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The synthetic cathinone psychostimulant α -PPP antagonizes serotonin 5-HT_{2A} receptors: In vitro and in vivo evidence

Yiming Chen¹, Bruce E. Blough², Kevin S. Murnane¹, and Clinton E. Canal¹

¹Department of Pharmaceutical Sciences, College of Pharmacy, Mercer University, Atlanta, GA, USA

²Center for Drug Discovery, Research Triangle Institute, Research Triangle Park, NC, USA

Abstract

Synthetic cathinones (SCs) are β -keto analogs of amphetamines. Like amphetamines, SCs target monoamine transporters; however, unusual neuropsychiatric symptoms have been associated with abuse of some SCs, suggesting SCs might possess additional pharmacological properties. We performed radioligand competition binding assays to assess the affinities of nine SCs at human 5-HT_{2A} receptors (5-HT_{2A}R) and muscarinic M₁ receptors (M₁R) transiently expressed in HEK293 cells. None of the SCs exhibited affinity at M₁R (minimal displacement of [$\sim K_d$] [3H]scopolamine up to 10 μ M). However, two SCs, α -pyrrolidinopropiophenone (α -PPP) and 4-methyl- α -PPP, had low μ M K_i values at 5-HT_{2A}R. In 5-HT_{2A}R–phosphoinositide hydrolysis assays, α -PPP and 4-methyl- α -PPP displayed inverse agonist activity. We further assessed the 5-HT_{2A}R functional activity of α -PPP, and observed it competitively antagonized 5-HT_{2A}R signaling stimulated by the 5-HT_{2R} agonist ($\pm$)-2,5- dimethoxy-4-iodoamphetamine (DOI; K_b = 851 nM). To assess in vivo 5-HT_{2A}R activity, we examined the effects of α -PPP on the DOI-elicited head-twitch response (HTR) in mice. α -PPP dose-dependently blocked the HTR with maximal suppression 10 mg/kg ($P < 0.0001$), which is a moderate dose used in studies investigating psychostimulant properties of α -PPP. To corroborate a 5-HT_{2A}R mechanism, we also tested 3,4-methylenedioxy- α -PPP (MDPPP) and 3-bromomethcathinone (3-BMC), SCs that we observed had 5-HT_{2A}R K_i s > 10 μ M. Neither MDPPP nor 3-BMC, at 10 mg/kg doses, attenuated the DOI HTR. Our results suggest α -PPP has antagonist interactions at 5-HT_{2A}R in vitro that may translate at physiologically-relevant doses in vivo. Considering 5-HT_{2A}R antagonism has been shown to mitigate effects of psychostimulants, this property may contribute to α -PPPs unpopularity compared to other monoamine transporter inhibitors.

Keywords

α -pyrrolidinopropiophenone (α -PPP); cathinone; psychostimulant; 5-HT_{2A}; 2,5-dimethoxy-4-iodoamphetamine (DOI)

1 | INTRODUCTION

Synthetic cathinones (SCs), exemplified by the schedule 1 drugs 4-methyl-*N*-methylcathinone (mephedrone), α -pyrrolidinopentiophenone (α -PVP), and methylenedioxypyrovalerone (MDPV), are β -keto analogs of amphetamines. They are powerful psychostimulants that produce effects similar to other illicit drugs, including cocaine, 3,4-methylenedioxymethamphetamine (MDMA), and methamphetamine.^{1–3} Like amphetamine, the cathinone scaffold is amenable to several, slight, chemical modifications that permit the synthesis of numerous, unique SCs that retain psychoactive properties. SCs have been surreptitiously marketed as “bath salts,” “research chemicals not for human consumption,” and “plant food,” and despite increased local and federal awareness of SCs and subsequent restriction of their distribution and use, certain SCs can still be purchased online. Desirable effects of SCs include euphoria, increased empathy, sociability, energy, and alertness. Grave side effects, typically manifested after administration of high doses or after prolonged use of SCs, can include rhabdomyolysis, autonomic symptoms such as tachycardia, hypertension, and hyperthermia, as well as many psychiatric symptoms such as anxiety, agitation, and fear that can progress, in some cases, to psychosis (delusions and hallucinations) and excited delirium.^{4–9} There have also been several fatalities associated with SC overdose.¹⁰

SCs, like amphetamines, primarily target monoamine transporters as inhibitors or substrate analogs.¹¹ Some SCs, however, seem to produce more intense psychiatric adverse events than amphetamines. For example, MDPV and α -PVP appear to have a propensity for inducing psychosis with excited delirium.⁹ Although chronic amphetamine abuse is well-known to cause psychosis in susceptible individuals,^{12–14} psychosis with excited delirium is less reported in the literature. MDPV is >20-fold and > 50-fold more potent than amphetamine and cocaine, respectively, at blocking uptake at the dopamine transporter (DAT).³ Similarly, α -PVP is 15-fold more potent than cocaine at inhibiting DAT.¹⁵ MDPVs and α -PVPs high potencies at DAT probably account for most of their perilous effects. Indeed, deficits in the dopamine transporter have been observed in brains of cocaine users who have died after displaying excited delirium.¹⁶

Nevertheless, SCs also have distinctive pharmacokinetic profiles, such as long half-lives¹⁷ and distinctive neuromolecular effects.¹⁸ Furthermore, some SCs interact with targets other than monoamine transporters.^{19,20} These properties may also contribute to the psychological and behavioral effects of SCs. Reports show that certain SCs have appreciable affinity at 7-transmembrane, G protein-coupled receptors,^{21–24} and one publication reports that 2,3-dimethylmethcathinone is a 5-HT_{2A} receptor (5-HT_{2A}R) agonist.²⁵ Few studies, however, have closely considered such off-targets as liabilities, and we are aware of no studies that have linked off-target activity to specific behavioral outcomes.

We hypothesized that at high doses, SCs can target G protein-coupled receptors, including activating serotonin 5-HT_{2A}R and inhibiting muscarinic M₁ receptors (M₁R), that, together with monoamine transporter perturbation, might contribute to psychosis and delirium associated with abuse of SCs.^{26,27} Our results from tests of nine SCs, including five pyrrolidine-containing and four ring-substituted SCs, however, did not support this hypothesis. In vitro pharmacology experiments showed that none of the nine SCs we tested

had affinity at M₁R (i.e, at concentrations up to 10 μ M, none displaced more than 10% of radioligand from M₁R), and only two, α -pyrrolidinopropiophenone (α -PPP) and 4-methyl- α -PPP, had affinities at 5-HT_{2A}R with $K_i < 3.5 \mu$ M. Unexpectedly, both α -PPP and 4-methyl- α -PPP consistently displayed inverse agonist activity at 5-HT_{2A}R. As α -PPP displayed higher affinity for 5-HT_{2A}R relative to 4-methyl- α -PPP, we further characterized its pharmacology at 5-HT_{2A}R. We observed that α -PPP competitively antagonized, at high nanomolar concentrations, human 5-HT_{2A}R expressed in vitro, and blocked the 5-HT_{2A}R-dependent, head-twitch response (HTR)^{28,29} elicited by the 5-HT₂R agonist, ($\pm$)-2,5-dimethoxy-4-iodoamphetamine (DOI) in mice.

Delivering an armamentarium of supportive evidence from the literature, we present the case that α -PPPs activity at 5-HT_{2A}R is physiologically relevant. We discuss this pharmacology in the context of the relative unpopularity of α -PPP in the illicit drug scene. α -PPP has been available on the illicit drug market for about two decades,³⁰ and yet relative to other SCs, for example MDPV, methylenedioxymethamphetamine (MDA), and *N*-ethylpentylone, there is relatively scant discussion about it on online drug forums, including Reddit, Bluelight, and Drugs-Forum. Moreover, it remains unscheduled by the Drug Enforcement Agency (DEA) of the United States, and in the last two years, it is one of the less common SCs identified in seizures by the DEA (DEA Emerging Threat Reports, 2016–2018). α -PPPs unpopularity is peculiar, as it has similar potency to cocaine as a DAT inhibitor, and it has high selectivity for DAT, relative to the serotonin transporter (SERT),¹⁵ which together, would seemingly make it a pervasive drug of abuse. We contend that the 5-HT_{2A}R antagonist activity of α -PPP may mitigate its effects at DAT.

2 | EXPERIMENTAL

2.1 | Compounds

Nine SCs (Figure 1) – 2-fluoromethcathinone, 2-(trifluoromethoxy) methcathinone, 3-bromomethcathinone (3-BMC), 4-bromomethcathinone, 3,4-methylenedioxy- α -pyrrolidinopropiophenone (MDPPP), α -PPP, 3-bromo- α -PPP, 4-bromo- α -PPP, 4-methyl- α -PPP, all racemic HCl salts – were synthesized by Bruce Blough at Research Triangle Institute. Racemic 2,5-dimethoxy-4-iodoamphetamine HCl (DOI) was purchased from Sigma-Aldrich (St Louis, MO, USA). MDL100907 HCl (volinanserin) was a gift from Kenner Rice. Chlorpromazine HCl was a gift from Raymond Booth. Atropine sulfate monohydrate was purchased from Alfa Aesar (Tewksbury, MA, USA). [³H]Ketanserin (specific activity 47.3 Ci/mmol) and N-methyl-[³H]Scopolamine (specific activity 80.1 Ci/mmol) were purchased from Perkin Elmer (Waltham, MA, USA).

2.2 | Cell culture and transfection

HEK293 cells (P < 18 from a procured stock, CRL-1573, ATCC, Manassas, VA, USA) were grown in 10 cm cell culture plates with Dulbecco's modification of Eagle's medium containing L-Glutamine and 4.5g glucose per liter (DMEM, BE12-604Q, Lonza, Basel, Switzerland) and supplemented with 10% regular fetal bovine serum (35-010-CV, Corning, NY, USA) at 37°C, 5% CO₂, and 95% humidity under sterile conditions. When cells reached ~90% confluency, they were transfected with human 5-HT_{2A}R or M₁R cDNA (cDNA

resource center, Bloomsburg, PA, USA) using lipid-based transfection (LipoD293 reagent, SignaGen Laboratories, Rockville, MD, USA) or electroporation (Neon transfection system, Invitrogen, Carlsbad, CA, USA) methods. For lipid-based transfections, 15 μL of LipoD293 reagent in 250 μL of DMEM was added to 5 μg of cDNA in 250 μL of DMEM, and the mixture was incubated at room temperature for 10 minutes before being added to the cells; medium was replaced after 18 hours. For electroporation transfections, cells were dissociated with 2 mL Hank's balance salt solution containing 0.25% trypsin (Lonza, Basel, Switzerland), collected, then centrifuged at 200 x g for 5 minutes at room temperature. Cells were then resuspended in phosphate buffered saline (without Ca^{2+} or Mg^{2+}) and centrifuged again, as just described. Cells were then resuspended in 100 μL "resuspension buffer R" (Invitrogen) at a final density of 2.0×10^7 cells/mL. The cell mixture was mixed with 10 μL of cDNA, and electroporation was performed with two pulses of 1100 V, at a width of 20 ms). Forty-eight hours after transfection with either method, cells were dislodged by forceful pipetting. Cells were centrifuged at 12,000 x g for 10 minutes at 4°C; pellet was homogenized in ice cold 50 mM Tris-HCl, 10 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 mM EDTA, pH 7.4 (assay buffer) with a glass homogenizer, followed by centrifugation under the same conditions two more times. Membrane pellets were stored at -80°C prior to being used for radioligand binding assays.

2.3 | Radioligand competition binding

Radioligand binding assays were conducted using methods we have previously published.^{31,32} Briefly, in 96-well plates, membranes expressing transfected receptors were incubated with 1.6 nM ($\sim K_d$ concentration) [^3H]Ketanserin to label 5-HT_{2A}R or 1 nM ($\sim K_d$ concentration) of N-methyl-[^3H]Scopolamine to label M₁R and increasing half-log unit concentrations of test compounds in assay buffer. DOI or volinanserin and atropine were included as positive controls for 5HT_{2A}R and M₁R, respectively, in each assay. Volinanserin or chlorpromazine for 5-HT_{2A} and atropine for M₁ at 10 μM were used to define non-specific binding. After a 90-minute incubation at room temperature on a shaker, contents from the plates were rapidly filtered through GF/B fiberglass filter mats, pre-soaked with ice cold 50 mM Tris buffer, using a Microbeta Filtermat-96 cell harvester (Perkin Elmer, Waltham, MA, USA). Approximately 200 mL of ice cold, 50 mM Tris buffer was then vacuumed through filter mats to wash away unbound radioligand. Filter mats were dried on a hot plate, then soaked in Betaplate (Perkin Elmer, Waltham, MA, USA) scintillation fluid in plastic bags. Bags were sealed and placed in cassettes. Radioactivity was measured in a Microbeta² Microplate Counter (Perkin Elmer, Waltham, MA, USA), and counts per minute (CPMs) corresponding to each well on the plates were recorded. For each assay plate, each concentration of the positive control was tested in duplicate, and each concentration of the test compounds was tested in triplicate.

2.4 | 5-HT_{2A}R phosphoinositide hydrolysis

The IP-One homogeneous time resolved fluorescence (HTRF) kit (Cisbio, Bedford, MA, USA) was used to measure 5-HT_{2A}R function of the SC cathinones that had $K_i < 3.5 \mu\text{M}$ at 5-HT_{2A}R (α -PPP and 4-methyl- α -PPP). This assay, which measures G α_q -mediated phosphoinositide hydrolysis via competition between endogenous inositol phosphate 1 (IP1) and fluorophore-labeled IP, was performed per the manufacturer's protocol with

optimization based on our published methods.^{33,34} Expression of 5-HT_{2A}R using LipoD293 reagent was performed as detailed in section 2.2, with one exception. Transfection reagent was replaced with fresh medium after 8 hours to improve cell viability. Twenty-four hours after transfection, cells were serum starved for 2 hours in DMEM. Cells were then collected and plated at 2500 cells per well in 384-well plates. Cells were immediately treated with α -PPP, 4-methyl- α -PPP, or DOI (positive control) at increasing half-log unit concentrations in stimulation buffer containing 50 mM LiCl, and then incubated at 37°C for 2 hours. For competitive antagonist experiments, cells were treated simultaneously with increasing half-log unit concentrations of DOI and 10, 30, or 100 μ M α -PPP. The reaction was terminated with lysis buffer containing d2-labeled IP1 and anti-IP1-Cryptate. HTRF resonance energy transfer was measured by a Mithras LB 940 Multimode Microplate Reader (Berthold Technologies, Bad Wildbad, Germany). For all assays, each concentration of the positive control and the test compounds was tested in triplicate.

2.5 | DOI-elicited HTR

Adult female FVB mice (stock 004828, Jackson Laboratory, Bar Harbor, ME, USA), between the ages of two and six months old (21–29 g) and bred in our vivarium, were used to assess the effects of α -PPP, MDPPP, and 3-BMC (the latter two as negative controls) on the DOI-elicited head-twitch response (HTR). Mice were housed in standard cages, up to four mice per cage, in Mercer University's Atlanta campus vivarium, on a 12-hour light–dark cycle (lights off at 7:00 PM) with ad libitum access to water and food. Prior to the start of testing, mice were transported in their home cages to a nearby mouse procedure room, and acclimated there for 30 minutes. Testing was performed between 11:00 AM and 4:00 PM, and consisted of subcutaneous (s.c.) administration of MilliQ (Millipore) water (vehicle), α -PPP (3 or 10 mg/kg, 0.3 or 1.0 mg/mL of vehicle), MDPPP (3 or 10 mg/kg, 0.3 or 1.0 mg/mL of vehicle), or 3-BMC (10 mg/kg, 1.0 mg/mL vehicle) followed 10 minutes later by s.c. injection of DOI (1 mg/kg, 0.1 mg/mL of vehicle). Ten min later, mice were placed into a clean, clear, plastic box (45×20×23 cm) for a 10-minute observation period. During this session, HTRs were counted by an observer (YC) blind to treatment. All drug solutions were freshly prepared by YC and CEC prior to their administration. Treatment groups were quasi-randomized prior to testing, so that each treatment was administered on each testing day until all tests were completed. Experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, and were approved by the Mercer University's Animal Care and Use Committee.

2.6 | Data analyses

All data were analyzed by GraphPad 8.0.1 software (San Diego, CA, USA). In vitro pharmacology data were analyzed using non-linear, curve-fitting models to determine K_i , pA_2/K_b , and EC_{50} values. Compounds exhibiting $K_i > 10 \mu$ M were reassessed in at least one additional assay for confirmation ($N = 2$). All compounds exhibiting $K_i < 10 \mu$ M were reassessed in a minimum of two additional assays ($N = 3$). At least three independent assays were performed for each of the 5-HT_{2A} phosphoinositide hydrolysis experiments ($N = 3$). Only data with curve fit $R^2 > 0.8$ were included in the data analyses. DOI HTR data were analyzed by ordinary one-way ANOVA with posthocDunnett's multiple comparisons tests, comparing individual treatment groups to the vehicle/DOI group. Significance was set to α

< 0.05. We designed DOI HTR experiments to include a minimum of five subjects per group, based on our previous experience and results from the literature that this number (or an approximate thereof) generates reliable, reproducible data that allow for determinations of potential treatment effects.^{35,36} Experiment and subject numbers included in the final data analyses are reported below. Data are reported as means $\pm$ SEMs.

3 | RESULTS AND DISCUSSION

3.1 | α -PPP is a 5-HT_{2A}R inverse agonist and competitive antagonist

Activation of 5-HT_{2A}R and inhibition of muscarinic receptors can precipitate psychotic symptoms and delirium,^{26,27} and these psychiatric adverse events are reportedly caused by abuse of certain SCs. Thus, we assessed the 5-HT_{2A}R and M₁R binding affinities of nine SCs, five representing pyrrolidine and four representing ring-substituted analogs (Figure 1). First, our observations that none of the nine SCs interacted with M₁R (less than 10% displacement of *N*-methyl-[³H]Scopolamine up to 10 μ M, *N* = 2–4, Table 1) suggest that direct modulation of M₁R is likely not involved in psychiatric adverse events associated with abuse of these SCs. [We highlight, however, that this set of SCs did not include MDPV or α -PVP, the major culprits in the reported associations between SC abuse and psychosis and delirium.] We also observed that only two SCs, α -PPP and 4methyl- α -PPP, had affinity at 5-HT_{2A}R < 3.5 μ M. The *pK_i* of α -PPP was 5.6 ± 0.7 (*K_i* = 2.5 μ M, *N* = 6), and the *pK_i* of 4-methyl- α -PPP was 5.5 ± 0.1 (*K_i* = 3.2 μ M, *N* = 3) (Table 1). Because MDPPP was tested in the DOI-elicited HTR assay as a primary negative control pyrrolidine SC, we tested it in competition binding assays at concentrations up to 100 or 300 μ M, in six independent experiments, to obtain its *pK_i* at 5-HT_{2A}R, which was 4.2 ± 0.1 (*K_i* = 63.6 μ M (Table 1)). Thus, MDPPP's affinity for 5-HT_{2A}R was ~25-fold lower than α -PPPs. The rank order of potencies at 5-HT_{2A}R among the five pyrrolidine SC derivatives were α -PPP > 4-methyl- α -PPP > 4bromo- α -PPP > 3-bromo- α -PPP ~ MDPPP (Table 1 and Figure 2). Three of the four ring-substituted SCs tested showed no appreciable affinity for 5-HT_{2A}R: Two displaced less than 10% of [³H]Ketanserin at 10 μ M concentrations (no binding), and one, 3-BMC, displaced between 10–20% of [³H]Ketanserin at 10 μ M concentrations (*pK_i* < 5). One ring-substituted SC, 4-bromomethcathinone, had an observed *pK_i* of 5.2 ± 0.1 (*K_i* = 6.3 μ M) at 5-HT_{2A}R (*N* = 2–3 for all ring-substituted SC, Table 1).

We further examined the functional effects of α -PPP and 4methyl-PPP at 5-HT_{2A}R. α -PPP displayed 5-HT_{2A}R inverse agonist activity, observed as dose-dependent decreases in IP1 production (Figure 3). 4-methyl- α -PPP produced similar results (data not shown). Thus, a second part of our original hypothesis, that the SCs we tested would activate 5-HT_{2A}R, was not supported. These data suggest that direct activation of 5-HT_{2A}R is likely not involved in psychiatric adverse effects associated with the SCs we tested. α -PPP and 4methyl- α -PPP functional activity at 5-HT_{2A}R was assessed in four separate assays, and in each assay, α -PPP and 4-methyl- α -PPP decreased production of IP1 relative to basal levels. However, results were variable, and nonlinear regression analyses did not adequately fit the data; *R*² values were typically less than 0.6, and 95% confidence intervals were very wide (the *R*² for representative data, Figure 3, was 0.72). Therefore, accurate IC₅₀ values were not obtained. This is not unusual, as 5-HT_{2A}R constitutive activity is low relative to other G protein-

coupled receptors, including the closely related 5-HT_{2B}R and 5-HT_{2C}R, and therefore difficult to detect and measure consistently.^{32,37} α -PPP was subsequently tested for competitive antagonist activity at 5-HT_{2A}R. As shown in Figure 4, α -PPP dose-dependently produced a dextral displacement of the DOI concentration-response curve, without altering its E_{MAX}. The pEC₅₀ of DOI, determined from all functional assays, was 9.4 ± 0.3 (0.4 nM, N = 7, which included four functional assays without competition, and three competitive antagonist assays). The pA₂ of α -PPP, calculated from competitive antagonism of DOI stimulated 5-HT_{2A} signaling, was 6.1 ± 0.2 SEM ($K_b = 851$ nM, N = 3), determined by Gaddum/Schild analyses.

3.2 | α -PPPs in vitro activity at 5-HT_{2A}R is physiologically relevant: A comparative analysis

The K_b (851 nM, competition with DOI) and K_i (2.5 μ M, competition with [³H]Ketanserin) values we obtained for α -PPP at 5-HT_{2A}R raise the question of whether or not α -PPPs affinity is physiologically relevant. Decisively, there are many other psychoactive substances with similar or even lower affinities at their well-recognized targets. For example, the prototypical psychostimulant amphetamine has been shown to bind to NET and DAT with 1.0 and 5.7 μ M affinity, respectively.²² MDMA has μ M affinity and IC₅₀ potency at SERT,^{21, 38} the putative, primary target underlying its psychoactive effects. Mephedrone, a popular SC,³⁹ has been shown to bind to DAT with 3.4 μ M affinity.²² Solriamfetol, a DAT/norepinephrine transporter (NET) inhibitor that has been used clinically for the treatment of excessive sleepiness has been shown to bind to DAT and NET with 14 μ M and 3.7 μ M affinities, respectively.⁴⁰

Micromolar affinities of psychoactive drugs at off-targets also might explain some of their toxicities. The synthetic opioid, fentanyl, for example, has 1.3 μ M affinity at 5-HT_{2A}R and 2.1 μ M affinity at 5-HT_{1A}R.⁴¹ Recent clinical studies report an association between fentanyl use and serotonin toxicity,^{42,43} and activation of 5-HT_{2A}R and/or 5-HT_{1A}R has been implicated in serotonin toxicity.^{44, 45} A recent preclinical study in rats showed that fentanyl dose-dependently exaggerates the serotonin toxicity-like responses induced by the 5-HT_{1A}R full agonist 8-OH-DPAT. Moreover, this effect is blocked by pre-treatment with the 5-HT_{1A}R antagonist WAY100653, but not by naloxone.⁴⁶ Most germane to our studies, the K_i of α -PPP at DAT is ~ 1.3 μ M,⁴⁷ an affinity that is only about two-fold higher than its affinity at 5-HT_{2A}R, and the IC₅₀ of α -PPP to inhibit DAT is ~ 332 nM,¹⁵ which is only about three-fold higher than its potency to antagonize 5-HT_{2A}R signaling, according to our observations. Still, until now, most effects produced by α -PPP have been attributed to its actions at DAT.

3.3 | On whether α -PPPs activity at 5-HT_{2A}R translates from in vitro to in vivo: Support from the 5-HT_{2A}R-dependent DOI-elicited HTR in mice

To examine in vivo 5-HT_{2A}R antagonist activity, we tested the effectiveness of α -PPP to reduce HTRs in mice elicited by DOI. To corroborate a 5-HT_{2A}R mechanism of α -PPP, we tested it in comparison to MDPPP and 3-BMC, which displayed negligible 5-HT_{2A}R affinities. As shown in Figure 5, FVB mice that received vehicle/DOI (1 mg/kg) exhibited 13 ± 1 HTRs (N = 10). This number is low relative to the number of DOI HTRs displayed by other mouse strains, such as C57BL/6J and DBA/2J mice, but akin to the number displayed

by the commonly used strains NIH Swiss and Swiss Webster (CFW) mice.²⁸ Pre-treatment with α -PPP dose-dependently blocked DOI-elicited HTRs. One-way ANOVA showed a treatment effect ($F(5, 38)=12.86, P<0.0001$), and post-hoc Dunnett's test confirmed that mice pre-treated with 10 mg/kg α -PPP ($N=8$) exhibited significantly fewer HTRs than mice treated with vehicle/DOI ($P<0.0001$). The average $\pm$ SEM number of HTRs in mice pre-treated with 10 mg/kg α -PPP followed by 1 mg/kg DOI was 1.5 ± 0.5 . Thus, 10 mg/kg α -PPP essentially abolished the DOI-elicited HTR. Mice pre-treated with 3 mg/kg α -PPP followed by 1 mg/kg DOI ($N=7$) also tended to exhibit fewer HTRs than the control group (Veh/ DOI), but the effect was not significant ($P=0.11$).

To provide evidence for in vivo 5-HT_{2A}R activity of α -PPP, we first chose to compare its effects to MDPPP. MDPPP is structurally similar to α -PPP but has ~25-fold lower affinity than α -PPP for 5-HT_{2A}R (Table 1 and Figures 1 and 2). MDPPP also has higher affinity and inhibitory potency at SERT, relative to α -PPP.^{47,48} Alterations in SERT activity can affect the DOI HTR.⁴⁹ For example, blocking SERT increases synaptic 5-HT levels which could compete with DOI for 5HT_{2A} binding sites, decreasing its effects. Pre-treatment with 3 or 10 mg/kg MDPPP ($N=7$ each), however, had no significant effect on the HTR elicited by DOI ($P=0.92$ and 0.19 , respectively, compared to vehicle/DOI).

Since MDPPP did not block the DOI HTR, despite having higher potency at SERT compared to α -PPP, this suggests that SERT inhibition was not the cause of α -PPP's effects on the DOI HTR. Nevertheless, 5-HT_{2A}R inverse agonist activity might alter SERT function by affecting the availability of phosphatidylinositol 4,5bisphosphate (PIP2). Direct PIP2 binding to SERT has been shown to mediate the formation of stable SERT oligomers expressed in the plasma membrane,⁵⁰ and in cells densely expressing 5-HT_{2A}R, 5-HT_{2A}R inverse agonists that decrease PLC activation could increase PIP2. Furthermore, the ability of SERT substrates, for example parachloroamphetamine, to stimulate sodium currents and induce 5HT efflux through SERT depends on the availability of PIP2.⁵¹ Thus, α -PPP's inverse agonist action at 5-HT_{2A}R might increase relative levels of stable SERT oligomers, and enhance their function via an increase in the relative availability of PIP2. It is yet to be revealed whether this is involved in α -PPP's efficacy to block the DOI HTR.

Since α -PPP has ~2 to 3-fold higher potency to inhibit NET compared to MDPPP,⁴⁷ we also considered the possibility that the effect of α -PPP to block the DOI-elicited HTR might be mediated by NET inhibition. Thus, we compared effects of α -PPP to 3-BMC, which has measurably higher potency to inhibit NET.⁵² If NET inhibition leads to a blockade of the DOI-elicited HTR, 3-BMC should be an effective inhibitor of the response. As shown in Figure 5, 10 mg/kg 3-BMC ($N=5$), had no effect on the DOI-elicited HTR ($P=0.95$, compared to Veh/DOI)—Reiterating, this dose of α -PPP abolished the response. Moreover, the highly selective SERT/NET inhibitor, venlafaxine, has about the same potency as α -PPP to inhibit NET, $IC_{50} \sim 316$ nM,⁵³ but acute administration of venlafaxine, at doses up to 30 mg/kg, does not inhibit the DOI HTR in mice.⁵⁴ These observations suggest that acute NET inhibition does not inhibit the DOI-elicited HTR, which argues that the effects of α -PPP in this assay were not mediated this pharmacological property. Likewise, it is unlikely the effect was mediated by DAT inhibition, as MDPPP has a measurably higher potency to inhibit DAT than α -PPP.¹⁵ Finally, none of the animals treated with DOI and α -PPP

behaved grossly differently from animals treated with DOI alone, DOI and MDPPP, or DOI and 3-BMC. All mice appeared hyperactive during testing; however, we did note that some mice treated with α -PPP appeared to exhibit less stereotypical behavior. In conclusion, though α -PPP could have activity at other currently undiscovered off-targets that can modulate the DOI-elicited HTR,²⁸ our most parsimonious explanation is that α -PPP blocks 5-HT_{2A} receptors in vivo.

3.4 | α -PPPs potency to abolish a 5-HT_{2A}R-mediated behavioral response is physiologically relevant

Preclinical studies that have examined α -PPPs psychostimulant properties, mediated by dopaminergic signaling, employed doses similar to or higher than doses needed to abolish the DOI-elicited HTR. For example, α -PPP increases locomotor activity in mice with peak effects at 25 mg/kg.⁵⁵ In rats, 30 mg/kg α -PPP produced significant increases in locomotor activity for 60 minutes (the entire test session), while a dose of 10 mg/kg only produced significant increases for the first 50 minutes of the session.⁵⁶ In another study examining the effects of pyrrolidine SCs on locomotor activity in mice, it was found that 10 mg/kg of the much more potent DAT inhibitor, α -PVP, produced significant increases in locomotor activity that lasted for 120 minutes, whereas 3 mg/kg effects lasted for 80 min.⁵⁷ Furthermore, we have detected brain concentrations of a behaviorally-active, phenylaminotetralin-type compound reaching ~5.8 μ M, 30 minutes after a s.c. injection of 3 mg/kg.³⁵

3.5 | On whether the potency of α -PPP at 5-HT_{2A}R is physiologically relevant in human drug users

Plasma and brain concentrations of α -PPP in human users have not been reported, to our knowledge; however, 8.07 μ M (1.43 mg/L) is the mean concentration of mephedrone obtained from post-mortem biological samples. The range in levels was 0.17 μ M (0.03 mg/L) to as high as 124.12 μ M (22.0 mg/L) in 10 cases where mephedrone was identified as the sole drug of abuse.^{5,10} Moreover, when interacting with lipid bilayers, cathinones are expected to have their pK_a values shifted,⁵⁸ which enable them to partition into membranes and circumvent limitations in bioavailability.⁵⁹ The high lipophilicity with high brain penetration and high volume of distribution means that cathinones can reach significantly greater concentrations in fatty tissues, such as the brain, than in the blood,^{22,60} which we have observed with another lipophilic compound.³⁵ Together with the fact that psychostimulant abusers often repeatedly administer drugs in a single drug-taking session,⁵ we infer that α -PPP is likely to reach micromolar levels in the brains of users, permitting it to act at 5-HT_{2A}R.

3.6 | α -PPP has high selectivity for DAT, but is not widely abused relative to other SCs: A role for its 5-HT_{2A}R antagonist property?

Recent, preclinical work shows that the reinforcing effectiveness of SCs is positively correlated with their selectivity for DAT over SERT.¹⁵ Relatedly, psychoactive drugs that block SERT tend to have reduced stimulant qualities.^{61–63} Based on this rationale, α -PPP might be expected to have a higher abuse potential than cocaine owing to its similar inhibitory potency at DAT, but much higher selectivity for DAT relative to SERT.¹⁵ It might

have also been predicted that α -PPP would match MDPV in its popularity among users. Anecdotal reports from drug forums and from DEA Emerging Threat Reports (Section 1), however, appear to suggest that this is not the case. Our observations that α -PPP has physiologically relevant antagonist activity at 5-HT_{2A}R might account for this discrepancy. With some exceptions,^{64,65} 5-HT_{2A}R antagonists can block behavioral effects of cocaine, at least pre-clinically. For example, pre-treating rats with either volinanserin or ketanserin reduces cocaine-induced hyperactivity and attenuates the discriminative stimulus effects of cocaine.^{66,67} Volinanserin also attenuates the cue- and cocaine-elicited reinstatement of cocaine seeking in rats.^{68,69} Furthermore, we have shown in nonhuman primates that volinanserin attenuates drug- and cue-induced reinstatement as well as cocaine-induced dopamine overflow in the dorsal striatum.⁷⁰ Therefore, it is reasonable to propose that the 5-HT_{2A}R antagonism property possessed by α -PPP offsets its high DAT to SERT ratio, thus making it unfavorable as a drug of abuse compared to other SCs and cocaine. It is also noteworthy that in⁷⁰, volinanserin failed to alter cocaine self-administration or cocaine-induced dopamine overflow in the ventral striatum, suggesting that 5-HT_{2A}R can modulate some but not all of the behavioral effects induced by cocaine.

4 | CONCLUSION

Our study evinces that α -PPP acts as a competitive antagonist at 5-HT_{2A}R with translationally relevant affinity and potency, a property that might explain its relative unpopularity as a drug of abuse. Finally, our observations that modifications at the benzene ring of pyrrolidine-containing SCs alter 5-HT_{2A}R affinity (Figure 2) suggest a structure–activity relationship worth exploring to develop novel 5-HT_{2A}R antagonists/inverse agonists that inhibit monoamine transporters. These compounds might possess psychotherapeutic properties with less abuse liability.

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FIGURE 1.
Chemical structures of synthetic cathinones tested

FIGURE 2.

Competition binding curves of representative synthetic cathinones and DOI, showing displacement of [^3H]Ketanserin from recombinant, human 5-HT $_2\text{A}$ R. The 10mM points on this graph were interpolated to generate complete curves for 4-bromo- α -PPP and MDPPP [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 3.
α-PPP exhibits inverse agonist activity at 5-HT_{2A}R [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 4.
 α -PPP exhibits competitive antagonist activity at 5HT_{2A}R [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 5.

α -PPP dose-dependently blocks the 5-HT_{2A}-dependent DOI-elicited HTR in mice, with significant effects at 10 mg/kg (**** $P < 0.0001$, compared to Veh). MDPPP, up to 10 mg/kg, and 3 BMC 10 mg/kg had no effect. Violin plots show the frequency distribution of the data, with the median and quartiles [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1

Affinities of test compounds at human serotonin 5-HT_{2A} and muscarinic M₁ receptors. N.B. = displaced less than 10% of radioligand at concentrations up to 10 μ M. N.T. = not tested. MDPPP was tested up to 300 μ M in 5-HT_{2A} receptor competition binding assays to calculate its p*K_i* to directly compare it to α -PPP

Affinities, p <i>K_i</i> (SEM)	5-HT _{2A}	M ₁
Cathinones		
α -PPP	5.6 (0.1)	N.B.
4-methyl- α -PPP	5.5 (0.1)	N.B.
4-bromo- α -PPP	5.4 (0.2)	N.B.
3-bromo- α -PPP	<5	N.B.
MDPPP	4.2 (0.1)	N.B.
4-bromomethcathinone	5.2 (0.1)	N.B.
2-fluoromethcathinone	N.B.	N.B.
2-(trifluoromethoxy)-methcathinone	N.B.	N.B.
3-bromomethcathinone	<5	N.B.
Positive controls		
DOI	7.9 (0.1)	N.T.
Volinanserin	9.3 (0.1)	N.T.
Atropine	N.T.	8.9 (0.1)