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Support for 5-HT2C receptor functional selectivity *in vivo* utilizing structurally diverse, selective 5-HT2C receptor ligands and the 2,5-dimethoxy-4-iodoamphetamine elicited head-twitch response model

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ABSTRACT

There are seemingly conflicting data in the literature regarding the role of serotonin (5-HT) 5-HT2C receptors in the mouse head-twitch response (HTR) elicited by the hallucinogenic 5-HT2A/2B/2C receptor agonist 2,5-dimethoxy-4-iodoamphetamine (DOI). Namely, both 5-HT2C receptor agonists and antagonists, regarding 5-HT2C receptor-mediated Gq-phospholipase C (PLC) signaling, reportedly attenuate the HTR response. The present experiments tested the hypothesis that both classes of 5-HT2C receptor compounds could attenuate the DOI-elicited-HTR in a single strain of mice, C57Bl/6J. The expected results were considered in accordance with ligand functional selectivity. Commercially-available 5-HT2C agonists (CP 809101, Ro 60-0175, WAY 161503, mCPP, and 1-methylpsilocin), novel 4-phenyl-2-N,N-dimethyl-aminotetralin (PAT)-type 5-HT2C agonists (with 5-HT2A/2B antagonist activity), and antagonists selective for 5-HT2A (M100907), 5-HT2C (SB-242084), and 5-HT2B/2C (SB-206553) receptors attenuated the DOI-elicited-HTR. In contrast, there were differential effects on locomotion across classes of compounds. The 5-HT2C agonists and M100907 decreased locomotion, SB-242084 increased locomotion, SB-206553 resulted in dose-dependent biphasic effects on locomotion, and the PATs did not alter locomotion. In vitro molecular pharmacology studies showed that 5-HT2C agonists potent for attenuating the DOI-elicited-HTR also reduced the efficacy of DOI to activate mouse 5-HT2C receptor-mediated PLC signaling in HEK cells. Although there were differences in affinities of a few compounds at mouse compared to human 5-HT2A or 5-HT2C receptors, all compounds tested retained their selectivity for either receptor, regardless of receptor species. Results indicate that 5-HT2C receptor agonists and antagonists attenuate the DOI-elicited-HTR in C57Bl/6J mice, and suggest that structurally diverse 5-HT2C ligands result in different 5-HT2C receptor signaling outcomes compared to DOI.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) type 2 receptors (5-HT2A, 2B, and 2C) regulate many complex brain functions, including sleep, feeding, emotion, perception, cognition, reward, and memory (Jensen et al., 2010; Landolt and Wehrle, 2009; Millan, 2005; Nichols, 2004). The functions of 5-HT2 receptors implicate them as potentially useful targets for treating neuropsychiatric disorders, however, drug discovery programs have yielded only one approved 5-HT2-selective medicine, the 5-HT2C-preferring agonist lorcaserin (BELVIQ®), which was approved for obesity recently. A number of factors explain the lack of drugs targeting 5-HT2 receptors. The amino acid sequences of the transmembrane regions of 5-HT2 receptors are highly homologous, creating a challenge for designing and developing ligands that activate very selectively one but not the other 5-HT2 receptor subtypes. This is a serious issue, since most 5-HT2 receptor agonists that may have clinically useful effects also produce hallucinations, mediated primarily by activation of the 5-HT2A receptor subtype (Nichols, 2004). Furthermore, prolonged activation of 5-HT2B receptors on heart valve leaflets has been associated with the development of cardiac pulmonary

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valvulopathy, as seen with fenfluramine (Hutcheson et al., 2011). Finally, selective 5-HT2 antagonists including 5-HT2A antagonists proposed as novel medications to treat schizophrenia, have exhibited only modest antipsychotic effects in clinical trials (Ebdrup et al., 2011), yet are effective for treating L-DOPA-induced psychosis (Abbas and Roth, 2008). Recent clinical studies have shown that 5-HT2A antagonists also increase slow wave sleep, and thus may treat certain sleep disorders (Monti, 2010; Teegarden et al., 2008).

Specific (distinguished from "selective") activation of 5-HT2C receptors may produce neuropharmacotherapeutic effects with few side-effects. For example, drugs specific for activating 5-HT2C receptors that have zero efficacy for activating 5-HT2A and/or 5-HT2B receptors may be useful for treating schizophrenia (Rosenzweig-Lipson et al., 2007) and substance abuse (Cunningham et al., 2011) by modulating central dopamine release. Extant 5-HT2C-selective agonists including BELVIQ®, however, produce 5-HT2C-dependent hypolocomotion effects in preclinical animal models and similarly can produce somnolence or fatigue in humans (Arena Pharmaceuticals, 2012; Halberstadt et al., 2009; Siuciak et al., 2007). Importantly, at higher concentrations, nearly all reported 5-HT2C selective agonists also activate 5-HT2A and/or 5-HT2B receptors, including BELVIQ® (Thomsen et al., 2008). Thus, the challenge remains to develop compounds that are specific 5-HT2C receptor agonists lacking sedative effects.

Most 5-HT2 receptor agonists (regardless of selectivity) produce a head-twitch response (HTR) in rodents (Canal and Morgan, 2012). Genetic ablation of 5-HT2A receptors or treatment with selective 5-HT2A antagonists abolishes the HTR in mice elicited by 5-HT2 agonist hallucinogens (Gonzalez-Maeso et al., 2007, 2003), such as 2,5-dimethoxy-4-iodoamphetamine (DOI), providing strong evidence that activation of 5-HT2A receptors is necessary for the HTR in rodents elicited by hallucinogenic 5-HT2 agonists. Several other neurotransmitter receptors also appear to modulate DOI-elicited-HTRs in mice, including 5-HT2C receptors (Canal et al., 2010; Fantegrossi et al., 2010). Interestingly, DOI produces HTRs at doses that also potently and efficaciously activate 5-HT2C receptors, yet selective 5-HT2C agonists, with lower potency and efficacy for activating 5-HT2A receptors, dose-dependently attenuate the DOI-elicited-HTR in rodents (Fantegrossi et al., 2010; Siuciak et al., 2007). Adding further intrigue, recent reports show that the selective 5-HT2C antagonist SB-242084 also attenuates the DOI-elicited-HTR in C57Bl/6J and DBA/2J mice (Canal et al., 2010). This effect was not observed, however, in NIH/Swiss mice, wherein SB-242084 actually enhanced the DOI-elicited-HTR (Fantegrossi et al., 2010).

One aim of the current studies was to resolve the seemingly conflicting observations in the literature by comparing directly the effects of multiple 5-HT2C agonists and antagonists on the DOI-elicited-HTR in a single animal model using identical experimental parameters and conditions. It was hypothesized that both classes of compounds could attenuate the DOI-elicited-HTR, and that the results could be in accordance with *in vivo* functional selectivity (Milligan, 1993; Moya et al., 2007; Urban et al., 2007). This hypothesis involves the notion that selective 5-HT2C agonists could activate 5-HT2C receptors *in vivo* in a different way compared to DOI, similar to reports of other 5-HT2 agonists acting at 5-HT2A receptors (Schmid and Bohn, 2010; Schmid et al., 2008). Therefore, like antagonists, selective 5-HT2C agonists could interfere with 5-HT2 receptor signaling that leads to the DOI-elicited-HTR by acting as functional antagonists of DOI *in vivo*.

Functional selectivity *in vivo* may indeed have therapeutic impact (Mailman, 2007). If 5-HT2C receptor ligands behave as functionally-selective agonists *in vivo*, then it may be feasible to develop therapeutic 5-HT2C receptor agonists that lack side-effects, such as sedation, by targeting specific 5-HT2C signaling patterns. To this end, in addition to effects on the DOI-elicited-HTR, several classes of selective 5-HT2C agonists were tested for their effects on locomotion following administration of DOI.

2. Materials and methods

2.1. Compounds and treatment doses

The chemical structures of all compounds used in the present studies are shown in Table 1. (±)-DOI (DOI), mCPP, SB-242084, SB-206553, and M100907 were purchased from Sigma-Aldrich (MO, USA). WAY 161503, Ro 60-0175, CP 809101, and 1-methylpsilocin were purchased from Tocris (Bristol, UK). Serotonin was purchased from Alfa Aesar (MA, USA). [^{3}H]-mesulergine, [^{3}H]-ketanserin, and [^{3}H]-myo-inositol were purchased from Perkin-Elmer (MA, USA). The (+)-(2*R*, 4*S*)- and (−)-(2*S*, 4*R*)-*trans* enantiomers of 4-phenyl-2-*N*,*N*-dimethylaminotetralin (PAT) and (−)-(2*S*, 4*R*)-*trans*-4-(4′-methyl)-*N*,*N*-dimethylaminotetralin (methyl-PAT) were synthesized in our laboratories as racemates that were resolved by chiral stationary-phase HPLC and converted to hydrochloride salts as previously described (Booth et al., 2009; Bucholtz et al., 1999; Vincek and Booth, 2009). All compounds, with the exceptions of SB-242084 and 1-methylpsilocin, were dissolved in sterile MilliQ water which served as the vehicle control. SB-242084 was dissolved in sterile MilliQ water containing Tween-80 (6% final v/v), and 1-methylpsilocin was dissolved in sterile MilliQ water containing acetic acid (5% v/v). Separate MilliQ water vehicles containing these additives were used for control groups for these compounds. All compounds were administered subcutaneously (sc) at a volume of 10 mL/kg. A single dose of DOI (1 mg/kg) was used for all experiments, as it is known to reliably elicit a robust and consistent number of HTRs in C57Bl/6J mice (Canal et al., 2010; Fox et al., 2010). Two doses were used to test the efficacy of 5-HT2C selective agonists for attenuating the DOI-elicited-HTR, 3 mg/kg and 5.6 mg/kg. Doses for antagonists were as follows: M100907, 0.0025, 0.025, and 0.25 mg/kg, SB-206553 and SB-242084, 0.03, 0.3, and 3 mg/kg. All compounds were weighed on a Mettler-Toledo (OH, USA) XP26 microanalytical balance, and solutions of all compounds were made fresh on the day of testing.

2.2. Animal subjects

Male C57Bl/6J mice, purchased at 60 days old from Jackson Laboratories (Bar Harbor, Maine) were used in all experiments. Mice were pair-housed in standard cages with *ad libitum* access to food and water, and were acclimated in the vivarium for at least 4 days prior to testing. Animal procedures were approved by the Institutional Animal Care and Use Committee and are in accordance with the principles in the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.3. Behavioral studies

Drug or vehicle was injected sc 10 min prior to injection of DOI. Mice were placed in an activity chamber (43 × 43 cm, Med Associates, Inc.) 10-min later, and head-twitches were counted for the next 10 min by an observer blind to experimental treatment. Locomotion (distance traveled, cm) was tracked with an overhead camera and Ethovision XT 7.0 software. A separate group of mice was administered DOI 10 min prior to injection of 0.25 mg/kg M100907 or 3 mg/kg SB-206553 to test whether the HTR becomes independent of 5-HT2 receptors after their activation, i.e. to test whether M100907 and/or SB-206553 remain effective at attenuating the DOI-elicited-HTR after 5-HT2 receptor activation.

2.4. Mouse 5-HT2A and 5-HT2C receptor constructs

Plasmid DNA containing either mouse htr2A or htr2C-vnv cDNA clones was purchased from Origene (MD, USA). cDNA clones were inserted between the EcoR 1 and Not 1 sites in pCMV6-Kan/Neo vectors. Plasmid DNA was transformed into Subcloning Efficiency DH5α competent cells following the manufacturer's protocol (Invitrogen, CA, USA). Selections of individual colonies of kanamycin-resistant cells were grown in sterile LB broth overnight which served to generate glycerol stocks that were kept stored at −80 °C. Following purification of DNA using Wizard Plus SV minipreps (Promega, USA), samples of htr2A or htr2C DNA were sequenced by the University of Florida DNA sequencing core. Verification of the open-reading frame sequences for the mouse htr2A and mouse htr2C DNA was performed by comparing the results obtained to results published on Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/). The sequences obtained were identical to the published sequences, and match the receptor htr2A and htr2C DNA sequences from the C57Bl/6 mouse. During the verification process, it was observed that the mouse htr2C cDNA from Origene coded for the VNV edited isoform of this receptor. cDNA coding for the human 5-HT2A or 5-HT2C-INI receptors were from previously transformed competent cells, as previously reported (Canal et al., 2011).