J Pharm Biopharm Res, 2024, 5(1): 396-411
DOI: 10.25082/JPBR.2023.01.004

RESEARCH ARTICLE

TAAR1 as a new target for the treatment of bipolar disorder: Anti-manic and anti-depressant activity of the novel agonist PCC0105004

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Received: January 2, 2024;
Accepted: March 21, 2024;
Published: March 25, 2024.

Citation: Yu L, Zhang W, Shi Y, *et al*. TAAR1 as a new target for the treatment of bipolar disorder: Anti-manic and anti-depressant activity of the novel agonist PCC0105004. *J Pharm Biopharm Res*, 2024, **5**(1): 396-411. https://doi.org/10.25082/JPBR.2023.01.004

Abstract: Background: Bipolar disorder (BD) is a deleterious psychiatric disorder, and the available pharmacotherapies have limited efficacy with significant side effects. Trace amine-associated receptor 1 (TAAR1) is an emerging drug target for neuropsychiatric disorders such as schizophrenia and substance user disorders. However, it is unknown whether TAAR1 is involved in the pathogenesis of BD. This study examined the effects and underlying mechanisms of a novel TAAR1 agonist, PCC0105004, in a rat model of ouabain (OUA)-induced BD. **Methods**: Intracerebroventricular (ICV) administration of OUA-induced BD model was established. The in vitro cell-based cAMP assay was used to examine TAAR1 agonism of PCC0105004. The receptor specificity of PCC0105004 was determined by an off-target panel assay that included radioligand binding and enzymatic assays. The effects of PCC0105004 on manic-like and depressive-like behaviors were evaluated in the rat BD model. TAAR1-mediated signaling and oxidative stress parameters were biochemically determined in the prefrontal cortex and the hippocampus of rats. **Results**: Western blotting revealed reduced TAAR1 expression level in the prefrontal cortex but unchanged in the hippocampus in model rats. PCC0105004, a TAAR1 agonist with the agonism EC_{50} value of 0.06182 μM, attenuated the manic-like behaviors on the 7th day and the depressive-like behaviors on the 14th day at doses that did not affect locomotor activity in the BD rats. Mechanistically, PCC0105004 exerted its behavioral effects via the reduction of ROS damage through the phosphorylation activation of the TAAR1/Akt/GSK3β/BDNF signaling pathway. **Conclusion**: These results demonstrated the potential antimanic-like and antidepressant-like efficacy of a novel TAAR1 agonist PCC0105004 in rats and revealed its underlying molecular basis, which supports the possibility of TAAR1 agonists as candidate pharmacotherapeutics for BD.

Keywords: bipolar disorder, TAAR1, PCC0105004, prefrontal cortex, oxidative stress

1 Introduction

Bipolar disorder (BD) is a complex psychiatric mood disorder influenced by multifactorial elements, including social, environmental, and genetic risk factors that contribute to its etiology. The disorder is characterized by fluctuating mood states, encompassing periods of mania and depression interspersed with intervals of wellbeing lasting days or weeks. BD encompasses various subtypes, such as BD type I, BD type II, and cyclothymia [1,2]. The World Mental Health Survey Initiative reported lifetime and 12-month prevalence estimates for bipolar disorders as 2.4% and 1.5%, respectively [3]. While psychological, social, and occupational interventions play crucial roles in managing BD, medication currently stands as the primary treatment modality [4]. Therapeutic options for BD are limited, with key categories including mood stabilizers like lithium and sodium valproate, as well as atypical antipsychotic medications such as aripiprazole and olanzapine. Most drugs are approved for maintenance therapy or treating the manic phase. Consequently, there is an urgent need to develop drugs proven effective in under-treated subpopulations, such as BD ll type depression. Moreover, there is a demand for drugs that enhance tolerance, particularly in mitigating weight gain and extrapyramidal reactions [5]. The search for new therapeutic mechanisms is crucial to identify drugs that are not only effective but also associated with minimal side effects and high tolerability.

Trace amine-associated receptor 1 (TAAR1) is a G-protein-coupled receptor (GPCR) expressed in various mammalian brain regions, including the prefrontal cortex, limbic and mesolimbic areas crucial for emotion regulation, cognition, and behavior. In individuals with BD, these

functions are often impaired. Evidence indicates that TAAR1 is involved in the modulation of dopaminergic, serotonergic and glutamatergic signaling, thereby influencing aspects of reward-processing, cognition and mood relevant to schizophrenia and other mental disorders [6]. The TAAR1 gene is located in the chromosomal area 6q23.2, adjacent to regions where susceptibility loci for BD have been identified. Thirteen missense variants of TAAR1 have been observed at a higher frequency in patients with mood disorders [6]. The identification of endogenous TAAR1 ligands and subsequent development of small-molecule agonists has revealed antipsychotic-, anxiolytic-, and antidepressant-like properties [7]. Therefore, TAAR1 could be a potential therapeutic target for BD [8], a possibility that has not been explored.

In this study, we reported the characterization of a novel TAAR1 agonist, PCC0105004 [(S)-4,7,8,9,10,10a-hexahydro-5H-thieno[2',3':3,4]pyrido[1,2-a]pyrazine] for its in vitro TAAR1 agonist property, in vivo pharmacokinetics, in vivo anti-BD efficacy and underlying molecular mechanisms.

2 Materials and Methods

2.1 Animals

Adult Wistar rats (Rattus norvegicus; 250–350 g) were purchased from a commercial vendor (Beijing Vital River Laboratory Animal Technology Co, Ltd, Beijing, China), and housed in a certified animal facility with a 12 h light/dark cycle, constant temperature and humidity (21-23°C and 40-60%), and free access to food and water. All animals were acclimated to the test environment for at least 2 days before any behavioral testing. All behavioral tests were conducted between 8:00 and 15:00 h by experimenters blinded to the treatment conditions. All experimental procedures in this study were conducted in accordance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson & Altman, 2010) and all animal protocols were approved by the Laboratory Animals Care and Use Committee of Yantai University (registration number is YTU20220329) (Yantai, China).

2.2 Drugs and Chemicals

PCC0105004 (formula $C_{10}H_{14}N_2S$, Figure 1) was provided by WuXi AppTec (Tianjin, China) with a purity of $> 95\%$. Valproate sodium was purchased from Sigma (United States). Ouabain (OUA) was purchased from MedChemExpress (United States). Artificial cerebrospinal fluid (aCSF) was purchased from Shanghai Xin Yu Biotech Co., Ltd (Shanghai, China). For in vivo studies, PCC0105004 and valproate were dissolved in physiologic saline (0.9% NaCl, Sal). OUA was dissolved in aCSF.

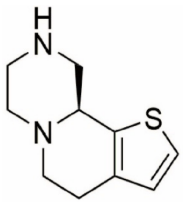

Figure 1 The chemical structure of PCC0105004, (S)-4,7,8,9,10,10a-hexahydro-5H-thieno[2',3':3,4]pyrido[1,2-a]pyrazine

2.3 cAMP Detection Assay

CHO-K1 cells expressing the human TAAR1 receptor (Accession number NP_612200.1) were cultivated in media (Advanced DMEM supplemented with 1% dialyzed fetal bovine serum) without antibiotics. The cells underwent detachment through gentle flushing with phosphate buffered saline containing 5 mM EDTA, followed by recovery through centrifugation and resuspension in assay buffer (Krebs-Ringers Henseleit buffer: 5 mM KCl, 1.25 mM $MgSO_4$, 124 mM NaCl, 25 mM HEPES, 13.3 mM Glucose, 1.25 mM KH2PO4, 1.45 mM $CaCl_2$, 0.5 g/l BSA, supplemented with 1mM Isobutylmethylxanthine). The testing was conducted in 384-well plates. With 10 μl of cells (6000 cells) mixed with 10 μl of the test compound diluted in assay buffer and then incubated for 30 minutes at 37°C. For the determination of the amount of cAMP formed, the HitHunter cAMP XS kit (DiscoveRx, Fremont, CA) was employed. The cAMP formation assay was concluded by the addition of 10 μl of cAMP XS antibody/lysis mix. After a 1-hour incubation, 10 μl of cAMP XS ED reagent was added, followed by an additional

1-hour incubation. Subsequently, 20 μl of cAMP XS EA/CL substrate mix was added, and the plates were covered with sealing tape. The plates were allowed to stand for 1-hour and then centrifuged at 200 g for 5 min. A further incubation of 14 hours in the dark ensues, followed by counting using a PerkinElmer 1450 MicroBeta TriLux counter (PerkinElmer Life and Analytical Sciences) in luminescence mode. A natural agonist of TAAR1 p-Tyramine(pTyr), was employed as a positive control.

2.4 In vitro off-target panel assay

This panel of assays comprises 71 radioligand binding assays and 16 enzymatic assays. In this preliminary screen, all assays were conducted using PCC0105004 at a concentration of 10 μM. The criteria for determining a significant effect were arbitrarily established at either 50% above or below the baseline.

2.5 Pharmacokinetic (PK) study

The concentration of PCC0105004 in the plasma and brain of rats was measured following a single oral dose administration of 5 mg/kgto healthy Wistar rats. Blood samples were collected from the orbital venous plexus of all rats (n=3 per group) at various timepoints, including pre-dose, 0.25, 0.5, 1, 2, 4, 6, 8 and 12 hours post-dose. Additionally, 0.3 mL of blood was collected from the orbital venous plexus, and whole brain tissues were obtained from all rats (n = 3/sex/group) following anesthesia and perfusion with physiological saline at timepoints of 0.25, 1, and 6 hours post-dose. Plasma was prepared by centrifugation at 8000 $\times$ g for 5 min, while brain tissues were homogenized with saline (1:4, w/v). The concentration of PCC0105004 in both plasma samples and brain tissues was determined using LC-MS/MS system, consisting of Agilent 1100 and TSQ Quantum Access (Thermo Electron Corporation, San Jose, CA, United States).

2.6 Stereotaxic surgery

Animals were intramuscularly anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). In a stereotaxic apparatus, the skin of the rat skull was removed, and a 27-gauge 9-mm guide cannula was positioned at 0.9 mm posterior to bregma, 1.5 mm right from the midline, and 1.0 mm above the lateral brain ventricle. A cannula was implanted 2.6 mm ventral to the superior surface of the skull through a 2-mm hole made at the cranial bone, and it was secured in place with dental acrylic cement. To minimize animal suffering, rats received an intramuscular injection of tramadol (10 mg/kg) after surgery for post-operational analgesia. Animals recovered from surgery within 3 days. On the fourth day after surgery, the animals received a single intracerebroventricular (ICV) injection of 5 μl of aCSF or OUA 10^{-3} M dissolved in aCSF [9, 10]. A 30-gage cannula was inserted inside the guide cannula and connected by a polyethylene tube to a microsyringe. The tip of the cannula infusion protruded 1.0 mm beyond the cannula guide, aiming at the right lateral brain ventricle.

2.7 Open-Field Test

Manic-like behaviors, including hyperactivity, are easily evaluated in the open-field test. The apparatus consists of a 60 $\times$ 60 $\times$ 50 cm (length $\times$ width $\times$ height) box, with the floor divided into nine equal squares (20 $\times$ 20 cm). In the test, the animals were placed to explore the area for 5 min. Locomotor activity was recorded and analyzed for 5 min using the TopScan monitoring system (CleverSys Inc. Reston, VA, United States). The parameters used in the test included: crossings (the total number of times rats crossed the squares during the entire test period), rearings (the total number of instances where the rats exhibited erect postures throughout the whole test period), and total activity (the distance the rat moved throughout the test period) [11, 12].

2.8 Forced Swimming Test

The forced swimming test (FST) is a procedure that involves two individual exposures to a cylinder filled with warm water. The cylinder designed with a height that prevents the animal from touching the floor of the apparatus or escaping. Composed of transparent acrylic and measuring 80 cm height and 30 cm diameter, the cylinder was filled with water (22–26°C) to a depth of 40 cm. The day before the official experiment, rats underwent an individual training session in the water-filled cylinder 15 min. On the day of the official experiment, rats were once again subjected to the FST for 6 min during the test session. A trained observer, blinded to the experimental groups, recorded the rats' immobility in seconds.

2.9 Brain Samples

After behavioral analysis, rats were euthanized by decapitation The skull was opened, and the cerebral content was excised and rapidly dissected on a chilled dish Petri. The prefrontal cortex and hippocampus were isolated and cleaned from subcortical structures and white matter. All samples were kept frozen at -150°C until the analyses were performed.

2.10 Western blot

The prefrontal cortex and the hippocampus tissues were rapidly dissected and homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer. Lysates were incubated on ice for 30 min, and the prefrontal cortex and the hippocampus samples were spun at 12000 rmin^{-1} for 20 min. Then, the supernatants were collected and transferred to fresh tubes, and the protein concentrations were analyzed using the bicinchoninic acid (BCA) Protein Assay kit (BioTek SYNERGY neo2, United States). A total of 50 μg of protein samples were resolved on a 10-12.5% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes for immunoblotting. After blocking for 2 h in 5% nonfat milk (m/v), the membranes were co-incubated at 4°C overnight with the following primary antibodies: rabbit anti-TAAR1 (1:1000; Abcam, Cambridge, United Kingdom, Cat#ab150646), rabbit anti-BDNF (1:1000; Abcam, Cambridge, United Kingdom, Cat#ab108319), rabbit anti-GSK-3β (GSK-3β) (1:1000; Abcam, Cambridge, United Kingdom, Cat#ab32391), rabbit anti-phospho-GSK-3β (Ser 9) (p-GSK-3β) (1:1000; Abcam, Cambridge, United Kingdom, Cat#ab75814), rabbit anti-total AKT (1:1000; Cell Signaling Technology, MA, United States, Cat#4691), rabbit anti-phospho-AKT (Ser 473) (p-AKT) (1:1000; Cell Signaling Technology, MA, United States, Cat#4060), mouse anti-GAPDH (1:1000, Beyotime Institute of Biotechnology, Shanghai, China, Cat#AF0006), and mouse anti-β-actin (1:1000, Beyotime Institute of Biotechnology, Shanghai, China, Cat#AF0003). After overnight incubation with primary antibodies, the membranes were then washed three times in TBST-Tween 20 and incubated for 1 h with the corresponding secondary antibodies: goat anti-mouse HRP (1:2000, Beyotime Institute of Biotechnology, Shanghai, China, Cat#A0216) and goat anti-rabbit HRP (1:2000, Beyotime Institute of Biotechnology, Shanghai, China, Cat#A0208), and exposed onto ChampChemi 610 (Sagecreation, Bejjing, China) with Sage CapturePro software (Sagecreation, Beijing, China). Signal intensities were quantified using Image J software and normalized to the corresponding β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) band.

2.11 Evaluation of Oxidative Stress in the prefrontal cortex in BD Rats

2.11.1 Measurement of lipid peroxidation

The levels of the thiobarbituric acid-reactive species (TBARS), specifically malondialdehyde (MDA), were measured in samples of cerebral structures using RIPA Lysis Buffer. The Lipid Peroxidation MDA Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China, Cat#S0131S) was employed for the direct quantitative measurement of MDA levels in the brain tissue samples.

2.11.2 Determination of protein carbonyl content

Carbonyl group content was determined using a Protein Carbonyl assay kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing Jiancheng Bioengineering Institute, Nanjing, China, Cat#A087-1-2).

2.11.3 Determination of antioxidant enzyme activity

Glutathione peroxidase (GPx) catalyzes the reduction of peroxides coupled to glutathione oxidation. The Total Glutathione Peroxidase Assay Kit with NADPH (Beyotime Institute of Biotechnology, Shanghai, China, Cat#S0058) was employed for the direct quantitative measurement of GPx activity levels.

2.12 Statistical analysis

All the data were presented as mean $\pm$ SEM. Data were analyzed using IBM SPSS Statistics version 21.0 (Chicago, IL, United States) and plotted using Graph Pad Prism software version 8.0 (San Diego, CA, United States). Data were analyzed by unpaired Student's t tests, or one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. Statistical significance was accepted at the level of $p < 0.05$. For detailed information, see the figure legends of the respective figures.

3 Results

3.1 Low expression of TAAR1 in the prefrontal cortex and hippocampus of OUA-induced BD rats

To investigate the association between TAAR1 and BD, we assessed protein expression in the prefrontal cortex and hippocampus of model rats. The timeline for the establishment of BD model, behavioral testing, and brain tissue collection is depicted in Figure 2A. Following 7 days of ICV administration of OUA, the open field test revealed increased numbers of crossings, rearings, and total activity in rats, signs of manic-like behaviors in this model (Figure B, C and D). After 14 days of ICV administration of OUA, depressive-like behaviors were produced, as evidenced by an increase in the time of immobility and a decrease in the time of swimming in the forced swimming test (Figure 2E). Western blotting was performed to examine the protein expression levels of TAAR1 in the prefrontal cortex and hippocampus during the manic and depressive phases, respectively. The results indicated that the BD model significantly reduced the expression of TAAR1 in the prefrontal cortex during both manic and depressive phases, whereas this change was not evident in the hippocampus (Figure 2F and 2G).

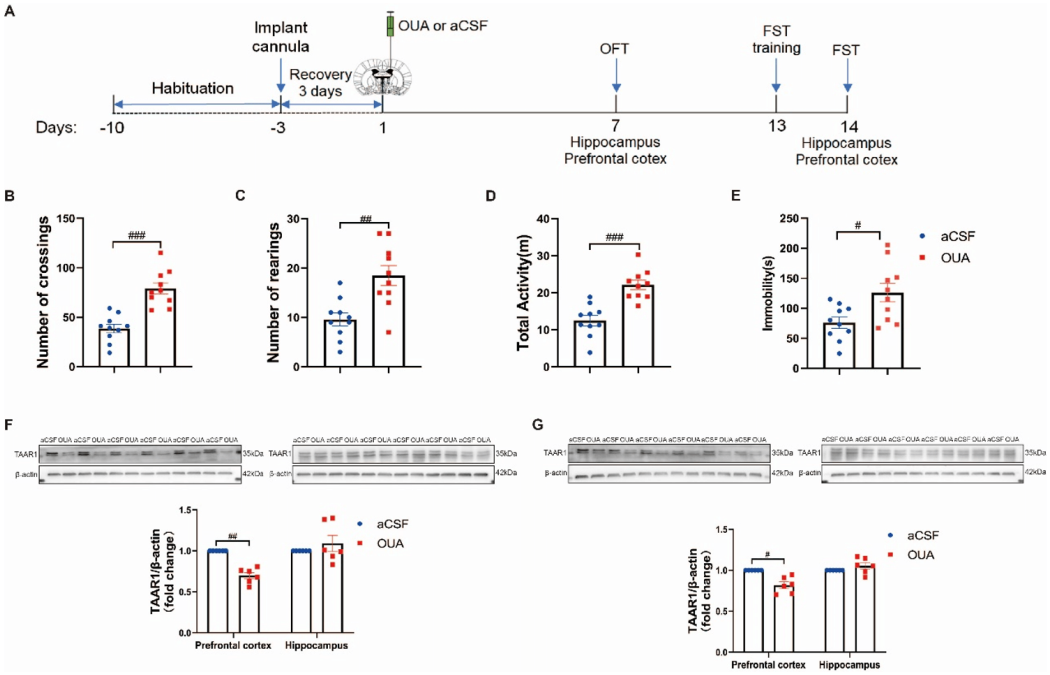

Figure 2 Expression of TAAR1 in the prefrontal cortex and hippocampus of OUA-induced BD rats. Scheme illustrating the experimental design used in this study (A). The number of crossings (B), rearings (C) and total activity (D) in the OFT of rats after ICV administration of OUA on the 7th day. The time of immobility in the FST of rats with ICV administration of OUA on the 14th day (E). Representative Western blot bands and quantitative analysis of TAAR1 in the prefrontal cortex and hippocampus of rats after injection of OUA 7 days (F) and 14 days (G) by densitometry. Data were expressed as percentage of value of aCSF group. Data were analyzed by Unpaired Student's t test. Values are expressed as mean ± SEM, n = 6-8 per group. $^{\#}p < 0.05$ $^{\#\#}p < 0.01$ compared to aCSF group.

3.2 In Vitro activity of PCC0105004

The EC50 values of pTyr and PCC0105004 for TAAR1 activity were determined using the cAMP Detection Assay. The experimental results are presented in Figure 3B and 3C. Both pTyr and the small molecule agonist PCC0105004 demonstrated TAAR1 agonistic activity, with EC_{50} of 1.235 μM and 0.06182 μM, respectively. PCC0105004 underwent a comprehensive binding selectivity assessment against an off-target panel comprising more than 80 receptors (Table 1) and enzymes (Table 2). At a concentration of 10 uM, PCC0105004 exhibited binding to Adrenergic α_{1A} (49%), Calcium Channel L-Type, Phenylalkylamine (43%), Serotonin (5-Hydroxytryptamine) 5-HT_{1B} (46%), Monoamine Oxidase MAO-B (32%) and Adrenergic α_{1B} (31%).

Table 1 Radioligand binding assays

Assay Name	Conc.	% Inhibition
Protein Tyrosine Kinase, EGF Receptor	10 μM	-7
Protein Tyrosine Kinase, Insulin Receptor	10 μM	-6
Tyrosine Hydroxylase	10 μM	-14
Adenosine A_1	10 μM	-6
Adenosine A_{2A}	10 μM	7
Adenosine A_{2B}	10 μM	2
Adenosine A_3	10 μM	8
Adrenergic α_{1A}	10 μM	49
Adrenergic α_{1B}	10 μM	31
Adrenergic β_1	10 μM	6
Adrenergic β_2	10 μM	-1
Androgen (Testosterone)	10 μM	3
Bradykinin B_2	10 μM	3
BZDp (TSPO)	10 μM	5
Calcitonin Gene-Related Peptide $CGRP_1$	10 μM	5
Calcium Channel L-Type, Benzothiazepine	10 μM	28
Calcium Channel L-Type, Dihydropyridine	10 μM	-5
Calcium Channel L-Type, Phenylalkylamine	10 μM	43
Calcium Channel N-Type	10 μM	6
Cannabinoid CB_1	10 μM	-9
Cannabinoid CB_2	10 μM	3
Cholecystokinin CCK_1 (CCK_A)	10 μM	5
Cholecystokinin CCK_2 (CCK_B)	10 μM	5
Dopamine D_1	10 μM	16
Dopamine D_{2L}	10 μM	8
Dopamine D_3	10 μM	12
Dopamine $D_{4.4}$	10 μM	-7
Endothelin ET_A	10 μM	-5
Estrogen ERα	10 μM	1
$GABA_A$, Chloride Channel, TBOB	10 μM	-10
$GABA_A$, Flunitrazepam, Central	10 μM	6
$GABA_{B1B}$	10 μM	0
Glucocorticoid	10 μM	2
Glutamate, AMPA	10 μM	0
Glutamate, Kainate	10 μM	10
Glutamate, Metabotropic, $mGlu_5$	10 μM	8
Glutamate, NMDA, Agonism	10 μM	11
Glutamate, NMDA, Phencyclidine	10 μM	-5
Glycine, Strychnine-Sensitive	10 μM	9
Histamine H_1	10 μM	31
Histamine H_3	10 μM	-16
Melatonin MT_1	10 μM	-3
Muscarinic M_1	10 μM	-5
Muscarinic M_2	10 μM	6
Muscarinic M_3	10 μM	-3
Muscarinic M_4	10 μM	-4
Muscarinic M_5	10 μM	8
Neuropeptide Y Y_1	10 μM	0
Neuropeptide Y Y_2	10 μM	-6
Nicotinic Acetylcholine $\alpha4\beta2$, Cytisine	10 μM	-3
Nicotinic Acetylcholine $\alpha7$, Bungarotoxin	10 μM	5
Opiate δ_1 (OP1, DOP)	10 μM	3
Opiate κ (OP2, KOP)	10 μM	0
Opiate μ (OP3, MOP) [3H]morphine	10 μM	10
Orexin OX_1	10 μM	-3
Orexin OX_2	10 μM	-6
Oxytocin	10 μM	0
Potassium Channel [SK_{CA}]	10 μM	0
Purinergic P2X	10 μM	-6
Serotonin (5-Hydroxytryptamine) 5-HT_{1B}	10 μM	46
Serotonin (5-Hydroxytryptamine) 5-HT_{2A}	10 μM	21
Serotonin (5-Hydroxytryptamine) 5-HT_{2B}	10 μM	36
Serotonin (5-Hydroxytryptamine) 5-HT_3	10 μM	17
Sodium Channel, Site 2	10 μM	19
Tachykinin NK_1	10 μM	1
Transporter, Adenosine	10 μM	-4
Transporter, Dopamine (DAT)	10 μM	-1
Transporter, GABA	10 μM	9
Transporter, Norepinephrine (NET)	10 μM	9
Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	10 μM	1
Vasopressin V_{1A}	10 μM	-7

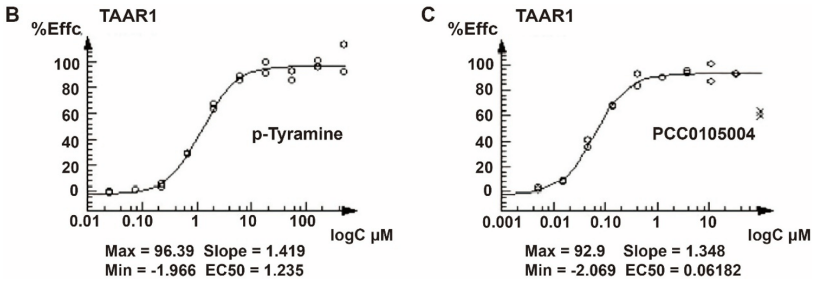

Figure 3 TAAR1 agonism of pTyr (B) and PCC0105004 (C). Mean drug concentration-time curve of rats after gavage with 5 mg/kg PCC0105004 (D). The content of PCC0105004 in plasma (E) and brain (F) and brain/plasma ratio (G) at 15, 60 and 360 min after administration in normal rats. Data were expressed as mean ± SEM, n = 3 per time point.

Table 2 Enzymatic assays

Assay Name	Conc.	% Inhibition
ATPase, Na^+/K^+, Heart, Pig	10 μM	1
Carbonic Anhydrase II	10 μM	-1
Catechol-O-Methyl Transferase (COMT)	10 μM	-3
Cholinesterase, Acetyl, ACES	10 μM	18
CYP450, 1A2	10 μM	2
CYP450, 2C19	10 μM	2
CYP450, 2D6	10 μM	4
CYP450, 3A4	10 μM	0
GABA Transaminase	10 μM	1
Monoamine Oxidase MAO-A	10 μM	-1
Monoamine Oxidase MAO-B	10 μM	32
Peptidase, Angiotensin Converting Enzyme	10 μM	1
Peptidase, CTSB (Cathepsin B)	10 μM	-2
Peptidase, CTSL (Cathepsin L)	10 μM	2
Phosphodiesterase PDE4D2	10 μM	0
Protein Serine/Threonine Kinase, MAPK14 (p38α)	10 μM	-9

3.3 The PK profile of PCC0105004 after a single oral administration in rats

To evaluate the mean plasma and brain concentration-time profiles, PK testing was conducted in rats. A preliminary study revealed that PCC0105004 could be detected in plasma at 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h following a single oral dose of 5 mg/kg in rats (Figure 4D). In plasma, C_{max} was 3730±846 nM, T_{max} was 0.417±0.144 h and $T_{1/2}$ was 1.85±0.217 h. In brain tissue distribution tests, PCC0105004 was detectable in both plasma and brain at 0.25, 1 and 6 h following a single oral dose of 5 mg/kg in rats. The highest concentrations of PCC0105004 in plasma and brain were observed at 0.25-1 h after administration, with the brain/plasma ratio ranging from 8.45 to 4.22 (Figure 4E to 4G). These results demonstrated that PCC0105004 can be readily absorbed into the blood, cross the blood-brain barrier and reach the brain.

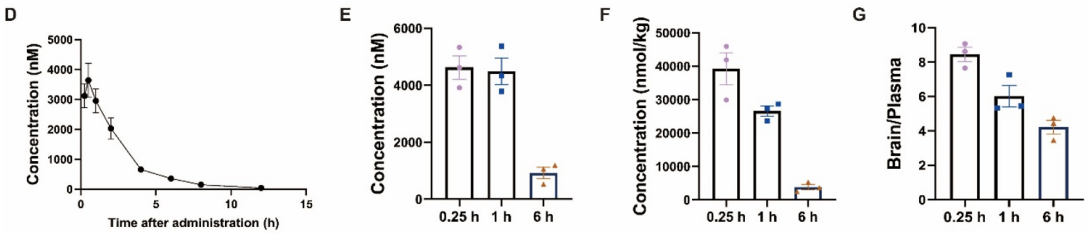

Figure 4 Mean drug concentration-time curve of rats after gavage with 5 mg/kg PCC0105004 (D). The content of PCC0105004 in plasma (E) and brain (F) and brain/plasma ratio (G) at 15, 60 and 360 min after administration in normal rats. Data were expressed as mean ± SEM, n = 3 per time point.

3.4 Effects of PCC0105004 on locomotor activity in normal rats

The open field test was used to evaluate the impact of PCC0105004 at doses ranging from 0.75 mg/kg to 6 mg/kg on locomotor activity in normal rats. The results indicated that PCC0105004 at 0.75 mg/kg and 1.5 mg/kg did not produce significant effects on the spontaneous activity of rats. However, when the dose exceeded 3 mg/kg, PCC0105004 exhibited pronounced sedative effects on rats (Figure 5).

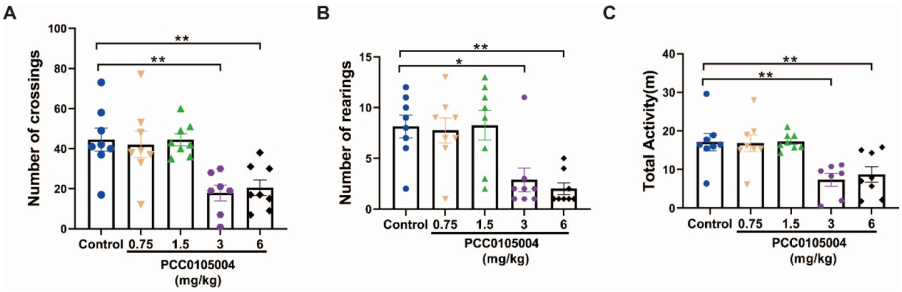

Figure 5 Effects of PCC0105004 on locomotor activity in normal rats. Effects of administration of PCC0105004 ranging from 0.75 mg/kg to 6 mg/kg on the number of crossings (A) and rearings (B) and total activity (C) in the OFT in normal animals. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean ± SEM, n = 8 per group. * $p < 0.05$ $^{**}p < 0.01$ compared to Control group.

3.5 Effects of PCC0105004 on manic-like and depressive-like behaviors in OUA-induced BD rats

To determine the optimal dose of PCC0105004 for subsequent studies in rats with BD, we conducted the open-field test on day 7 after the OUA injection. The timeline for establishing BD models and behavioral testing is depicted in Figure 6A. Our results revealed that PCC0105004 at doses ranging from 0.75 mg/kg to 3 mg/kg dose-dependently improved manic-like behaviors in BD rats (Figure 6B, 6C and 6D). Considering the results of the open field test in normal rats, a dose of 1.5 mg/kg of PCC0105004 was selected for the subsequent studies.

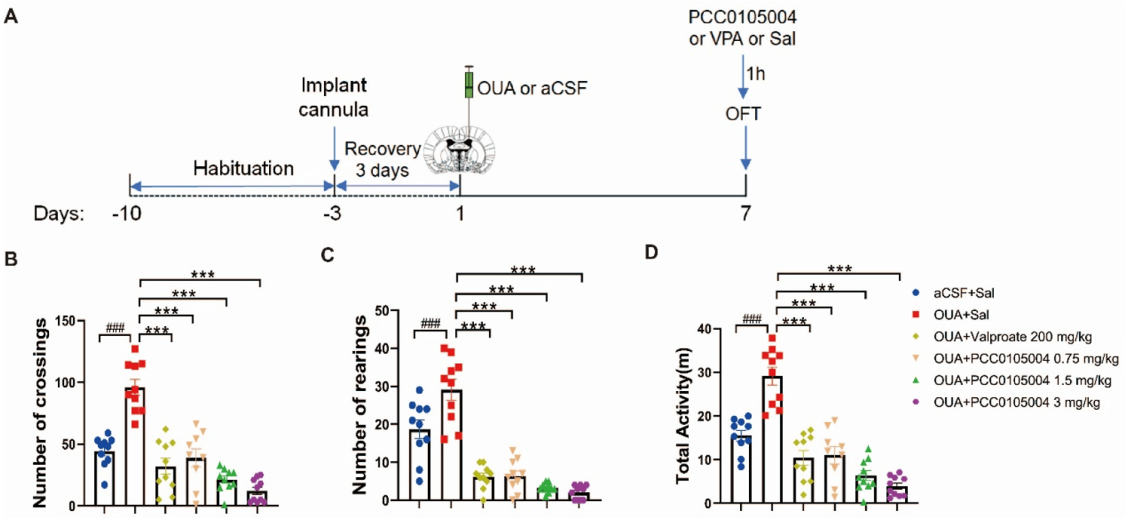

Figure 6 The dose-dependent effects of PCC0105004 on manic-like behaviors in OUA rats. Scheme illustrating the experimental design used in this study (A). Effects of administration of valproate (200 mg/kg) or PCC0105004 from 0.75 mg/kg to 3 mg/kg on the 7th day on the number of crossings (B), rearings (C) and total activity (D) in the OFT in animals treated with OUA. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean ± SEM, n = 10 per group. $^{\#\#\#}$ $p <$ 0.001 compared to aCSF+Sal group. *** $p < 0.001$ compared to OUA+Sal group.

To assess the effects of PCC0105004 on manic-like and depressive-like behaviors in rats with BD, we performed the open-field test and forced swimming test. The timeline for establishing BD models and conducting behavioral tests is depicted in Figure 7A. Compared to the artificial

cerebrospinal fluid + physiologic saline (aCSF + Sal) group, both PCC0105004 (1.5 mg/kg) and valproate (200 mg/kg) demonstrated no significant effects on locomotor activity in sham rats on day 7 and day 14 after the OUA injection, indicating that their effects on rats with BD were not associated with sedation. In contrast to the ouabain + physiologic saline (OUA+ Sal) group, both 1.5 mg/kg PCC0105004 and 200 mg/kg valproate significantly reduced the number of crossings, rearings, and total activity in OUA-induced BD rats in the open-field test on day 7 (Figure 7B, 7C and 7D) and decreased the immobility time in the forced swimming test on day 14 (Figure 7E).

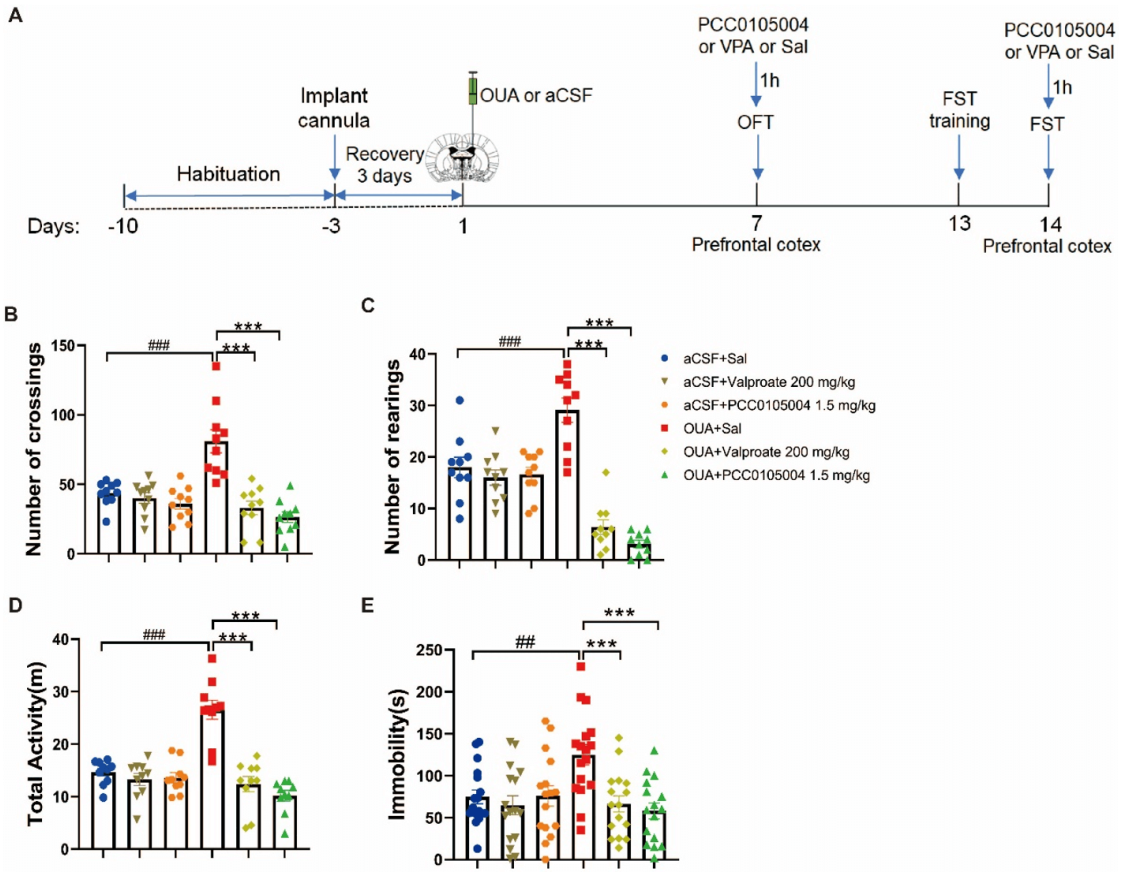

Figure 7 Effects of PCC0105004 on manic-like and depressive-like behaviors in OUA rats. Scheme illustrating the experimental design used in the manic phase and depressive phase study (A). Effects of 200 mg/kg valproate or 1.5 mg/kg PCC0105004 on the 7th day on the number of crossings (B), rearings (C) and total activity (D) in the OFT in animals treated with OUA (n = 10 per group). Effects of 200 mg/kg valproate or 1.5 mg/kg PCC0105004 on the 14th day on the time of immobility (E) in animals treated with OUA (n = 15 per group). Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean ± SEM. $^{\#\#}p < 0.01$ $^{\#\#\#}p < 0.001$ compared to aCSF + Sal group. $^{***}p < 0.001$ compared to OUA +Sal group.

3.6 Mood-stabilizing effect of PCC0105004 in OUA-induced BD rats

To assess the mood-stabilizing effect of PCC0105004 in rats with BD, we conducted the open-field test and forced swimming test after a continuous dosing period. The timeline for establishing BD model and conducting behavioral testing is depicted in Figure 8A. Following 6 days of continuous administration, both 1.5 mg/kg PCC0105004 and 200 mg/kg valproate effectively reversed manic-like behaviors on day 7 after the OUA injection, evidenced by the reduction in the number of crossings, rearings, and total activity of rats in the open field test (Figure 8B, 8C and 8D). After 13 days of continuous administration, both 1.5 mg/kg PCC0105004 and 200 mg/kg valproate continued to reverse depressive-like behavior on day 14 after the OUA injection, as evidenced by the decrease in immobility time in the forced swimming test (Figure 8E).

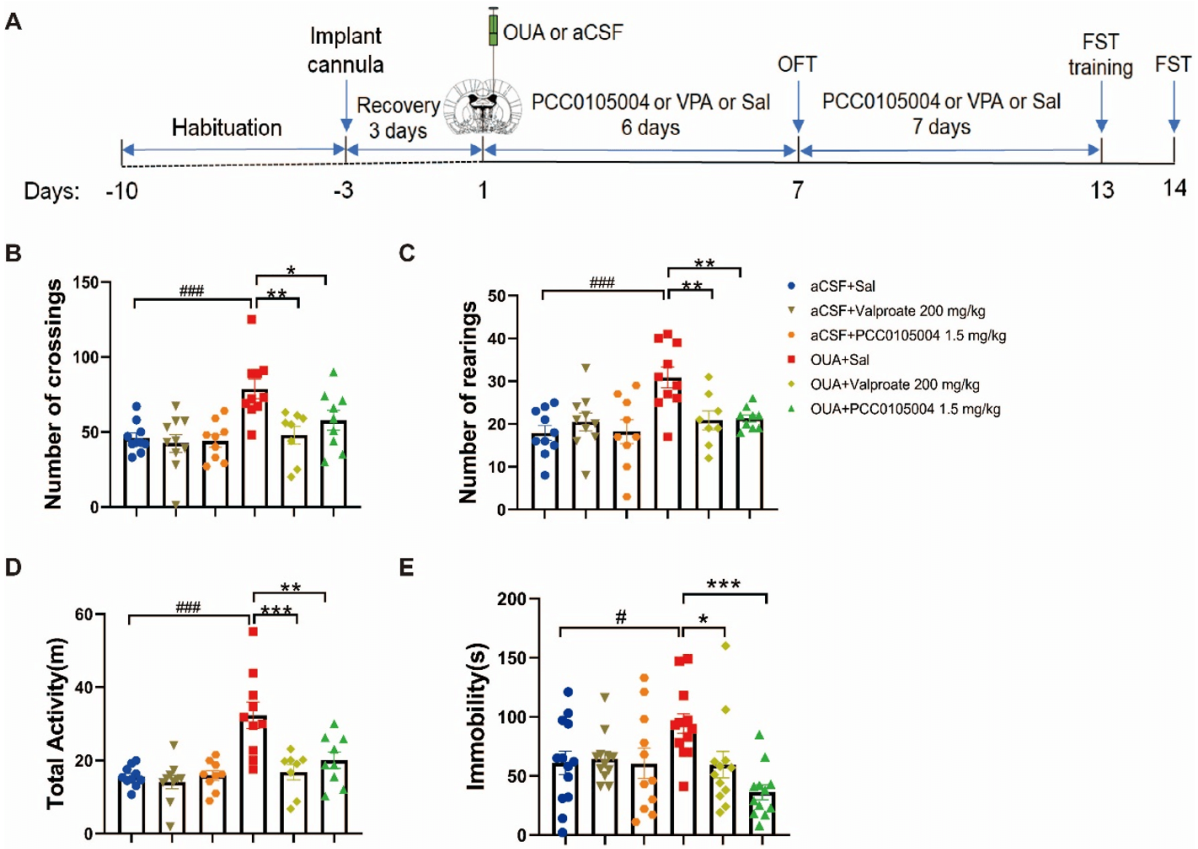

Figure 8 Mood-stabilizing effect of PCC0105004 in OUA-induced BD rats. Scheme illustrating the experimental design used in the study of maintenance therapy in the manic phase and maintenance therapy in the depressive phase (A). Effects of 200 mg/kg valproate or 1.5 mg/kg PCC0105004 for 6 days of continuous dosing on the number of crossings (B) and rearings (C) and total activity (D) in the OFT on the day 7 in animals treated with OUA. Effects of 200 mg/kg valproate or 1.5 mg/kg PCC0105004 for 13 days of continuous dosing on the time of immobility (E) on the day 14 in animals treated with OUA. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean ± SEM, n = 10 per group. $^{\#}p < 0.05$ $^{\#\#\#}p < 0.001$ compared to aCSF+Sal group. $^{*}p < 0.05$ $^{**}p < 0.01$ $^{***}p < 0.001$ compared to OUA +Sal group.

3.7 Effects of PCC0105004 on oxidative stress in the prefrontal cortex of OUA-induced BD rats

To demonstrate alterations in oxidative stress in the prefrontal cortex of BD rats, we assessed the levels of protein carbonyl content, MDA and GPx. During the manic and depression phases, compared with the aCSF + Sal group, protein carbonyl content (Figure 9A and 9D), MDA activity (Figure 9B and 9E) and GPx activity (Figure 9C and 9F) in the prefrontal cortex of BD rats were all significantly increased. However, these changes were effectively reversed by treatment with 1.5 mg/kg PCC0105004.

3.8 Effects of PCC0105004 on the TAAR1/Akt/GSK-3β/BDNF pathway in the prefrontal cortex of OUA-induced BD rats

As PCC0105004 was a TAAR1 agonist, we subsequently examined its effects on the TAAR1 signaling pathways through western blot analysis in the prefrontal cortex. This assessment included the expression levels of TAAR1 and its downstream molecules including BDNF, phosphorylated AKT at Ser473 and GSK3β at Ser9. During the manic and depression phases, in comparison to the aCSF + Sal group, the expression of TAAR1 (Figure 10A and 10E), BDNF (Figure 10B and 10F) and phosphorylated AKT at Ser473 (Figure 10C and 10G), as well as GSK3β at Ser9 (Figure 10D and 10H), in the prefrontal cortex of BD rats were all significantly downregulated. However, these changes were significantly reversed with 1.5 mg/kg PCC0105004 treatment.

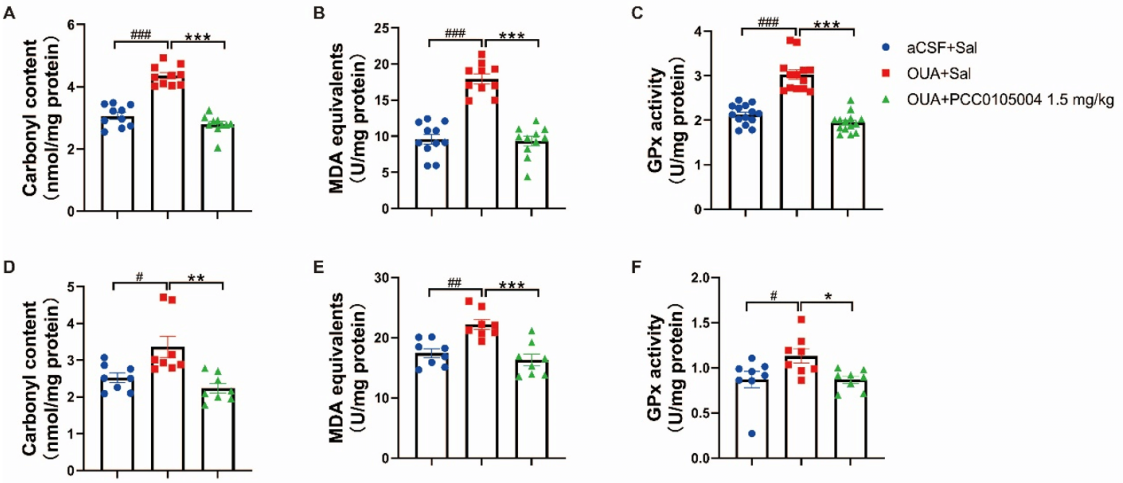

Figure 9 Effects of PCC0105004 on the oxidative stress in the prefrontal cortex of OUA-induced BD rats. Effects of administration of PCC0105004 1.5 mg/kg on the levels of protein carbonyl (A) and the activity of MDA (B) and glutathione peroxidase (C) in the prefrontal cortex of rats after injection of OUA for 7 days(n = 10-12 per group). Effects of PCC0105004 treatment on the levels of protein carbonyl (D) and the activity of MDA (E) and glutathione peroxidase (F) in the prefrontal cortex of rats after injecting with OUA for 14 days (n = 8 per group). Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean ± SEM. $^{\#}p < 0.05$ $^{\#\#}p < 0.01$ $^{\#\#\#}p < 0.001$ compared to aCSF+ Sal group. $^{*}p < 0.05$ $^{**}p < 0.01$ $^{***}p < 0.001$ compared to OUA+ Sal group.

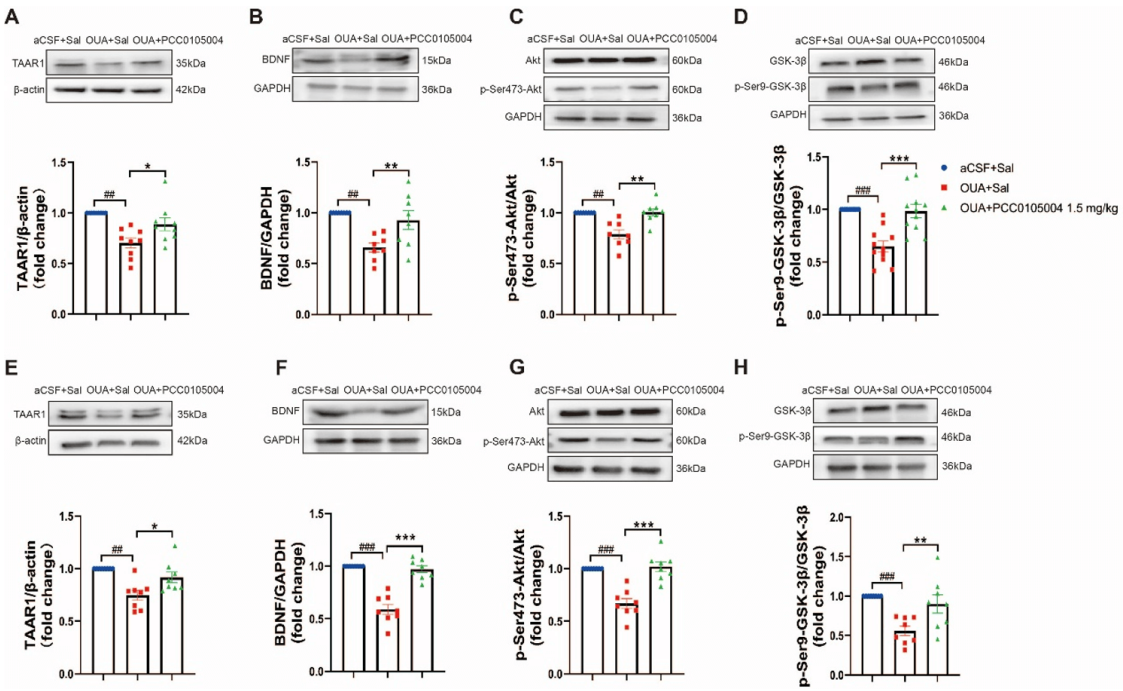

Figure 10 Effects of PCC0105004 on the TAAR1/BDNF and TAAR1/Akt/GSK-3β pathway in the prefrontal cortex of OUA-induced BD rats. The representative western blot level of TAAR1 (A), BDNF (B), p-Ser473-Akt/Akt (C) and p-Ser9- GSK-3β/ GSK-3β (D) in the prefrontal cortex of rats after injecting with OUA for 7 days. The representative western blot level of TAAR1 (E), BDNF (F), p-Ser473-Akt/Akt (G) and p-Ser9-GSK-3β/ GSK-3β (H) in the prefrontal cortex of rats after injecting with OUA for 14 days. Data were quantified by densitometric analysis and expressed as percentage of value of aCSF+ Sal group. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean ± SEM, n = 8-10 per group. $^{\#\#}p < 0.01$ $^{\#\#\#}p < 0.001$ compared to aCSF+ Sal group. $^{*}p < 0.05$ $^{**}p < 0.01$ $^{***}p < 0.001$ compared to OUA+ Sal group.

4 Discussion

BD is a challenging clinical condition and currently the available treatments only have limited efficacy but have various adverse effects. In an effort to explore novel drug targets for the development of new therapies of BD, we focus on TAAR1 in this study, which is a novel GPCR that may be involved in BD pathophysiology [13]. Here, we report that, using a rat OUA-induced BD model, we observed a reduced TAAR1 receptor expression in the model rats, suggesting the direct involvement of TAAR1 in BD. Then, we reported a small molecule TAAR1 agonist, PCC0105004, which showed high efficacy, good potency (EC_{50} value of 0.06182 μM), good receptor selectivity and favorable pharmacokinetics. In the in vivo efficacy study, PCC0105004 demonstrated clear efficacy against both the manic-like and depressive-like behaviors in the OUA-induced rats at the doses that did not affect general locomotion. In an effort to decipher the mechanistic underpinnings of PCC0105004 effects, we found that PCC0105004 alleviated oxidative stress possibly via the up-regulation of the Akt/GSK3β/BDNF pathway. Combined, these results revealed the important role of TAAR1 in BD and suggested that TAAR1 agonists such as PCC0105004 have the potential as novel pharmacotherapies for BD. A schematic figure depicting the potential mechanisms was provided in Figure 11.

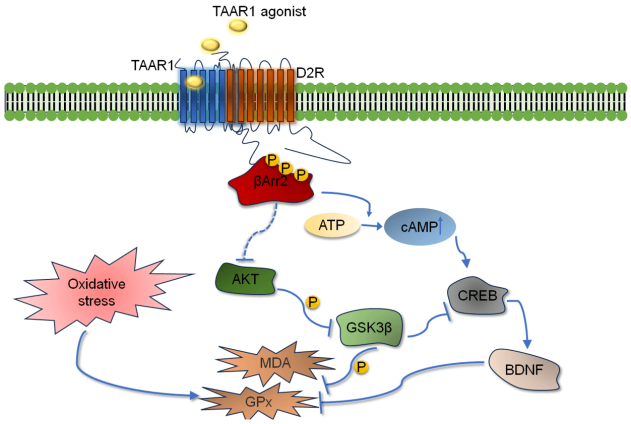

Figure 11 A schematic figure depicts proposed mechanism of oxidative stress modulation by TAAR1/Akt/GSK3β/BDNF signaling pathway

Evidence suggests that sodium and potassium-activated adenosine triphosphatase (Na^+, K^+-ATPase) is involved in BD pathophysiology [14]. OUA is a digitalis-like compound that inhibits Na^+, K^+-ATPase activity [15–17]. ICV administration of OUA in rats leads to a behavioral phenotype that mimics specific manic and depressive-like symptoms and consequently this model has been proposed as one of the best to mimick human BD [18, 19]. Therefore, to test our hypothesis that TAAR1 is involved in the pathophysiology of BD, we first established an OUA-induced model of BD, and examined the TAAR1 protein expression levels in the prefrontal cortex and hippocampus. Our results were consistent with the literature [20] which showed that OUA-induced BD rats exhibited manic-like symptoms on day 7 and depressive-like symptoms on day 14. Western blotting analyses showed that TAAR1 expression was down-regulated in both manic-like and depressive-like phases in the prefrontal cortex, respectively. Taken together, these results suggest that TAAR1 may be involved in the pathophysiology of BD.

We then characterized a small molecule TAAR1 agonist, PCC0105004 [(S)-4,7,8,9,10,10a-hexahydro-5H-thieno[2',3':3,4]pyrido[1,2-a]pyrazine] in both in vitro and in vivo assays. PCC0105004 demonstrated a strong agonistic activity in cells expressing TAAR1 with an estimulated EC_{50} of 0.06182 μM. Subsequent pharmacokinetics study showed that PCC0105004 had good in vivo pharmacokinetics prolife and blood-brain barrier permeability. In vitro counter-selectivity assay showed that PCC0105004 was highly selective against a broad panel of enzymes and receptors in a pan-screen assay. These results support further evaluation of PCC0105004 as a TAAR1 agonist.

One confounding factor for evaluating the in vivo efficacy of compounds using behavioral animal models is its behavioral specificity. In models wherein the suppression of the expression of certain behaviors is anticipated evidence of efficacy, general behavioral inhibition such as significant sedation will lead to difficulty in interpretating the behavioral data. Therefore, we evaluated the potential sedative effects of PCC0105004 both in normal rats and in BD rats. PCC0105004 at the dose of 1.5 mg/kg displayed specific antimanic-like effects without affecting

the general spontaneous activity in normal rats. Thus, the antimanic-like effects of PCC0105004 was behaviorally-specific. Similar results were also observed in the FST study where 1.5 mg/kg PCC0105004 reduced the immobility time in OUA-induced BD rats. As such, these results suggest that the antimanic-like and anti-depressant-like effects of PCC0105004 in BD rats were behaviorally specific. Additionally, PCC0105004 also demonstrated mood-stabilizing effect similar to the classical mood stabilizer valproate. Together, these results suggest that PCC0105004 has the potential to specifically treat BD-related symptoms.

Oxidative stress is known to be associated with the pathophysiology of BD and alterations in the activity of antioxidant enzymes and other oxidative disturbances have been reported in BD patients [21]. The oxidative stress occurs when the levels of reactive oxygen species (ROS) exceed the capacity of the antioxidant defense mechanisms, which may lead to protein oxidation, lipids peroxidation, and cell death. It was previously shown that, in OUD-induced BD model, the manic-like behavior and depressive-like behavior were accompanied by increased oxidative stress [20, 22]. Here, we found that the levels of protein carbonyl content, MDA, and GPx increased in OUA-induced BD rats, consistent with the literature [20]. Additionally, PCC0105004 attenuated OUA-induced increases in protein carbonyl content, MDA and GPx activity. Thus, PCC0105004-induced relief of oxidative stress may be the underlying mechanism attributing to its antimanic and antidepressant-like effects.

TAAR1 is the primary target of trace amines such as pTyr and β-phenylethylamine (β-PEA), which are endogenous amines closely related to classical monoamines such as dopamine and serotonin [23]. Emerging evidence suggests that TAAR1 is a promising target for the treatment of neuropsychiatric disorders such as schizophrenia, depression, and substance abuse [8, 24–31]. Although the exact molecular mechanisms of TAAR1-assocaited signaling remains to be elucidated, TAAR1 interacts with and modulates other GPCRs by the formation of heterodimers. Putative partners include dopamine, serotonin, adrenergic and glutamate receptors, although the exact machinery mechanisms of such interactions remain elusive [32]. TAAR1 reportedly interacts with dopamine D2 receptor, resulting in decreased cAMP signaling and enhanced β-arrestin2 (βArr2) signaling. This results in the formation of βArr2-dependent cascade which includes the phosphorylation of Akt and subsequent inactivation of GSK3β signaling by its phosphorylation. This signaling pathway is particularly relevant because it was shown that the dopamine signaling/Akt/GSK3β phosphorylation was inhibited in BD [32]. BD patients were found to have lower serum levels of BDNF [33–35]. The literature demonstrates the involvement of neurotrophic factors in BD pathophysiology since they regulate neurotransmitter release and neuronal excitability [36, 37]. Recent studies show that TAAR1 activation increases the production of BDNF both in vivo [38] and ex vivo, which was abolished by TAAR1 antagonism [39]. GSK-3β inactivates cAMP response element-binding protein (CREB) by phosphorylation [40]. CREB, a regulator of BDNF expression, can directly regulate BDNF transcription [41]. Previous studies have shown that GSK-3β plays an essential role in oxidative stress [42–46]. Decreased BDNF can also cause oxidative stress, providing another way through which it may drive changes associated with BD [47]. In this study, we found that the expression of TAAR1 protein in the prefrontal cortex of BD rats was down-regulated during the manic and depressive phases, which were associated with AKT/GSK3β/BDNF signaling pathways. These changes were reversed by PCC0105004 treatment. These findings suggest that the effect of PCC0105004 in the treatment of BD may be achieved by reducing oxidative stress through the TAAR1/Akt/GSK3β/BDNF pathways.

In conclusion, this study is the first to describe reduced TAAR1 expression in the prefrontal cortex of OUA-induced BD rats and the first to demonstrate the antimanic-like and antidepresssive-like activities of a TAAR1 agonist. Combined with the favorable PK profiles of PCC0105004, these results suggest that PCC0105004 may represent a novel candidate with new mechanisms of action to treat BD and warrant further investigations.

Acknowledgements

We sincerely thank the school of Pharmacy, Key Laboratory of Molecular Pharmacology and Drug Evaluation Yantai University, for their support during the study.

Author contributions

The main idea of this study was from Chunmei Li and Jingwei Tian. Linyao Yu, Wei Zhang, Yaoqin Shi, Yingtian Zhang, Min Xu and Yang Xu conducted the experiments. Linyao Yu, Wei Zhang, and Chunmei Li designed the study, conducted the data analysis. Linyao Yu and

Chunmei Li wrote the first draft of the manuscript. Jingwei Tian revised the content of the paper. All authors contributed to and approved the final version of the manuscript.

Funding

This work was supported by the Natural Science Foundation of Shandong Province (No. ZR2020MH411).

Data availability

The data presented in this study are available on request from the corresponding authors.

Conflict of interests

The authors have no conflicts of interest to declare in relation to this work.

Ethical approval

All experimental procedures in this study were conducted in accordance with the National Institutes of Health Guidelines for Care and Use of Laboratory Animals and all animal protocols were approved by the Laboratory Animals Care and Use Committee of Yantai University (registration number is YTU20220329) (Yantai, China). Efforts were made to minimize the number of animals used and to limit their suffering.

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