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Original article

Effects of Zhengtian Pills on Migraine Headache in Rats via Transient Receptor Potential Vanilloid 1

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ABSTRACT

Objective To investigate the effects and molecular mechanism of Zhengtian Pills (ZTP) on migraine headache. **Methods** All rats were randomly divided into control, positive control, migraine model, low- and high-dose ZTP groups, and glyceryl trinitrate was injected to induce migraine headache. The time of ears turning red, frequency of scratching head, climbing the cage, and head-twitching were used to evaluate rat behaviors. After 10 d administration of ZTP, the expression levels of transient receptor potential vanilloid 1 (TRPV1) both in cortex and hippocampus were determined by Western blotting. **Results** After 2 min of glyceryl trinitrate injection, rats showed headache phenomena that parallels the clinical symptoms of migraine, which peaked in 30 min, and lasted for 60 min. Frequency of head-twitching and numbers of scratching head in glyceryl trinitrate (GTN) group were significantly increased. In contrast, after ZTP (1.08 g/kg, ig) treatment, the numbers of scratching head with fore-limb, hind-limb and the frequency of head-twitching were significantly decreased. Flunarizine (FLU) and low-dose ZTP (0.54 g/kg) also showed a trend to decrease the numbers of scratching head and head-twitching frequency, but no significant difference. Besides, ZTP significantly decreased the up-regulated TRPV1 protein expression level both in cortex and hippocampus. **Conclusion** The present study shows that ZTP could significantly improve the migraine symptoms of headache in rats and TRPV1 might be one of the important molecular mechanisms. This is the first report about the effect of ZTP on TRPV1 protein expression level both in cortex and hippocampus of rats.

Key words

behavior; glyceryl trinitrate; migraine; TRPV1; Zhengtian Pills

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1. Introduction

Migraine is a very common primary headache disorder associated with intermittent attacks and great suffering. Migraine has a prevalence of 10% in the general population and its societal costs are high. Typically, the headache affects one half of the head and is pulsating in nature. The precise mechanisms underlying the pathophysiology of migraine are still elusive (Seng and Seng, 2016; Cameron et al, 2015; Khan et al, 2015). Considerable debate has surrounded the cause and underlying pathology of migraine and several theories have been proposed, for example, depolarization theory, vascular theory, serotonin theory, and hypothyroidism.

Generally, the acute treatment of migraine attacks has been limited to the use of analgesics, combinations of analgesics with caffeine, ergotamines, and the triptans. Some new approaches for the treatment of acute migraine target the calcitonin gene-related peptide, serotonin receptors, and glutamate, $GABA_A$ receptors, or a combination of $5\text{-}HT_{1B/1D}$ receptors and neuronal nitric oxide synthesis also show promise as both acute and preventive therapies. Besides, humanized antibodies against CGRP or CGRP receptor, calcitonin gene-related peptide, non-invasive and invasive neuromodulation approaches were investigated, further studies are needed to define the appropriate candidates for these therapies and none of them has been successful in clinical trials thus far. Collectively, for migraine therapy, the available chemical drugs are either ineffective or poorly tolerated thus far (Diener et al, 2015; Ferrari et al, 2015).

Traditional Chinese medicine (TCM) is one of the oldest healing systems, and herbal medicine is the important part of TCM. The holistic philosophy of TCM shares much with the key ideas of emerging network pharmacology and network biology, and meets the requirements of overcoming complex diseases in a systematic manner. Zhengtian Pills (ZTP), a traditional potential and effective therapeutic tool for migraine and other types of headaches, such as tension headache and sinus headache in China, which consists of 15 medicinal herbs such as *Ramulus Uncariae cum Uncis* (Gouteng), *Paeoniae Alba Radix* (Baishao), *Angelicae Sinensis Radix* (Danggui), *Chuanxiong Rhizoma* (Chuanxiong), *Rehmanniae Radix* (Dihuang), *Saposhnikoviae Radix* (Fangfeng), *Angelicae Dahuricae Radix* (Baizhi), *Notopterygii Rhizoma* et *Radix* (Qianghuo), *Persicae Semen* (Taoren), *Carthami Flos* (Honghua), *Asari Radix* et *Rhizoma* (Xixin), *Angelicae Pubescentis Radix* (Duhuo), *Ephedrae Herba* (Mahuang), *Aconiti Lateralis Radix Praeparata* (Fupian), and *Caulis Spatholobi* (Jixueteng). "Jun-Chen-Zuo-Shi", also known as sovereign-minister-assistant-courier, was followed to prescribe this component herb according to that of the TCM. ZTP has been used in clinic for over 30 years and holds a great promise for treating headaches in an integrative and holistic way. However, the detailed molecular mechanism was not clear yet. Therefore, in the present study, the effects and molecular mechanism of ZTP on migraine headache will be investigated.

2. Materials and methods

2.1 Animals

Fifty Sprague-Dawley (SD) rats [male, (200 ± 20) g] were provided by Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were housed in standard laboratory conditions [under 12 h light/dark cycles and at a temperature of (23 ± 1) ^oC] and could freely access to food and water. Experimental protocols and procedures were handled in accordance with Chinese Animal Use and Care Committees and executed according to National Animal Law.

2.2 Drugs and reagents

Glyceryl trinitrate injections (No. 20140310) were purchased from Beijing Yimin Pharmaceutical Co., Ltd. and sc given at dose of 10 mg/kg. ZTP was supplied by Sanjiu Medical & Pharmaceutical Company, and was re-suspended in ddH_2O. Flunarizine (FLU) (No. 140414122) was obtained from Xi'an Janssen Pharmaceutical Ltd. and also dissolved in ddH_2O. FLU was ig given at dose of 0.9 mg/kg. All other chemical reagents in our study were of analytical grade.

2.3 Experimental groups

All rats were randomly divided into five groups such as control group (saline), glyceryl trinitrate-induced migraine model (GTN) group, low- and high-dose (0.54 and 1.08 g/kg) ZTP groups, and FLU group (positive control), formed by 10 animals in each group. The animals were prepared as previously described (Wang et al, 2011). After 3 d habituation, rats orally received either ddH_2O (control and GTN groups) or various concentration of ZTP and FLU (0.9 mg/kg) for 10 d. After 30 min of last administration, glyceryl trinitrate (10 mg/kg) was sc given to establish migraine model.

2.4 Glyceryl trinitrate-induced headache in rats

The animals were acclimatized for 3 d in the test chamber before testing. After 10 d of administration, the computer-aided controlling system for open field test was conducted to appraise the general behavior and symptom during 90 min. The general behavior and symptom, including the time of turning red of rats' ears, frequency of scratching head, climbing cage, and head-twitching, which were used to evaluate the different categories of headache in rats. After detection of the basal level of general behavior, glyceryl trinitrate (10 mg/kg) was sc given to duplicate the animal model of migraine, and the GTN rats were immediately placed into open field chambers to detect the numbers of scratching head, climbing cage, and head-twitching. The behaviors and symptoms were real-time recorded and continuously observed at various time intervals.

2.5 Western blotting

After the migraine model was established, the cortex

and hippocampus from brains were quickly dissected and stored at −80 °C for analysis. These tissues were added with RIPA buffer at a ratio of 1:10 to form homogenate and smashed for 2 min by Ultrasonic Cell Disruptor (Ningbo Scientz Biotechnology Co., Ltd.). The protein concentration was determined by BCA Assay Kit (CW Biotech, Beijing, China). Equal amounts of protein samples (40 μg/well) were separated by electrophoresis in 8% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, USA) in Tris-glycine buffer at 60 V for 40 min and 100 V for 80 min. The membranes were blocked in Tris-buffer containing 0.04% Tween-20 (TBST) with 5% non-fat milk powder for 1 h at room temperature. We incubated the membranes overnight at 4 °C with the following primary antibodies: TRPV1 rabbit polyclonal antibody (1:1000, Abcam, USA) and β-Actin rabbit polyclonal antibody (1:2000, Easybio, Korea). The membranes were washed thrice with TBST and incubated with secondary goat anti-rabbit IgG-HRP conjugated antibody (1:5000, Easybio, Korea) for 2 h at room temperature (Chen et al, 2015). The protein blots were developed using a Super ECL Plus Chemiluminescence Solution (HXBC biotech, Beijing, China) after rewashing thrice with TBST. Protein expression levels were visualised with a ChemiDoc XRS Chemiluminescence Detector (Bio-Rad, USA). Gel-Pro analyzer 4.0 was used to analyze the gray value of each protein band.

2.6 Statistical analysis

All data were shown as $\bar{x} \pm s$. Student's *t*-test was used to compare the two groups in Western blotting. ANOVA was used to compare multiple groups. $P < 0.05$ was considered as statistically significant.

3. Results

3.1 Effect of ZTP on general behavior and symptom in glyceryl trinitrate-induced migraine rats

Neither of control and GTN groups had significant change on general behavior and symptom before modeling. Except control group, the behavioral parameters and symptoms in each group were different after 2 min of glyceryl trinitrate injection, for example, red ears, scratching head frequently, head-twitching, and climbing cage. The phenomena reached the peak in 30 min, and was continued for 60 min and then the frequencies of these behaviors were decreased gradually.

3.2 Effect of ZTP on scratching head in glyceryl trinitrate-induced migraine rats

As shown in Figure 1, compared with the control group, the rats in the GTN group obviously increased the numbers of scratching head with fore-limb and hind-limb after the model was established ($P < 0.001$, 0.001), all behavior parameters in details were shown in Tables 1 and 2. In contrast, the numbers of scratching head with fore-limb in ZTP (0.54 and 1.08 g/kg) and FLU groups decreased significantly compared with the GTN group ($P < 0.05$, 0.001, and 0.001). The numbers of scratching head with hind-limb in the ZTP (1.08 g/kg) group decreased obviously compared with the GTN group ($P < 0.01$). The numbers of scratching head with hind-limb in ZTP (0.54 g/kg) and FLU group decreased compared with GTN group, but there was no significant difference ($P > 0.05$).

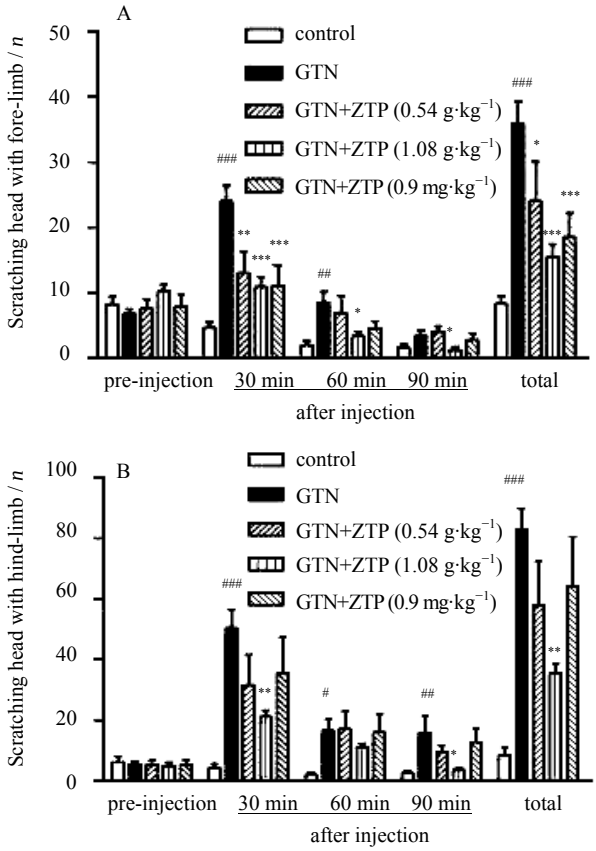

Figure 1 Effect of ZTP on scratching head with fore-limb (A) and hind-limb (B) in glyceryl trinitrate-induced migraine rats ($\bar{x} \pm s$, $n = 10$)

$^{\#}P < 0.05$ $^{\#\#}P < 0.01$ $^{\#\#\#}P < 0.001$ *vs* control group;
$^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$ *vs* GTN group; same as below

Table 1 Effect of ZTP on scratching head with fore-limb in glyceryl trinitrate-induced migraine rats

Groups	Doses	Pre-injection (n)	Post-injection (n)			
			30 min	60 min	90 min	Total
control	−	8.22 ± 1.16	4.67 ± 0.85	2.00 ± 0.64	1.67 ± 0.44	8.33 ± 1.13
GTN	−	6.80 ± 0.73	24.00 ± 2.38$^{\#\#\#}$	8.50 ± 1.68$^{\#}$	3.40 ± 0.83	35.90 ± 3.35$^{\#\#\#}$
GTN+ZTP (g·kg^{-1})	0.54	7.56 ± 1.47	13.00 ± 3.37**	6.89 ± 2.61	4.11 ± 0.77	24.00 ± 6.06*
GTN+ZTP (g·kg^{-1})	1.08	10.33 ± 0.96	10.89 ± 1.48***	3.44 ± 0.62*	1.22 ± 0.55*	15.56 ± 1.93***
FLU (mg·kg^{-1})	0.90	7.90 ± 5.92	11.10 ± 3.07***	4.60 ± 1.03	2.80 ± 0.95	18.50 ± 3.71***

Table 2 Effect of ZTP on scratching head with hind-limb in glyceryl trinitrate-induced migraine rats

Groups	Doses	Pre-injection (n)	Post-injection (n)			
			30 min	60 min	90 min	Total
control	−	6.00 ± 1.96	4.11 ± 1.45	1.89 ± 0.71	2.44 ± 0.73	8.44 ± 2.52
GTN	−	5.40 ± 0.95	50.40 ± 6.03$^{\#\#\#}$	16.50 ± 3.63$^{\#}$	15.90 ± 5.42$^{\#\#}$	82.80 ± 7.08$^{\#\#\#}$
GTN+ZTP (g·kg^{-1})	0.54	5.22 ± 1.42	31.56 ± 9.92	17.00 ± 5.83	9.44 ± 2.24	58.00 ± 14.41
GTN+ZTP (g·kg^{-1})	1.08	4.89 ± 1.05	21.11 ± 2.05**	11.00 ±1.16	3.44 ± 0.90*	35.56 ± 3.08**
FLU (mg·kg^{-1})	0.90	5.20 ± 1.49	35.50 ±11.85	16.00 ±5.99	12.60 ± 4.65	64.10 ± 17.35

3.3 Effect of ZTP on head-twitching in glyceryl trinitrate-induced migraine rats

As shown in Figure 2, compared with the control group, GTN group obviously increased the head-twitching numbers after model was established ($P < 0.001$). In contrast, the numbers of head-twitching in ZTP (0.54 and 1.08 g/kg) and FLU groups decreased significantly compared with GTN group ($P < 0.05$, 0.01, 0.001). The numbers of head-twitching in all groups were shown in Table 3.

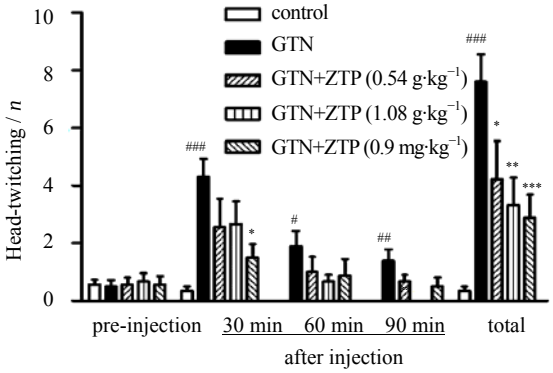

Figure 2 Effects of ZTP on head-twitching in glyceryl trinitrate-induced migraine rats ($\bar{x} \pm s$, $n = 10$)

3.4 Effect of ZTP on climbing cage in glyceryl trinitrate-induced migraine rats

As shown in Figure 3, compared with the control group, the rats in the GTN group had not increased the climbing the cage numbers ($P > 0.05$). Compared with the GTN group, the numbers of climbing the cage of rats in ZTP (0.54 g/kg) and FLU groups did not decrease ($P > 0.05$). The rats in the ZTP (1.08 g/kg) group showed a trend to decrease the numbers of scratching head, but no meaning of statistical significance ($P > 0.05$). The climbing data of rats in all cages were shown in details in Table 4.

Table 3 Effect of ZTP on head-twitching in glyceryl trinitrate-induced migraine rats

Groups	Doses	Pre-injection (n)	Post-injection (n)			
			30 min	60 min	90 min	Total
control	−	0.56 ± 0.18	0.33 ± 0.17	0	0	0.33 ± 0.17
GTN	−	0.50 ± 0.22	4.30 ± 0.63$^{\#\#\#}$	1.90 ± 0.53$^{\#}$	1.40 ± 0.39$^{\#\#}$	7.60 ± 1.01$^{\#\#\#}$
GTN+ZTP (g·kg^{-1})	0.54	0.56 ± 0.24	2.56 ± 0.99	1.00 ± 0.53	0.67± 0.24	4.22 ± 1.32*
GTN+ZTP (g·kg^{-1})	1.08	0.67 ± 0.29	2.67 ± 0.78	0.67 ± 0.23	0.00 ± 0.00**	3.33 ± 0.94**
FLU (mg·kg^{-1})	0.90	0.56 ± 0.29	1.50 ± 0.47*	0.88 ± 0.57	0.50 ± 0.31	2.88 ± 0.80***

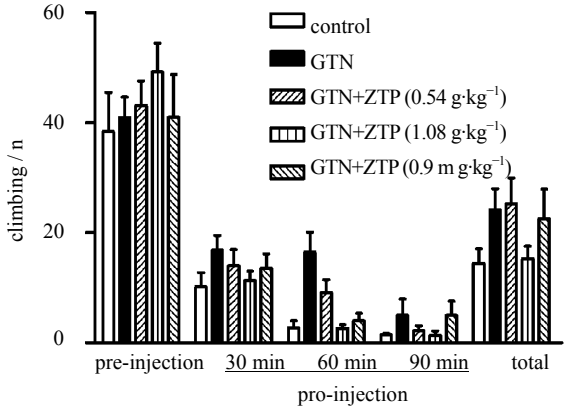

Figure 3 Effects of ZTP on climbing cage in glyceryl trinitrate-induced migraine rats ($\bar{x} \pm s$, $n = 10$)

3.5 Effect of ZTP on TRPV1 expression level in cortex and hippocampus

As shown in Figure 4, the expression of TRPV1 was significantly enhanced in cortex and hippocampus of GTN group compared with the control group (Student' *t*-test, $P < 0.01$). Compared with GTN group, the relative values of TRPV1 were obviously decreased in cortex and hippocampus of ZTP (1.08 g/kg) group (Student' *t*-test, $P < 0.05$, 0.01).

4. Discussion

Nitric oxide (NO) is known to play a key role in migraine pathogenesis, which is a very important molecule in the regulation of cerebral and extra cerebral cranial blood flow and arterial diameters. It is also involved in nociceptive processing (Greco et al, 2015; Olesen, 2008). Therefore, in the present study, glyceryl trinitrate was used to prepare for the experimental animal model of migraine. Exogenous NO,

Table 4 Effect of ZTP on climbing cage in glyceryl trinitrate-induced migraine rats

Groups	Dose	Pre-injection (n)	Post-injection (n)			
			30 min	60 min	90 min	Total
control	−	38.44 ± 7.10	10.22 ± 2.54	2.78± 1.23	1.44 ± 0.29	14.44 ± 2.61
GNT	−	40.90 ± 3.79	16.90 ± 2.69	2.20 ± 0.77	5.10 ± 2.88	24.20 ± 3.83
GNT+ZTP ($g \cdot kg^{-1}$)	0.54	43.11 ± 4.45	14.00 ± 2.98	9.11± 2.37	2.22 ± 0.86	25.33 ± 4.66
GNT+ZTP ($g \cdot kg^{-1}$)	1.08	49.22 ± 5.24	11.33 ± 1.67	2.67 ± 0.64	1.33 ± 0.83	15.33 ± 2.26
FLU ($mg \cdot kg^{-1}$)	0.90	41.00 ± 7.76	13.56 ± 2.61	4.00 ± 1.39	5.00 ± 2.60	22.56 ± 5.38

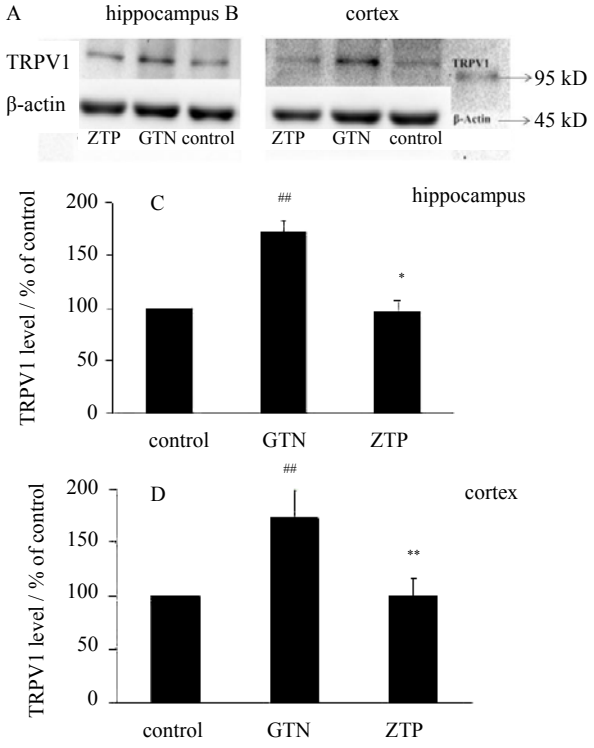

Figure 4 Effect of ZTP (1.08 $g \cdot kg^{-1}$) on TRPV1 expression levels in cortex and hippocampus

(A) In hippocampal area, representative blots of TRPV1 and β-Actin with the similar protein loading in each group. (B) In cortical area, representative blots of TRPV1 and β-Actin with the similar protein loading in each group. (C) Relative amounts of TRPV1 in the hippocampus and blots were digitized and quantified using densitometric analysis. (D) Relative amounts of TRPV1 in the cortex and blots were digitized and quantified using densitometric analysis

released by glyceryl trinitrate, which also activates the NO synthetic pathway, induces migraine-like headache in predisposed subjects (Ashina et al, 2011; Shina et al, 2004).

ZTP was effective in relieving the behavior and symptom of glyceryl trinitrate induced headache in migraine model rats. This finding suggested that ZTP decreased the expression of TRPV1 which may play an important role in the pathogenesis of migraine.

TRPV1 is a Ca^{2+} permeable channel and gated by noxious heat, oxidative stress, and capsaicin (McGarvey et al, 2014). TRPV1 belongs to transient receptor potential (TRP) family of cation channels. It was first characterized in primary afferent fibres as a receptor for capsaicin. Peripheral TRPV1 has a very well-described role in nociception, and TRPV1 in brain is emerging as an important molecular substrate which is dually implicated in both pain and psychiatric disorders, and represents a novel therapeutic target for these conditions and their comorbidity (Déciga-Campos et al, 2015; Madasu et al, 2015; Ren et al, 2015).

However, TRPV1 is now recognized to have a broader distribution and function, with supraspinal/brain TRPV1 known to modulate pain processing. Studies employing histological, genetic, and pharmacological approaches have provided the evidence that supraspinal TRPV1 also modulates brain neurobiology and behaviours related to anxiety, depression, and schizophrenia. In the present study, we found that ZTP significantly down-regulated the enhanced TRPV1 expression both in cortex and hippocampus of rats. Obviously, TRPV1 is one of the important molecular target of ZTP.

Except the effect of ZTP on TRPV1 protein expression level, in the present study, we also observed that ZTP showed significant efficacy on LTP induction both in the dentate gyrus *in vivo* and in CA1 of rats. LTP in the hippocampal area was induced by high-frequency stimulation (data not shown here). It is quite interesting that Puente, Canduela et al. reported that TRPV1 immunoparticles are highly concentrated in postsynaptic dendritic spines to asymmetric perforant path synapses in the outer 2/3 of the molecular layer, poorly at the excitatory hilar mossy cell synapses in the inner 1/3 of this layer, and the TRPV1 pattern distribution disappeared in the molecular layer of TRPV1-knockout mice. Therefore, the presence of TRPV1 in a brain region where the channel has been shown to have a functional role, such as the perforant path synapses in the hippocampal dentate molecular layer (Canduela et al, 2015; Puente et al, 2015). Tahmasebi et al reported that the cannabinoid agonist reduced both field excitatory post-synaptic potential slope and population spike amplitude after high frequency stimulation (HFS) with respect to the control group, whereas the TRPV1 agonists increased these parameters along with the increased induction of LTP. The co-administration of cannabinoid and TRPV1 agonists had different effects on fEPSP slope and PS amplitude. They concluded that TRPV agonists system could modulate the cannabinoid outputs that cause an increase in synaptic plastisity, while in contemporary consumption of two agonist, TRPV1 agonist can change the production of endocannabinoid, which in turn results to enhancement of LTP induction (Canduela et al, 2015; Puente et al, 2015; Tahmasebi et al, 2015). These findings suggest that the two

systems may interact or share certain common signaling pathways in the hippocampus. These reports are coincidence with what we have observed in the present study, which gives a clue that TRPV1 could be an important molecular mechanism of the effect of ZTP on migraine headache.

Conflict of interest statement

All authors declare no conflicts of interest.

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