Effects of Rhynchophylline on Behavior and Mechanisms in Methamphetamine-Dependent Zebrafish (Postprint)

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Abstract

Objective: To observe the effects of rhynchophylline on methamphetamine-induced conditioned place preference in zebrafish and explore the underlying mechanisms. Methods: Experimental groups comprised a blank control group, a methamphetamine model group, a model + low-dose rhynchophylline group (50 mg/kg), a model + high-dose rhynchophylline group (100 mg/kg), and a model + ketamine group (150 mg/kg). A methamphetamine-induced zebrafish CPP model was established via conditioned place preference training. The Noldus Ethovision XT system was employed to measure zebrafish residence time in the non-preferred compartment (drug-paired compartment) and movement trajectories within the CPP apparatus. Western blotting was utilized to detect the expression of NR2B, TH, and GLUR2 proteins in zebrafish brain tissue. Results: Compared with the blank group, the model group exhibited significant differences in changes of residence time in the drug-paired compartment before and after training, as well as in locomotor distance ($P<0.05$). The OD values for NR2B, TH, and GLUR2 protein expression also differed significantly ($P<0.05$). Compared with the model group, the high-dose rhynchophylline group showed significant differences in changes of residence time in the drug-paired compartment and locomotor distance ($P<0.05$), with significant differences also observed in the OD values for NR2B, TH, and GLUR2 protein expression ($P<0.05$). Conclusion: Rhynchophylline demonstrates an inhibitory effect on methamphetamine dependence in zebrafish, and its mechanism is associated with modulating the expression of TH, NR2B, and GLUR2 proteins in the brain.

Full Text

Preamble

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Abstract

Objective: To investigate the effect of rhynchophylline on methamphetamine-induced conditioned place preference (CPP) in zebrafish and explore its underlying mechanisms. **Methods:** Zebrafish were divided into five groups: blank control, methamphetamine model, model + low-dose rhynchophylline (50 mg/kg), model + high-dose rhynchophylline (100 mg/kg), and model + ketamine (150 mg/kg). A methamphetamine-induced CPP model was established using conditioned place preference training. The Noldus Ethovision XT system was used to measure the time spent in the drug-paired compartment (non-preferred side) and to track movement patterns in the CPP apparatus. Western blotting was employed to detect the expression of three proteins—NR2B, TH, and GLUR2—in zebrafish brain tissue. **Results:** Compared with the control group, the model group exhibited significant differences in both the change in residence time in the drug-paired compartment before and after training and in locomotor distance ($P<0.05$), along with significant alterations in the optical density values of NR2B, TH, and GLUR2 protein expression ($P<0.05$). High-dose rhynchophylline treatment significantly reversed these behavioral changes and reduced protein expression differences compared with the model group ($P<0.05$). **Conclusion:** Rhynchophylline demonstrates inhibitory effects on methamphetamine dependence in zebrafish, likely through modulation of TH, NR2B, and GLUR2 protein expression in the brain.

Keywords: zebrafish; rhynchophylline; methamphetamine; drug dependence; conditioned place preference

Introduction

Methamphetamine (MA) is a widely abused psychostimulant that produces powerful central nervous system excitation. Chronic use leads to profound psychological dependence and significant damage to the central nervous system, with withdrawal symptoms including depression, anxiety, and anhedonia. Research has demonstrated that MA exerts notable neurotoxic effects on dopaminergic neurons in both animals and humans [1-2]. The underlying toxic mechanisms involve multiple pathways: disruption of dopamine signal transduction and dopamine oxidation [3-4], glutamate-mediated excitotoxicity [5], oxidative stress and cytokine formation [6-7], mitochondrial dysfunction with induction

of neuronal apoptosis [8], and activation of glial cells [4]. Tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, serves as a reliable marker for changes in dopamine neurotransmission [9].

Our previous studies have shown that in rats with MA-induced CPP, expression of the N-methyl-D-aspartate (NMDA) receptor subunit in brain glutamate systems is significantly enhanced [10-11]. Additionally, AMPA receptor expression increases in the nucleus accumbens but decreases in the hypothalamus of CPP rats.

Currently, no highly effective pharmacological treatments exist for amphetamine-type stimulant dependence. Rhynchophylline, the principal active component of *Uncaria rhynchophylla* (Miq) Miq. ex Havil., is commonly used in traditional medicine for treating drug addiction. Our prior work has demonstrated that rhynchophylline effectively antagonizes amphetamine-induced place preference in rats, suppresses MA-induced upregulation of NR2B protein expression, and normalizes GLUR2/3 expression levels in MA-dependent rat brains [10-12]. These findings suggest that rhynchophylline represents a promising traditional Chinese medicine-derived compound for treating amphetamine-type substance dependence.

While rhynchophylline' s effects on drug dependence have been extensively studied in rodent models, its impact on zebrafish models remains unreported. Zebrafish are emerging as a valuable model organism in addiction research. In this study, we employed our previously established zebrafish CPP model [13] to examine rhynchophylline' s effects on MA-induced place preference formation, characterize behavioral alterations in MA-dependent zebrafish, and investigate changes in NR2B, TH, and GLUR2 protein expression in the zebrafish brain, thereby providing deeper insights into MA-induced dependence and the therapeutic potential of this active herbal component.

Methods

Reagents and Materials

Methamphetamine hydrochloride was obtained from the National Narcotics Laboratory (Batch No. 1212-9802). Rhynchophylline was purchased from Wako Pure Chemical Industries, Ltd. (Batch No. 184-01931). Tricaine methanesulfonate (MS222) was acquired from Sigma. Anti-NR2B (AB1557P) and Anti-GLUR2 (MAB397) antibodies were sourced from Millipore. Magic Mark (LC5602) was obtained from Invitrogen. Ketamine hydrochloride injection was purchased from Jiangsu Hengrui Medicine Co., Ltd. (Batch No. KH090701). Fish physiological saline was prepared in-house.

Experimental Animals

Wild-type AB strain male zebrafish were provided by the Zebrafish Experimental Center of Southern Medical University. Fish were 3–6 months old and

weighed 0.5–1.0 g. The aquatic system (Beijing Aisheng Company) maintained water temperature at 28.5–29.5°C, salinity at 0.03%–0.04%, and pH at 7.2–7.6. The light cycle was controlled at 14 h light (8:30 am–10:30 pm) and 10 h dark.

Experimental Apparatus

The conditioned place preference tank [13] measured 16 cm × 9 cm × 9 cm and featured a removable partition dividing it into two equal compartments. One side was brown-colored, while the other was transparent with two 2-cm-diameter black dots on the bottom. The Noldus Ethovision XT system (Noldus Information Technology, Netherlands, http://www.noldus.com) was used for behavioral tracking and analysis.

Establishment of Methamphetamine-Induced CPP Model and Behavioral Testing

Animal Grouping: Based on previous literature [13] and pilot experiments, adult zebrafish naturally prefer the brown compartment; therefore, the transparent side was designated as the drug-paired compartment. Prior to drug administration, baseline place preference was assessed using the behavioral analysis system, and animals not conforming to natural preference patterns were excluded. For testing, the partition was removed and individual fish were observed for 15 minutes, with residence time in the white compartment recorded when the fish' s head entered the area. Fifty zebrafish meeting the natural preference criteria were randomly assigned to five groups (n=10 each): (1) blank control, (2) methamphetamine model, (3) model + low-dose rhynchophylline (50 mg/kg), (4) model + high-dose rhynchophylline (100 mg/kg), and (5) model + ketamine (150 mg/kg).

CPP Model Establishment: The complete protocol spanned nine days. (1) **Baseline measurement:** Zebrafish were placed individually in CPP training tanks with water depth 5 cm to ensure adequate hydrostatic pressure and acclimated for two days. On day 3, baseline place preference was tested (15-minute observation), and movement patterns were tracked for 5 minutes using Noldus Ethovision XT software. (2) **Training sessions:** On days 4, 6, and 8, all groups except the blank control were lightly anesthetized in 200 mg/L MS222 solution and intraperitoneally injected with methamphetamine (40 g/g body weight) before being confined to the non-preferred (drug-paired) compartment for 45 minutes. A transparent barrier prevented movement between compartments while allowing visual contact. After 45 minutes, fish were transferred to larger blue-environment tanks, and the black/white compartments were cleaned with 70% ethanol followed by system water rinse. Blank control fish received equivalent volumes of fish physiological saline (10 L/fish) following the same anesthesia procedure. (3) **Saline sessions:** On days 5 and 7, all groups received intraperitoneal saline injections (10 L/fish) and were placed in the preferred (non-drug-paired) compartment for 45 minutes, following identical procedures. (4) **Testing:** Twenty-four hours after the final injection (day 9), the Noldus

Ethovision XT system measured residence time in the drug-paired compartment and tracked movement patterns, comparing pre- and post-training differences.

Drug Administration: Twelve hours after daily methamphetamine or saline training sessions, treatment groups were lightly anesthetized with MS222 and intraperitoneally injected with respective drug solutions (low-dose rhynchophylline 50 mg/kg, high-dose rhynchophylline 100 mg/kg, or ketamine 150 mg/kg) before transfer to blue-environment tanks. Blank control and model groups received equivalent volumes of fish physiological saline (10 L/fish) following identical procedures for five consecutive days.

Protein Expression Analysis of NR2B, TH, and GLUR2 in Zebrafish Brain

Following behavioral testing, zebrafish were euthanized, brains were dissected, homogenized, and proteins were extracted. Protein concentrations were determined, and Western blotting was performed to detect NR2B, TH, and GLUR2 expression levels, with -actin serving as the internal control.

Results

Behavioral Analysis of Methamphetamine-Induced CPP

The model group exhibited a significant difference in residence time change in the drug-paired compartment before and after training compared with the control group ($P<0.01$), confirming robust conditioned place preference formation. High-dose rhynchophylline and ketamine groups showed significantly reduced time differences compared with the model group ($P<0.05$), whereas low-dose rhynchophylline produced no significant effect ($P>0.05$) [Figure 1: see original paper]. Similarly, locomotor distance in the drug-paired compartment differed significantly between the model and control groups post-training ($P<0.01$). High-dose rhynchophylline and ketamine significantly attenuated this increase ($P<0.05$), while low-dose rhynchophylline remained ineffective ($P>0.05$) [Figure 2: see original paper]. Representative movement trajectories in the CPP apparatus are shown in [Figure 3: see original paper].

Protein Expression of NR2B, TH, and GLUR2 in Zebrafish Brain

Western blotting analysis revealed significantly elevated expression of TH, NR2B, and GLUR2 in the model group compared with controls ($P<0.05$). High-dose rhynchophylline and ketamine significantly reduced these protein levels ($P<0.05$ or $P<0.01$), whereas low-dose rhynchophylline showed no significant effect ($P>0.05$) [Figure 4: see original paper], [Figure 5: see original paper].

Discussion

Zebrafish share remarkable developmental similarities with humans, with approximately 85% genetic homology in blood, visceral, visual, and central nervous systems. Their high reproductive capacity, embryonic transparency, rapid growth, and genetic similarity make them increasingly attractive to biologists. Internationally, researchers have successfully employed zebrafish to study drug dependence withdrawal symptoms and addiction-induced conditioned place preference, validating their utility in addiction medicine. Caffeine, ethanol, morphine, and diazepam have all been shown to produce addiction and withdrawal behaviors in zebrafish, affecting both behavior and endocrine function [14-15]. Bretaud et al. [16] demonstrated that morphine-pre-exposed larvae exhibited significantly increased residence time in morphine-containing water, an effect reducible by opioid or dopamine receptor antagonist pretreatment. Our group previously established a methamphetamine-induced zebrafish CPP model [13], which we utilized here to examine rhynchophylline' s effects. The significant increases in drug-paired compartment residence time and locomotor distance in MA-treated zebrafish indicate strong conditioned place preference formation. High-dose rhynchophylline and ketamine markedly reduced these behavioral indices, suggesting effective inhibition of MA-induced place preference. Low-dose rhynchophylline showed minimal effect. These findings demonstrate the applicability of the MA-induced zebrafish CPP model for investigating traditional Chinese medicine-based addiction interventions.

The mesolimbic dopamine system (MLDS), endogenous opioid system, and GABA/glutamate neural networks are critically involved in drug abuse and psychological dependence. Key neurotransmitters including dopamine [17], glutamate, and serotonin [18] play essential roles in addiction development. Glutamate receptors (GluRs), particularly NMDA and AMPA receptors, are abundantly expressed in the brain and intimately associated with drug dependence [19]. AMPA receptors comprise four subunits (GluR1-4), with GluR2/3 heteromers predominating in the central nervous system [20]. These receptors are crucial for neurodevelopment, signal transduction, and synaptic plasticity underlying learning and memory. NMDA receptors are ligand-gated cation channels formed by essential NR1 subunits and at least one NR2 regulatory subunit. Kato et al. [21] reported that NR2B phosphorylation plays a critical role in morphine-induced reward, with tyrosine kinases such as Src family kinases enhancing NMDA receptor function through increased tyrosine phosphorylation of NR2B C-terminal residues. As a key central nervous system neurotransmitter, dopamine influences voluntary movement, hormone secretion, and emotional regulation. Tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, serves as a marker for dopaminergic neurons and reflects dopamine content [22].

We selected NR2B, GLUR2, and TH as biochemical markers relevant to methamphetamine dependence to elucidate the associated neurochemical mechanisms. Our results show significantly enhanced expression of TH, NR2B, and GLUR2

in MA-treated zebrafish brains, suggesting that dependence formation may involve upregulation of these proteins. High-dose rhynchophylline markedly reduced their expression, while low-dose treatment showed minimal effect. These biochemical findings align with the behavioral results, indicating that rhynchophylline inhibits methamphetamine dependence, possibly by modulating TH, NR2B, and GLUR2 expression, thereby providing experimental support for its therapeutic potential.

Ketamine, a non-competitive NMDA receptor antagonist, served as a positive control. At 150 mg/kg, ketamine significantly suppressed MA-induced place preference and attenuated the associated increase in TH, NR2B, and GLUR2 expression. While various NMDA receptor antagonists can inhibit reward effects of addictive substances like morphine, ketamine's own potential for psychological dependence and abuse complicates interpretation of its anti-addiction properties, necessitating further investigation.

References

[1] El Ayadi A, Zigmond MJ. Low concentrations of methamphetamine can protect dopaminergic cells against a larger oxidative stress injury: mechanistic study[J]. PLoS One, 2011, 6(10): e24722.

[2] Shin EJ, Bach JH, Nguyen TT, et al. Gastrodia elata bl attenuates methamphetamine-induced dopaminergic toxicity via inhibiting oxidative burdens[J]. Curr Neuropharmacol, 2011, 9(1): 118-21.

[3] Karila L, Weinstein A, Aubin HJ, et al. Pharmacological approaches to methamphetamine dependence: a focused review[J]. Br J Clin Pharmacol, 2010, 69(6): 578-92.

[4] Abdul MM, Salikunju S, Szlatchetka A, et al. Methamphetamine inhibits the glucose uptake by human neurons and astrocytes: stabilization by acetyl-L-carnitine[J]. PLoS One, 2011, 6(4): e19258.

[5] Izquierdo A, Belcher AM, Scott L, et al. Reversal-specific learning impairments after a binge regimen of methamphetamine in rats: possible involvement of striatal dopamine[J]. Neuropsychopharmacology, 2010, 35(2): 505-14.

[6] Hozumi H, Asanuma M, Miyazaki I, et al. Protective effects of interferon-gamma against methamphetamine-induced neurotoxicity[J]. J Pharmacol Sci, 2008, 106(1): 175P.

[7] Zhang X, Dong F, Mayer GE, et al. Selective inhibition of cyclooxygenase-2 exacerbates methamphetamine-induced dopamine depletion in the striatum in rats[J]. Neuroscience, 2007, 150(4): 950-8.

[8] Kanthasamy K, Gordon R, Jin HJ, et al. Neuroprotective effect of resveratrol against methamphetamine-induced dopaminergic apoptotic cell death in a cell culture model of neurotoxicity[J]. Curr Neuropharmacol, 2011, 9(1): 49-53.

[9] Jing XU, LI Yan, XF Zeng, et al. Dopaminergic toxicity in related brain areas of rats after methamphetamine intoxication[J]. Progress in Modern Biomedicine, 2011, 11(12): 2230-3.

[10] Liu W, QX Peng, X L Lin, et al. Effect of rhynchophylline on the expression of p-CREB and c-Fos in striatum and hippocampal CA1 area of methamphetamine-induced conditioned place preference rats[J]. Fitoterapia, 2014, 92(3): 16-22.

[11] Li JK, Liu W, Peng QX, et al. Effect of rhynchophylline on conditioned place preference expression of NR2B in methamphetamine-dependent mice[J]. Biochem Biophys Res Commun, 2014, 452(3): 695-700.

[12] Zhou JY, Mo ZX, Zhou SW. Effect of rhynchophylline on central neurotransmitter levels in amphetamine-induced conditioned place preference rat brain[J]. Fitoterapia, 2010, 81(7): 844-8.

[13] 陈毅飞, 翁建霖, 张文清, 等. 甲基苯丙胺诱导的斑马鱼位置偏爱模型研究 [J]. 中华行为医学与脑科学杂志, 2011, 20(9): 772-4.

[14] Cachat J, Canavello P, Elegante M, et al. Modeling withdrawal syndrome in zebrafish[J]. Behav Brain Res, 2010, 208(2): 371-6.

[15] Tran S, Chatterjee D, Gerlai R. An integrative analysis of ethanol tolerance and withdrawal in zebrafish (Danio rerio)[J]. Behav Brain Res, 2015, 276(SI): 161-70.

[16] Lau B, Bretaud S, Huang Y, et al. Dissociation of food and opiate preference by a genetic mutation in zebrafish[J]. Genes Brain Behav, 2006, 5(7): 497-505.

[17] Parsegian A, See RE. Dysregulation of dopamine and glutamate release in the prefrontal cortex and nucleus accumbens following methamphetamine self-administration and during reinstatement in rats[J]. Neuropsychopharmacology, 2014, 39(4): 811-22.

[18] Thomas DM, Perez MA, Francescutti-Verbeem DM, et al. The role of endogenous serotonin in methamphetamine-induced neurotoxicity to dopamine nerve endings of the striatum[J]. J Neurochem, 2010, 115(3): 605-605.

[19] Yuan J, Darvas M, Sotak B, et al. Dopamine is not essential for the development of methamphetamine-induced neurotoxicity[J]. J Neurochem, 2010, 114(4): 1135-42.

[20] 周吉银, 莫志贤. 谷氨酸和受体与药物依赖相关的研究进展 [J]. 中国药物依赖性杂志, 2006, 15(4): 255-9.

[21] Kato H, Narita M, Suzuki M, et al. Role of tyrosine kinase-dependent phosphorylation of NR2B subunit-containing NMDA receptor in morphine reward[J]. Nihon Arukoru Yakubutsu Igakkai Zasshi, 2007, 42(1): 13-20.

[22] Volkoff H. The effects of amphetamine injections on feeding behavior and the brain expression of orexin, CART, tyrosine hydroxylase (TH) and thyrotropin re-

leasing hormone (TRH) in goldfish (Carassius auratus)[J]. Fish Physiol Biochem, 2013, 39(4): 979-91.

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Note: Figure translations are in progress. See original paper for figures.