Pharmacology, Biochemistry and Behavior 148 (2016) 136–144

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

The effect of the sigma-1 receptor selective compound LS-1-137 on the DOI-induced head twitch response in mice

Maninder Malik [a], Claudia Rangel-Barajas [a,1], Robert H. Mach [b], Robert R. Luedtke [a,*]

[a] University of North Texas Health Science Center, The Center for Neuroscience Discovery, 3500 Camp Bowie Boulevard, Fort Worth, TX 76107, United States
[b] Radiochemistry Laboratory, Department of Neurology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, United States

ARTICLE INFO

Article history:
Received 1 March 2016
Received in revised form 17 June 2016
Accepted 6 July 2016
Available online 8 July 2016

Keywords:
Head twitch response
Sigma-1 receptors
2,5-dimethoxy-4-iodoamphetamine (DOI)
Neuropsychiatric disorders
Antipsychotic activity

ABSTRACT

Several receptor mediated pathways have been shown to modulate the murine head twitch response (HTR). However, the role of sigma receptors in the murine (±)-2,5-dimethoxy-4-iodoamphetamine (DOI)-induced HTR has not been previously investigated. We examined the ability of LS-1-137, a novel sigma-1 *vs.* sigma-2 receptor selective phenylacetamide, to modulate the DOI-induced HTR in DBA/2J mice. We also assessed the *in vivo* efficacy of reference sigma-1 receptor antagonists and agonists PRE-084 and PPCC. The effect of the sigma-2 receptor selective antagonist RHM-1-86 was also examined. Rotarod analysis was performed to monitor motor coordination after LS-1-137 administration. Radioligand binding techniques were used to determine the affinity of LS-1-137 at 5-HT2A and 5-HT2C receptors. LS-1-137 and the sigma-1 receptor antagonists haloperidol and BD 1047 were able to attenuate a DOI-induced HTR, indicating that LS-1-137 was acting *in vivo* as a sigma-1 receptor antagonist. LS-1-137 did not compromise rotarod performance within a dose range capable of attenuating the effects of DOI. Radioligand binding studies indicate that LS-1-137 exhibits low affinity binding at both 5-HT2A and 5-HT2C receptors. Based upon the results from these and our previous studies, LS-1-137 is a neuroprotective agent that attenuates the murine DOI-induced HTR independent of activity at 5-HT2 receptor subtypes, D2-like dopamine receptors, sigma-2 receptors and NMDA receptors. LS-1-137 appears to act as a sigma-1 receptor antagonist to inhibit the DOI-induced HTR. Therefore, the DOI-induced HTR can be used to assess the *in vivo* efficacy of sigma-1 receptor selective compounds.

1. Introduction

Previous behavioral pharmacology studies suggest that hallucinogenic drugs are capable of inducing psychotic-like episodes and cognitive deficits in humans that resemble the disordered thoughts, auditory and/or visual hallucinations (positive symptoms) associated with schizophrenia (Moreno and González-Maeso, 2013; Corne and Pickering, 1967; Arnedo et al., 2014). Stimulation of serotonin 5-HT2 receptor subtypes has been implicated in the mechanism of action of several hallucinogenic drugs (Glennon et al., 1984). In addition, a number of known hallucinogenic compounds, including the ergoline lysergic acid diethylamide (LSD) (Maj et al., 1978; Halberstadt and Geyer, 2013; Moreno et al., 2013a, b), the arylcyclohexylamine phencyclidine (PCP) (Nabeshima et al., 1987), the psychedelic tryptamine 5-methoxy-N,N-diisopropyltryptamine (5-MeO-DIPT) (Fantegrossi et al., 2006) and the substituted amphetamine 2,5-dimethoxy-4-iodoamphetamine (DOI) (Canal and Morgan, 2012) induce a head twitch response (HTR), which manifests as a rapid side-to-side head movement in mice.

The DOI-induced HTR has been discussed as an animal model for examining 5-hydroxytryptamine 5-HT2 receptor activity *in vivo* (Canal and Morgan, 2012). DOI binds with high affinity at 5-HT2 receptors (Knight et al., 2004; McKenna and McKenna and Peroutka, 1989; Shannon et al., 1984) and has been reported to be a full agonist at 5-HT2A receptors and a partial agonist at 5-HT2C receptors (Porter et al., 1999). However, DOI is likely functionally selective (Urban et al., 2007) because it has been reported to be either a partial or a full agonist for 5-HT2A, 5-HT2B and 5-HT2C receptors, depending on the signaling pathway that is used for the evaluation (Marek and Aghajanian, 1996; Porter et al., 1999; Berg et al., 1998, 2001; Cussac et al., 2002; Moya et al., 2007).

The symptoms of schizophrenia have been historically categorized as positive (abnormal thoughts/perceptions, auditory hallucinations, delusions) and negative (decreased social functions) symptoms (Kay et al., 2004). However, cognitive measures have also been documented to be impaired in schizophrenia, including a) attention, b) working memory, c) verbal and visual learning, d) reasoning/problem solving and e) social cognition (*i.e.*, emotion perception and social cue

Abbreviations: DOI, 2,5-dimethoxy-4-iodoamphetamine; HTR, Head twitch response; TS, Tourette Syndrome; 5-hydroxytryptamine, 5-HT2; Dimethyl sulfoxide, DMSO.

* Corresponding author at: University of North Texas Health Science Center, 3500 Camp Bowie, Fort Worth, TX 76107, United States.
E-mail address: robert.luedtke@unthsc.edu (R.R. Luedtke).
[1] Current address: Neuropsychology, Biological Psychology, Behavioural Science, Indiana University at Bloomington.

http://dx.doi.org/10.1016/j.pbb.2016.07.001
0091-3057/

interpretation) (Michalopoulou et al., 2013; Rodriguez-Jimenez et al., 2012). Cognitive impairment represents a major impediment to the recovery of schizophrenic patients. One might anticipate that the administration of a hallucinogen, such as DOI, might also affect cognitive performance.

At a low dose (0.1 mg/kg) DOI has been reported to enhance conditioned response-based memory formation in rats (Meneses, 2002), while at a similar dose DOI interfered with a delayed non-matching to position working memory task in 5,7-dihydroxytryptamine lesioned rats (Ruotsalainen et al., 1998). At doses of 0.1 mg/kg and 0.25 mg/kg, DOI did not affect rat learning or the error rate in an "alleys and door" Morris water maze test (Kant et al., 1998). At moderate doses (0.25 to 0.625 mg/kg) DOI was found to increase temporal discrimination, where the length of time for a stimulant is the controlling variable, which the authors suggest may reflect an impairment of sustained attention (Hampson et al., 2010). However, at a higher dose, DOI (2.0 mg/kg) was reported to reduce accuracy and impaired responding in a n-back working memory task in rodents (Ko and Evenden, 2009).

Antipsychotic drugs, which are used clinically to attenuate the frequency and intensity of the positive symptoms of schizophrenia, are also capable of attenuating the HTR in rodents (Hayslett and Tizabi, 2005; Moreno et al., 2013b). For example, the butyrophenone haloperidol, which is a typical antipsychotic used for the treatment of Tourette Syndrome (TS), schizophrenia and obsessive compulsive disorder, has been shown to inhibit the murine DOI-dependent HTR in a dose-dependent manner (Hayslett and Tizabi, 2005; Rangel-Barajas et al., 2014). Although haloperidol is generally thought to mediate its antipsychotic activity primarily by antagonizing dopamine D2 receptors, it also binds with high (nM) affinity at the D3 dopamine receptor subtype (Luedtke et al., 2012). In addition, the affinity of haloperidol at sigma-1 receptors is comparable to its affinity at D2 and D3 dopamine receptors (Largent et al., 1984; Tam and Cook, 1984; Su et al., 1986; Weissman et al., 1990; Luedtke et al., 2012). Several other neuroleptics, including chlorpromazine and pimozide, have appreciable affinity for sigma-1 receptors and have also been shown to attenuate the DOI-induced HTR (Deutsch et al., 1988; Tam and Cook, 1984; Walker et al., 1990; Rangel-Barajas et al., 2014).

The studies in this communication were performed to further investigate the *in vivo* pharmacological properties of LS-1-137, a sigma-1 *vs.* sigma-2 receptor selective phenylacetamide that we developed (Huang et al., 2001), by examining its ability to attenuate the DOI-induced HTR in mice. We recently reported studies on the characterization of the DOI-induced HTR in male DBA/2J mice and the ability of a panel of D2-like dopamine receptor selective ligands, that we developed, to attenuate the murine HTR. That panel of compounds included a) haloperidol, b) D2 *vs.* D3 dopamine receptor selective compounds (Rangel-Barajas et al., 2014) and c) D3 *vs.* D2 dopamine receptor selective compounds (Rangel-Barajas et al., 2015). Although haloperidol is a high affinity D2 dopamine, D3 dopamine and sigma-1 receptor antagonist, the D2 and D3 receptor selective compounds that we used to inhibit the HTR bind with low affinity at both sigma-1 and sigma-2 receptors.

In this communication we investigated whether LS-1-137, a sigma-1 selective compound devoid of D2-dopaminergic and 5-HT2 binding activity, could modulate the DOI-induced HTR in DBA/2J mice. We previously reported that LS-1-137 has neuroprotective properties *in vivo* using a transient middle cerebral artery occlusion (t-MCAO) model of stroke (Schetz et al., 2007; Luedtke et al., 2012). More recently we reported that LS-1-137 could a) partially reverse the cognitive deficits associated with muscarinic antagonist administration in mice and b) trigger the release of brain-derived neurotrophic factor (BDNF) from astrocytes (Malik et al., 2015). These effects of LS-1-137 appear to be independent of the involvement of NMDA and muscarinic receptors. We now report that LS-1-137 can inhibit the DOI-dependent HTR in a dose-dependent manner. We compared its inhibitory activity to reference sigma-1 receptor compounds to determine if LS-1-137 was acting *in vivo* as an agonist or antagonist at sigma-1 receptors.

2. Methods

2.1. Animals

All animal procedures were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) at University of North Texas Health Science Center. Male DBA/2J (6–8 weeks) mice were obtained from Jackson Laboratories. The weight of the animals ranged from 20 to 28 g. Mice were habituated for 1 week in the animal facility before behavioral studies were initiated. Mice were housed at ≤5 animals per cage under standard 12 h light/12 h dark conditions with free access to food and water. The animals were randomly assigned into different groups. Animal care and housing were in adherence with the conditions set forth in the "Guide for the Care and Use of Laboratory Animals".

2.2. Preparation of drugs

LS-1-137 was synthesized by NIMH Chemical Synthesis. DOI, 4-[4-(p-chlorophenyl)-4-hydroxypiperidino]-4′-fluorobutyrophenone (haloperidol) and 2-morpholin-4-ylethyl 1-phenylcyclohexane-1-carboxylate (PRE-084) were purchased from Sigma-Aldrich (St. Louis, MO, USA). (1*R*,2*S*/1*S*,2*R*)-2-[(4-Hydroxy-4-phenylpiperidin-1-yl)methyl]-1-(4-methylphenyl) cyclopropanecarboxylate ((±)-PPCC) and N′-[2-(3,4-dichlorophenyl)ethyl]-*N*,*N*,*N*′-trimethylethane-1,2-diamine (BD 1047) were purchased from Tocris Bioscience (Bristol, UK).

DOI and all test drugs for animal experiments were dissolved in sterile, Millipore filtered distilled water containing 5% dimethyl sulfoxide (DMSO). Test drugs were prepared on the day of the experiment. All drugs were administered *via* intraperitoneal (i.p.) injection. Test drugs were given 5 min prior to the DOI injection. The quantification of the HTR was started immediately following DOI administration. Animals were allowed a 6 day drug-free period of time between experiments.

2.3. Binding assays

The binding properties of membrane-associated receptors were characterized using a competitive radioligand filtration binding assay (Clarke et al., 2001). Human 5-HT2A receptors were expressed in HEK 293 cells and human 5-HT2C receptors were expressed in Chinese hamster ovary (CHO) cells. For 5-HT2A receptor binding studies, cells were incubated with [^3H]ketanserin and 1 μM of methysergide was used to define the non-specific binding. For 5-HT2C receptor binding studies, cells were incubated with [^3H]mesulergine and 1uM of mianserin was used to define the non-specific binding. The cells were incubated at 37 °C for 60 min and the final assay volume for both the 5-HT2A and 5-HT2C receptor binding assays was 300 μl.

D2long, D3 and D4.4 dopamine receptors were expressed in HEK 293 cells. Membrane homogenates (50 μl) expressing the D2-like dopamine receptors were suspended in 50 mM Tris–HCl/150 mM NaCl/10 mM EDTA buffer, pH = 7.4 and incubated with 50 μl of ^{125}I-IABN (Luedtke et al., 2000) in the presence or absence of the competitive inhibitor (50 μl) at 37 °C for 60 min, using 2.5 M (+)-butaclamol to define the non-specific binding.

For each competition curve, two concentrations of inhibitor per decade were used and each assay was performed in triplicate. Binding was terminated by the addition of cold wash buffer (10 mM Tris–HCl/150 mM NaCl, pH = 7.4) and filtration over a glass-fiber filter (Pall A/B filters, #66198). For these binding studies the IC50 values were determined using a one site fit analysis. The IC50 values were converted to equilibrium dissociation constants (Ki values) using the Cheng and Prusoff equation (Cheng and Prusoff, 1973). Mean Ki values ± S.E.M. are reported for at least three independent experiments. The Kd values that were used to calculate the Ki values were a) 1.1 nM for 5HT2A receptors and b) 0.56 nM for 5-HT2C receptors.

2.4. Head twitch protocol

On the day of testing, mice were weighed and placed individually in an open ended Plexiglas cylinder (21 × 34 cm) with a clean paper towel floor in a dimly lighted room. Animals were allowed to habituate to the cylinder for 15 min prior to the i.p. injection of vehicle (5% DMSO in sterile water) or test drug. Five minutes later the DOI was administered to the animal by i.p. injection and the mouse was immediately returned to the cylinder. The HTR was defined as a rapid left to right (or right to left) movement of the head, without the involvement of the front paws. After our initial observation that LS-1-137, at a dose of 5 mg/kg, could attenuate the HTR when administered 5 min prior to the administration of 5 mg/kg DOI, we continued to screen sigma receptor related compounds for activity using the same conditions.

Two observers counted the number of head twitches by visual examination and the number of head twitches was recorded in 5-min intervals. The data points are presented as the mean values obtained by two observers. In our initial studies (Rangel-Barajas et al., 2014, 2015) both observers were blinded to the dose of the test drugs. As we continued these studies, we monitored the number of head twitches that were recorded by the two observers and found the reproducibility of the number of head twitches recorded by the two observers was very consistent. In all subsequent studies at least one observer was blinded to the dose of test drug.

The estimated IC50 value for the inhibition of the HTR by LS-1-137 was determined using Prism Graph Pad Version 6.0a, using a four-parameter sigmoidal variable slope model with the value for no inhibition constrained using experimental data and an unconstrained value for maximum inhibition.

2.5. Rotarod studies

A rotarod behavioral test was performed to evaluate the drug's effect on motor performance and coordination. The rotarod procedure for mice has been previously described (Kumar et al., 2009; Jung and Metzger, 2013; Rangel-Barajas et al., 2015). The rotarod apparatus (AccuScan Instruments Inc., Columbus, OH) consists of four cylinders that are mounted 35.5 cm above a padded surface. Mice were placed on the cylinder and a timer switch was activated to rotate the cylinders. Acceleration continued until it reached 44 rpm for a maximum of 90 s or until animals fell to the padded surface. Mice were trained for 3 days. Each training day was comprised of two sessions, one in the morning and one in the afternoon. Each session consisted of five trials. The data is presented as mean performance of the animals on a testing day. On day 4 LS-1-137 was administered at the approximate IC50 dose (5 mg/kg) for only the morning session. On day 5, haloperidol (3 mg/kg) was tested as a positive control (one session). Animals were given vehicle (5% DMSO in sterile water) or test drugs *via* i.p. injection 5 min before the beginning of each first session.

2.6. Statistical analysis

All results are presented as mean ± S.E.M. Data was analyzed using Analysis of Variance (ANOVA) when appropriate. When the main effect was obtained, Fisher's Least Significant Difference post-hoc test was performed. Significance for all statistical comparisons was set at $p \leq 0.05$. The data was analyzed using Prism 6.0a; GraphPad Software (San Diego, CA, USA).

3. Results

Based upon our previously reported studies, we selected male DBA/2J mice for the current studies because DBA/2J mice elicit a substantial number of DOI-induced head twitches over a reasonable amount of time (90–100 twitches/30 min) that can be readily quantified (Rangel-Barajas et al., 2014, 2015). Although it has been reported that the effect of DOI on the HTR is dose-dependent, low doses (0.05–0.1 mg/kg) of DOI do not elicit a HTR. A robust HTR was found using doses of DOI ranging from 2 to 10 mg/kg (Darmani et al., 1990, 1991; Fox et al., 2010; Canal and Morgan, 2012). In our initial studies (Rangel-Barajas et al., 2014), we examined two different doses (1 and 5 mg/kg) of DOI and we found that 5 mg/kg, allowed for the quantification of the HTR with a consistent frequency throughout the observation period (30 to 120 min). For both doses the duration of the response lasted approximately 2 h, with the head twitches starting at about 90 s after DOI administration. During that 90 s we did not observe any head twitch-like movements, suggesting that LS-1-137 and the other test compounds were not capable of inducing this type of movement.

The 5 mg/kg dose of DOI was selected for the current studies because the intensity of the HTR, in our opinion, would be more accurate when studying the inhibitory effects of our compounds. In addition, if we used the same dose of DOI in this study that we used for our studies examining the effects of D2-like receptor selective ligands (Rangel-Barajas et al., 2014, 2014) we could directly compare the efficacy of LS-1-137 to inhibit the HTR with our dopaminergic ligands.

For the initial studies the DOI-induced HTR was monitored for a duration of 2 h using a dose of 5 mg/kg of DOI in the presence or absence of the D2-like, sigma-1 and sigma-2 receptor antagonist haloperidol (Fig. 1A). Haloperidol (1 mg/kg) was given 5 min prior to DOI injection. Two-way repeated measure ANOVA indicated that haloperidol rapidly attenuated the frequency (number of twitches/unit of time) of the DOI-induced HTR (Fig. 1A and B) ($F(23, 276) = 31.55$; $p < 0.0001$). A significant difference between the two groups (DOI plus vehicle and DOI plus haloperidol) was seen as early as 10 min and was maintained throughout the duration of the observation period of 120 min. For subsequent experiments the effect of test compounds to modulate the DOI-induced HTR was evaluated over a 30 min time period.

We then compared the ability of a panel of sigma-1 and sigma-2 receptor compounds to attenuate the DOI-induced HTR. The pharmacological profile of the test compounds is shown in Table 1. We found that all of the sigma-1 and sigma-2 selective test compounds, except haloperidol, bind with low affinity at the D2-like (D2, D3 and D4) dopamine receptor subtype.

For these studies test compounds were administered at a dose of 5 mg/kg five minutes prior to DOI (5 mg/kg) administration, with the exception of haloperidol (1 mg/kg). The sigma-2 selective antagonist RHM-1-86 did not significantly attenuate the DOI-induced HTR (80.0 ± 4.1 head twitches/30 min) when compared to DOI vehicle control (85.0 ± 3.6 head twitches/30 min) ($p = 0.4030$; $n = 8$). However, LS-1-137 significantly inhibited the DOI-induced HTR (55.6 ± 2.2 head twitches/30 min) ($p < 0.0001$; $n = 7$). One-way ANOVA indicated a significant treatment effect between groups ($F(3, 39) = 22.20$; $p < 0.0001$) (Fig. 2A).

To further define the role of sigma-1 receptors in inhibiting the DOI-induced HTR we compared the effect of LS-1-137 to commercially available sigma-1 receptor agonists (PRE-084 and PPCC) (Maurice et al., 1994; Prezzavento et al., 2010) and antagonists (BD1047 and haloperidol). The sigma-1 receptor antagonists haloperidol and BD1047 both attenuated the DOI-induced HTR (36.0 ± 5.4 and 56.0 ± 2.6 head twitches/30 min, respectively) when compared to their respective vehicle controls (85.0 ± 3.6 and 84.5 ± 1.7 head twitches/30 min, respectively) ($p < 0.001$; $n = 6$) (Fig. 2A and B). However, the agonists PRE-084 and PPCC did not significantly attenuate the DOI-induced HTR (84.5 ± 3.5 and 78.1 ± 4.5 head twitches/30 min, respectively) ($p > 0.05$; $n = 8$) when compared to the vehicle controls. One-way ANOVA indicated a significant treatment effect between groups ($F(6, 80) = 21.70$; $p < 0.0001$) (Fig. 2B).

To further confirm that LS-1-137 is acting as a sigma-1 receptor antagonist in the DOI-induced HTR, a competition experiment was undertaken to determine if the effect of LS-1-137 could be blocked by the sigma-1 receptor agonist PRE-084. LS-1-137 and PRE-084 were given simultaneously *via* i.p. injection. PRE-084 was tested at a dose of either

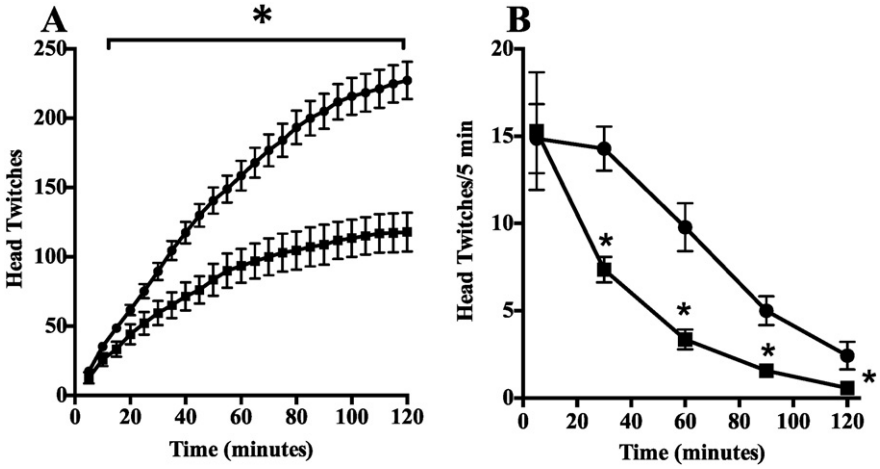

Fig. 1. Temporal studies using haloperidol. Male DBA/2J mice were administered either vehicle (5% DMSO in sterile water) plus 5 mg/kg of DOI (●) or haloperidol (1 mg/kg) plus 5 mg/kg of DOI (■) and the number of head twitches were recorded over 5-min intervals. A. Data is plotted as the mean cumulative number of head twitches ± S.E.M. over a 2 h time period. B. Data is plotted as the mean number of head twitches over 30 min intervals for a 2 h time period. Significance was determined using two-way repeated measure ANOVA. Data is reported as mean ± S.E.M. for $n = 7$ animals per group. * Compared with DOI group. $p < 0.05$ is considered significant.

5 mg/kg or 10 mg/kg. LS-1-137 was tested at a constant dose of 5 mg/kg. PRE-084 was found to dose dependently inhibit the effect of LS-1-137 (Fig. 3). PRE-084 at a dose of 10 mg/kg (81.8 ± 5.5 head twitches/30 min) significantly blocked the effect of LS-1-137 (56.6 ± 3.1 head twitches/30 min, $p = 0.1483$; $n \geq 6$) ($F(3, 31) = 19.56$; $p < 0.0001$).

We then characterized the potency of LS-1-137 in the HTR model by performing a dose-effect response analysis (Fig. 4). LS-1-137 was administered at varying doses (0.5 mg/kg, 5 mg/kg, 10 mg/kg and 30 mg/kg) 5 min prior to DOI administration (5 mg/kg). LS-1-137 dose-dependently inhibited the DOI-induced HTR with an IC50 of 4.7 mg/kg.

The data presented thus far indicate that LS-1-137 is capable of attenuating the HTR caused by the administration of DOI. Since DOI is a 5-HT2A and 5-HT2C receptor agonist/partial agonist, one possible explanation for the inhibitory effect of LS-1-137 might be that it is a high affinity competitive inhibitor for the binding of DOI at 5-HT2A and/or 5-HT2C receptors *in vivo*. To test this possibility we determined the affinity of LS-1-137 for 5-HT2A and 5-HT2C receptors using a competitive radioligand binding assay. For these assays a) human 5-HT2A receptors expressed in HEK 293 cells were used in conjunction with the radioligand [^3H]ketanserin and b) 5-HT2C receptors expressed in CHO cells were radiolabeled with [^3H]mesulergine. We found that there was a > 80-fold difference in the Ki values of LS-1-137 for binding sites 5-HT2A receptors (Ki value >15,000 nM) and >200-fold difference for 5-HT2C binding (Ki value >15,000 nM) compared to the binding of DOI at 5-HT2A receptors (Ki value = 70.5 ± 8.1 nM) and 5-HT2C receptors (Ki value = 182 ± 23.1 nM) (Fig. 5**)**.

To obtain a more detailed insight into the pharmacological profile of LS-1-137 we submitted a sample to the NIMH Psychoactive Drug Screening Program (PDSP). The receptor/transporter binding sites that were evaluated included a) 5-HT receptor subtypes (1A, 1B, 1D, 2A, 2B 2C, 5A and 7), b) alpha-adrenergic receptor subtypes (1A, 1B, 1D, 2A, 2B and 2C), c) beta-adrenergic receptor subtypes (1, 2 and 3), d) dopamine receptor subtypes (1, 2, 3, 4) d) muscarinic receptor subtypes (1, 2, 3, 4 and 5), e) histamine receptor subtypes (1, 2 and 3), f) opioid receptor subtypes (delta, kappa and mu) and g) sigma receptor subtypes (1 and 2). Binding at GABAA receptor and the biogenic amine transporters (dopamine (DAT), serotonin (SERT) and norepinephrine (NET) transporters) were also evaluated. Table 2 lists the mean Ki values ($n = 3$) for those binding sites with Ki values <10,000 nM. Ki values for the rest of the binding sites were not determined because they did not meet the minimum threshold criteria of >50% inhibition at a dose concentration of 1×10^{-6} M.

Of the binding sites tested, sigma-1 receptors exhibited the highest affinity. The Ki values and the values for test compound *vs.* sigma-1 receptor binding selectivity obtained by the NIMH/PDSP for dopamine and serotonin receptors are in reasonable agreement with the values we obtained (Table 1). Of the binding sites tested the histamine-1, 5-HT2B and alpha2b-adrenergic receptor binding sites exhibited a < 100-fold test binding site:sigma-1 receptor binding selectivity ratio (34-fold, 44-fold and 99-fold, respectively).

Finally, we conducted rotarod studies to investigate the possibility that the observed LS-1-137-dependent decrease in the HTR might be due to an inhibition of motor performance. After a training session,

Table 1
Pharmacological properties of sigma-1 and sigma-2 receptor selective compounds tested in this study.

Ki values (nM)						
Compound	D2L	D3	D4	Sigma-1	Sigma-2	Sigma-2:Sigma-1
LS-1-137[a]	997 ± 99	622 ± 85	>1000	3.2 ± 0.1	257 ± 30	80
Haloperidol[a]	1.1 ± 0.1	12.7 ± 3.9	ND	1.5 ± 0.3	24.2 ± 3.0	16
RHM-1-86[a]	>1000	627 ± 244	>1000	>1000	8.2 ± 1.4	<0.008
PRE-084[b]	>1000	>1000	>1000	45.8 ± 11.5	>1000	>500
PPCC[c]	>1000	>1000	>600	1.5 ± 0.06	50.8 ± 3.0	34
BD 1047[d]	>1000	>1000	>1000	0.93 ± 0.14	47 ± 0.6	51

Ki values (nMolar) for the human D2L, D3 and D4.4 dopamine receptor subtypes were determined using stably transfected HEK cells and the radioligand [^{125}I]IABN.
Affinity at sigma-1 receptors (nMolar) was determined using guinea pig brain membrane homogenates and [^{3}H](+)-pentazocine. Affinity at sigma-2 receptors was determined using rat liver membranes and 5 nM [^3H]DTG in the presence of 1 μM (+)-pentazocine.

[a] Data taken from Luedtke et al. (2012).
[b] Data taken from Entrena et al. (2009).
[c] Data taken from Prezzavento et al. (2007).
[d] Data taken from Matsumoto et al. (1995).

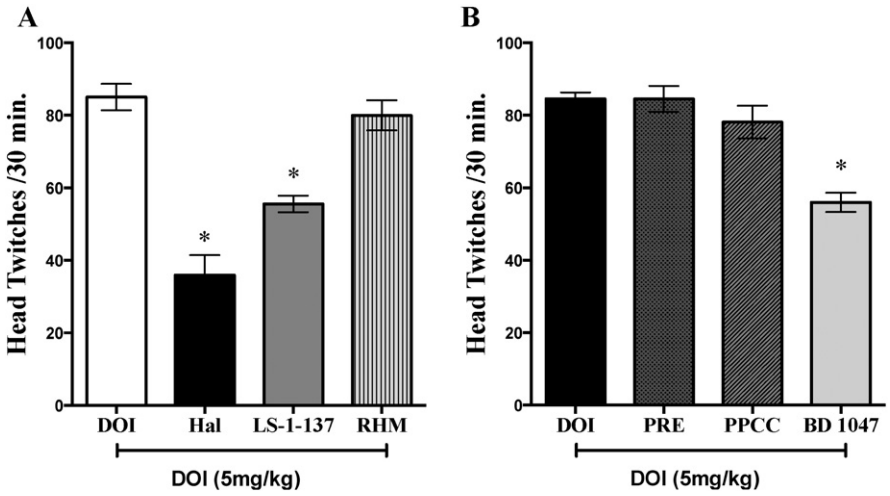

Fig. 2. Effect of sigma-1 receptor compounds on the DOI-induced HTR. Male DBA/2J mice were injected with DOI at a dose of 5 mg/kg in the presence or absence (DOI) of test drug. Except for haloperidol (Hal), all test drugs were tested at a dose of 5 mg/kg. Haloperidol was tested at 1 mg/kg. Data represents the mean number of head twitches over a 30 min time period ± S.E.M. for $n \geq 6$ animals per group. A. Haloperidol and LS-1-137 were able to significantly attenuate the DOI-induced HTR. There was no significant difference observed when the sigma-2 receptor selective antagonist, RHM-1-86 treatment was compared to vehicle control (DOI). B. Commercially available sigma receptor agonists and antagonists were tested. Sigma-1 receptor agonists (PRE-084, PPCC) were not able to significantly attenuate the DOI-induced HTR. BD1047, sigma-1 receptor antagonist was able to attenuate DOI-induced HTR. Significance was determined using one-way ANOVA. Data is reported as mean ± S.E.M. for $n \geq 7$ animals per group. * Compared with vehicle group. $p < 0.05$ is considered significant.

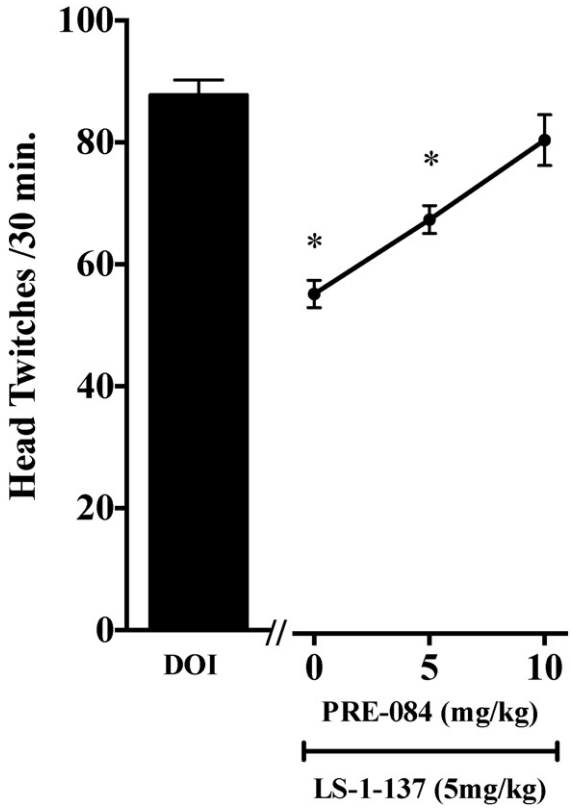

Fig. 3. Sigma-1 receptor agonist PRE-084 blocks the effect of LS-1-137. Male DBA/2J mice were injected with DOI at a dose of 5 mg/kg in the presence or absence of test drugs. LS-1-137 and PRE-084 were given simultaneously *via* intraperitoneal injection. Test drugs were given 5 min ahead of DOI administration. Data represents the mean number of head twitches over a 30 min time period ± S.E.M. for $n > 7$ animals per group. LS-1-137 was tested at 5 mg/kg. PRE-084 was tested at 5 mg/kg and 10 mg/kg. PRE-084 at dose of 5 mg/kg was not able to significantly attenuate the DOI-induced HTR compared to DOI vehicle treatment. When PRE-084 was given at dose of 10 mg/kg, it significantly blocked the effect of LS-1-137. Significance was determined using one-way ANOVA. Data is reported as mean ± S.E.M. for $n > 7$ animals per group. * Compared with vehicle group. $p < 0.05$ is considered significant.

the effect of LS-1-137 on rotarod performance (latency to fall) was evaluated. The rotarod studies were conducted at the approximate IC50 dose (5.0 mg/kg) for LS-1-137 (Fig. 4). Animals were given vehicle (5% DMSO in sterile water) or test drug 5 min prior to each session. LS-1-137 did not affect the latency to fall time (18.2 ± 3.1 s) when compared to the last day of the training session (17.9 ± 2.6 s) (Fig. 6**)**. One-way ANOVA indicated the effect of treatment was not significant ($F(1.739, 13.91) = 4.910$; $p = 0.0280$; $n = 9$). On the last day of the experiment the effect of the administration of haloperidol (3.0 mg/kg) was evaluated as a positive control. Haloperidol significantly impaired the ability of the animals to perform (7.5 ± 0.4 s) ($F(1.244, 9.949) = 15.12$; $p = 0.0021$).

4. Discussion

Based upon our studies using commercially available sigma-1 receptor agonists and antagonists as reference compounds, LS-1-137 acts as an antagonist *in vivo* to inhibit the DOI-dependent HTR in a dose-dependent manner. The sigma-1 receptor antagonist BD 1047, which binds with low affinity at 5-HT2 receptor subtypes (Matsumoto et al., 1995), also attenuates the DOI-induced HTR. The sigma-1 receptor agonist PRE-084, which also binds with low affinity at the 5-HT2 receptor (Su et al., 1991), was unable to attenuate the HTR but it was able to reverse the inhibitory effect of LS-1-137 on the DOI-induced HTR.

Based upon the results of our competitive radioligand binding studies it appears unlikely that LS-1-137 directly blocks the binding of DOI at 5-HT2 receptors because LS-1-137 binds with low affinity at both the 5-HT2A and 5-HT2C receptors. These findings are strengthened by the data provided by the NIMH Psychoactive Drug Screening Program. Furthermore, based upon the results of our rotarod studies (Fig. 6) and our previous studies on the effect of LS-1-137 on swim speed (Malik et al., 2015), it appears unlikely that LS-1-137 is attenuating the HTR by compromising the animal's motor performance.

The DOI-induced HTR has been proposed as a strategy to investigate 5-HT2A/C receptor-associated behaviors. DOI is thought to mediate the HTR by activating cortical postsynaptic 5-HT2 receptors. Cortical 5-HT2 receptor stimulation leads to the activation of phospholipase C and the formation of diacylglycerol (DAG) and inositol triphosphate (IP3). IP3, in turn, activates IP3 receptors on the endoplasmic reticulum, releasing Ca^{2+} from intracellular stores. DOI was reported to modulate the firing rate of neurons in the cortex, thus interfering with the cortical–basal

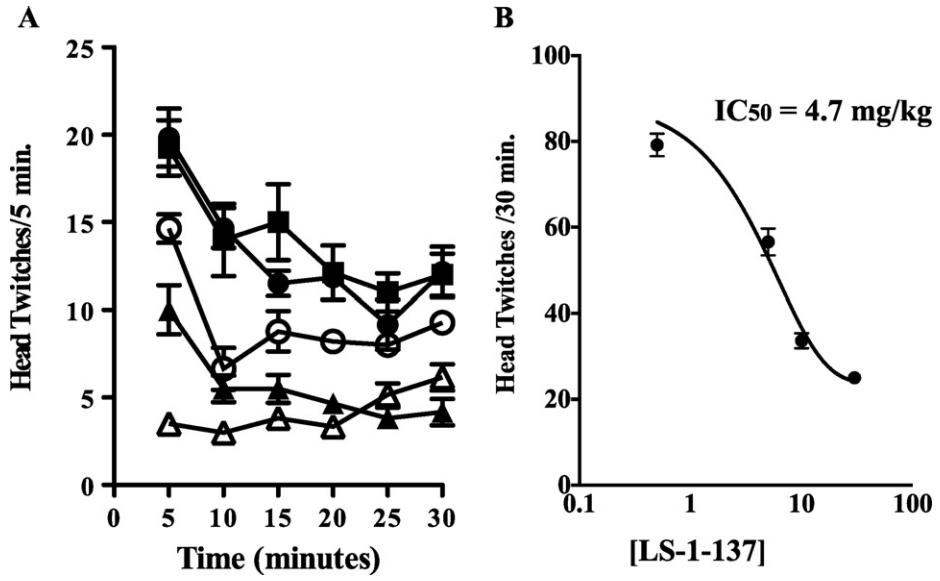

Fig. 4. LS-1-137 dose-effect study. The dose-dependent effect curve for LS-1-137 on DOI-induced head twitches in DBA/2J mice is shown. The DOI dose is 5 mg/kg. A. A temporal plot of the data is show as a function of the mean number of head twitches observed for each consecutive 5 min increment for each dose of LS-1-137: a) 0 mg/kg (■), b) 0.05 mg/kg (●), c) 5 mg/kg (○), d)10 mg/kg (▲) and e) 30 mg/kg (△). B. The data is plotted as the mean number of head twitches over a 30 min time period as a function of test compound dose (mg/kg) using 5 mg/kg of DOI. The IC50 value for LS-1-137 was found to be 4.7 mg/kg.

ganglia loop (Puig et al., 2003; González-Maeso et al., 2003, 2007; Ashby et al., 1990).

The hallucinogen *N*,*N*-dimethyltryptamine (DMT), which is found in trace amounts in the mammalian brain, has been postulated to be the endogenous ligand for the sigma-1 receptor (Su et al., 2009; Fontanilla et al., 2009). The sigma-1 receptor, which exhibits no homology to other mammalian proteins (Hanner et al., 1996), does not directly transduce extracellular signals into an intracellular response. Rather, they appear to regulate a variety of signaling pathways (Su et al., 2010). The sigma-1 receptor regulates the amplification of NMDA-sensitive, dopaminergic and IP3-related metabotropic receptor activity and neurotransmitter release (Hayashi and Su, 2005; Su and Hayashi, 2003). The sigma-1 receptor also regulates the cellular redox environment, cellular survival and synaptogenesis (Fujimoto et al., 2012; Hayashi and Su, 2007).

Sigma-1 receptors localized at the mitochondria-associated endoplasmic reticulum (ER) membrane (MAM) regulate Ca^{2+} signaling between the ER and mitochondria by chaperoning IP3 receptors at the ER and mitochondrion interface to ensure proper Ca^{2+} signaling from the ER into the mitochondrion (Ortega-Roldan et al., 2013).

Haloperidol is known to be a high affinity antagonist at sigma-1 and sigma-2 receptors, as well as at D2 and D3 dopamine receptors. With the knowledge that D2 dopamine receptor (Rangel-Barajas et al., 2014), D3 dopamine receptor (Rangel-Barajas et al., 2015) and sigma-1 receptor selective blockade can independently attenuate the DOI-dependent HTR, it becomes clearer how haloperidol can effectively inhibit the DOI-dependent HTR.

Given the complexity and diversity of how sigma-1 receptors function, we can only speculate how LS-1-137 and the other sigma-1 receptor antagonists might attenuate the DOI-induced HTR. Since sigma-1 receptors play a modulatory role in the stabilization of the flow of Ca^{2+} from intracellular stores (Hayashi and Su, 2007), LS-1-137 might be inhibiting a DOI-mediated increase in intracellular Ca^{2+}, leading to the observed attenuation of the HTR. Alternatively, since sigma-1 receptors regulate glutamate NMDA receptors and the release of other neurotransmitters (Hayashi and Su, 2004), LS-1-137 might modulate

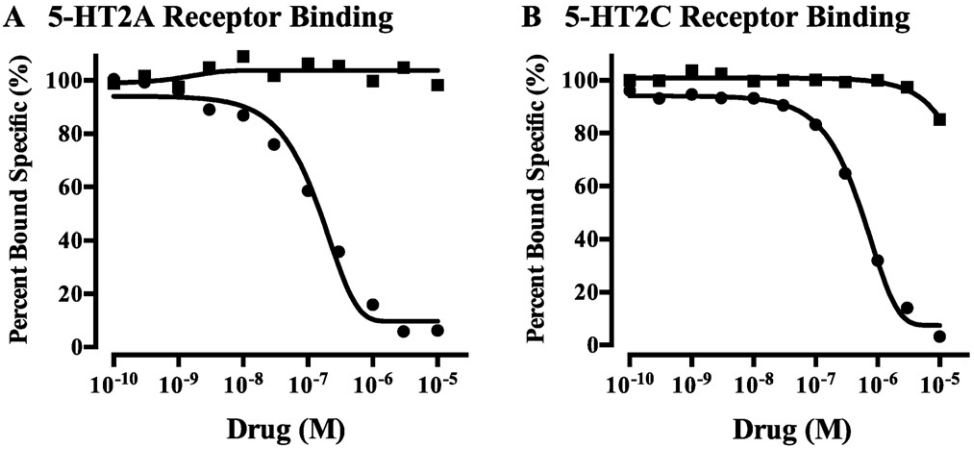

Fig. 5. Binding of LS-1-137 to 5-HT2 receptor subtypes. This figure shows a composite competition curve for the specific binding of (A) [^3H]ketanserin to 5-HT2A receptor and (B) [^3H]mesulergine binding to 5-HT2C receptors as a function of inhibitor concentration, where each point is the mean inhibition for ≥3 independent experiments. IC50 values for each independent experiment were converted to Ki values using the Cheng and Prusoff (1973). There was a > 80-fold difference in the Ki values of LS-1-137 (■) for binding sites 5-HT2A receptors (Ki value >15,000 nM) and >200-fold difference for 5-HT2C binding (Ki value >15,000 nM) compared to the binding of DOI (●) at 5-HT2A receptors (Ki value = 70.5 ± 8.1 nM) and 5-HT2C receptors (Ki value = 182 ± 23.1 nM).

Table 2
Binding profile for LS-1-137.

Receptors	Mean Ki values (nM)	Receptor:Sigma-1
Sigma-1	5.6 ± 0.59	1.0
Histamine-1	192 ± 42.0	34
5-HT2B	244 ± 41.0	44
α-2b adrenergic	554 ± 108	99
Sigma-2	695 ± 119	124
Mu-opiate	1288 ± 141	230
α-2c adrenergic	1474 ± 277	263
5-HT transporter	1984 ± 374	354
α-2a adrenergic	2835 ± 527	506
D3 dopamine	3981 ± 350	712
Muscarinic 2	3981 ± 448	714
D4 dopamine	4460 ± 752	796
Histamine 3	5129 ± 661	916
Muscarinic −5	5420 ± 799	968
D2 dopamine	8547 ± 1432	1526
D1 dopamine	8598 ± 1432	1535

The mean Ki values (n = 3) for the binding of LS-1-137 were provided by the NIMH Psychoactive Drug Screening Program. The receptor *vs.* sigma-1 receptor binding selectivity is also shown.

glutamate release. Furthermore, sigma-1 receptors appear to directly interact with the D2 dopamine receptor (Navarro et al., 2013) and we have recently reported that selective blockade of the D2 receptor subtype can attenuate the HTR (Rangel-Barajas et al., 2014). Therefore, an alternative mechanism may involve the modulation of D2 receptor function. These mechanisms of action are not mutually exclusive.

Our collective pharmacological data at this point suggests that LS-1-137 is a neuroprotective (Luedtke et al., 2012) compound that mediates its inhibitory action of the rodent DOI-dependent HTR independent of a) the 5-HT2A/C receptor subtypes, b) D1-like, D2-like dopamine receptors or the dopamine transporter, c) NMDA or GABA receptors d) beta-adrenergic receptors e) muscarinic receptors or f) the sigma-2 receptor (Malik et al., 2015). The NIMH PDSP binding data indicates that LS-1-137 binds with >100-fold selectivity at all components tested except for the histamine-1 receptor (34-fold selective), the 5-HT2B receptor (44-fold selective) and the α2b-adrenergic receptor (99-fold selective).

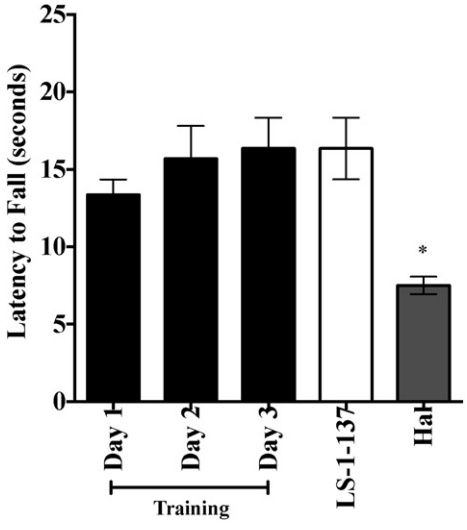

Fig. 6. Rotarod evaluation of LS-1-137. A rotarod apparatus was used to assess the effect of LS-1-137 on motor coordination and agility of male DBA/2J mice. The data is presented as the mean amount of time the animal remained on the rotarod before falling (Latency to Fall; in seconds) ± S.E.M. The acceleration conditions for the rotarod test were 0 to 44 rpm in 90 s. This experiment was performed on 5 consecutive days. Days 1–3 were training sessions that were conducted in the morning and afternoon. After the training session was completed, LS-1-137 was administered (i.p.) at a dose approximately equal to the IC50 value (*as determined in* Fig. 5). Rotarod evaluation was initiated at 5 min following the drug administration (day 4). The data for LS-1-137 is presented as the mean of 5 trials ± S.E.M. LS-1-137 administration did not impair the ability of the animals to perform on the rotarod. Haloperidol (**Hal**) was included as a positive control (3 mg/kg, day 5). A one way repeated measure ANOVA analysis was used for the statistical evaluation of the data using Graphpad software. * indicates significance ($p < 0.05$) when comparing the effect of the mean values for the haloperidol to the mean values of the training sessions.

Based upon the results of an *in vitro* sigma-1/Bip association assay (Ortega-Roldan et al., 2013), we would categorize LS-1-137 as a sigma-1 receptor selective agonist (Malik et al., 2015). That characterization was consistent with the *in vivo* studies that we published indicating that LS-1-137 was capable of partially reversing the cognitive deficits observed in C57BL/6 mice caused by the muscarinic antagonist scopolamine. In those studies reference sigma-1 agonists, but not antagonists, exhibited similar *in vivo* properties (Malik et al., 2015). It is also consistent with our observation that LS-1-137 can act as a sigma-1 receptor agonist when it enhances the release of BDNF from astrocytes (Malik et al., 2015; Xu et al., 2015).

However, in our original publication on the effects of LS-1-137 we reported that a) LS-1-137 exhibited neuroprotective properties in both *in vitro* and *in vivo* model systems related to stroke and b) this neuroprotective property was similar to that observed for sigma-1 antagonists, but not for sigma-1 receptor agonists (Luedtke et al., 2012; Schetz et al., 2007). The results from this communication on the attenuation of the DOI-HTR again suggest that LS-1-137 is acting *in vivo* like an antagonist, when compared to the same reference compounds used by Malik et al., 2015. Therefore, in some animal behavioral paradigms LS-1-137 appears to act *in vivo* as a sigma-1 receptor antagonist, while in other animal models it appears to have agonist activity.

We do not understand the basis of this anomaly but we suggest the following possible explanations. First, the sigma-1 receptor modulates signaling responses rather than initiating the activation of a signaling pathway. Therefore, it may not consistently function under the operational terms generally used to describe a conventional agonist or antagonist associated with GPCRs or ligand gated ion channels. Second, LS-1-137 may be acting like a partial agonist *in vivo*. To our knowledge there has been no discussion in the literature of the possibility that a partial agonist at sigma-1 receptors could exist, how that efficacy might be quantitated, or how such a compound might act *in vivo*. Third, the cellular environments where a sigma-1 receptor/LS-1-137 interaction can occur *in vivo* likely vary and the observed consequences of that interaction may differ depending upon the behavioral paradigm that is being utilized. In these different cellular milieus LS-1-137 may be modulating different combinations of signaling pathways working in concert to dictate the behavioral effects.

In conclusion, the DOI-induced HTR model represents a novel assay to explore the *in vivo* efficacy of sigma-1 receptor selective ligands and might prove to be useful for screening compounds for antipsychotic activity because neuroleptics have been found to attenuate the murine DOI-induced HTR (Moreno and González-Maeso, 2013; Rangel-Barajas et al., 2014). To our knowledge this is the first study that describes the effect of sigma-1 selective compounds on the HTR. Our cumulative results suggest that LS-1-137 may have some potential for use as a novel antipsychotic or in conjunction with currently used antipsychotics because it a) attenuates the effect of a hallucinogenic compound, b) appears to ameliorate cholinergic-dependent cognitive deficits (Malik et al., 2015), c) exhibits neuroprotective properties and d) enhances the release of BDNF from astrocytes (Malik et al., 2015). Furthermore, the *in vivo* pharmacological properties of LS-1-137 appear to be unique compared to other commercially available sigma-1 receptor selective ligands because in some *in vivo* scenarios it exhibits agonist-like properties while in other scenarios it appears to act like an antagonist.

Conflict of interest

The authors have no conflicts of interest in conducting this research. This study complies with the laws of the United States of America.

Acknowledgements

The authors would like to thank Michelle Taylor for her technical assistance with the binding assays and for her editorial assistance in the preparation of this manuscript. The authors would also like to thank Dr. Marianna Jung and Mr. Daniel Metzger for their technical assistance with the rotarod studies. LS-1-137 was generously provided to us by Chunyang Jin, Ph.D. and Kenneth Rehder, Ph.D. of the NIMH Chemical Synthesis and Drug Supply Program. We would also like to thank Drs. William P. Clarke and Kelly A. Berg in the Department of Pharmacology at the University of Texas Health Science Center at San Antonio for providing us with the transfected cell lines expressing 5-HT2A and 5-HT2C receptors and for providing us with the technical information related to the radioligand binding protocols for these receptors. **Addendum** Recently, the crystal structures of the human sigma-1 receptor in complex with two chemically divergent ligands, PD144418 and 4-IBP have been reported (Schmidt et al., 2016). A trimeric architecture with a single transmembrane domain for each protome was reported. Schmidt HR, Zheng S, Gurpinar E, Koehl A, Manglik A, Kruse AC. (2016) Crystal structure of the human σ1 receptor. Nature. 532, 527-30.

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