

REGULAR RESEARCH ARTICLE

A Novel Synthetic Interfering Peptide Tat-3L4F Attenuates Olanzapine-Induced Weight Gain Through Disrupting Crosstalk Between Serotonin Receptor 2C and Protein Phosphatase and Tensin Homolog in Rats

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ABSTRACT

Background: Accompanied with profound efficacy, atypical antipsychotics (AAPs) contribute to metabolic adverse effects with few effective strategies to attenuate. Serotonin 5-HT_{2C} receptor (HTR_{2C}) plays a critical role in hyperphagia and weight gain induced by AAPs, and expression of phosphatase tensin homolog (PTEN) in the hypothalamus also affects feeding behavior and weight change. Moreover, PTEN has a physical crosstalk between PTEN and a region in the third intracellular loop (3L4F) of the HTR_{2C}. Tat-3L4F has the property to disrupt crosstalk between PTEN and HTR_{2C}. This is the first study to our knowledge to investigate the effect of Tat-3L4F on olanzapine-induced metabolic abnormalities and PTEN/ phosphatidylinositol 3-kinase/ protein kinase B expression in the hypothalamus in rats.

Methods: The effects of Tat-3L4F were investigated through measuring body weight, food intake, and blood glucose. In addition, PTEN/phosphatidylinositol 3-kinase/protein kinase B level in the hypothalamus was detected by immunofluorescence assay and western blot. Metabolites in the liver tissue were detected by liquid chromatography-mass spectrometry and analyzed by multivariate analyses and pairwise comparison.

Results: Our results showed that hyperphagia and weight gain were evident in the olanzapine alone-fed rats but was attenuated after Tat-3L4F treatment. In addition, oral glucose tolerance test indicated blood glucose at 120 minutes was higher in the olanzapine alone-treated group than in groups treated with vehicle and olanzapine + Tat-3L4F (10 $\mu\text{mol kg}^{-1}$ per day). Furthermore, compared with olanzapine alone treatment, treatment with Tat-3L4F (10 $\mu\text{mol kg}^{-1}$ per day) significantly inhibited PTEN expression in the hypothalamus. The olanzapine alone-treated group had the highest bile acid level, followed by the olanzapine with Tat-3L4F (1 $\mu\text{mol kg}^{-1}$) group, olanzapine with Tat-3L4F (10 $\mu\text{mol kg}^{-1}$) group, and vehicle group.

Conclusions: Our present results reveal that Tat-3L4F is a potential pharmacological strategy for suppressing hyperphagia and weight gain induced by olanzapine, which acts through disrupting crosstalk between HTR_{2C} and PTEN as a result of PTEN downregulation in the hypothalamus.

Keywords: 5-HT_{2C} receptors, metabolic disorder, olanzapine, PTEN

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Significance Statement

Blockade of HTR2C is known to be a critical mechanism of weight gain and hyperphagia induced by AAPs, and agonism on HTR2C contributes to suppressing weight gain and hyperphagia. However, the property, antagonism of HTR2C, of AAPs was needed for improving cognitive and negative symptoms in schizophrenia. Our results revealed Tat-3L4F (1 or 10 $\mu\text{mol kg}^{-1}$ per day) presented the potential of alleviating weight gain and hyperphagia induced by olanzapine through disruption of crosstalk between PTEN and HTR2C instead of directly binding to HTR2C. Furthermore, PTEN expression was significantly downregulated in the hypothalamus and food intake was suppressed after Tat-3L4F (10 $\mu\text{mol kg}^{-1}$ per day) treatment.

Introduction

Atypical antipsychotics (AAPs) have been widely prescribed to treat a variety of severe mental illnesses, notably schizophrenia and bipolar disorder (Meltzer, 1999; Keck et al., 2000). Despite the documented efficacy, AAPs have important detrimental effects, including weight gain, impaired glucose tolerance and new-onset diabetes, hyperlipidemia, and increased cardiovascular risk (Meyer et al., 2005; Henderson et al., 2015; Vancampfort et al., 2015). The beneficial psychotropic effects of AAPs may be attributed to lower levels of dopamine D_2 receptor occupancy and higher affinity for serotonin 2A receptors (Miyamoto et al., 2005). However, the molecular mechanisms underlying AAP-induced metabolic side effects are not fully understood (Henderson et al., 2015). There were various pharmacological interventions, including anti-diabetes agents (such as metformin, glucagon-like peptide-1 receptor agonists [GLP-1Ras], etc.), melatonin, topiramate, nizatidine, and other agents, to lower the risk of metabolic malfunctions secondary to AAPs (Mizuno et al., 2014; Siskind et al., 2019). Indeed, these drugs have a counteracting effect on antipsychotic-induced weight gain and other metabolic adversities; however, most of them exert pharmacological effects in the peripheral tissues. In addition, some of them have a weight loss effect through agonism on the receptor in the central nervous systems but were not specific to AAPs. For instance, lorcaserin, a selective 5-HT_{2C} receptor (HTR2C) agonist, can promote weight loss in mice treated with AAPs (Lord et al., 2017) and in high-fat diet-induced obese rats (Saravanan et al., 2014). In general, the mechanisms that underlie the metabolic malfunctions secondary to AAPs have not been fully understood, and thus it is necessary to investigate more about the mechanism and potential intervention.

Mounting evidence from animal studies has suggested the untoward metabolic effects induced by AAPs are mediated by multiple monoamine receptors, including the histamine H_1 receptors, α -adrenergic receptors, and serotonin (5-HT) 2C receptors (Bymaster et al., 1999; Berglund et al., 2013; Ikegami et al., 2013; Lord et al., 2017). Remarkably, among them, HTR2C has been proven to be a key mediator of weight gain induced by the atypical antipsychotic olanzapine (OLZ) (Lord et al., 2017), and blockade of HTR2C leads to hyperphagia (Tecott et al., 1995). In addition, lorcaserin could suppress OLZ-induced hyperphagia and weight gain (Lord et al., 2017). Therefore, agonism of HTR2C could present a weight loss effect (Bohula et al., 2018).

Tat-3L4F peptide consists of HIV Tat protein and 3L4F peptide derived from the third intracellular loop of the HTR2C (3L4F [third loop], fourth fragment) (Julius et al., 1988; Ji et al., 2006). Furthermore, it could traverse the blood-brain barrier to prevent phosphatase tensin homolog (PTEN)-mediated dephosphorylation of HTR2C in the central nervous system (Aarts et al., 2002; Ji et al., 2006). Initially, Tat-3L4F was observed to effectively suppress the rewarding effects induced

by drugs of abuse through suppression of the firing rate of dopaminergic neurons in the ventral tegmental area (Maillet et al., 2008). Recently, there is evidence supporting that Tat-3L4F can allosterically augment HTR2C-mediated signaling in live cells (Anastasio et al., 2013). Unlike the orthosteric agonist lorcaserin, which directly binds to the specific HTR2C domain as the endogenous agonist, the allosteric modulator Tat-3L4F of HTR2C binds to a site that is topographically different from the orthosteric binding site (Anastasio et al., 2013). Notably, Tat-3L4F is not directly involved in the neurotransmission process and hence usually does not induce a compensatory effect (Anastasio et al., 2013; Soto et al., 2017). Additionally, most AAPs have inverse agonist properties at HTR2C, which contributes to alleviate negative symptoms and cognitive impairment of schizophrenia patients (Sullivan et al., 2015). Is there a strategy to preserve HTR2C inverse agonism and meanwhile regulate HTR2C-mediated downstream signaling associated with metabolic side effects? On the basis of the above evidence, we developed a hypothesis that HTR2C inverse agonism of AAPs may trigger the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway via crosstalk with PTEN (Cai et al., 2013). To test peptide disruption of HTR2C coupling with PTEN as a putative protective mechanism against AAP (e.g., OLZ) induced metabolic side effects, we firstly examined the effect of Tat-3L4F on body weight change, feeding behavior, and blood glucose. To investigate whether Tat-3L4F could affect the downstream signaling pathway of HTR2C, we measured PTEN/PI3K/AKT signaling in the hypothalamus (closely relevant to feeding behavior) using western blot and immunofluorescence stain. To comprehensively screen differentially expressed metabolites in the peripheral tissues after treatment with Tat-3L4F, we used untargeted metabolomics to detect metabolic profiling of liver tissues.

MATERIALS AND METHODS

Experimental Animals

Female Sprague-Dawley rats (approximately 160–180 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The animals were individually housed in a temperature-controlled room with a 12-h-light/dark cycle (lights on 8 AM to 8 PM) at the Shanghai Key Laboratory of Psychotic Disorders at Shanghai Mental Health Center. Food consisting of 4% fat, 20.5% protein, and 50% carbohydrate from Puluteng Co., Ltd. (Shanghai, China) and water were provided ad libitum unless otherwise noted. Wire cages were used to feed animals. The spacing between the wires was approximately 3–4 mm and the diameter of the food particles was about 1 cm. All the experimental animals were acclimated to laboratory conditions for at least 1 week before individual experiments and

singly maintained for 1 week before drug administration. After acclimatization before singly maintained, rats were randomly assigned through a computer-generated table into 1 of the 6 treatments, including vehicle (saline), OLZ alone, co-treatment with vehicle and Tat-3L4F (1 $\mu\text{mol kg}^{-1}$ per day), co-treatment with vehicle and Tat-3L4F (10 $\mu\text{mol kg}^{-1}$ per day), co-treatment with OLZ and Tat-3L4F (1 $\mu\text{mol kg}^{-1}$ per day) and co-treatment with OLZ and Tat-3L4F (10 $\mu\text{mol kg}^{-1}$ per day), respectively. The number of rats in each study group was set at 10. The cages of each group were located in parallel in the metallic shelves of the individual ventilated cage system. All animal studies were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academies Press, 2011), the China National standards for Laboratory Animal Quality, and the Chinese Guidelines for Care and Use of Laboratory and Animals.

Drug Preparation and Administration

OLZ (Sigma-Aldrich, St. Louis, USA) powder was added to 0.9% saline. Before suctioning, an electronic mixer was used to mix the drug in the saline to form a relatively stable fluid suspension. In groups treated with OLZ alone and co-treatment with OLZ and Tat-3L4F (1 $\mu\text{mol kg}^{-1}$ per day or 10 $\mu\text{mol kg}^{-1}$ per day), 4 mg kg^{-1} per day OLZ was administered via gavage between 9:00 AM and 2:00 PM for 10 days. Tat-3L4F was synthesized by Nanjing Peptide Biotech Co., Ltd (Nanjing, China) according to the reported sequence (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Asn-Pro-Asp-Gln-Lys-Pro-Arg-Arg-Lys-Lys-Lys-Glu-Lys-Arg) (Ji et al., 2006); the reports of peptide synthesis are shown in Supplemental Material (supplemental Figures 3–5). Tat-3L4F peptide was supplied with a purity of >95% as acetate salt to reduce toxicity of residual trifluoroacetic acid in the initially synthesized peptide. Tat-3L4F solution was prepared by dissolving the peptide in 0.9% saline solution reaching the adequate concentration. Tat-3L4F (1 or 10 $\mu\text{mol kg}^{-1}$ per day) and vehicle (saline) was respectively injected i.p. within 1 hour before the dark phase (7:00 PM to 8:00 PM) for 10 days. The researchers were not blinded to intervention. When conducting drug administration, one prepared the drugs and calculated the administration volume according to the body weight and the other one (blinded to the study design) performed only the administration.

Body Weight

Body weight was monitored daily during the first day and 10th day of drug administration. One researcher caught the rat and put it on the electronic balance and the other one (blinded to the study design) recorded the value.

Food Consumption

Food intake was measured every 12 hours during the first day and 10th day of drug administration. One researcher put the remaining food on the electronic balance and the other one (blinded to the study design) recorded the value.

Oral Glucose Tolerance Test

For the oral glucose tolerance test (OGTT), all the rats were fasted for 12 hours with water provided ad libitum from 9 PM before the experimental day. Glucose level at baseline was measured 1 hour prior to OGTT. During OGTT, blood glucose levels at 15, 30, 60, and 120 minutes after 2 mg kg^{-1} glucose

administration via gavage were monitored using Contour Glucometer (Bayer Pharma AG). One researcher caught the rats and cut the tail and the other one (blinded to the study design) used the glucometer to measure blood glucose and recorded the value.

Liver Samples Collection, Pretreatment, and Untargeted Analysis by liquid chromatography-mass spectrometry

After completing 10 days of treatment, rats were perfused by phosphate buffered saline under isoflurane anesthesia. Liver samples of 10 rats from each of Veh-, OLZ-, OLZ+Tat1-, and OLZ+Tat10- groups were collected, washed with 0.9% saline solution, and immediately quenched in liquid nitrogen ($n=10$). Samples were stored at -80°C prior to analysis. Quality samples were pooled by mixing 10 μL of extraction mixture from different samples.

A high-resolution tandem mass spectrometer Triple TOF5600plus (SCIEX, California, USA) was used to detect metabolites. The Q-TOF operated in both positive and negative ion modes. An ACQUILITY UPLC HSS T3 Column (100 A, 1.8 μm , 2.1 mm \times 100 mm, Waters, Massachusetts, USA) was used for the reversed phase separation of the prepared samples. The mobile phase was composed of solvent A (water, 0.1% formic acid) and solvent B (acetonitrile, 0.1% formic acid). Detailed gradient elution conditions and the data analysis process are shown in the supplemental Material.

Western-Blot Analysis

Hypothalamus tissues were collected from each of the 6 groups ($n=3$). Hypothalamic tissues were homogenized and lysed by 100:1 radio-immune precipitation assay lysis buffer with protease inhibitor cocktail (Beyotime, China). Protein concentrations were measured with a BCA Protein Assay Kit (BIOTECH WELL, Shanghai, China). Protein extracts were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis. Immunoblots were blocked with 5% nonfat milk for 1 hour and washed with Tris buffered saline with 0.1% Tween 20 at room temperature. Blots were then incubated overnight at 4°C in primary antibodies against PTEN, PI3K (PI3K p85 alpha) (Abcam, #ab170941, #ab191606, respectively), and Phospho-AKT (ser-437) (Cell Signaling Technology, #4060). After washing with Tris buffered saline with 0.1% Tween 20, blots were incubated for 1 hour with secondary antibodies at room temperature and then detected using enhanced chemiluminescence (Tanon Science & Technology, Shanghai, China). PTEN, PI3K, and p-AKT levels were normalized with glyceraldehyde 3-phosphate dehydrogenase.

Immunofluorescence

The whole brain was collected from each of the 6 groups ($n=3-4$). Immunofluorescence staining was conducted on rat brain sections (4 μm), which were blocked by bovine serum albumin for 30 minutes at room temperature and incubated for 24 hours at 4°C with primary antibodies against PTEN, PI3K (Abcam, #ab191606, #170941), and p-AKT (Cell Signaling Technology, #4060). After washing 3 times, 5 minutes each time with phosphate buffered saline, sections were incubated for 50 minutes at room temperature with goat anti-rabbit 488 (Shanghai Eubio Technology, 1:400). The images were captured with a Nikon fluorescence microscope.

Statistical Analyses

Data represent the mean \pm SE. Before conducting parameter test or nonparameter test, normality plots with test would first be generated to estimate the normality of data. Repeated-measure data including weight gain and food intake were analyzed by linear mixed-models repeated-measures analysis (Maurissen and Vidmar, 2017), and Bonferroni multiple comparison post-test was used when necessary. Blood glucose levels at different time points among the 4 treatment groups were analyzed using multivariate analysis. One-way ANOVA with Dunnett-t (2-sided) multiple comparison post-test was applied in semiquantitative analysis of data from western blot. All analyses were performed using the statistical software SPSS (version 24.0). Values $P \leq .05$ were regarded as statistically significant.

For metabolomic analysis, we differentiated metabolites against the selection criteria of variable importance in the projection ≥ 1 , false discovery rate (Benjamini-Hochberg) adjusted $P \leq .05$, and fold change ≥ 2 or ≤ 0.5 . The liquid chromatography-mass spectrometry raw data were converted and processed using XCMS software, metaX software, and R 3.5.1 software. More details were shown in supplemental Materials.

RESULTS

Tat-3L4F Attenuates Weight Gain and Hyperphagia Induced by OLZ

Throughout 9 days of treatment, body weight change differed significantly among groups, with higher weight gain in the OLZ alone treatment group than in the other groups (Figure 1A; Table 1). However, rats treated with OLZ and Tat-3L4F (1 $\mu\text{mol kg}^{-1}$ per day or 10 $\mu\text{mol kg}^{-1}$ per day) also had significantly higher weight gain compared with rats treated with vehicle during 9 days of treatment (all $P < .001$). Tat-3L4F did not significantly affect food intake, body weight, and blood glucose in vehicle treated rats (Figure 1A, B, D; Table 1). In addition, food conversion efficiency (cumulative weight gain divided by food consumed) of the OLZ alone-treated group was significantly higher than the vehicle-treated group over the 9-day treatment (mean \pm SD, 0.59 ± 0.35 vs 0.86 ± 0.35 vs 0.87 ± 0.47 vs 1.69 ± 0.34 , for the Veh-, Veh+Tat1-, Veh+Tat10-, and OLZ- group, respectively) (Figure 1C; Table 1). Furthermore, food conversion efficiency did not differ among OLZ-, O+Tat1-, and O+Tat10- groups after 9 days of treatment (mean \pm SD, 1.69 ± 0.34 vs 1.30 ± 0.36 vs 1.25 ± 0.41 , for the OLZ-, OLZ+Tat1-, and OLZ+Tat10- group, respectively) (Figure 1C; Table 1). OLZ has been proven to be associated with abnormal eating behavior (food craving, binge eating) in humans, mice, and rats (Kluge et al., 2007; Weston-Green et al., 2011; Lord et al., 2017). Consistent with previous studies, we found an increase in food consumption in OLZ-treated rats compared with vehicle-treated rats during the dark phase throughout the 9 days of treatment ($P < .001$; Figure 1B; Table 1). However, hyperphagia induced by OLZ was restored close to control levels by co-treatment with OLZ and Tat-3L4F (10 $\mu\text{mol kg}^{-1}$ per day) (Figure 1B; Table 1). However, food intake during the light phase and dark phase of a day (24 hours) was increased in groups treated with OLZ alone, co-treatment with OLZ, and Tat-3L4F (1 $\mu\text{mol kg}^{-1}$ per day) and co-treatment with OLZ and Tat-3L4F (10 $\mu\text{mol kg}^{-1}$ per day) than in groups treated with vehicle (supplemental Figure 1). Furthermore, OGTT results showed blood glucose at 120 minutes was higher in the OLZ alone-treated group than in the other groups (Figure 1D; Table 1).

Metabolomic profiling of alteration in liver metabolome of rats with metabolic syndrome induced by OLZ after treatment with Tat-3L4F

The raw data were preprocessed as described above, and then multivariate analysis methodologies including principal component analysis were performed to investigate possible clustering and outlier detection. The following analysis results were based on data acquired from positive ion mode. The heatmap showed the intensity of every detected metabolite in each sample, which could be used to analyze the correlation between groups (Figure 2B). Additionally, it was shown that degree of dispersion in the metabolite level from either the OLZ group or OLZ+Tat10 group was higher than from the other 2 groups (Figure 2A). That indicated that after treatment with OLZ alone or co-treatment with OLZ and Tat-3L4F (10 $\mu\text{mol kg}^{-1}$ per day), different treatment responses existed in each individual rat within the same treatment group. In addition, although a few overlapping parts existed between treatment groups, the 4 groups can be discriminated distinctly. For further analysis of pairwise comparison in effect of Tat-3L4F on OLZ-induced metabolic change, we calculated principal component score and investigated separation between the OLZ and OLZ+Tat1 groups and between the OLZ and OLZ+Tat10 groups (Figure 2C–D). Clear separation was shown between the OLZ and OLZ+Tat1 groups and between the OLZ and OLZ+Tat10 groups. Similar cross comparison results were obtained by using supervised partial least squares discriminant analysis (supplemental Figure 2A–B). A total of 5352 high-quality ion features were detected quantitatively in the first mass spectrometry. A total of 662 and 475 differential metabolites were screened and identified for OLZ vs OLZ+Tat1 and OLZ vs OLZ+Tat10, respectively. Each metabolite can be fragmented in the mass spectrometer, and fragments of 508 differential metabolites were identified by the secondary mass spectrometry database. Most identified differential metabolites were classified as lipids and lipid-like molecules (10 and 9 differential metabolites classified as lipids and lipid-like molecules for OLZ vs OLZ+Tat1 and OLZ vs OLZ+Tat10, respectively) (supplemental Tables 1 and 2). Among the identified metabolites from the first mass spectrometry, bile acid is a metabolite that is highly associated with cholesterol metabolism (Schroor et al., 2019). Additionally, we found intensity of bile acids in the liver tissues significantly increased in the OLZ alone-treated group compared with each of the other 3 groups (supplementary Figure 2C).

Tat-3L4F Could Induce Downregulation of PTEN in Hypothalamus

PTEN expression was firstly localized via immunofluorescence stain and scanning analysis. Because it is closely related to feeding behavior and glucose and energy regulation (Hausen et al., 2016), hypothalamus was the brain area of our interest and PTEN immunofluorescence was confirmed to express in the hypothalamus (Figure 3B). We further quantitatively detected PTEN expression in the hypothalamus. The semiquantitative analysis of western blots indicated the PTEN level tended to be lower in Veh+Tat10- and OLZ+Tat10-treated rats than in OLZ- and Veh-treated rats (Figure 3A). In spite of no statistical significance, there was tendency of downregulated PTEN expression in the other 2 groups after administration of Tat-3L4F compared with the Veh- and OLZ-treated groups (Figure 3A).

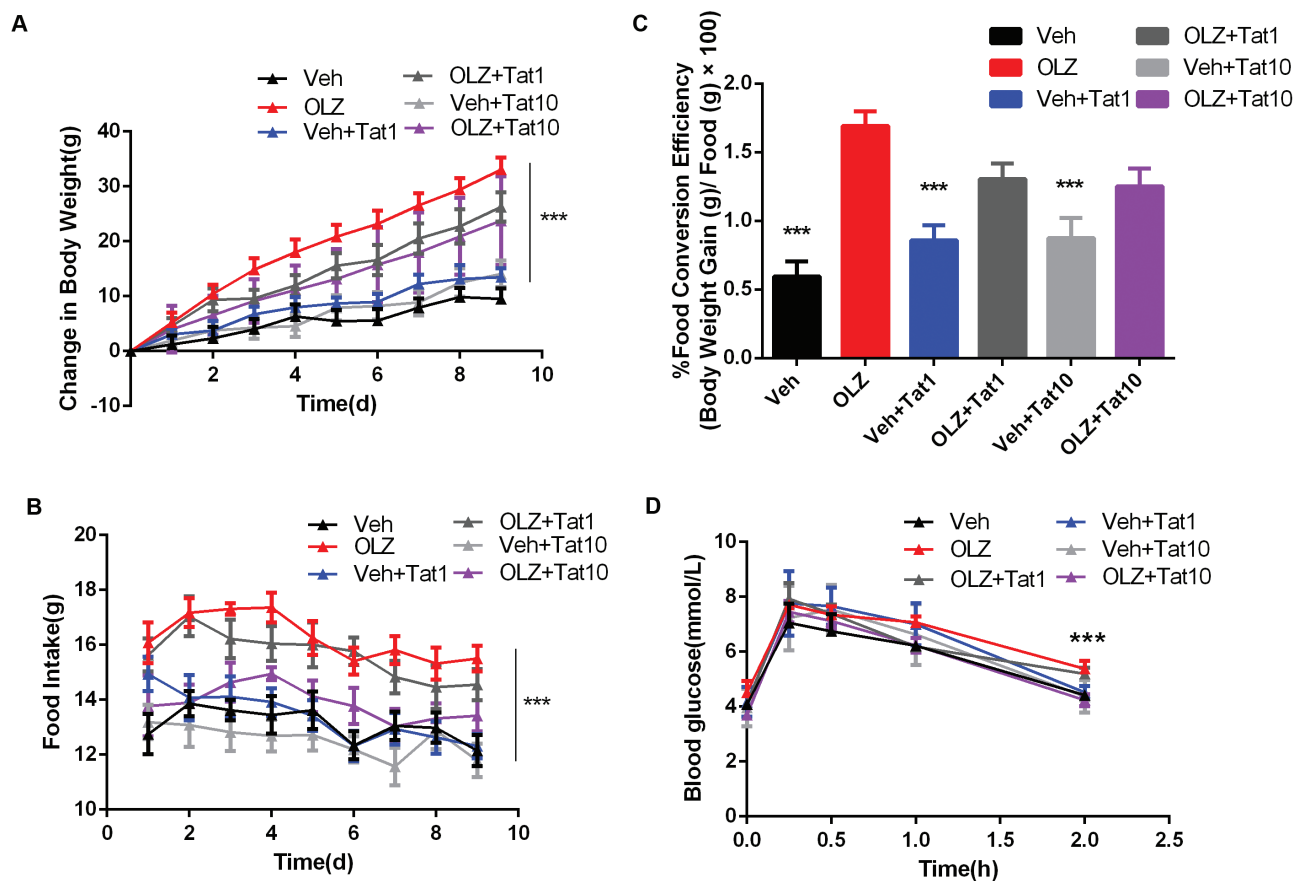


Figure 1. Effects of Tat-3L4F on body weight, feeding behavior, and oral glucose tolerance. (A) Tat-3L4F significantly attenuated weight gain induced by olanzapine (OLZ) ($n=10$). (B) Food intake during the dark phase ($n=10$). (C) Tat-3L4F did not attenuate increased percent average food-conversion efficiency (weight gain [g] per food consumed [g] $\times 100$) induced by OLZ ($n=10$). (D) Oral glucose tolerance test ($n=9-10$). Statistical significance was defined as $P < .05$ ($*P < .05$; $***P < .001$). Data represent the mean \pm SE. OLZ, OLZ alone; OLZ+Tat1, OLZ + $1 \mu\text{mol kg}^{-1}$ per day Tat-3L4F; OLZ+Tat10, OLZ + $10 \mu\text{mol kg}^{-1}$ per day Tat-3L4F; Veh, vehicle alone; Veh+Tat1, vehicle + $1 \mu\text{mol kg}^{-1}$ per day Tat-3L4F; Veh+Tat10, vehicle + $10 \mu\text{mol kg}^{-1}$ per day Tat-3L4F.

Table 1. Summary of statistical results of body weight change, food intake, food conversion efficiency and blood glucose

	Group effect		Time effect		Group \times time effect		P value				
	F	P	F	P	F	P	Veh vs Veh+Tat1	Veh vs OLZ	OLZ vs Veh+Tat1	OLZ vs OLZ+Tat1	OLZ vs OLZ+Tat10
	value	value	value	value	value	value					
Weight change	59.342	<.001*	54.702	<.001*	1.706	.016	1.000	1.000	<.001*	<.001*	<.001*
Food intake (dark phase)	51.235	<.001*	6.565	<.001*	0.525	.988	1.000	.939	<.001*	.469	<.001*
Food conversion efficiency	10.552	<.001*	-	-	-	-	1.000	1.000	<.001*	.427	.198
Blood glucose at 120 min	5.633	<.001*	-	-	-	-	1.000	1.000	.024*	1.000	.003*

Abbreviations: OLZ, olanzapine alone; OLZ+Tat1, olanzapine + $1 \mu\text{mol kg}^{-1}$ per day Tat-3L4F; OLZ+Tat10, olanzapine + $10 \mu\text{mol kg}^{-1}$ per day Tat-3L4F; Veh, vehicle; Veh+Tat1, vehicle + $1 \mu\text{mol kg}^{-1}$ per day Tat-3L4F; Veh+Tat10, vehicle + $10 \mu\text{mol kg}^{-1}$ per day Tat-3L4F.

Significant group differences are indicated by asterisks (*). Columns represent effects of time, group, and group \times time interaction. Pairwise comparison was conducted by Bonferroni post hoc analysis.

Expression of PI3K and p-AKT in Hypothalamus After Treatment With Tat-3L4F

PI3K and p-AKT expression was also localized by immunofluorescence stain assay, and we respectively confirmed PI3K and p-AKT expressed in the hypothalamus (Figure 4B-C).

Semiquantitative analysis of western blots showed accompanied with PTEN downregulation after treatment with Tat-3L4F, there was a trend that PI3K expression increased in the 4 groups with Tat-3L4F treatment compared with the other 2 groups without Tat-3L4F treatment. A significantly lower p-AKT

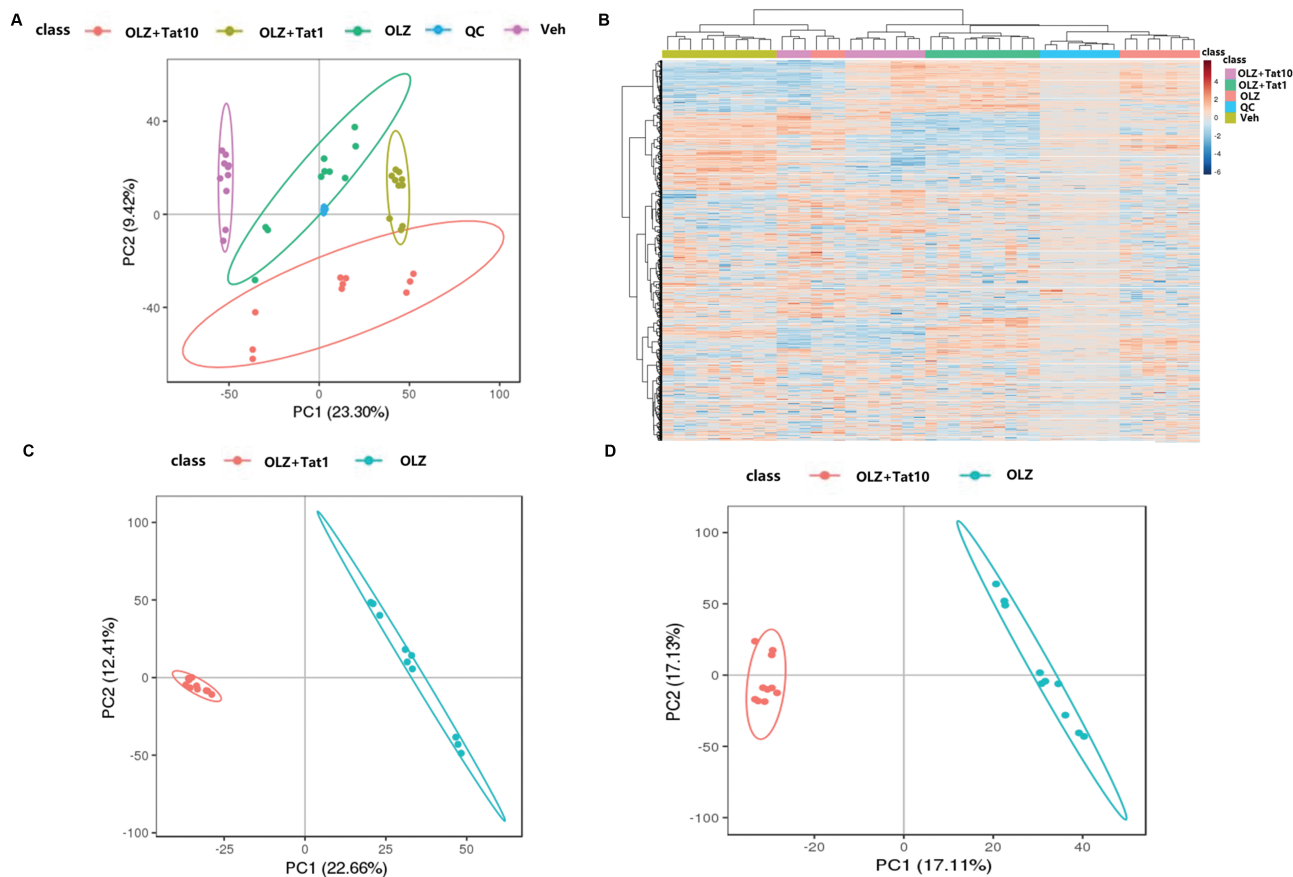


Figure 2. Untargeted metabolomic analysis of liver tissues from Veh-, OLZ-, OLZ+Tat1-, and OLZ+Tat10-treated rats by LC/MS-MS from positive ion mode ($n=10$). (A) Clustering analysis of 4 treatment groups by principal component analysis (PCA). (B) Heatmap analysis of metabolites from all the detected samples. (C–D) PC1 \times PC2 score plot of between OLZ and OLZ+Tat1 samples and between OLZ and OLZ+Tat10 samples. OLZ, olanzapine alone; OLZ+Tat1, olanzapine + $1 \mu\text{mol kg}^{-1}$ per day Tat-3L4F; OLZ+Tat10, olanzapine + $10 \mu\text{mol kg}^{-1}$ per day Tat-3L4F; Veh, vehicle alone; Veh+Tat1, vehicle + $1 \mu\text{mol kg}^{-1}$ per day Tat-3L4F; Veh+Tat10, vehicle + $10 \mu\text{mol kg}^{-1}$ per day Tat-3L4F.

level in the hypothalamus was investigated in rats treated with OLZ (including OLZ, OLZ+Tat1, and OLZ+Tat10 groups) than in rats treated with vehicle (Figure 4A). Moreover, there was no significant difference in p-AKT expression among the 3 OLZ-treated groups, which suggests Tat-3L4F could not restore OLZ-induced p-AKT downregulation.

Discussion

To the best of our knowledge, this is the first study on the effect and mechanism of Tat-3L4F on OLZ-induced metabolic abnormalities. The present study has provided new evidence of the preventive effect of Tat-3L4F on metabolic disorders (including weight gain and glucose and lipid dysregulation) induced by OLZ in vivo and revealed a mechanism of the action of Tat-3L4F on the PTEN/PI3K/AKT signaling pathway. Our results indicated the potential of Tat-3L4F treatment, administered after OLZ, for suppressing hyperphagia and weight gain along with PTEN downregulation in the hypothalamus of rats. Notably, Tat-3L4F did not affect body weight and feeding behavior in the control group, which indicated this interfering peptide was specific to OLZ-induced weight gain and hyperphagia. Additionally, 4 treatment groups were distinctly differentiated through clustering the features detected by liquid chromatography-mass spectrometry with multivariate analyses, and this reveals metabolic profiling of rats has changed after Tat-3L4F treatment.

Our results suggest Tat-3L4F can mitigate OLZ-induced weight gain and hyperphagia through allosterically modulating downstream signaling of HTR2C instead of directly binding to HTR2C. In addition, PTEN expression in the hypothalamus downregulated after co-treatment with OLZ and Tat-3L4F ($10 \mu\text{mol kg}^{-1}$ per day) compared with treatment with OLZ alone. A recent study showed that lorcaserin, a selective 5-HT_{2C} receptor agonist, could also suppress hyperphagia and weight gain induced by AAPs (Lord et al., 2017). However, agonism on HTR2C inhibited dopamine efflux, which detrimentally influenced cognitive function and negative symptoms in schizophrenic patients (Meltzer and Massey, 2011). Therefore, regulating downstream signaling of HTR2C may be a better strategy for alleviating AAP-induced metabolic abnormalities.

In the present study, the gender of animals might have influenced our results. The body weight and feeding behavior of female rats may be influenced by sex hormone fluctuation (Skrede et al., 2017). Moreover, the female sex hormone estradiol has an important role in regulation of body weight, and metabolic impacts of OLZ are dependent on ovarian secretion (Skrede et al., 2017). Furthermore, the male rats and mice do not develop weight gain when treated with OLZ (Lord et al., 2017; Skrede et al., 2017). That is why we chose female rats to conduct this pharmacological study on OLZ-induced weight gain.

Our results also showed expression of downstream p-AKT and PI3K in the hypothalamus was not significantly affected

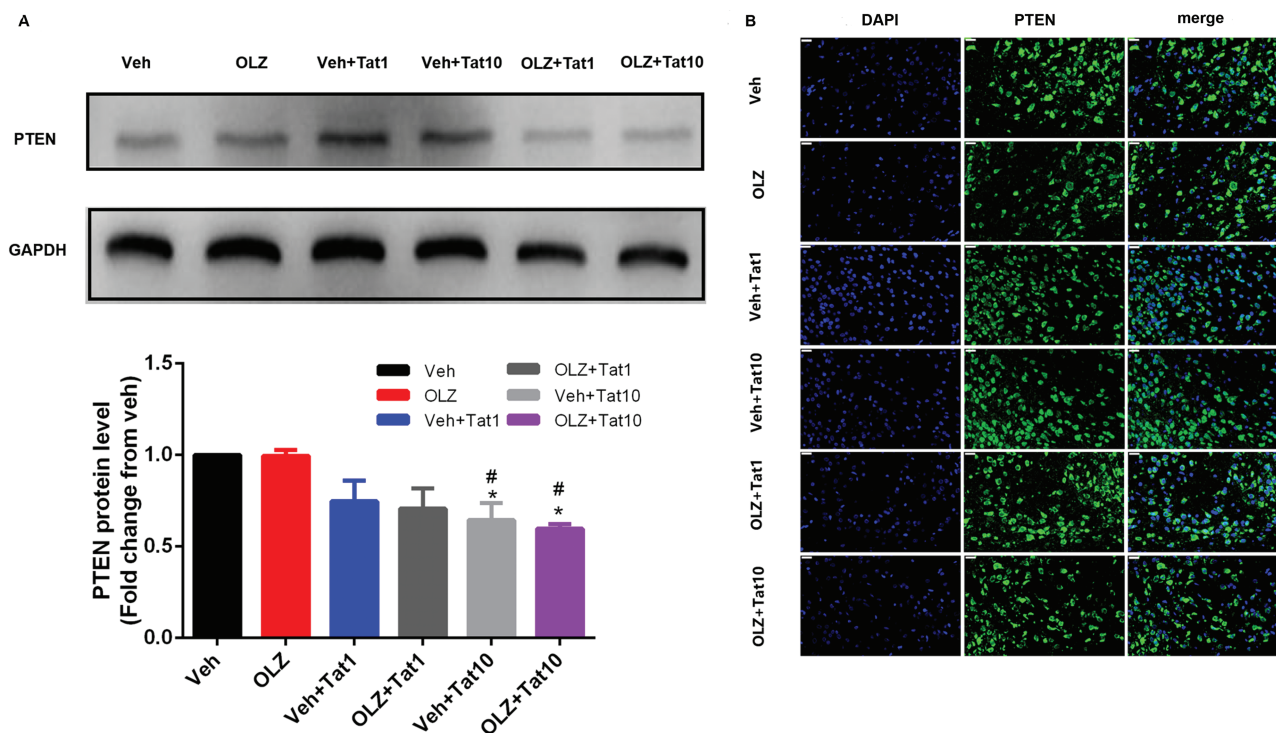


Figure 3. PTEN level in hypothalamus tissues from 6 group. (A) Similar expression of PTEN in hypothalamus as shown by western-blot analysis of protein lysates of tissues from Veh-, OLZ-, Veh+Tat1-, Veh+Tat10, OLZ+Tat1-, and OLZ+Tat10-treated rats. Blots were probed with PTEN and GAPDH as loading control ($n=3$). (B) The expression of PTEN in the hypothalamus as shown by immunofluorescence stain. In the immunofluorescence panel, the left column represents the cell nucleus (blue) stained by 4',6-diamidino-2-phenylindole (DAPI); the next column presents the target protein PTEN (green) stained by goat anti-rabbit 488. Merged images are shown at right ($n=3-4$). Scale bar = 20 μm . Statistical significance was defined as $P < .05$ ($^{\#}P < .05$ vs OLZ; $^*P < .05$ vs Veh). Data represent the mean \pm SE. OLZ, olanzapine alone; OLZ+Tat1, olanzapine + 1 $\mu\text{mol kg}^{-1}$ per day Tat-3L4F; OLZ+Tat10, olanzapine + 10 $\mu\text{mol kg}^{-1}$ per day Tat-3L4F; Veh, vehicle alone; Veh+Tat1, vehicle + 1 $\mu\text{mol kg}^{-1}$ per day Tat-3L4F; Veh+Tat10, vehicle + 10 $\mu\text{mol kg}^{-1}$ per day Tat-3L4F.

by Tat-3L4F treatment. The past studies reported deficiency or inhibition of PTEN in the hypothalamus could suppress hyperphagia and weight gain (Choi et al., 2008; Sumita et al., 2014). Remarkably, in the present study, we found OLZ treatment unexpectedly inhibited p-AKT expression without significantly upregulating PTEN expression in the hypothalamus. This may be associated with various upstream molecules to regulate PI3K/AKT signaling pathway, such as insulin receptor and insulin receptor substrate-1, insulin-like growth factor, and so on (Paz et al., 1999; Ma et al., 2017). Additionally, according to a recent cytology experiment, OLZ (0.25, 2.5, 25, and 100 μM) had no effect on pAKT expression in immortalized hypothalamic rodent neurons (Kowalchuk et al., 2019). The difference of pAKT expression between our present study and the recent study may be attributed to the source of subjects used for western blot. Our present study was in vivo, while the other study was in vitro.

The pharmacological effect of Tat-3L4F on feeding behavior was most significant within 12 hours (8 PM to 8 AM) of administration, and food intake during the light phase presented rebound phenomenon, which led to similar total food intake during 24 hours (the dark phase and light phase of a day) among groups treated with OLZ alone, co-treatment with OLZ and low-dose Tat-3L4F (1 $\mu\text{mol kg}^{-1}$ per day), and co-treatment with OLZ and high-dose Tat-3L4F (10 $\mu\text{mol kg}^{-1}$ per day), respectively. This phenomenon may be associated with the relatively short half-life of the peptide drug (Pearson et al., 2019). Surprisingly, although food intake during 24 hours was similar among groups respectively treated with OLZ alone, co-treatment with OLZ and

low-dose Tat-3L4F (1 $\mu\text{mol kg}^{-1}$ per day), and co-treatment with OLZ and high-dose Tat-3L4F (10 $\mu\text{mol kg}^{-1}$ per day), weight gain was still alleviated after treatment with either low-dose or high-dose Tat-3L4F. Consistently, a recent study showed OLZ treatment reduced locomotor activity, which may contribute to lower energy consumption and more weight gain in the OLZ alone-treated group (Lord et al., 2017).

OGTT showed that the blood glucose level at 120 minutes was higher in the OLZ alone-treated group than in each of the other groups, which indicated the potential of Tat-3L4F for improving glucose tolerance in OLZ-treated rats. In the past studies, OLZ treatment has been proven to induce impaired glucose tolerance in both human and rodents, and anti-diabetic agents could improve the glucose tolerance (Albaugh et al., 2011; Correll et al., 2019; Lord et al., 2017).

Metabolomic profiling indicated that small metabolic molecules (especially lipids and lipid-like molecules) in OLZ-treated rats changed after treatment with Tat-3L4F. Furthermore, we found bile secretion increased in the OLZ alone-treated group compared with each of the other 3 treatment groups. This revealed Tat-3L4F treatment regulated increased bile secretion induced by OLZ. It has been known that bile acids are synthesized from cholesterol in the liver and implicated in metabolic regulation mainly through activating the nuclear receptor farnesoid X receptor (FXR) (Zaborska and Cummings, 2018; Lazarevic et al., 2019). FXR activation could contribute to lipid dysregulation and hence increased bile acids could activate FXR, resulting in lipid dysregulation through activation on FXR (Jiang et al., 2015;

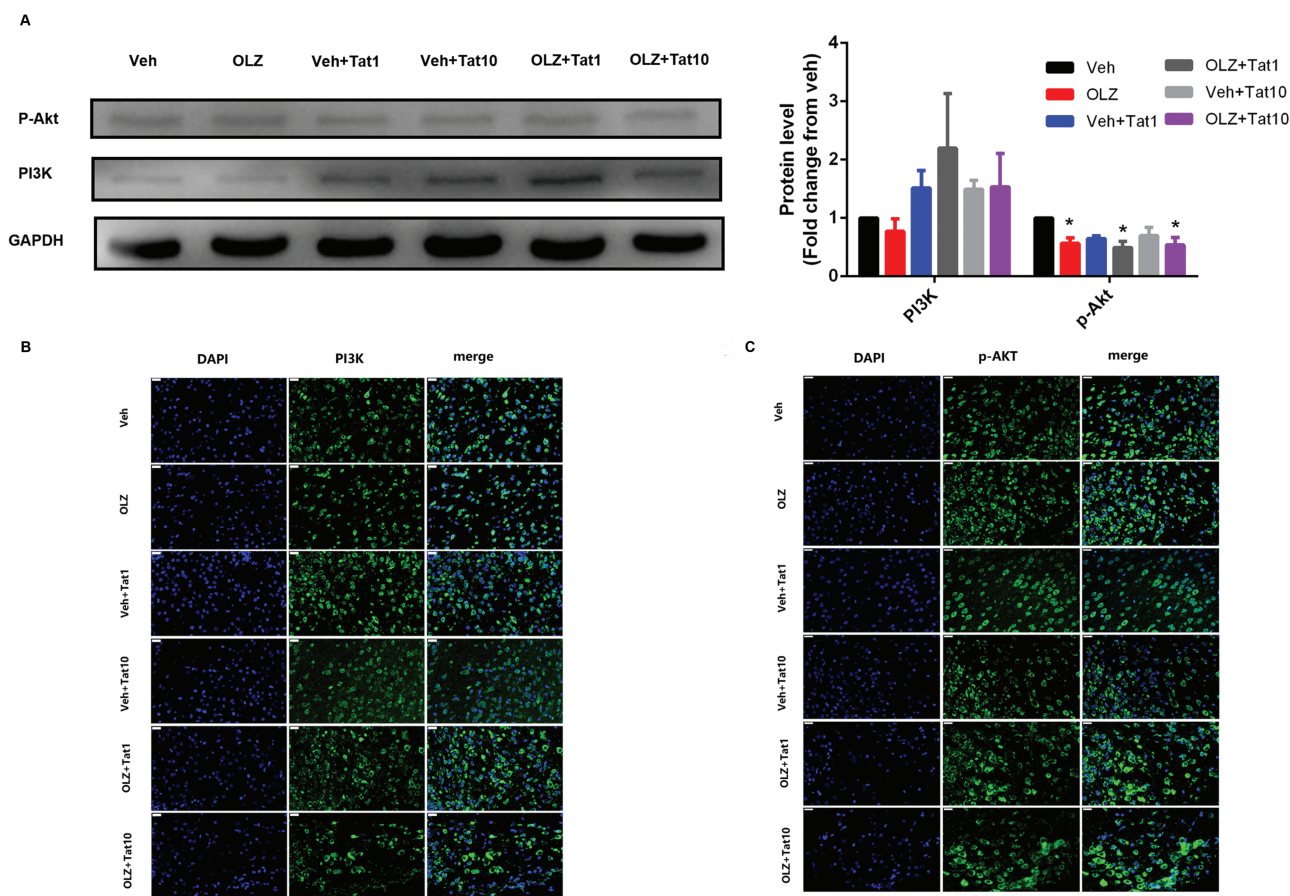


Figure 4. PI3K and p-Akt expression in hypothalamus tissues from 6 groups. (A) The expression of p-Akt was significantly lower in OLZ-, OLZ+Tat1-, and OLZ+Tat10-treated rats than in Veh-treated rats. Similar expression of PI3K and p-Akt was measured among Veh+Tat1-, Veh+Tat10-, OLZ-, OLZ+Tat1-, and OLZ+Tat10-treated rats. Blots were probed with PI3K, p-Akt, and GAPDH as loading control. Statistical significance was defined as $P < .05$ ($*P < .05$ vs Veh). Data represent the mean \pm SE (n=3). (B–C) PI3K and p-Akt expression in the hypothalamus were respectively confirmed by immunostaining of PI3K or p-Akt (green) immunofluorescence. Scale bar = 20 μ m. OLZ, olanzapine alone; OLZ+Tat1, olanzapine + 1 μ mol kg⁻¹ per day Tat-3L4F; OLZ+Tat10, olanzapine + 10 μ mol kg⁻¹ per day Tat-3L4F; Veh, vehicle alone; Veh+Tat1, vehicle + 1 μ mol kg⁻¹ per day Tat-3L4F; Veh+Tat10, vehicle + 10 μ mol kg⁻¹ per day Tat-3L4F.

Zaborska and Cummings, 2018). Although there was no significant difference in cholesterol in the liver among the 4 treatment groups, this may be attributed to short treatment duration and thus increased bile acids or salts could make a compensatory action for cholesterol.

This study has several limitations that should be taken into account. First, we did not validate the pharmacological effects (agonism, antagonism, or inverse agonism) of HTR2C after administration of Tat-3L4F, although our present results on weight change and feeding behavior indicated Tat-3L4F treatment presented a HTR2C agonism-like effect. Ideally, Tat-3L4F should not affect HTR2C antagonism caused by AAPs; however, there was no empirical evidence to support this hypothesis. Second, we did not investigate the other upstream molecules (IGF-1 and IRS-1) that may affect PI3K/AKT signaling and metabolic parameters, including insulin, apolipoprotein, and cholesterol in the peripheral blood. Third, the researchers were not blinded to intervention and this may have resulted in higher risk of bias. In addition, more trials, such as glucose clamp and serum insulin level detection, should be conducted to prove the effect of Tat-3L4F on glucose metabolism and homeostasis. Moreover, wire cages were used to feed animals in the present study and there may be risk of food falling through, which may result in some measurement errors. In the present study,

serum OLZ concentration was not monitored due to protecting the animals from stress.

Taken together, this study hypothesized that Tat-3L4F may be a potential pharmacological intervention through disrupting HTR2C coupling with PTEN for AAP-induced metabolic abnormalities. Our results showed that co-treatment with Tat-3L4F and OLZ alleviated OLZ-induced changes in body weight, food intake, blood glucose, and lipid in rats. We also found that Tat-3L4F regulated OLZ-induced metabolic adverse effects through disrupting crosstalk between HTR2C and PTEN, which contributed to PTEN downregulation in the hypothalamus. Our findings implied that Tat-3L4F may be a putative therapeutic strategy for AAP-induced metabolic disorders through regulating HTR2C downstream signaling. Compared with an HTR2C agonist like lorcaserin, Tat-3L4F has a pharmacological advantage in that it presents pharmacological effect without directly binding to HTR2C. Finally, we focused on whether Tat-3L4F could effectively improve metabolic abnormalities induced by OLZ because the following research about how Tat-3L4F works depends on it. Therefore, additional studies investigating the change of signaling after Tat-3L4F administration should contain other signaling pathways in addition to PTEN/PI3K/AKT and verify whether Tat-3L4F treatment influences the pharmacological effect of HTR2C.

Supplemental Materials

Supplemental data are available at *International Journal of Neuropsychopharmacology (IJNPPY)* online.

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Interest Statement: None.

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