ACS Chemical Neuroscience

Research Article

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An Orally Active Phenylaminotetralin-Chemotype Serotonin 5-HT$_7$ and 5-HT$_{1A}$ Receptor Partial Agonist That Corrects Motor Stereotypy in Mouse Models

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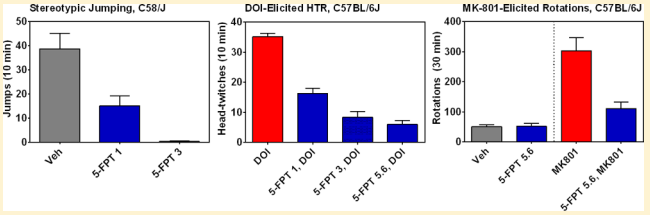
Supporting Information

ABSTRACT: Stereotypy (e.g., repetitive hand waving) is a key phenotype of autism spectrum disorder, Fragile X and Rett syndromes, and other neuropsychiatric disorders, and its severity correlates with cognitive and attention deficits. There are no effective treatments, however, for stereotypy. Perturbation of serotonin (5-HT) neurotransmission contributes to stereotypy, suggesting that distinct 5-HT receptors may be pharmacotherapeutic targets to treat stereotypy and related neuropsychiatric symptoms. For example, preclinical studies indicate that 5-HT$_7$ receptor activation corrects deficits in mouse models of Fragile X and Rett syndromes, and clinical trials for autism are underway with buspirone, a 5-HT$_{1A}$ partial agonist with relevant affinity at 5-HT$_7$ receptors. Herein, we report the synthesis, *in vitro* molecular pharmacology, behavioral pharmacology, and pharmacokinetic parameters in mice after subcutaneous and oral administration of (+)-5-(2′-fluorophenyl)-*N*,*N*-dimethyl-1,2,3,4-tetrahydronaphthalen-2-amine ((+)-5-FPT), a new, dual partial agonist targeting both 5-HT$_7$ (K_i = 5.8 nM, EC_{50} = 34 nM) and 5-HT$_{1A}$ (K_i = 22 nM, EC_{50} = 40 nM) receptors. Three unique, heterogeneous mouse models were used to assess the efficacy of (+)-5-FPT to reduce stereotypy: idiopathic jumping in C58/J mice, repetitive body rotations in C57BL/6J mice treated with the NMDA antagonist, MK-801, and repetitive head twitching in C57BL/6J mice treated with the 5-HT$_2$ agonist, DOI. Systemic (+)-5-FPT potently and efficaciously reduced or eliminated stereotypy in each of the mouse models without altering locomotor behavior on its own, and additional tests showed that (+)-5-FPT, at the highest behaviorally active dose tested, enhanced social interaction and did not cause behaviors indicative of serotonin syndrome. These data suggest that (+)-5-FPT is a promising medication for treating stereotypy in psychiatric disorders.



KEYWORDS: *Stereotypy, 5-HT$_7$, 5-HT$_{1A}$, receptor, partial agonist, autism, mice*

Although two drugs (the antipsychotic medications, risperidone and aripiprazole) are approved to treat irritability associated with autism spectrum disorder (ASD), there are no drugs approved to treat core symptoms of ASD, which include deficits in social communication as well as restricted and repetitive patterns of behavior, such as stereotypy. Stereotypy is observed as uncontrolled, rigid and repetitive, low-order, motor behavior, e.g., hand waving and body rocking, and is considered the most robust diagnostic marker of ASD in children.[1] Despite the severe impact stereotypy has on daily life functioning for persons with ASD and its close correlation with cognitive and attention deficits, it has received relatively scant attention regarding development of drug therapy.[2] Moreover, repetitive motor behavior, including stereotypy and higher-order restrictive behaviors (e.g., compulsions), are also observed in other neurodevelopmental and neuropsychiatric disorders, such as Asperger syndrome (stereotypy), Fragile X syndrome (FXS, stereotypy), Prader–Willi syndrome (self-injurious behaviors (SIB) and compulsions), Rett syndrome (stereotypy, compulsions, SIB), Tourette syndrome (tics, SIB, compulsions), attention-deficit hyperactivity disorder (ADHD), obsessive-compulsive disorder, psychotic disorders, psychostimulant addiction, and generalized anxiety (e.g., akathisia), with many instances of comorbidity. The commonalities suggest that repetitive motor behaviors observed in many neuropsychiatric disorders may involve shared neurobiological mechanisms[3] and may be categorized within a single spectrum.

On the basis of observations of hyperkinesia and stereotypy that occur as a result of prolonged psychostimulant use or levodopa treatment as well as tremor and akathisia resulting from first-generation antipsychotics that prominently block dopamine D_2-type receptors, dopaminergic mechanisms in basal ganglia–thalamus–cortex motor circuits are thought to

Special Issue: Serotonin Research

Received: March 25, 2015
Revised: May 20, 2015
Published: May 26, 2015

DOI: 10.1021/acschemneuro.5b00099
ACS Chem. Neurosci. 2015, 6, 1259–1270

underlie uncontrollable motor perturbations, such as stereotypy.[3,4] Many converging lines of evidence, however, also point to a prominent, direct or modulatory role for the serotonin (5-hydroxytryptamine, 5-HT) system. For example, blood 5-HT levels and 5-HT transporter genotype correlate with the presence of stereotypy,[5,6] and diet-induced reduction of 5-HT (via depletion of its precursor amino acid tryptophan) in persons with ASD increases stereotypy.[7] Furthermore, in some clinical trials for ASD, selective serotonin reuptake inhibitors (SSRIs) showed positive effects on stereotypy and compulsions. The effects of SSRIs, however, are mixed, as other studies reported that SSRIs worsened stereotypy.[1,10−12] Side effects from SSRI treatment are also highly prevalent,[1,8−10] potentially as a result of the shotgun approach, i.e., nondiscriminant elevation of 5-HT levels that could have nontherapeutic interactions at multiple 5-HT receptors. Targeting selected 5-HT receptors may provide better treatment outcomes for stereotypy.

The 5-HT_7 G_s-coupled receptor was cloned in 1993[11,12] and shown to be highly expressed in the hypothalamus.[13] Reports from observations of 5-HT_7 receptor knockout (KO) mice and rodents administered selective 5-HT_7 receptor agonists and antagonists provided evidence for a prominent role in regulating circadian rhythms,[14] which are disturbed in most neuropsychiatric conditions, including ASD. 5-HT_7 receptors are similarly bountiful in the thalamus,[13] a neural system critical for regulating motor behavior,[15,16] in addition to sensory processing, attention, and executive function, depending on specific subregions.[17,18] Recently, it was observed that *N*-(4-cyanophenylmethyl)-4-(2-diphenyl)-1-piperazinehexanamide (LP-211, Figure 1), a 5-HT_7 partial agonist that is highly selective for 5-HT_7 over other 5-HT receptors,[19] corrects several phenotypic deficits in an *Mecp2* KO mouse model of Rett syndrome, including improving cognition, reducing anxiety, and restoring a reduction of cortical 5-HT_7 receptors observed in drug-free *Mecp2* KO mice.[20] Meanwhile, (*R*)-(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin ((*R*)-(+)-DPAT; Figure 1), a low-potency 5-HT_7 partial agonist[21] and a potent 5-HT_{1A} full agonist,[22] reversed exacerbated long-term depression (LTD) in an *Fmr1* KO mouse model of FXS with applicability to ASD.[23]

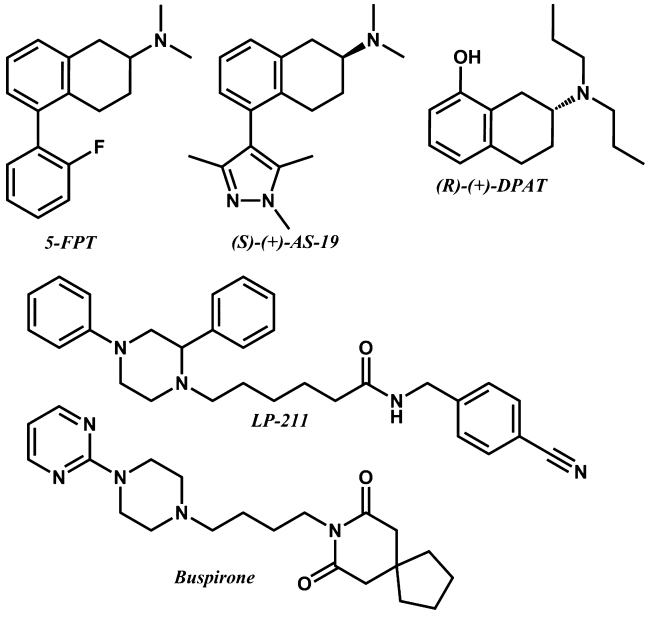


Figure 1. 5-FPT and other 5-HT_7 and/or 5-HT_{1A} agonists. Racemic 5-FPT (shown) was resolved into the (+) and (−) optical enantiomers. Similar to (*S*)-(+)-AS-19 and (*R*)-(+)-DPAT, (+)-5-FPT was the more active enantiomer.

Importantly, targeting 5-HT_{1A} G_i-coupled receptors may also attenuate synaptic plasticity deficits in ASD and related neurodevelopmental and neurological disorders to improve motor symptoms.[24] The 5-HT_{1A} receptor is widely expressed in the brain and is central to regulating 5-HT neuron firing. 5-HT neuronal activity suppresses striatal dopamine neurons, and this effect is inhibited by activation of 5-HT_{1A} receptors, resulting in disinhibition of dopamine neural activity. Recently, it was suggested that activation of 5-HT_{1A} receptors may treat catalepsy and L-DOPA-induced dyskinesia in Parkinson's disease.[25] Furthermore, in ASD patients, 5-HT_{1A} receptor binding site density is reduced in certain cortico−limbic systems,[26] and buspirone (Figure 1), a moderate affinity (K_i ∼20 nM with agonist radiolabel)[27] 5-HT_{1A} receptor partial agonist, is in clinical trials to treat children with ASD.[28,29] The activity of buspirone at the human 5-HT_7 receptor has not been reported to our knowledge, but it binds appreciably with unknown function at the rat 5-HT_7 receptor (K_i ∼ 400 nM with agonist radiolabel).[12] Thus, we considered that coactivating 5-HT_7 and 5-HT_{1A} receptors could treat stereotypy commonly observed in FXS, Rett, ASD, and other neurodevelopmental and neuropsychiatric disorders. It is noted, however, that full 5-HT_{1A} agonists such as (*R*)-(+)-DPAT are not appropriate for clinical development due to their induction of 5-HT syndrome, which can be life-threatening (e.g., hyperthermia, cardiac arrhythmia, seizures, loss of consciousness).

Extending our work to develop 4-phenyl-2-dimethylaminotetralin (PAT) derivatives that target 5-HT_2 receptors to treat neuropsychiatric disorders without liability for sedation or weight gain,[30−32] we designed and synthesized novel PAT analogues with the phenyl moiety at the 5-position (5-PAT), in an effort to target 5-HT_7 receptors, in accordance with available structure−activity relationship (SAR) information.[33] 5-PATs affinity at 5-HT_2 receptors was diminished, and the new chemotype provided stereoselective high-affinity binding at 5-HT_7 receptors, similar to the structurally related, selective 5-HT_7 partial agonist, (2*S*)-(+)-5-trimethylpyrazolyl-2-dimethylaminotetralin (AS-19; Figure 1).[34,35] During exploration of the activity of 5-PATs, we discovered that several analogues also have high affinity at 5-HT_{1A} receptors, similar to that of other 2-aminotetralin-type 5-HT_7 ligands such as (*R*)-(+)-DPAT and AS-19. Herein, we highlight and describe the preclinical development of one of our lead 5-PAT analogues from this endeavor, (+)-5-(2′-flourophenyl)-2-dimethylaminotetralin or (+)-5-(2′-fluorophenyl)-*N*,*N*-dimethyl-1,2,3,4-tetrahydronaphthalen-2-amine ((+)-5-FPT; Figure 1), a high-affinity 5-HT_7 and 5-HT_{1A} partial agonist. (+)-5-FPT potently and efficaciously attenuates stereotypy in three unique, heterogeneous mouse models, without altering locomotor behavior on its own. Furthermore, (+)-5-FPT increases social interactions. When administered at behaviorally active doses, (+)-5-FPT was observed to have diminutive liability to cause symptoms of serotonin syndrome, potentially due to its partial activation of serotonin receptors. Finally, (+)-5-FPT has a favorable pharmacokinetic profile, with efficacy after oral administration, suggesting that it is an appropriate lead for development as a novel pharmacotherapy to treat ASD and related conditions.

DOI: 10.1021/acschemneuro.5b00099
ACS Chem. Neurosci. 2015, 6, 1259−1270

Table 1. Affinity Values of (+)-5-FPT and (-)-5-FPT at a Select Panel of G Protein-Coupled Receptors[a]

	5-HT$_7$	5-HT$_{1A}$	5-HT$_{2A}$	5-HT$_{2B}$	5-HT$_{2C}$	α_{1A}	α_{1B}	H$_1$	D$_2$	m5-HT$_{2A}$	m5-HT$_{2C}$
(+)-5-FPT	5.8 (0.7)	22 (2.5)	886 (64)	60 (9)	269 (18)	>10 μM	>10 μM	>1 μM	>1 μM	632 (43)	644 (92)
(−)-5-FPT	460 (53)	>1 μM	119 (22)	684 (131)	>1 μM	>10 μM	>10 μM	>1 μM	>1 μM	147 (14)	>1 μM

[a]Each data value is expressed as the mean (SEM) K_i in nM. All receptors were human, except for m = mouse. 5-HT$_{2C}$ = 5-HT$_{2C-INI}$, m5-HT$_{2C}$ = m5-HT$_{2C-VNV}$, 5-HT$_7$ = 5-HT$_{7a}$.

Table 2. Functional Activity of (+)-5-FPT at Human 5-HT$_7$ and 5-HT$_{1A}$ Receptors (cAMP Detection) and 5-HT$_2$ Receptors (IP1 Detection)[a]

		5-HT$_7$	5-HT$_{1A}$	5-HT$_{2A}$	5-HT$_{2B}$	5-HT$_{2C}$
(+)-5-FPT	EC$_{50}$	34 (13)	40 (15)	3526 (826)	inactive	230 (69)
	E_{max}	33 (11)	48 (10)	39 (4)	inactive	87 (13)

[a]Each data value is expressed as the mean (SEM). EC$_{50}$ is given in nM, and E_{max} is the percent of maximal response relative to AS-19 (5-HT$_7$), 5-CT (5-HT$_{1A}$), and 5-HT (5-HT$_2$). Inactive = no activation up to 100 μM.

RESULTS AND DISCUSSION

The presence of motor stereotypy is a core diagnostic criterion in ASD, FXS, Rett syndrome, and other neurodevelopmental disorders. Currently, however, there is a lack of effective treatments for motor stereotypy, despite its negative impact on daily life functions. Recent preclinical findings report that activating 5-HT$_7$ receptors corrects LTD synaptic deficits in *Fmr1* KO mice, a genetic model of FXS,[20,23] and behavioral and molecular deficits in *Mecp2* transgenic mice, a genetic model of Rett syndrome. These findings, together with the known dense localization of 5-HT$_7$ receptors in the thalamus, a neural system involved in regulating stereotypy,[15] led us to hypothesize that targeted activation of the 5-HT$_7$ receptor would be pharmacotherapeutic in mouse models of stereotypy associated with ASD and related disorders.

(+)-5-FPT Is a High-Affinity 5-HT$_7$ and 5-HT$_{1A}$ Partial Agonist. On the basis of our work developing 4-substituted-phenyl-2-dimethylaminotetralin compounds targeting 5-HT$_2$ receptors[30,31] and based on activity of 5-substituted-phenyl-2-dimethylaminotetralins reported in the literature, including the 5-HT$_7$ agonist AS-19,[33−35] we designed and synthesized novel 5-substituted-phenyl-2-dimethylaminotetralin compounds to target 5-HT$_7$ receptors. Reported here are data obtained with the novel 5-(2′-fluorophenyl) analogue, 5-FPT (Figure 1).

HEK293 cells stably expressing human 5-HT$_7$ receptors were generated to assess 5-HT$_7$ pharmacology of the 5-FPT enantiomers. Receptor binding site density in the clone with the highest specific binding (CHTR7beta) was assessed with [^{3}H]5-CT saturation binding, which revealed a mean (SEM) receptor binding site density, B_{max}, of 7.7 (0.4) pmol/mg protein (Supporting Information Figure S1). Even at such a high 5-HT$_7$ receptor density, a 5-HT$_7$ partial agonist is not expected to appear as a full agonist, because 5-HT$_7$ receptor reserve does not appear to be an issue.[36] In addition, 5-FPT pharmacology was evaluated in HEK293 cells transiently expressing relevant human 5-HT receptors (5-HT$_{1A,2A,2B,2C}$) and potential off-targets, including the dopamine D$_2$, adrenergic $\alpha_{1A,1B}$, and histamine H$_1$ receptors. Notably, D$_2$ can display high affinity for the 2-aminotetralin scaffold, depending on substitution pattern and stereochemistry, and α_1 and H$_1$ receptors are common off-targets of antipsychotic drugs used in ASD. Studies also were conducted using HEK293 cells transiently expressing the mouse 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, given their relevance to the *in vivo* translational studies. Unfortunately, murine versions of the other receptors were not procured.

As shown in Table 1, 5-FPT demonstrated high affinity at 5-HT$_7$ receptors that was enantioselective; the (+)-enantiomer (K_i = 5.8 nM) was about 80-times more potent than the (−)-enantiomer (K_i = 460 nM). (+)-5-FPT behaved as a 5-HT$_7$ partial agonist regarding G$_s$-cyclic AMP (cAMP) signaling (EC$_{50}$ = 34 nM, E_{max} vs AS-19 = 33%; see Table 2 and Figure 2), and the (−)-enantiomer also demonstrated partial agonism

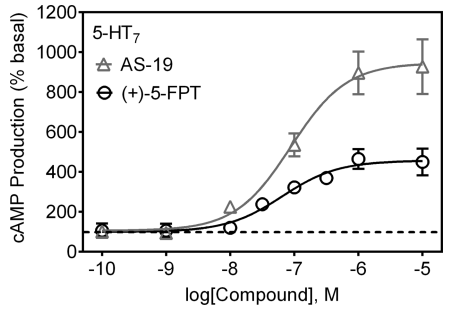


Figure 2. Representative 5-HT7-G$_s$-cAMP functional assay results show 5-HT$_7$ partial agonist effects of (+)-5-FPT, relative to AS-19.

(EC$_{50}$ = 378 (82) nM, E_{max} vs AS-19 = 29 (6.8)%) with similar kinetics, albeit with about 11 times less potency than that of the (+)-isomer (Table 1 and Figure 4). Further *in vitro* pharmacological studies with 5-FPT revealed that the (+)-enantiomer also had high-affinity partial agonist properties at 5-HT$_{1A}$ receptors (K_i = 22 nM, EC$_{50}$ = 40 nM, E_{max} vs 5-CT = 48%; Tables 1 and 2 and Figure 3). In contrast, Table 1 shows that the (−)-enantiomer of 5-FPT did not display appreciable affinity at 5-HT$_{1A}$ receptors. The striking high

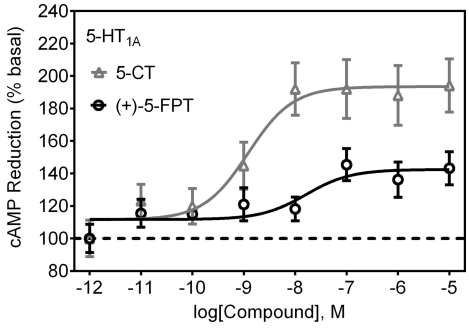


Figure 3. Representative 5-HT1A-G$_i$-cAMP functional assay results show 5-HT$_{1A}$ partial agonist effects of (+)-5-FPT, relative to 5-CT.

DOI: 10.1021/acschemneuro.5b00099
ACS Chem. Neurosci. 2015, 6, 1259−1270

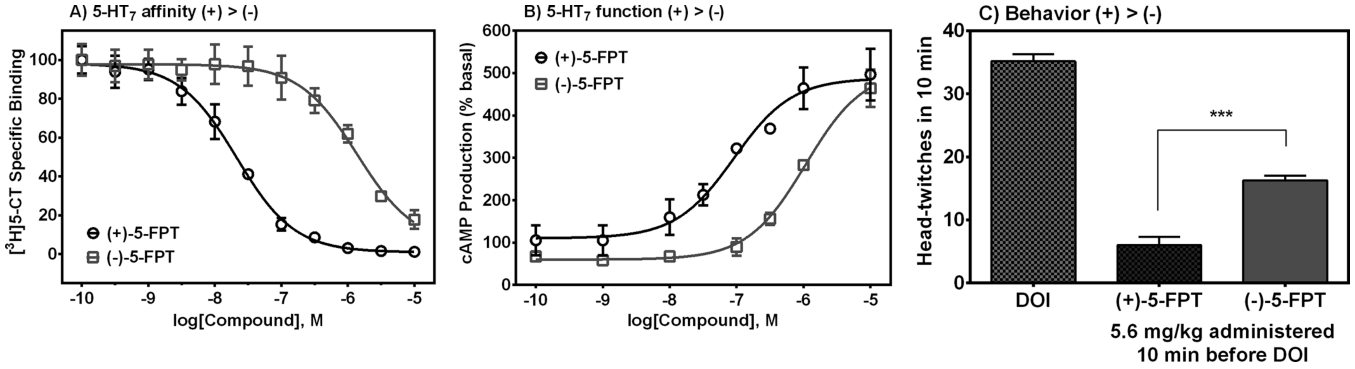


Figure 4. Effects of 5-FPT are enantioselective. Relative to the (−) enantiomer, the (+) enantiomer has (A) higher affinity at 5-HT$_7$ receptors (and 5-HT$_{1A}$ receptors, see Table 1), (B) greater potency for activating 5-HT$_7$ receptors, and (C) greater potency for reducing the DOI (1 mg/kg)-elicited head-twitch behavioral response. Checkerboard bars include data shown in Figure 7 for comparison. Note, for competition binding shown in (A), 2.37 nM (calculated) [^{3}H]5-CT was used to label 5-HT$_7$ receptors, and its K_D was set at 0.7 nM. Data are expressed as means ± SEMs.

affinity of (+) over (−)-5-FPT at 5-HT$_7$ and 5-HT$_{1A}$ receptors indicated that subsequent translational studies to assess efficacy to modulate stereotypy in mice should focus on the (+)-enantiomer (see Figure 4).

Regarding activity at other 5-HT receptors, as shown in Table 1, (+)-5-FPT affinity at 5-HT$_{2A}$ receptors was very low, and it was a very low potency partial agonist (Table 2 and Supporting Information Figure S2A). In contrast to 5-HT$_{2A}$ receptors, (+)-5-FPT bound with appreciable affinity at 5-HT$_{2B}$ (K_i = 60 nM) and 5-HT$_{2C}$ (K_i = 269 nM) receptors. In functional assays, (+)-5-FPT was devoid of 5-HT$_{2B}$ activity up to 100 μM, suggesting neutral antagonism (Table 2 and Supporting Information Figure S2B). It is noted that 5-HT$_{2B}$ agonist activity is untenable regarding drug development because it can lead to cardiac valvulopathy.[37] 5-HT$_{2B}$ antagonism, on the other hand, is proving to be useful to treat attention deficits, as illustrated by the efficacy of the 5-HT$_{2B}$ antagonist, metadoxine, in clinical trials of adults with ADHD.[38] At 5-HT$_{2C}$ receptors, (+)-5-FPT was a nearly full-efficacy agonist (Table 2 and Supporting Information Figure S2C), with modest potency (EC_{50} = 230 nM), consistent with its affinity. At the mouse 5-HT$_{2A}$ receptor, (+)-5-FPT had low affinity (K_i = 632 nM), similar to the human receptor; however, its affinity at the mouse 5-HT$_{2C}$ receptor (K_i = 644 nM) was nearly 2.5 times lower than that at the human receptor.

Unexpectedly, we observed that at mouse and human 5-HT$_{2A}$ receptors, the (−)-5-FPT enantiomer had ∼4- and 8-fold higher affinity, respectively, than (+)-5-FPT (Table 1). In functional assays, (−)-5-FPT was a low-potency 5-HT$_7$ and 5-HT$_{2C}$ agonist, but it did not activate 5-HT$_{2A}$ or 5-HT$_{2B}$ receptors up to 100 μM (data not shown). Neither 5-FPT enantiomer had appreciable affinity at α_{1A}, α_{1B}, H_1, and D_2 receptors (i.e., K_i > 1 μM; Table 1).

(+)-5-FPT Attenuates Motor Stereotypy without Affecting Locomotion. We tested (+)-5-FPT in three heterogeneous models of stereotypy, each with different scales of validity: (1) idiopathic stereotypic jumping in C58/J mice, (2) (±)-2,5-dimethoxy-4-iodoamphetamine (DOI)-elicited stereotypic head twitching in C57BL/6J mice, (3) and (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5,10-imine (MK-801)-elicited stereotypic rotations in C57BL/6J mice. (+)-5-FPT was also tested for efficacy to attenuate *d*-amphetamine (AMP)-elicited hyperlocomotion in C57BL/6J mice.

C58/J mice exhibit robust stereotyped jumping that appears early in development (modeling the early developmental nature of stereotypy in ASD). C58/J mice also possess genes that are associated with ASD, including tryptophan hydroxylase 2 (rate-limiting enzyme in brain 5-HT synthesis), that are in different loci relative to C57BL/6J mice.[39,40] Furthermore, jumping in C58/J mice has been used as a model of stereotypy responsive to drug treatment.[41] As shown in Figure 5, (+)-5-FPT potently

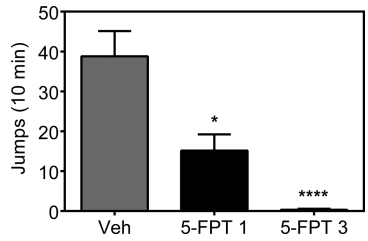


Figure 5. (+)-5-FPT (1 and 3 mg/kg) dose-dependently eliminates idiopathic stereotypic jumping in C58/J mice. Bar graphs show the means ± SEMs.

eliminated stereotypic jumping in C58/J mice in a dose-dependent manner, without altering locomotor behavior (see Figure 8). (+)-5-FPT showed greater efficacy in this model than that of the recently reported mGluR5 negative allosteric modulator, GRN-529,[41] which was under development to treat ASD.

Regarding glutamate neurotransmission in stereotypy, the NMDA receptor antagonist MK-801 characteristically elicits stereotypic rotations that appear to mimic monogenetic stereotypy observed in *Fmr1* KO mice.[42] Furthermore, mutations and autoantibodies of the NMDA glutamate receptor that decrease its function are causally linked to ASD, intellectual disabilities, and psychiatric symptoms in humans.[43−45] As shown in Figure 6A, (+)-5-FPT (5.6 mg/kg) significantly reduced MK-801-elicited rotations in C57BL/6J mice. Note that neither (+)-5-FPT nor AMP caused stereotypic rotational behavior (Figure 6A). Additionally, (+)-5-FPT (5.6 mg/kg) significantly decreased hyperlocomotion caused by MK-801, but it did not significantly reduce hyperlocomotion caused by AMP (Figure 6B). Importantly, (+)-5-FPT also did not alter locomotion in mice when administered alone (Figures 6B and Figure 8).

The DOI-elicited head-twitch response (HTR) is a behavioral model of cortical 5-HT$_{2A}$ activation and also has

DOI: 10.1021/acschemneuro.5b00099
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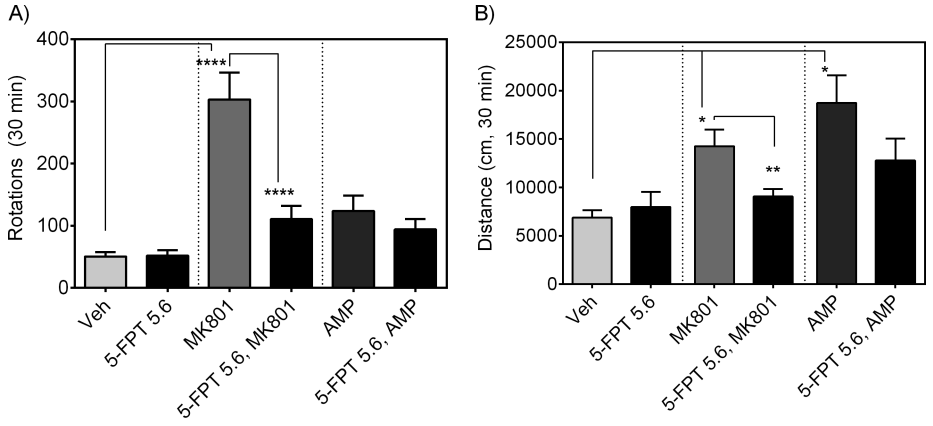

Figure 6. (A) (+)-5-FPT (5.6 mg/kg) significantly reduces stereotypic rotations elicited by MK-801 (0.3 mg/kg) in C57BL/6J mice. (B) (+)-5-FPT (5.6 mg/kg) blocks MK-801 (0.3 mg/kg) but not amphetamine (AMP, 3 mg/kg) hyperlocomotion, and (+)-5-FPT does not affect locomotor behavior on its own. AMP does not cause stereotypic rotational behavior despite significantly increasing locomotion. Bar graphs show the means ± SEMs.

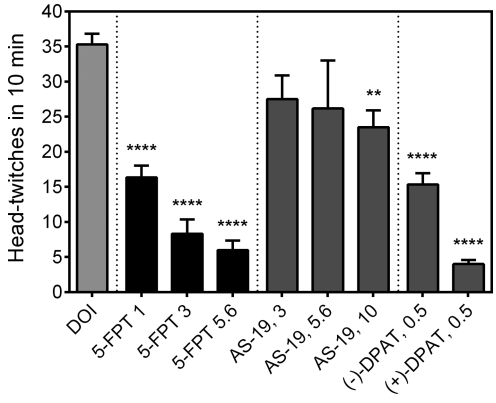

Figure 7. (+)-5-FPT dose-dependently blocks the DOI-elicited HTR in C57BL/6J mice, with effects similar to those of (*R*)-(+)-DPAT (0.5 mg/kg) and (*S*)-(−)-DPAT (0.5 mg/kg). In comparison, AS-19 (10 mg/kg) attenuates the HTR. Numbers on the x-axis represent mg/kg doses. Bar graphs represent the means ± SEMs.

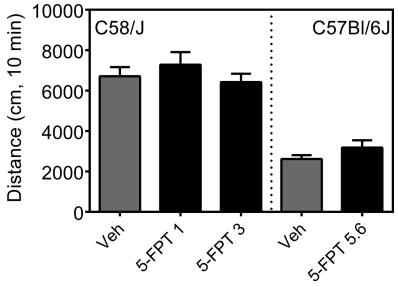

Figure 8. (+)-5-FPT does not alter locomotor behavior on its own. Data are from stereotypic jumping experiments with C58/J mice (left) and DOI-elicited HTR experiments with C57BL/6J mice ((+)-5-FPT plus vehicle treated control group) (right). (+)-5-FPT doses were 1, 3, and 5.6 mg/kg. Bar graphs show the means ± SEMs.

face validity for stereotyped tics.[46] The 5-HT_{2A} receptor is a predominant 5-HT receptor in the cortex and serves important excitation modulation functions on glutamate pyramidal and GABA neurons.[46,47] 5-HT_{2A} receptor function in cortical neurons is altered in *Fmr1* KO mice,[48] and 5-HT_{2A} function also is disrupted in persons with ASD and Tourette syndrome.[49,50] Moreover, 5-HT_{2A} antagonists such as ketanserin treat tics in Tourette syndrome, and when infused in subthalamic nuclei, they reduce stereotypy in rats,[51,52] supporting the DOI-elicited HTR as a model of stereotypy and/or tics. Relevant, too, is that the 5-HT_{1A} receptor partial agonist buspirone, in clinical trials to treat children with ASD,[28,29] has clinically germane affinity (K_i ∼ 140 nM) at 5-HT_{2A} receptors. As shown in Figure 7, (+)-5-FPT dose-dependently attenuated the DOI-elicited HTR, with significant attenuating effects observed with each dose. Notably, DOI has weak affinity at both 5-HT_{1A} and 5-HT_7 receptors (K_i > 1 μM, unreported observations), and (+)-5-FPT has weak activity at 5-HT_{2A} receptors, which mediate the DOI-elicited HTR in C57BL/6J mice.[46] These observations suggest that the effect of (+)-5-FPT was not due to competition with DOI for receptor sites but that (+)-5-FPT was indirectly modulating DOI-elicited 5-HT_{2A} receptor activity to impact behavior. Importantly, although (+)-5-FPT showed weak partial agonist activity at human 5-HT_{2A} receptors, it did not elicit an HTR on its own (Table 3). To further support the assertion that (+)-5-FPT reduced the DOI-elicited HTR via receptor mechanisms other than 5-HT_{2A}, tests of (−)-5-FPT, AS-19, (*R*)-(+)-DPAT, and (*S*)-(−)-DPAT in this assay were also conducted. The (−)-5-FPT enantiomer that has the same physicochemical properties as those of (+)-5-FPT, but substantially higher affinity at human and mouse 5-HT_{2A} receptors, with neutral antagonist function, was substantially less efficacious than (+)-5-FPT at reducing the HTR at the 5.6 mg/kg dose (see Figure 4). Furthermore, AS-19 (10 mg/kg) and both enantiomers of

Table 3. Test for Serotonin Syndrome[a]

treatment	flat body	forepaw tread	head weave	HTR (*n*)	moon walk	piloerection	rears (*n*)	Straub tail	tremor
vehicle	0 (0)	0 (0)	0 (0)	1.1 (0.5)	0.1 (0.1)	0 (0)	27 (5)	0 (0)	0 (0)
(+)-5-FPT, 5.6 mg/kg	0 (0)	0 (0)	0 (0)	0 (0)	0.3 (0.2)	0 (0)	9 (3)****	0 (0)	0 (0)

[a]Shown are the mean (SEM) number of sessions, from a total of six 1 min observation sessions, in which mice displayed the behavior (score/6 possible), except for (*n*) = the mean of the total number of responses across all six sessions.

DOI: 10.1021/acschemneuro.5b00099
ACS Chem. Neurosci. 2015, 6, 1259−1270

DPAT (0.5 mg/kg), all of which have weak affinity at 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors but clinically relevant affinity at 5-HT_7 and 5-HT_{1A} receptors, suppressed the DOI-elicited HTR (Figure 7). Notably, (*R*)-(+)-DPAT, a 5-HT_{1A} full agonist, caused severe hypolocomotion and obvious behaviors indicative of serotonin syndrome in this assay, whereas neither (*S*)-(−)-DPAT, a 5-HT_{1A} partial agonist, nor AS-19 affected locomotor behavior or caused obvious serotonin syndrome (data not shown). Overall, our results support previous assertions that DPAT, via 5-HT_{1A} activation, suppresses DOI-elicited HTR.[53] Furthermore, relative to full agonists, 5-HT_{1A} partial agonists appear to translate with fewer untoward effects, such as behaviors associated with serotonin syndrome (see below).

(+)-5-FPT Increases Social Interactions and Does Not Cause Symptoms of Serotonin Syndrome. As shown in Figure 9, (+)-5-FPT (5.6 mg/kg) significantly increased the number of initiated social interactions in C57BL/6J mice littermates while also decreasing grooming. Furthermore, as shown in Table 3, (+)-5-FPT, at the highest behaviorally effective dose tested (5.6 mg/kg), did not result in symptoms of serotonin syndrome, including flat body, forepaw treading, moon walking, piloerection, Straub tail, or tremor, but did significantly decrease rearing, suggestive of 5-HT_{1A} activation. Of note, after behavioral testing was complete, blind scorers categorized mice into two groups based on number of rears and initiated social interactions, and the two groups differentiated vehicle from (+)-5-FPT-treated mice with 100% accuracy. These data warrant investigation of the potential therapeutic effects of (+)-5-FPT in ASD and other neuropsychiatric models that involve social deficits, including social behavior models of the negative symptoms of schizophrenia.

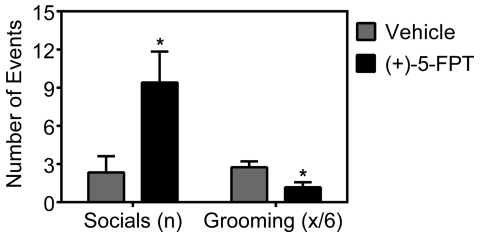

Figure 9. (+)-5-FPT (5.6 mg/kg) increases social interactions with vehicle-treated littermates while subsequently decreasing grooming. Shown are the mean (SEM) number of social interactions across six 1 min observation sessions and mean (SEM) number of sessions (out of six) in which mice displayed grooming. Data are from C57BL/6J mice.

(+)-5-FPT Is Orally Active and Readily Crosses the Blood−Brain Barrier. As shown in Figures 7 and 11, respectively, (+)-5-FPT significantly attenuated the DOI-elicited HTR after subcutaneous (sc) and oral administration. In addition, (+)-5-FPT readily crosses the blood−brain barrier, as evidenced by detection of microgram levels 30, 60, and 90 min after systemic administration (Table 4). Notably, levels of (+)-5-FPT were substantially lower in plasma relative to those in brain tissue as soon as 30 min postadministration, suggesting that (+)-5-FPT is rapidly cleared in the periphery. Meanwhile, the attenuating effects of (+)-5-FPT (5.6 mg/kg) on the DOI-elicited HTR remained significant for up to 2 h postadministration; at 3 h postadministration, (+)-5-FPT did not block DOI-elicited HTR (Figure 10).

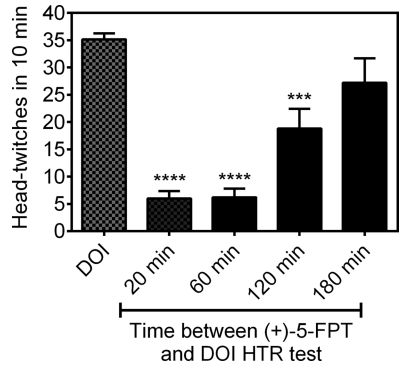

Figure 10. Efficacy time course of (+)-5-FPT (5.6 mg/kg). (+)-5-FPT retains efficacy for blocking the DOI-elicited (1 mg/kg) HTR when administered 120 min, but not 180 min, before testing. Data are from C57BL/6J mice. Checkerboard bars include data shown in Figure 7 for comparison. Bar graphs show the means ± SEMs.

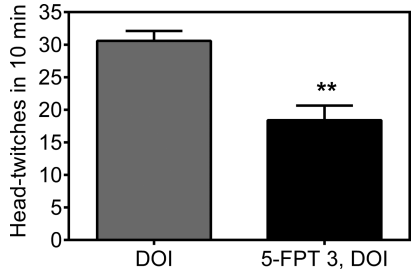

Figure 11. (+)-5-FPT is orally active. Oral gavage administration of 3 mg/kg (+)-5-FPT 10 min prior to sc administration of 1 mg/kg DOI attenuates the HTR in C57BL/6J mice. Bar graphs show the means ± SEMs.

Table 4. Plasma and Brain Concentrations of (+)-5-FPT after 3.0 mg/kg Subcutaneous Administration[a]

	time after injection		
	30 min	60 min	90 min
plasma (μg/mL)	0.114 (0.03)	0.118 (0.01)	0.070 (0.01)
brain (μg/g)	1.78 (0.24)	2.16 (0.17)	1.46 (0.09)

[a]Data are expressed as means (SEMs).

CONCLUSIONS AND PERSPECTIVES

The high affinity of (+)-5-FPT at 5-HT_{1A} and 5-HT_7 receptors and its efficacy to block stereotypic motor behavior elicited by MK-801 and DOI suggest (+)-5-FPT acts *in vivo* via 5-HT_{1A} and 5-HT_7 partial agonism mechanisms to regulate glutamatergic and/or 5-HT_2 receptor signaling. (+)-5-FPT activity at 5-HT_{2B} (antagonism) and 5-HT_{2C} (agonism) receptors also may contribute to its effects on stereotypy. Given the poor affinity of (+)-5-FPT at D_2 receptors and our observations that (+)-5-FPT (5.6 mg/kg) did not significantly decrease hyperlocomotion elicited by amphetamine or affect locomotor behavior on its own, we surmise that (+)-5-FPT does not work directly through dopaminergic mechanisms to suppress stereotyped motor behaviors. There could, however, be other potential off-targets of (+)-5-FPT that contribute to its effects. Thus, future studies will involve screening (+)-5-FPT at a comprehensive panel of G protein-coupled receptors (GPCRs). Nevertheless, 5-FPT represents an important, new lead for development of compounds with varying degrees of 5-HT_{1A} relative to 5-HT_7 partial agonism to help delineate mechanisms

DOI: 10.1021/acschemneuro.5b00099
ACS Chem. Neurosci. 2015, 6, 1259−1270

underlying (+)-5-FPT's pharmacotherapeutic effects in models of stereotypy. Furthermore, since social interaction and both MK-801- and DOI-elicited behaviors are also used as preclinical models of negative and positive symptoms of schizophrenia, respectively, we are alerted to the possibility that (+)-5-FPT could have potential as a novel antipsychotic medication.

Drug Discovery and Development To Treat Stereotypy. Traditionally, target validation and drug development for neuropsychiatric disorders have focused on preclinical animal models that attempt to recapitulate a disorder's entire phenotypic spectrum. Unfortunately, for a number of reasons, including disagreements regarding defining mental health disorders, such as ASD, many animal models suffer from poor translational validity. An innovative strategy here was to simplify the approach by using converging results from multiple etiologically unique mouse models of a single phenotype of many nervous system disorders, stereotypy. Stereotypy in rodents is an easily quantifiable behavior, has excellent face and etiological validity, is common across mammalian species, and shares common neurobiological mechanisms.[15] Our approach to focus on stereotypy was based in part on the recent failures of the mGluR5 antagonist, mavoglurant (AFQ056), and the GABA-B agonist, arbaclofen, for meeting primary therapeutic end points in clinical trials to treat ASD, despite success in preclinical models. Clinical experts opine that clinical heterogeneity may explain these failures,[54] and stratifying the population in clinical trials, for instance, carefully choosing patients with ASD who display marked and similar forms of stereotypy, may have revealed therapeutic benefit.

We contend that motor alterations associated with mental health disorders, including stereotypy in FXS, ASD, and Rett syndromes, tics in Tourette syndrome, ritualistic motor behavior in OCD, akathisia or fidgety motor behavior in ADHD, psychoses, generalized anxiety disorder, and psychostimulant addiction can be categorized within a spectrum. Abnormal, repetitive motor behavior that associates closely with a mental health disorder (that is not a result of neurodegeneration, e.g., as seen in Huntington's disease) may be the result of exaggerated psychological agitation (neural noise), involving neurobiological mechanisms shared across nervous system disorders, and therefore may serve as an endophenotype.[55] We are unaware of studies that have examined whether siblings or parents of persons with diagnosed stereotypy show relatively increased abnormal motor behaviors, but as mechanistic support of this hypothesis, the protein expressed from *Top3β*, a gene strongly implicated in ASD, schizophrenia, and intellectual disability, was found to form a complex with the Fragile X Mental Retardation Protein, FMRP (Top3*β*, TDRD3, and FMRP), which is important for regulating activity of several RNAs associated with several diverse neuropsychiatric disorders.[56] Also, a recent study reports a powerful phenotypic, environmental and genetic link between ADHD and stereotypy in ASD,[57] a finding that bolsters the hypothesis that stereotypy is a robust preclinical model applicable to ASD and other psychiatric disorders and amenable to drug target discovery, such as the 5-HT_7 and 5-HT_{1A} receptors.

In conclusion, we argue that stereotypy may be an expression of a disordered mental state that is amenable to novel drug discovery and suggest that (+)-5-FPT, via 5-HT_7 and 5-HT_{1A} partial agonism, combined with therapeutically appropriate 5-HT_{2B} and 5-HT_{2C} polypharmacology, for exceptional drug benefits,[58] may be an expedient treatment for stereotypy observed in ASD and many other nervous system disorders. The work we presented here provides a foundation for performing comprehensive preclinical studies with advanced translational validity to corroborate (+)-5-FPT as a potential treatment for stereotypy. For example, we plan to assess the activity of (+)-5-FPT in chronic administration regimens that more closely model once-daily dosing treatments in humans. In addition, (+)-5-FPT will be tested in genetic mouse models of human disorders that include stereotypy as a core symptom.

METHODS

Synthesis of (+)- and (−)-5-FPT. Full details on the synthesis of (+)- and (−)-5-FPT, including absolute stereochemistry determined from single X-ray crystallographic structure, will be reported separately in connection with a series of analogues and their structure−affinity analysis at 5-HT_7 receptors (Vemula et al., in preparation). Briefly, 5-bromo-1-tetralone was reduced with sodium borohydride to give the corresponding alcohol that underwent an acid-catalyzed dehydration to obtain the C(1)−C(2) olefin compound. *m*-Chloroperbenzoic acid was reacted with the olefin to obtain the C(1)−C(2) epoxide that underwent an acid-catalyzed epoxide opening to yield 5-bromo-2-tetralone.[59] Suzuki−Miyaura cross-coupling with 2-fluorobenzeneboronic acid, followed by reductive amination with dimethylamine, gave racemic 5-FPT, which was converted to the hydrochloride (HCl) salt for characterization (^{1}H NMR [Varian 500 MHz, $CDCl_3$]: δ 1.90−1.76 [m, 1H], 2.38 [dd, J = 11.0, 5.0 Hz, 1H], 2.65−2.56 [m, 1H], 2.84−2.74 [brs, 7H], 3.20 [t, J = 12.0 Hz, 1H], 3.38−3.33 [m, 1H], 3.56−3.47 [m, 1H], 7.11−7.07 [m, 2H], 7.23−7.15 [m, 4H], 7.36−7.31 [m, 1H], 12.82 [bs, 1H]; mp 232−235 °C). The free base racemate was resolved to (+)- and (−)-5-FPT by semipreparative polysaccharide-based chiral stationary phase (CSP)-HPLC[60] (EtOH/hexane [1:9] + 0.1% of diethylamine modifier + 0.1% trifluoroacetic acid modifier; flow rate = 2.0 mL/min), and the HCl salt form of each enantiomer was characterized for optical (stereochemical) purity: (+)-5-FPT: CSP-HPLC t = 24.2 min, $[\alpha]_D^{22}$ (PerkinElmer 343 series polarimeter) = (+) 5.65° (c 0.32, CH_2Cl_2); (−)-5-FPAT: CSP-HPLC t = 26.5 min, $[\alpha]_D^{25}$ = (−) 5.45° (c 0.22, CH_2Cl_2).

Commercial Compounds. (±)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride (DOI), (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate (MK-801), *d*-amphetamine sulfate (AMP), AS-19, and (*R*)-(+)- and (*S*)-(−)-8-hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide ((*R*)-(+)- and (*S*)-(−)-DPAT) were used for *in vivo* experiments. 5-HT hydrochloride, mianserin hydrochloride, mepyramine maleate, and spiperone were used for *in vitro* experiments. These compounds were obtained from Sigma-Aldrich (St. Louis, MO) and Tocris (Bristol, BS11 0QL, UK). The tritiated radioligands 5-CT, ketanserin, mesulergine, mepyramine, raclopride, and prazosin were purchased from PerkinElmer (Waltham, MA).

***In Vitro* Affinity and Functional Pharmacology.** Human embryonic kidney 293 cells (HEK293, ATCC no. CRL-1573), fed Corning cellgro Dulbecco's modified Eagle's medium (DMEM, MT-10-013) with 8% fetal bovine serum and 1% penicillin−streptomycin in 10 cm plates, were grown in a humidified incubator at 37 °C with 5% carbon dioxide. cDNA encoding the human 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C^-INI}, 5-HT_{7a}, dopamine D_2, histamine H_1, adrenergic α_{1A}, and α_{1B} receptors was obtained from UMR cDNA Resource Center (Rolla, MO), and mouse 5-HT_{2A} and 5-HT_{2C^-VNV} cDNA was from Origene Technologies (Rockville, MD). For production of human 5-HT_7 stable expressing HEK293 cells, cells at pass number three (P3) were grown to ∼50% confluence in a 10 cm plate, transfected with 10 μg of HTR_{7a} cDNA together with 20 μL TurboFect transfection reagent (Thermo Scientific, Pittsburgh, PA) in 5 mL of Ultra-MEM (Thermo) and 5 mL of DMEM containing 5% dialyzed FBS, and placed in an incubator overnight. [Note that alternative splicing at the second intron of HTR_7 leads to at least four different 5-HT_7 isoforms, depending on species; however, the full-length 5-HT_{7a} isoform is not significantly different across species and from other 5-HT_7 isoforms with regard to agonist binding, membrane localization, and G_s-cAMP function.[61]] Cells were then selected with 500 μg/mL G418 in

DOI: 10.1021/acschemneuro.5b00099
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DMEM with 5% dialyzed FBS, which was refreshed every other day. On day six post-transfection, cells were serial-diluted (1:2 to 1:1000) and passed into 6-well plates, and 10 individual colonies were selected 3 weeks later and grown to confluence in individual 10 cm plates. Membranes were collected and screened for receptor expression using 4 nM [^{3}H]5-CT with 10 μM 5-HT to define nonspecific binding. Receptor binding site density in the clone with the highest specific binding in an initial screen (CHTR7beta) was assessed with [^{3}H]5-CT saturation binding using established methods (Supporting Information Figure S1). CHTR7beta was used for all remaining 5-HT$_7$ pharmacology studies.

Transiently transfected HEK293 cells at less than P20 were used for all other GPCR assays, with the exception of tests of 5-HT$_{1A}$ function, wherein CHO-K1 cells (ATCC no. CCL-61) less than P10 were used; attempts to measure 5-HT$_{1A}$ agonist signaling in transiently expressing HEK293 cells were fruitless. Receptor competition binding experiments were performed as previously described,[30,62] based on firmly established methods.[63] Affinity was assessed a minimum of three times with samples in triplicate or greater, unless the initial K_i was greater than 1 μM, in which case, only a second confirmatory screen was performed. Radioligands, used at approximate K_D concentrations for competition binding experiments, were tritriated 5-CT (5-HT$_{1A}$, 5-HT$_7$), ketanserin (5-HT$_{2A}$), mesulergine (5-HT$_{2B}$, 5-HT$_{2C}$), mepyramine (H$_1$), raclopride (D$_2$), and prazosin (α_{1A}, α_{1B}). Nonspecific binding was determined in the presence of 10 μM 5-HT (5-HT$_{1A}$, 5-HT$_7$), mianserin (5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$), mepyramine (H$_1$), or spiperone (D$_2$) or 20 μM mianserin (α_{1A}, α_{1B}).

5-HT$_7$-G$_s$-mediated cAMP production and 5-HT$_{1A}$-G$_i$-mediated reduction of 1.0 μM (EC$_{90}$) forskolin-stimulated cAMP were measured using PerkinElmer's Lance Ultra cAMP kit, with methods described by the manufacturer.[64] HEK293 or CHO-K1 cells expressing 5-HT$_7$ or 5-HT$_{1A}$ receptors, respectively, were harvested in HBSS (Lonza, Hopkinton, MA) and then pelleted by centrifugation at 200g at 37 °C for 5 min. Cells were resuspended in stimulation buffer (1× HBSS, 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA, pH 7.4). Cells were counted using a hemacytometer (Hausser Scientific, Horsham, PA) and diluted to 500 (5-HT$_7$) or 600 (5-HT$_{1A}$) cells/μL in stimulation buffer. Five microliters of cell suspension and 5 μL of test compounds diluted in stimulation buffer with (5-HT$_{1A}$ CHO-K1) or without (5-HT$_7$ HEK293) 1.0 μM forskolin or stimulation buffer alone (to define baseline activity) were added to each well of a 384-well plate (Greiner Bio-One, Monroe, NC). The plate was incubated at room temperature for 30 min. After incubation, the reaction was terminated by adding 5 μL of the Eu-cAMP tracer and 5 μL of ULight-anti-cAMP mixed in cAMP detection buffer (from assay kit). The plate was incubated at room temperature for 1 h to reach equilibrium. cAMP levels were detected by the FRET emission at 665 nM using Synergy H1 reader with a Lance filter cube (BioTek, Winooski, VT). 5-HT$_2$-G$_q$ signaling was measured using the Cisbio (Bedford, MA) IP-One HTRF assay (which detects inositol phosphate 1 (IP1)), and FRET data were collected using the Synergy H1 reader with an HTRF filter cube as we previously described.[65] All *in vitro* ligand pharmacology assays were performed a minimum of three times, with a minimum of three data points per ligand concentration.

***In Vivo* Pharmacology.** All mouse subjects were males and were obtained from the Jackson Laboratory (Bar Harbor, ME) as adults (60 days old). Upon arrival at Northeastern University, mice were housed four/cage and were then maintained in a vivarium on a 12 h light/dark cycle (lights on at 0800) with *ad libitum* access to chow and water. Behavioral testing began a minimum of 2 weeks later and involved transporting mice in their home cages to a temperature (70−73 °F)-controlled testing room two floors above their vivarium. Mice were habituated, in their home cages, to the testing room for a minimum of 2 h prior to commencing testing. During this habituation phase, compounds were prepared in vehicle and sterile-filtered. Vehicle was Milli-Q water (EMD Millipore, Billerica, MA) for all compounds, except AS-19, which was prepared in a maximum (i.e., for the 10 mg/kg dose) of 5% DMSO in water (2.8 and 1.5% DMSO for the 5.6 and 3 mg/kg doses, respectively). Compounds were then administered at 0.1 mL/10 g body weight. Mice were placed into an open-field (43 × 43 cm, Med Associates, St. Albans, VT) for behavioral observations 10 min after the last compound was administered. The open-field was cleaned with AccelTB disinfectant and dried prior to the testing of each mouse. We have found this injection and timing strategy to be effective for several 2-aminotetralin compounds.[30] Automatic measuring of behaviors, including distance traveled (cm) and rotations (360° body rotations around an animal's axis, "threshold" set at 90°), was performed by Noldus Ethovision XT9 software and an overhead camera tracking system (Noldus Information Technology, Leesburg, VA) linked to a Dell Precision T5600 PC. All other behaviors were scored by an observer(s) blind to treatment. All behavioral procedures were approved by Northeastern University Division of Laboratory Animal Medicine and were performed in accordance with the Guide for the Care and Use of Laboratory Animals.[66]

Test of (+)-5-FPT on Idiopathic Stereotypic Jumping. Sixteen adult (∼75 days old) C58/J mice were used for assessing the effects of (+)-5-FPT on idiopathic stereotypy. Mice received a sc injection of vehicle or (+)-5-FPT (1 or 3 mg/kg) and were then placed in the open field 10 min later. A jump was defined as a discrete act of pushing upward with hind limbs until both limbs simultaneously left the ground, and the number of jumps was counted over a period of 10 min, using a tally counter. Each C58/J mouse was tested four times, using a randomized grouping strategy (Supporting Information Table S1) that involved an 8 day washout period for each dose of (+)-5-FPT. Each mouse received vehicle treatment two times, matched for each dose of (+)-5-FPT used (1 and 3 mg/kg), i.e., once between each dose. Thus, each mouse served as its own control. Prior to testing, an average minimum number of 10 jumps/10 min after vehicle was set for inclusion criterion; one mouse did not meet inclusion criterion. The average number of jumps/10 min after each vehicle treatment was not statistically different and therefore these numbers were averaged before statistical analyses for treatment effects. One mouse with a bottom right incisor malocclusion that was eight grams lighter than cage mates at the time of testing was sacrificed. Two mice were found dead in their home cages, and the cause of death was unknown (no physical trauma was apparent). Thus, 12 mice completed the study.

Test of (+)-5-FPT on MK-801-Elicited Stereotypic Rotations and Hyperlocomotion. C57BL/6J mice were used to test the effects of (+)-5-FPT on MK-801-elicited behavior. In addition to the often reported MK-801-elicited increase in locomotor behavior, C57BL/6J mice administered 0.3 mg/kg MK-801 sc also display disrupted coordination and balance and repetitive rotational behavior that persists for at least 30 min. As NMDA receptor dysfunction has been linked with ASD,[43−45] MK-801-elicited repetitive rotation was used as a model of drug-induced stereotypy. Vehicle or (+)-5-FPT (5.6 mg/kg) was administered sc 10 min prior to injection of vehicle or MK-801 (0.3 mg/kg) sc. Ten minutes later, mice were placed in the open field for a 30 min observation and recording period. Mice were tested only one time in this model. Two mice were excluded from analyses, one due to skin lesions from in-cage fighting and one that showed abnormal hypolocomotion and corner-sitting. The final number of subjects/group was as follows: vehicle plus MK-801 (0.3 mg/kg) = seven; (+)-5-FPT (5.6 mg/kg) plus MK-801 (0.3 mg/kg) = eight; (+)-5-FPT (5.6 mg/kg) plus vehicle = six; vehicle plus vehicle = six. [The vehicle plus vehicle group was also used for the AMP study below.]

Test of (+)-5-FPT on Amphetamine-Induced Hyperlocomotion. The methods for AMP-induced hyperlocomotion in treatment-naïve C57BL/6J mice were as previously described, with no alterations.[30] A single dose of (+)-5-FPT, 5.6 mg/kg, was tested in this model. The observation period was 30 min. Mice were tested only one time in this model, and no mice were excluded from analyses. The number of subjects/group was as follows: vehicle plus AMP (3 mg/kg) = eight; (+)-5-FPT (5.6 mg/kg) plus AMP (3 mg/kg) = six.

Test of (+)-5-FPT and (−)-5-FPT on DOI-Elicited Stereotypic Head Twitching. Alterations in 5-HT$_{2A}$ receptors have been observed in Tourette and Asperger syndromes as well as in *Fmr1* KO mice.[48−50] Furthermore, 5-HT$_2$ antagonists decrease stereotypy when administered centrally to subthalamic nuclei[52] and decrease tics in children with Tourette syndrome.[51] We used these lines of evidence

DOI: 10.1021/acschemneuro.5b00099
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to include the head-twitch response (HTR) elicited by the 5-HT_2 agonist DOI as a model of stereotypy. The methods for this assay using treatment-naïve C57BL/6J mice were as described in the literature with no alterations.[30] During the 10 min observation session, DOI (1 mg/kg)-elicited HTRs, defined as rapid, discrete, paroxysmal twitches of the head were counted, using a tally counter. Mice were tested only one time in this model, and no mice were excluded from analyses. Three doses of (+)-5-FPT were used: 1, 3, and 5.6 mg/kg. (−)-5-FPT (5.6 mg/kg) was also tested in this assay for comparison because of its higher affinity at 5-HT_{2A} receptors, relative to that of (+)-5-FPT (see Results and Discussion). The commercially available, selective 5-HT_7 agonist AS-19 and the high potency 5-HT_{1A}/moderate potency 5-HT_7 agonists (*R*)-(+)-DPAT and (*S*)-(−)-DPAT , were also tested for comparison in this assay. No mice were excluded from analyses. The number of subjects/group was as follows: vehicle plus DOI (1 mg/kg) = 19; (+)-5-FPT (1, 3, or 5.6 mg/kg) plus DOI (1 mg/kg) = six per dose; AS-19 (3, 5.6, or 10 mg/kg) plus DOI (1 mg/kg) = six per dose; (*R*)-(+)-DPAT (0.5 mg/kg) plus DOI (1 mg/kg) = six; (*S*)-(−)-DPAT (0.5 mg/kg) plus DOI (1 mg/kg) = six; (−)-5-FPT (5.6 mg/kg) plus DOI (1 mg/kg) = four; vehicle plus vehicle = five; (+)-5-FPT (5.6 mg/kg) plus vehicle = five. HTR was not observed at significant levels in these latter two groups, although vehicle-treated C57BL/6J mice occasionally exhibit an HTR. For clarity, HTR data from subjects of these groups are not shown in Figure 7 (which displays the DOI-elicited HTR results); however, locomotor data (distance traveled) from these subjects are shown in Figure 8. HTR data were also collected from subjects in the serotonin syndrome study (next section) and are shown in Table 3.

Test of (+)-5-FPT To Elicit Serotonin Syndrome. For each session, two C57BL/6J littermates were injected sc with either vehicle or 5.6 mg/kg (+)-5-FPT and placed into the open field for observation 10 min later. Two observers blind to treatment recorded the occurrence of serotonin syndrome-like responses (SSR) during six 1 min sessions, each separated by 5 min (6 min of recorded observation over a 30 min period), for each mouse. SSR were determined as not present (=0) or present (=1) during each observation session, for a total score of between 0 and 6. SSR scored in this manner included flat body posture, forepaw treading, grooming, head weaving, hind limb abduction, moon walking, piloerection, Straub tail, and tremor, as previously described.[67] In addition, the total number of HTR and rears displayed across all six observation sessions were also tallied. No mice were excluded from analyses. The number of subjects/group was seven.

Test of (+)-5-FPT on Social Interactions. During initial observations of pairs of littermates for SSR, we noticed that some mice appeared to show more social engagements than others. Thus, we tested whether (+)-5-FPT also affected social interactions with littermates. During the SSR observation period (above), we scored the number of social interactions produced by each mouse, with a social interaction being predefined as one mouse approaching the other mouse, resulting in direct nose-to-body (including nose-to-nose, nose-to-torso, etc.) contact between the approaching mouse and the recipient mouse, respectively. One mouse walking by the other mouse that involved body contact was not scored as a social interaction. The same blind observers scoring SSR also scored social interactions blind to treatment. No mice were excluded from analyses. The number of subjects/group was vehicle = six, and (+)-5-FPT, 5.6 mg/kg = five.

Behavioral Duration of Action of (+)-5-FPT Using the DOI-Elicited Head-Twitch Model. After a minimum 4 week drug washout period, C57BL/6J mice from earlier studies were reused and were approximately 4 months old at the time of testing. Mice were injected sc with (+)-5-FPT (5.6 mg/kg) 180, 120, or 60 min prior to testing in the DOI HTR model. DOI (1 mg/kg) was injected sc 10 min prior to testing. Mice were placed into the open field chamber, and HTRs were counted as above. Two mice were excluded from analyses due to abnormal hypolocomotion and corner-sitting. Five mice per time point were included in the final analyses.

Plasma and Whole Brain (+)-5-FPT Concentrations after Systemic Administration. Adult male C57BL/6J mice, approximately 6 months old, and treatment-naïve for at least 6 weeks prior to testing, were injected sc with (+)-5-FPT (3.0 mg/kg) and returned to their home cages. At 30, 60, or 90 min later, mice were euthanized by rapid cervical dislocation and decapitation. Trunk blood was collected in prechilled, heparin-coated tubes. Brains were quickly excised and frozen in liquid nitrogen. Plasma was collected from blood after centrifugation for 5 min at 13 000*g*. Whole brain samples were wrapped in foil, and brain and plasma samples were labeled and stored at −80 °C until liquid chromatography−mass spectrometry/mass spectrometry (LC-MS/MS) assays were performed.

Frozen brain samples were weighed and homogenized in phosphate buffered saline (PBS), pH 7.4. After the first analysis, the extra brain homogenate was stored at −80 °C until they were thawed for a second, more dilute, analysis. Plasma samples were used directly upon arrival. The proteins from each plasma sample and a portion of each brain homogenate were immediately precipitated with 1:1 methanol/acetonitrile (4× starting volume) and internal standard ((−)-MBP[68]) followed by centrifugation at 14 000*g* for 5 min at 4 °C. The resulting supernatants from each sample were dried under nitrogen. Each sample was reconstituted in methanol, vortexed, sonicated briefly, and centrifuged prior to LC-MS/MS analysis. Calibration curves were constructed from the ratios of the peak areas of 5-FPT versus (−)-MBP in extracted standards made in mouse plasma or homogenized mouse brain.

LC-MS/MS analysis was performed using an Agilent 1100 series HPLC and a Thermo Finnigan Quantum Ultra triple quad mass spectrometer. The mobile phases used were 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) in a 5 min gradient. Samples of 10 μL each were injected onto a Phenomenex Gemini C18 column (2 × 50 mm, 5 μ) with a C18 guard column. 5-FPT and its internal standard ((−)-MBP) were ionized in ESI+ and detected in SRM mode. Internal standards were used for quantification of the compound level per gram of tissue or per microliter of plasma. Four mice were included per group, but plasma levels from one mouse were not detectable due to a low volume of blood collected.

Test of Orally Administered (+)-5-FPT on DOI-Elicited Head Twitching. Treatment-naïve male C57BL/6J mice approximately 2.5 months old were administered vehicle or (+)-5-FPT (3.0 mg/kg) orally (0.1 mL/10 g body weight) via the gavage method 10 min prior to a sc injection of DOI (1 mg/kg). Ten minutes later, mice were placed into the open field, and HTRs were counted for 10 min as above. No mice were excluded from analyses, and five mice per group were tested.

Statistics. All data were analyzed using GraphPad Prism 6.05 software (La Jolla, CA). Comparisons of mean stereotypy scores obtained from mice treated with vehicle or test compound(s) were performed with one-way repeated-measure ANOVA for the C58/J stereotypic jumping study and ordinary one-way ANOVA for DOI-elicited HTR tests; Dunnett's posthoc tests were used for multiple comparisons. Ordinary one-way ANOVA tests with Tukey's multiple comparison were used to assess differences in mean stereotypy scores and locomotion (distance traveled) in MK-801 and amphetamine (AMP) models. A statistically significant difference was defined as $P < 0.05$. *P* values are noted with asterisks in figures and are defined as *, $P < 0.05$; **, $P < 0.005$; ***; $P < 0.0005$; and ****, $P < 0.0001$. All asterisks in figures represent differences from vehicle group unless explicitly shown. *In vitro* pharmacology data were analyzed using nonparametric curve-fitting algorithms in Prism to obtain K_i, K_D, B_{max}, EC_{50}, and E_{max} values, as previously described.[30,62]

■ ASSOCIATED CONTENT

Supporting Information

Supporting information includes: a representative graph showing [^{3}H]5-CT 5-HT_7 saturation binding isotherms obtained from CHTR7beta, human 5-HT_7 stable-expressing cells; a representative graph showing (+)-5-FPT function at 5-HT_2 receptor subtypes; a table showing the grouping strategy for C58/J jumping assays; and ^{1}H and ^{13}C NMR data for 5-FPT. The Supporting Information is available free of charge on

DOI: 10.1021/acschemneuro.5b00099
ACS Chem. Neurosci. 2015, 6, 1259−1270

the ACS Publications website at DOI: 10.1021/acschemneuro.5b00099.

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Author Contributions

C.E.C. and R.G.B. designed the experiments. C.E.C. wrote and R.G.B. edited the manuscript. C.E.C., D.E.F., W.Z., and Y.L. conducted pharmacological experiments and analyzed data. J.T.W. performed the LC/MS-MS experiments measuring plasma and brain concentrations of (+)-5-FPT after samples were collected by C.E.C. C.E.C. and D.E.F. conducted behavioral experiments; C.E.C. performed drug administrations, and D.E.F. was the observer and scorer blind to treatment. C.K.P. also was also a scorer in the serotonin syndrome and social interaction experiments and was also blind to treatment. C.E.C. analyzed behavioral data. R.V. developed the synthetic schemes, synthesized the initial batches of (+)- and (−)-5-FPT, and verified the purity of the compounds. 5-FPT compounds synthesized by R.V. were used for all but the serotonin syndrome and social interaction experiments; C.K.P. synthesized the (+)-5-FPT used for these latter experiments.

Funding

This work was funded by USPHS (NIH) R01MH081193, R01MH068655, and R01DA030989 awarded to R.G.B.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

MK-801, dizocilpine; AMP, *d*-amphetamine; DOI, (±)-(2,5)-dimethoxy-4-iodoamphetamine; HTR, head-twitch response; SIB, self-injurious behavior; ASD, autism spectrum disorders; FXS, fragile X syndrome; ADHD, attention deficit hyperactivity disorder; 5-FPT, 5-(2′-fluorophenyl)-2-dimethylaminotetralin; DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; AS-19, (2*S*)-(+)-5-trimethylpyrazolyl-2-dimethylaminotetralin; GPCR, G protein-coupled receptor

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DOI: 10.1021/acschemneuro.5b00099
ACS Chem. Neurosci. 2015, 6, 1259−1270