, 315.12587; observed $[M + H]^+$, 315.12666

*N-(3-Iodobenzyl)-2-(5-methoxy-1H-indol-3-yl)ethan-1-amine Maleate (**5i**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 84%; mp 131−2 °C. ^{1}H NMR (300 MHz, CD_3OD): δ ppm 7.87 (1H, brs, Ph-H), 7.81 (1H, d, J = 7.9 Hz, Ph-H), 7.45 (1H, d, J = 7.7 Hz, Ph-H), 7.27 (1H, d, J = 8.3 Hz, In−H), 7.21 (1H, t, J = 7.8 Hz, Ph-H), 7.13 (1H, s, In−H), 7.01 (1H, d, J = 2.3 Hz, In−H), 6.80 (1H, dd, J = 8.9, 2.3 Hz, In−H), 6.24 (2H, s, maleate), 4.18 (2H, s, NB-CH_2), 3.82 (3H, s, OCH_3), 3.31−3.36 (2H, m, α-CH_2, overlapping with solvent), 3.11−3.16 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 155.39 (In-Cq), 139.98 (Ph−CH), 139.88 (Ph−CH), 136.76 (maleate), 134.98 (Ar−Cq), 133.58 (Ar−Cq), 132.03 (Ph−CH), 130.31 (Ph−CH), 128.46 (Ar−Cq), 124.99 (In-CH), 113.36 (In-CH), 113.08 (In-CH), 109.80 (Ar−Cq), 101.03 (In-CH), 95.42 (Ar−Cq-iodine), 56.42 (OCH_3), 51.41 (NB-CH_2), 49.1 (α-CH_2), 23.37 (β-CH_2). HRMS calculated for $C_{18}H_{20}IN_2O$ $[M + H]^+$, 407.06148; observed $[M + H]^+$, 407.06188.

*N-(3-Methylbenzyl)-2-(5-methoxy-1H-indol-3-yl)ethan-1-amine Maleate (**5j**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 78%; mp 125−7 °C. ^{1}H NMR (300 MHz, CD_3OD): δ ppm 7.22−7.35 (5H, m, overlapping 4 x Ph-H and 1 x In−H), 7.13 (1H, s, In−H), 6.99 (1H, d, J = 2.3 Hz, In−H), 6.80 (1H, dd, J = 8.9, 2.4 Hz, In−H), 6.24 (2H, s, maleate), 4.17 (2H, s, NB-CH_2), 3.82 (3H, s, OCH_3), 3.29−3.35 (2H, m, α-CH_2, overlapping with solvent), 3.10−3.15 (2H, m, β-CH_2), 2.36 (3H, s, CH_3). ^{13}C NMR (CD_3OD): δ ppm 170.90 (maleate), 155.38 (In-Cq), 140.47 (Ar−Cq), 136.80 (maleate), 133.61 (Ar−Cq), 132.46 (Ar−Cq), 131.49 (Ph−CH), 131.40 (Ph−CH), 130.27 (Ph−CH), 128.46 (Ar−Cq), 127.93 (Ph−CH), 125.00 (In-CH), 113.35 (In-CH), 113.06 (In-CH), 109.87 (Ar−Cq), 101.07 (In-CH), 56.40 (OCH_3), 52.24 (NB-CH_2), 48.9 (α-CH_2), 23.37 (β-CH_2), 21.36 (CH_3). HRMS calculated for $C_{19}H_{23}N_2O$ $[M + H]^+$, 295.18049; observed $[M + H]^+$, 295.18090.

*N-(3-Methylthiobenzyl)-2-(5-methoxy-1H-indol-3-yl)ethan-1-amine Maleate (**5j**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 80%; mp 151−2 °C. ^{1}H NMR (300 MHz, CD_3OD): δ ppm 7.31−7.39 (3H, m, Ph-H), 7.26 (1H, d, J = 8.9 Hz, In−H), 7.19 (1H, dt, J = 7.0, 1.9 Hz, Ph-H), 7.13 (1H, s, In−H), 7.00 (1H, d, J = 2.4 Hz, In−H), 6.80 (1H, dd, J = 8.9, 2.4 Hz, In−H), 6.24 (2H, s, maleate), 4.19 (2H, s, NB-CH_2), 3.81 (3H, s, OCH_3), 3.30−3.35 (2H, m, α-CH_2, overlapping with solvent), 3.11−3.16 (2H, m, β-CH_2), 2.48 (3H, s, CH_3). ^{13}C NMR (CD_3OD): δ ppm 170.91 (maleate), 155.40 (Ar−Cq), 141.95 (Ar−Cq), 136.78 (maleate), 133.60 (Ar−Cq), 133.34 (Ar−Cq), 130.74 (Ph−CH), 128.46 (Ar−Cq), 128.40 (Ph−CH), 128.30 (Ph−CH), 127.20 (Ph−CH), 125.00 (In-CH), 113.36 (In-CH), 113.08 (In-CH), 109.84 (Ar−Cq), 101.04 (In-CH), 56.41 (OCH_3), 52.05 (NB-CH_2), 49.1 (α-CH_2), 23.38 (β-CH_2), 15.37 (CH_3). HRMS calculated for $C_{19}H_{23}N_2OS$ $[M + H]^+$, 327.15256; observed $[M + H]^+$, 327.15362.

*N-(3-Trifluoromethylbenzyl)-2-(5-methoxy-1H-indol-3-yl)ethan-1-amine Maleate (**5k**).* Obtained as needles following crystallization from acetone/EtOAc/Et_2O; yield 62%; mp 161−2 °C. ^{1}H NMR (300 MHz, CD_3OD): δ ppm 7.84 (1H, brs, Ph-H), 7.62−7.78 (3H, m, Ph-H), 7.26 (1H, d, J = 8.8 Hz, In−H), 7.14 (1H, s, In−H), 7.02 (1H, d, J = 2.1 Hz, In−H), 6.80 (1H, dd, J = 8.9, 2.3 Hz, In−H), 6.24 (2H, s, maleate), 4.32 (2H, s, NB-CH_2), 3.81 (3H, s, OCH_3), 3.35−3.40 (2H, m, α-CH_2), 3.13−3.18 (2H, m, β-CH_2). ^{13}C NMR (CD_3OD): δ ppm 170.91 (maleate), 155.41 (In-Cq), 136.74 (maleate), 134.85 (Ph−CH), 134.04 (Ph−Cq), 133.60 (In-Cq), 132.56 (Ph-Cq, d, J = 32.3 Hz), 131.24 (Ph−CH), 128.46 (In-Cq), 127.84 (Ph−CH, q, J = 4.0 Hz), 127.46 (Ph−CH, q, J = 3.8 Hz), 125.4 (CF_3, q, J = 272 Hz), 125.01 (In-CH), 113.36 (In-CH), 113.06 (In-CH), 109.79 (In-Cq), 101.06 (In-CH), 56.39 (OCH_3), 51.63 (NB-CH_2), 49.20 (α-CH_2), 23.41 (β-CH_2). HRMS calculated for $C_{19}H_{20}F_3N_2O$ $[M + H]^+$, 349.15222; observed $[M + H]^+$, 349.15259

Pharmacology. *Receptor Affinity.* Receptor affinity values for a panel of human serotonin receptors were obtained for all compounds through the NIMH-sponsored PDSP program (www.pdsp.med.unc.edu). Affinity data from screening are reported in Table 1. Following

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the initial screen, more detailed values were obtained for affinity at the human 5-HT_{2A} and 5-HT_{2C} receptors using both an antagonist radioligand ([^{3}H]ketanserin for 5-HT_{2A}) and ([^{3}H]mesulergine for 5-HT_{2C}) and an agonist radioligand ([^{3}H]-DOI) for both receptors. Those data are reported in Table 2.

Receptor Efficacy and Potency in the Ca^{2+} Mobilization Assay. Changes in intracellular Ca^{2+} levels were measured using a Fluorometric Imaging plate reader (FLIPR^{TETRA}, Molecular Devices), essentially as described in the PDSP (NIMH Psychoactive Drug Screening Program) Assay Protocol Book (www.pdsp.med.unc.edu). PO1C cells stably transfected with r5-HT_{2C} or r5-HT_{2A} receptors, and HEK 293 cells stably transfected with h5-HT_{2A}, h5-HT_{2B}, or h5-HT_{2C} receptors were plated (20,000 cells/well) into poly-L-lysine coated 394-well clear-bottom black-walled microplates (Greiner Bio-one) with 50 μL of media (DMEM media supplemented with 500 μg/mL Geneticin sulfate (G-418), 10% dialyzed fetal bovine serum, and 50 U of penicillin/50 μg of streptomycin) and incubated overnight (37 °C, 5% CO_2). The following day, media were replaced with 20 μL of FLIPR Calcium 4 Assay Kit (Molecular Devices) diluted in assay buffer (HBSS, 2.5 mM probenecid, and 20 mM HEPES, pH 7.4−7.8) and incubated for 45 min at 37 °C and 15 min at room temperature. Compounds were initially dissolved in DMSO. The 16-point curves were prepared as 3× serial dilutions for each compound with final concentrations ranging from 10 μM to 0.003 nM. Basal fluorescence was measured for 10 s, then 10 μL of test or control compounds was added followed by continued fluorescence measurement for an additional 120 s. Raw data were normalized to baseline fluorescence (0%) and 5HT at 10 μM (100%), expressed as percent activation, and plotted as a function of molar concentration of test compound using Prism 5.0 (GraphPad Software). These data are reported in Table 3.

Mouse Head Twitch Response. *Animals.* Male C57BL/6J mice (6−8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and housed in a vivarium at the University of California, San Diego, an AAALAC-approved animal facility that meets Federal and State requirements for the care and treatment of laboratory animals. Mice were housed up to four per cage in a climate-controlled room with a reversed light-cycle (lights on at 1900 h, off at 0700 h). Food and water were provided ad libitum, except during behavioral testing. Testing was performed between 1000 and 1830 h. Experiments were conducted in accord with NIH guidelines and were approved by the UCSD animal care committee.

Procedures. The HTR was assessed using a head-mounted magnet and a magnetometer detection coil. Mice were anesthetized (100 mg/kg ketamine, 3 mg/kg acepromazine, and 20 mg/kg xylazine, IP), and a neodymium magnet (4.57 × 4.57 × 2.03 mm, 375 mg) was attached to the skull using dental cement. The magnet was positioned so that the N−S axis was parallel to the dorsoventral plane of the head. Mice were allowed to recover for 2 weeks after surgery. HTR experiments were conducted in a well-lit room. Test compounds were dissolved in water containing 5% Tween-80 and administered SC (5 or 10 mL/kg). Mice were injected with drug or vehicle and placed in a glass cylinder surrounded by a magnetometer coil. Head movements were recorded and analyzed for HTR as described previously.[23,31] Coil voltage was low-pass filtered (5−10 kHz), amplified, and digitized (40 kHz sampling rate) using a Powerlab/8SP with LabChart v 7.3.2 (ADInstruments, Colorado Springs, CO, USA). The data were filtered off-line (40−200 Hz band-pass), and HTRs were identified by manually searching for sinusoidal wavelets possessing at least two bipolar peaks, spectrum in the 40−160 Hz range, amplitude exceeding the background noise level, and duration <0.15 s, with stable coil voltage during the period immediately before and after each response.

Analysis. HTR counts were analyzed using one-way analyses of variance (ANOVAs). Post-hoc comparisons were made using Tukey's studentized range method. Significance was demonstrated by surpassing an α-level of 0.05. ED_{50} values and 95% confidence limits were calculated using nonlinear regression. These data are reported in Table 4.

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Author Contributions

D.N. directed the project, synthesized all of the compounds, supervised the integration of the various studies, and was responsible for the writing and final editing of the manuscript, F.S. carried out the calcium mobilization functional assays, A.H. supervised the mouse head twitch assays, L.M.K. assisted with the mouse assays, S.D.B. and S.P.E. carried out the analytical chemistry assays, and W.F. made key suggestions for the project and edited the manuscript.

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Notes

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CHAPTER 2

RECEPTOR BINDING PROFILES AND BEHAVIORAL PHARMACOLOGY OF RING-SUBSTITUTED *N,N*-DIALLYLTRYPTAMINE ANALOGS

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Receptor binding profiles and behavioral pharmacology of ring-substituted *N,N*-diallyltryptamine analogs

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ABSTRACT

Substantial effort has been devoted toward understanding the psychopharmacological effects of tryptamine hallucinogens, which are thought to be mediated by activation of 5-HT_{2A} and 5-HT_{1A} receptors. Recently, several psychoactive tryptamines based on the *N,N*-diallyltryptamine (DALT) scaffold have been encountered as recreational drugs. Despite the apparent widespread use of DALT derivatives in humans, little is known about their pharmacological properties. We compared the binding affinities of DALT and its 2-phenyl-, 4-acetoxy-, 4-hydroxy-, 5-methoxy-, 5-methoxy-2-methyl-, 5-fluoro-, 5-fluoro-2-methyl-, 5-bromo-, and 7-ethyl-derivatives at 45 receptor and transporter binding sites. Additionally, studies in C57BL/6J mice examined whether these substances induce the head twitch response (HTR), a 5-HT_{2A} receptor-mediated response that is widely used as a behavioral proxy for hallucinogen effects in humans. Most of the test drugs bound to serotonin receptors, σ sites, α_2-adrenoceptors, dopaminergic D_3 receptors, histaminergic H_1 receptors, and the serotonin transporter. DALT and several of the ring-substituted derivatives were active in the HTR assay with the following rank order of potency: 4-acetoxy-DALT > 5-fluoro-DALT > 5-methoxy-DALT > 4-hydroxy-DALT > DALT > 5-bromo-DALT. 2-Phenyl-DALT, 5-methoxy-2-methyl-DALT, 5-fluoro-2-methyl-DALT, and 7-ethyl-DALT did not induce the HTR. HTR potency was not correlated with either 5-HT_{1A} or 5-HT_{2A} receptor binding affinity, but a multiple regression analysis indicated that 5-HT_{2A} and 5-HT_{1A} receptors make positive and negative contributions, respectively, to HTR potency ($R^2 = 0.8729$). In addition to supporting the established role of 5-HT_{2A} receptors in the HTR, these findings are consistent with evidence that 5-HT_{1A} activation by tryptamine hallucinogens buffers their effects on HTR.

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1. Introduction

Over the past decade there has been a renewed focus on the pharmacology and effects of serotonergic hallucinogens (Halberstadt, 2015; Nichols, 2016). This focus has been driven, in part, by accumulating evidence that serotonergic hallucinogens may have therapeutic efficacy against anxiety, depression, substance abuse, and obsessive-compulsive disorder (Bogenschutz and Ross, 2017). Additionally, although hallucinogen use has remained relatively stable over the past few decades, there has been a marked increase in the availability and diversity of hallucinogens in recent years that has resulted in numerous reports of untoward effects. Some of these hallucinogens are derived from *N,N*-diallyltryptamine (DALT). 5-Methoxy-*N,N*-diallyltryptamine (5-MeO-DALT), for example, was first synthesized by Alexander T. Shulgin (A.T. Shulgin, personal communication), and was first marketed via the Internet in 2004 (Corkery et al., 2012). According to Shulgin, oral doses of 12–20 mg produce psychoactive effects with a rapid onset and a relatively brief duration of 2–4 h (Shulgin and Shulgin, 2004). Subsequently, 5-MeO-DALT and other DALT derivatives

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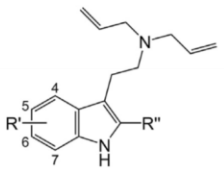
have become popular recreational hallucinogen; 5-MeO-DALT has been identified in many seized samples (Nagai et al., 2007; Rasanen et al., 2014; Strano Rossi et al., 2014; Odoardi et al., 2016; Brunt et al., 2017) and DALT and 4-acetoxy-*N*,*N*-diallyltryptamine (4-AcO-DALT) have also been detected (EMCDDA, 2008,2013,2015).

Despite the widespread distribution and nonmedical use of diallyltryptamines (DALTs), very little is known about their pharmacology. It was previously reported that six DALT compounds bind non-selectively to 27 different receptors including 5-HT receptors (Cozzi and Daley, 2016), and 5-MeO-DALT has been shown to act as a 5-HT_{2A} agonist (Arunotayanun et al., 2013). However, few animal behavioral assessments have been performed with these compounds, and the resulting information could provide insight into the relationship between receptor binding and the behavioral effects of these drugs. Hence, the binding of DALT and nine ring-substituted DALTs (see Fig. 1) were assessed at 45 receptor and transporter binding sites, followed by behavioral evaluation using the head twitch response (HTR).

Serotonergic hallucinogens produce the HTR, a brief paroxysmal head rotation in rats and mice, via activation of the 5-HT_{2A} receptor (Schreiber et al., 1995; Canal and Morgan, 2012; Halberstadt and Geyer, 2013), the same receptor responsible for the psychedelic effects of hallucinogens in humans (Quednow et al., 2012; Kometer et al., 2013; Valle et al., 2016; Kraehenmann et al., 2017; Preller et al., 2017a,b). The HTR is widely used as a behavioral proxy in rodents for human hallucinogenic effects because it is one of only a few behaviors that can reliably distinguish hallucinogenic and non-hallucinogenic 5-HT_{2A} receptor agonists (Gonzalez-Maeso et al., 2007). We employed HTR studies with the ten DALT compounds in C57BL/6 J mice to test whether these tryptamines produce LSD-like behavioral effects *in vivo*.

In addition to producing effects via the 5-HT_{2A} receptor, tryptamine hallucinogens also bind to 5-HT_{1A} receptors with moderate to high affinity and efficacy (McKenna et al., 1990; Blough et al., 2014; Rickli et al., 2016). The HTR induced by hallucinogens is attenuated by administration of 5-HT_{1A} receptor agonists such as 8-OH-DPAT, ipsapirone, and buspirone (Darmani et al., 1990; Schreiber et al., 1995; Kleven et al., 1997), which is consistent with evidence for countervailing interactions between 5-HT_{1A} and 5-HT_{2A} receptors (Araneda and Andrade, 1991; Ashby et al., 1994; Krebs-Thomson and Geyer, 1998; Amargos-Bosch et al., 2004; Li et al., 2011). In light of this apparent cross-talk, one unanswered question is whether the ability of tryptamine hallucinogens to induce the HTR via 5-HT_{2A} activation is modulated by their concurrent effects on 5-HT_{1A} receptors. Pretreatment with the mixed 5-HT_{1A}/β-adrenergic antagonist pindolol markedly augments the subjective response induced by the hallucinogen *N*,*N*-dimethyltryptamine (DMT) in human volunteers, suggesting that 5-HT_{1A} activation by DMT may blunt its 5-HT_{2A}-mediated effects (Strassman, 1996). Based on those findings, we hypothesized that 5-HT_{1A} activation by tryptamine hallucinogens may buffer their ability to induce the HTR in mice.

One way to gauge the involvement of 5-HT_{1A} receptors in the behavioral response to hallucinogens is to assess the effect of combined administration with a 5-HT_{1A} antagonist. The possibility exists, however, that 5-HT_{1A} antagonists might alter the potency of 5-HT_{2A} receptor-mediated responses due to interactions that are known to occur between the receptors (Krebs-Thomson and Geyer, 1998; Salmi and Ahlenius, 1998; Li et al., 2011). Indeed, 5-HT_{1A} antagonists can augment the HTR induced by hallucinogen administration (Willins and Meltzer, 1997), and under certain conditions can even induce head twitches through a mechanism involving indirect activation of 5-HT_{2A} receptors (Darmani and Reeves, 1996; Darmani, 1998; Fox et al., 2010). As an alternative to conducting antagonist blockade studies, receptor binding studies were conducted with DALT derivatives and regression analyses were performed to determine whether potency in the HTR assay is correlated with 5-HT_{2A} and/or 5-HT_{1A} receptor affinities.



R'	R"	Abbreviation
H	H	DALT
H	C_6H_5	2-Ph-DALT
4-OAc	H	4-AcO-DALT
4-OH	H	4-HO-DALT
5-OCH_3	H	5-MeO-DALT
5-OCH_3	CH_3	5-MeO-2-Me-DALT
5-F	H	5-F-DALT
5-F	CH_3	5-F-2-Me-DALT
5-Br	H	5-Br-DALT
7-C_2H_5	H	7-Et-DALT

Fig. 1. Chemical structures of *N*,*N*-diallyltryptamine (DALT) and several ring-substituted derivatives.

2. Materials and methods

2.1. Subjects

Male C57BL/6 J mice (6–8 weeks old) obtained from Jackson Laboratories (Bar Harbor, ME, USA) were housed in a vivarium at the University of California San Diego, an AAALAC-approved animal facility that meets all Federal and State requirements for care and treatment of laboratory animals. Mice were housed up to four per cage in a climate-controlled room on a reverse-light cycle (lights on at 1900 h, off at 0700 h) and were provided with *ad libitum* access to food and water, except during behavioral testing. Testing was conducted between 1000 and 1800 h. All animal experiments were conducted in accordance with NIH guidelines and were approved by the UCSD animal care committee.

2.2. Drugs

The following drugs were tested: *N*,*N*-diallyltryptamine hydrochloride (DALT), 5-methoxy-*N*,*N*-diallyltryptamine hydrochloride (5-MeO-DALT), 5-fluoro-*N*,*N*-diallyltryptamine hydrochloride (5-F-DALT), 5-bromo-*N*,*N*-diallyltryptamine hydrochloride (5-Br-DALT), 4-hydroxy-*N*,*N*-diallyltryptamine hemifumarate (2:1) (4-HO-DALT), 4-acetoxy-*N*,*N*-diallyltryptamine fumarate (4-AcO-DALT), 2-phenyl-*N*,*N*-diallyltryptamine hydrochloride (2-Ph-DALT), 5-methoxy-2-methyl-*N*,*N*-diallyltryptamine hydrochloride (5-MeO-2-Me-DALT), 5-fluoro-2-methyl-*N*,*N*-diallyltryptamine hydrochloride (5-F-2-Me-DALT), and 7-ethyl-*N*,*N*-diallyltryptamine hydrochloride (7-Et-DALT). 4-AcO-DALT fumarate and 4-HO-DALT hemifumarate were obtained from Scientific Supplies (London, UK); the other tryptamines were synthesized, fully characterized, and available from previous studies (Meyer et al., 2014; Michely et al., 2015; Dinger et al., 2016; Brandt et al., 2017; Michely et al., 2017; Caspar et al., 2018).

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2.3. Binding studies

A screening at 45 receptor and transporter binding sites was performed by the NIMH Psychoactive Drug Screening Program (NIMH PDSP). Most of these screenings were performed with cloned human receptors; exceptions are listed in Table 1. Test compounds were dissolved in DMSO and were tested at 10 μM in competition assays against radioactive probe compounds. Sites exhibiting > 50% inhibition at 10 μM were tested in secondary assays at the identified receptor or transporter using 12 concentrations of the test compound, measured in triplicate, to generate competition binding isotherms. K_i values were obtained from nonlinear regression of these binding isotherms from best-fit IC_{50} values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). K_i values were converted to pK_i values for data analysis. The radioligands used were as follows: [^{3}H]8-OH-DPAT (5-HT_{1A}), [^{3}H]GR125743 (5-$HT_{1B/1D}$), [^{3}H]5-HT (5-HT_{1E}), [^{3}H]ketanserin (5-HT_{2A}), [^{3}H]LSD (5-$HT_{2B/5A/6/7}$), [^{3}H]mesulergine (5-HT_{2C}), [^{3}H]citalopram (serotonin transporter), [^{3}H]prazocin ($\alpha_{1A/1B/1D}$), [^{3}H]rauwolscine ($\alpha_{2A/2B/2C}$), [^{125}I]pindolol (β_1), [^{3}H]CGP12177 (β_2, β_3), [^{3}H]nisoxetine (norepinephrine transporter), [^{3}H]SCH23390 (D_1, D_5), [^{3}H]*N*-methylspiperone ($D_{2/3/4}$), [^{3}H]WIN35428 (dopamine transporter), [^{3}H]DAMGO (μ-opioid), [^{3}H]DADLE (δ-opioid), [^{3}H]U69593 (κ-opioid), [^{3}H]muscimol ($GABA_A$), [^{3}H]flunitrazepam (central benzodiazepine), [^{3}H]PK11195 (peripheral benzodiazepine), [^{3}H]pyrilamine (H_1), [^{3}H]tiotidine (H_2), [^{3}H]α-methylhistamine (H_3), [^{3}H]histamine (H_4), [^{3}H]QNB (M_{1-5}), [^{3}H](+)-pentazocine (σ_1), and [^{3}H]DTG (σ_2). The experimental protocols are available from the NIMH PDSP website (Roth, 2013).

2.4. Head-twitch response

The head twitch response (HTR) was assessed using a head-mounted magnet and a magnetometer detection coil (Halberstadt and Geyer, 2013, 2014; Nichols et al., 2015). Briefly, mice were anesthetized and a small neodymium magnet was attached to the dorsal surface of the cranium using dental cement. Following a two-week recovery period, HTR experiments were carried out in a well-lit room with at least 7 days between sessions to avoid carryover effects. Test compounds were dissolved in water containing 5% Tween 80 and administered IP at a volume of 5 or 10 mL/kg body weight immediately prior to testing. Mice ($n = 5–6$/group) were injected with drug or vehicle and then HTR activity was recorded in a glass cylinder surrounded by a magnetometer coil for 30 min. Coil voltage was low-pass filtered (2−10 kHz cutoff frequency), amplified, and digitized (20 kHz sampling rate) using a Powerlab/8SP with LabChart v 7.3.2 (ADInstruments, Colorado Springs, CO, USA), then filtered off-line (40−200 Hz band-pass). Head twitches were identified manually based on the following criteria: 1) sinusoidal wavelets; 2) evidence of at least two sequential head movements (usually exhibited as bipolar peaks) with frequency ≥ 40 Hz; 3) amplitude exceeding the level of background noise; 4) duration < 0.15 s; and 5) stable coil voltage immediately preceding and succeeding each response.

2.5. Data analysis

Head twitch counts were analyzed using one-way analyses of variance (ANOVA). *Post hoc* pairwise comparisons between selected groups were performed using Tukey's studentized range method. The entire 30-min recordings were examined for head twitches, but in some cases a shorter block of time was used for analysis to accommodate compounds with a brief duration-of-action (potency calculations can be confounded by extended periods of inactivity). ED_{50} values and 95% confidence limits were calculated using nonlinear regression. Relationships between HTR potency and binding affinities were assessed using linear regression and ordinary least-squares regression. For all analyses, significance was demonstrated by surpassing an α-level of 0.05.

3. Results

3.1. Receptor binding

DALT and 9 ring-substituted derivatives were submitted to the NIMH PDSP for examination of their binding profiles at 45 neurotransmitter receptors and transporters. K_i values were determined for compounds that produced > 50% displacement of a radioactive probe compound at a concentration of 10,000 nM. The results are summarized in Table 1. The data for DALT and several of its 5-substituted derivatives (5-MeO-DALT, 5-F-DALT, and 5-Br-DALT) were reported in a previous publication (Cozzi and Daley, 2016). All of the compounds were devoid of 50% displacement at M_1-M_5 muscarinic, β_1-β_3 adrenergic, H_4 histaminergic, central benzodiazepine sites (labeled with [^{3}H]flunitrazepam), and $GABA_A$ receptors.

As reported previously (Cozzi and Daley, 2016), DALT binds relatively non-selectively to 5-HT_1 and 5-HT_2 subtypes, σ_1 and σ_2 sites, α_2-adrenoceptors, dopaminergic D_3 receptors, histaminergic H_1 receptors, and the 5-HT transporter (SERT). DALT had the highest measured affinities for 5-HT_{2B} ($K_i = 61$ nM), 5-HT_{1A} ($K_i = 100$ nM), σ_1 ($K_i = 101$ nM), α_{2A} ($K_i = 124$ nM), H_1 ($K_i = 127$ nM) and SERT ($K_i = 150$ nM). Incorporation of an oxygenated substituent at the 4-position altered the binding pattern of DALT. Compared to DALT, the 4-hydroxy and 4-acetoxy derivatives showed several-fold lower affinities for 5-HT_{1A}, 5-HT_{2C}, α_{2A}-adrenergic receptors, σ_1 and σ_2 sites, and SERT, whereas 5-HT_7 receptor affinity was increased by at least an order of magnitude. 4-Hydroxy-DALT also had low affinity for 5-HT_{2B} receptors ($K_i = 2593$ nM) and moderately high affinity for 5-HT_6 receptors ($K_i = 213$ nM).

The 2-phenyl-substituted DALT derivative (2-Ph-DALT) showed a notable binding profile. The 5-HT_{2A} binding affinity of 2-Ph-DALT ($K_i = 13$ nM) was 54-fold higher than the affinity of DALT ($K_i = 701$ nM) and at least 10-fold higher than the affinity of any other DALT derivative. According to a previous report (Stevenson et al., 2000), 2-aryl-tryptamines such as 2-phenyl-*N*,*N*-dimethyltryptamine and 2-phenyl-*N*,*N*-diethyltryptamine act as 5-HT_{2A} receptor antagonists and have high affinity (K_i values of 4.4 nM and 2.8 nM, respectively, vs. [^{3}H]ketanserin). 2-Ph-DALT was the only compound tested herein that bound to D_1, D_4, D_5, H_2, δ-opioid, and peripheral benzodiazepine receptors with a K_i value < 10 μM. Compared to the other compounds, 2-Ph-DALT also had relatively high affinity for α_{1A} and α_{1D} adrenoceptors and D_2 receptors. By contrast, 2-phenyl substitution abolished binding to σ_1 sites and SERT.

The 2-methyl derivatives of 5-MeO-DALT and 5-F-DALT were also examined. Incorporation of a 2-methyl group tended to reduce the affinity of those DALT derivatives for 5-HT receptors and SERT. The affinities of 5-MeO-DALT and 5-F-DALT for 5-HT_{1A}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{2A}, and 5-HT_{2C} receptors were consistently reduced by 2-methylation (see Table 1). Likewise, the binding of 5-MeO-DALT to SERT ($K_i = 499$ nM) was abolished by 2-methylation (5-MeO-2-Me-DALT: < 50% displacement at 10,000 nM), whereas the affinity of 5-F-DALT ($K_i = 36$ nM) was reduced almost 30-fold (5-F-2-Me-DALT; $K_i = 983$ nM).

Although 7-ethyl-substitution tended to reduce the binding affinity of DALT for most sites (including 5-HT_{1A} and 5-HT_{2A}

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Table 1
Summary of binding data for *N,N*-diallyltryptamine (DALT) and ring-substituted derivatives at 33 receptors and transporters.

Site	Species[a]	Binding Affinity (K_i, nM)									
		DALT	5-MeO	5-F	5-Br	4-HO	4-AcO	2-Ph	5-MeO-2-Me	5-F-2-Me	7-Et
5-HT_{1A}	Human	100	19	80	11	319	383	402	267	318	1013
5-HT_{1B}	Human	>10,000	735	1787	950	2494	>10,000	273	2267	2011	>10,000
5-HT_{1D}	Human	689	107	816	130	693	801	204	900	1592	2691
5-HT_{1E}	Human	378	500	474	512	238	467	>10,000	1594	1273	>10,000
5-HT_{2A}	Human	701	218	247	477	652	565	13	1153	655	1515
5-HT_{2B}	Human	61	59	16	53	2593	63	192	241	17	65
5-HT_{2C}	Human	385	456	102	358	2113	1515	278	>10,000	541	443
5-HT_{5A}	Human	>10,000	3312	4299	2389	>10,000	5844	1670	1822	1916	>10,000
5-HT_6	Human	1718	153	74	133	213	1791	68	206	168	>10,000
5-HT_7	Human	>10,000	90	402	49	600	724	>10,000	>10,000	493	>10,000
SERT	Human	150	499	36	127	5210	1089	>10,000	>10,000	983	795
α_{1A}	Human	1663	>10,000	1251	637	>10,000	>10,000	75	1198	1570	>10,000
α_{1B}	Human	1369	>10,000	>10,000	2050	>10,000	>10,000	904	>10,000	>10,000	>10,000
α_{1D}	Human	>10,000	>10,000	>10,000	1124	>10,000	>10,000	243	2405	>10,000	>10,000
α_{2A}	Human	124	215	119	83	1206	342	85	189	53	141
α_{2B}	Human	305	726	218	227	>10,000	170	78	335	108	489
α_{2C}	Human	901	1467	848	356	>10,000	748	159	888	184	682
NET	Human	1121	>10,000	1818	964	>10,000	>10,000	420	>10,000	>10,000	1879
D_1	Human	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	2793	>10,000	>10,000	>10,000
D_2	Human	>10,000	>10,000	2463	4349	>10,000	>10,000	388	>10,000	4416	>10,000
D_3	Human	672	>10,000	120	240	1570	>10,000	342	2399	414	1082
D_4	Human	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	1000	>10,000	>10,000	>10,000
D_5	Human	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	2003	>10,000	>10,000	>10,000
DAT	Human	1406	3378	2150	2455	>10,000	>10,000	746	2413	2208	1725
MOR	Human	>10,000	>10,000	>10,000	1726	>10,000	>10,000	>10,000	>10,000	>10,000	2674
DOR	Human	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	6789	>10,000	>10,000	>10,000
KOR	Human	2477	1132	2184	898	>10,000	5235	589	391	580	580
PBR	Rat kidney[b]	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	1929	>10,000	>10,000	>10,000
H_1	Human	127	505	83	106	>10,000	353	79	847	435	913
H_2	Human	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	367	>10,000	>10,000	>10,000
H_3	Guinea pig	>10,000	1712	2093	1495	>10,000	>10,000	>10,000	1134	1397	>10,000
σ_1	Rat brain[b]	101	301	86	101	2765	299	>10,000	427	531	22
σ_2	Rat PC12[b]	356	253	303	224	>10,000	>10,000	717	1235	396	136

Abbreviations **2-Ph**, 2-phenyl-*N,N*-diallyltryptamine; **4-AcO**, 4-acetoxy-*N,N*-diallyltryptamine; **4-HO**, 4-hydroxy-*N,N*-diallyltryptamine; **5-Br**, 5-bromo-*N,N*-diallyltryptamine; **5-F**, 5-fluoro-*N,N*-diallyltryptamine; **5-F-2-Me**, 5-methoxy-2-fluoro-*N,N*-diallyltryptamine; **5-MeO**, 5-methoxy-*N,N*-diallyltryptamine; **5-MeO-2-Me**, 5-methoxy-2-methyl-*N,N*-diallyltryptamine; **7-Et**, 7-ethyl-*N,N*-diallyltryptamine; **DALT**, *N,N*-diallyltryptamine; **DAT**, dopamine transporter; **DOR**, δ-opioid receptor; **KOR**, κ-opioid receptor; **MOR**, μ-opioid receptor; **NET**, norepinephrine transporter; **PBR**, peripheral benzodiazepine receptor; **SERT**, serotonin transporter.

[a] The experiments were performed using cloned receptors from the species indicated.

[b] The experiment was performed using tissues or cells natively expressing the receptor.

receptors), the affinity of 7-Et-DALT for σ_1 sites ($K_i = 22$ nM) was nearly 5-fold higher than the parent compound.

3.2. Head twitch response

DALT induced the HTR in mice with an ED_{50} of 3.42 mg/kg. Compared to other *N,N*-disubstituted tryptamines such as *N,N*-dipropyltryptamine and *N,N*-diisopropyltryptamine (Smith et al., 2014), DALT had relatively low potency. Similar to other tryptamine derivatives (Fantegrossi et al., 2008a), the response to DALT followed an inverted-U-shaped dose-response function (see Table 2).

Ring-substitution on the DALT molecule resulted in active compounds, some of which were more potent than DALT (see Table 2). The 4-hydroxy and 5-methoxy derivatives induced the HTR with almost twice the potency of DALT. 4-Acetoxy- or 5-fluoro-substitution produced even greater increases in potency. By contrast, 5-bromo substitution did not significantly alter HTR potency relative to DALT. Substitution at the 2-position with either a methyl or a phenyl group (e.g., 2-Ph-DALT, 2-Me-5-MeO-DALT, 2-Me-5-F-DALT) abolished activity in the HTR assay. Similarly, 7-Et-DALT did not induce the HTR. In addition to having higher potency than DALT, the 4-hydroxy and 4-acetoxy derivatives produced a HTR with an extremely rapid onset (data not shown).

For DALT and its active derivatives, there was no correlation between HTR potency (ED_{50} values) and 5-HT_{1A} receptor affinity ($R^2 = 0.2804$; $F\ (1,4) = 1.56$, *NS*) or 5-HT_{2A} receptor affinity ($R^2 = 0.1646$; $F\,(1,4) = 0.79$, *NS*). A multiple regression analysis was performed to test whether HTR potency is predicted by both 5-HT_{1A} and 5-HT_{2A} affinity. The ordinary least-squares (OLS) regression revealed that 5-HT_{1A} and 5-HT_{2A} binding affinities significantly predicted HTR potency ($R^2 = 0.8729$; $F\ (2,3) = 10.31$, $p < 0.05$; Fig. 2). Both 5-HT_{2A} affinity ($\beta = 0.741$, $t\,(3) = 3.74$, $p < 0.04$) and 5-HT_{1A} affinity ($\beta = -0.279$, $t\ (3) = -4.09$, $p < 0.03$) contributed significantly to the prediction, indicating that 5-HT_{2A} and 5-HT_{1A} receptors make positive and negative contributions, respectively, to HTR potency. In addition to 5-HT_{1A} and 5-HT_{2A} receptors, several other monoaminergic sites can influence HTR expression, including 5-HT_{2C} receptors (Fantegrossi et al., 2010), SERT (Basselin et al., 2009), and α_2-adrenoceptors (Schreiber et al., 1995). To test whether these other receptors play a role in the HTR induced by DALT derivatives, additional regression analyses were performed for sites with $K_i < 10{,}000$ nM. There was no correlation between HTR potency and affinity at 5-HT_{2C} ($R^2 = 0.0292$; $F\,(1,4) = 0.12$, *NS*), SERT ($R^2 = 0.0661$; $F\ (1,4) = 0.28$, *NS*), or α_{2A} sites ($R^2 = 0.2197$; $F\ (1,4) = 1.12$, *NS*). Furthermore, affinity for these sites did not significantly predict HTR potency when analyzed in combination with 5-HT_{2A} receptor affinity using multiple regression (data not shown).

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Table 2
Summary of head twitch response (HTR) data for *N,N*-diallyltryptamine (DALT) and ring-substituted derivatives.

Drug	One-Way ANOVA	Duration (min)	*N*	Dose (mg/kg)	HTR Counts (mean ± SEM)	ED_{50} (95% CI) (mg/kg)	ED_{50} (95% CI) (μmol/kg)
DALT	$F(5,24) = 5.71$, $p < 0.002$	30	5	0	3.6 ± 0.9	3.42 (2.44–4.79)	12.3 (8.8–17.3)
			5	0.875	8.2 ± 2.8		
			5	1.75	6.8 ± 2.6		
			5	3.5	14.2 ± 4.3		
			5	7	21.8 ± 4.4 **		
			5	14	20.6 ± 2.7 **		
5-MeO-DALT	$F(5,24) = 6.63$, $p = 0.0005$	20	5	0	3.0 ± 1.5	2.25 (1.82–2.78)	7.3 (5.9–9.1)
			5	1.75	6.6 ± 1.0		
			5	3.5	19.8 ± 1.5 **		
			5	7	8.8 ± 2.6		
			5	14	8.0 ± 4.9		
5-F-DALT	$F(5,24) = 5.12$, $p < 0.003$	30	5	0	4.4 ± 0.6	1.58 (1.09–2.28)	5.4 (3.7–7.7)
			5	0.875	9.8 ± 2.6		
			5	1.75	21.0 ± 5.7		
			5	3.5	36.0 ± 6.8 **		
			5	7	26.8 ± 7.1 *		
			5	14	21.0 ± 4.0		
5-Br-DALT	$F(5,24) = 5.21$, $p < 0.003$	30	5	0	3.4 ± 0.5	4.80 (2.70–8.54)	13.5 (7.6–24.0)
			5	3.5	5.0 ± 0.3		
			5	7	10.8 ± 2.7 *		
			5	14	8.6 ± 2.7		
			5	28	1.4 ± 0.4		
			5	56	1.4 ± 1.4		
4-HO-DALT	$F(5,24) = 12.07$, $p < 0.0001$	5	5	0	1.2 ± 0.4	2.60 (2.01–3.35)	8.3 (6.4–10.6)
			5	0.875	4.0 ± 3.3		
			5	1.75	9.2 ± 3.7		
			5	3.5	28.6 ± 4.1 **		
			5	7	31.6 ± 4.9 **		
			5	14	24.6 ± 4.7 **		
4-AcO-DALT	$F(5,24) = 6.87$, $p = 0.0004$	30	5	0	4.8 ± 1.0	1.99 (1.35–2.95)	4.8 (3.3–7.1)
			5	0.875	10.4 ± 1.4		
			5	1.75	42.0 ± 9.5 *		
			5	3.5	39.0 ± 14.1 *		
			5	7	65.0 ± 8.4 **		
			5	14	47.8 ± 10.0 **		
2-Ph-DALT	$F(5,24) = 2.20$, *NS*	30	5	0	3.8 ± 0.8	*ND*[a]	*ND*
			5	0.875	2.8 ± 0.5		
			5	1.75	3.6 ± 1.3		
			5	3.5	1.4 ± 0.5		
			5	7	2.0 ± 0.5		
			5	14	1.2 ± 0.5		
2-Me-5-MeO-DALT	$F(5,24) = 1.02$, *NS*	30	5	0	3.8 ± 1.3	*ND*	*ND*
			5	0.875	4.4 ± 0.2		
			5	1.75	7.4 ± 2.2		
			5	3.5	4.2 ± 1.0		
			5	7	4.2 ± 0.9		
			5	14	5.0 ± 1.4		
2-Me-5-F-DALT	$F(5,24) = 0.19$, *NS*	30	5	0	5.4 ± 1.7	*ND*	*ND*
			5	0.875	6.2 ± 1.0		
			5	1.75	6.8 ± 0.9		
			5	3.5	5.8 ± 2.5		
			5	7	6.4 ± 1.6		
			5	14	7.2 ± 0.8		
7-Et-DALT	$F(1,10) = 0.11$, *NS*	30	6	0	10.7 ± 1.7	*ND*	*ND*
			6	15	9.8 ± 1.8		

*$p < 0.05$, **$p < 0.01$, significant difference from the vehicle control group (Tukey's test).
[a] *ND* = not determined (the compound was not active within the dose range tested).

4. Discussion

The potency and 5-HT receptor affinities of tryptamine hallucinogens are influenced by the substituent groups present on the indole nucleus and amine nitrogen. Most compounds in this structural class contain *N,N*-dialkyl substituents, but tryptamines containing *N,N*-diallyl groups have also been synthesized (Brandt et al., 2017). Although the structure-activity relationships and pharmacology of dialkyltryptamines such as DMT and psilocybin have been widely investigated, relatively little is known about the comparative properties of diallyltryptamines. The present studies were conducted to investigate the pharmacology and behavioral effects of DALT and a variety of ring-substituted derivatives, some of which are used recreationally as new psychoactive substances or "research chemicals" and reportedly have hallucinogenic effects.

Consistent with the effects of other tryptamine hallucinogens (Fantegrossi et al., 2006, 2008b; Halberstadt et al., 2011; Carbonaro et al., 2015; Nichols et al., 2015), DALT and several of its derivatives substituted at the 4- or 5-position induced head twitches in mice. Although our studies measured 5-HT_{2A} binding affinity and did not include a functional assessment of receptor activation, DALT, 4-HO-DALT, 4-AcO-DALT, 5-Br-DALT, 5-F-DALT and 5-MeO-DALT are likely to be 5-HT_{2A} agonists based on their effects in the HTR assay. Importantly, 5-MeO-DALT was previously reported to act as an

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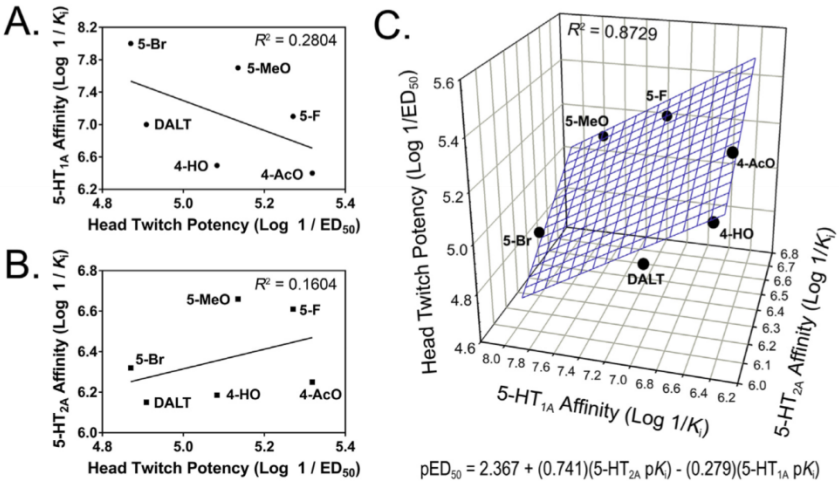

Fig. 2. Correlation between potency in the head twitch response (HTR) assay (pED_{50} values) and serotonin receptor binding affinities (pK_i values) for *N,N*-diallyltryptamine (DALT) and five ring-substituted derivatives. (A) Correlation between HTR potency and 5-HT_{1A} receptor affinity. (B) Correlation between HTR potency and 5-HT_{2A} receptor affinity. (C) Correlation between HTR potency and 5-HT_{1A} and 5-HT_{2A} receptor affinity.

agonist at recombinant human 5-HT_{2A} receptors (Arunotayanun et al., 2013). Similarly, it was recently reported (Gatch et al., 2017) that 5-MeO-DALT produces full substitution in rats trained to discriminate the hallucinogenic 5-HT_{2A} receptor agonist 2,5-dimethoxy-4-methylamphetamine (DOM). Since the head twitch assay is routinely used to test whether 5-HT_{2A} agonists produce LSD-like behavioral effects (Gonzalez-Maeso et al., 2007), the ability of diallyltryptamines to induce the HTR and produce DOM-like stimulus effects is thus consistent with their classification as serotonergic hallucinogens. However, few details have been published regarding the effects of these compounds in humans.

Notably, the potency of the diallyltryptamines in the HTR assay is not correlated with 5-HT_{2A} receptor binding affinity alone but is dependent on activity at *both* 5-HT_{1A} and 5-HT_{2A} receptors. According to the multiple regression analysis, there is a positive relationship between HTR potency and 5-HT_{2A} affinity and a negative relationship between HTR potency and 5-HT_{1A} affinity; in other words, HTR potency increases as 5-HT_{2A} affinity increases and decreases as 5-HT_{1A} affinity increases. As noted earlier, the hallucinogen HTR occurs as a result of 5-HT_{2A} activation and can be suppressed by concurrent administration of a 5-HT_{1A} agonist (Darmani et al., 1990; Schreiber et al., 1995; Kleven et al., 1997). Based on the roles that 5-HT_{1A} and 5-HT_{2A} receptors are known to play in the hallucinogen HTR, the regression analysis can be interpreted as showing that 5-HT_{2A} activation by DALT and its derivatives mediates the HTR, whereas their interaction with the 5-HT_{1A} receptor has a countervailing influence that inhibits expression of head twitch behavior. Hence, the potency of diallyltryptamines in the HTR assay may ultimately be determined by their combined activities at 5-HT_{1A} and 5-HT_{2A} receptors. These findings support the hypothesis that 5-HT_{1A} activation by tryptamine hallucinogens buffers their effects on the HTR.

Based on the ability of 5-HT_{1A} agonists to inhibit the HTR, there has been speculation that 5-HT_{1A} stimulation by nonselective tryptamine and lysergamide hallucinogens may reduce or inhibit the frequency of their induced head twitch behavior (Darmani et al., 1990). Our recent work has demonstrated that the LSD analog and non-selective 5-HT_{1A}/5-HT_{2A} agonist lysergic acid morpholide (LSM-775) does not induce the HTR in mice unless the animals are pretreated with the 5-HT_{1A} antagonist WAY-100635 (Brandt et al., 2018), indicating that 5-HT_{1A} activation by LSM-775 masks its ability to induce the HTR. As far as we are aware, however, the present study is the first to show that the *potency* of the HTR induced by tryptamine hallucinogens may be influenced by their 5-HT_{1A} interactions. Nevertheless, these findings remain tentative given to the small number of compounds tested; follow-up studies with a larger group of tryptamines are necessary to achieve more definitive results.

One potential confound for the regression analysis is that the binding studies were performed with cloned human 5-HT receptors whereas the behavioral experiments were performed in mice. Sequence differences between rodent and human 5-HT receptors can result in ligand binding affinity differences (Kao et al., 1992; Oksenberg et al., 1992; Parker et al., 1993; Smolyar and Osman, 1993). There are reportedly species differences in the affinities of 4-hydroxytryptamines for the 5-HT_{2A} receptor, which are potentially relevant to our studies with 4-HO-DALT and 4-AcO-DALT. Specifically, according to Gallaher et al. (1993), who studied human and rat 5-HT_{2A} receptors labeled with [^{3}H]ketanserin, 4-hydroxy-DMT (psilocin) has 15-fold higher affinity for the human receptor ($K_i = 340$ nM) than for the rat receptor ($K_i = 5100$ nM), whereas its 5-hydroxy isomer bufotenine has nearly equal affinities for the human and rat receptors (K_i values of 300 nM and 520 nM, respectively). The human 5-HT_{2A} receptor contains a serine at position 242 in helix V whereas alanine is present in the receptor in rodents, leading Gallaher et al. (1993) to speculate that psilocin may have higher affinity for the human receptor because Ser-242$^{(5.42)}$ can form a hydrogen-bond with the 4-hydroxyl group in psilocin. Other studies, however, failed to confirm their findings. Another group reported that both psilocin and bufotenine displace [^{125}I]*R*-(−)-DOI binding to 5-HT_{2A} receptors in rat cortex with high affinity and have nearly equivalent IC_{50} values (McKenna et al., 1990). Furthermore, Ser-242$^{(5.42)}$ in the human 5-HT_{2A} receptor is believed to form a hydrogen-bond with the indole *N*1 nitrogen of

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tryptamines and ergolines based on mutagenesis experiments and molecular modeling (Nelson et al., 1993; Johnson et al., 1994; Almaula et al., 1996; Wacker et al., 2017), abrogating the structural basis for the species differences posited by Gallaher. Therefore, although there is no clear evidence indicating that differences between human and mouse 5-HT receptors are likely to confound our regression analysis, especially with regard to 4-substituted DALT derivatives, the potential existence of cross-species differences in 5-HT receptor pharmacology must be acknowledged as a source of potential error for the regression.

DALT and derivatives substituted at the 5-position have been shown to bind to multiple 5-HT receptors, as well as α_2 adrenergic subtypes, σ_1 and σ_2 sites, histamine H_1 receptors, and SERT (Cozzi and Daley, 2016). As shown in the present investigation, substitution at other positions in the indole ring can markedly alter the binding profile of DALT. The 4-substituted derivatives displayed reduced affinity at 5-HT_{1A} receptors compared to DALT and the 5-substituted derivatives. This is consistent with reports demonstrating that 4-hydroxy-DMT (psilocin) binds to 5-HT_{1A} sites with 20-fold lower affinity compared to its 5-hydroxy isomer (bufotenine) or the 5-hydroxy *O*-methyl derivative (5-methoxy-DMT), whereas there is little difference between their 5-HT_{2A} receptor affinities (McKenna et al., 1990; Blair et al., 2000).

Addition of a methyl group to the 2-position of 5-MeO-DALT reduced its affinity for most 5-HT binding sites, including 5-HT_{1A} and 5-HT_{2A} receptors, and abolished its ability to induce the HTR in mice at doses up to 14 mg/kg. These findings parallel those of Glennon et al. (2000), who found that 2-methylation or 2-ethylation of 5-methoxy-DMT reduced its affinity for 5-HT_{2A} receptors. Similarly, although 2-methyl-5-methoxy-DMT is a hallucinogen in humans, it reportedly has significantly lower potency than 5-methoxy-DMT (Shulgin and Shulgin, 1997). The 5-HT_{2A} receptor apparently has difficulty accommodating tryptamines with a 2-alkyl substituent.

2-Ph-DALT did not induce the HTR despite having the highest 5-HT_{2A} affinity of any compound screened ($K_i = 13$ nM). According to Stevenson et al. (2000), various 2-phenyl-*N*,*N*-dialkyltryptamines including the *N*,*N*-dimethyl, *N*,*N*-diethyl, and *N*-methyl-*N*-ethyl homologues bind to the 5-HT_{2A} receptor with high (nM) affinities. However, all of these compounds blocked the stimulatory effect of 5-HT on phosphoinositide hydrolysis in CHO cells expressing the human 5-HT_{2A} receptor. In light of the fact that other 2-phenyl-*N*,*N*-disubstituted tryptamines act as antagonists, the failure of 2-Ph-DALT to induce the HTR suggests that it may also act as a 5-HT_{2A} antagonist.

The 7-ethyl-substituted derivative of DALT also had low affinity for 5-HT_{1A} and 5-HT_{2A} receptors and did not induce the HTR in mice when tested at 15 mg/kg. These findings are consistent with the behavioral effects of other 7-ethyl-substituted tryptamines. 7-Ethyl-DMT produces only partial substitution in rats trained to discriminate 5-MeO-DMT from vehicle (Glennon et al., 1980a). Rats trained to discriminate the interoceptive cue produced by 5-MeO-DMT generalize to other serotonergic hallucinogens (Glennon et al., 1980b; Young et al., 1982); hence, the absence of full substitution with 7-ethyl-DMT indicates that it does not produce hallucinogen-like stimulus effects in rodents.

The present findings also suggest that while 4- and 5-substituted DALT compounds may produce hallucinogenic effects in humans, 2- and 7-substituted DALT compounds may lack hallucinogenic effects, although further studies are necessary to test this hypothesis. While DALT, 5-MeO-DALT, and 4-AcO-DALT have already been detected by the European Early-Warning System and reported to the European Monitoring Center for Drugs and Drug Addiction (EMCDDA, 2013, 2015), no such reports have arisen for 2- or 7-substituted DALT compounds.

To our knowledge, this analysis is the first to quantify the relative contributions of 5-HT_{2A} and 5-HT_{1A} receptors to the induction of HTR by a class of tryptamine hallucinogens. These findings may allow us to better predict the psychoactive potential of DALT derivatives based on their behavioral pharmacology, and suggest that similar analyses could be attempted for other classes of tryptamine hallucinogens. However, although 5-MeO-DALT produces hallucinogen-like behavioral responses in rodent behavioral paradigms including mouse HTR (the present studies) and rat drug discrimination (Gatch et al., 2017), it is not yet clear whether DALT derivatives can fully mimic the psychedelic effects produced by classical hallucinogens, allowing the possibility of subtle pharmacological differences relative to other tryptamine hallucinogens. Hence, it is not known whether the observed relationship between HTR potency and 5-HT_{2A} and 5-HT_{1A} binding affinities is consistent across the entire class of tryptamine hallucinogens. Nevertheless, if similar relationships do exist for other tryptamines, performing similar analyses on those classes should improve our understanding of their complex pharmacology and facilitate predictions regarding their psychoactive potencies.

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CHAPTER 3

HYPERSENSITIVITY TO BEHAVIORAL EFFECTS OF HALLUCINOGENS IN MICE LACKING THE $mGlu_5$ RECEPTOR

1. Introduction

Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors that modulate glutamate signaling (Gupta et al., 2005; Kew and Kemp, 2005). Dysfunctional glutamatergic signaling has been implicated in a variety of neuropsychiatric disorders including schizophrenia, depression, and addiction (Conn and Jones, 2009; Gupta et al., 2005; Krivoy et al., 2008; Olive, 2010). Accordingly, the functional roles of mGluRs in maintaining normal neural function are of particular therapeutic interest, since directly targeting ionotropic glutamate receptors (i.e. NMDARs, AMPARs) can produce dangerous side effects including seizures and neurotoxicity (Conn and Jones, 2009). Allosteric modulators of inhibitory $G_{i/o}$-coupled $mGlu_{2/3}$ and excitatory $G_{q/11}$-coupled $mGlu_5$ receptors have shown particular promise in preclinical models of psychosis, and are currently subject to ongoing clinical trials with varying results (Conn and Jones, 2009; Gregory et al., 2013; Hovelsø et al., 2012; Patil et al., 2007; Rook et al., 2015).

Serotonergic signaling, and specifically signaling at the 5-HT_{2A} receptor, has also been implicated in psychosis (Halberstadt and Geyer, 2013a). Most agonists acting at 5-HT_{2A} (e.g. hallucinogens) produce perceptual and behavioral effects similar to the positive symptoms of schizophrenia, and antagonists at 5-HT_{2A} (e.g. atypical antipsychotics) have shown efficacy in treatment of psychotic symptoms in both preclinical and clinical paradigms (Halberstadt and Geyer, 2013a; Meltzer, 1999, 1991; Seeman, 2002). Since both mGluRs and 5-HT receptors are associated with psychotic phenotypes, significant interest has arisen regarding functional crosstalk between these neurotransmitter systems.

Evidence for functional interactions between $mGlu_{2/3}$ and 5-HT_{2A} is robust. $mGlu_{2/3}$ agonists antagonize the HTR induced by the 5-HT_{2A} agonist DOI, and $mGlu_{2/3}$ antagonists potentiate DOI-induced HTR (Benneyworth et al., 2007; Gewirtz and Marek, 2000; González-Maeso et al., 2008; Kłodzinska et al., 2002; Moreno et al., 2013, 2011). Additionally, $mGlu_{2/3}$ agonists suppress 5-HT_{2A}-induced increase in the frequency of spontaneous EPSPs in layer V pyramidal cells in prefrontal cortex (PFC) (Benneyworth et al., 2007; Kłodzinska et al., 2002; Marek et al., 2000). Furthermore, expression of

$mGlu_2$ has been shown as necessary for the induction of several hallucinogen-mediated responses. $mGlu_2$-KO mice do not display DOI-induced HTR, nor do they show DOI-induced expression of the immediate early gene *egr-2* (González-Maeso et al., 2008; Moreno et al., 2011). These findings, alongside evidence of propinquity of 5-HT_{2A} and $mGlu_2$ receptors in cells, have led to the hypothesis that $mGlu_2$ may interact directly with 5-HT_{2A}, forming functional heterocomplexes in frontal cortex that are relevant to psychosis and the effects of hallucinogens (González-Maeso et al., 2008).

Though less thoroughly explored, mounting evidence also supports possible functional interactions between $mGlu_5$ and 5-HT_{2A} receptors. $mGlu_5$ and 5-HT_{2A} show similar regional distributions, as both are heavily expressed in prefrontal cortex, striatum, and hippocampus (Cornea-Hébert et al., 1999; Romano et al., 1995a; Shigemoto et al., 1993; Testa et al., 1994). $mGlu_5$ negative allosteric modulators (NAMs) increase 5-HT release in hippocampus and frontal cortex, and the anxiolytic- and antidepressant-like effects of $mGlu_5$ NAMs can be blocked by a 5-HT_{2A}/2C antagonist (Smolders et al., 2008; Stachowicz et al., 2007). Previous work in our lab has demonstrated that the $mGlu_5$ NAM 2-methyl-6-(phenylethynyl)pyridine (MPEP) or genetic knockout of $mGlu_5$ induces locomotor hyperactivity, a behavioral effect also induced by 5-HT_{2A} agonists (Halberstadt et al., 2011b). Both effects can be blocked by the highly selective 5-HT_{2A} inverse agonist M100907, suggesting that these effects are dependent on 5-HT_{2A} activation. The locomotor response to the 5-$HT_{2A/2C}$ agonist and hallucinogen DOM was augmented in $mGlu_5$-KO mice, further supporting a functional interaction for the two receptors, and suggesting a possible modulating role for $mGlu_5$ in the behavioral effects of hallucinogens (Halberstadt et al., 2011b).

The physiology underlying this sensitization is still unclear. Locomotion is a complex behavior that can be mediated by various pharmacological targets. For instance, non-serotonergic hallucinogens such as phencyclidine (PCP) and ketamine induce hyperlocomotion by stimulating subcortical dopamine release in a process independent of direct 5-HT_{2A} activation (De Deurwaerdère and Di Giovanni, 2017; Maurel-Remy et al., 1995). The locomotor hyperactivity induced by PCP, cocaine, and amphetamine can be blocked by M100907 despite minimal serotonergic activity of these drugs of abuse, including at 5-

HT_{2A} (Maurel-Remy et al., 1995; McMahon and Cunningham, 2001; Neill et al., 1999). Furthermore, mice lacking the $mGlu_5$ receptor in cortical pyramidal neurons show hypersensitivity to the locomotor effects of methylphenidate (Jew et al., 2013), a selective DA/NE reuptake inhibitor with little or no affinity for serotonergic targets (Han and Gu, 2006). Consequently, verifying hypersensitivity to the effects of hallucinogens in $mGlu_5$-KO mice requires consistency across multiple behaviors closely tied to the pharmacology underlying hallucinogen effects. The head-twitch response (HTR) is a brief, paroxysmal head rotation induced by 5-HT_{2A} activation in rats and mice. HTR is often used as a behavioral proxy for the effects of hallucinogens in rodents due to its ability to distinguish hallucinogenic from non-hallucinogenic 5-HT_{2A} agonists, as well as its high correlation with reported hallucinogen potencies in humans (Halberstadt et al., unpublished data). Here we demonstrate that the hypersensitivity to hallucinogen-mediated locomotor effects in $mGlu_5$-KO mice generalizes to the HTR, a mouse behavior associated with both the 5-HT_{2A} receptor and hallucinogenic properties, and that the selective 5-HT_{2A} agonist *N*-(2-hydroxybenzyl)-2,5-dimethoxy-4-cyanophenethylamine (25CN-NBOH) is sufficient to demonstrate this hypersensitivity.

5-HT_{2A} and $mGlu_5$ receptors activate similar $G_{\alpha q}$-dependent signaling pathways, including phospholipase-C (PLC) mediated signaling cascades that result in inositol triphosphate (IP_3) turnover and phospholipase-A_2 mediated cascades that result in the generation of arachidonic acid (AA). The proportion of PLC to PLA_2 mediated signaling depends on the chemistry of the specific ligand and its interaction with the receptor, a process known as agonist-directed trafficking. Though the significance of these respective signaling pathways to hallucinogen-mediated behaviors has not been identified, one or both of these signaling pathways may be involved in the functional interaction between 5-HT_{2A} and $mGlu_5$ receptors as they pertain to sensitization of the hallucinogen response in $mGlu_5$-KO mice. Experiments with DOM do little to identify the primary signaling pathway(s) responsible for these effects, as its efficacy at inducing IP accumulation is roughly equivalent to its efficacy at inducing AA release via 5-HT_{2A} receptor activation (Moya et al., 2007). In contrast, [(*7R*)-3-bromo-2,5-dimethoxy-bicyclo[4.2.0]octa-1,3,5-trien-7-yl]methanamine (TCB-2) is significantly biased toward the PLC

signaling cascade, with (R)-(-)-TCB-2 displaying ~65-fold selectivity over PLA_2 and (S)-(+)-TCB-2 at least 22-fold selective for PLC (EC_{50} for AA release was >10000 nM and thus not specifically determined) (McLean et al., 2006). Furthermore, racemic TCB-2 produced intrinsic activity for IP_3 accumulation equivalent to 94% of the response stimulated by 5-HT, suggesting that TCB-2 acts as a full agonist for the PLC signaling cascade (McLean et al., 2006). The experiments presented here expand on previous locomotor experiments by recapitulating the effect with TCB-2 to assess whether preferential activation of PLC-mediated signaling over PLA_2-mediated signaling by a 5-HT_{2A} agonist preserves the hypersensitivity to locomotor activation in $mGlu_5$-KO mice.$mGlu_5$

2. Materials and methods

2.1. Subjects

Male and female $mGlu_5$ wild-type (WT) and knockout (KO) mice on a C57BL/6J background were bred in-house. Mice heterozygous for $mGlu_5$ were used for breeding in order to generate both WT and KO mice from the same parents. $mGlu_5$ KO mice were generated as previously described (Jia et al., 1998). Upon weaning (p21-p24), a small segment of the tail was removed for genotyping by polymerase chain reaction (PCR) to confirm the absence of the *GRM5* gene. Mice were housed up to 4 per cage by genotype in an AAALAC-approved animal facility at the University of California, San Diego (UCSD) that meets all Federal and State requirements for the care and treatment of laboratory animals. *Ad libitum* access to food and water was provided and mice were housed on a reversed light cycle (lights on at 1900h, off at 0700h). Adult mice (3-9 months) were used for all experiments. All efforts were made to minimize animal suffering, and all animal experiments were carried out in accordance with NIH guidelines and the approval of the UCSD animal care committee.

2.2. Drugs

Drugs used were 1-(2,5-dimethoxy-4-methylphenyl)-propan-2-amine hydrochloride (DOM; National Institute on Drug Abuse, Bethesda, MD); [(*7R*)-3-bromo-2,5-dimethoxy-bicyclo[4.2.0]octa-1,3,5-trien-7-yl]methanamine hydrobromide (TCB-2; Tocris Bioscience, Ellisville, MO); and 4-[2-[(2-hydroxyphenyl)methylamino]ethyl]-2,5-dimethoxybenzonitrile hydrochloride (25CN-NBOH; Tocris Bioscience, Ellisville, MO). DOM and TCB-2 were dissolved in isotonic saline and administered intraperitoneally (i.p.). 25CN-NBOH was dissolved in water containing 5% Tween 80 and was administered subcutaneously (s.c.). All drugs were administered at a volume of 5 mL/kg body weight.

2.3. Locomotor activity

Locomotor activity was assessed using the mouse behavioral pattern monitor system (BPM; San Diego Instruments, San Diego, CA) as described in Halberstadt et al 2010. Briefly, each unit consisted of a 30.5 x 61 x 38 cm Plexiglas chamber equipped with a 12 x 24 array of photobeams 1 cm above the floor to determine the location of the mouse via x-y-coordinates of beam breaks with a resolution of 1.25 cm. Testing occurred in the dark with 65 dB white noise in the testing room. Data for rearing, investigatory behavior (i.e. hole pokes into one of three holes on each wall and the floor of the chamber), and locomotor path complexity were collected concurrently, but were not included in these analyses. Mice were tested in a three-way crossover design with one week between tests. Each animal received vehicle, 0.3, or 1 mg/kg TCB-2 in a pseudo-randomized, counterbalanced order, and was immediately placed in the BPM chamber for 60 min. All animals received all treatments over the course of three weeks for within-subjects comparison. The cohort for these experiments consisted of three male WT and nine male KO mice, and six female WT and five female KO mice. Since no significant sex effects were detected (see *Results*), analyses were collapsed across sex for a total of n = 9 WT and n = 14 KO mice. $mGlu_5$ WT and KO mice for all experiments were littermates derived from heterozygous pairings. Horizontal locomotor activity was quantified as distance traveled in centimeters per 10-min block and was analyzed using one-, two-, or three-way analyses of variance (ANOVAs) with sex and genotype as between-subject

variables and drug treatment and time as within-subject variables. One-way ANOVAs for each 10-min block were used for post hoc analyses. Significance was demonstrated by exceeding an α-level of 0.05.

2.4. Head-twitch response

The head twitch response (HTR) was assessed using a magnet adhered to the skull of the mouse and a magnetometer coil to precisely detect head movements via deflections in coil voltage, as described in Klein et al. 2018 (see also Halberstadt & Geyer, 2013, 2014). In two separate experiments, mice were administered vehicle, 0.25, or 0.5 mg/kg DOM and vehicle, 1, or 3 mg/kg 25CN-NBOH, respectively, immediately prior to placement in the magnetometer coil. HTR was assessed for 30 min, at which point mice were immediately returned to their home cage. At least one week was allowed between test sessions, and all mice received all doses of DOM in pseudorandom, counterbalanced order, followed by all doses of 25CN-NBOH similarly assigned with one month between experiments in a within-subject design. For both experiments, mean total HTR counts for each group were analyzed using one-, two-, or three-way ANOVAs with gene and sex as between-subject variables and drug treatment as a repeated measure. Due to the lack of significant sex effects (see *Results*), groups were collapsed across sex for all further analyses. Post-hoc analyses were carried out using Student's t-tests for each dose to compare WT and KO groups. Significance was demonstrated by exceeding an α-level of 0.05.

2.5. 5-HT_{2A} *receptor mRNA quantification*

To assess relative expression of 5-HT_{2A} receptors in $mGlu_5$ WT and KO mice, quantitative real-time PCR was carried out on samples from experimentally naive mice. Expression of *htr2a* mRNA was assessed in frontal cortex, striatum, and hippocampus, as these regions typically demonstrate robust expression of both 5-HT_{2A} and $mGlu_5$ receptors (Cornea-Hébert et al., 1999; Romano et al., 1995a; Shigemoto et al., 1993; Testa et al., 1994). Briefly, male and female $mGlu_5$ WT and KO littermates were sacrificed and regions of interest were dissected on an ice cold platform immediately following brain extraction. Samples were placed in 1.5 ml tubes containing 500 µl of RNA Later (Life Technologies,

Carlsbad, CA). The RNeasy Lipid Kit (Qiagen, Valencia, CA) was used to extract RNA according to manufacturer instructions, followed by on-column DNase digestion using the RNase-Free DNase Kit (Qiagen, Valencia, CA). A Nanodrop 2000 (Thermo Scientific, Wilmington, DE) was used to assess quality and quality of RNA in each sample. Total RNA was standardized across samples and the Superscript III first-strand kit (Life Technologies, Carlsbad, CA) was used for first-strand cDNA synthesis. The resulting cDNA was transferred to the UCSD core facility (http://cfar.ucsd.edu/) for TaqMan quantitative PCR (Applied Biosystems, Carlsbad, CA) using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Inc, Foster City, CA). *htr2a* was assessed using custom probes designed and ordered from Applied Biosystems (Applied Biosystems, Carlsbad, CA), and was compared with the housekeeping gene *ACTb*. The $2^{-\Delta\Delta(Ct)}$ method (Livak & Schmittgen, 2001) was used to normalize *htr2a* gene expression relative to *ACTb*, and normalized expression values for WT and KO mice within each region of interest were compared using Student's t-tests. Because no significant sex effects were detected in behavioral experiments, groups were collapsed across sex, yielding n=4 WT and n=5 KO samples per region.

3. Results

3.1. Head-twitch response

3.1.1. DOM-induced HTR in $mGlu_5$*-WT and -KO mice*

To determine whether hypersensitivity to the behavioral effects of DOM extends to other 5-HT_{2A}-mediated behaviors, we assessed the HTR in response to DOM (0, 0.25, or 0.5 mg/kg, i.p.) in male and female $mGlu_5$ WT and KO mice. We detected main effects of DOM treatment ($F(2,32) = 53.93$, $p < 0.0001$) and genotype ($F(1,16) = 10.47$, $p < 0.01$) on mean number of head twitches detected over a 30 min session, with both DOM treatment and $mGlu_5$-KO significantly increasing the HTR. Although we detected a trend level main effect of sex on HTR ($F(1,16) = 3.80$, $p = 0.0691$) with females producing

slightly more head twitches during the session, sex did not interact significantly with either DOM treatment ($F(2,32) = 2.02$, *NS*), genotype ($F(1,16) = 0.15$, *NS*), or the interaction between genotype and drug treatment ($F(2,32) = 0.68$, *NS*). Since interactions between genotype and drug treatment are of specific interest, the remainder of analyses were collapsed across sex.

There was a highly significant interaction between DOM treatment and genotype ($F(2,36) = 5.50$, $p < 0.01$) with DOM treatment inducing a more pronounced increase in the HTR in $mGlu_5$-KO relative to WT mice (Fig. 1A). Independent student's t-tests were then carried out to compare HTR between genotypes at each dose and indicated a dose-dependent interaction between genotype and drug treatment. HTR counts induced by DOM were significantly greater in $mGlu_5$-KO mice compared to WT at a dose of 0.5 mg/kg ($F(1,18) = 9.38$, $p < 0.001$), but not at 0.25 mg/kg despite increasing HTR counts at this dose. $mGlu_5$-KO mice also produced significantly more HTR counts following vehicle treatment than their WT littermates ($F(1,18) = 5.08$, $p < 0.05$), with 5.14 ± 0.88 and 10.46 ± 1.65 for $mGlu_5$-KO and $mGlu_5$-WT mice, respectively.

3.1.2. Selective 5-HT_{2A} *agonist 25CN-NBOH-induced HTR in* $mGlu_5$*-WT and -KO mice*

To assess the sufficiency of effects at 5-HT_{2A} for inducing hypersensitivity to the HTR in $mGlu_5$-KO mice, we administered 25CN-NBOH (0, 1, or 3 mg/kg, s.c.), a selective 5-HT_{2A} agonist, immediately prior to testing in the HTR assay. 25CN-NBOH was previously purported to be as high as 100-fold selective for 5-HT_{2A} over 5-HT_{2C} (Hansen et al., 2014), though experiments conducted by our lab suggest 23-fold selectivity *in vitro* (Halberstadt et al., 2016). We detected significant main effects of 25CN-NBOH treatment ($F(2,28) = 104.89$, $p < 0.0001$) and genotype ($F(1,14) = 5.69$, $p < 0.05$) with 25CN-NBOH or $mGlu_5$-KO independently increasing the mean number of head-twitches detected over the 30 min session (Fig. 1B). Since no main effect of sex ($F(1,14) = 0.06$, *NS*) or interaction between sex and drug treatment ($F(2,28) = 1.70$, *NS*), genotype ($F(1,14) = 2.12$, *NS*), or drug x genotype ($F(2,28) = 0.31$, *NS*) was detected, subsequent analyses collapsed across sex.

There was a trend level interaction between 25CN-NBOH treatment and genotype ($F(2,32) = 3.03$, $p = 0.0623$) reflecting a similar hypersensitivity to the effects of 25CN-NBOH as was seen with DOM. Independent Student's t-tests comparing HTR counts between $mGlu_5$-KO and WT mice at each dose indicated a highly significant increase in HTR counts in $mGlu_5$-KO relative to WT mice at a dose of 3 mg/kg 25CN-NBOH ($F(1,16) = 9.54$, $p < 0.01$) and a trend level increase at a dose of 1 mg/kg ($F(1,16) = 3.68$, $p = 0.0733$). No difference between genotypes was detected with vehicle treatment in this experiment, though similar means were detected for $mGlu_5$-KO and WT mice relative to vehicle treatment in the DOM experiment (6.71 ± 1.52 and 9.46 ± 3.16 for KO and WT mice, respectively).

3.2. 5-HT_{2A} receptor mRNA quantification in $mGlu_5$-WT and -KO mice

To assess whether the hypersensitivity to the behavioral effects of 5-HT_{2A} agonists in $mGlu_5$-KO mice results from a compensatory increase in 5-HT_{2A} receptor expression in the absence of $mGlu_5$ receptors, we performed quantitative PCR (qPCR) analysis for *htr2a* mRNA, which encodes the 5-HT_{2A} receptor, on tissue samples collected from both $mGlu_5$-WT and -KO mice. We focused specifically on frontal cortex, hippocampus, and striatum, as these regions have been shown to robustly express both 5-HT_{2A} and $mGlu_5$ receptors and may be involved in locomotor hyperactivity and the HTR (Cornea-Hébert et al., 1999; Romano et al., 1995a; Shigemoto et al., 1993; Testa et al., 1994). However, we detected no significant differences in 5-HT_{2A} receptor $mGlu_5$ expression relative to the housekeeping gene *ActB* in frontal cortex ($F(1,7) = 2.07$, *NS*), hippocampus ($F(1,7) = 0.03$, *NS*), or striatum ($F(1,7) = 0.89$, *NS*) (Fig. 2) between WT and $mGlu_5$-KO mice, suggesting that hallucinogen hypersensitivity in $mGlu_5$-KO mice does not result from a compensatory increase in 5-HT_{2A} receptor expression in behaviorally-relevant regions.

3.3. Sensitivity to the locomotor activating effects of TCB-2 in $mGlu_5$-KO mice

We probed the sufficiency of the 5-HT_{2A}-mediated PLC signaling pathway in the hypersensitivity of $mGlu_5$-KO mice to hallucinogen-induced hyperlocomotion by assessing locomotor activity following administration of TCB-2, a 5-HT_{2A} agonist that is functionally selective for PLC over PLA_2 signaling. Because we detected no significant main effect of sex, or interaction between sex and drug treatment ($F(1,19) = 0.04$, *NS*), sex and genotype ($F(1,19) = 0.05$, *NS*), or sex and drug treatment x genotype ($F(2,38) = 0.14$, *NS*), we collapsed analyses across sex. TCB-2 produced a significant main effect on locomotor activity ($F(2,38) = 11.37$, $p < 0.001$), increasing total distance traveled across all 10 min time blocks and at both 0.3 and 1 mg/kg doses relative to vehicle. There was also a significant main effect of $mGlu_5$ genotype on distance traveled ($F(1,21) = 14.40$, $p < 0.01$) with $mGlu_5$-KO mice traveling significantly greater distances than WT mice across the final 50 min of the session according to post-hoc analyses.

Importantly, we detected a significant drug treatment x genotype interaction ($F(2,42) = 4.71$, $p < 0.05$), indicating a significant increase in sensitivity to the locomotor activating effects of TCB-2 in $mGlu_5$-KO mice (Fig. 3), as well as a significant drug treatment x genotype x block interaction ($F(10,210) = 2.34$, $p < 0.05$). Symbols in Figure 3 indicate the levels of significance for the drug treatment x genotype interaction detected by post hoc analyses for each 10 min block.

4. Discussion

Metabotropic glutamate receptors are of particular interest as possible targets for treatment of psychosis due to their ability to modulate glutamatergic signaling (Conn and Jones, 2009; Gupta et al., 2005; Krivoy et al., 2008). However, mounting evidence indicates that altering signaling at mGluRs can also affect neuromodulatory signaling that may be relevant to symptoms associated with psychosis (Benneyworth et al., 2007; Gewirtz and Marek, 2000; González-Maeso et al., 2008; Kłodzinska et al., 2002; Marek and Zhang, 2008; Moreno et al., 2011; Smolders et al., 2008; Stachowicz et al., 2007). $mGlu_{2/3}$ receptors have been closely associated with hallucinogen effects via functional interactions with

5-HT_{2A} (Benneyworth et al., 2008, 2007; Carbonaro et al., 2015; Gewirtz and Marek, 2000; González-Maeso et al., 2008; Kłodzinska et al., 2002; Moreno et al., 2013, 2011), but little work has been done to investigate possible functional interactions between 5-HT_{2A} and other mGluRs. Previous work in our lab indicated a baseline increase in locomotor activity in $mGlu_5$-KO mice that was sensitive to blockade by the highly selective 5-HT_{2A} inverse agonist M100907, as well as a hypersensitivity to the locomotor activating effects of the hallucinogenic amphetamine DOM (Halberstadt et al., 2011a). Here, we expanded on these findings to show that the hypersensitivity of $mGlu_5$-KO mice to the behavioral effect of DOM extends to another 5-HT_{2A}-mediated behavior, the head-twitch response, and that 5-HT_{2A} activation alone is sufficient for this hypersensitivity since it also exists for HTRs induced by 25CN-NBOH, a selective agonist at 5-HT_{2A}. We also detected baseline differences in HTR counts between $mGlu_5$-WT and KO mice when treated with vehicle, but the significance of this effect was not consistent across experiments. Interestingly, we showed that this hypersensitivity to the effects of hallucinogens does not result from a compensatory increase in 5-HT_{2A} receptor expression, as no differences in *htr2a* mRNA were detected between WT and KO mice in qPCR. To probe the relative contributions of canonical downstream signaling cascades at 5-HT_{2A} to the hypersensitivity of $mGlu_5$-KO mice to hallucinogen effects, we recapitulated the findings of our previous locomotor experiments using the 5-HT_{2A} agonist TCB-2, which is highly selective for activating PLC-dependant signaling pathways relative to PLA_2 (McLean et al., 2006), improving our molecular understanding of these effects. Taken together, these findings suggest that the behavioral effects of hallucinogens are modulated by the presence or absence of the $mGlu_5$ receptor, and that signaling via PLC-dependent pathways is particularly pertinent to this interaction.

Although previous experiments identified the 5-HT_{2A} receptor as the most likely candidate for mediating the locomotor hyperactivity induced by DOM in $mGlu_5$-KO mice, two caveats limited the conclusions that could be drawn from these studies. First, DOM is an agonist at both 5-HT_{2A} and 5-HT_{2C} receptors, with ~12-fold selectivity for 5-HT_{2A} over 5-HT_{2C} in mice . As such the dose of DOM used in those studies (0.5 mg/kg) has appreciable activity at 5-HT_{2C} receptors, which can influence locomotor

activity. Second, though the 5-HT_{2A} inverse agonist used to block the locomotor effects of DOM in $mGlu_5$-KO mice, M100907, is highly selective for 5-HT_{2A} over 5-HT_{2C} receptors, it nonetheless possesses moderate affinity for 5-HT_{2C} receptors. Since the dose of M100907 required to block the effects of DOM in those experiments was relatively high (1 mg/kg) compared to doses that are typically used to block other 5-HT_{2A}-mediated behaviors in mice (Benneyworth et al., 2005; Fantegrossi et al., 2006; Kehne et al., 1996; Winter et al., 2005), it is thus possible that activity at 5-HT_{2C} rather than 5-HT_{2A} was responsible for blocking the effect. 5-HT_{2C} receptor blockade typically increases locomotor activity, but at high doses, the 5-HT_{2C} inverse agonist SB206553 reduced locomotor activity in C57BL/6J mice (Browne et al., 2017). The capacity for blockade of 5-HT_{2C} to produce similar effects to blockade of 5-HT_{2A} in assays of locomotor activity therefore complicates interpretation of these findings.

To resolve this complication, we demonstrated that hypersensitivity to hallucinogen-induced behaviors in $mGlu_5$-KO mice extends to the HTR, a 5-HT_{2A}-mediated behavior in which 5-HT_{2A} and 5-HT_{2C} ligands consistently produce opposing effects, with 5-HT_{2C} agonists suppressing HTR and 5-HT_{2C} antagonists enhancing the behavior. The HTR is reliably induced by a broad range of hallucinogenic 5-HT_{2A} agonists (Fantegrossi et al., 2010, 2008, 2006, Halberstadt and Geyer, 2014, 2013b; Schreiber et al., 1995), correlates strongly with reported potencies of hallucinogens in humans (Halberstadt et al., unpublished data), and can also distinguish between hallucinogenic and non-hallucinogenic 5-HT_{2A} agonists (Egan et al., 1998; González-Maeso et al., 2007, 2003, Halberstadt and Geyer, 2013a, 2013b), supporting its use as a rodent behavioral proxy for human hallucinogen effects (Halberstadt and Geyer, 2013a, 2013b; Hanks and González-Maeso, 2013). M100907 suppresses the hallucinogen-induced HTR (Halberstadt and Geyer, 2014, 2013b; Schreiber et al., 1995; Vickers et al., 2001), while administration of the highly selective 5-HT_{2C} antagonist SB242084 augments the effects of hallucinogens (Fantegrossi et al., 2010). The hypersensitivity of $mGlu_5$-KO mice to the effect of DOM in the HTR assay is thus likely mediated by activity at 5-HT_{2A} rather than 5-HT_{2C} receptors, which further supports the role of 5-HT_{2A} in our previous studies on locomotor effects (Halberstadt et al., 2011a). The extension of the hypersensitivity to DOM-induced HTR to HTRs induced by the selective 5-HT_{2A} agonist 25CN-NBOH in

$mGlu_5$-KO mice lends additional support to the predominance of 5-HT_{2A} receptors in these effects. Although initial reports indicated > 100-fold selectivity for 5-HT_{2A} over 5-HT_{2C} with 25CN-NBOH (Hansen et al., 2014), studies in our lab indicated ~23-fold selectivity, and negligible affinity for non-5-HT_2 sites (Halberstadt et al., 2016). Nonetheless, 25CN-NBOH is roughly twice as selective for 5-HT_{2A} over 5-HT_{2C} compared to DOM, further validating the role of 5-HT_{2A} in the hallucinogen hypersensitivity of $mGlu_5$-KO mice.

The ability of subthreshold doses of TCB-2 to produce significant locomotor effects in $mGlu_5$-KO mice indicates a essential role for PLC-mediated signaling in the enhancement of hallucinogen-mediated behaviors in the absence of $mGlu_5$. Although this suggests that PLC-mediated signaling alone may be sufficient for the locomotor effects of 5-HT_{2A} agonists in mice lacking the $mGlu_5$ receptor, caution should be taken with this interpretation. TCB-2 is at least 22-fold selective for PLC-mediated signaling over PLA_2-mediated signaling (McLean et al., 2006), but the selectivity of TCB-2 against various non-canonical signaling pathways has not been assessed. Under certain conditions, for instance, 5-HT_{2A} agonists can stimulate G_i-mediated signaling that results in decreased cAMP, and the capacity for activation of this signaling pathway has been hypothesized to differentiate hallucinogenic from non-hallucinogenic 5-HT_{2A} agonists (González-Maeso et al., 2007). Furthermore, although TCB-2 minimally activates signaling via PLA_2, the relative degree of PLA_2- versus PLC-mediated signaling that is behaviorally appreciable is unclear, and TCB-2 may thus nonetheless produce effects via PLA_2 signaling. Indeed, the potency of various 5-HT_{2A} agonists at inducing the HTR does not seem to correlate with the proportion of PLC- to PLA_2-mediated signaling (Moya et al., 2007), and thus the roles of these pathways in hallucinogen effects remains opaque. Still, given that hallucinogenic and non-hallucinogenic 5-HT_{2A} agonists produce distinct transcriptomic fingerprints (González-Maeso et al., 2003), it is probable that functional selectivity for particular signaling pathways is crucial to understanding how hallucinogens produce their effects, and how interactions between $mGlu_5$ and 5-HT_{2A} receptors play into those effects. It has been previously reported that agonists and antagonists at $mGlu_2$ receptors can alter downstream effectors of agonists acting at 5-HT_{2A} receptors (Moreno et al., 2011). As such, future experiments should

aim to quantify relative PLC-mediated signaling between $mGlu_5$-KO and -WT mice to determine whether the absence of $mGlu_5$ affects signaling bias in a manner that could be relevant to the behavioral effects of hallucinogens.

There are a number of possible mechanisms that could be responsible for hypersensitivity to the behavioral effects of hallucinogens in $mGlu_5$-KO mice. As has been suggested for $mGlu_2$ and 5-HT_{2A} receptors (González-Maeso et al., 2008), one possibility is the existence of functional heterodimers formed by $mGlu_5$ and 5-HT_{2A} receptors. $mGlu_5$ and 5-HT_{2A} receptors are robustly expressed in similar regions including PFC, striatum, and hippocampus, and both can be expressed postsynaptically on perisomatic dendritic shafts (Cornea-Hébert et al., 1999; Miner et al., 2003; Morilak et al., 1993; Romano et al., 1995a; Shigemoto et al., 1993; Testa et al., 1994). $mGlu_5$ receptors have been shown to regulate the cellular response to DOI in slices of mouse frontal cortex, as administration of the $mGlu_5$ negative allosteric modulator MPEP attenuates the stimulation of IP hydrolysis by DOI (Molinaro et al., 2009). Notably, genetic knockout of $mGlu_2$ receptors abolishes the behavioral response to hallucinogens in mice (Moreno et al., 2011). Given the opposing effects on cellular activity by $mGlu_2$ and $mGlu_5$ receptors (Abe et al., 1992; Marek and Zhang, 2008; Mateo and Porter, 2007), it is feasible that opposite interactions between the receptors occur with putative $mGlu_5$-5-HT_{2A} heterodimers compared to those in putative $mGlu_2$-5-HT_{2A} heterodimers, in which case elimination of $mGlu_5$ receptors would enhance rather than abolish the behavioral response to hallucinogens.

Activity of DOI at presynaptic 5-HT_{2A} receptors has also been shown to inhibit evoked glutamate release from rat cerebrocortical synaptosomes, as the effect can be blocked by the 5-HT_{2A}/5-HT_{2C} antagonist ketanserin, but not the selective 5-HT_{2C} antagonist RS102221 (Wang et al., 2006). Pretreatment of synaptosomes with U73122, a PLC inhibitor, or Ro320432 or GF109203X, protein kinase C inhibitors, also blocked the inhibitory effect of DOI, suggesting it is mediated by the PLC pathway. $mGlu_5$ receptors can be expressed presynaptically on glutamatergic terminals, where they can function as autoreceptors to regulate glutamate release (Romano et al., 1995b; Shigemoto et al., 1993; Testa et al., 1994). If $mGlu_5$-5-HT_{2A} heterodimers exist presynaptically, elimination of $mGlu_5$ receptors may

compromise the function of these presynaptic 5-HT_{2A} receptors in much the same way that $mGlu_2$-KO mice show altered downstream signaling with DOI and LSD relative to WT mice (González-Maeso et al., 2008, 2007, 2003). As such, this inhibitory role for presynaptic 5-HT_{2A} receptors would not be served, increasing glutamate release. The result would be augmentation of behaviors mediated by excitation in PFC, which include locomotor hyperactivity and the head twitch response. Still, this inhibitory role for presynaptic 5-HT_{2A} has not been confirmed *in vivo*, and the overwhelming majority of 5-HT_{2A} receptors in PFC are expressed postsynaptically on pyramidal neurons (Cornea-Hébert et al., 1999), tempering the likelihood of this hypothesis. Further research is necessary to assess the proximity and coexpression of $mGlu_5$ and 5-HT_{2A} receptors, as well as their possible physical interaction.

Another possible set of mechanisms involves direct or indirect inhibition by $mGlu_5$ of 5-HT_{2A}-mediated glutamate release. Bath application of 5-HT in prefrontal cortex increases the frequency and amplitude of spontaneous excitatory postsynaptic potentials/currents (EPSP/Cs) in layer V pyramidal cells, and these effects can be blocked by selective antagonists at 5-HT_{2A} receptors (Aghajanian and Marek, 1997; Béïque et al., 2007; Lambe et al., 2000; Lambe and Aghajanian, 2007). These spontaneous currents can also be blocked entirely by antagonism of AMPA receptors, by the fast sodium channel blocker tetrodotoxin (TTX) which impedes synaptic transmission, or by perfusing slices with artificial cerbrospinal fluid lacking calcium and high in magnesium, all of which suggest that the mechanism for these EPSP/Cs is dependent on glutamate release (Aghajanian and Marek, 1997). Application of hallucinogenic 5-HT_{2A} agonists LSD or DOI to PFC slices followed by electrical stimulation of the slice also results in a fast EPSC followed by induction of a cortical "UP state" in which the probability of additional EPSCs is increased, as well as recurrent activity within the network (Aghajanian, 2009), further supporting this role for 5-HT_{2A}. These effects of 5-HT_{2A} activation on glutamate release and recurrent network activity result primarily from direct excitation of pyramidal neurons in deep layers of PFC, as restoration of 5-HT_{2A} exclusively in PFC pyramidal neurons is sufficient to restore these electrophysiological effects in slices obtained from 5-HT_{2A}-KO mice (Béïque et al., 2007).

Administration of hallucinogenic 5-HT_{2A} agonists thus creates conditions of hyperexcitation in PFC via increased glutamate release.

Several lines of evidence associate increased glutamate release in PFC with the HTR and locomotor hyperactivity. Infusion of DOI into mPFC in rats is sufficient to induce the HTR (Willins and Meltzer, 1997), and is also associated with local increases in glutamatergic signaling (Muschamp et al., 2004; Scruggs et al., 2003). A wide range of neurotransmitter receptor ligands that attenuate 5-HT-induced EPSCs in PFC can also suppress the HTR induced by hallucinogens; μ-opioid agonists, adenosine A_1 receptor agonists, AMPA receptor antagonists, $mGlu_4$ receptor PAMs, $mGlu_5$ receptor NAMs, and α_1-adrenergic receptor antagonists, for instance, have all been found to suppress the hallucinogen-induced HTR (Marek, 2017). Though few studies have specifically explored the role of PFC in the locomotor activating effects of phenylalkylamine hallucinogens, increased glutamate efflux in PFC has been associated with locomotor hyperactivity induced by psychomotor stimulants and dissociatives. Individual glutamate dialysate levels assessed via microdialysis positively correlated with locomotor activity induced by the partial NMDA antagonist MK-801 (Zuo et al., 2006), and selective potentiation of $mGlu_2$ receptors, which typically suppress glutamate release, attenuates both the hyperlocomotion induced by the partial NMDA antagonist phencyclidine (PCP) and the amplitude of the blood oxygenation level-dependent (BOLD) response to PCP in rats (Hackler et al., 2010). Intra-PFC infusion of l-allylglycine (LAG), an inhibitor of GABA synthesis, also resulted in locomotor hyperactivity in rats as well as an increase in expression of Fos, a marker for neuronal activation (Asinof and Paine, 2013). Locomotor hyperactivity induced by dissociatives and psychomotor stimulants such as PCP, cocaine, and amphetamine can be blocked by the highly selective 5-HT_{2A} inverse agonist M100907 (Maurel-Remy et al., 1995; McMahon and Cunningham, 2001; O'Neill et al., 1999), suggesting that altered levels of 5-HT_{2A} signaling can modulate the locomotor effects of these drugs. It is thus likely that the locomotor effects of 5-HT_{2A} agonists are mediated by changes in PFC activity in rodents. Similarly, changes in PFC activity in humans have also been associated with hallucinogen effects. The magnitude of subjective effects of psilocybin correlate with occupancy of 5-HT_{2A} receptors in PFC, and psilocybin

increases metabolic activity in PFC in human PET imaging studies (Vollenweider et al., 1997). fMRI studies also demonstrate changes in activity in PFC after administration of both psilocybin and LSD, though the direction of these effects is unclear due to technical inconsistency (Carhart-Harris et al., 2017, 2016; Lewis et al., 2017). Together, these data support a significant role for glutamate signaling in PFC in the behavioral and perceptual effects of hallucinogens across species. If the effects of $mGlu_5$ ablation on these behavioral measures in mice is dependent on changes in PFC glutamatergic signaling, it is thus possible that $mGlu_5$ may play a general role in modulating hallucinogen effects in rodents and humans alike.

Though $mGlu_5$ receptors couple primarily to G_q and result in depolarization via downstream increases in intracellular calcium, various studies have demonstrated a role for $mGlu_5$ receptors in preventing excessive, potentially toxic excitation through direct and indirect inhibitory mechanisms. For instance, tonic elevation of extracellular glutamate or recent activation of group I mGluRs has been shown to temporarily switch subsequent activation of $mGlu_{1/5}$ receptors from excitatory to inhibitory, likely through a change in preferred G-protein coupling from the excitatory G_q to the inhibitory G_i (Herrero et al., 1998). Later studies confirmed the $mGlu_5$ receptor to be specifically responsible for these effects seen using general agonists for group I mGluRs (Bruno et al., 2001). Consequently, 5-HT_{2A} agonist administration may produce conditions under which $mGlu_5$ serves a presynaptic inhibitory function to curb further glutamate release following 5-HT_{2A}-mediated elevation of extracellular glutamate. In the absence of $mGlu_5$, this mechanism would not exist to suppress additional glutamate release, permitting more robust elevations in 5-HT_{2A}-mediated glutamate release and presumably increasing the potency of 5-HT_{2A} agonists in eliciting associated behaviors.

In addition to their presynaptic role in suppressing excess glutamate release, $mGlu_5$ receptors also play a postsynaptic inhibitory role through release of an inhibitory retrograde signal. Activation of group I mGluRs stimulates the biosynthesis of the endocannabinoid 2-arachidonoylglycerol (2-AG) in primary cultures of corticostriatal and hippocampal slices from rat brains (Jung et al., 2005). Biosynthesis of 2-AG depends on activation of $mGlu_5$ as well as diacylglycerol lipase (DGL), which physically interact via

Homer-1b and Homer-2 scaffolding proteins, and is catalyzed by PLC (Gregg et al., 2012; Jung et al., 2007). 2-AG acts a retrograde inhibitory neurotransmitter by binding to presynaptic CB1 receptors, which couple preferentially to G_i (Lafourcade et al., 2007; Wilson and Nicoll, 2001). Activation of these DGL-associated postsynaptic $mGlu_5$ receptors thus plays a role in the inhibition of glutamate release in the presence of high levels of synaptic glutamate through endocannabinoid synthesis. If $mGlu_5$ is not present, this signaling mechanism cannot function, and the impeded synthesis of 2-AG would permit increased glutamate release following 5-HT_{2A} agonist administration, since the retrograde signal responsible for suppressing excessive release is no longer present. This would in turn lead to hypersensitivity to hallucinogen-associated behaviors that depend on the release of glutamate in PFC.

A simpler, circuit level explanation for these effects could involve projection of $mGlu_5$-expressing inhibitory interneurons onto 5-HT_{2A}-expressing pyramidal cells in PFC. $mGlu_5$ is ubiquitously expressed across various cell types, though the relative expression on inhibitory versus excitatory neurons is not well quantified (Romano et al., 1995b; Shigemoto et al., 1993; Testa et al., 1994). It is thus possible that, as described above, $mGlu_5$ receptor activation typically serves a role in exciting inhibitory interneurons to provide tonic feed-forward inhibition onto principal neurons in PFC that express 5-HT_{2A}. Consequently, in the absence of $mGlu_5$ receptors, inhibitory tone onto 5-HT_{2A} expressing principal neurons would be reduced, allowing increased excitation upon 5-HT_{2A} activation, and increasing sensitivity to ensuing behavioral effects. Given the predominate expression of $mGlu_5$ receptors on postsynaptic elements (Shigemoto et al., 1993), these latter two hypotheses are most probable, but presynaptic mechanisms could also contribute. It should be noted that because qPCR quantifies mRNA rather than surface protein expression, it is also possible that while the absolute expression of *htr2a* mRNA remains consistent with $mGlu_5$-KO, compensatory increases in protein translation or surface expression of the 5-HT_{2A} receptor could occur in the absence of $mGlu_5$ receptors, resulting in hypersensitivity despite the absence of differences detected by qPCR. Future studies should explore this hypothesis by assessing surface protein expression through autoradiography with radiolabeled 5-HT_{2A} agonists, alongside these other neurochemical and circuit level hypotheses.

Due to recent interest in mGluRs as possible targets for antipsychotic treatment, improving our understanding of the interactions of this neurochemical system with other chemical signals is imperative to optimize efficacy and minimize untoward side effects. These studies are a strong step toward building a comprehensive model of interactions between serotonergic and glutamatergic signaling, particularly with respect to the effects of serotonergic hallucinogens. The results of these experiments suggest that $mGlu_5$ receptors may play a tonic role in mitigating hyperexcitation induced by 5-HT_{2A} agonists, and in their absence, the behavioral effects of 5-HT_{2A} agonists are more pronounced. It remains to be seen whether $mGlu_5$ receptors play a similar role in the effects of hallucinogens in humans, and additional experiments are necessary to better establish the mechanisms underlying this hypersensitivity. Nonetheless, these experiments provide valuable insight into the functional relationship between $mGlu_5$ and 5-HT_{2A} receptors by demonstrating the consistency of hypersensitivity of $mGlu_5$-KO mice across multiple behavioral effects of hallucinogens, indicating the sufficiency of 5-HT_{2A} activation in producing these effects, and identifying a key role for PLC-mediated signaling in the enhancement of hallucinogen effects.

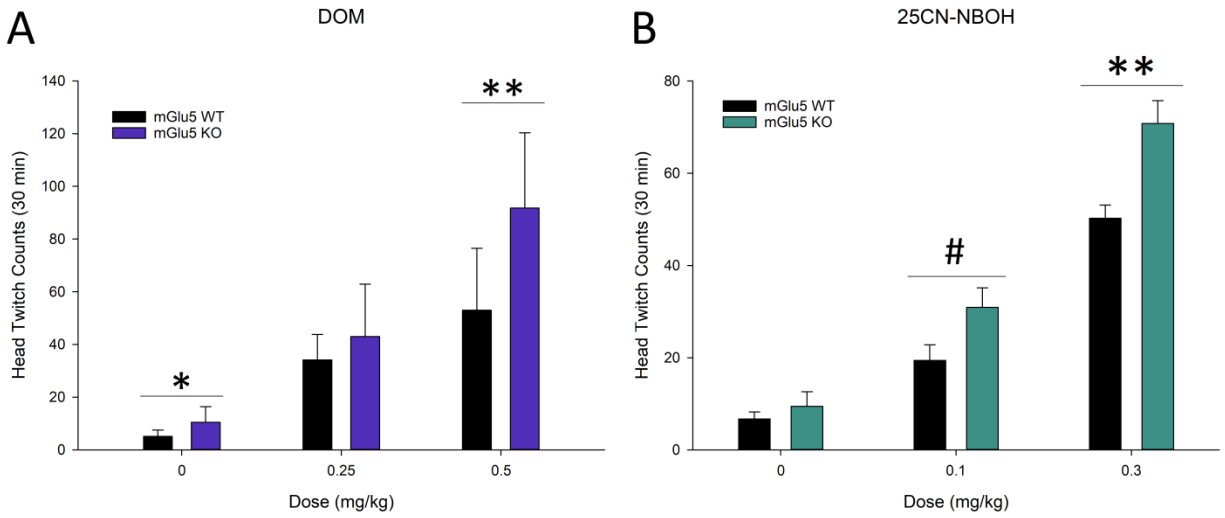

Figure 3.1. $mGlu_5$-KO mice are hypersensitive to the HTR induced by **(A)** the hallucinogen DOM and **(B)** the selective 5-HT_{2A} agonist 25CN-NBOH relative to mGlu5-WT mice. # $p < 0.1$, * $p < 0.05$, ** $p < 0.01$ in independent Student's t-tests comparing WT to KO mice at each dose.

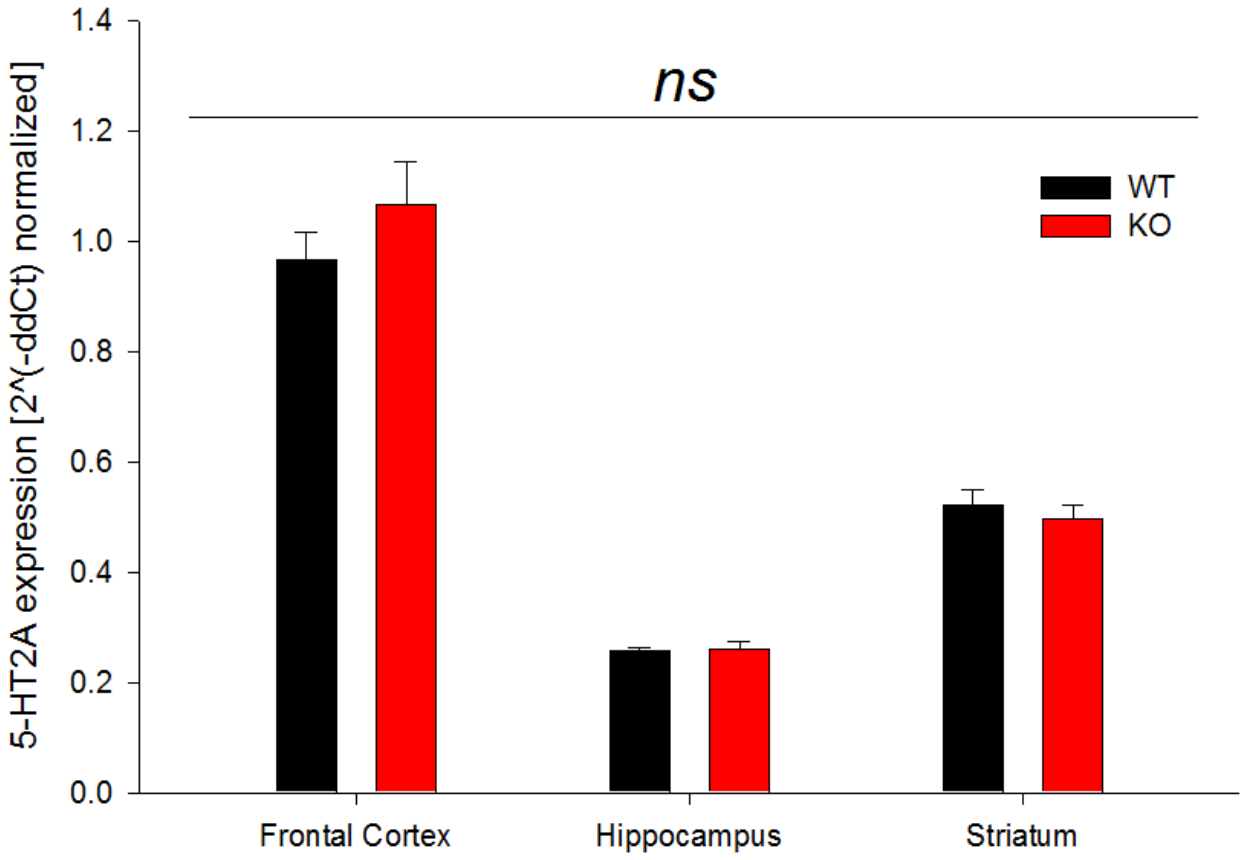

Figure 3.2. Real-time PCR results quantifying expression of *htr*2a mRNA, which encodes for the 5-HT_{2A} receptor, relative to the housekeeping gene *Actb* in $mGlu_5$-KO and -WT mice. *htr2a* expression does not differ between KO and WT mice in frontal cortex, hippocampus, or striatum ($p > 0.05$, Student's t-test).

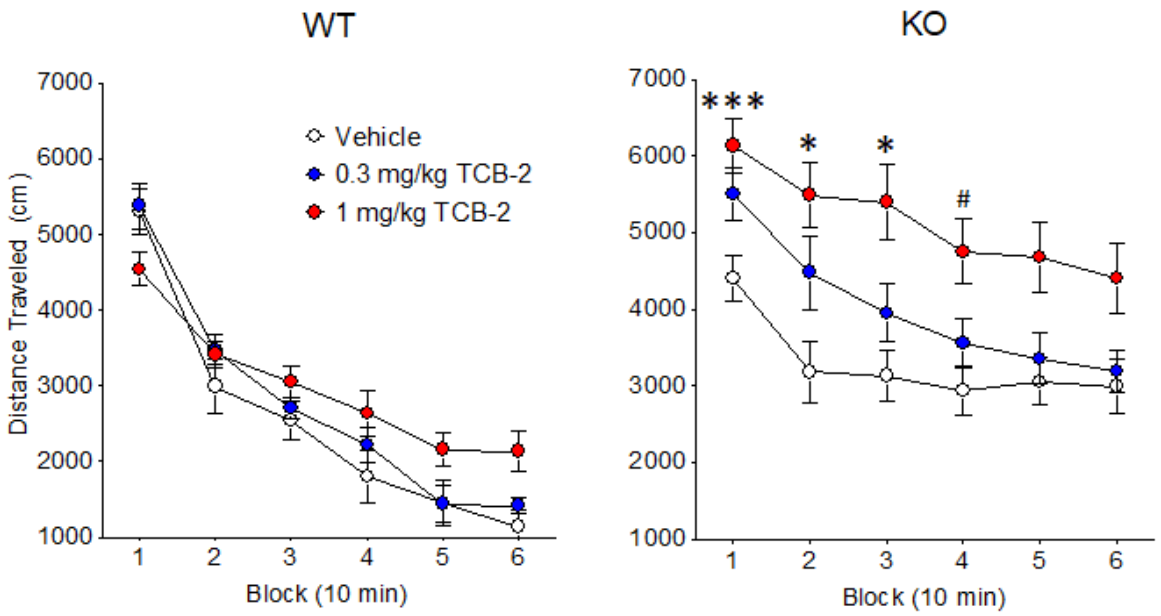

Figure 3.3. Effects of the PLC-selective 5-HT_{2A} agonist TCB-2 on locomotor activity in $mGlu_5$-WT and -KO mice. TCB-2 increases locomotor activity in KO mice at doses that do not affect locomotor activity in WT mice. # $p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to vehicle within genotype (Dunnett's test).

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CHAPTER 4

CONSISTENT ALTERATIONS OF INTERVAL TIMING IN MICE BY 5-HT_{2A} RECEPTOR AGONISTS AND INVERSE AGONISTS

1. Introduction

The perception of time is essential for survival and is necessary for organizing behaviors, determining relationships between sensory stimuli, planning and anticipating events, and myriad other cognitive and behavioral functions. Several distinct types of temporal processing occur over multiple timescales, ranging from sensorimotor timing on the scale of milliseconds to circadian timing occurring over hours or days (Buhusi and Meck, 2005). Interval timing occurs between those scales and refers to the perception of durations in the seconds to minutes range. Given the dependence of behavior and executive function on the ability to time intervals, impairments of interval timing are thought to contribute to the symptoms and deficits associated with a variety of neuropsychiatric disorders. Timing abnormalities have been demonstrated in patients suffering from attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), obsessive-compulsive disorder (OCD), and schizophrenia (Allman et al., 2011; Allman and Meck, 2012; Carroll et al., 2009, 2008; Davalos et al., 2011; Gu et al., 2011; Pollak et al., 2009; Rammsayer, 1990; Szelag et al., 2004; Toplak et al., 2006; Volz et al., 2001). In schizophrenia, impaired temporal processing constitutes a core deficit and is thought to contribute to symptoms such as cognitive dysfunction, hallucinations, delusions, and inappropriate behavior by interfering with the assessment of causal relationships between stimuli (Carroll et al., 2008; Ward et al., 2012). The severity of timing deficits in schizophrenia correlates with the severity of the first-rank symptoms of the disorder (Waters and Jablensky, 2009); importantly, a recent meta-analysis confirmed that the timing deficits in schizophrenic patients are independent of impairments in attention, memory, and cognition (Ciullo et al., 2016). Understanding the pathophysiology underlying this core deficit of schizophrenia may thus prove critical in treating the debilitating positive symptoms associated with schizophrenia.

Serotonergic hallucinogens have been used as models of the positive symptoms of schizophrenia (Halberstadt and Geyer, 2013a). Similar to schizophrenia, serotonergic hallucinogens can alter the subjective experience of time (Halberstadt, 2015). Anecdotal reports of intoxication with hallucinogens

such as lysergic acid diethylamide (LSD) and mescaline often include accounts of dilation or contraction of subjective time, or feelings of timelessness (Beringer, 1927; Deshon et al., 1952; Hoch et al., 1952; Šerko, 1913). Recent controlled studies with psilocybin, the active ingredient of "magic mushrooms," corroborated these reports, demonstrating disruption of both the accuracy and precision of timing in human volunteers (Wackermann et al., 2008; Wittmann et al., 2007). Most of the characteristic effects of hallucinogens are mediated by the 5-HT_{2A} receptor (Halberstadt, 2015; Nichols, 2004; Preller et al., 2017; Vollenweider et al., 1998), but the pharmacological and circuit mechanisms of many perceptual effects of hallucinogens have yet to be investigated due to the technical limitations of human research and the difficulty of probing subjective perceptual experience in animal models. As such, a complete understanding of the neural mechanisms responsible for hallucinogen effects on interval timing requires a translatable timing task that can be similarly conducted in both humans and rodents and permits pharmacological and circuit-level manipulations.

Recently, a mouse version of the discrete-trials interval timing task (DTT), a retrospective timing task involving differential responding based on the duration of an elapsed interval, was developed by Halberstadt et al. (2016). In the mouse DTT, the hallucinogen and 5-$HT_{2A/2C}$ agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) increased both T_{50}, an accuracy measure corresponding to the point at which the subject is equally likely to respond to a duration as being long or short, and the difference limen (DL), a precision measure reflecting the minimal detectable difference in stimulus magnitude. These effects were blocked by M100907, a highly selective inverse agonist at the 5-HT_{2A} receptor. SB242084, a selective 5-HT_{2C} receptor antagonist, failed to block DOI effects on T_{50} and DL, suggesting that the effect of DOI on timing is mediated by 5-HT_{2A} and not 5-HT_{2C} receptors. Halberstadt et al. also demonstrated that the selective 5-HT_{2A} agonist *N*-(2-hydroxybenzyl)-2,5-dimethoxy-4-cyanophenethylamine (25CN-NBOH) increased T_{50} and DL similarly to DOI (Halberstadt et al., 2016), supporting the sufficiency of 5-HT_{2A} activation for altering timing in the DTT.

Phenylalkylamine hallucinogens such as DOI and 25CN-NBOH act primarily via 5-HT_2 receptors. By contrast, ergoline and tryptamine hallucinogens such as LSD and psilocybin bind to most

5-HT receptors (Nichols, 2004), some of which have been shown to modulate timing. For instance, tryptamines have significant affinity for 5-HT_{1A} receptors (Blough et al., 2014; Mckenna et al., 1990; Rickli et al., 2016), which have been shown to contribute to the behavioral effects of tryptamine hallucinogens in both rodents and humans (Halberstadt and Geyer, 2011; Klein et al., 2018; Pokorny et al., 2016; Strassman, 1995). The 5-HT_{1A} agonist 8-OH-DPAT produces effects on interval timing in rats (Asgari et al., 2006; Body et al., 2002b; Chiang et al., 2000), raising the possibility that activity at 5-HT_{1A} receptors may influence effects of tryptamine hallucinogens on timing. Whether hallucinogens from multiple structural classes produce similar behavioral profiles in the mouse DTT and whether the 5-HT_{1A} receptor plays a role in the effects of tryptamine hallucinogens on timing remain to be established. Here, we administered psilocin acutely to mice trained on the DTT in order to assess whether the timing effects of DOI generalize to tryptamine hallucinogens, and then probed the role of both 5-HT_{1A} and 5-HT_{2A} receptors in the resulting effects. To our knowledge, this is the first experiment to assess the timing effects of a tryptamine hallucinogen in rodents.

The PFC is an important target for hallucinogens and is a potential locus for 5-HT_{2A}-mediated alterations of interval timing. Psilocybin increases metabolic activity in PFC in human PET imaging studies and the magnitude of the hallucinogenic state induced by psilocybin correlates with occupancy of 5-HT_{2A} receptors in PFC (Vollenweider et al., 1997). Human fMRI studies have also demonstrated changes in activity in PFC after administration of both psilocybin and LSD (Carhart-Harris et al., 2017, 2016; Lewis et al., 2017). Electrophysiological studies in rats show that DOI increases recurrent network activity in PFC and increases the frequency of excitatory post-synaptic potentials (EPSPs) in layer V pyramidal neurons by stimulating glutamate efflux both *in vitro* and *in vivo*, effects that are mediated by 5-HT_{2A} receptors (Aghajanian and Marek; Béïque et al., 2007; Lambe et al., 2000; Lambe and Aghajanian, 2007; Marek et al., 2006; Marek and Aghajanian, 1998). Direct infusion of DOI into mPFC is sufficient to induce the head-twitch response in rats (Willins and Meltzer, 1997), a 5-HT_{2A}-mediated rodent behavior often used as a behavioral proxy for hallucinogenic activity in humans (Halberstadt, 2015). Additionally, studies in both humans and rats indicate reductions in low frequency oscillatory

power and reductions in gamma-band synchrony in PFC with administration of DOI (Celada et al., 2008), psilocybin (Muthukumaraswamy et al., 2013), and 5-MeO-DMT (Riga et al., 2014). Since the PFC is integrally involved in both hallucinogen effects and interval timing, we tested whether infusion of DOI directly into the PFC of mice trained on the DTT can mimic the effects produced by systemic administration of hallucinogens.

2. Materials and methods

2.1. Subjects

Mice were housed in an AAALAC-approved animal facility at UCSD that meets all Federal and State requirements for laboratory animal care and treatment. Male C57BL/6J mice obtained from Jackson Labs (Bar Harbor, ME) were housed up to 4 per cage in a climate controlled room on a reversed light cycle (lights on at 1900h, lights off at 0700h). Mice were allowed to acclimate for 1 week after arrival. Water was provided *ad libitum* except during behavioral training and testing. All testing and training occurred between 1000 and 1800h and was conducted in accordance with the 'Principles of Laboratory Animal Care' NIH guidelines and approval of the UCSD animal care committee.

2.2. Drugs

Drugs used were 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI; Cayman Chemical Co., Ann Arbor, MI); psilocin (National Institute on Drug Abuse, Rockville, MD), (*R*)-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol (M100907; Hoechst Marion Roussel Inc., Kansas City, MO); α-phenyl-1-(2-phenylethyl)-4-piperidinemethanol (MDL 11,939; Tocris Bioscience, Ellisville, MO); AC90393 (ACADIA Pharmaceuticals Inc, San Diego, CA); 3-{2-[4-(4-fluorobenzoyl)piperidin-1-yl]ethyl}quinazoline-2,4(1*H*, 3*H*)-dione tartrate (Ketanserin; Research Biochemical International, Natick, MA); 2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine hydrochloride (25I-NBOMe; Cayman Chemical Co., Ann Arbor,

MI); and *N*-[2-[4-(2-methoxyphenyl-1-piperazinyl]ethyl]-*N*-(2-pyridyl)cyclo-hexanecarboxamide maleate (WAY100635; Sigma Chemical Co., St. Louis, MO). DOI, AC90393, and 25I-NBOMe were dissolved in isotonic saline. Psilocin was dissolved in 0.05 mM tartaric acid and the pH was adjusted to 5-6 using 1N NaOH. M100907 and MDL 11,939 were dissolved in sterile water containing 5% Tween 80. WAY100,635 was dissolved in sterile water.

2.3. Discrete-trials timing task

The discrete-trials timing task was carried out as described in Halberstadt et al (2016). See Halberstadt et al (2016) for detailed descriptions of the apparatus and training procedures. A brief summary is provided below.

2.3.1. Apparatus

Mice were trained and tested in 2-lever operant chambers (21.6 x 17.8 x 12.7 cm, Med Associates Inc, St. Albans, VT). One wall in each chamber contained a food delivery magazine (Lafayette Instruments, Lafayette, IN), 2 motor-driven retractable levers 2.2 cm above the grid floor and spaced 10.4 cm apart, and an incandescent house-light near the ceiling. Two white LEDs mounted horizontally near the top of the magazine were used to signify reward availability and a green LED was mounted above each lever. A peristaltic pump (Lafayette Instruments, Lafayette, IN) delivered liquid reinforcement (Strawberry Nesquik® in non-fat milk, 20 µL) to a well in the floor of the magazine. Magazine entries were monitored using an infrared beam mounted horizontally inside the magazine. The chamber was located in a sound-attenuating box, ventilated by a fan that also provided a low level of background noise. Stimuli were controlled and responses were recorded using a SmartCtrl Package 8-In/16-Out interfacing with MED-PC for Windows (Med Associates Inc., St. Albans, VT) using custom programming.

2.3.2. Procedure

Mice were maintained at 85% of their free-feeding weight and trained 5–7 days a week at the same time each day during the dark (awake) phase of their light-dark cycle. Mice were first trained using a session in which reinforcement was dispensed every 15 s into the magazine, which was simultaneously illuminated. Magazine entries resulted in the light being extinguished until the next reinforcement was delivered. This training was repeated daily until there were ≥ 30 magazine entries in 10 min for 2 consecutive days. In the second phase, mice were trained to respond on either lever in order to obtain reinforcement under an FR1 schedule. This training phase was repeated daily until all mice made > 70 lever presses within a 30 min session on 2 consecutive days.

Mice were then trained on the discrete-trials task (Fig. 1A). At the beginning of each training session, the house light was extinguished and the magazine light and the green LEDs above the levers were illuminated. Trials were initiated when the mouse entered and exited the food magazine. At the beginning of each trial, the magazine light was extinguished, and then, following a variable duration (2.5, 5.0, 8.0, or 10.5 s, in pseudorandom order), the levers were presented for 10 s. Responding on lever A was reinforced if the interval between trial initiation and lever presentation was < 6.5, and responding on lever B was reinforced if the interval was > 6.5 s; the position of the two levers (left vs. right) was counterbalanced across subjects. An incorrect response or failure to respond within the 10 s response window (i.e. an omission) resulted in a time-out punishment where the levers were retracted, the house light was illuminated, and no aperture was responsive for 4 s. One-fifth of the trials were forced choice trials where only one lever was presented and was not retracted until a response was made. Sessions lasted until 30 min had elapsed or 120 trials had been completed. Training continued until all mice reached a criterion of 85% correct performance [correct/(correct + incorrect)*100] for the 2.5 and 10.5 s trials. Test sessions were identical to training sessions except a larger number of intervals (2.5, 3.4, 4.3, 5.2, 6.1, 6.9, 7.8, 8.7, 9.6, and 10.5 s) were used, there were no forced choice trials, and sessions lasted 45 min or 200 trials. Training was also conducted between experimental test sessions to maintain task performance at criterion levels.

2.4. Systemic pharmacology

2.4.1. Experimental Design

Group assignments were randomized, with a minimum of 7 days between experiments to avoid carry-over effects. Dose-response experiments were conducted using a between-subjects design. Antagonist blockade experiments were performed using a partial crossover within-subjects design where the animals always received the same pretreatment but the drug treatment varied between sessions (n = 5-10/group). Pretreatment times were: psilocin: 2 min; M100907: 30 min; WAY100,635: 25 min; MDL 11,939: 30 min; ketanserin: 10 min; AC90393: 20 min.

2.5. Infusion of compounds into mPFC

2.5.1. Surgical methods

Mice in a second cohort (n=52) were trained to perform the DTT; bilateral guide cannulae were implanted into the mPFC when performance reached criterion levels. Mice were anesthetized with ketamine (100 mg/kg IP) and xylazine (20 mg/kg IP) and placed in a stereotaxic apparatus. Burr holes were drilled in the skull and 23-gauge 8-mm guide cannulae were implanted bilaterally over the target region in the mPFC (1.7 mm anterior to bregma, ±0.9 mm lateral to bregma, 1.75 mm below the surface of the skull; 10° angle away from the midline). The cannulae were secured with a skull screw and dental cement and closed with a removable stylet. Meloxicam (2 mg/kg IP) was administered immediately following surgery to minimize discomfort.

2.5.2. Microinfusion procedure

After > 10 days of recovery, food deprivation was reinstated and DTT training resumed. Once retraining was complete, DOI (10 µg/µL) or a 3:1 solution of aCSF to isotonic saline was microinfused bilaterally into the mPFC through a 33-guage injection cannulae extending 1-mm below the tip of the guide cannulae. Injection cannulae were inserted and mice were acclimated for 1 min. Microinfusions (0.2 µL/side) were then made over 2 min using a 10-µL Hamilton microsyringe and an electronic syringe

pump, and then the injection cannulae was left in place for another 1 min. Animals were placed in the operant chamber for experimental testing 5 min after removal of the injection cannulae. This experiment was carried out using a pseudorandom, between-subjects design with *n*=10-20 mice/group. .

2.5.4. Histology

Following completion of experimental testing, mice were anesthetized and 2% Evan's Blue dye was infused bilaterally using the same protocol and instruments used for DOI infusion. Brains were removed immediately following removal of injectors and post-fixed in 4% paraformaldehyde (PFA) for at least 24 h. The brains were then placed in PBS containing 30% sucrose and stored at 4°C overnight. perfused transcardially with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PFA) in PBS. Fifty-micrometer coronal sections were cut on a freezing-sliding microtome, mounted on slides, and viewed under a microscope to confirm microinfusion locations. Mice were excluded from analysis if the microinfusion sites were located outside of the mPFC.

2.6. Data analysis

The percentage of responses made on the long duration lever (arbitrarily selected; %B responding) was analyzed by two- or three-way ANOVA. For dose-response experiments, pretreatment and/or treatment were between-subjects factors and stimulus duration was a repeated measure. For antagonist blockade experiments, pretreatment was a between-subjects factor and treatment and stimulus duration were repeated measures. Post-hoc analyses used Tukey's test or Dunnett's test and significance was determined by exceeding an α-level of 0.05.

A 2-parameter logistic function was used to fit the data: $\%B = \frac{1}{1+exp^{-\varepsilon(t-T_{50})}}$ where ε is the slope of the function and t is the stimulus duration. The difference limen was calculated as: $\frac{T_{75}-T_{25}}{2}$. The difference limen, slope (ε), T_{50}, goodness of fit (r^2), and number of trials completed were analyzed by one- or two-way ANOVA with pretreatment and/or treatment as between-subject factors for dose-responses

and local pharmacology, and with pretreatment as a between-subjects factor and treatment as a repeated measure for systemic blockade experiments. Post-hoc analyses were performed using Dunnett's test or Tukey's test. Subjects were excluded from analysis if they failed to complete at least 100 trials, if the logistic function could not be fit to the data from the subject, or if the cannulae placement was not located within the mPFC.

2.7. Head twitch response

The dose response for blockade of the 25I-NBOMe-induced head twitch response by AC90393 was conducted and analyzed using a head-mounted magnet and magnetometer coil as described previously (Halberstadt and Geyer, 2014, 2013b; Klein et al., 2018; Nichols et al., 2015). AC90393 (0, 0.1, 1, or 10 mg/kg) was administered 20 min prior to testing, and 25I-NBOMe (0.3 mg/kg SC) was administered immediately prior to testing.

2.8. In vitro functional assays

R-SAT™ (Receptor Selection and Amplification Technology) assays were performed as described previously (Vanover et al., 2006; Weiner et al., 2001), with the following modifications. Briefly, NIH-3T3 cells were grown in 96-well tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (Hyclone Laboratories, Logan, UT) and 1% penicillin/streptomycin/glutamine (Invitrogen, Carlsbad, CA) to 70% to 80% confluence. Cells were transfected for 12 to 16 h with plasmid DNAs using Superfect Reagent (QIAGEN, Valencia, CA) as per the manufacturer's protocols. R-SAT assays were generally performed with 1 to 50 ng/well of receptor and 20 ng/well of β-galactosidase plasmid DNA. After overnight transfection, media were replaced with Assay Medium (serum-free Dulbecco's modified Eagle's medium (DMEM) containing 30% Ultraculture synthetic supplement (BioWhittaker, Rockland, MD), 0.4% calf serum (Hyclone Laboratories), 1% penicillin/streptomycin/glutamine) and varying concentrations of drug. Cells were then grown in a humidified atmosphere with 5% ambient CO_2 for 5 days. Media were then removed from the plates, and

β-galactosidase activity was measured by the addition of o-nitrophenyl β-D-galactopyranoside (in phosphate-buffered saline with 5% NP-40 detergent). Alternatively, cells were grown in roller bottles in DMEM supplemented as described above and transfected in the roller bottles for 18 h with the relevant G protein-coupled receptor gene and the gene for β-galactosidase using Superfect Reagent as described above. After transfection, cells were trypsinized, harvested, and frozen at -135◦C until use. Aliquots of frozen cell batches were thawed and tested for response to reference agonists (5-carboxy tryptamine or 5-CT) and antagonists/inverse agonists (ritanserin) ensuring pharmacologically appropriate responses. To initiate an assay, cells were thawed rapidly and prepared in Assay Medium, and then they were added to 96-well microtiter plates containing either test compounds or reference ligands. Antagonist assays were conducted with 0.3 μM 5-CT added to all wells in addition to the test compound. After 5 days in culture, media were removed from the wells, and the cells were incubated at room temperature in 200 μl of phosphate-buffered saline, pH 7.4, with 3.5 mM o-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich, St. Louis, MO) and 0.5% Nonidet P-40 (Sigma-Aldrich). For either protocol, the resulting colorimetric reaction was measured in a spectrophotometric plate reader (Bio-Tek Instruments, Winooski, VT) at 420 nM. All data were analyzed using the computer program Excel Fit, and EC_{50}, IC_{50}, and IEC_{50} (inverse agonist EC_{50}) determinations were made using least-squares fit analysis with GraphPad Software Inc. (San Diego, CA) software. K_i values were derived from IC_{50} values using the method of Cheng and Prusoff (Cheng and Prusoff, 1973). pK_i and $pIEC_{50}$ values were calculated as the negative logarithm of the pK_i and $pIEC_{50}$ values.

3. Results

3.1. Effects of psilocin on DTT performance

To determine the effect of a tryptamine hallucinogen on interval timing in mice, we tested mice in the DTT after systemic administration of psilocin (0, 0.5, 1, or 2 mg/kg, s.c.). In addition to a main effect of stimulus interval on %B responding ($F(9,180) = 134.03$, $p < 0.0001$), there was a main effect of

psilocin treatment ($F(3,20) = 7.76$, $p = 0.0013$) as well as an interaction between interval and psilocin ($F(27,180) = 3.45$, $p < 0.0001$). Post-hoc analyses revealed significant effects of 1 mg/kg psilocin on %B responding relative to vehicle for the 2.5, 6.9, 7.8, 8.7, 9.6, and 10.5 s trials ($p < 0.05$, Dunnett's test), resulting in a rightward shift of the psychometric curve. Although administration of 0.5 mg/kg psilocin did not significantly affect %B responding at any stimulus interval, this dose produced an intermediate effect, both flattening and producing a rightward shift in the psychometric curve, further suggesting a dose-dependent effect (Fig. 1B).

At a dose of 1 mg/kg, psilocin significantly increased T_{50} from 7.2 ± 0.3 (mean ± SEM) to 9.2 ± 0.5 s ($p < 0.01$, Dunnett's test) and the difference limen from 1.132 ± 0.115 to 2.309 ± 0.617 ($p < 0.05$, Dunnett's test) relative to vehicle, suggesting that psilocin affected both accuracy and precision of responding on the DTT. Psilocin also significantly decreased the slope of the psychometric curve from 1.077 ± 0.138 to 0.607 ± 0.127 ($p < 0.05$, Dunnett's test) at 1 mg/kg relative to vehicle treatment. However, the effect of psilocin on the Weber Fraction (WF) did not reach significance at any dose tested ($F(3,20) = 1.85$, $p = 0.17$, *NS*). Treatment with psilocin significantly reduced the number of trials completed from 165.9 ± 6.1 to 114.2 ± 23.7 ($p < 0.05$, Dunnett's test), although sufficient trials were completed to accurately assess DTT performance.

3.2. Interaction between psilocin and M100907

To determine whether the 5-HT_{2A} receptor mediates the effect of psilocin in the DTT, we examined whether pretreatment with vehicle or the highly selective 5-HT_{2A} inverse agonist M100907 (0.03 mg/kg, s.c.) alters the response to psilocin (0.8 mg/kg, s.c.) in the DTT (Fig. 2A). With respect to %B responding, there was a significant interaction between M100907 and psilocin ($F(1,28) = 6.25$, $p < 0.05$) as well as a significant three-way interaction between M100907, psilocin, and stimulus interval ($F(9,252) = 2.32$, $p < 0.05$). Post-hoc analyses showed there were significant interactions between M100907 and psilocin for the 6.9 and 8.7 s stimulus intervals ($p < 0.05$, Tukey's test). These results suggest that pretreatment with M100907 significantly attenuated the effect of psilocin on %B responding,

particularly at longer intervals. As anticipated, there was a significant main effect of psilocin ($F(1,28) = 5.01$, $p < 0.05$) as well as a significant interaction between psilocin and stimulus interval ($F(9,252) = 3.38$, $p < 0.001$). Specific comparisons confirmed that 0.8 mg/kg psilocin significantly altered %B responding to the 7.8, 9.6, and 10.5 s stimuli ($p < 0.05$, 0.01, Tukey's test). Notably, there was not a main effect of M100907 pretreatment on %B responding ($F(1,28) = 2.27$, *NS*), but there was a significant interaction between stimulus interval and M100907 pretreatment ($F(9,252) = 3.39$, $p < 0.001$). Post-hoc comparisons also revealed significant effects of 0.03 mg/kg M100907 on %B responding for stimulus durations of 6.9 and 7.8 s ($p < 0.05$, 0.01, Tukey's test).

Consistent with the effects of other hallucinogens in previous studies, there was a main effect of psilocin on T_{50} ($F(1,28) = 4.58$, $p < 0.05$), with 0.8 mg/kg psilocin significantly increasing T_{50} relative to vehicle. There was also a significant interaction between M100907 and psilocin with respect to T_{50} ($F(1,28) = 5.28$, $p < 0.05$), suggesting M100907 antagonized the effect of psilocin on clock speed. Pretreatment with M100907 (0.03 mg/kg) did not produce a significant main effect on T_{50} in this experiment ($F(1,28) = 1.85$, *NS*). There were also main effects of psilocin on the difference limen and the slope of the curve, but no significant interactions between M100907 and psilocin were detected for these metrics or for the number of trials completed (data not shown).

3.3. Interaction between psilocin and WAY-100,635

Since tryptamine hallucinogens interact with 5-HT_{1A} receptors, we examined whether pretreatment with the highly selective 5-HT_{1A} antagonist WAY-100,635 alters the response to psilocin in the DTT (Fig. 2B). There were no significant interaction between WAY-100,635 and psilocin for %B responding ($F(1,24) = 0.23$, *NS*), T_{50} ($F(1,24) = 0.03$, *NS*), difference limen ($F(1,24) = 0.50$, *NS*), or the slope of the response curve ($F(1,24) = 0.77$, *NS*). The absence of an interaction between WAY100,635 and psilocin indicates that the 5-HT_{1A} receptor is not responsible for mediating the effects of psilocin on timing. Similar to other experiments, psilocin significantly altered %B responding ($F(1,24) = 5.61$, $p < 0.05$), difference limen ($F(1,24) = 13.97$, $p < 0.01$), and curve slope ($F(1,24) = 7.70$, $p < 0.05$), whereas

T_{50} was only marginally affected ($F(1,24) = 3.75$, $p = 0.0645$). There were main effects of WAY100,635 pretreatment on %B responding ($F(1,24) = 7.53$, $p < 0.05$), T_{50} ($F(1,24) = 7.48$, $p < 0.05$), and curve slope ($F(1,24) = 5.26$, $p < 0.05$).

3.4. Effect of 5-HT_{2A} *inverse agonists on DTT performance*

Because we detected an effect of M100907 on timing in EXP 3.2, we examined the dose-dependence of the response to M100907 (Fig. 3A). There was a main effect of M100907 on %B responding ($F(3,34) = 5.57$, $p < 0.01$), as well as a significant interaction between interval and M100907 ($F(27,306) = 1.96$, $p < 0.01$). Post hoc analyses demonstrated that the effect of M100907 was dose-dependent and was only significant at a dose of 0.1 mg/kg, which selectively affected the response to longer stimulus intervals ($p < 0.05$ and 0.01, Dunnett's test). Administration of 0.1 mg/kg M100907 increased T_{50} from 6.7 ± 0.2 s to 8.1 ± 0.3 s ($p < 0.01$, Dunnett's test). Although 0.1 mg/kg M100907 reduced the slope of the psychometric curve from 0.957 ± 0.018 to 0.863 ± 0.038 ($p < 0.05$, Dunnett's test), it did not significantly affect the difference limen or the Weber Fraction.

To determine whether other 5-HT_{2A} inverse agonists produce similar effects on DTT performance, we also tested the selective 5-HT_{2A} inverse agonist MDL11,939 (Fig. 3B) and the 5-$HT_{2A/2C}$ inverse agonist ketanserin in the DTT (Fig. 3C). Although there was only a trend level effect of MDL11,939 on %B responding ($F(2,29) = 2.66$, $p = 0.0867$), there was a significant interaction between MDL11,939 and stimulus interval ($F(18,261) = 2.23$, $p < 0.01$). Post hoc analyses indicated that 1 mg/kg MDL11,939 had significant effects on %B responding to the 2.5 ($p < 0.05$, Tukey's test) and 7.8 s ($p < 0.01$, Tukey's test) intervals. At a dose of 0.3 mg/kg, MDL11,939 also produced a significant effect on %B responding to the 7.8 s stimulus ($p < 0.05$, Tukey's test). There was also a main effect of MDL 11,939 on T_{50} ($F(2,29) = 3.39$, $p < 0.05$); T_{50} increased from 6.5 ± 0.1 to 6.9 ± 0.3 ($p > 0.1$, Tukey's test) and 7.2 ± 0.1 ($p < 0.05$, Tukey's test) after administration of MDL 11,939 at 0.3 and 1.0 mg/kg, respectively. No significant effects on slope, difference limen, or Weber Fraction were observed in mice treated with MDL11,939.

There was a main effect of ketanserin on %B responding ($F(3,33) = 6.16$, $p < 0.01$) and an interaction between ketanserin and stimulus interval ($F(27,297) = 3.45$, $p < 0.0001$). Post hoc analyses indicated that %B responding was significantly altered by the highest dose of ketanserin at longer intervals, with 1 mg/kg significantly altering the response to the 6.1, 6.9, 7.8, 8.7, 9.6, and 10.5 s trials ($p < 0.05$, Dunnett's test), whereas 0.3 mg/kg primarily affected the response to intervals near the middle of the stimulus range, significantly altering %B responding to the 6.1 ($p < 0.05$, Dunnett's test) and 6.9 s stimuli ($p < 0.01$, Dunnett's test). Ketanserin also produced significant main effects on T_{50} ($F(3,33) = 7.14$, $p < 0.001$), the slope of the curve ($F(3,33) = 5.14$, $p < 0.01$), the difference limen ($F(3,33) = 9.26$, $p < 0.001$), and the Weber Fraction ($F(3,33) = 3.19$, $p < 0.05$); post hoc analyses indicated that these measures were only significantly altered after administration of 1 mg/kg ketanserin ($p < 0.05$, Dunnett's test).

3.5. Effect of a 5-HT_{2A} *neutral antagonist on DTT performance*

Our DTT studies demonstrate that 5-HT_{2A} inverse agonists alter interval timing. The effects of 5-HT_{2A} inverse agonists on DTT performance could potentially reflect blockade of 5-HT_{2A} activation by 5-HT; alternatively, 5-HT_{2A} receptors are known to exhibit significant constitutive activity (Berg et al., 2005) and it is possible that 5-HT_{2A} inverse agonists alter DTT by dampening the constitutive activity of the receptor. To test the latter explanation, we examined whether DTT performance is affected by AC90393, a novel 5-HT_{2A} neutral antagonist. To verify that AC90393 acts as a neutral antagonist, Ketanserin, MDL 100,907 and AC90393 were profiled using the R-SAT functional assay for antagonist and inverse agonist activity against the human 5-HT_{2A} serotonin receptor (Table 1). Ketanserin and MDL 100907 were each approximately equally potent as antagonists and inverse agonists. In contrast, AC90393 was an effective 5-HT_{2A} antagonist, but inactive as an inverse agonist.

We then identified doses of AC90393 that are capable of interacting with 5-HT_{2A} receptors *in vivo*, by examining the ability of AC90393 to block the head twitch response induced by the hallucinogen 25I-NBOMe in C57 mice (Fig. 4B). There was a significant main effect of AC90393 ($F(3,20) = 47.26$, p

< 0.001). Pretreatment with AC90393 at doses of 1 mg/kg and 10 mg/kg significantly attenuated the response to 25I-NBOMe (0.3 mg/kg SC) ($p < 0.01$, Dunnett's test). Nonlinear regression demonstrated that AC90393 blocked the response to 25I-NBOMe with an ED_{50} = 2.2 mg/kg (95% CI 1.3–3.7 mg/kg).

For logistical reasons, different doses of AC90393 were tested in separate between-subjects experiments (Fig. 4A). In contrast to 5-HT_{2A} agonists and inverse agonists, the neutral antagonist AC90393 did not produce any significant effects on any measures of DTT performance, including %B responding, T_{50}, slope, difference limen, or Weber Fraction.

3.6. DTT performance after microinfusion of a hallucinogen directly into the mPFC

Given the significance of the PFC to both hallucinogen effects and interval timing, we examined whether DTT performance is affected by microinfusion of DOI directly into mPFC (Fig. 5). Infusion of DOI (10 µg/µl) into the mPFC bilaterally (0.2 µl/side) had a significant main effect on %B responding ($F(1,31) = 8.84$, $p < 0.01$), and there was a significant interaction between DOI treatment and interval ($F(9.279) = 3.88$, $p < 0.0001$). Post hoc analyses confirmed that %B responding was significantly altered in the 5.2, 6.9, 7.8, 8.7, and 9.6 s intervals ($p < 0.05$, Tukey's test) after infusion of DOI. Infusion of DOI into the mPFC also significantly increased T_{50} from 6.6 ± 0.1 to 7.2 ± 0.2 ($F(1,31) = 10.75$, $p < 0.01$), but did not significantly affect the difference limen, Weber Fraction, or the slope of the curve ($p > 0.05$, Tukey's test). In addition, DOI infusion reduced the number of trials completed ($F(1,31) = 8.23$, $p < 0.01$) from 190.3 ± 1.9 to 169.4 ± 6.9 for DOI treated mice.

4. Discussion

By training mice on a discrete-trials temporal discrimination task, we expanded on previous work investigating hallucinogen effects on interval timing in rodents. As was shown previously with the phenylalkylamine hallucinogen DOI and the selective 5-HT_{2A} agonist 25CN-NBOH (Halberstadt et al., 2016), we found that the tryptamine hallucinogen psilocin alters interval timing in mice, reducing %B

responding at long durations and altering both the accuracy and precision of duration judgments as reflected in increases in T_{50} and the difference limen, respectively. This is one of the first studies to assess the effect of a tryptamine hallucinogen on interval timing in rodents. As with DOI, the effect of psilocin on interval timing are dependent on 5-HT_{2A} receptor activation, as evidenced by the ability of M100907 to block the response to psilocin. Furthermore, although the 5-HT_{1A} receptor is targeted by psilocin (Mckenna et al., 1990) and is known to play a role in interval timing (Asgari et al., 2006; Body et al., 2002; Chiang et al., 2000), the present experiments nonetheless demonstrated that the 5-HT_{1A} receptor does not play a significant role in the effects of psilocin on timing. Lastly, we showed that infusion of a DOI directly into the PFC is sufficient to mimic the effects of systemic administration of hallucinogens on interval timing.

Because M100907 alone had a marginal effect on temporal discrimination that resembled the effects observed with hallucinogens, we also conducted dose-response experiments with other 5-HT_{2A} inverse agonists. In addition to M100907, the highly selective 5-HT_{2A} inverse agonist MDL11,939 and the 5-$HT_{2A/2C}$ inverse agonist ketanserin also produced hallucinogen-like effects on interval timing in the DTT. These effects include a reduction of %B responding at longer intervals and increases of T_{50} and the difference limen. In contrast, at doses sufficient to block induction of the head twitch response (a 5-HT_{2A}-mediated behavior), the neutral 5-HT_{2A} antagonist AC90393 did not affect performance on the DTT, suggesting that inverse agonists alter interval timing by reducing 5-HT_{2A} constitutive activity, as opposed to blocking receptor activation in response to 5-HT release. These findings indicate that constitutive activation of the 5-HT_{2A} receptor is required to preserve accurate and precise interval timing.

Though phenylalkylamine and tryptamine hallucinogens produce similar subjective effects in humans (Hoch et al., 1952; Shulgin and Shulgin, 1997, 1995), their distinct pharmacological profiles can result in different behavioral effects in rodent assays. For instance, phenylalkylamine hallucinogens reliably increase locomotor activity in mice via effects on 5-HT_{2A} receptors (Halberstadt et al., 2013), whereas tryptamine hallucinogens decrease locomotor activity via effects on 5-HT_{1A} receptors

(Halberstadt and Geyer, 2011). 5-HT_{2A} and 5-HT_{1A} receptors also exert countervailing effects on the head twitch response (Darmani et al., 1990; Schreiber et al., 1995). Although most of the subjective effects of hallucinogens, including psilocybin and LSD, can be blocked by co-administration of the 5-$HT_{2A/2C}$ antagonist ketanserin (Preller et al., 2017; Vollenweider et al., 1998), the effects of certain hallucinogens may be modulated by activity at 5-HT_{1A} receptors. For example, pretreatment with pindolol, a mixed 5-HT_{1A}/β-adrenergic antagonist, increases the magnitude of subject effects induced by the tryptamine *N*,*N*-dimethyltryptamine (DMT) in human volunteers (Strassman, 1995). Likewise, administration of the 5-HT_{1A} agonist buspirone has been shown to blunt the visual effects of psilocybin (Pokorny et al., 2016). On the other hand, numerous drug discrimination studies have shown that tryptamine and phenylalkylamine hallucinogens reliably produce cross-substitution in rats (Halberstadt, 2015), demonstrating that different structural classes of hallucinogens produce similar interoceptive cues in rodents. Nevertheless, because the 5-HT_{1A} receptor agonist 8-OH-DPAT has been shown to alter interval timing in rats (Chiang et al., 2000), it was surprising to find the selective 5-HT_{1A} antagonist WAY-100,635 did not modulate the effects of psilocin on timing. Since there was a significant effect of WAY100,635 on timing performance alone, it appears that while changes in activity at both receptors can alter timing, there is not significant functional crosstalk between 5-HT_{2A} and 5-HT_{1A} within timing circuitry.

Although the effects of tryptamine hallucinogens on interval timing had not been previously assessed in rodents, human studies indicate that multiple structural classes hallucinogens are capable of altering the perception of time. Anecdotally, both phenylalkylamine hallucinogens, such as mescaline and DOM (Hoch et al., 1952; Shulgin and Shulgin, 1995), and indoleamine hallucinogens, such as LSD and DMT (Aronson et al., 1959; Boardman et al., 1957; Heimann, 1994; Hoch et al., 1952; Shulgin and Shulgin, 1997), can induce subjective experiences of time dilation or contration, as well as feelings of timelessness. Controlled human studies with psilocybin, a pro-drug that is rapidly dephosphorylated into psilocin *in vivo* (Hasler et al., 1997), confirmed that it produces significant changes in time perception and timing-dependent behavior. Specifically, psilocybin impaired the ability of subjects to reproduce

intervals longer than 2.5 s, impaired their capacity to synchronize tapping to inter-beat intervals longer than 2 s, and caused the subjects to have a slower preferred tapping rate (Wackermann et al., 2008; Wittmann et al., 2007). The slowing of time in these human studies with psilocybin is reminiscent of the increased internal clock speed induced by DOI in rats when an immediate rather than a retrospective timing schedule is used (Body et al., 2013, 2006a, 2006b, 2003; Cheung et al., 2007).

Our studies recapitulate the aforementioned human findings by demonstrating decreases in the accuracy and precision of duration judgments, particularly at longer durations, in mice treated with psilocin and DOI. By contrast, however, our results indicate a rightward shift of the psychometric curve and an increase of T_{50} after administration of psilocin and DOI to mice, indicating a decrease in clock speed (i.e., a contraction or "speeding up" of time perception). These findings are consistent with previous studies using discrete-trials and other retrospective timing tasks in rats (Asgari et al., 2006; Hampson et al., 2010), as well as our previous DTT studies with systemic DOI and 25CN-NBOH in mice (Halberstadt et al., 2016). Notably, the effects of hallucinogens on timing in humans have not been assessed using a discrete-trials timing task similar to the design used in this study.

There are several factors related to the specific design of our DTT that may be responsible for the rightward shift of the timing curve. One property of interval timing is that temporal uncertainty scales linearly as the magnitude of the interval increases, which is known as the "scalar" property of timing (Gibbon, 1991, 1977; Gibbon et al., 1984). Our task relies on the judgment of intervals that are equidistant from the criterion interval regardless of whether they are shorter or longer than that criterion. Because temporal uncertainty increases linearly with the magnitude of the interval timed, uncertainty in control conditions is inherently higher for longer intervals than shorter ones. Consequently, hallucinogen administration, which putatively impairs timing uniformly across intervals, is more likely to result in incorrect responses for longer intervals than shorter ones, as the uncertainty in these responses is intrinsically higher.

Additionally, due to a shorter delay period between trial initiation and reward delivery on correct trials when the stimulus interval is shorter, it is possible that a stronger reward value is associated with the

short duration lever, as reward value decreases as delay between stimulus and reward increases (Fiorillo et al., 2008; Kobayashi and Schultz, 2008). As a result, when temporal information is uncertain, test subjects may be more inclined to respond on the short duration lever, resulting in incorrect responses only when the duration is long. Increasing uncertainty through hallucinogen administration would then magnify the disparity in responses by increasing the frequency of trials with insufficient temporal information. These hypotheses are supported by the fact that mice generally reached performance criteria for the short duration lever more rapidly during training (data not shown). This disparity in performance decrement between long and short durations results in an increase in T_{50} that may or may not reflect a change in clock speed, since the acquisition of more temporal information (i.e. the passage of more time) is necessary to prompt a decision to respond on the long duration lever.

Importantly, a similar outcome could result from a general loss of stimulus control at longer durations unrelated to timing, since stimulus control decreases as the delay between stimulus and reward increases. This would manifest as a general impairment of operant responding that is particularly robust at longer durations. Activation of 5-HT_{2A} receptors can disrupt the spatial tuning of pyramidal neurons in PFC during working memory tasks in rodents (Williams et al., 2002), and high doses of DOI have been shown to impair attention and vigilance in operant tasks (Nakamura and Kurasawa, 2000), suggesting that non-temporal processes associated with performance on the DTT could be impaired by these manipulations. However, since performance of rats on a light-intensity discrimination task modeled after the DTT to match for difficulty was not affected by doses of DOI sufficient to alter timing in the DTT (Asgari et al., 2006), it is unlikely that the putative timing effects of hallucinogens in the DTT result from a general deficit in the capacity for operant performance. Furthermore, if a loss of stimulus control was responsible for the effects seen at longer durations, we would expect to see an increase in the number of omissions (i.e. trials initiated but not completed) as the stimulus interval increased. The number of omissions was extremely low for all stimulus intervals tested and did not differ between short and long intervals (data not shown) suggesting that a loss of stimulus control is not responsible for the effects of hallucinogens on performance in the DTT.

The striatal beat frequency (SBF) model of interval timing, posited by Meck et al., is a scalar-expectancy theory-based model of neural substrates that could comprise a central timing circuit. The model posits that coincident input from pyramidal PFC projections to dorsal striatal medium spiny neurons (MSNs) constitutes "counts" to keep track of ongoing intervals in the seconds to minutes range. This coincident input occurs as a result of constructive interference between cortical ensembles that intrinsically oscillate at different frequencies (Buhusi and Meck, 2005; Coull et al., 2011; Gibbon et al., 1984; Matell et al., 2003; Matell and Meck, 2004; Meck et al., 2008). Subsets of cortical neurons projecting to different MSNs thus interfere at regular intervals that vary depending on the specific cortical ensemble, allowing specific MSNs to encode distinct temporal *receptive fields* (Fig. 6A) (Chiba et al., 2008; Gu et al., 2015; Matell et al., 2003; Matell and Meck, 2004). MSN activity has been shown to predict responding in an interval bisection timing task, suggesting that the activity of these neurons is strongly tied to interval timing (Chiba et al., 2008; Soares et al., 2016). However, infusion of DOI into rat DS does not alter timing performance (Body et al., 2006a), indicating that hallucinogen effects on interval timing are mediated at other central sites.

The collective results of our experiments are consistent with the hypothesis that hallucinogens affect interval timing by altering patterns of activity in PFC, thereby disrupting the integrity of concurrent output to DS. First, the alteration of DTT performance resulting from infusion of DOI directly into PFC supports the sufficiency of hallucinogen action in prefrontal cortex to affect interval timing, and the effects of infusion into PFC mimicked those of systemic DOI administration on most measures (Halberstadt et al., 2016). Although DOI in PFC did not recapitulate the effects of systemic DOI on precision measures in the DTT, this was likely a result of incomplete diffusion of the drug throughout the entirety of mPFC, affecting only certain components of the relevant timing circuitry within this region. Although our infusions were targeted toward infralimbic-prelimbic region of PFC, other areas such as anterior cingulate cortex, have been implicated in hallucinogen effects (Carhart-Harris et al., 2012; Carhart-Harris et al., 2016; Gresch et al., 2007; Lewis et al., 2017; Vollenweider et al., 1997; Wood et al., 2012), send dense projections to MSNs in DS (Gabbott et al., 2005; Haber, 2016), and may also

contribute to effects on timing (Pouthas et al., 2005; Stevens et al., 2007). Further experiments are needed to confirm the necessity of PFC to the timing effects of hallucinogens and to determine whether other neural structures contribute to the effects of hallucinogens on interval timing.

The capacity for both agonists and inverse agonists at the 5-HT_{2A} receptor to produce similar effects on timing further supports this hypothesis, as it demonstrates consistent effects of two manipulations known to similarly affect oscillatory synchrony in PFC. Studies in anesthetized rats show that reductions in oscillatory power in PFC via stimulation of 5-HT release from the dorsal raphe nucleus result from changes in the activity of fast-spiking interneurons that are dependent on activation of 5-HT_{2A} (Puig et al., 2010). Consistent with these findings, DOI reduced low frequency oscillations in anesthetized rats based on local field potential recordings collected from PFC (Celada et al., 2008). The 5-HT_{2A}/5-HT_{2C} inverse agonist ritanserin, which significantly reduced inositol triphosphate production below baseline in HEK 293 cells expressing a constituitively active mutant form of the 5-HT_{2A} receptor (Muntasir et al., 2006), also reduced the power of slow wave (< 2 Hz) oscillations in PFC (Puig et al., 2010). The highly selective 5-HT_{2C} antagonist SB-242084, however, had no effect on the power of these oscillations, suggesting that inverse agonist activity at the 5-HT_{2A} receptor is responsible for these effects (Puig et al., 2010). Notably, SB-242084 produced distinct effects in the DTT from the 5-HT_{2A} agonists and inverse agonists tested here and previously, shifting the psychometric curve to the left and reducing T_{50} with no significant effect on measures of precision (Halberstadt et al., 2016). Since SB-242084 does not affect the power of oscillations in PFC, the effects of SB-242084 on timing likely correspond to a unique mechanism of timing alteration mediated elsewhere.

It is also of note that the neutral 5-HT_{2A} antagonist AC90393 did not alter timing in the DTT at doses that produced comparable blockade of the hallucinogen-induced head twitch response to doses of M100907 used in these studies (Halberstadt and Geyer, 2014). This observation further supports the notion that maintenance of normal patterns of activity in cells expressing 5-HT_{2A} receptors is integral to accurate and precise timing behavior, since, in contrast to agonists and inverse agonists, a neutral antagonist does not significantly affect the activity of these cells under basal conditions. Though previous

studies did not indicate significant effects of M100907 on timing in the DTT in rats, these experiments were not full dose-responses but rather blockade experiments, and as such, the dose of M100907 was likely selected to minimize independent effects (Asgari et al., 2006; Body et al., 2006a, 2006b).

A population coding theory built off of the SBF posited by Gu et al. (2015) suggests that oscillatory multiplexing, or the simultaneous conveyance of domain-specific information within distinct frequency bands, allows projections from PFC to DS to act as the nexus of both temporal and working memory information transfer (Gu et al., 2015). In this theory, temporal information is primarily carried in signals in the delta, or low frequency, band while faster oscillations carry information about the item and its relation to other items, including temporal order. Indeed, low frequency oscillations play a demonstrated role across a variety of timing processes in both humans and animals, including rhythmic anticipation (Stefanics et al., 2010), temporal offset of multimodal sensory information (Kösem et al., 2014), and attention to stimuli presented at regular intervals (Lakatos et al., 2008). In addition, low frequency oscillations can determine the momentary power of higher frequency (e.g. gamma) oscillations via hierarchical cross-frequency coupling (Lakatos et al., 2008, 2005). Gamma band oscillations have been associated with performance on beat processing and rhythmic movement-based tasks (Fujioka et al., 2009) and thus may also play a role in the conveyance of temporal information, either as an independent information stream or as a hub for integrating multiple streams of lower frequency input.

Interestingly, while only 5-HT_{2A} receptors were implicated in the reduction of slow wave oscillatory power in the aforementioned study, the power of gamma band oscillations was reduced by ritanserin or WAY100,635, indicating that 5-HT_{1A} blockade can also reduce oscillatory power in PFC (Puig et al., 2010). Since our findings indicate timing effects of WAY100,635 that resemble but do not interact with those of psilocin, it is possible that disruption of either gamma or delta oscillations can impact timing, either via parallel information transfer or through integration of multiple information streams influencing behavior via the gamma band, one of which is driven by delta band temporal information. Notably, manipulation of 5-HT_{2A} signaling can also impair working memory in a non-monotonic fashion, with both inverse agonists and high doses of agonists producing similar effects

(Williams et al., 2002), possibly via similar structures to those involved in interval timing (Gu et al., 2015). Given that balanced activation of 5-HT_{2A} is necessary for stable synchronization of slow wave oscillations in PFC and that these waves are hypothesized to carry temporal information via projections from PFC to DS, our results are consistent with a role for altered PFC oscillatory activity in the timing effects of hallucinogens (Fig. 6B). Still, further experiments are needed to confirm these electrophysiological mechanisms.

Interval timing is an essential function for appropriate behavior and cognition. Consequently, disruption of interval timing across a variety of psychiatric illnesses can be extremely debilitating, and can potentially lead to the emergence of even more pronounced cognitive and perceptual symptoms. By better establishing the pharmacological and anatomical characteristics of the circuitry relevant to hallucinogen effects on interval timing, we can identify optimal targets for treatment, improving the lives of those suffering from symptoms associated with conditions ranging from Parkinson's Disease to schizophrenia.

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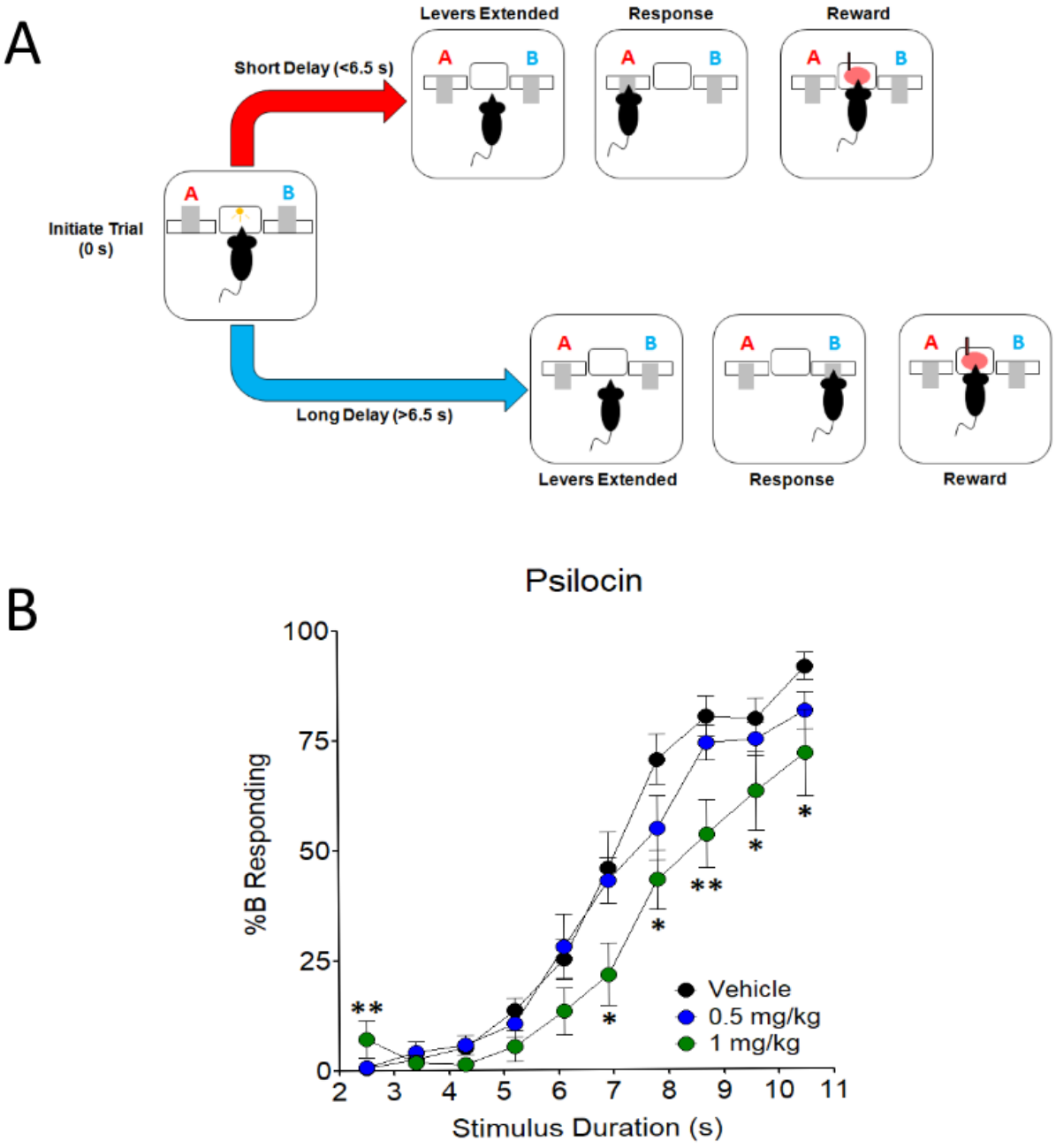

Figure 4.1. **(A)** Design for the discrete-trials task. **(B)** Effects of psilocin on temporal discrimination. The data shown (group means ± SEM) represent the proportional choice of lever B as a function of stimulus duration (%B responding). *$p < 0.05$, **$p < 0.01$ compared to vehicle (Dunnett's test).

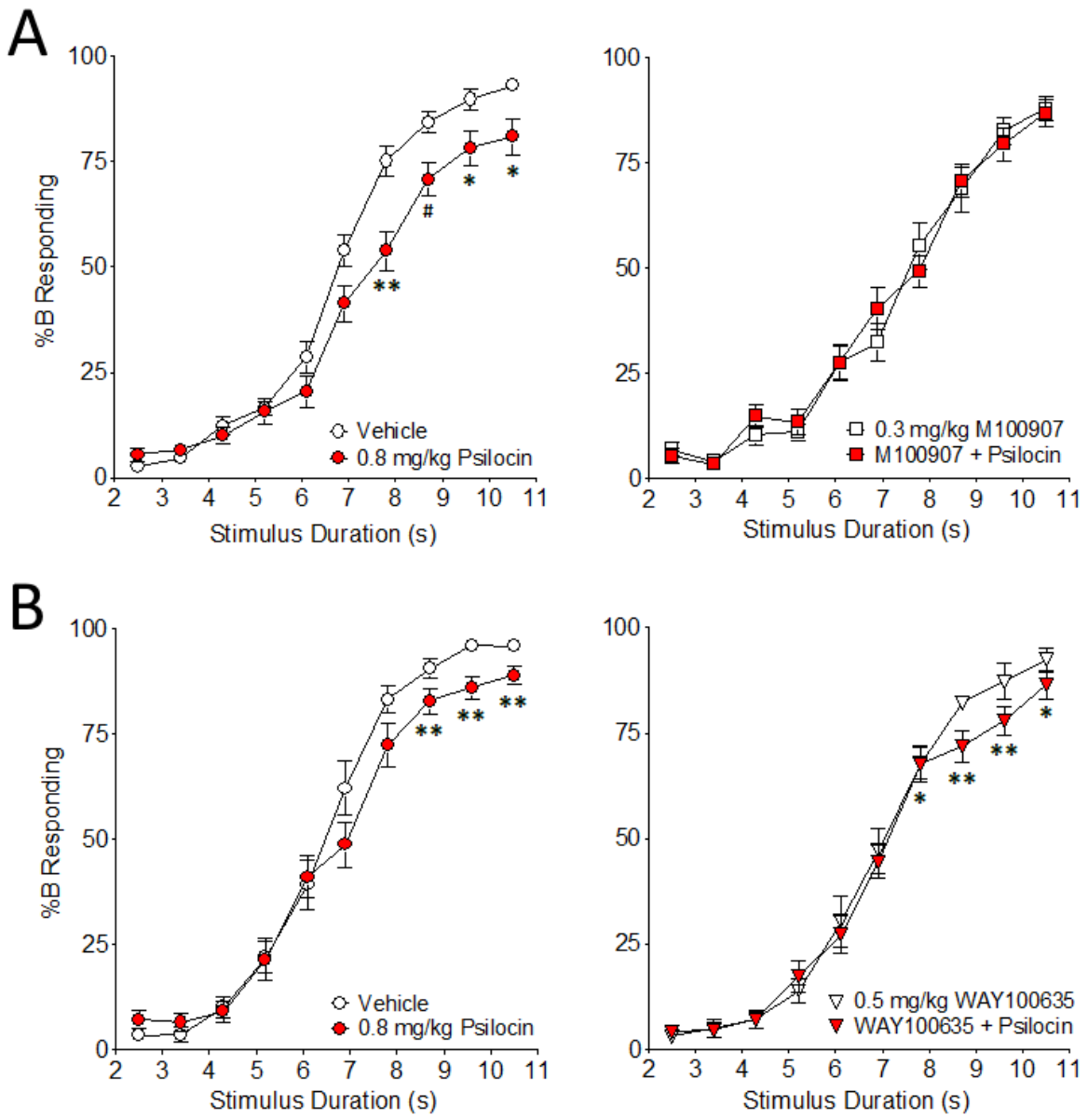

Figure 4.2. Effect of pretreatment with **(A)** 0.03 mg/kg M100907 or **(B)** 0.5 mg/kg WAY100,635 on the response to 0.8 mg/kg psilocin in the discrete-trials temporal discrimination task. Mice pretreated with vehicle are displayed in the left panel and mice pretreated with antagonist are displayed in the right panel to facilitate comparison of psilocin effects in each condition. The data shown (group means ± SEM) represent the proportional choice of lever B as a function of stimulus duration (%B responding). # $p < 0.1$, *$p < 0.05$, **$p < 0.01$ compared to vehicle + vehicle group (Dunnett's test).

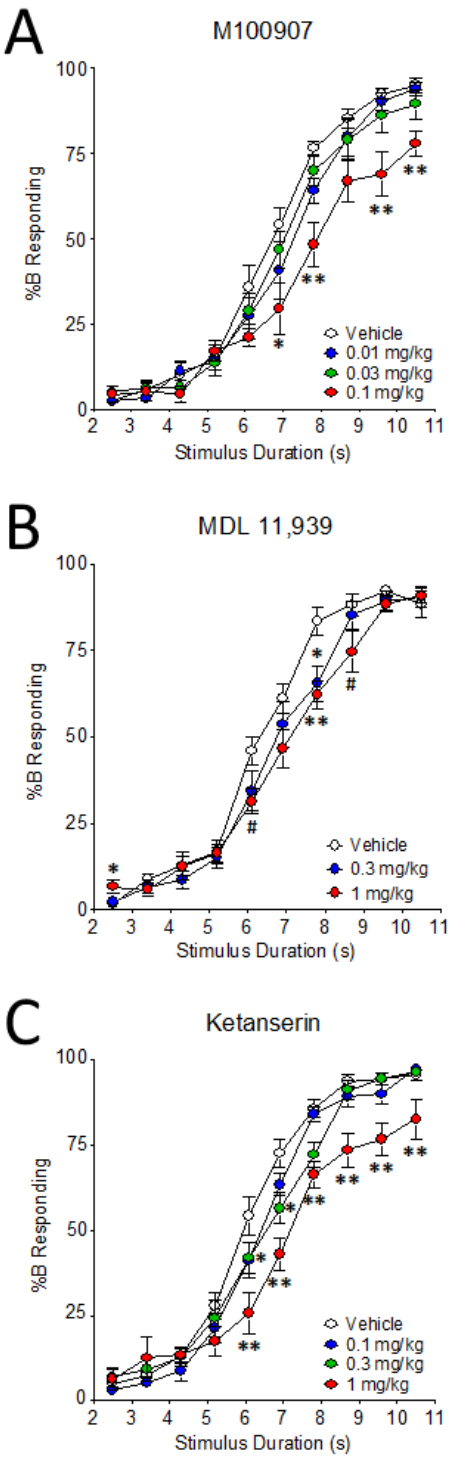

Figure 4.3. Effects of 5-HT_{2A} inverse agonist **(A)** M100907, **(B)** MDL 11,939, or **(C)** ketanserin on temporal discrimination. The data shown (group means ± SEM) represent the proportional choice of lever B as a function of stimulus duration (%B responding). # $p < 0.1$, *$p < 0.05$, **$p < 0.01$ compared to vehicle (Dunnett's test).

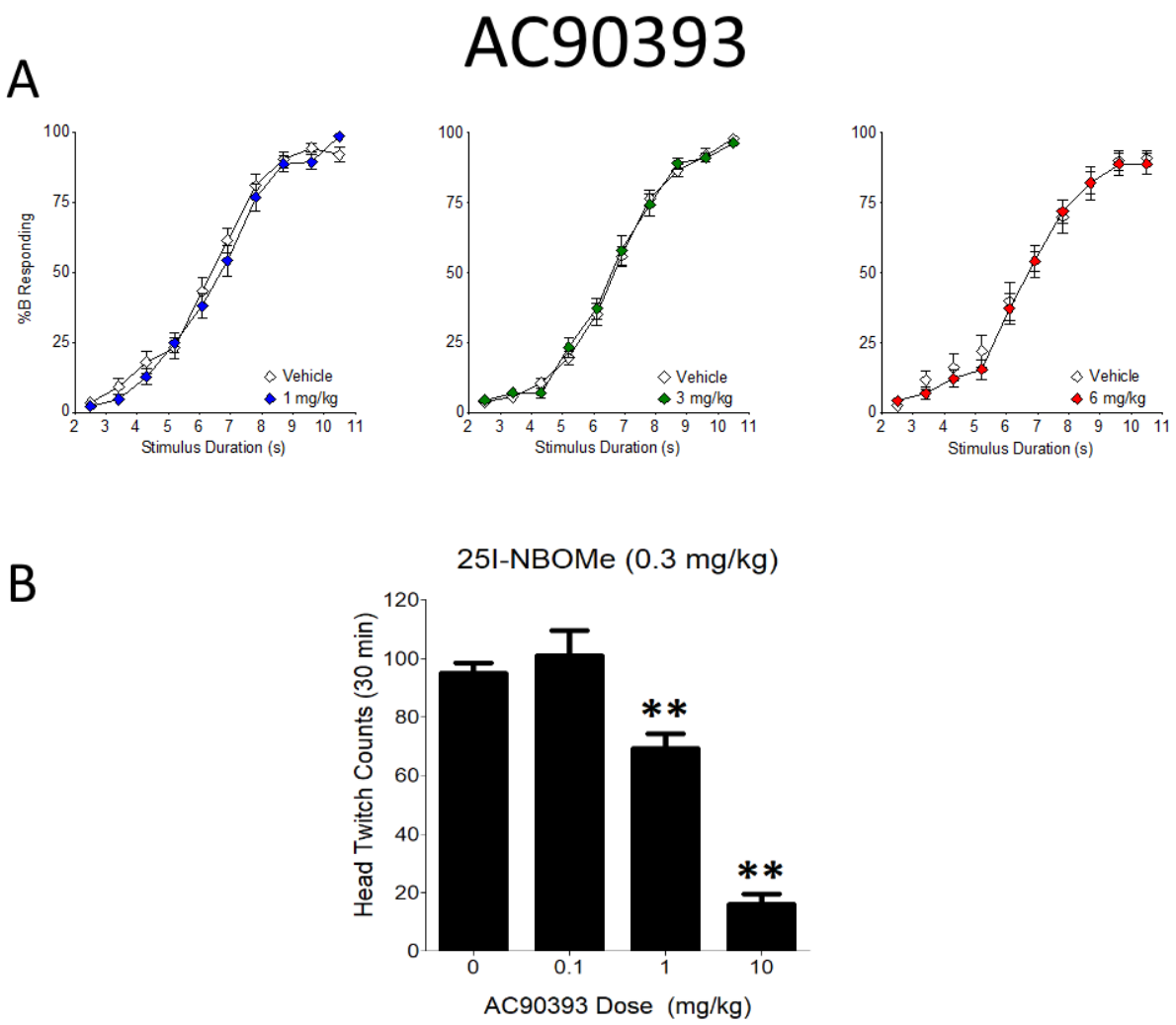

Figure 4.4. **(A)** Effects of the neutral 5-HT_{2A} antagonist AC90393 on temporal discrimination. The data shown (group means ± SEM) represent the proportional choice of lever B as a function of stimulus duration (%B responding). **(B)** Effects of AC90393 pretreatment on the head twitch response induced by 0.3 mg/kg 25I-NBOMe. The doses of AC90393 tested in the discrete-trials task are sufficient to block the hallucinogen-induced HTR. $^{**}p < 0.01$ compared to 25I-NBOMe alone (Dunnett's test).

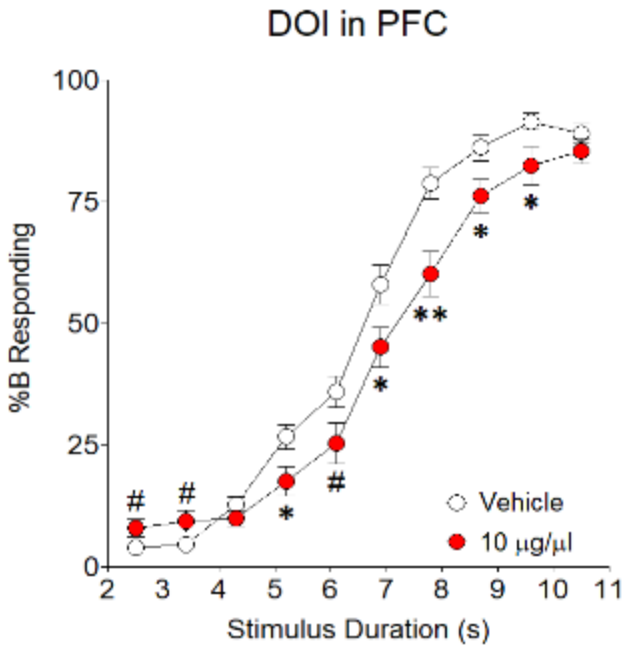

Figure 4.5. Effects of intra-PFC infusion of 10 µg/µl (0.2 µl/side) DOI on temporal discrimination. The data shown (group means ± SEM) represent the proportional choice of lever B as a function of stimulus duration (%B responding). # $p < 0.1$, *$p < 0.05$, **$p < 0.01$ compared to vehicle (Dunnett's test).

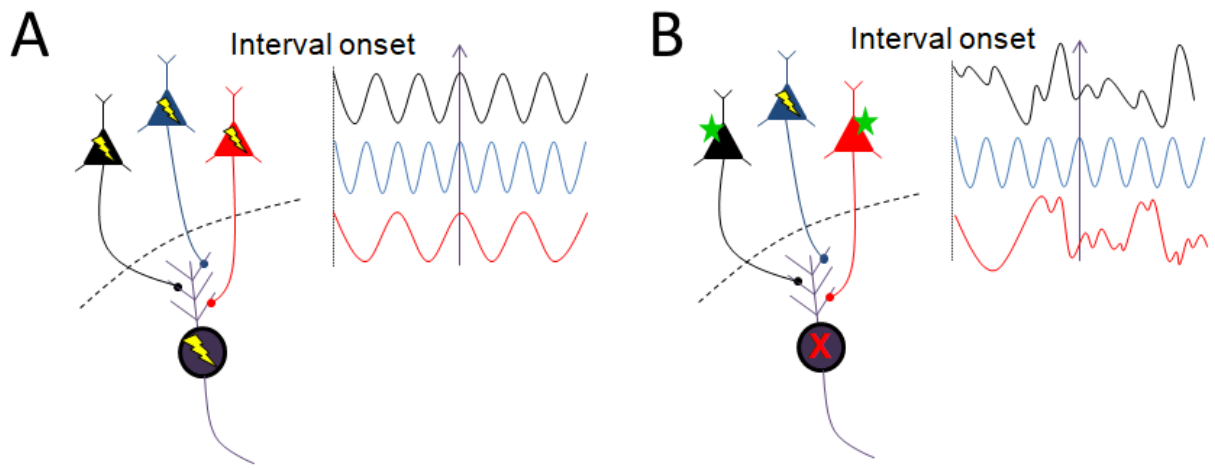

Figure 4.6. Schematic representation of a hypothetical timing circuit following **(A)** vehicle or **(B)** hallucinogen treatment. *Inset*: activity patterns of pyramidal neurons; *Triangles*: PFC pyramidal cells; *Circles*: DS MSNs; *Bolt*: neuron is firing at the time represented by the arrow on inset; *X*: neuron is not firing at the time represented in inset; *Star*: hallucinogen is bound to 5-HT_{2A} receptors on neuron. Hallucinogens alter patterns of oscillatory activity in PFC resulting in incoherent input to DS MSNs. Input is insufficient to induce interval-associated firing by MSN.

Table 4.1. *In vitro* profiling of putative 5-HT_{2A} inverse agonists and a neutral antagonist at the human 5-HT_{2A} receptor. SD denotes standard deviation. N denotes number of independent experiments. NA denotes no activity.

Ligand	5-HT_{2A} Antagonist			5-HT_{2A} Inverse Agonist		
	pKi	SD	N	$pIEC_{50}$	SD	N
AC-90393	7.1	0.3	6	NA	-	6
Ketanserin	8.5	0.2	3	8.4	0.1	3
MDL 100907	9.4	0.3	3	9.2	0.2	4

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