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Effects of Electroacupuncture on Expression of D1 Receptor (D1R), Phosphorylation of Extracellular-Regulated Protein Kinase 1/2 (p-ERK1/2), and c-Fos in the Insular Cortex of Ketamine-Addicted Rats

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Background: The aim of this study was to investigate the effects of electroacupuncture (EA) on expression of the D1 receptor (D1R), phosphorylation of extracellular-regulated protein kinase 1/2 (p-ERK1/2) and c-Fos in the insular cortex (IC) of ketamine-addicted rats.

Material/Methods: Sprague-Dawley rats were randomly divided into 7 groups: the normal group, the normal saline (NS) group, the ketamine (Ket) group, the U0126+Ket group, the SCH23390+Ket group, the Ket+acupoints EA (EA1) group, and the Ket+ non-acupoints EA (EA2) group. We used immunohistochemistry to detect the expression of D1R, p-ERK1/2, and c-Fos. We also used Nissl staining techniques to study the morphology of IC neurons.

Results: Our study demonstrated that the ketamine group had sparsely distributed neurons, large intracellular vacuoles, nuclei shift, and unclear nucleolus. The number of Nissl-positive (neuronal) cells in the ketamine group were decreased than in the normal group. Our results also indicated that there was significantly lower expression of D1R, p-ERK1/2, and c-Fos in the IC of the U0126+Ket group, SCH23390+Ket group, and Ket+EA1 group as compared with that of the Ket group.

Conclusions: Ketamine addiction induces c-Fos overexpression in the IC by increasing the expression of D1R and p-ERK1/2. Acupoints EA downregulate D1R and p-ERK1/2 by reducing the overexpression of c-Fos.

MeSH Keywords: **Cerebral Cortex • Electroacupuncture • Extracellular Signal-Regulated MAP Kinases • Genes, fos • Ketamine • Receptors, Dopamine D1**

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Background

Ketamine is a commonly used anesthetic drug in clinical settings. However, it is also abused and is addictive. Previous studies have shown that ketamine increases the expression of c-Fos in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) [1,2]. Studies have reported that electroacupuncture (EA) reduces the overexpression of c-Fos in NAc and mPFC [1,2], but the mechanisms by which EA downregulates c-Fos expression in the addictive brain region is largely unknown. In this study we demonstrate that EA downregulates D1R and p-ERK1/2, thus reducing the overexpression of c-Fos in the insular cortex of ketamine-addicted rats.

Ketamine, a phencyclidine-based intravenous anesthetic, has become one of the most widely abused drugs in recent years due to its strong hallucinogenic effects and addictive properties. It is commonly known as “K” powder, being used for entertainment and abuse [3]. There is widespread concern about the abuse of ketamine as a drug of addiction in many countries. This concern has triggered investigations into the mechanism by which ketamine induces addiction, eventually laying a foundation for ketamine addiction management. Current research regarding ketamine addiction and treatment mechanisms is still limited.

The insular cortex (IC) is closely related to drug addiction behavior, and the fiber connection between IC and other addictive brain regions may be one of the important foundations for its regulation of drug-seeking behavior [4]. It has been reported that the expression of immediate early genes in IC is related to the cocaine-seeking behavior [5]. It has been found that ketamine can induce abnormal expression of the c-Fos protein in various brain regions, especially in the posterior cingulate cortex and splenium of the corpus callosum cortex. Furthermore, ketamine can cause vacuolar damage to neurons in this region, which is also the region involved in the onset of schizophrenia. Therefore, it is thought that the adverse reactions caused by ketamine may be related to damage in these areas [6].

Studies have shown that phosphorylation of extracellular-regulated protein kinase 1/2 (p-ERK1/2) can cause c-Fos to enter the nucleus and cause damage to cells [7]. Studies on drug addiction and abuse have shown that dopamine D1 receptor (D1R) plays a key role in cocaine, methamphetamine, and propofol addiction [8]. Also, the D1R antagonist SCH23390 inhibits propofol self-administration and decreases the expression of p-ERK1/2 in NAc [9]. It has also been found that U0126 is the specific blocker of the extracellular-regulated protein kinase 1/2 (ERK1/2) pathway, and it can also attenuate propofol self-administration behavior [10].

Our previous studies showed that ketamine caused an increase in the expression of c-Fos in NAc and mPFC. In addition, our studies also demonstrated that electroacupuncture (EA) at “Sanyinjiao” and “Zusanli” acupoints can reduce the overexpression of c-Fos in NAc and mPFC induced by ketamine [1,2]. However, it is unclear whether it is caused by D1R-mediated p-ERK1/2. The present study investigated the mechanism by which EA downregulates c-Fos overexpression in the brain region caused by ketamine addiction.

Material and Methods

Animals and grouping

A total of 42 male Sprague-Dawley (SD) rats, weight 200 ± 20 g, were purchased from the Experimental Animal Center of Zhejiang province (Animal certificate No.: SCXK (Zhejiang) 2014-0001). Rats were randomly divided into 7 groups: normal group, normal saline (NS) group, ketamine (Ket) group, U0126+ketamine (U0126+Ket) group, SCH23390+ketamine (SCH23390+Ket) group, ketamine+acupoints electroacupuncture (“Sanyinjiao” (SP 6) and “Zusanli” (ST 36), Ket+EA1) group, and ketamine+non-acupoints electroacupuncture group (outside the middle of the thigh and calf, Ket+EA2). There were 6 rats in each group. The treatment of rats in this study was in accordance to relevant provisions of the “Guiding Opinions on Treating Experimental Animals” issued by the Ministry of Science and Technology of China.

The main reagents and instruments

Rabbit anti-D1R, rabbit anti-p-ERK1/2, and rabbit anti-c-Fos were purchased from Beijing Bioss Company; SABC immunohistochemistry kits were purchased from Wuhan Boster Company; Nissl staining solution was purchased from Beyotime Biotechnology; SCH23390 and U0126 were purchased from MedChem Express USA; ketamine hydrochloride injection was purchased from GuTian Pharma, Fujian, China (specifications 2 ml: 100 mg, product batch number: 1709292). We used an Electronic Acupuncture Treatment Instrument (SDZ-II). We also used a frozen section machine (Thermo Fisher Scientific), an OLYMPUS BX51 fluorescence microscope, and the ImageJ image analysis system (Japan).

The experimental method

The ketamine addiction model was established by intraperitoneal administration of ketamine (50 mg/kg) once a day for 7 days [11]. U0126 (10 mg/kg) and SCH23390 (0.5 mg/kg) were given intraperitoneally (ip) 30 min before ketamine administration, once a day for 7 days, and once daily for 7 days after ketamine withdrawal. Acupoint EA (2 Hz, 1mA) was applied to unilateral “Zusanli” (ST 36, near the knee joint, 5 mm lateral

to the anterior tubercle of the tibia) and “Sanyinjiao” (SP 6, 10 mm above the medial malleolus) acupoints for 30 min [12] once daily for 7 days and once daily for 7 days after ketamine withdrawal. In addition to acupoints areas, needling was also done at the non-acupoints areas at the lateral part of the thigh and calf. Rats in the normal saline group were given normal saline (2 ml/kg ip), once a day for 7 days. The normal rats group acted as blank control. The expression of D1R, p-ERK1/2, and c-Fos proteins in IC were detected by immunohistochemistry. Nissl staining method was used to investigate IC neurons.

Nissl staining method

Nissl staining was used to distinguish viable neurons from apoptotic or neurotic neurons [13]. Frozen sections (thickness 20 μ m) were put in 4% paraformaldehyde for 15 min, then washed twice in distilled water for 2 min each time. Sections were immersed in Nissl staining solution for 6 min in a warm box (37°C), then rinsed with distilled water, dehydrated in ethanol, and cleared in xylene. The morphology and counts of Nissl-positive neurons in IC were observed under a microscope at 400 \times .

Immunohistochemical staining method

After the experiment, the rats were injected with 10% chloral hydrate (300 mg/kg) anesthesia [14]; brains were then dissected, further fixed in 4% PFA for another 24 h, and equilibrated in 20% and 30% sucrose-PBS solutions [15]. We removed the IC segment containing brain tissue, and serial sections (thickness 20 μ m) were cut. Sections were treated with 3% H₂O₂ dropwise for removal of endogenous peroxidase for 20 min, followed by antigen repair using PBT punching for 1 h. We used 5% normal sheep

serum closed at 37°C in a warm box for 40-min dropping D1R, p-ERK1/2, and c-Fos antibody (dilution ratio 1: 100) according to the SABC kit instructions, stained them with DAB, and mounted them. We randomly selected 2 slices from each rat, for a total of 12 slices in each group. We used image analysis system (Image J analysis system, Japan) values to measure the immunoreactive products of D1R, p-ERK1/2, and c-Fos immunoreactive cells with intact cell membranes of the insular cortex observed at 400 \times .

Statistical analysis

The experimental data were analyzed using SPSS 16 statistical software. One-way ANOVA was used for comparison of the number of D1R-, p-ERK1/2-, and c-Fos-positive cells among different groups. $P < 0.05$ was defined as a statistically significant difference.

Table 1. Comparison of number of Nissl-positive cells among different groups ($\bar{x} \pm s$, $n=6$).

Group	Count of Nissl-positive cells
Normal group	52.17 \pm 2.14
NS group	51.33 \pm 2.88
Ket group	31.17 \pm 2.32*
U0126+Ket group	49.50 \pm 3.21**
SCH23390+Ket group	51.50 \pm 2.43**
Ket+EA1 group	44.67 \pm 2.16**,*
Ket+EA2 group	31.17 \pm 2.32***

* Compared with the normal group, $P < 0.05$; ** compared with the Ket group, $P < 0.05$; *** compared with the Ket+EA1 group, $P < 0.05$.

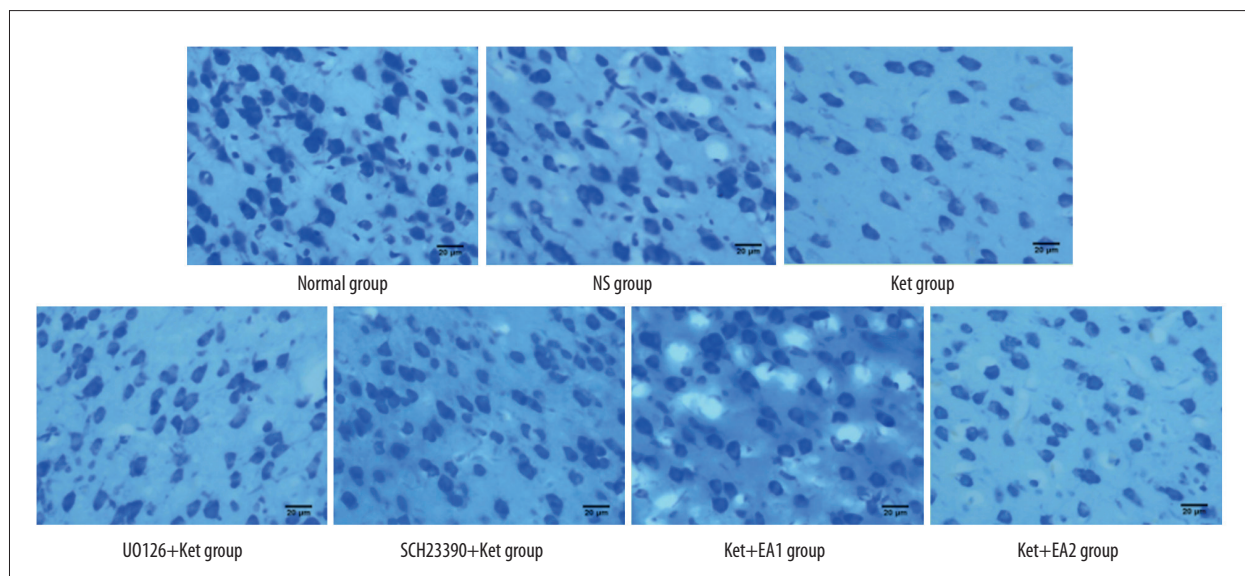


Figure 1. The expression of Nissl-positive cells in IC in each group of rats (Nissl, $\times 400$).

Table 2. Comparison of number of D1R positive cells among different groups ($\bar{x}\pm s$, n=6).

Group	Count of D1R-positive cells
Normal group	8.83±1.47
NS group	9.67±1.75
Ket group	20.17±2.14*
U0126+Ket group	4.33±1.03***
SCH23390+Ket group	4.17±0.98***
Ket+EA1 group	13.33±1.03***
Ket+EA2 group	21.50±1.52***

* Compared with the normal group, P<0.05; ** compared with the Ket group, P<0.05; *** compared with the Ket+EA1 group, P<0.05.

Results

Nissl staining

Nissl staining showed that the IC neurons were densely distributed, the Nissl-positive neurons structure was clear and complete, and the nuclei were centered and clear in the normal group and NS group. The Ket group had sparse distribution of neurons, large intracellular vacuoles, nucleus shift, and the nucleolus display was unclear. Compared with the normal group, the number of Nissl-positive cells was decreased (P<0.05) in the Ket group. The morphology of neurons in the U0126+Ket group, SCH23390+Ket group, and Ket+EA1 group were similar to that of the normal group. In the U0126+Ket group, SCH23390+Ket group, and Ket+EA1 group, the number of Nissl-positive cells increased (P<0.05) as compared to the Ket group. The morphology

Table 3. Comparison of number of p-ERK1/2 positive cells among different groups ($\bar{x}\pm s$, n=6).

Group	Count of p-ERK1/2-positive cells
Normal group	7.33±1.21
NS group	8.67±1.37
Ket group	13.33±1.37*
U0126+Ket group	3.83±0.75***
SCH23390+Ket group	4.33±1.03***
Ket+EA1 group	8.67±1.63**
Ket+EA2 group	14.17±1.17***

* Compared with the normal group, P<0.05; ** compared with the Ket group, P<0.05; *** compared with the Ket+EA1 group, P<0.05.

Table 4. Comparison of number of c-Fos positive cells among different groups ($\bar{x}\pm s$, n=6).

Group	Count of c-Fos-positive cells
Normal group	5.33±1.03
NS group	5.67±0.82
Ket group	16.33±1.03*
U0126+Ket group	4.33±1.03**
SCH23390+Ket group	5.50±1.05**
Ket+EA1 group	6.17±1.17**
Ket+EA2 group	13.83±0.98***

* Compared with the normal group, P<0.05; ** compared with the Ket group, P<0.05; *** compared with the Ket+EA1 group, P<0.05.

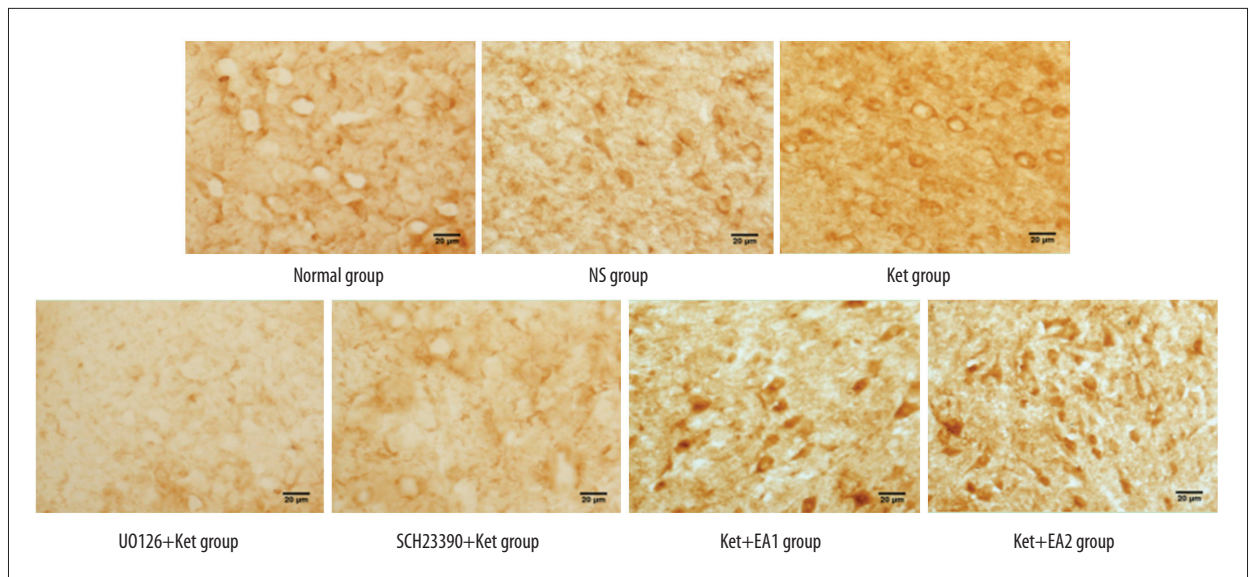


Figure 2. The expression of D1R positive cells in IC in each group of rats (IR, ×400).

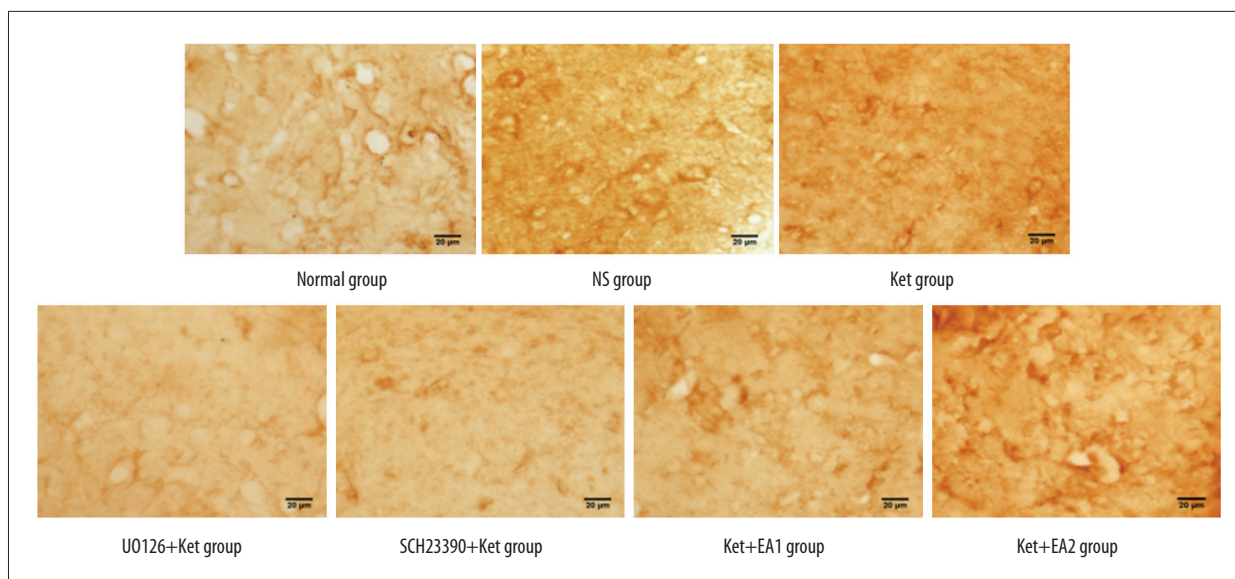


Figure 3. The expression of p-ERK1/2 positive cells in IC in each group of rats (IR, $\times 400$).

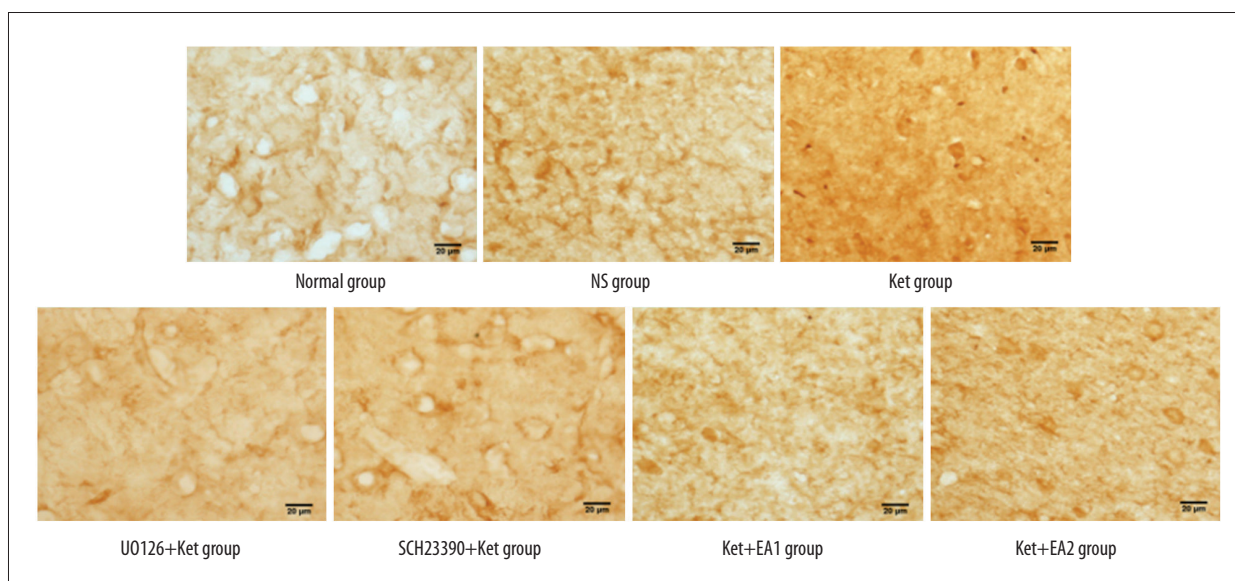


Figure 4. The expression of c-Fos positive cells in IC in each group of rats (IR, $\times 400$).

and the number of Nissl-positive neurons in the Ket+EA2 group were almost the same to that of the Ket group (Table 1, Figure 1).

Immunohistochemical staining

The expression of D1R, p-ERK1/2, and c-Fos immunoreactive protein was observed in the IC of each group. The positive cells were brownish-yellow and were mainly stained in the cytoplasm and membranes. In the normal group, the D1R-, p-ERK1/2-, and c-Fos-positive cells were small and sparse. There was no significant difference in the number of D1R-, p-ERK1/2-, and c-Fos-positive cells between the NS group and the normal group ($P > 0.05$). Compared with the normal group, the number of D1R-, p-ERK1/2-,

and c-Fos-positive cells were increased ($P < 0.05$) in the Ket group, and the cells were deeply stained. In comparison with the Ket group, the number of D1R-, p-ERK1/2-, and c-Fos-positive cells were decreased in the U0126+Ket group, SCH23390+Ket group, and Ket+EA1 group ($P < 0.05$). There were more D1R-, p-ERK1/2-, and c-Fos-positive cells in the Ket+EA2 group than in the Ket+EA1 group ($P < 0.05$). (Tables 2–4, Figures 2–4).

Discussion

The IC is a part of the cerebral cortex and is hidden deep in the brain. The role of the IC in nicotine addiction has gradually

gained attention since the discovery that IC damage caused nicotine addicts to avoid recurrence of smoking and stop relapsing [16]. The results of Nissl staining showed that ketamine addiction can cause neuronal damage in the IC, suggesting that IC might be one of the target sites of nerve damage caused by ketamine addiction.

The results of immunohistochemistry showed that D1R, p-ERK1/2, and c-Fos immunoreactive products had abnormally high expression in IC neurons of ketamine-addicted rats. However, high expression of D1R, p-ERK1/2, and c-Fos immunoreactive products was not observed in the IC when treated with SCH23390 (D1R antagonist) and U0126 (a specific blocker of the ERK1/2 pathway). Previous studies on drug addiction and abuse [8–10] have shown that D1R plays a key role in cocaine, methamphetamine, and propofol addiction. SCH23390 and U0126 inhibit propofol self-administering behavior by reducing the expression of p-ERK1/2 in NAc. Some researchers reported that phosphorylation of extracellular-regulated protein kinase (ERK1/2) can cause c-Fos to enter the nucleus and damage cells [7]. Ketamine is a non-competitive N-methyl-d-aspartic (NMDA) receptor antagonist that often causes undesirable psychotic symptoms such as nightmares and hallucinations. It has been found that ketamine can induce abnormal expression of the c-Fos protein in various brain regions, especially in the posterior cingulate cortex and cortex in the corpus callosum, and can cause vacuolar damage in this area. This site is also related to the onset of schizophrenia, so it is believed that the adverse reactions caused by ketamine may be related to damage in these areas [6]. The availability of D1R in the dorsal prefrontal cortex of ketamine addicts was significantly increased [17], and the pharmacological activation of D1R significantly promoted ERK activity and phosphorylation in different nerve cells [18]. Our results suggest that ketamine addiction is induced by D1R-mediated activation of p-ERK1/2, resulting in abnormally high expression of c-Fos protein in the IC region, which in turn causes neurological damage to the IC. Studying the expression of the c-fos gene in the addiction-related part of the brain and understanding the changes in the central nervous system after addiction can reveal the drug addiction mechanism and help develop new therapeutic methods.

The use of acupuncture therapy is common in China in the field of drug addiction prevention and treatment and shows great efficacy. Since the introduction of acupuncture and detoxification in the 1970s, this therapy has spread widely around the world, opening up a new approach to detoxification. Its efficacy has been widely recognized by clinicians and patients [19]. Studies have shown that heroin addiction causes chronic and persistent damage to brain tissue, and can cause extensive damage to the brain of rats, mainly by neuronal degeneration. Acupuncture can interfere with the behavioral changes of conditioned position preference in heroin-relapse rats, reduce the brain tissue

damage caused by heroin, and protects neurons [20]. It has been observed experimentally that heroin addicts have increased cerebral blood flow and oxygen consumption in brain areas such as the IC after viewing heroin in a clear glass bottle, which indicates that stimulation by environmental clues can induce specific activation of heroin craving and increased neurological activity. After acupuncture at the Zusanli point, activation of the brain area associated with craving was decreased, the blood flow and perfusion in the activated brain area was reduced, and the activity of local neurons was weakened. These results suggest that acupuncture can inhibit the brain area induced by heroin cue activation and craving, and to some extent the cue-induced craving can produce immediate intervention effects [21]. The “Sanyinjiao” point is a point of meridian intersection of foot and 3 yin, which can strengthen the spleen and dampness, and replenish the liver and kidney. “Zusanli” is a strong point for the whole body; it can raise and clear turbidness, invigorate spleen and stomach, regulate qi, and stop vomiting, and acupuncture can achieve the effect of regulating the balance of yin and yang in drug addicts [22]. In addition, some scholars have shown that “Zusanli” and other electroacupuncture acupoints have a benign regulatory effect on the expression of Bcl-2, Bax, and caspase-3 in cerebral cortex neurons of rats with focal cerebral ischemia, but non-acupoint electroacupuncture does not have this regulation effect [23]. In the present study, the experimental EA group, the “Sanyinjiao” and “Zusanli” points were selected as EA groups, and a non-acupoint EA control group was established. The results of Nissl staining and immunohistochemistry showed that acupoint EA can protect neurons of the IC and significantly inhibit the overexpression of D1R, p-ERK1/2, and c-Fos caused by ketamine addiction in the IC region, but the non-acupoint EA had no effect.

Studies have shown that EA can reduce the expression of p-ERK1/2 in the anterior cingulate cortex of rats with neuropathic pain, which may be related to the change of anxiety induced by neuropathic pain [24]. EA can downregulate the expression of D1R in the striatum of rats with cerebral ischemia reperfusion, and plays a protective role in the brain [25]. In summary, the results of this study show that EA downregulates the abnormal expression of c-Fos in the IC region induced by ketamine addiction and protects neurons. The mechanism may be related to the benign regulation of the expression of key factors such as D1R and p-ERK1/2 by EA at acupoints. One limitation of our study is that at present we do not have the experimental conditions for establishing a rat model of ketamine self-administration. The other limitation is that the mechanism by which EA downregulates the overexpression of c-Fos and the inhibition of neuronal apoptosis in the brain area of subjects with drug addiction is not clear. In addition, there may be other mechanisms by which EA downregulates the overexpression of c-Fos in the brain region of drug addicts, which also needs further research.

Conclusions

Ketamine addiction induces overexpression of c-Fos protein in the IC by increasing expression of D1R and p-ERK1/2. Acupoints

EA reduces the overexpression of c-Fos protein in IC by down-regulating the expression of D1R and p-ERK1/2. This study therefore provides theoretical insight into clinical treatment of ketamine addiction.

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