Levo-Tetrahydroberberrubine Produces Anxiolytic-Like Effects in Mice through the 5-HT\textsubscript{1A} Receptor

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Abstract  
Tetrahydroprotoberberines (THPBs) are isoquinoline alkaloids isolated from the Chinese herb Corydalis yanhusuo. In the present study, we performed competitive binding assays to examine the binding of l-THBr to neurotransmitter receptors known to be involved in sedation, hypnosis and anxiety. Our results show that l-THBr does not interact with GABAergic receptors but has binding affinities for dopamine and serotonin receptors. In addition, cAMP and [\textsuperscript{35}S]GTP\textsubscript{γ}S assays were used to determine the agonist or antagonist properties of l-THBr at dopamine (D\textsubscript{1}, D\textsubscript{2}) or serotonin (5-HT) receptors. Our results show that l-THBr displays D\textsubscript{1} and D\textsubscript{2} antagonist and 5-HT\textsubscript{1A} agonist properties. Moreover, l-THBr-treated rodents exhibit anxiolytic-like effects in the light/dark box and elevated plus-maze tests, and the anxiolytic effect of l-THBr can be reduced by WAY-100635, a selective 5-HT\textsubscript{1A} receptor antagonist. Our results suggest that l-THBr may produce potent anxiolytic-like effects mainly through serotonin receptors.

Introduction  
Anxiety is a common mental state provoked in anticipation of a threat or potential threat, which may become an illness when excessive or inappropriate [1, 2]. The major physical and mental symptoms of anxiety include racing thoughts, nervousness, tremor, insomnia, emotional discomfort and agitation [3, 4]. As one of the most common psychiatric illnesses, anxiety disorders cause a prominent health care problem worldwide. 

For over a century, researchers have searched for effective and safe agents to treat anxiety disorders. Benzodiazepines have been the mainstay of treatment since chlordiazepoxide was introduced in 1960 [5]. However, their therapeutic efficacy is limited due to unwanted side effects such as sedation, muscle relaxation, retrograde amnesia [6, 7] and dependency liability [8]. Another class of drugs, partial agonists of the serotonergic 5-HT\textsubscript{1A} receptor, such as buspirone, gepirone, and ipsapirone, was identified as valuable for improving the clinical management of anxiety [9], but their therapeutic effects are delayed for 1–3 weeks [10]. Therefore,
there is a demand for robust anxiolytic compounds that have fewer side effects and a more immediate onset of action.

Tetrahydropseudoberberines (THPBs) are isoquinoline alkaloids isolated from the Chinese herb *Corydalis yanhusuo*. *l*-Tetrahydropalmatine (*l*-THP), the main active ingredient of *C. yanhusuo*, has been used for more than 40 years in China as a treatment for chronic pain and anxious insomnia [11,12]. *l*-THP displays D1 and D2 antagonist properties and shows anti-addictive effects in animal models [13–16]. In addition, *l*-stepholidine (*l*-SPD), another derivative of tetrahydropseudoberberines, displays D1 agonist and D2 antagonist effects [17]. *l*-SPD has attracted much attention for its potential efficacy as a schizophrenia treatment [18–20]. However, *l*-SPD’s poor bioavailability and high industrial production cost limits its use [21]. Therefore, a new derivative of THPBs, levo-tetrahydroberberine (*l*-THBr) (Fig 1) was synthesized. In the present study, we examined the binding features of *l*-THBr to neurotransmitter receptors using competitive binding assays to address possible interactions with these receptors. Moreover, we characterized the functional activity of *l*-THBr at cloned D1 and D2 dopamine receptors and rat hippocampal 5-HT1A serotonin receptors. In addition, the anxiolytic-like effects of *l*-THBr in two experimental animal models of anxiety were evaluated.

**Materials and Methods**

**Animals**

The experimental procedures were approved by the Beijing Institute of Basic Medical Science Institutional Committee on Animal Care and Use, and all efforts were made to minimize animal suffering and reduce the number of animals used for experiments. Male Sprague-Dawley
(SD) rats and male CD-1 ICR mice (body mass of 18–22 grams) were purchased from Vitalriver Experimental Animal Center (Beijing, China). All animals were maintained under standard laboratory conditions and kept in temperature- and humidity-controlled rooms (21–22°C, 50%-60% humidity) on a 12 hour light-dark cycle (lights on from 7:00 am to 7:00 pm). All mice were used only once, and all behavioral experiments were performed between 8:00 and 12:00 am.

Reagents and drug treatments
Quinpirole, buspirone, 8-OH-DPAT, SCH 23390, \[^{35}\text{S} \text{GTP}\gamma\text{S}\] and GTP\(\gamma\text{S}\) were purchased from Sigma (St. Louis, MO, USA). SKF 38393 was purchased from Tocris (Bristol, United Kingdom). The cAMP Assay Kit was purchased from CISBIO (Catalog Number: 62AM4PEC). WAY100635 was obtained from Selleck (Texas, USA) and was dissolved in 0.9% saline. l-THBr was synthesized by the Department of Complex Prescription of Traditional Chinese Medicine (TCM), China Pharmaceutical University. It was dissolved in 0.1 mol/L H\(_2\)SO\(_4\), diluted with sterile water and adjusted to pH 5–6 with 0.1 mol/L NaOH. The vehicle was prepared as above without drug. Diazepam (DZP) was purchased from Tianjin Jinyao Amino Acid Co., Ltd. (Tianjin, China) and was dissolved with control vehicle. For in vitro assays, all compounds were dissolved in DMSO and diluted with a solution of HBSS plus 20 mM HEPES.

Radio-ligand binding assay
To determine the possible targets of l-THBr action, the binding affinities of l-THBr to neurotransmitter receptors known to be involved in sedation, hypnosis and anxiety were investigated. Radio-ligand binding assays were performed by Caliper Lifescience (Hopkinton, MA, USA). The screening was carried out at a concentration of 10 \(\mu\text{M}\) l-THBr to test its ability to inhibit the binding of radioligands to their corresponding receptors. The results were expressed as percentage of inhibition of labeled ligand binding to individual receptors. Significant binding activity was defined as \(\geq 50\%\).

cAMP assay for binding properties at D\(_1\) receptor
Dopamine receptors can be categorized into two classes: G\(\alpha_s\) protein coupled receptors (D\(_1\) and D\(_3\)), or G\(\alpha_i\) protein coupled receptors (D\(_2\), D\(_3\), and D\(_4\)). Activation of D\(_1\) receptors can excite adenylate cyclase activity and increase cyclic adenosine monophosphate (cAMP). cAMP assays were used to determine the agonist or antagonist properties of l-THBr at the dopamine D\(_1\) receptor.

CHO K1 cells stably expressing D\(_1\) receptors were purchased from Genscript (Catalogue Number: M00247, Gene Number NM_000794). The cells were seeded in Ham’s F12 containing 10% fetal bovine serum and 200 \(\mu\text{g/mL}\) zeocin. On the day of the assay, 5 \(\mu\text{l}\) of cell suspension (3000 cells) was seeded on a 384-well plate. The assay was performed according to the manufacturer’s instructions. To test for agonist effects at the D1 receptor, the compounds (a known D\(_1\) agonist SCH38393 or l-THBr) were added from stocks two-fold more concentrated than the final concentration. In another experiment to test for antagonist effects, the known D\(_1\) receptor antagonist SCH23390 or l-THBr was added to the system in the presence of the D\(_1\) receptor agonist SCH38393 (10 \(\mu\text{M}\)). After incubation (30 min at room temperature), 10 \(\mu\text{l}\) of HTRF reagents (cAMP-XL665 and anti-cAMP cryptate) were added. The signal was quantified after one hour of incubation at room temperature. The fluorescence intensity ratio \(\frac{A_{665\text{nm}}}{A_{620\text{nm}}} \times 10^4\) was calculated.
[\textsuperscript{35}S]GTP\gamma S assay for binding properties at D\textsubscript{2} and 5-HT\textsubscript{1A} receptors

D\textsubscript{2}-D\textsubscript{4} dopamine receptors and 5-HT\textsubscript{1A} serotonin receptors are G\alpha-i-coupled receptors that mediate inhibitory neurotransmission. We conducted [\textsuperscript{35}S]GTP\gamma S assays to determine the agonist or antagonist properties of L-ThBr at D\textsubscript{2} dopamine receptors or 5-HT\textsubscript{1A} serotonin receptors. HEK293 cells stably expressing D\textsubscript{2} receptors were provided by the Beijing Institute of Pharmacology and Toxicology (Beijing, China). The membrane preparation of D\textsubscript{2}-expressing HEK293 cells and rat hippocampal tissues highly expressing 5-HT\textsubscript{1A} receptors were prepared as previously described [22–23]. Briefly, 10 SD rats were anesthetized and decapitated, and the hippocampi were quickly dissected and stored at -80°C until use. D\textsubscript{2} receptor-expressing HEK293 cells and the hippocampal tissue were each homogenized at 4°C in 50 mM Tris-HCl buffer (pH 7.4). The homogenates were centrifuged at 2500 \times g for 6 min, and the supernatant was further centrifuged for 20 min at 40000 \times g. Membranes were re-suspended in Tris-HCl buffer and stored at -80°C until use.

[\textsuperscript{35}S]GTP\gamma S assays were performed in a total volume of 0.5 ml at 4°C. The incubation mixtures were prepared in glass tubes and consisted of membrane preparations (20 \mu g of protein), GDP (15 \mu M) and [\textsuperscript{35}S]GTP\gamma S (0.2 nM). Nonspecific binding was determined in the presence of unlabeled GTP\gamma S (40 \mu M) following a 60 min incubation period at 30°C in the absence or in the presence of different concentrations of drugs (L-ThBr, quinpirole and 8-OH-DPAT: 10^{-10}–10^{-5} M; or quinpirole: 10 \mu M). The reactions were stopped with ice-cold Tris-HCl buffer and rapidly filtered through Whatman GF/B filters. Filters were quickly washed five times with 3 ml ice-old Tris-HCl and placed in scintillation cocktail solution. Bound radioactivity was determined by liquid scintillation counting. Drug effects were expressed as drug-induced increase in binding over basal binding (binding in the absence of drugs). Curves were fitted by non-linear regression analysis to the equation \[ Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{(\text{EC}_{50} - X)} 	imes \text{Hill Slope}} \], where Top and Bottom are plateaus in the units of the Y axis, EC\textsubscript{50} is the concentration of agonist that gives a response halfway between Bottom and Top, and Hill Slope describes the steepness of the family of curves. The inhibitory effect of L-ThBr was determined by a similar equation to obtain the IC\textsubscript{50} values.

Locomotor activity test

The spontaneous activity video analysis system consists of 8 sound-attenuated chambers (40 cm \times 40 cm \times 65 cm) with a built-in infrared camera. (JL Behave, Shanghai Ji-Liang Software Technology Co., Ltd). During the behavioral tests, the experimenter was outside the testing room, and the chambers were cleaned between successive runs. Forty male CD-1 ICR mice were allowed to acclimate for three days in home cages and were handled for another three days to minimize stress after arrival in the animal facility. On the following day, after habituation to the activity chambers for 60 min, mice were administered vehicle, diazepam (DZP, 2 mg/kg, i.p.) or L-ThBr (1, 5, or 10 mg/kg, i.p.) and immediately placed into the test chambers to record their locomotor activity for 60 min.

Light-dark box test

The light-dark box test is a sensitive model to detect activity in disorders related to anxiety, based on the innate aversion of rodents to brightly lit areas and on their spontaneous exploratory behavior in response to a novel environment [24]. The light-dark transition box is a polypropylene animal cage (44 cm \times 21 cm \times 21 cm), which is divided into two compartments, a light box (illuminated by a 60 W light source with 1000 lx light intensity) and a dark box. Forty-eight male CD-1 ICR mice were placed in the light box 30 min after L-ThBr or DZP...
injection and allowed to move freely to both boxes for 5 min. The number of transitions between the two boxes were recorded by a video camera.

Elevated plus-maze test
The elevated plus-maze test is widely used for the screening and evaluation of anxiolytic drugs [25–26]. The apparatus consists of two open arms (30 cm × 5 cm) and two enclosed arms (30 cm × 5 cm × 15 cm), which is elevated 45 cm above the ground. The entire maze was made of clear Plexiglas and illuminated by four 30 W white lights with 300 lx light intensity arranged as a cross 100 cm above the maze. 48 male CD-1 ICR mice were randomly divided into either the vehicle group, one of three doses of l–THBr groups, or the diazepam group. 30 min after injection, mice were gently placed on the center platform facing an open arm, and the number of entries and the time spent in both arms were recorded by a video camera for 5 min. The results were expressed as the percentage of entries into the open arms (%) = (the number of entries into the open arms / the total number of entries into the four arms) × 100%; and the percentage of time spent in the open arms (%) = (time spent in the open arms / total time spent in the four arms) × 100%.

In a separate experiment, WAY100635 (a 5-HT₁A antagonist) was used to test whether the anxiolytic-like activity of l–THBr is mediated by the activation of the 5-HT₁A receptor. Forty male CD-1 ICR mice were randomly divided into one of four groups: vehicle, WAY100635 (3 mg/kg, i.p.), l–THBr (5 mg/kg, i.p.) or WAY100635 (3 mg/kg, i.p.) + l–THBr (5 mg/kg, i.p.). Mice were administered vehicle or WAY100635 followed by vehicle or l–THBr (5 mg/kg) injections 15 min later. The test was performed 30 min after the administration of l–THBr or vehicle.

Statistical analysis
All data sets were initially checked for normality and homogeneity of variance. The data were expressed as the mean ± S.E.M and assessed using one-way ANOVA followed by Bonferroni post hoc comparisons. A 2 x 2 factorial ANOVA was used to determine interaction effects for WAY100635 and l–THBr. P < 0.05 was defined as a statistically significant difference.

Results

Binding affinity of l–THBr to neurotransmitter receptors
The in vitro receptor competitive binding data illustrate that l–THBr at a concentration of 10 μM has a high binding affinity for D₁, D₂, and D₃ dopamine and 5-HT₁A serotonin receptors but not for GABA or glutamate receptors. The inhibition of ligand binding to D₁, D₂, and D₃ dopamine and serotonin 5-HT₁A receptors was 100.6%, 98.41%, 70.63% and 79.3%, respectively (Table 1).

cAMP assay for D₁ receptor activity
As shown in Fig 2A, the D₁ receptor agonist SKF 38393 but not l–THBr induced a dose-dependent increase in cAMP production in CHO cells stably expressing the D₁ receptor, with an EC₅₀ of 49.1 nM. In contrast, the D₁ receptor antagonist SCH23390 inhibited the production of cAMP induced by SKF 38393 (10 μM) in a dose-dependent manner with an IC₅₀ of 1.42 nM. l–THBr also inhibited cAMP production with an IC₅₀ of 361 nM (Fig 2B), indicating that l–THBr is a D₁ receptor antagonist. The maximum inhibition by l–THBr is 98.75% ±3.49.
Table 1. The Inhibition of ligand binding to neurotransmitter receptors by I-THBr.

| Receptor | Radioligand | Reference compound | Average inhibition percentage | Activity |
|----------|-------------|--------------------|-------------------------------|----------|
| Dopamine Transporter | [3H]WIN 35,428 | GBR12909 | 8.8% | No |
| Dopamine, D1 (h) | [3H]-SCH23390 | SCH23390 | 100.61% | Yes |
| Dopamine, D2s (h) | [3H]-Raclopride | Haloperidol | 96.43% | Yes |
| Dopamine, D3 | [3H]-7-OH-DAPT (+/-)-7-OH-DAPT HBr | 77.61% | Yes |
| Dopamine, D4.4 (h) | [3H]-YM-09151-2 | Haloperidol | 79.55% | Yes |
| GABA A, Agonist Site | [3H]GABA | GABA | 7.89% | No |
| GABA A, BDZ, Alpha 1 site | [3H]Flunitrazepam | Ro5-1788 | -3.30% | No |
| GABA-B | [3H]CGP 54626A (+/-) | Baclofen | -6.94% | No |
| Glutamate, AMPA Site (Ionotropic) | [3H]AMPA (+/-) | AMPA HBr | 0.31% | No |
| Glutamate, Kainate Site (Ionotropic) | [3H]Kainic acid | Kainic Acid | -0.89% | No |
| Glutamate, MK-801 (Ionotropic) | [3H] MK-801 (+)-MK-801 HMaleate | 6.41% | No |
| Glutamate, NMDA Agonist Site (Ionotropic) | [3H]CGP 39653 | NMDA | 4.79% | No |
| Glutamate, NMDA, Phencyclidine Site (Ionotropic) | [3H]TCP (+)-MK-801 Hydrogen | -12.74% | No |
| Glutamate, NMDA, Glycine (stry-Insensitive Site (Ionotropic) | [3H]-MDL-105,519 | MDL-105,519 | 1.79% | No |
| Glycine, Strychnine-sensitive | [3H]Strychnine | Strychnine nitrate | 21.26% | No |
| Serotonin Transporter | [3H]Citalopram,N-Methyl | Imipramine HCl | 34.43% | No |
| Serotonin, 5HT1A (h) | [3H]-8-OH-DPAT | 8-OH-DPAT | 78.31% | Yes |
| Serotonin, 5HT1D | [3H]5-CT | 5-CT | 42.28% | No |
| Serotonin, 5HT2A | [3H]Ketanserin | Methysergide maleate | 40.90% | No |
| Serotonin, 5HT2C | [3H]Mesulergine | Mianserin | 30.65% | No |
| Serotonin, 5HT3 | [3H]GR 65630 | MDL 72222 | 23.02% | No |
| Serotonin, 5HT4 | [3H] 113808 | Serotonin | 32.84% | No |
| Serotonin, 5HT5A (h) | [3H]-LSD | Methiothepin mesylate | 23.79% | No |
| Serotonin, 5HT6 (h) | [3H]-LSD | Methiothepin mesylate | 22.79% | No |
| Serotonin, 5HT7 (h) | [3H]-LSD | 5-CT | 54.82% | Yes |

Significant binding affinity of I-THBr was defined as greater than 50% inhibition of ligand binding. The concentration of I-THBr and ligands was 10 μmol/L. Each value was determined by two independent experiments.

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Fig 2. I-THBr acts as an antagonist at D1 dopamine receptors. (A) The effects of the D1 receptor agonist SKF 38393 and I-THBr on cAMP formation in CHO cells expressing the D1 receptor. The EC50 of SKF 38393 was calculated. (B) The inhibition of cAMP formation induced by SKF 38393 (10 μM) by the D1 receptor antagonist SKF 23390 and I-THBr in CHO cells expressing the D1 receptor. Curves were fitted by non-linear regression analysis. The half maximal inhibitory concentration (IC50) was calculated. The means ± S.E.M. from three independent experiments performed in duplicate are shown.

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\[ ^{35}\text{S} \text{GTP}\gamma \text{S} \text{ assay for D}_2 \text{ receptor activity} \]

As shown in Fig 3A, the D\textsubscript{2} receptor agonist quinpirole but not l-THBr induced a dose-dependent increase in \[^{35}\text{S} \text{GTP}\gamma \text{S} \] binding in HEK293 cells expressing the D\textsubscript{2} dopamine receptor, with an EC\textsubscript{50} of 63.71 nM. l-THBr significantly attenuates the effect of 10 \(\mu\text{M}\) quinpirole on \[^{35}\text{S} \text{GTP}\gamma \text{S} \] binding to D\textsubscript{2} receptors in a concentration-dependent manner with an IC\textsubscript{50} of 5.264 nM, indicating that l-THBr is a D\textsubscript{2} receptor antagonist (Fig 3B).

\[ ^{35}\text{S} \text{GTP}\gamma \text{S} \text{ assay for 5-HT}_{1A} \text{ receptor activity} \]

As shown in Fig 4, both l-THBr and the 5-HT\textsubscript{1A} agonist 8-OH-DPAT increased \[^{35}\text{S} \text{GTP}\gamma \text{S} \] binding to 5-HT\textsubscript{1A} receptors in rat hippocampus in a dose-dependent manner, with an EC\textsubscript{50}
of 234.7 nM for \( \text{l-THBr} \) 98.2 nM for 8-OH-DPAT, indicating that \( \text{l-THBr} \) is an agonist of the 5-HT\(_{1A}\) receptor.

### The effects of \( \text{l-THBr} \) on spontaneous locomotor activity

Mice were administered \( \text{l-THBr} \) (1, 5, or 10 mg/kg, i.p.), diazepam (DZP 2 mg/kg, i.p.) or vehicle and immediately placed into locomotor chambers to test their locomotor activity for 60 min. Travel distance (in cm) was calculated every 10 min. Diazepam or \( \text{l-THBr} \) administration had no influence on locomotor activity at the test doses (\( F_{(3, 35)} = 1.914, P > 0.05 \)). The results are shown in Fig 5.

### The effects of \( \text{l-THBr} \) in the light/dark box test

One-way ANOVA revealed significant differences among treatment groups (\( F_{(4, 43)} = 6.068, P < 0.001 \)). The administration of DZP (2 mg/kg) or \( \text{l-THBr} \) (1 or 5 mg/kg) increased the number of transitions between the light/dark sides (\( P < 0.05 \) compared with the vehicle group). The results are shown in Fig 6.

### The effects of \( \text{l-THBr} \) in the elevated plus maze

A one-way ANOVA revealed a significant difference among the treatment groups in the percentage of entries into the open arms (\( F_{(4, 43)} = 6.141, P < 0.001 \)) and the percentage of time spent in the open arms (\( F_{(4, 43)} = 5.557, P < 0.01 \)). Diazepam (2 mg/kg, i.p.) produced a significant increase in the percentage of arm entries and the percentage of time spent in the open arms (\( P < 0.05 \) compared with the control group), indicating the predictive validity of the elevated plus maze model. \( \text{l-THBr} \) (1 or 5 mg/kg, i.p.) increased the ratio of entries into the open arms and the ratio of time spent in the open arms (\( P < 0.05 \) compared with the control group), indicating that \( \text{l-THBr} \) has anxiolytic effects in this animal model. The results are shown in Fig 7A and 7B.

Fig 7C and 7D show the effects of WAY-100635, a 5-HT\(_{1A}\) receptor antagonist, on the anxiolytic effects of \( \text{l-THBr} \). A 2\( \times \)2 factorial ANOVA revealed a significant interaction between WAY-100635 (3 mg/kg, i.p.) and \( \text{l-THBr} \) (5 mg/kg, i.p.) (for percentage of open arm entries: \( F_{(1,33)} = 6.33, P < 0.05 \); for percentage of open arm time: \( F_{(1,33)} = 6.38, P < 0.05 \)). Subsequent analysis of single treatment effects indicated that there was a difference between the control
Fig 6. Effects of \(\text{I-THBr}\) on the number of transitions in the light/dark box test. \(\text{I-THBr}\) (1, 5 or 10 mg/kg i.p.), DZP (2 mg/kg), or vehicle was administered 30 min before the test. Administration of DZP (2 mg/kg) or \(\text{I-THBr}\) (1 or 5 mg/kg) increased the number of transitions between the light/dark sides \((P < 0.05\) compared with the vehicle group). The data are represented as the means ± S.E.M. *\(P < 0.05\), **\(P < 0.01\), compared with the vehicle group.

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Fig 7. The effects of \(\text{I-THBr}\) on the elevated plus maze task. (A) Effects of diazepam (DZP) or \(\text{I-THP}\) on the percentage of entries into open arms. (B) Effects of diazepam (DZP) or \(\text{I-THP}\) on the percentage of time spent in the open arms during a 5 min period. (C) The anxiolytic-like effects of \(\text{I-THBr}\) were reversed by co-administration of WAY100635, a 5-HT\(_{1A}\) receptor antagonist. The data were expressed as the percentage of entries into the open arms. (D) The anxiolytic-like effects of \(\text{I-THBr}\) were reversed by co-administration of WAY100635. The data were expressed as the percentage of the time spent in the open arms. In the WAY100635 antagonist groups, mice were administered saline or WAY100635 (3 mg/kg, i.p.) followed by administration of a vehicle or \(\text{I-THBr}\) (5 mg/kg, i.p.) 15 min later. The test was performed 30 min after the administration of \(\text{I-THBr}\) or vehicle. The data are represented as the means ± S.E.M. *\(P < 0.05\), **\(P < 0.01\), compared with the vehicle group. \(*^\circ P < 0.05\), compared with the \(\text{I-THBr}\) group.

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and l-THBr-treated groups (for percentage of open arm entries: $F_{(1, 33)} = 6.22, P < 0.05$; for percentage of open arm time: $F_{(1, 33)} = 5.94, P < 0.05$). The analysis also indicated a significant difference between the l-THBr (5 mg/kg) and WAY100635 + l-THBr groups (for percentage of open arm entries: $F_{(1, 33)} = 8.95, P < 0.01$; for percentage of open arm time: $F_{(1, 33)} = 7.12, P < 0.05$). These results show that the anxiolytic-like effects of l-THBr were significantly reversed by co-administration of the 5-HT$_{1A}$ antagonist WAY100635 (3 mg/kg, i. p.).

**Discussion**

In the present study, we evaluated the anxiolytic-like effects of l-THBr in behavioral models of anxiety. We found that intraperitoneal administration of l-THBr produced anxiolytic-like effects in the elevated plus maze and light-dark box tests. In addition, l-THBr had a high affinity for D$_1$, D$_2$-like dopamine and serotonin 5-HT$_{1A}$ receptors and exhibited D$_1$, D$_2$ antagonist and 5-HT$_{1A}$ agonist properties.

The anxiolytic mechanism of diazepam occurs mainly through benzodiazepine receptors, which are present in the GABA receptor pentameric complex. Thus, diazepam induces sedative effects by increasing the opening frequency of the associated chloride ion channel and hyperpolarizing the membrane [27]. In the present study, diazepam showed a significant and stable anxiolytic-like effect in the male ICR mice, consistent with some previous studies [28]. The in vitro receptor competitive test results demonstrated that l-THBr does not interact with inhibitory GABAergic receptors at benzodiazepine (BDZ) sites but mainly binds to dopamine and serotonin receptors. Thus, l-THBr works well at relieving anxiety without causing sedative effects.

Anxiety disorders are associated with the dysfunction of a number of neurotransmitters and their receptors, including dopamine and serotonin [29–33]. An increase in dopaminergic transmission has been demonstrated to aggravate anxiety [34], and the D$_1$ receptor antagonist SCH23390 exhibits clear anxiolytic-like effects [33, 35, 36]. However, D$_2$ receptor ligands can produce either anxiogenic [33, 37] or anxiolytic-like effects in animal models [28, 38, 39]. D$_1$ dopamine receptors are mainly found at postsynaptic sites, whereas D$_2$ dopamine receptors are localized both presynaptically (where they act as autoreceptors) and postsynaptically. Therefore, D$_2$ antagonists may block presynaptic D$_2$ dopamine autoreceptors and increase the release of dopamine, which in turn modulate anxiety-like behaviors by acting on postsynaptic D$_2$ dopamine receptors [40–41]. Whether D$_2$ antagonists exert effects through presynaptic D$_2$ receptor or postsynaptic D$_2$ receptors may largely depend on the test doses used [39–42]. However, in our studies, the anxiolytic-like effect of l-THBr in the elevated plus maze test was blocked by the 5-HT$_{1A}$ antagonist WAY100635. Thus, our results suggest that the anxiolytic-like effects of l-THBr are probably mediated through a 5-HT$_{1A}$ receptor mechanism.

Previous studies indicate that injection of 5-HT into the brain stem produces anxiety [43]. Moreover, the anxiolytic activities of 5-HT$_{1A}$ full or partial agonists are thought to be the result of decreased 5-HT outflow and a reduction of serotonergic neuron activity via the activation of 5-HT$_{1A}$ autoreceptors at presynaptic sites [44].

In addition, the interaction of D$_2$ receptors and 5-HT$_{1A}$ receptors plays an important role in mental disorders [45]. For example, the 5-HT$_{1A}$ agonist buspirone at low doses of 1.25–5.0 mg/kg (which are relevant doses for the anxiolytic effects of buspirone) blocks presynaptic D$_2$ autoreceptors [46, 47]. In addition, aripiprazole or SSR181507 (a combined D$_2$ antagonist and a 5-HT$_{1A}$ partial agonist, respectively) improve depression and anxiety symptoms in patients with schizophrenia [48, 49]. Based on these findings, it has been proposed that a combination of D$_2$ antagonistic and 5-HT$_{1A}$ agonistic properties would offer additional advantages in
treated some mental disorders, such as anxiety, depression (for fast onset anti-depressants) and schizophrenia [50, 51].

In conclusion, l-THBr exhibits anxiolytic activity in two animal models of anxiety. Activation of 5-HT$_{1A}$ autoreceptors and a decrease in serotonergic activity most likely contributes to the anxiolytic activity of l-THBr in these tests. The ability of l-THBr to exert effective anxiolytic activity without sedative effects suggests a potential use for l-THBr as a superior treatment for anxiety.

Author Contributions

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