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Decrease of morphine-CPP by sinomenine via mediation of tyrosine hydroxylase, NMDA receptor subunit 2B and opioid receptor in the zebrafish brain

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Abstract: To study the effects of sinomenine on conditioned place preference (CPP) zebrafish induced by morphine and expression levels of intracephalic tyrosine hydroxylase (TH), NMDA receptor subunit 2B (NR2B), μ-opioid receptor (zfmor) and δ-opioid receptors (zfdor1 and zfdor2), morphine (40mg/kg) was administrated to zebrafish and the effect of CPP was detected in these zebrafish treated with sinomenine. The expression of TH and NR2B was detected by immunohistochemistry; and the mRNA expression of opioid receptors zfmor, zfdor1 and zfdor2 in the zebrafish brain was assayed by RT-qPCR. In the CPP test, morphine induced significant behavioral alteration, while pretreatment with sinomenine or methadone, resulted in decreased activity time in the morphine-paired compartment significantly. Morphine also increased the integral optical density value of TH- and NR2B-positive cells in the zebrafish brain, and reduced the amount of opioid receptors. However, the compound sinomenine could attenuate these effects. These findings demonstrate that sinomenine (80mg/kg) decreased the CPP effects of zebrafish induced by morphine significantly, downregulated expression of TH and NR2B, and upregulated μ-opioid (zfmor) and δ-opioid (zfdor1 and zfdor2) receptor expression in the CPP zebrafish brains.

Keywords: Sinomenine, morphine, conditioned place preference, tyrosine hydroxylase, opioid receptors.

INTRODUCTION

Sinomenine is the main effective component of the Menispermaceae plant family, *Caulis Sinomenii*. *Caulis Sinomenii* is a dried plant stem of *Sinomenium acutum* and *Sinomenium acutum var. cinereum*. As a type of traditional Chinese medicine, *Caulis Sinomenii* is primarily used for the treatment of rheumatism and for analgesia and sedation. Pharmacological studies have demonstrated that sinomenine could reduce the release of intracephalic monoamine neurotransmitters and regulate the concentration of intracellular Ca^{2+} and thereby possesses a therapeutic effect for morphine withdrawal symptoms (Zhang *et al.,* 2009). It can also inhibit the contraction of isolated guinea pig ileum induced by naloxone-precipitated withdrawal in a dose-dependent manner (Wang *et al.,* 2003). However, sinomenine itself neither induces conditioned place preference (CPP) behavior in mice nor produces psychological or physical dependence (Mo *et al.,* 2004; Wang *et al.,* 2002). Taken together, these results indicate that sinomenine is a potential active ingredient with good application prospects for treating morphine dependence.

Opioid drugs (opiates) include opium, morphine and heroin, which are made from raw material from the opium poppy. In 1973, the high affinity receptor for opioid was found in the brain (Pert *et al.,* 1973; Simon *et al.,* 1973). Opioid receptors belong to the G protein-coupled receptor family, and can be classified into four subtypes: μ-opioid receptor (zfmor), δ-opioid receptor (zfdor1, zfdor2), κ-opioid receptor (zfkor) and opioidreceptor-like receptor (zforl), which are widely distributed in the brain and spinal cord (Gavril *et al.,* 1998). By activating the intracellular $G_{i/o}$ protein, opioid receptors can transmit extracellular signals into the cell and activate adenosine cyclase, thereby inhibiting the cyclic adenosine monophosphate (cAMP) signaling pathway (Bian *et al.,* 2012). Studies on related receptor agonists, inhibitors and gene knockout animals have demonstrated that opioid receptors mediate the rewarding effects of opioids and are associated with the formation of opioid dependence (Le Merrer *et al.,* 2011; Nguyen *et al.,* 2012). In addition, dopamine, glutamate, serotonin and other neurotransmitters in the central nervous system have a close relationship with drug dependence (Helmuth, 2001; Tassin, 2002; Thomas *et al.,* 2010).

Although the zebrafish nervous system is relatively simple, it can control learning, dependence, depression, and other complex behaviors of zebrafish, which is advantageous in studies of drug abuse and dependence (Guo, 2009; Klee *et al.,* 2012). There are relatively few reports of sinomenine on drug dependence. For example, sinomenine does not result in physical or psychological dependence or addiction in rats and mice, which suggests

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that sinomenine is safe for use (Wang *et al.,* 2003; Mo *et al.,* 2004). Another study reported that sinomenine could inhibit morphine withdrawal syndromes in mice or rats (Hu *et al.,* 2003). Moreover, one study reported that an ethanol-induced zebrafish CPP model has been successfully established (Mathur *et al*. 2011). Our team previously established the morphine-induced zebrafish CPP model in the early stage of study (Peng *et al.,* 2013). Methadone is an opioid receptor agonist with morphine-like pharmacological effects and methadone maintenance treatment (MMT) is high acceptability and stable efficacy, so, we used methadone as the positive drug (Joseph *et al.,* 2000).

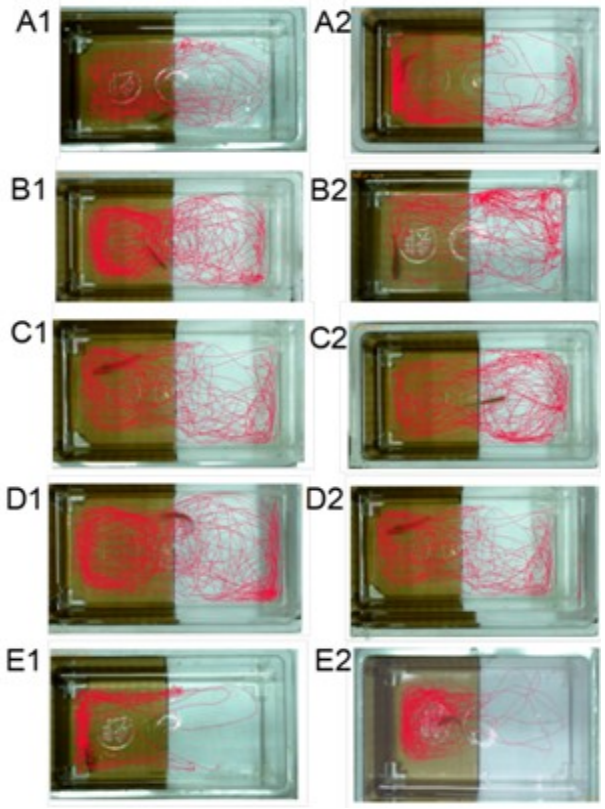

Fig. 1: Representative photographs of the swimming trajectories of zebrafish in the CPP compartment; each group consisted of 10 zebrafish. A1-E1 are the activity routes of zebrafish in the CPP compartment before CPP training (A1, control group; B1, morphine group; C1, low dose of sinomenine group; D1, high dose of sinomenine group; E1, methadone group); A2-E2 are the activity routes of zebrafish in the CPP compartment after CPP training (A2, control group; B2, morphine group; C2, low dose of sinomenine group; D2, high dose of sinomenine group; E2, methadone group).

In this study, we established a CPP model of zebrafish induced by morphine, to observe the effects of sinomenine on behavioral indicators of morphine-induced CPP zebrafish. We investigated the expression level of N-methyl-D-aspartate (NMDA) receptor subunit 2B (NR2B), glutamate receptor (GluR), tyrosine hydroxylase (TH), μ-opioid receptor (zfmor), and δ-opioid receptors (zfdor1, zfdor2) in morphine-induced CPP zebrafish brains. The experimental results will allow us to better understand the mechanism of sinomenine intervention on morphine dependence.

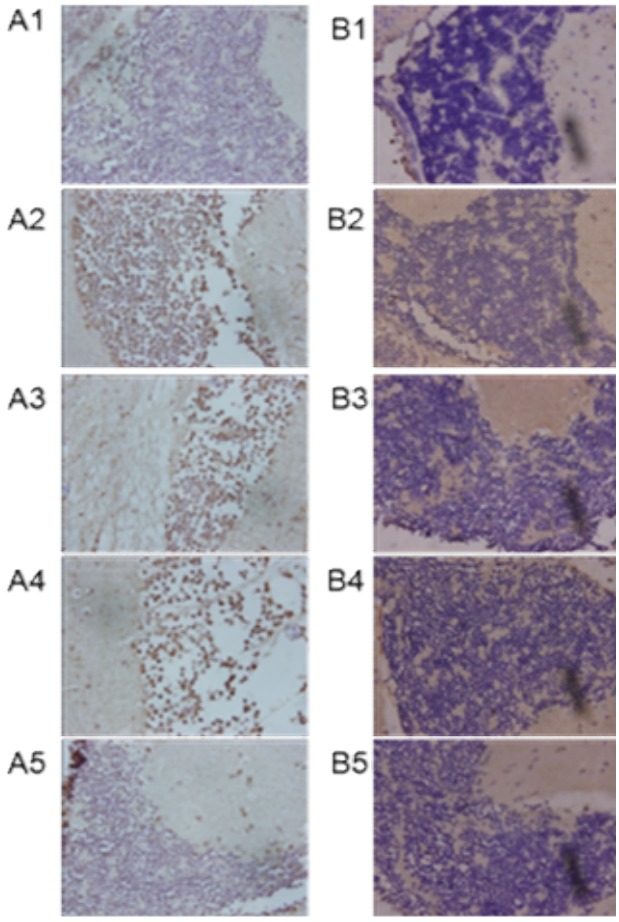

Fig. 2: Results of TH and NR2B immunohistochemistry of the zebrafish whole brain tissue. a1-a5 are TH-positive cells in zebrafish brain (A1, control group; A2, morphine group; A3, low dose sinomenine group; A4, high dose sinomenine group; A5, methadone group); B1-B5 are NR2B-positive cells in zebrafish brain (B1, control group; B2, morphine group; B3, low dose sinomenine group; B4, high dose sinomenine group; B5, methadone group).

MATERIALS AND METHODS

Animals

AB wild-type strain zebrafish (0.5-1g, 4-7 months old) were used in this research. All experimental operations were in accordance with the rules for use of laboratory animals of Southern Medical University and National Institutes of Health (NIH, USA). This research was allowed by the Southern Medical University Experimental Animal Ethics Committee.

Table 1: Primers used in real-time PCR

Gene	Sequence	Length/bp
EF1α	Sense:5'-GTACTTCTCAGGCTGACTGTG-3'	136
	Antisense:5'-ACGATCAGCTGTTTCACTCC-3'	
Zfmor	Sense:5'-ACGAGCTGTGCAAGATTGTG-3'	187
	Antisense:5'-CCGATTGCAGATGAAAGGAT-3'	
Zfdor1	Sense:5'-ACTATGAGAGCGTGGACCGTT-3',	116
	Antisense:5'-GCGGAGGAGAGGATCCAGAT-3'	
Zfdor2	Sense:5'-TCAGGCAAAACAATCTGCATG-3'	138
	Antisense: 5'-CAGGATCATCAGGCCGTAGC-3'	

Equipment and instruments

Table 2: Change in the time spent in the non-preferred compartment after injection of morphine. Data are expressed as mean ± SD, n=10. $^{\#\#}p<0.01$, v.s. morphine group; $^{**}p<0.01$, $^{*}p<0.05$, vs. control group.

Group	Dose (mg/kg)	Zebrafish time spent in non-preferred compartment (s)	
		pre-training	post-training
Control	--	239 ± 60	201 ± 60
Morphine	40	237 ± 103	531 ± 120**
Low dose of Sinomenine	40	222 ± 80	292 ± 138*
High dose of Sinomenine	80	260 ± 99	229 ±74 ##
Methadone	40	217 ± 100	261 ± 89##
F value		0.178	54.492
P value		0.512	0.009

Sinomenine decreased the expression of TH and NR2B

Table 3: The IOD values of TH- and NR2B-positive cells in zebrafish whole brain in each group. Data are expressed as mean ± SD, n=3. $^{\#\#}p<0.01$, vs. morphine group; $^{**}p<0.01$, vs. control group

Group	Dose(mg/kg)	TH (IOD) ($\times10^3$)	NR2B (IOD) ($\times10^3$)
Control	--	13.68 ± 2.47	26.59 ± 14.37
Morphine	40	62.36 ± 24.68**	85.49 ± 74.53 **
Low dose of Sinomenine Sinomenine	40	43.47 ± 14.90**	73.41 ± 25.78 **
High dose of Sinomenine	80	25.36 ± 3.68##	37.91 ± 34.688 ##
Methadone	40	20.47 ± 6.81##	27.68 ± 33.58 ##
F value		41.895	45.229
P value		0.000	0.000

Sinomenine increased the expression of opioid receptors zfmor, zfdor1 and zfdor2 in zebrafish brains.

Table 4: The relative mRNA expression of opioid receptors in the zebrafish whole brain. Data are expressed as mean ± SD, n=3. $^{\#\#}p<0.01$, vs. morphine group; $^{**}p<0.01$, $^{*}p<0.05$, vs. control group.

Group	Dose(mg/kg)	zfmor	zfdor1	zfdor2
Control	--	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Morphine	40	0.42 ± 0.05**	0.47 ± 0.05 **	0.47 ± 0.06 **
Low dose of Sinomenine	40	0.89 ± 0.12 ##	0.49 ± 0.06**	0.51 ± 0.08**
High dose of Sinomenine	80	0.90 ± 0.09 ##	0.81 ± 0.04** ##	0.82 ± 0.09** ##
Methadone	40	1.09 ± 0.08##	0.88 ± 0.11* ##	1.02 ± 0.14##
F value		60.231	55.383	75.254
P value		0.000	0.000	0.000

Drugs and reagents

Morphine hydrochloride (Lot# 710303, with purity ≥ 98%) was provided by pharmaceutical plant of PLA General Logistics Department; methadone (Lot# 020111) was bought from Tianjin Central Pharmaceutical Co., Ltd.; sinomenine (Lot# 20000528) was obtained from Hunan Zhengqin Medicine Co., Ltd., China. Tricaine methanesulfonate (MS222) was purchased from Sigma. Primers were synthesized by Genomics Institute of Beijing and the sequences are presented in table1. Total RNA extraction reagent, RNAiso Plus (TaKaRa company, Japan); reverse transcription kit (Lot# DRR037S, TaKaRa company, Japan); fluorescence quantitative reagent, SYBR®Select Master Mix dyes (Lot# 4472908, Life

Technologies); diethyl pyrocarbonate (Amresco company, USA); Anti-NR2B (Lot# AB1557P, Millipore); Anti-TH (Lot# MAB397, Millipore).

The CPP apparatus used in this study was consistent with the equipment reported in the previous literature of our research team (Jiang *et al.,* 2016). It is a rectangular tank and consisted of two equal-sized compartments. There was a transparent door in the neutral area of the tank providing access to each compartment. Swimming trajectories of zebrafish in CPP apparatus were detected by software of Noldus Ethovision XT 8.0 (Noldus, Netherlands).

CPP procedure

Zebrafish were randomly divided into five groups with 10 qualified zebrafish in each group: control group, morphine-only group, sinomenine 40mg/kg and 80mg/kg groups, and methadone 40mg/kg group. They prefer black versus than white versus, as confirmed by our pilot test (Jiang *et al.,* 2016). So, we considered the white side as non-preferred side. A baseline preference of zebrafish was detected by video cassette recorder for calculating activity distance and time in non-preferred side. They were tested in a CPP apparatus and the method of CPP paradigm was established by our previous reports (zhu *et al.,* 2017).

Immunohistochemistry for TH and NR2B expression

After the CPP test, we anesthetized zebrafish by chloral hydrate and euthanized them in water. The whole brain tissues of zebrafish were excised with 4% paraformaldehyde in 0.1M phosphate buffer saline for 24 h at 4°C. The method of immunohistochemistry was reported by our previous work (Jiang *et al.,* 2016).

RT-qPCR detection of zfmor, zfdor1, and zfdor2 expression in zebrafish brains

The total RNA in zebrafish whole brain tissue from each of the three samples per group was extracted individually according to the total RNA extraction reagent RNAiso Plus (TaKaRa Company) manual. The total RNA concentration was measured by an ultraviolet spectrophotometer, the ratio of A260/A280 nm was between 1.8-2.0, and the ratio of A260/A230 nm was between 2.0-2.2. All of the total RNA extractions were kept at -80°C until use. A 1μl total RNA of every extraction was synthesized individually into cDNA by the reverse transcription in line with instructions of reverse transcription kit (TaKaRa Company). A 1μl cDNA was assessed in line with manual of master mix dye by real-time PCR. We selected EF1α as a reference gene. All experiments were repeated three times.

STATISTICAL ANALYSIS

All data processing and statistical analysis are carried out by SPSS 17.0 software. Data were expressed as mean ± SD and analyzed by one-way analysis of variance. $p<0.05$ was considered statistically significant.

RESULTS

Sinomenine reduced the CPP effect of zebrafish caused by morphine

The effect of sinomenine on CPP zebrafish caused by morphine is presented in fig. 1 and analyzed in table 2. Zebrafish receiving morphine spent significantly more time on the white compartment than did the control zebrafish given fish physiological saline ($p<0.01$). The high doses of sinomenine or methadone spent significantly less time in the white compartment than did the morphine group ($p<0.01$), indicating that high doses of sinomenine and methadone reversed the CPP behavior induced by morphine in zebrafish.

As illustrated in fig. 2, the integral optical density (IOD) value of TH- and NR2B-positive cells in the zebrafish brains was significantly increased in the morphine group compared to the control group ($p<0.01$), and no significant difference was observed in the expression of TH and NR2B in the brains of zebrafish in the low dose sinomenine group compared to the morphine group. However, the high dose sinomenine reduced the IOD value of TH- and NR2B-positive cells ($p<0.01$), and similar results were observed in zebrafish treated with methadone ($p<0.01$). The IOD values of TH and NR2B immunoreactivity cells in the zebrafish brains are analyzed in table 3.

As is shown in table 4, the relative mRNA expression of opioid receptors in the brains of morphine-induced zebrafish was significantly lower than that of the control zebrafish ($p<0.01$). The high dose of sinomenine reversed the decreased relative mRNA expression of the intracorporal μ-opioid receptor (zfmor) and δ-opioid receptors (zfdor1 and zfdor2) in the morphine-induced zebrafish to similar levels to the control group, which indicated that a continuous injection of morphine could suppress the expression of opioid receptors in zebrafish brains. The high doses of sinomenine and methadone could upregulate the expression of zfmor, zfdor1, and zfdor2 in zebrafish brains, compared to that of the morphine group, and the difference was significant ($p<0.01$). The low dose of sinomenine reduced the expression of zfmor to normal levels, but no significant difference was observed in the expression of zfdor1 and zfdor2 compared to that of the morphine group ($p > 0.05$).

DISCUSSION

CPP is a classical conditioning task for assessing drug dependence (Cart, 1989). Our previous studies have investigated the optimal conditions for the formation of morphine-induced CPP in zebrafish (Peng *et al.,* 2013),

suggesting that morphine could induce zebrafish to prolong their activity time in non-preferred compartment (Khor *et al.,* 2011; Mathur *et al.,* 2011). The results of this research (fig.1) also indicate that we have successfully established a morphine-induced CPP model of zebrafish. The mesolimbic dopamine reward circuit is a common pathway of drug addiction, and consists of dopaminergic neuron and its projection targets including the hippocampus, amygdala and nucleus accumbens (Nestler, 2001). Functional upregulation of dopaminergic neurons is the neurobiological basis of triggering positive reinforcement effects and causing drug addiction (Berke *et al.,* 2000).

Tyrosine hydroxylase, as the key enzyme in the process of catecholamine biosynthesis, is the marker enzyme of dopamine neurons, the amount of which can reflect dopamine content (Rao *et al.,* 2007). GluRs are abundant in the brain, and can be classified into two large families: the ionotropic family and metabotropic family (mGluRs). There are two receptor subtypes of ionotropic GluRs: the NMDA and AMPA receptors which are closely related to drug dependence (Aitta *et al.,* 2012; Shen *et al.,* 2014). In this experiment, the expression of TH and NR2B was enhanced in the brains of zebrafish in morphine group (table 3) compared to the control group.

The CPP effect increased correspondingly, which confirmed that the formation of the morphine-induced CPP effect in the zebrafish brain is related to increasing protein expression of TH and NR2B. Additionally, the positive expression of TH and NR2B in the brains of zebrafish in the high dose sinomenine group and the methadone group was significantly decreased (table 3), while the CPP effect was also reduced accordingly (fig. 1). The results suggest that sinomenine can suppress zebrafish morphine-induced CPP effect to some extent, in addition to methadone, and that its mechanism may be related to modulating the expression of TH and NR2B. Our previous results demonstrated that *Sinomenium acutum* and sinomenine can return dopamine and other neurotransmitters in rat brains to normal levels after morphine withdrawal (Zhang *et al.,* 2009; Liu *et al.,* 2014). The NR2B receptors are involved in inducing a series of tolerance and withdrawal syndromes due to opioids abuse (Narita *et al.,* 2000). Phosphorylation of the NR2B amino acid residues plays an important role in the morphine-induced reward effect (Kato *et al.,* 2007). Ifenprodil, a highly selective antagonist of NR2B, is associated with a dose-dependent decrease in morphine-induced CPP effect in zebrafish, which is not caused by the environment or food (Ma *et al.,* 2006). Therefore, sinomenine may affect the expression of TH and NR2B or sequentially regulate the amount of dopamine in the brain or the interaction between glutamic acid and its receptors to inhibit the development of morphine dependence.

Dopaminergic neurons are dominated by inhibiting neurons of γ-aminobutyric acid (GABA) in different brain regions. Opioids are coupled with opioid receptors on the presynaptic membrane of GABA neurons, which can activate opioid receptors to produce an inhibitory effect on GABA neurons and reduce the release of the inhibitory neurotransmitter GABA in the ventral tegmental area. The decreased GABA level can trigger the dopamine level in the nucleus accumbens to rise and result in the rewarding effects and euphoria. Under normal physiological conditions, endogenous opioid peptides regulate and maintain a functional balance between the various systems of the body via interacting with opioid receptors. Long-term use of opioids results in the adaptive upregulation of the reward threshold, and breaking the steady state of endogenous opioid peptides, leading to drug tolerance, dependence, and addiction (Bretaud *et al.,* 2007; Le *et al.,* 2009).

There are opioid receptors in zebrafish that are functionally similar to those in mammals, which are known to be related to morphine addiction (Ninkovic *et al.,* 2006). The morphine-induced CPP effect in zebrafish could be antagonized by intraperitoneal injection of antagonists of opioid receptors or dopamine receptors (Bretaud *et al.,* 2007). In the μ-opioid receptor gene knockout mice, the CPP effect of morphine disappeared (Nguyen *et al.,* 2012). Therefore, the μ-opioid receptor is an important factor in the development of morphine dependency. The δ-opioid and μ-opioid receptors are present in the same neuron. The δ-opioid receptor found in the large, dense, core vesicles of the cytoplasm and is transported to the plasma membrane by vesicles when stimulated by morphine or pain (He *et al.,* 2002). The δ-opioid receptor does not directly regulate the rewarding effects of morphine, but it may promote contextual learning (Chefer *et al.,* 2006; Le *et al.,* 2011; Le *et al.,* 2012). In the process of drug dependence, the δ-opioid receptor plays an important role in regulating the μ-opioid receptor (Rozenfeld *et al.,* 2007). The δ-opioid and μ-opioid receptors can form a heterodimer, the formation of which is associated with morphine tolerance and dependence (Daniels *et al.,* 2005; Ananthan, 2006; Billa *et al.,* 2010). Therefore, pharmacological targeted drug delivery of the δ/μ heterodimer is a potential novel method for treatment of chronic pain and drug dependence (Stockton *et al.,* 2012).

Morphine can affect the expression of the opioid receptor gene in zebrafish (Sanchez *et al.,* 2010). The present study indicated that the relative expression of the μ-opioid receptor (zfmor) and δ-opioid receptors (zfdor1, zfdor2) were significantly decreased in the brains of zebrafish in morphine group compared to the control group (table 4), indicating that a continuous injection of morphine can suppress the expression of the μ-opioid and δ-opioid receptors in the zebrafish brain, resulting in down

regulation of opioid receptors. This is likely to be an adaptive adjustment of the organism for long-term use of morphine, but it will result in the dysfunction of the endogenous opioid peptides system after morphine withdrawal.

CONCLUSION

In this study, sinomenine and methadone were demonstrated to inhibit the reduction of the relative mRNA expression of zfmor, zfdor1, zfdor2 and return the levels to normal. This indicated that sinomenine may suppress the effect of morphine on opioid receptor expression in addition to methadone, and therefore maintain the balance of normal metabolic and organ functions after morphine withdrawal. The chemical structure of sinomenine consists of a hydrogen phenanthrene nucleus and ethylamine bridge, which is similar to the chemical structure of morphine; thus, sinomenine may play an effective role by acting on opioid receptors. In summary, the results of this experiment demonstrated that the decrease of morphine-CPP by sinomenine is associated with down-regulating the expression of TH and NR2B and up regulating the expression of zfmor, zfdor1, and zfdor2 in the zebrafish brain, but the specific mechanism requires further study.

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