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The adrenergic receptor antagonist carvedilol interacts with serotonin 2A receptors both *in vitro* and *in vivo*

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ABSTRACT

There is increasing support for the potential clinical use of compounds that interact with serotonin 2A (5-HT_{2A}) receptors. It is therefore of interest to discover novel compounds that interact with 5-HT_{2A} receptors. In the present study, we used computational chemistry to identify critical ligand structural features of 5-HT_{2A} receptor binding and function. Query of compound databases using those ligand features revealed the adrenergic receptor antagonist carvedilol as a high priority match. As carvedilol is used clinically for cardiovascular diseases, we conducted experiments to assess whether it has any interactions with 5-HT_{2A} receptors. *In vitro* experiments demonstrated that carvedilol has high nanomolar affinity for 5-HT_{2A} receptors. *In vivo* experiments demonstrated that carvedilol increases the ethanol-induced loss of the righting reflex and suppresses operant responding in mice, and that these effects are attenuated by pretreatment with the selective 5-HT_{2A} receptor antagonist M100907. Moreover, carvedilol did not induce the head-twitch response in mice, suggesting a lack of psychedelic effects. However, carvedilol did not activate canonical 5-HT_{2A} receptor signaling pathways and antagonized serotonin-mediated signaling. It also reduced the head-twitch response induced by 2,5-Dimethoxy-4-iodoamphetamine, suggesting potential *in vivo* antagonism, allosteric modulation, or functional bias. These data suggest that carvedilol has functionally relevant interactions with 5-HT_{2A} receptors, providing a novel mechanism of action for a clinically used compound. However, our findings do not clearly delineate the precise mechanism of action of carvedilol at 5-HT_{2A} receptors, and additional experiments are needed to elucidate the role of 5-HT_{2A} receptors in the behavioral and clinical effects of carvedilol.

1. Introduction

Serotonin 2A (5-HT_{2A}) receptor 5-HT_{2A} receptors are widely expressed in the brain (Pompeiano et al., 1994), elicit important physiological effects, and are capable of considerable cross talk with other important neurotransmitter systems as they are expressed on dopamine (Ikemoto et al., 2000) and glutamate (Jakab and Goldman-Rakic, 1998) neurons. Numerous studies have linked activation of the 5-HT_{2A} receptor to hallucinogenic activity (Johanson et al., 2006; Ortmann et al., 1982; Peroutka and Snyder, 1981; Sadzot et al., 1989; Tancer and Johanson, 2003), which can induce clinically limiting adverse effects for therapeutic agents. Previous studies have supported the use of 5-HT_{2A} receptor antagonists for substance use disorders (Auclair et al., 2004; Broderick et al., 2004; Murnane et al., 2013a; Murnane et al., 2013b) and other disease states (Murnane, 2019). Likewise, ongoing studies are investigating the therapeutic potential of psychedelic 5-HT_{2A} receptor agonists. For example, psilocybin treatment in heavily tobacco-dependent smokers resulted in approximately 80% abstinence rates at 6 months (Johnson et al., 2014) and 67% abstinence rates at 12 months (Johnson et al., 2017). A recent clinical trial in 51 patients

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with a life-threatening cancer diagnosis reported that psilocybin decreased clinician- and self-rated measures of depression and anxiety, increased quality of life, life meaning, and optimism, and decreased death anxiety (Griffiths et al., 2016). Another recent study, reported significant decreases in alcohol consumption after psilocybin treatment in 10 alcohol-dependent subjects using an open-label design (Bogenschutz et al., 2015). It is not yet known whether the clinical benefits of psychedelics are mediated by stimulation of 5-HT_{2A} receptors or persistent agonist-mediated downregulation of these receptors. Given the potential role of the 5-HT_{2A} receptor in therapeutic and adverse drug effects (Murnane, 2018), the discovery of interactions between this receptor and both novel and existing compounds is of interest.

To date, a number of compounds used clinically have been described as non-psychedelic 5-HT_{2A} receptor agonists. Putative non-psychedelic 5-HT_{2A} receptor agonists include the anti-parkinsonian agent lisuride and the anti-migraine agent ergotamine (Pokorny et al., 2016). Previous research has attempted to catalog the signaling cascades induced by psychedelic and non-psychedelic 5-HT_{2A} receptor agonists, and such investigations often used lisuride and ergotamine as the prototypical non-psychedelic 5-HT_{2A} receptor agonists (Gonzalez-Maeso and Sealfon, 2009; Gonzalez-Maeso et al., 2007). These studies have identified promising molecular signaling markers for predicting psychedelic activity, but have thus far yielded no clearly defined signaling pathways that differentiate psychedelic and non-psychedelic 5-HT_{2A} receptor agonists (Canal, 2018). In reviewing such studies, it has become apparent to us that the dearth of non-psychedelic 5-HT_{2A} receptors is an important limiting factor in efforts to create comprehensive signaling profiles of psychedelic and non-psychedelic 5-HT_{2A} receptor agonists. It was also apparent that psychedelic and non-psychedelic 5-HT_{2A} receptor agonists often show substantial structural overlap. In particular, the psychedelic 5-HT_{2A} receptor agonist lysergic acid diethylamide (LSD) and the non-psychedelic 5-HT_{2A} receptor agonist lisuride have substantial structural similarity, with the main difference being the isomeric and rotational relationships of the amide functional group (Fig. 1). In the present study, we sought to take advantage of that structural similarity by using computational chemistry and pharmacophore molecular modeling methods to determine the structural features of LSD and lisuride predictive of 5-HT_{2A} receptor affinity and psychedelic (or conversely non-psychedelic) activity. We used these structural features to query compound databases, which identified the adrenergic receptor antagonist carvedilol (Fig. 2) as a high priority match to the structural features of compound likely to bind to 5-HT_{2A} receptors and with possible non-psychedelic agonist activity. As carvedilol is used clinically for cardiovascular disease, we sought to conduct a series of experiments to assess whether it has any interactions with 5-HT_{2A} receptors. Our findings demonstrate a novel mechanism for a brain-penetrant adrenergic receptor antagonist that has considerable clinical utility for congestive heart failure and other cardiovascular disease states.

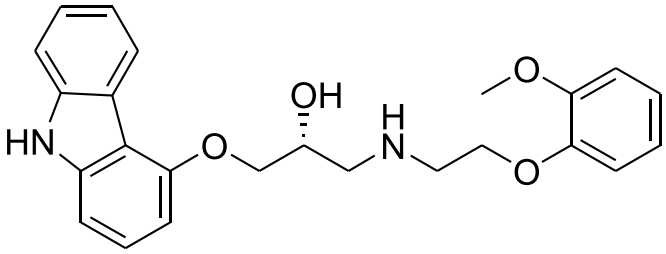


Fig. 2. Chemical structure of carvedilol.

2. Methods

2.1. Pharmacophore modeling

Our initial studies have focused on LSD, ergotamine, and lisuride, as these three ergotamine derivatives share structural similarities and, as noted, have widely divergent psychoactive effects. Indeed, the primary structural difference between lisuride and LSD is an inverse amide group. Our modeling studies utilized the Schrödinger molecular modeling software that includes various small-molecule modeling capabilities, including pharmacophore modeling, quantitative structure-activity relationships, property prediction tools, as well as structure-based design tools, including docking, scoring, and homology modeling (Schrödinger, 2014). We have also developed a multi-conformational 3D structural database of over 7 million compounds for use in virtual high-throughput screening. Using these techniques, in this study, we developed several pharmacophore models to understand how ligands bind to the 5-HT_{2A} receptor in different ways, some of which result in psychedelic agonist effects and some of which result in non-psychedelic agonist effects. Pharmacophore models were generated by aligning features of the prototypical compounds lysergic acid diethylamide (LSD - psychedelic), ergotamine (mildly psychedelic), and lisuride (non-psychedelic) using the Phase module of Schrödinger (Dixon et al., 2006). This approach takes into consideration conformational variances, tautomeric and stereochemical variations, and different ionization states. The essential pharmacophoric features were a hydrophobic moiety, an H-bond acceptor (A), an H-bond donor (D), a positive ionizable group (P), and an indole aromatic ring (R).

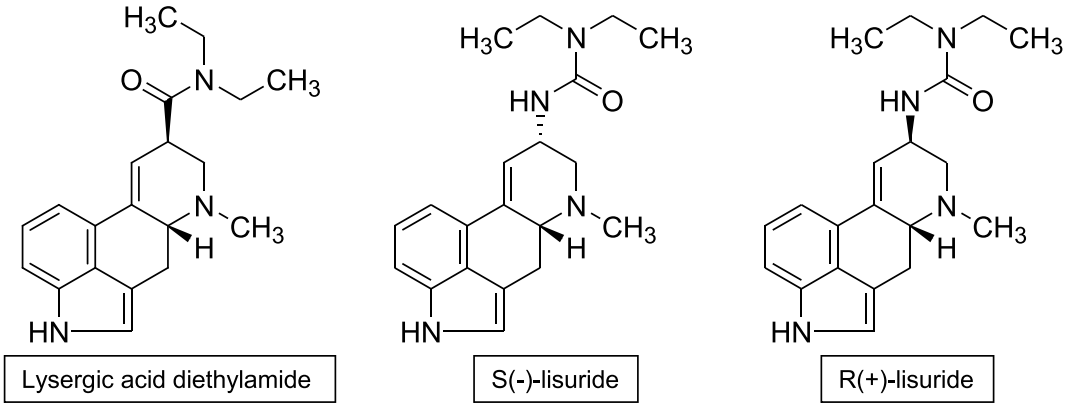


Fig. 1. Chemical similarity between lysergic acid diethylamide (left), S(−)-lisuride (middle), and R(+)-lisuride (right). The psychedelic 5-HT_{2A} receptor agonist lysergic acid diethylamide and the non-psychedelic 5-HT_{2A} receptor agonist lisuride have substantial structural similarity, with the main difference being the rotation of their amide linker. The structural overlap between these compounds was used to generate our lead pharmacophore model.

2.2. Drugs

Carvedilol, 5-HT, and (+/−)-2,5-Dimethoxy-4-iodoamphetamine (DOI) are commercially available compounds; they were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in normal saline. Carvedilol was used in the base form, whereas DOI was used as a hydrochloride salt. SB-242084 (Kennett et al., 1997) was purchased as a dihydrochloride salt from Tocris (Bristol, United Kingdom) and dissolved in 5% Tween 80, 5% ethanol, and brought to final volume in saline (Murphy and Murnane, 2019). OmniPur®, pure 200 proof, ethanol was commercially purchased (VWR, Radnor, PA) and was diluted to 30% with distilled water. M100907 was synthesized at the Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism at the National Institutes of Health (Bethesda, MD). M100907 was dissolved in sterile water and 0.1 N hydrochloric acid. All injections were administered intraperitoneally at a volume of 1 ml/100 g.

2.3. Molecular studies

2.3.1. Cell culture and transient transfection

HEK293 cells were obtained from ATCC (Manassas, VA) and cultured in 100 mm plates containing Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies, Grand Island, NY). Transfections were performed using LipoD293 reagent (Signagen Laboratories, Gaithersburg, MD) and 5 μg of pcDNA3.1 + -humanHTR2A-3x-N-HA-tagged plasmid from the cDNA Resource Center (www.cdna.org). All experiments were conducted 48 h after transfection.

2.3.2. Immunoblotting

Immunoblotting was performed as described previously (Burns and Moniri, 2010; Cheshmehkani et al., 2017). Briefly, cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 10 mM Na_2HPO_4, pH 7.4) plus protease inhibitor cocktail for 20 min and cleared for insoluble debris by centrifugation at 15,000 × *g* for 15 min at 4 °C. Protein concentrations were standardized using DC Protein Assay (Bio-Rad, Hercules, CA) and an aliquot of the lysate was denatured in 2× SDS-sample buffer and boiled for 5 min. Equivalent concentrations of lysates were resolved by SDS–PAGE, followed by transfer to PVDF membranes, and immunoblotted with rabbit anti-phospho- or total- ERK1/2 antibodies (Cell Signaling Technologies (Beverly, MA).

2.3.3. Radioligand binding assays

Radioligand binding assays were performed with membrane homogenates prepared from HEK293 cells that transiently express the 5-HT_{2A} receptor, as we've described previously (Moniri et al., 2004; Moniri and Daaka, 2007), but with Kd concentrations (1 nM) of the 5-HT_{2A} receptor antagonist radioligand [^{3}H]-ketanserin (Bonhaus et al., 1997). N-(4-isothiocyanatophenethyl)spiperone (NIPS; 10 μM) was used to define non-specific binding. Inhibition data using DOI or carvedilol were analyzed by nonlinear regression using the sigmoidal curve-fitting algorithms in Prism (GraphPad Software Inc., San Diego, CA) to determine EC_{50} and Hill coefficients (nH) and ligand affinity, accounting for radioligand concentration, is expressed as Ki (Cheng and Prusoff, 1973). Each experimental condition was run in triplicate and each experiment was performed a minimum of three times.

2.3.4. Inositol phosphate formation

Formation of inositol phosphates was measured using [^{3}H]-myo-inositol (0.4 μCi/well) in the presence of 50 mM lithium chloride, followed by formic acid extraction and column chromatography, as described in detail previously (Moniri et al., 2004; Moniri and Daaka, 2007). Cells (1×10^6/well) were treated with carvedilol or 5-HT alone for 45 min, or were first preincubated with 660 nM (~Ki) carvedilol for 5 min prior to stimulation with 5-HT for 45 min.

2.4. In vivo studies

2.4.1. Subjects

Male, Swiss-Webster mice (Charles River Laboratories, Inc., Wilmington, MA) served as subjects. Swiss-Webster mice were chosen for these studies because these mice are a general purpose strain that has been used extensively to study behavior, physiology, and neurochemistry (Murnane et al., 2012; Murphy and Murnane, 2019; Oppong-Damoah et al., 2019; Ray et al., 2018). Animals were housed 2 or 3 per cage in a temperature-controlled room. Animals had access to food (Laboratory Rodent Diet #5001, PMI Feeds, Inc., St. Louis, MO, USA) and water *ad libitum*. All studies were carried out in accordance with the Guide for Care and Use of Laboratory animals as adopted and promulgated by the National Institutes of Health, and experimental protocols were approved by the Institutional Animal Care and Use Committee at Mercer University.

2.4.2. Loss of righting reflex (LORR)

The LORR assay is a behavioral model of the direct physiological effects of ethanol. We used this assay to test the effects of carvedilol because we have previously found that DOI increases the LORR, suggesting interactions between ethanol and 5-HT_{2A} receptors that may make this assays useful for assessing whether a compound interacts with 5-HT_{2A} receptors *in vivo*. We therefore chose this assay as it is sensitive to 5-HT_{2A} receptor stimulation. The LORR procedure was conducted using procedures modified from those that have been previous described by others (Al Mansouri et al., 2014). Mice were injected with a dose of ethanol that we established as a sedative dose in preliminary studies (2.6 g/kg) from a 30% solution in isotonic saline. Ten minutes after the injection, each mouse was placed in the supine position in a plastic trough and tested to ensure the presence of the righting reflex. The time to recovery from the ethanol-induced LORR was recorded. Recovery from the ethanol-induced LORR was defined as the time required for the mouse to right themselves three times in 30 s following placement in the supine position. Mice were given an injection of saline or carvedilol (10 mg/kg) 15 min prior to the ethanol injection. M100907 was administered 15 min prior to the carvedilol injection. The 10 mg/kg dose of carvedilol was chosen as the starting dose based on previous rodent studies (Liu et al., 2012) and the relative affinities of DOI and carvedilol for the 5-HT_{2A} receptor. The human dose range of carvedilol for angina, congestive heart failure, and hypertension is between 0.18 and 1.18 mg/kg as documented in the Micromedex https://www.micromedexsolutions.com/) and Lexicomp (https://www.wolterskluwercdi.com/lexicomp-online/) drug information references, which based on interspecies dose scaling (Nair and Jacob, 2016) equates to doses of 2.21 and 14.51 mg/kg in mice.

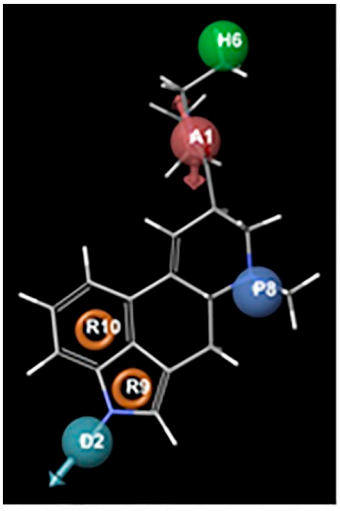
2.4.3. Suppression of operant responding

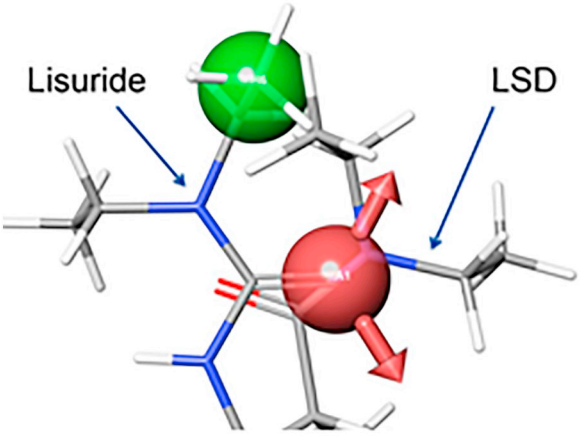
As a secondary *in vivo* assay, we examined whether suppression of food-maintained operant responding by carvedilol involves interactions with 5-HT_{2A} receptors. We chose this assay because suppression of operant responding has been used to document novel drug interactions across a broad range of substances and pharmacological mechanisms, and is particularly useful for assessing compounds with poorly understood pharmacologies or when assessing novel drug interactions. All rate suppression studies were conducted in two-lever operant-conditioning chambers (model ENV-307W-CT; MED Associates, St. Albans, VT) that were individually enclosed in larger lightproof Malaguard sound-attenuating cubicles (model ENV-022MD; MED Associates). Operant responding was maintained through the delivery of 14 mg, rodent purified, Dustless Precision Pellets® (FO5684; BioServ, Flemington, NJ). The side wall of each operant chamber was equipped with a receptacle through which pellets was delivered, driven by a pedestal pellet dispenser mounted outside the chamber but within the

sound attenuated cubicle. The receptacle was centered between two ultra-sensitive mouse retractable levers and positioned just beneath a stimulus light, which was illuminated during reinforcer delivery. Acquisition of food-maintained lever-pressing behavior training was carried out as described in previous studies (Goeders et al., 2009; Murnane et al., 2009; Yarosh et al., 2007). Briefly, mice were trained five days per week under a fixed-ratio schedule of reinforcement wherein completion of the response requirement on either lever was reinforced by pellet delivery. Each reinforcer delivery was followed by a 10 s time-out (TO) before programmed consequences were reinitiated. Once a response requirement was met on either lever, that lever was retracted and the subjects were required to meet the response requirement on the other lever. When the response requirement was met on each of the levers, both levers were reintroduced following the TO. In this manner, mice received equivalent reinforcement from each lever, and no subsequent biases for one lever or the other were noted. The mice acquired food-maintained lever-pressing behavior under a fixed ratio (FR) 1 schedule of reinforcement in sessions lasting 60 min or until 60 reinforcers had been earned (whichever came first). The FR value increased by one every 20th reinforcer earned within a given session, with a terminal parameter of an FR 10. When the subject obtained 20 pellets within a session, the initial session FR value was incremented, until the subject reached the terminal parameters. The following day, the initial FR value was incremented by one. This segment of the training was complete when mice performed stably over five consecutive FR10 sessions, which typically occurred within a month of training. Once terminal responding was achieved, subjects received pre-session drug injections whenever the number of reinforcers obtained in each of the three previous baseline (non-drug injected) sessions did not vary by > 20% in any one of the three sessions compared to the average of those 3 sessions. As 10 mg/kg of carvedilol was ineffective in this assay, the dose was increased up to 30 mg/kg.

2.4.4. Head-twitch response (HTR)

The drug-elicited HTR is a selective behavioral model of 5-HT_{2A} receptor agonism that is highly predictive of psychedelic and non-psychedelic activity in humans. This high-throughput behavioral test is based on twitch behaviors that occur in rodents spontaneously, but are increased in frequency by the administration of various psychedelic drugs. Studies have established that psychedelic 5-HT_{2A} receptor agonists induce this effect, 5-HT_{2A} receptor antagonists selectively block the HTR, and the potency with which they do so is highly correlated with the antagonist's affinity for 5-HT_{2A} receptors (Fantegrossi et al., 2008; Gonzalez-Maeso et al., 2007). Sessions began by placing mice individually into acrylic chambers and activating a video camera mounted above the chamber to record behavior for subsequent offline scoring. HTR behavior in each subject was recorded from 5 to 15 min after the injection of each drug. For the drug interaction study, carvedilol was administered 15 min prior to DOI. In addition, to assess the role of pharmacokinetics in any HTR induced by carvedilol, the HTR was again recorded from 50 to 60 min after the injection of carvedilol. As there is strong homology between the 5-HT_{2A} and 5-HT_{2C} receptors (Cordova-Sintjago et al., 2012), it is possible that the ligand properties that allow carvedilol to interact with the 5-HT_{2A} receptor may also allow for interactions with the 5-HT_{2C} receptor. Furthermore, there is evidence that activation of the 5-HT_{2C} receptor acts in opposition to the 5-HT_{2A} receptor and suppresses the HTR (Fantegrossi et al., 2010). Therefore, any activity of carvedilol at 5-HT_{2C} receptors may suppress its HTR. To test this hypothesis, we pretreated carvedilol with vehicle or 0.3 mg/kg of the selective 5-HT_{2C} receptor agonist SB-242084 (Kennett et al., 1997), and assessed its HTR at 5–15 min and 50–60 min after the injection of carvedilol, administered at 10 or 30 mg/kg. The 0.3 mg/kg of SB-242084 was chosen based on our previous studies (Murphy and Murnane, 2019). Each session was scored by a trained observer that had demonstrated 90% or greater reliability to master





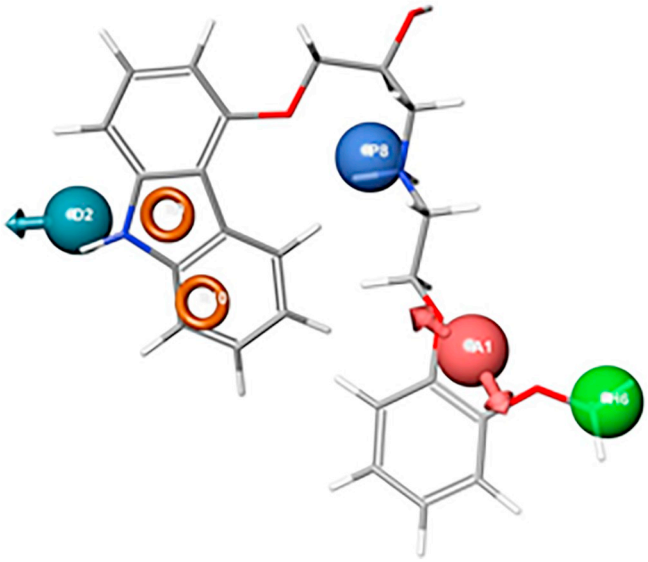


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Fig. 3. (Top) The six pharmacophore features predicted from our lead model are overlaid on the 3D structure of lysergic acid diethylamide. (Middle) Differences in the fit between lisuride and lysergic acid diethylamide (LSD) in our pharmacophore model of the binding site of the 5-HT_{2A} receptor. The carbonyl group of lisuride has a good fit with the A1 feature of the pharmacophore model. In contrast, the carbonyl group of LSD is out of phase with the A1 feature of the pharmacophore model. (Bottom) Carvedilol mapped onto the lead pharmacophore model. Carvedilol has a good fit with several structural features of the model including the A1 feature.

files generated from previous data.

2.5. Data analysis

Graphical presentation of all data depicts mean ± standard error of the mean (SEM). All graphical data presentations and statistical tests were generated using GraphPad Prism (La Jolla, CA), and significance was arbitrated at a $p < 0.05$.

3. Results

3.1. Pharmacophore modeling

Our lead model has six pharmacophore features that are important for receptor agonism (Fig. 3, top). The D2 (light blue, H-bond donor), P8 (dark blue, positive ionizable), R9 and R10 (orange, aromatic rings), and H6 (green, hydrophobic group) features appear to be important for receptor binding and biological activity. They are shared by all three reference compounds. The A1 (pink, H-bond acceptor) feature appears to critically influence psychedelic *versus* non-psychedelic agonism of 5-HT_{2A} receptors (Fig. 3, middle). The primary structural differences altering the ligand fit with the A1 feature are twofold: lisuride has a urea functional group instead of the amide in LSD, and the stereochemistry of the functional group relative to the ring is reversed. Whereas lisuride fits all six of the pharmacophoric features of the lead pharmacophore model, LSD fits five of the features, missing the A1 H-bond acceptor feature. We postulated that the existence of this H-bond donor feature in any compound is necessary to dampen the psychedelic characteristics. Ergotamine, on the other hand, has the amide linker positioned in a structurally similar manner to LSD. Due to conformational adjustments that are presumably needed to bind to the receptor site, the amide carbonyl group is moved out of phase with the A1 H-bond acceptor site. Instead an ether oxygen moves in phase with the A1 site providing an overall poor fit, which explains the depressed level of psychedelic activity of ergotamine. Lisuride, as would be expected, fits very well to the A1 site and is fully non-psychedelic.

Using this model, we have determined the "goodness of fit" across the 6 pharmacophore features for all three of our reference compounds, including both stereoisomers of lisuride. As ergotamine can have mild psychedelic-like effects at high doses, our findings generate the hypothesis that a fitness value of 2.0 is a critical threshold for a ligand to fit within the binding pocket of the 5-HT_{2A} receptor in a manner that does not result in agonist activity that results in psychedelic effects. After the lead pharmacophore model was determined, the objective shifted to lead identification. The strategy was to search our multi-conformational 3D structural database of over 7 million compounds, which we developed for virtual high-throughput screening, to identify commercially available compounds that fit all six pharmacophore features well. Additionally, we wanted to identify compounds that do not possess the ergotamine ring scaffold in order to achieve structural diversity. Queries of our 3D-structural databases identified a series of new compounds with a range of fit values. Carvedilol fit the model for ligand features predictive of 5-HT_{2A} receptor binding, but did not fit the model of a non-psychedelic agonist as well as other structures identified. Nevertheless, in this study, we decided to examine the *in vitro* and *in vivo* pharmacology of carvedilol at 5-HT_{2A} receptors given its clinical importance. We therefore examined this compound both because of its match to our model and because it is a well know known non-selective adrenergic receptor blocker with marked clinical utility. The conformation of carvedilol that aligns with the pharmacophore model is displayed in Fig. 3, bottom.

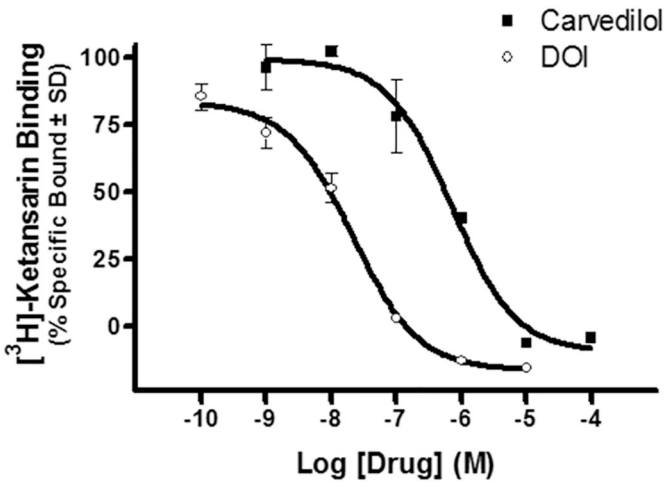

Fig. 4. Competition binding curves of carvedilol and DOI at 5-HT_{2A} receptors labeled by [^{3}H]-ketanserin on HEK293 cell membranes. Carvedilol was able to fully displace [^{3}H]-ketanserin (1 nM) binding to the 5-HT_{2A} receptor with a Ki of 546.5 ± 89 nM and Hill coefficient of −1.2 ± 0.3. DOI was used as a reference compound and fully displaced [^{3}H]-ketanserin binding with a Ki of 7.1 ± 0.8 nM and Hill coefficient of −0.76 ± 0.2.
DOI = 2,5-Dimethoxy-4-iodoamphetamine.

3.1.1. 5-HT_{2A} receptor binding studies

To quantitate carvedilol binding to 5-HT_{2A} receptors, competition binding assays were performed using the 5-HT_{2A} receptor radioligand [^{3}H]ketanserin (~1 nM) and the ability of carvedilol to compete for the radiolabeled site was assessed in membranes of HEK293 cells transiently expressing the receptor. As shown in Fig. 4, carvedilol dose-dependently displaced [^{3}H]ketanserin from the 5-HT_{2A} receptor with an EC_{50} of 2.15 ± 0.08 μM and a Ki of 546.5 ± 89 nM. Displacement of [^{3}H]ketanserin by carvedilol produced a sigmoidal and steep curve with a Hill coefficient (n_H) close to unity ($n_H = -1.20 \pm 0.31$). The psychedelic agonist DOI bound with nearly 80-fold greater affinity than carvedilol to the 5-HT_{2A} receptor, with an EC50 of 23.5 ± 2.7 μM and a Ki of 7.1 ± 0.8 nM. DOI produced a shallow and sloped displacement curve, with an n_H of −0.76 ± 0.20, consistent with agonist binding to non-uniform receptor populations.

3.1.2. Molecular signaling pathways

To determine the effects of carvedilol on 5-HT_{2A} receptor G-protein signaling, we assessed the formation of inositol phosphates (IP), which are generated following agonism of the receptor *via* canonical Gαq/11 cascades, and are stimulated by psychedelic agonists such as LSD (Kurrasch-Orbaugh et al., 2003b). Carvedilol failed to elicit any notable formation of IP at concentrations up to 100 μM alone (Fig. 5). When cells were preincubated for 5 min with ~Ki concentrations of carvedilol (660 nM) prior to stimulation with 5-HT, carvedilol failed to inhibit IP formation induced by the endogenous agonist; and in fact, at lower doses of 5-HT, carvedilol somewhat potentiated the effects of the agonist, suggesting an allosteric interaction between carvedilol and 5-HT. Since 5-HT_{2A} receptor agonism is also known to influence phosphorylation of ERK1/2 mitogen-activated protein kinases (Aringhieri et al., 2017; Knauer et al., 2009; Kurrasch-Orbaugh et al., 2003a; Marinova et al., 2013; Tsuchioka et al., 2008), we then assessed the capacity of carvedilol to modulate this pathway. Carvedilol (1 μM) produced a slight but statistically insignificant increase in ERK1/2 phosphorylation

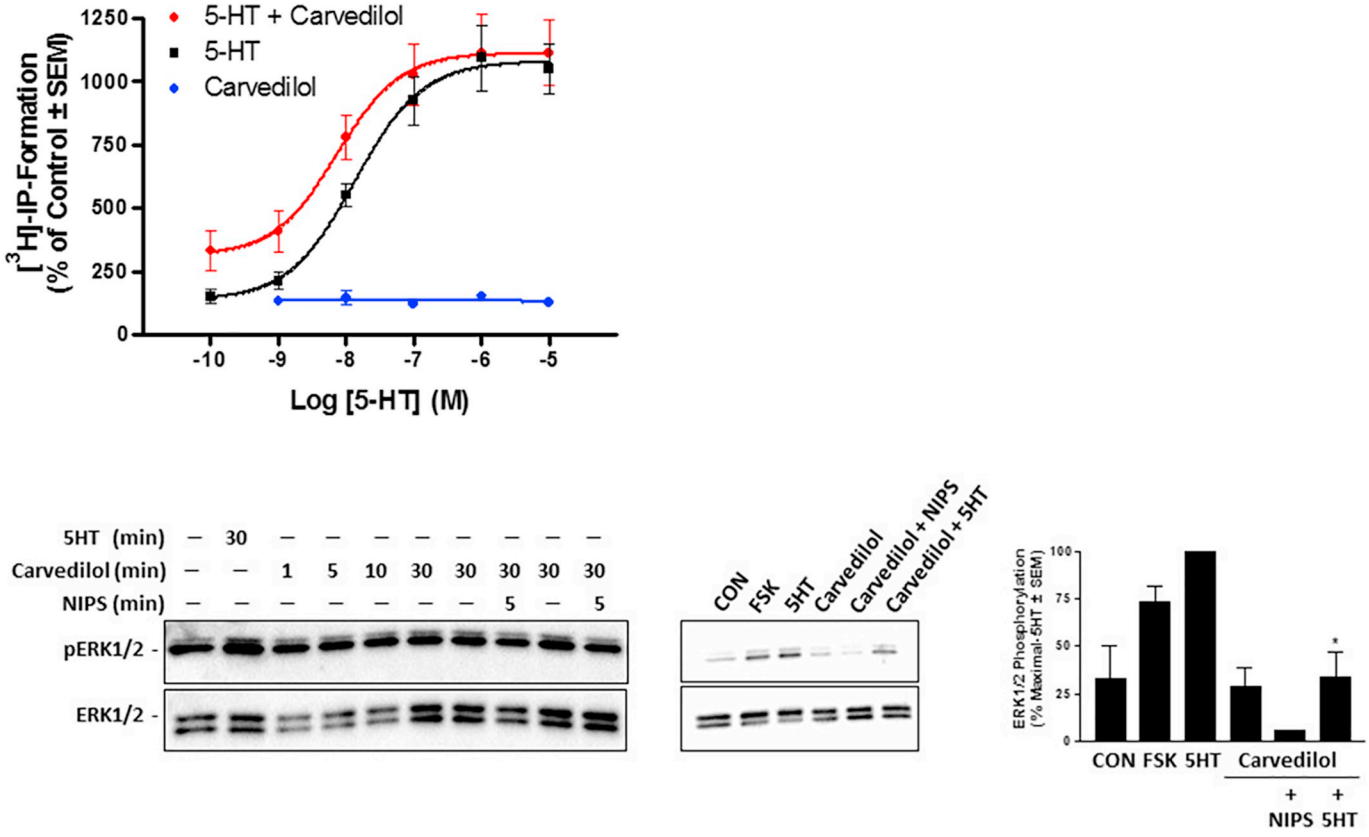

Fig. 5. (Top) Serotonin, but not carvedilol, produced dose-dependent formation of inositol phosphates in HEK293 cells expressing 5-HT_{2A} receptors. In cells pre-incubated for 5 min with ~Ki concentrations of carvedilol (660 nM) prior to stimulation with 5-HT, carvedilol failed to inhibit IP formation induced by the endogenous agonist; and in fact, at lower doses of 5-HT, carvedilol modestly potentiated the effects of 5-HT. (Bottom) Carvedilol (1 μM) did not significantly increase ERK1/2 phosphorylation. Carvedilol (1 μM) preincubation for 5 min prior to 5-HT (1 μM) treatment resulted in a statistically significant blockade of the agonist effect of 5-HT. FSK (forskolin; 10 μM) is used as an experimental positive control to elicit maximal phospho-ERK1/2 signal. * denotes $p < 0.05$ compared to 5-HT alone.

at stimulation times of 1–30 min (Fig. 5). This observable but insignificant response produced at 30 min was lower following administration of the 5-HT_{2A} receptor antagonist NIPS. When carvedilol was assessed in the presence of 5-HT (1 μM), it significantly ($p < 0.05$) blocked the effects of the endogenous agonist.

3.2. In vivo behavioral studies

As carvedilol exhibited appreciable affinity for 5-HT_{2A} receptor *in vitro*, we conducted experiments to determine whether carvedilol has any relevant *in vivo* interactions with 5-HT_{2A} receptors using two distinct assays. First, we determined whether carvedilol increases the duration of the LORR response induced by ethanol, and whether this effect is significantly reduced by pretreatment with the selective 5-HT_{2A} antagonist M100907 (Fig. 6). In this experiment, there was a significant main effect of treatment ($F_{4,29} = 8.941$; $p < 0.0001$) as assessed by one-way analysis of variance (ANOVA). Post-hoc analysis by Tukey's test revealed a significant difference when ethanol was administered after vehicle or 10 mg/kg of carvedilol ($p < 0.05$). Moreover, this effect of carvedilol was significantly decreased when carvedilol was administered after M100907 at 0.003, 0.03, or 0.3 mg/kg ($p < 0.05$). This reversal of the effects of carvedilol by M100907 was not related to changes in the LORR induced by M100907 itself, as there was no significant difference ($T_5 = 0.01$; $p = 0.99$) in the duration of the ethanol-induced LORR when ethanol was administered after M100907 (0.03 mg/kg) or vehicle. Second, we examined whether suppression of food-maintained operant responding by carvedilol involves interactions with 5-HT_{2A} receptors (Fig. 7). In this experiment, there was no significant difference in response rates when the subjects were

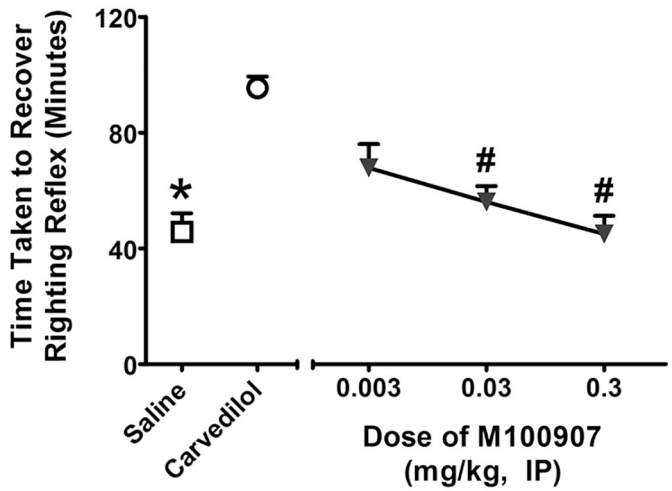

Fig. 6. Carvedilol increases the duration of the loss of the righting reflex induced by ethanol, and this effect is significantly reduced by the selective 5-HT_{2A} antagonist M100907. Abscissae: Dose of the M100907 pretreatment given before the carvedilol treatment expressed in milligrams per gram of body weight. Ordinates: Total time taken to recover the righting response following ethanol administration expressed in minutes. * = $p < 0.05$ compared to saline; # = $p < 0.05$ compared to carvedilol; as assessed by one-way analysis of variance and Tukey's post-hoc test.

administered vehicle or M100907 at 0.03 mg/kg ($T_{14} = 0.37$; $p = 0.71$) indicating that M100907 does not alter response rates at this dose. There was a significant main effect of carvedilol treatment

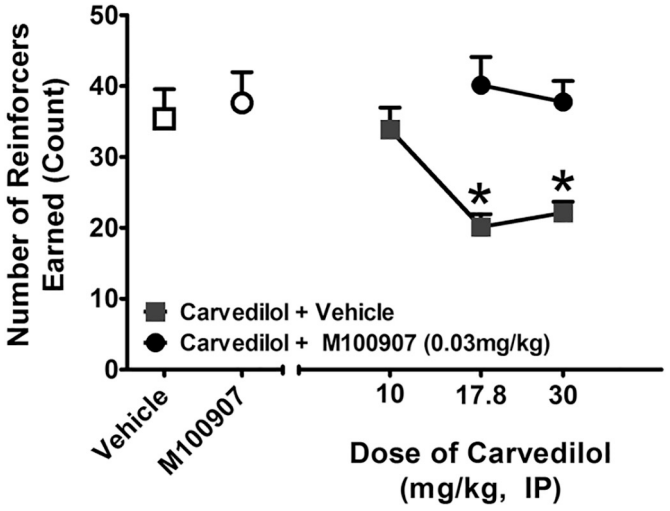

Fig. 7. Carvedilol suppresses operant responding mice and this effect is reversed by pretreatment with the selective 5-HT_{2A} antagonist M100907. Abscissae: Dose of carvedilol expressed in milligrams per gram of body weight. Ordinates: Number of reinforcers earned on an FR10 schedule of reinforcer delivery expressed as a raw count. * = $p < 0.05$ compared to vehicle administration and vehicle pre-treatment as assessed by one-way analysis of variance and Dunnett's post-hoc test.

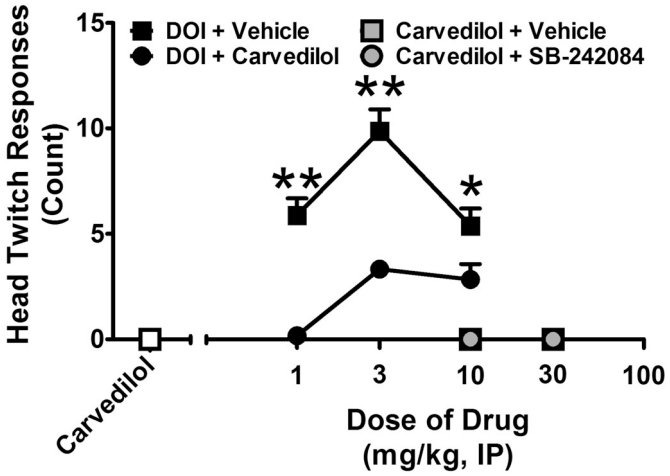

Fig. 8. Carvedilol administered alone at 10 mg/kg produces no observable head-twitch response and when administered as a pretreatment suppresses the head-twitch response induced by DOI. This assay is highly predictive of psychedelic effects and is believed to be centrally mediated, indicating that carvedilol has minimal psychoactive effects yet can interact with central 5-HT_{2A} receptors. Following pretreatment with vehicle or SB-242084, carvedilol produced no observable HTR at a dose of 10 or 30 mg/kg. Abscissae: Dose of the DOI or carvedilol expressed in milligrams per gram of body weight. Ordinates: Number of head twitches exhibited expressed as a raw count. ** = $p < 0.01$ compared to DOI + carvedilol as assessed by student's *t*-test and corrected by the Bonferroni method for multiple comparisons.
DOI = 2,5-Dimethoxy-4-iodoamphetamine.

($F_{3,31} = 7.520$; $p < 0.001$) when the carvedilol treatment followed pretreatment with vehicle, as assessed by one-way ANOVA. Post-hoc analysis by Dunnett's test revealed a significant difference at doses of 17.8 and 30 mg/kg of carvedilol ($p < 0.05$). In contrast, there was no significant main effect of carvedilol treatment ($F_{2,23} = 0.1376$; $p = 0.87$) when the carvedilol treatment followed pretreatment with M100907, as assessed by one-way ANOVA. As carvedilol appeared to interact with 5-HT_{2A} receptors *in vivo*, we went on to assess the effects of carvedilol in the HTR assay (Fig. 8). The drug-elicited HTR is a selective behavioral model of 5-HT_{2A} receptor agonism that is highly predictive of psychedelic and non-psychedelic activity in humans, with an increased HTR indicating potential psychedelic effects. We found that carvedilol at 10 mg/kg does not induce any HTR. Moreover, carvedilol suppressed the HTR induced by DOI at 1 mg/kg ($T_{12} = 5.96$; $p < 0.001$), 3 mg/kg ($T_{12} = 2.97$; $p < 0.01$), and 10 mg/kg ($T_{12} = 2.33$; $p < 0.05$), as assessed by student's *t*-test and corrected by the Bonferroni method for multiple comparisons. Following pretreatment with vehicle or SB-242084, carvedilol produced no observable HTR at a dose of 10 or 30 mg/kg and at 5–15 min or 50–60 min after the injection of carvedilol (Fig. 8).

4. Discussion

The major findings of this study are that query of several compound databases using computational modeling identified the adrenergic receptor antagonist carvedilol as a high priority match for binding to 5-HT_{2A} receptors. Consistent with this prediction, radioligand binding experiments demonstrated that carvedilol displayed appreciable affinity at 5-HT_{2A} receptors. Moreover, carvedilol increases the ethanol-induced loss of the righting reflex and suppresses operant responding in mice, and these effects are attenuated by pretreatment with the selective 5-HT_{2A} receptor antagonist M100907. Carvedilol did not activate canonical 5-HT_{2A} receptor $G\alpha_{q/11}$ signaling and antagonized serotonin-mediated signaling at lower concentrations. Moreover, carvedilol did not induce the head-twitch response in mice, suggesting a lack of psychedelic effects, and reduced the head-twitch response induced by DOI. The apparent antagonist-like effects of carvedilol against 5-HT-mediated induction of canonical signaling pathways as well as the HTR response induced by DOI could be mediated by the propensity of DOI and carvedilol to activate distinct post-receptor signaling cascades. Further work in our laboratories is underway to better understand the carvedilol-induced signaling axis, fully elucidate the mechanism of action of carvedilol at 5-HT_{2A} receptors, and the resultant behavioral and physiological outcomes of these processes.

Our findings support the use of our computational procedure to identify compounds that interact with 5-HT_{2A} receptors. This computational procedure could potentially be iteratively refined using the results of *in vitro* and *in vivo* studies to make stronger and more specific predictions of compounds with a variety of mechanisms of action at 5-HT_{2A} receptors. Computer-aided drug design (CADD) is an essential aspect of the design and discovery of new biologically active chemicals as lead candidate drugs. Pharmacophore modeling is one of the most successful tools of CADD, with it being noted that “Perceiving a pharmacophore is the most important first step in understanding the interaction between a receptor and a ligand” (Güner, 2000). Although such modeling has allowed for significant advances in classical pharmacology, to the best of our knowledge, the proposed studies are the first to use such an approach to elucidate the properties of ligands that are critical drivers of non-psychedelic agonism. Our modeling studies identified carvedilol as a high priority match for interacting with 5-HT_{2A} receptors. By and large, this prediction has been borne out by the *in vivo* and *in vitro* pharmacology data. Carvedilol exhibited behavioral effects that were attenuated by a selective 5-HT_{2A} receptor antagonist in two distinct behavioral assays. Moreover, it did not induce effects consistent with psychedelic-like activity. These data would have been bolstered by a comprehensive assessment of the activity of carvedilol at a variety of 5-HT_{2A} receptor linked signaling pathways. As it stands, we cannot yet make a strong determination of whether carvedilol is acting as an allosteric modulator, functionally bias agonist, or antagonist. However, this is a difficult objective to complete because many signaling pathways have been linked to 5-HT_{2A} receptor activation, such as Gαq/11-PLC, PLA2/AA, PLD, ERK 1 and 2, JNK, AKT, PI3K, p38, Ras, Rac1, Rho, and β-arrestin 1 and 2. There are an abundance of signaling pathways that we could pursue to identify critical signaling pathways, if any, for the interactions between carvedilol and 5-HT_{2A}

receptors. We intend to pursue this line of research in future studies. The identification of these signaling pathways may allow us to further refine our models in an iterative fashion such that even stronger predictions can be made in the future.

There is a growing body of literature describing therapeutic benefits of psychedelics in a variety of psychiatric disorders, including alcohol-use disorder (Abuzzahab and Anderson, 1971; Bogenschutz and Johnson, 2016; Dyck, 2005; Grinspoon and Bakalar, 1986; Mangini, 1998). Likewise, adrenergic receptor antagonists have shown potential therapeutic utility for alcohol-use disorder. The beta-blocker propranolol is known to reduce physical withdrawal symptoms and anxiety during alcohol withdrawal (Bailly et al., 1992), and in a recent study in alcohol-dependent rats, the combination of the beta-blocker propranolol and the alpha 1 antagonist prazosin additively suppressed alcohol drinking during both alcohol withdrawal and following prolonged imposed abstinence (Rasmussen et al., 2014). Moreover, the alpha 1 antagonist prazosin attenuates ethanol but not sucrose self-administration in rats (Verplaetse et al., 2012) and its combination with propranolol increases propranolol-induced suppression of ethanol self-administration (Verplaetse and Czachowski, 2015). Interestingly, prazosin has been shown to suppress ethanol self-administration in rats made dependent through vapor exposure, but increase self-administration in rats not exposed to ethanol vapor (Walker et al., 2008). Carvedilol is known to antagonize alpha and beta-adrenergic receptors, we herein demonstrate that it also apparently interacts with 5-HT_{2A} receptors, and its high lipophilic characteristics (logP > 4) suggests that it has amenable properties for brain bioavailability. As there is evidence that alpha receptor antagonism, beta receptor antagonism, and 5-HT_{2A} receptor agonism or antagonism may have therapeutic utility for alcohol-use disorder, if carvedilol is indeed a polypharmacological agent showing a combination of all three mechanisms of action, it may have clinical utility as a novel treatment of alcohol-use disorder. Bolstering this argument, it has been reported that carvedilol attenuates the progression of alcohol-induced fatty liver disease in rats (Liu et al., 2012). Furthermore, carvedilol pretreatment stabilized cardiovascular function during alcohol withdrawal, and as such, may prevent alcohol-withdrawal-induced mortality (Shirafuji et al., 2010). Likewise, interactions with 5-HT_{2A} receptors may have important implications for the use of carvedilol in cardiovascular disease. This is interesting as the doses that we tested are in the upper bounds of the clinically used dose range for angina, congestive heart failure, and hypertension, based on interspecies dose scaling (Nair and Jacob, 2016). These issues should be explored in future studies.

Our findings suggest that carvedilol interacts with 5-HT_{2A} receptors, but does not appear to activate canonical pathways. In the IP experiments, carvedilol did not induce IP formation by itself, yet it induced a modest potentiation of 5-HT-induced IP formation at lower concentrations. *In vivo*, carvedilol induced a downward shift in the DOI HTR dose-response curve, and not a competitive rightward shift in the dose-effect curve. Two possible explanations for these effects are allosteric interactions between carvedilol and DOI at the 5-HT_{2A} receptor and orthosteric interactions that result in activation of distinct post-receptor signaling pathways. We attempted to identify the carvedilol-critical signaling pathway by examining ERK1/2. However, as one moves away from canonical Gq-mediated IP formation, it becomes increasingly difficult to interpret the findings of signaling studies. For example, ERK1/2 production through 5-HT_{2A} receptors has been linked to the application of the psychedelic 5-HT_{2A} receptor agonists DOI and LSD (Knauer et al., 2009; Marinova et al., 2013; Tsuchioka et al., 2008) as well as the 5-HT_{2A} receptor antagonist clozapine (Aringhieri et al., 2017). Nevertheless, future studies should continue to elucidate the signaling pathways that underlie non-psychedelic agonist signaling. It may be fruitful to compare the binding and activity of carvedilol, non-psychedelic 5-HT_{2A} receptor agonists such as lisuride, classic psychedelics such as DOI and LSD, and some of the newer agents that are selective for the 5-HT_{2A} receptor such as 25CN-NBOH. The promise of such studies is that carvedilol to may serve as a useful prototypical agent for the study of such non-canonical 5-HT_{2A} receptor signaling, and it may have potential as a chemical backbone for the synthesis of new agents with reduced psychoactivity.

It remains possible that adrenergic receptors have a direct or indirect role in the effects that we measured. The selective β2-adrenergic receptor agonist clenbuterol, for example, is known to suppress the DOI-induced HTR in rats, and this effect is attenuated by the β2-adrenergic receptor antagonist ICI-118,553 (Marek and Ramos, 2018). Somewhat in contrast, however, it is also known that the beta-adrenergic/5-HT_1 receptor antagonist alprenolol has no significant effect on the DOI-induced HTR (Darmani et al., 1991), suggesting that while adrenergic agonists may impact the DOI-induced HTR, it is unclear whether adrenergic agonists can alter this response. The same study reported that alpha 1 and alpha 2 receptor agonists have been reported to inhibit the 5-HT-induced HTR, and that the alpha 1 receptor antagonists prazosin and thymoxamine inhibited the 5-HT-induced HTR (Handley and Brown, 1982). Nevertheless, our findings suggest, but do not definitively establish, that carvedilol activates 5-HT_{2A} receptors in an atypical manner. To the best our knowledge, this is the first study to indicate that the well-known and commonly clinically used agent carvedilol has any interactions with 5-HT receptors. These findings warrant additional experiments to elucidate the role of 5-HT_{2A} receptors in the behavioral and clinical effects of carvedilol.

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