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ORIGINAL INVESTIGATION

The serotonin 2C receptor potently modulates the head-twitch response in mice induced by a phenethylamine hallucinogen

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Abstract
Rationale Hallucinogenic serotonin 2A (5-HT_{2A}) receptor partial agonists, such as (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI), induce a frontal cortex-dependent head-twitch response (HTR) in rodents, a behavioral proxy of a hallucinogenic response that is blocked by 5-HT_{2A} receptor antagonists. In addition to 5-HT_{2A} receptors, DOI and most other serotonin-like hallucinogens have high affinity and potency as partial agonists at 5-HT_{2C} receptors.
Objectives We tested for involvement of 5-HT_{2C} receptors in the HTR induced by DOI.
Results Comparison of 5-HT_{2C} receptor knockout and wild-type littermates revealed an approximately 50% reduction in DOI-induced HTR in knockout mice. Also, pretreatment with either the 5-HT_{2C} receptor antagonist SB206553 or SB242084 eradicated a twofold difference in DOI-induced HTR between the standard inbred mouse strains C57BL/6J and DBA/2J, and decreased the DOI-induced HTR by at least 50% in both strains. None of several measures of 5-HT_{2A} receptors in frontal cortex explained the strain difference, including 5-HT_{2A} receptor density, $G\alpha_q$ or $G\alpha_{i/o}$ protein levels, phospholipase C activity, or DOI-induced expression of *Egr1* and *Egr2*. 5-HT_{2C} receptor density in the brains of C57BL/6J and DBA/2J was also equivalent, suggesting that 5-HT_{2C} receptor-mediated intracellular signaling or other physiological modulators of the HTR may explain the strain difference in response to DOI.
Conclusions We conclude that the HTR to DOI in mice is strongly modulated by 5-HT_{2C} receptor activity. This novel finding invites reassessment of hallucinogenic mechanisms involving 5-HT_2 receptors.

Keywords Serotonin 2A receptor (5-HT_{2A}) · Serotonin 2C receptor (5-HT_{2C}) · Hallucinogens · Head-twitch response (HTR) · Phospholipase C (PLC) · Phospholipase A (PLA)

Introduction

Serotonin-based hallucinogens, such as the phenethylamine 2,5-dimethoxy-4-iodoamphetamine (DOI), the indoleamine psilocin, and ergoline d-lysergic acid diethylamide (LSD) profoundly alter sensory perception, mood, cognition, and behavior in humans. These effects inspired long ago the idea that hallucinogens could be models for understanding mental illnesses, such as schizophrenia (Cohen 1953; Fischer et al. 1951; Hollister 1964). A common denominator of serotonin-based hallucinogens is their high affinity for serotonin type 2 (5-HT_2) receptors (Nichols 2004), including 5-HT_{2A} and 5-HT_{2C} receptors that are expressed

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densely in regions of the brain known to regulate emotion and memory, such as the frontal cortex and limbic structures (Lopez-Gimenez et al. 1997, 2001, 2002). 5-HT_{2A} and 5-HT_{2C} receptors are altered in psychiatric conditions, ranging from obsessive-compulsive disorder and depression (Benedetti et al. 2008; Millan 2005) to schizophrenia (Herrick-Davis et al. 2000; Kang et al. 2009; Quednow et al. 2009). In addition, atypical antipsychotics as well as novel antidepressants and anxiolytic medications target 5-HT_{2A} and/or 5-HT_{2C} receptors.

Although it is well appreciated that both 5-HT_{2A} and 5-HT_{2C} receptors are altered in psychiatric conditions, whether 5-HT_{2A} or both 5-HT_{2A} and 5-HT_{2C} receptors mediate the effects of hallucinogens remains to be determined. Several lines of evidence suggest that the behavioral and cognitive effects of hallucinogens are driven primarily through activation of 5-HT_{2A} receptors in the frontal cortex (Gonzalez-Maeso et al. 2007; Vollenweider et al. 1997; Willins and Meltzer 1997). The hallucinogenic indoleamine psilocybin causes significant increases in cerebral metabolic rate in the frontal cortex and anterior cingulate cortex (Vollenweider et al. 1997), and pretreatment with the 5-HT_{2A} antagonist ketanserin dose-dependently prevents hallucinations caused by psilocybin in humans (Vollenweider et al. 1998). Also, the 5-HT_{2A} antagonists MDL100907 and ketanserin block a frontal cortex-mediated hallucinogenic behavioral proxy in rodents, the head-twitch response (HTR; Vickers et al. 2001; Willins and Meltzer 1997), as well as the increase in synaptic activity in frontal cortex neurons caused by application of the 5-$HT_{2A/2C}$ agonists α-methyl-5-HT and DOI (Beique et al. 2007; Marek and Aghajanian 1996). Furthermore, mice devoid of the 5-HT_{2A} receptor do not exhibit an HTR to DOI (Gonzalez-Maeso et al. 2007), and 5-HT_{2A} antagonists attenuate the discriminative properties of several hallucinogens in rodents (Benneyworth et al. 2005; Fiorella et al. 1995b; Schreiber et al. 1994).

Although all known serotonin hallucinogens bind with high affinity and activate with high potency the 5-HT_{2C} receptor, this receptor has largely been dismissed as a potential modulator of the hallucinogenic response for a number of reasons. Despite similar coupling to G-proteins and second messenger systems as 5-HT_{2A} receptors, there is evidence that 5-HT_{2C} receptor activity may oppose activity of the 5-HT_{2A} receptor. Neurochemically, 5-HT_{2C} receptors tonically inhibit dopamine release in the frontal cortex, while 5-HT_{2A} receptor stimulation increases dopamine release in the frontal cortex (Gobert and Millan 1999; Gobert et al. 2000; Millan et al. 1998). However, a recent report shows that injections of the 5-HT_{2C} agonist RO 60-0175 into the medial prefrontal cortex of rats enhance dopamine outflow in nucleus accumbens caused by cocaine administration (Leggio et al. 2009).

Behaviorally, 5-HT_{2A} receptor agonism increases, whereas 5-HT_{2C} agonism decreases locomotor activity, inferred from experiments testing 5-$HT_{2A/2C}$ receptor agonists in 5-HT_{2A} knockout mice (Halberstadt et al. 2009) and selective 5-HT_{2C} receptor agonists in 5-HT_{2C} knockout mice (Fletcher et al. 2009). Also, systemic injections of selective 5-HT_{2C} receptor agonists with low efficacy at 5-HT_{2A} receptors, such as mCPP and MK212, do not cause an HTR in rats. However, when rats are pretreated with the 5-HT_{2C} receptor antagonist, SB242084, both mCPP and MK212 induce an HTR (Vickers et al. 2001). Further suggesting that 5-HT_{2C} receptors are not involved in the behavioral effects of hallucinogens are the observations that the HTR caused by direct injections of DOI into the frontal cortex of rats is blocked by systemic injections of MDL100907, yet not the 5-$HT_{2C/2B}$ antagonist, SDZ-SER 082 (Willins and Meltzer 1997). Finally, systemic injections of MK212 and mCPP have been reported to inhibit a DOI-induced HTR in rats (Berendsen and Broekkamp 1990). However, these latter findings may be confounded by the observations that 5-HT_{2C} agonists dose-dependently decrease locomotor activity, perhaps disguising a role for 5-HT_{2C} receptors in the DOI-induced HTR.

Other findings support the possibility of 5-HT_{2C} receptor involvement in the hallucinogenic response. For example, direct injections of mCPP and MK212 into the frontal cortex of rats induce an HTR, an effect that is attenuated by systemic pretreatment with the 5-HT_{2C} antagonist SDZ-SER 082 (Willins and Meltzer 1997). In addition, enhanced sensitivity to LSD drug discrimination in rats following p-chlorophenylalanine depletion of serotonin is associated with enhanced 5-HT_{2C}, yet not 5-HT_{2A} receptor-mediated phospholipase C (PLC) activity (Fiorella et al. 1995a).

Also, although having greater affinity for 5-HT_{2A} receptors than 5-HT_{2C} receptors, MDL100907, ketanserin, and other 5-HT_{2A} "selective" antagonists that block the effects of hallucinogens have appreciable affinity for 5-HT_{2C} receptors (Knight et al. 2004; Roth et al. 1992), cautioning interpretation of observations with these drugs as evidence for specific 5-HT_{2A} receptor dependence. Furthermore, caution should be present when interpreting data using "selective" 5-HT_{2C} antagonists, such as SB242084, as recent studies show that it has inverse agonist properties at the 5-HT_{2C}→phospholipase A2→arachidonic acid pathway, yet agonist properties at the 5-HT_{2C}→PLC→inositol phosphate pathway in Chinese hamster ovary (CHO) cells (De Deurwaerdere et al. 2004). The second messengers activated by serotonin hallucinogens that are necessary for the hallucinogenic response are still unknown, although it has been shown that DOI-induced head-twitch responses are only partially prevented in Gq knockout mice, suggesting multiple second messenger pathways may be involved (Garcia et al. 2007). Finally, at least part of the electrophysiological effects of

hallucinogens in the frontal cortex is mediated by the 5-HT_{2C} receptor (Beique et al. 2007).

In the present study, we use pharmacological evidence, evidence from genetically modified mice lacking the 5-HT_{2C} receptor, and evidence from a robust and reproducible mouse strain difference in HTR to DOI to show convincingly that 5-HT_{2C} receptors potently modulate hallucinogen-induced head-twitch. These converging results suggest the possibility that coordinated activity of both 5-HT_{2A} and 5-HT_{2C} receptors underlie the hallucinogenic behavioral response in mice.

Methods

Mice

All experimental procedures were performed in accordance with (1) the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication 86–23) and (2) Institutional Animal Care and Use Committee. Eight-week-old male C57BL/6J and DBA/2J mice were purchased from Jackson Laboratories and allowed 2 weeks acclimation time in the vivarium prior to testing. Twenty-week-old 5-HT_{2C} receptor null mice (Tecott et al. 1995) and wild-type littermates on a mixed 129 and C57BL/6 background, as well as 12–16-week-old 129SJ mice, were from established colonies in our laboratory. Mice were housed three to five per cage in standard rodent cages and had unlimited access to rodent chow and fresh water. All experiments involving live mice were performed between 1000 and 1600 hours.

Drugs

The 5-$HT_{2A/2C}$ receptor agonist, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI), and the irreversible alkylating agent, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) were purchased from Sigma-Aldrich (USA). The 5-HT_{2A} receptor antagonist, MDL100907, was a gift from Merrill-Dow Corp (USA). The 5-HT_{2C} receptor agonist, MK212, the 5-HT_{2C} receptor inverse agonist/antagonist, SB206553, and the 5-HT_{2C} receptor antagonist, SB242084, were each purchased from Tocris (USA). The mixed 5-$HT_{1/2}$ receptor antagonist, methysergide, was also purchase from Tocris. [^{3}H]-ketanserin and [^{125}I]-DOI were purchased from Perkin-Elmer (USA). [^{3}H]-mesulergine was purchased from G.E. Healthcare, Inc.

Head-twitch response

5-HT_{2A} receptor hallucinogens induce a quantifiable HTR in rodents that is not induced by the non-hallucinogenic 5-HT_{2A} receptor agonist lisuride (Gonzalez-Maeso et al. 2007). In addition, the HTR is not observed in 5-HT_{2A} knockout mice administered 5-HT_{2A} hallucinogens, and in wild-type animals, it is blocked by MDL100907 (Gonzalez-Maeso et al. 2007; Vickers et al. 2001). Also, 5-HT_{2A} antagonists block the hallucinogenic response in humans. These data support the HTR as a valid behavioral proxy of hallucinogen action in mice. The role of 5-HT_{2A} and 5-HT_{2C} receptors in DOI-induced HTR was evaluated using both experimental genetic and pharmacologic manipulations. First, 5-HT_{2C} receptor null mice (N=7) and littermate control mice (N=5) were treated with the hallucinogenic 5-HT_2 receptor agonist DOI (1 mg/kg). Second, C57BL/6J and DBA/2J mice (N=6 per treatment group) were treated with DOI (1 mg/kg) alone or after pretreatment with 5-HT_{2A} (MDL100987, 0.25 mg/kg) or 5-HT_{2C} (SB206553, 0.3 and 3.0 mg/kg; SB242084, 3.0 mg/kg) receptor antagonists 10 min prior to the DOI treatment. To evaluate possible pharmacokinetic effects, C57BL/6J and DBA/2J mice (N=3 per treatment group) were also evaluated for dose–response to DOI at 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 mg/kg without any pretreatment. DOI was injected intraperitoneally (IP: inbred strain dose–response and MDL100907 experiments) or subcutaneously (SC: 5-HT_{2C} null mice and SB206553 and SB242084 experiments) at a volume of 10 ml/kg.

An HTR was defined as a clear, rapid, left to right or right to left tic movement of the head of the mouse following DOI treatment. Ten minutes after DOI injection, mice were placed inside a clean 3-L transparent glass beaker with a thin layer of fresh rodent bedding at the bottom. At least two observers counted HTRs in a 10-min period—observer's scores were averaged before analysis. Analyses of the data were by t test or analysis of variance (ANOVA; Stata, v.11).

EEDQ blockade of 5-HT_{2A} receptor binding sites

To test whether SB206553 blocks 5-HT_{2A} receptors in addition to 5-HT_{2C} receptors in vivo, SB206553 was examined for its ability to prevent EEDQ binding to 5-HT_{2A} receptors. EEDQ is an alkylating agent that binds irreversibly to 5-HT_{2A} receptor binding sites (Kettle et al. 1999), blocking binding of [^{3}H]-ketanserin to 5-HT_{2A} receptors in membrane preparations. Mice were injected SC with saline (N=6), SB206553 (3 mg/kg, 10 ml/kg, N=4), or MDL100907 (0.25 mg/kg, 10 ml/kg, N=4) 30 min prior to EEDQ injections (10 mg/kg, 10 ml/kg). Twenty-four hours later, mice were killed, and frontal cortex brain tissue was processed for [^{3}H]-ketanserin radioligand binding (as described above). Ten-micromolar SB206553 was added to the radioligand assay buffer to block [^{3}H]-ketanserin binding to 5-HT_{2C} receptor sites. A single

concentration of [^{3}H]-ketanserin (5 nM) was used, and nonspecific binding was determined with the addition of 100 μM methysergide. All experiments were carried out in triplicate. Data were transformed to assess 5-HT_{2A} receptor specific binding in drug-treated animals as percent of 5-HT_{2A} receptor specific binding in saline-treated animals. A one-way ANOVA was performed to compare groups.

Saturation binding

5-HT_{2A} receptor binding site density (Bmax) and antagonist affinity (Kd) were determined with [^{3}H]-ketanserin, comparing membranes from frontal cortex of C57BL/6J and DBA/2J mice. Adult male C57BL/6J and DBA/2J mice were killed by brief isoflurane anesthesia and decapitation. Brains were quickly dissected free of the head, and olfactory bulbs were removed. The brain was placed into a cold, clean brain matrice, and frontal cortex, defined as cortex 2–3 mm caudal to the anterior pole of the brain, was cut free coronally using a clean razor blade. The tissue was homogenized in ice-cold 50-mM Tris assay buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 0.1 mM EDTA (pH 7.3)). The homogenate was centrifuged at 20,000 g for 20 min at 4°C. The supernatant was discarded, and the pellet was resuspended in assay buffer. The centrifugation step was repeated two more times. Finally, the supernatant was discarded, the pellet was resuspended in assay buffer, briefly homogenized, and protein concentration was measured by Bradford assay (Bio-Rad).

Membrane preparations (200 μg/tube) were incubated with a range of concentrations of [^{3}H]-ketanserin (0.25–8 nM) for 60 min at 37°C to determine total binding. Nonspecific binding was determined with 100 μM methysergide. All experiments were performed in duplicate. Following incubation, free radioligand was separated from bound by vacuum filtration through Whatman GF/C glass filters (Brandel, Gaithersburg, MD, USA). Filters were placed in vials with scintillation cocktail and counted 12 h later in a liquid scintillation counter.

Whole brain minus cerebellum was used for analysis of 5-HT_{2C} receptor binding site density. Brain tissue was homogenized in cold assay buffer and centrifuged at 20,000 g for 20 min at 4°C. Supernatant was decanted, pellet was re-homogenized in fresh, cold assay buffer, then spun again at 20,000 g; this step was repeated, then samples were incubated for 30 min at 37°C to remove endogenous ligand. A final spin was performed, and the pellet was re-homogenized in ice-cold assay buffer. Protein concentration was determined using the BCA method. Samples were incubated at 37°C for 90 min with increasing concentrations of [^{3}H]-mesulergine for 5-HT_{2C} receptor binding (0.077–22.1 nM). One hundred nanomolar spiperone was added to block 5-HT_{2A} receptor sites, and nonspecific binding was determined with the addition of 100 μM methysergide. All experiments were performed in duplicate. The reaction was terminated by adding ice-cold assay buffer, and membranes were collected, using a Brandel harvester, on Whatman GF/B glass filters presoaked with 0.3% polyethyleneimine. Filters were washed three times with cold 50 mM Tris–HCl buffer. Filters containing membrane-bound radioligand were placed in 5 mL scintillation fluid (Aquasol-2, Perkin-Elmer, USA), vortexed vigorously, and allowed to set overnight in the dark at room temperature. Bound radioactivity was determined by liquid scintillation spectrometry.

Nonlinear regression analysis (Stata, v.11, and Graphpad 5.02, USA) was used to determine Bmax and Kd estimates by simultaneously fitting the total and nonspecific curve data for six independent experiments per strain. The Bmax and Kd estimates for the C57BL/6J and DBA/2J samples were then compared by *t* test.

5-HT_{2A} and 5-HT_{2C} receptor autoradiography

Mice were anesthetized with isoflurane anesthesia then decapitated. Brains were removed and frozen quickly in isopentane on dry ice, then dabbed dry with a Kim Wipe and stored at −80°C until sectioning. Brains were thawed to −20°C inside a Cryostat chamber. Twenty-micrometer sections were thaw-mounted onto Superfrost Plus slides, then stored again at −80°C. At the start of the autoradiography experiments, slides were brought to room temperature then washed twice, 10 min each at room temperature in assay buffer containing 100 nM SB269770 to block 5-HT7 receptors. Slides were transferred to slide boxes containing assay buffer with 0.14 nM [^{125}I]-DOI. Additional slides were incubated in radioligand plus 100 nM spiperone to define 5-HT_{2C} receptors or 30 μM methysergide to define nonspecific binding. Slides were incubated for 60 min at room temperature then washed thrice, 10 min in ice-cold assay buffer, and briefly washed in cold deionized water, prior to drying with a stream of dehumidified air. Slides were exposed to Biomax MR film for 24, 48, or 72 h prior to developing. Autoradiograms were digitized using a CanoScan 4400F scanner (Canon, USA). Regional analysis of 5-HT_{2A} or 5-HT_{2C} receptor binding was quantified using NIH ImageJ version 1.42q (Abramoff et al. 2004). Brain areas, including anterior cingulate cortex, prefrontal cortex, somatosensory cortex, anterior striatum, and posterior striatum, were outlined based on a mouse brain atlas (Paxinos and Watson 2004). Average pixel density was determined and converted to microcuries per gram protein using 14C standards (ARC, Inc., St. Louis, MO, USA). Data from six C57BL/6J male mice were compared with data from six DBA/2J male mice using two-tailed unpaired *t* tests.

Western blots of $G\alpha_{q/11}$ and $G\alpha_{i/o}$ subunits

To examine whether levels of G-proteins that couple to 5-HT_2 receptors relate to the strain differences in the DOI-induced HTR, Western blots for $G\alpha_{q/11}$ and $G\alpha_{i/o}$ in the frontal cortex were performed. C57BL/6J and DBA/2J mice (N=12, eight for each strain for $G\alpha_{q/11}$ and $G\alpha_{i/o}$, respectively) were anesthetized with isoflurane and decapitated. Brains were removed quickly and sectioned in a chilled brain block. Cortex tissue was placed in ice-cold cell lysis buffer, containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton-X, 1 mM freshly prepared PMSF, and Roche Complete® protease inhibitors and phosphatase inhibitors. Tissue was homogenized using a Kontes pellet pestle motor and pestle then centrifuged at 13,000 rpm for 20 min. Supernatant was aliquoted and stored at −80°C until Western blot procedures. Protein concentrations were determined using the BCA method. Twenty micrograms of protein was run on 10% acrylamide gels and transferred to PVDF membranes overnight. Membranes were washed in Tris-buffered saline (TBS), incubated in 5% non-fat dry milk with 0.1% Tween-20 in TBS, then incubated with $G\alpha_{q/11}$ (rabbit, 1 to 500 dilution, Santa Cruz Biotechnology, USA) or $G\alpha_{i/o/t/z/gust}$ (rabbit, 1 to 500 dilution, Santa Cruz Biotechnology, USA), and α-tubulin (mouse, 1 to 7500, Sigma-Aldrich, USA) primary antibodies for 90 min. Membranes were then incubated in 4% non-fat dry milk with 0.1% Tween-20 in TBS, then with horseradish peroxidase labeled donkey anti-rabbit secondary antibody (1 to 100,000 dilution, G.E. Healthcare, USA) and sheep anti-mouse (1 to 100,000 dilution, G.E. Healthcare, USA) for 90 min, followed by several washes in TBS with 0.1% Tween-20. Protein was visualized using chemiluminescence and exposure of membranes to film. Film was scanned onto a PC, and densitometry analyses were performed using ImageJ software (Abramoff et al. 2004).

Serotonin-induced PLC activity

A comparison of basal and 5-HT-stimulated PLC activity was made in ex vivo cortex membrane preparations from six young adult male C57BL/6J mice and six young adult male DBA/2J mice. Frontal cortex was homogenized in 20 volumes of 25 mM HEPES-Tris, pH 7.4, containing 1 mM EGTA ("homogenization buffer"). Homogenates were centrifuged at 20,000 g for 10 min at 4°C. Buffer was decanted. Membranes were resuspended in 20 volumes of homogenization buffer and centrifuged at 20,000 g for 10 min at 4°C, four additional times. Membranes were then resuspended in 25 mM HEPES-Tris, pH 7.4, containing 3 mM EGTA, 10 mM LiCl, and centrifuged at 20,000 g for 10 min at 4°C. The supernatant was discarded, and the membrane pellet(s) were stored at −80°C. On the day of the assay, pellets were thawed, and protein concentration was measured by Bradford assay.

The assay protocol was based on methods previously described (Damjanoska et al. 2004; Wolf and Schutz 1997). One hundred micrograms of membrane protein was diluted on ice into 100 µl of total volume with a buffer containing 25 mM HEPES-Tris pH 7.4, 3 mM EGTA, 10 mM LiCl, 12 mM $MgCl_2$, 1.44 mM sodium deoxycholate, 200 nM Ca^{2+}, 1 µM GTPγS, 3 µM 5-HT, and 1 mM unlabeled phosphatidyl inositol. 5-HT was not included for basal measures. After addition of 100 µM [^{3}H]-phosphatidyl inositol, the reaction tubes were incubated in a 37°C water bath for 20 min. The reactions were terminated by the addition of 0.9 ml 1:2 chloroform:methanol mixture, followed by 0.3 ml chloroform and 0.3 ml 0.25 M HCl, and 2 min of vigorous shaking. Tubes were then centrifuged at 8,000 g for 2 min. Of the upper aqueous phase, 0.3 ml was mixed with 6 ml scintillation cocktail and counted 1 h later on a liquid scintillation counter. PLC activity for each biological sample (mouse) was measured in triplicate under basal and 5-HT-stimulated conditions. Triplicates were averaged by mouse before analysis.

The comparison of basal and 5-HT-stimulated PLC activity was made between strains by use of an ANOVA model that included one within mouse factor (basal versus stimulated), a factor to account for correlated measures by mouse (mouse), one between mouse factor (C57BL/6J versus DBA/2J), and an interaction between stimulation and strain factors. Of interest in this ANOVA is the interaction that tests whether PLC activity differs between strains.

DOI-induced Egr1 and Egr2 gene expression

Early growth response 1 (*Egr1*) and 2 (*Egr2*), two gene markers induced by hallucinogenic 5-HT_{2A} receptor agonists (Gonzalez-Maeso et al. 2007), were measured to test for potential differences in DOI-induced cellular signaling in frontal cortex of DBA/2J and C57BL/6J. *Egr1* and *Egr2* RNA transcript relative abundance was compared by real-time polymerase chain reaction (PCR) between saline (0.9%)- and DOI (3 mg/kg, IP route)-treated young adult male C57BL/6J and DBA/2J mice (N=5 mice per condition). Tissue was collected 45 min after injection.

Frontal cortex was placed immediately into RNAlater and stored until RNA isolation. The tissue was homogenized in TRI Reagent (Sigma), chloroform was mixed with the lysate, and the mixture was separated into three phases by centrifugation. The RNA was then precipitated from the aqueous phase with isopropanol. DNA-free DNase treatment and removal reagents (Ambion) were used to remove contaminating genomic DNA. RNA quality control and assessment was checked by measuring the 260/280

absorption ratio in 10 mM Tris using a Nanodrop ND-1000 spectrophotometer. In addition, an RNA integrity number (RIN) using an Agilent 2100 Bioanalyzer was determined. All 20 samples used for real-time PCR were high quality, intact RNA, without significant protein or DNA contamination.

FAM-labeled Taqman probes for relative quantification of *Egr1* (assay ID Mm00656724 m1) and *Egr2* (assay ID Mm00456650 m1) were purchased from Applied Biosystems, along with two control gene probes (mouse ACTB and mouse GAPD). The real-time PCR assay was run by the Vanderbilt Microarray Shared Resource core facility (http://array.mc.vanderbilt.edu/services/realtime.vmsr) using the ABI 7900HT Fast real-time PCR system. All samples were run in quadruplicate, and cycle times were calculated using ABI SDS software.

Cycle times and sample identifier data for target and control genes were exported from ABI SDS software to a tab-delimited text file. Data were imported to Stata (v.11) for statistical analyses. Because there is no justification for paired comparisons between individual C57BL/6J mice and DBA/2J mice, the 2^(−ΔΔ Ct) method cannot be used (Schmittgen and Livak 2008). Instead, statistical comparisons are made on 2^(−Δ Ct) data, for example, on 2^(−(CtEgr1–CtGapdh)) (Schmittgen and Livak 2008). Analysis of the target genes was by comparison to *Gapdh*, because it did not appear to be regulated by DOI as did *Actb*. Target genes were tested jointly by multivariate analysis of variance (MANOVA) for an interaction between strain and DOI treatment, because both *Egr1* and *Egr2* are reportedly downstream readouts of DOI-induced 5-HT_{2A} receptor activation (Gonzalez-Maeso et al. 2007).

Results

DOI induces a 5-HT_{2A} receptor-dependent head-twitch response that is modulated by the 5-HT_{2C} receptor

As shown in Fig. 1, 5-HT_{2C} knockout mice exhibited a significantly lower number of HTR in 10 min relative to their wild-type littermates (*t* test, $p=0.01$; mean ± SEM number of HTR: WT ($N=5$), 25±3; 5-HT_{2C} knockout ($N=7$), 14±3).

To assess head-twitch dose–response to DOI, HTRs were counted in three C57BL/6J and three DBA/2J mice for each of eight DOI dose levels (48 total mice): 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 mg/kg (Fig. 2a). Group means (with ±1 SEM error bars) are shown for each strain in Fig. 2a. A 2×8 factorial ANOVA produced a significant interaction between strain and dose level ($F(7,32)=4.93$, $p<0.01$). Bonferroni corrected simple main effects (SME) analysis resolved the interaction into strain differences at

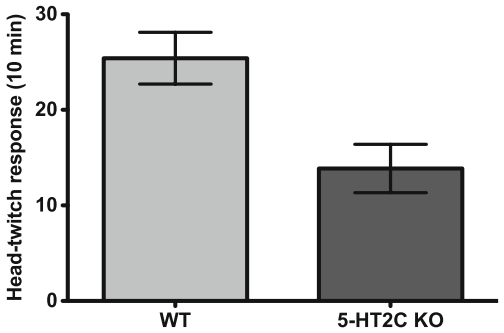

Fig. 1 5-HT_{2C} receptor knockout male mice ($N=7$) exhibit significantly fewer head-twitch responses compared to littermate control mice ($N=5$) after 1 mg/kg DOI treatment

DOI doses of 0.4 mg/kg and above (*$p<0.05$ as indicated in Fig. 2a), but not at either 0.1 or 0.2 mg/kg. Both strains appear to reach maxima in HTR between 0.8 and 1.6 mg/kg DOI dose and maintain this response at higher doses. A small decrement in DBA/2J response was noticed at the higher doses and was accompanied by apparent reduction of overall activity during the observation period. When these data are fit by a four-parameter logistic (dose–response) equation, including parameters for top, bottom, ED50, and Hill slope, only the strain difference in the maximum was significant ($t=7.57$, $p<0.001$, C57BL/6J top=38 head-twitches, DBA/2J top=64 head-twitches, model fit not shown). The dose–response profiles suggest a robust strain difference in DOI-induced HTR that is not readily explained by pharmacokinetic or metabolic factors presenting as a left–right shift in the ED50 but similar maxima.

The role of 5-HT_{2A} receptors in the HTR to DOI was assessed using the antagonist MDL100907. When MDL100907 was given as a pretreatment (0.25 mg/kg) 10 min prior to 1 mg/kg DOI, the DOI-induced HTR was completely blocked in both strains (C57BL/6J=0(0) (mean (SEM)), $N=6$; DBA/2J=1.25(0.54), $n=6$; 10 min observation; data not shown). Neither C57BL/6J nor DBA/2J mice head-twitch following only saline injection (C57BL/6J=2.3 (0.44), $N=6$; DBA/2J=0.75(0.36), $N=6$; 10 min observation; data not shown).

The role of 5-HT_{2C} receptors in the HTR to DOI was assessed using the inverse agonist/antagonist SB206553 and the antagonist SB242084. Figure 2b shows head-twitches after saline pretreatment, 3 mg/kg SB206553 pretreatment, or 3 mg/kg SB242084 pretreatment prior to a 1-mg/kg DOI treatment in C57BL/6J and DBA/2J mice ($N=6$ per group). SB206553 was also used at a pretreatment dose of 0.3 mg/kg, and it produced similar results to 3 mg/kg (data not shown). As expected from the non-parallel lines in Fig. 2b, a 2×3 factorial ANOVA produced a significant interaction between strain and pretreatment ($F(2,30)=10.41$, $p<0.01$). Bonferroni corrected SME analysis resolved the interaction into a significant strain difference

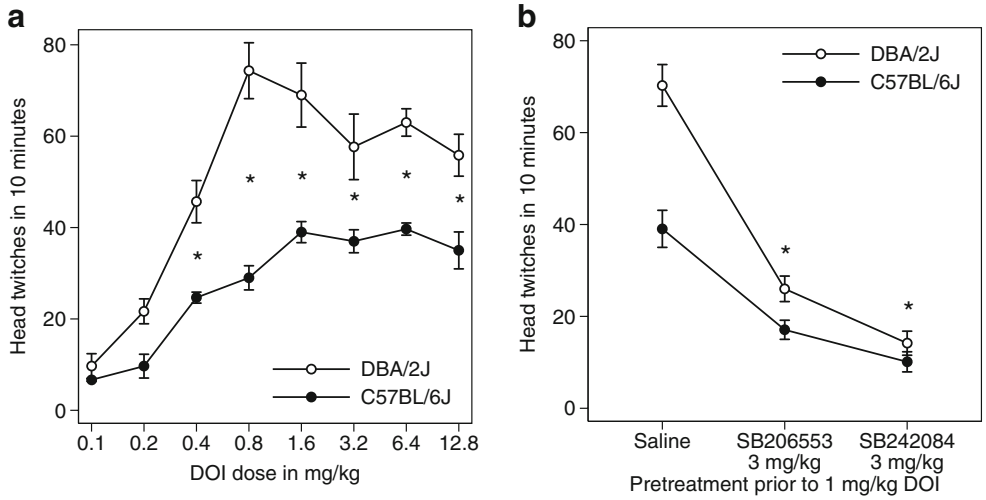

Fig. 2 **a** C57BL/6J and DBA/2J male mice exhibit a robust difference in head-twitch response to DOI (N=3 mice per dose level per strain). Dose–response profiles show a significant difference in maxima between strains. DBA/2J has approximately 1.5- to twofold greater head-twitch response than C57BL/6J. A 2×8 factorial analysis of variance reveals a significant strain by dose interaction that is resolved into differences between strains at dose levels of 0.4 mg/kg or higher, as indicated by *stars*. **b** DOI-induced head-twitch in C57BL/6J and DBA/2J mice is reduced but not eliminated by 5-HT_{2C} antagonist pretreatment, and the strain difference in head-twitch is eradicated by 5-HT_{2C} antagonist pretreatment (N=6 per group). The *stars* indicate both a significant reduction in head-twitch response after SB206553 or SB242084 pretreatment compared to saline pretreatment, and significant reduction in the difference between strains after 5-HT_{2C} antagonist pretreatment compared to saline (see text for details)

after saline pretreatment (F(1,30)=48.34, p<0.01), but not after SB206553 pretreatment (F(1,30)=3.94, p=NS), nor after SB242084 pretreatment (F(1,30)=0.82, p=NS). Alternatively, Bonferroni corrected SME analysis describes differences within each strain between saline and either antagonist pretreatments as significantly different (p<0.05), but that head-twitch between antagonist pretreatments do not differ (p=NS). Finally, the difference in HTR between C57BL/6J and DBA/2J mice after saline pretreatment is significantly different than the difference between strains after either SB206553 pretreatment (F(1,30)=18.30, p<0.01) or SB242084 pretreatment (F(1,30)=12.34, p<0.01), indicated by the stars in Fig. 2b. We conclude that DOI-induced head-twitch in C57BL/6J and DBA/2J mice is reduced but not eliminated by 5-HT_{2C} antagonist pretreatment. Furthermore, we conclude that the strain difference in head-twitch is eradicated by 5-HT_{2C} antagonist pretreatment.

Reduction in DOI-induced head-twitch response by pretreatment with SB206553 is not mediated by 5-HT_{2A} receptors

Figure 3 shows the results of an EEDQ inactivation study, a classic pharmacological demonstration of drug specificity. One-way ANOVA analysis revealed a significant main effect of drug treatment on 5-HT_{2A} specific binding (F(2,13)=13.09, p<0.01). Pretreatment of mice (N=6) with EEDQ significantly reduced [^{3}H]-ketanserin binding to 5-HT_{2A} sites after 24 h, eliminating 65% of specific binding. Pretreatment with 0.25 mg/kg MDL100907 (N=4) 30 min prior to EEDQ significantly prevented EEDQ inactivation of 5-HT_{2A} receptors (p<0.05), restoring 5-HT_{2A} specific [^{3}H]-ketanserin binding to 90% of controls. In contrast, pretreatment with 3.0 mg/kg SB206553 (N=4) 30 min prior to EEDQ treatment did not alter the ability of EEDQ to reduce 5-HT_{2A} specific [^{3}H]-ketanserin binding, suggesting that this dose of SB206553 does not appreciably bind 5-HT_{2A} receptor binding sites in the mouse brain in vivo.

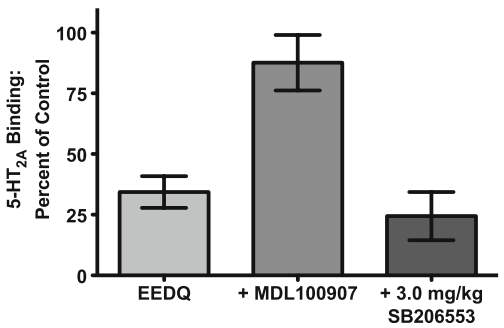

Fig. 3 EEDQ treatment inactivates 5-HT_{2A} receptor binding (N=6) in the mouse brain which is prevented by pretreatment with MDL100907 (0.25 mg/kg, N=4), but not SB206553 (3.0 mg/kg, N=4), suggesting that 3.0 mg/kg SB206553 does not bind to 5-HT_{2A} receptor binding sites at this dose or lower doses

Table 1 $5HT_{2A}$ and $5HT_{2C}$ receptor saturation binding Bmax (femtomoles per milligram protein) and Kd (nanomolar)

Strain	*n*	$5HT_{2A}$		$5HT_{2C}$	
		Bmax (SEM)	Kd (SEM)	Bmax (SEM)	Kd (SEM)
C57BL/6J	6	410 (28.7)	1.3 (0.11)	40 (4.0)	2.2 (0.40)
DBA/2J	6	384 (39.5)	1.3 (0.24)	39 (4.3)	3.0 (0.56)

Candidate mechanisms explaining the C57BL/6J and DBA/2J difference in head-twitch

5-HT_{2A} and 5-HT_{2C} receptor saturation binding and autoradiography

Saturation binding was used to test for differences in 5-HT_{2A} receptor density in the frontal cortex and 5-HT_{2C} receptor density in whole brains, from C57BL/6J and DBA/2J mice. Nonlinear regression analysis was used to determine the Bmax and Kd estimates from total binding and nonspecific binding data for each sample (Table 1). Comparisons of the 5-HT_{2A} Bmax values (femtomoles per milligram protein) between strains (*t* test, t=0.54, df=10, p=NS) and of the 5-HT_{2A} Kd values (nanomolar) between strains (*t* test, t=0.06, df=10, p=NS) showed no significant differences. Comparisons of the 5-HT_{2C} Bmax values between strains (*t* test, t=0.21, df=10, p=NS) and of the 5-HT_{2C} Kd values between strains (*t* test, t=1.34, df=10, p=NS) also showed no significant differences.

[^{125}I]-DOI autoradiography for cortical and striatal brain areas in C57BL/6J and DBA/2J did not reveal obvious differences in either 5-HT_{2A} or 5-HT_{2C} receptor high-affinity binding (Fig. 4a, b, respectively). Kruskal–Wallis tests of prefrontal cortex and two other cortical regions, as well as two striatal regions, were not significant (p>0.05).

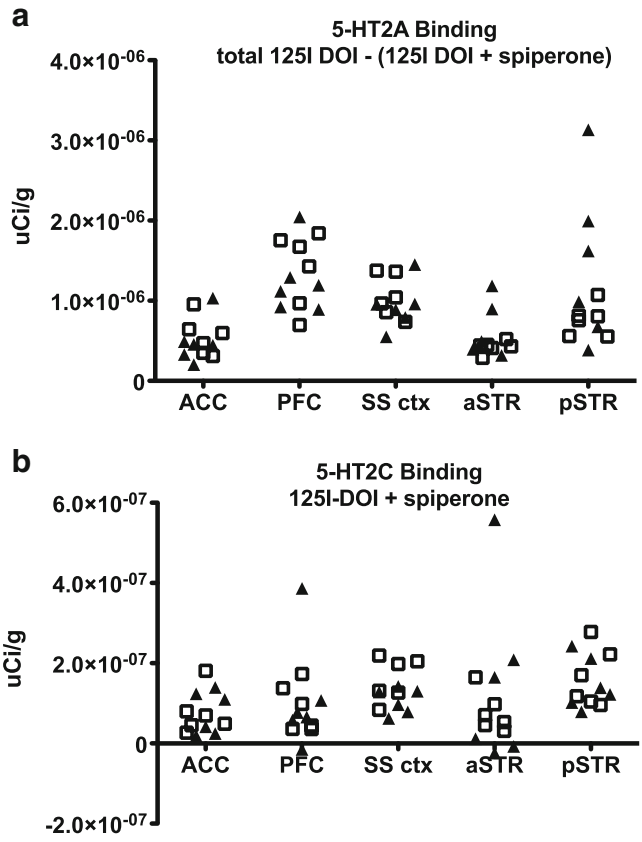

Fig. 4 [^{125}I]-DOI autoradiography data showing 5-HT_{2A} and 5-HT_{2C} receptor high-affinity binding in cortical and striatal brain regions comparing six C57BL/6J mice (*squares*, N=6) and six DBA/2J mice (*triangles*, N=6). The *y*-axis is microcuries per gram of tissue, and the *x*-axis defines the brain regions anterior cingulate cortex (ACC), prefrontal cortex (PFC), somatosensory cortex (SS ctx), anterior striatum (ASTR), and posterior striatum (PSTR). **a** [^{125}I]-DOI 5-HT_{2A} receptor binding. **b** [^{125}I]-DOI 5-HT_{2C} receptor binding

Western blot for $G\alpha_{q/11}$ or $G\alpha_{i/o}$

No evidence could be established to support a difference in levels of $G\alpha_{q/11}$ or $G\alpha_{i/o}$ in frontal cortex between C57BL/6J and DBA/2J mice. Because α-tubulin appeared to be regulated between strains (data not shown; however, see Fig. 5 inset for an example), we compared raw density measures for $G\alpha_{q/11}$ and $G\alpha_{i/o}$ between C57BL/6J and DBA/2J mice. Frontal cortex from 12 mice of each strain was used for $G\alpha_{q/11}$ Western blots, and frontal cortex from eight mice of each strain was used for $G\alpha_{i/o}$ Western blots. Unpaired *t* tests comparing mean protein densities in C57BL/6J and DBA/2J mice showed no significant differences between groups ($G\alpha_{q/11}$, p=0.94; $G\alpha_{i/o}$, p=NS).

PLC activity

Serotonin-stimulated PLC activity was measured by the amount of [^{3}H]-inositol phosphate produced by PLC in the

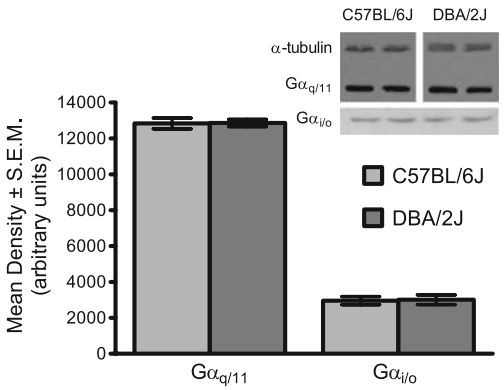

Fig. 5 There were no detectable differences in densities of $G\alpha_{q/11}$ and $G\alpha_{i/o}$ proteins in cortex of C57BL/6J and DBA/2J. *Inset* shows a representative photomicrograph of Western blots of frontal cortex from C57BL/6J and DBA/2J mice showing equivalent levels of $G\alpha_{q/11}$ and $G\alpha_{i/o}$ proteins

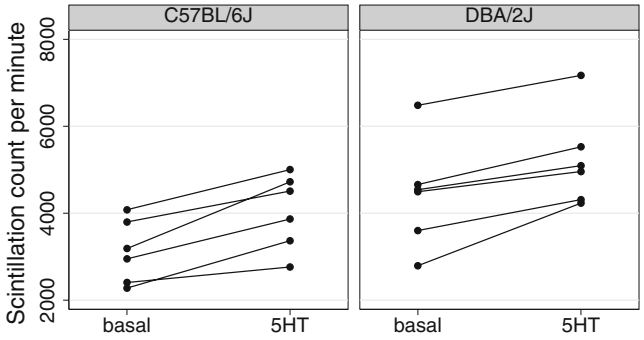

Fig. 6 The figure plots the basal and stimulated counts connected by mouse for each strain. Despite induction of significant 5-HT-stimulated [^{3}H]-inositol phosphate levels produced by phospholipase C in frontal cortex from C57BL/6J and DBA/2J mice (N=6 per strain), there were no differences between strains

membrane fractions of C57BL/6J (N=6 mice) and DBA/2J (N=6 mice) frontal cortex. Figure 6 shows PLC activity measures for basal and 5-HT-stimulated conditions by mouse and strain. A strain difference in PLC activity would be evident as a difference in the slope from basal to stimulated conditions. While ANOVA confirms significant 5-HT-stimulated PLC activity across strains (F(1,10)=63.89, p<0.01), showing that 5-HT stimulated PLC activity, the interaction between strain and PLC activity was not significant (F(1,10)=0.40, p=NS). There is no evidence for differing PLC activity in frontal cortex between C57BL/6J and DBA/2J mice. PLC activity induced by 5-HT$_{2A}$ receptors does not likely underlie DOI-induced head-twitch differences between C57BL/6J and DBA/2J mice.

DOI-induced Egr1 and Egr2 gene expression

The relative RNA transcript abundance of *Egr1* and *Egr2* genes in the frontal cortex of C57BL/6J and DBA/2J mice after saline and DOI (3 mg/kg) injections was measured using real-time PCR and analyzed by the comparative C*T* method (n=5 mice per group). Figure 7 shows in panels (a) and (b) the delta cycle times for *Egr1* and *Egr2* relative to *Gapdh*. A MANOVA predicting *Egr1* and *Egr2* expression from strain and DOI treatment confirmed the strain (F(2,15)=17.39, p<0.01) and DOI (F(2,15)=20.18, p<0.01) effects apparent in panels (a) and (b). The interaction between strain and DOI was not significant (F(2,15)=0.10, p=NS). *Egr1* and *Egr2* expression in the frontal cortex of C57BL/6J and DBA/2J mice are increased equivalently by DOI, independent of a basal strain difference in gene expression.

Discussion

5-HT type 2 receptors are central mechanistic components of the psychoactive response to serotonin-based hallucinogenic drugs, and it is accepted that the 5-HT$_{2A}$ receptor subtype is the most essential receptor mediating the hallucinatory effects (Nichols 2004; Winter 2009). In humans, antagonists of 5-HT$_{2A}$ receptors such as chlorpromazine dampen the psychoactive effects of hallucinogens (Freedman 1986), and pretreatment in humans with the 5-HT$_{2A}$ antagonist ketanserin dose-dependently prevents the hallucinogenic effects caused by psilocybin ingestion (Vollenweider et al. 1998). In addition, mice devoid of the 5-HT$_{2A}$ receptor do not exhibit a regularly observed behavioral response to hallucinogens, the HTR (Gonzalez-Maeso et al. 2007). Fairly selective 5-HT$_{2A}$ antagonists, such as MDL100907, also prevent this response, and block drug discrimination of DOI and the related hallucinogenic analogue, DOB (Benneyworth et al. 2005; Schreiber et al. 1994).

However, several findings confound the interpretation that the psychoactive response to serotonin-like hallucino-

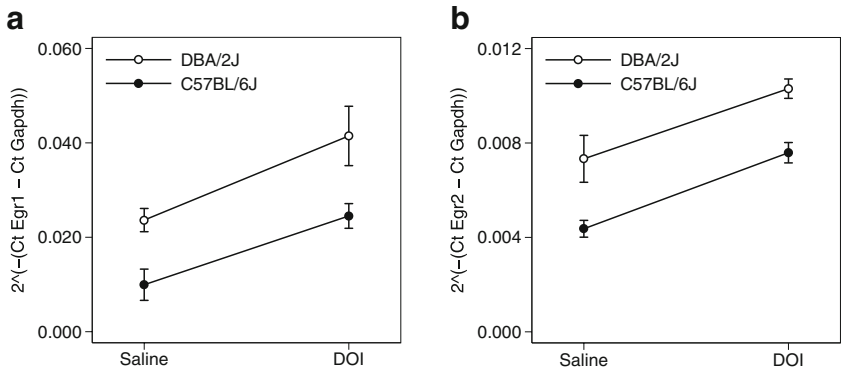

Fig. 7 Interaction plots are shown for relative gene expression from (**a**) *Egr1* and (**b**) *Egr2* real-time polymerase chain reaction experiments. Means ± SEM for gene expression (y-axis) are shown against DOI condition (x-axis) and strain (*white or black symbols*, N=5 per group, 20 mice total). *Egr1* and *Egr2* expression in the frontal cortex of C57BL/6J and DBA/2J mice are equivalently increased by DOI, independent of a basal strain difference in gene expression; there is no indication of a strain difference

gens is mediated solely by the 5-HT_{2A} receptor. Results from radioligand binding studies show that the 5-HT_{2A} receptor antagonists ketanserin, chlorpromazine, and MDL100907 have appreciable affinity for 5-HT_{2C} receptors (PDSP, http://pdsp.med.unc.edu/). In addition, all known 5-HT_2 receptor agonists that are hallucinogenic have equal or greater affinities and potencies at 5-HT_{2C} receptors relative to 5-HT_{2A} receptors. These data leave open the possibility that 5-HT_{2C} receptors functionally participate in inducing the hallucinogenic response (Fiorella et al. 1995a; Winter 2009).

Despite speculation that activation of the 5-HT_{2C} receptor could modulate the psychoactive effects caused by hallucinogens, the collective results of drug discrimination studies suggest that 5-HT_{2C} receptors are not central to the subjective effects of serotonin-like hallucinogens (Winter 2009). 5-HT_{2C} receptor antagonists do not block drug discrimination for DOI in mice (Schreiber et al. 1994; Smith et al. 2003). We have also observed previously that 3.0 mg/kg of the 5-HT_{2C} inverse agonist/antagonist SB206553 does not reduce the ability of mice to discriminate LSD from vehicle (data not shown). Hence, we were surprised to observe a blunted DOI-induced HTR in 5-HT_{2C} knockout mice, our initial hint that 5-HT_{2C} receptors may be involved in this response elicited by hallucinogens. The reduced HTR to DOI in 5-HT_{2C} knockout mice was likely not due to developmental alterations in 5-HT_{2A} receptor levels, as it has been shown that 5-HT_{2C} knockout mice have equivalent levels of 5-HT_{2A} receptors as their wild-type littermates (Lopez-Gimenez et al. 2002). Similarly, 5-HT_{2A} knockout mice do not have alterations in 5-HT_{2C} levels (Weisstaub et al. 2006), suggesting that genetically manipulating either 5-HT_{2C} or 5-HT_{2A} receptors does not alter expression of the other.

In a separate set of experiments, we observed that DBA/2J and C57BL/6J mice have dramatically different HTRs to DOI with DBA/2J mice showing 75% more head-twitches. This difference was likely not due to pharmacokinetic or metabolic factors, as it was maintained across a wide range of doses. The HTR was completely eliminated by pretreating either strain of mouse with MDL100907, suggesting it is 5-HT_{2A} mediated. However, several measures aimed at discovering a difference between the strains in 5-HT_{2A} receptor function in the frontal cortex, including receptor and G-protein density, *Egr1* and *Egr2* expression, and PLC activity, proved futile. Collectively, these data suggested that the 5-HT_{2A} receptor was not mediating the strain differences in response to DOI.

We next tested whether the 5-HT_{2C} receptor mediated the strain differences. The 5-HT_{2C} selective inverse agonist and antagonist SB206553 completely eliminated the strain differences in response to DOI at two doses, 3.0 mg/kg and a tenfold lower dose, 0.3 mg/kg. This effect was not due to non-selective blockade of 5-HT_{2A} receptors, as pretreatment of mice with 3.0 mg/kg SB206553 did not prevent EEDQ inactivation of 5-HT_{2A} receptors. Recent reports show that SB206553 is a positive allosteric modulator of nicotinic $\alpha 7$ receptors (Dunlop et al. 2009), which called into question whether other targets of SB206553 could be mediating its effects on the DOI HTR. However, a different 5-HT_{2C} antagonist, SB242084, also attenuated DOI HTR and eliminated the strain differences; these data conflict with a previous report using rats (Vickers et al. 2001), suggesting there may be species-related differences in mechanisms controlling the DOI-induced HTR in rodents. The observation that at high concentrations SB242084 activates 5-HT_{2C} receptor→PLC signaling in CHO cells may also be pertinent to species differences in the effects of SB242084 (De Deurwaerdere et al. 2004). In summary, our data showing suppressive effects of 5-HT_{2C} receptor antagonists on DOI-induced HTR in mice combined with the attenuated response to DOI in 5-HT_{2C} knockout mice strongly support the conclusion that the 5-HT_{2C} receptor potently modulates the behavioral response in mice induced by the hallucinogenic 5-HT_2 agonist DOI.

We attempted to discover a difference in 5-HT_{2C} density or cellular function in C57BL/6J and DBA/2J mice but were unsuccessful. [^{3}H]-mesulergine saturation binding did not reveal a difference in total 5-HT_{2C} density in brains of the two strains. Also, [^{125}I]-DOI autoradiography which preferentially labels high-affinity agonist sites did not reveal a significant difference between the strains in 5-HT_{2C} binding sites in regions thought to underlie the DOI-induced HTR. A forward genetic screen of DOI-induced HTR in experimental crosses of mice derived from C57BL/6J and DBA/2J may point to the genetic loci that underlie the strain difference. Speculative possibilities that may underlie the different HTR induced by DOI in C57BL/6J and DBA/2J mice include alterations in glutamatergic, dopaminergic, and/or 5-HT1A receptors or the serotonin transporter, which have been shown to modulate the stimulus properties and head-twitch-inducing properties of hallucinogens in rodents (Jennings et al. 2008; Krall et al. 2008; Winter 2009).

The role of the 5-HT_{2C} receptor in modulating the behavioral response to DOI in mice is interesting in light of observations that the 5-HT_{2A} receptor agonist, lisuride, does not have LSD-like hallucinatory effects in humans (Freedman and Boggan 1982; Herrmann et al. 1977) and does not cause an HTR in mice (Gonzalez-Maeso et al. 2007), an observation confirmed in our laboratory (data not shown). These findings have been interpreted to support the idea that 5-HT_{2A} receptor agonists with hallucinogenic properties stabilize a unique conformation of the 5-HT_{2A} receptor that is not induced by non-hallucinogenic 5-HT_{2A} agonists, suggesting that serotonin-based hallucinogens are conformationally selective ligands, activating specific signal-

ing pathways downstream of the 5-HT_{2A} receptor (Gonzalez-Maeso and Sealfon 2009; Gonzalez-Maeso et al. 2007).

An alternative or additional possibility is that lisuride differentially interacts with 5-HT_{2A} and 5-HT_{2C} receptors. In cultured cells of the rat choroid plexus, lisuride behaves as an antagonist at 5-HT_{2C} receptors, whereas the hallucinogens LSD, DOB, DOM, and 5-methoxy-DMT are potent 5-HT_{2C} agonists (Burris et al. 1991; Sanders-Bush and Breeding 1991). This is in contrast to in vitro studies using 5-HT_{2C}-VSV receptors stably expressed at high levels in CHO cells, where lisuride behaves as a weak partial agonist (Cussac et al. 2002) or agonist (Cussac et al. 2008). The differences in 5-HT_{2C} receptor expression levels and edited isoforms between native cells from the choroid plexus and CHO cells transfected with 5-HT_{2C}-VSV (Burns et al. 1997; Cussac et al. 2008; Sanders-Bush and Breeding 1991) could account for this discrepancy; alterations in 5-HT_{2C} receptor levels and RNA editing of 5-HT_{2C} receptor can alter ligand pharmacology at 5-HT_{2C} receptors (Berg et al. 2008; Fitzgerald et al. 1999). Also, weak partial agonist activity in vitro may translate to antagonism in vivo. The inability of lisuride to induce LSD-like hallucinations may relate to its antagonism of 5-HT_{2C} receptors expressed in vivo. Taken together, the converging evidence suggests that the behavioral response in mice elicited by the hallucinogen DOI is potently modulated by 5-HT_{2C} receptors.

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