Penehyclidine hydrochloride improves cognitive function of rats with brain injury via CAMP/CREB signaling pathway

Yuting Wang1,2, Wen Zhao1,2, Shuhui Zhu2, Liqun Wei1,*

1 Department of Anesthesiology, Huashan Hospital, Fudan University, Shanghai, China
2 Shanghai Medical College, Fudan University, Shanghai, China

Abstract

This study explored the impact of penehyclidine hydrochloride on cognitive function in rats with brain injury. Sprague-Dawley rats (n=36) were randomly assigned to sham-operation, model, and penehyclidine hydrochloride groups. Rats in the sham-operation group underwent craniotomy, while the model and penehyclidine hydrochloride groups received brain injury models and interventions with normal saline and penehyclidine hydrochloride, respectively. Specimens were obtained two weeks post-intervention. Neurological deficits were evaluated using Zea-Longa scores, and memory was assessed with the Morris water maze test. ELISA determined brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) content. mRNA expressions of BDNF and NGF were assessed via qPCR, and phosphorylated CREB (p-CREB) protein expression was measured by Western blotting. Compared to the sham-operation group, both model and penehyclidine hydrochloride groups showed increased Zea-Longa scores. Escape latencies were longer and platform crossings were fewer in model and penehyclidine hydrochloride groups compared to the sham-operation group, but penehyclidine hydrochloride demonstrated a shorter latency and more platform crossings than the model group. BDNF and NGF content decreased in model and penehyclidine hydrochloride groups compared to the sham-operation group, with an increase in the penehyclidine hydrochloride group compared to the model group. mRNA expression levels declined in model and penehyclidine hydrochloride groups but were higher in the latter. p-CREB protein expression was lower in model and penehyclidine hydrochloride groups compared to the sham-operation group but higher in the penehyclidine hydrochloride group than the model group. Penehyclidine hydrochloride exhibited neuroprotective effects by upregulating the cAMP/CREB signaling pathway, improving cognitive function in rats with brain injury.

Keywords: Brain injury, Penehyclidine hydrochloride, cAMP/CREB signaling pathway, Cognitive function

1. Introduction

Brain injury is a prevalent and debilitating condition, characterized by severe trauma to brain tissue, leading to cognitive impairment, memory deterioration, dementia, anxiety, and apathy in affected individuals [1-3]. The rising incidence of injuries from events such as traffic accidents and high-altitude falls has contributed to an increasing morbidity rate associated with brain injury, posing significant threats to patients' work capacity, quality of life, and overall safety.

The pathology of brain injury encompasses intricate mechanisms and responses that significantly impact the central nervous system's progression and recovery following injury [4,5]. Ischemic and hypoxic changes, along with ischemia/reperfusion injury in brain tissues, constitute critical elements of this complex process. In the quest for effective management, various treatments have been employed, each with its strengths and limitations. Common treatments for brain injury include pharmacological interventions, such as anti-inflammatory drugs and neuroprotective agents, aimed at mitigating the inflammatory response and reducing secondary damage. Surgical interventions, such as decompressive craniectomy, may be implemented to alleviate intracranial pressure. Rehabilitation therapies, including physical, occupational, and speech therapy, play pivotal roles in facilitating recovery and restoring functionality. Additionally, supportive measures like nutritional support and psychological counseling contribute to holistic patient care. While these treatments have demonstrated varying degrees of efficacy, they are not without shortcomings. Pharmacological interventions may have limited success in addressing the multifaceted nature of brain injury pathology. Surgical interventions, while relieving intracranial pressure, may pose risks and complications. Rehabilitation therapies often face challenges in achieving optimal patient participation and adherence. Furthermore, the individualized nature of brain injury responses necessitates a tailored and comprehensive approach, which may be challenging to implement consistently.
Penecyclidine hydrochloride, a commonly used selective anti-cholinergic drug in clinical practice, has demonstrated its potential to improve microcirculation, dilate capillaries, and protect brain tissues. However, the precise mechanisms underlying these effects remain unclear [6, 7]. The cyclic adenosine monophosphate (cAMP)/cAMP-responsive element-binding protein (CREB) signaling pathway is intricately linked to brain tissue development, nervous system repair, and cognitive function. Therefore, this research aims to investigate the impact of penecyclidine hydrochloride on the cognitive function of rats with brain injury by modulating the cAMP/CREB signaling pathway.

Through this exploration, we seek to unravel the specific mechanisms by which penecyclidine hydrochloride influences cognitive function in the context of brain injury. The findings from this study may provide valuable insights into the development of therapeutic strategies aimed at enhancing cognitive recovery and quality of life for individuals affected by brain injury.

2. Materials and methods

2.1. Laboratory animals

A total of 36 SPF-grade laboratory Sprague-Dawley (SD) rats of either sex (1 month old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. [license No.: SCXK (Shanghai, China) 2014-0003]. Those rats were fed in the Laboratory Animal Center with normal diet and sterile filtered water every day under a 12/12 h light/dark cycle, conventional room temperature and humidity. This study was approved by the Animal Ethics Committee of Fudan University Animal Center.

2.2. Reagents and instruments

Penecyclidine hydrochloride was bought from Chengdu List Pharmaceutical Co., Ltd. (Chengdu, China), anti-phosphorylated (p)-CREB primary antibody and secondary antibody were provided by Abcam (Cambridge, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kit and quantitative polymerase chain reaction (qPCR)-related kits were offered by Vazyme (Nanjing, China). A light microscope was purchased from Leica (Wetzlar, Germany), a fluorescence qPCR instrument was bought from ABI (Applied Biosystems, Foster City, CA, USA), and a water maze was provided by the Chinese Academy of Sciences.

2.3. Grouping and intervention

The 36 SD rats of either sex were assigned to sham-operation group (n=12), model group (n=12) and penecyclidine hydrochloride group (n=12) using a random number table. The rat model of brain injury was established in penecyclidine hydrochloride group, and penecyclidine hydrochloride solution (0.01 mg/kg) was intraperitoneally injected daily after operation. The rats in model group were fed with 3% pentobarbital sodium solution (5 mL/kg) injected intraperitoneally. After successful anesthesia, the head of the rat was deplated and disinfected, followed by skin preparation. Then the rats were fixed on an operation table, a longitudinal incision (about 3 cm) was made along the midline of scalp, and the periosteum was stripped. Next, a bone window was prepared at the middle of lambdoid suture and right anterior fontanelle, so as to expose the cerebral dura mater. Later, the cerebral dura mater in the bone window was hit by an impactor (height: 2 mm, velocity: 2 m/s, contact time: 80 s). Finally, the wound was sutured, and the rats were raised in separate cages.

2.4. Modeling methods

The rats were anesthetized with 3% pentobarbital solution (5 mL/kg) injected intraperitoneally. After successful anesthesia, the head of the rat was deplated and disinfected, followed by skin preparation. Then the rats were fixed on an operation table, a longitudinal incision (about 3 cm) was made along the midline of scalp, and the periosteum was stripped. Next, a bone window was prepared at the middle of lambdoid suture and right anterior fontanelle, so as to expose the cerebral dura mater. Later, the cerebral dura mater in the bone window was hit by an impactor (height: 2 mm, velocity: 2 m/s, contact time: 80 s). Finally, the wound was sutured, and the rats were raised in separate cages.

2.5. Evaluation of neurological deficit of rats using Zea-Longa score

At 1 h after the last intervention, the neurological deficit of the rats was independently assessed by 2 researchers familiar with the Zea-Longa scoring rules according to the symptoms and manifestations of the rats.

2.6. Evaluation of rat memory through Morris water maze test

The rats were put into the water maze after the last intervention, where they were allowed to move freely. The escape latency and the times of crossing the platform in the water maze of the rats were recorded. After that, the platform in the water maze was withdrawn, and the rats were put into the water maze from any place. As the rats moved freely, the times of crossing the original platform and the residence time in the original quadrant were recorded within 70 s.

2.7. Specimen acquisition

The rats were anesthetized with 3% pentobarbital sodium solution (5 mL/kg) via intraperitoneal injection. Then the brain tissues were directly obtained from 6 randomly selected rats in each group, flushed with normal saline, put into Eppendorf (EP) tubes and stored at -80°C for later use. As for the remaining 6 rats in each group, the thoracic cavity was cut open to expose the heart, and 400 mL of 4% paraformaldehyde was perfused from the left atrial appendage. Subsequently, the brain tissues were taken out and immediately immersed with 4% paraformaldehyde solution, followed by fixation for 48 h.

2.8. Detection of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) content in brain tissues via ELISA

The freshly obtained brain tissues were mashed. Based on the instructions of the ELISA kit, samples were loaded, and standard substance, biotinylated antibody working solution and enzyme conjugate working solution were added. Finally, the plate was washed, and the tissues were detected using a microplate reader at 450 nm.

2.9. qPCR assay

The fresh brain tissues were added with ribonucleic acid (RNA) extraction reagent to extract the total RNA in the specimens. Next, the total RNA obtained was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using a reverse transcription kit and a designed reaction system (20 μL) under the following conditions: reaction at 53°C for 5 min, pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s and annealing at 62°C.
for 30 s for 35 cycles. ΔCt value was calculated first, and then the expression difference of target genes was calculated. The detailed primer sequences are shown in Table 1.

### 2.10. Western blotting assay

The lysis buffer was added into the cryopreserved brain tissues for 1 h of ice bath, then the tissues were centrifuged at 14,000 g for 10 min in a centrifuge, and the proteins were quantified using biocinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Next, the absorbance and standard curve of the proteins were obtained through the microplate reader, based on which the protein concentration in tissues was calculated. Subsequently, the proteins in tissue specimens were subjected to denaturation and separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The position of the Marker proteins was observed, and the electrophoresis was stopped when the Marker proteins reached the bottom of glass plate in a straight line. Later, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and reacted with blocking buffer for 1.5 h. After that, anti-p-CREB primary antibody (1:1000) and secondary antibody (1:1000) were added in sequence. Finally, the image was fully developed with chemiluminescent reagent in the dark for 1 min after rinsing.

### 2.11. Statistical analysis

In this research, Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was adopted for statistical analysis. The measurement data were expressed as mean ± standard deviation. t-test was performed for the data meeting normal distribution and homogeneity of variance, corrected t-test was used for the data meeting normal distribution and heterogeneity of variance, and non-parametric test was conducted for the data not meeting normal distribution and homogeneity of variance. The rank sum test was utilized for the ranked data, and the enumeration data were examined by chi-square test.

### 3. Results

#### 3.1. Zea-Longa score in each group

Compared with sham-operation group, model group and penehyclidine hydrochloride group had notably increased Zea-Longa scores, with statistically significant differences (P<0.05), while penehyclidine hydrochloride group exhibited a remarkably decreased Zea-Longa score in comparison with model group, and the difference was statistically significant (P<0.05) (Figure 1).

#### 3.2. Water maze test results in each group

The model group and penehyclidine hydrochloride group displayed obviously longer escape latencies and markedly fewer times of crossing the original platform than sham-operation group, showing statistically significant differences (P<0.05). However, the escape latency was evidently shortened, but the times of crossing the original platform were increased distinctly in penehyclidine hydrochloride group in contrast model group, and there were statistically significant differences (P<0.05) (Figure 2).

### Table 1. Primer sequences.

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
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<tbody>
<tr>
<td>NGF</td>
<td>5’TGTTAAAAAAGCGGCGACTCC3’</td>
<td>5’TCCTTGCCCTTGTAGTCTGG3’</td>
</tr>
<tr>
<td>BDNF</td>
<td>5’CTGTATCAAAAAAGCCAAGCTGAA3’</td>
<td>5’GTGTCTATCCTTATGAAATCGGCA3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’ACGGCAAGTTCAACGGACACAG3’</td>
<td>5’GAAGACGCCAGTAGACTCCACGAC3’</td>
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</table>
Penecyclidine hydrochloride group compared with that in sham-operation group, and it was raised clearly in penecyclidine hydrochloride group in comparison with that in model group. All the differences were statistically significant (P<0.05).

3.4. Messenger RNA (mRNA) expressions of BDNF and NGF in brain tissues in each group determined through qPCR

The relative mRNA expression levels of BDNF and NGF in brain tissues declined prominently in model group and penecyclidine hydrochloride group in contrast with those in sham-operation group, and the differences were statistically significant (P<0.05). Furthermore, the opposite results were observed between penecyclidine hydrochloride group and model group, with statistically significant differences (P<0.05) (Figure 4).

3.5. Protein expression of p-CREB in brain tissues in each group measured by Western blotting

The protein expression of p-CREB was high in sham-operation group but low in model group (Figure 5A). According to the statistical results (Figure 5B), the relative protein expression level of p-CREB in brain tissues was apparently lower in model group and penecyclidine hydrochloride group than that in sham-operation group, displaying statistically significant differences (P<0.05), whereas it was remarkably higher in penecyclidine hydrochloride group than that in model group, and the difference was statistically significant (P<0.05).

4. Discussions

Brain injury is a common outcome of traumatic cranio-cerebral injury, in which trauma and violence usually lead to a series of pathological lesions in brain tissues, including hemorrhage, ischemia, hypoxia and ischemia/reperfusion injury, resulting in apoptosis, necrosis, autophagy and other pathological responses of brain neurons and damaging the normal physiological functions of the central nervous system. Studies have demonstrated that the greatest impact of brain injury on the physiological functions of the brain is to affect the cognitive function of patients so that the patients manifest memory deterioration, cognitive impairment, anxiety and apathy, which severely influence patients’ work capacity and quality of life and even threaten their lives [8-10]. Therefore, it is essential to deeply investigate the related pathological mechanisms and responses after brain injury as well as therapeutic methods for the disease. It has been discovered through studies that neuronal damage (apoptosis, necrosis, autophagy, etc.) in the brain induced by brain injury is not only an important cause of affected brain functions but also a crucial pathological factor for cognitive impairment in patients [11,12]. Hence, how to protect the brain neurons after brain injury is one of the vital strategies to repair the nervous system and ameliorate cognitive impairment following brain injury. Both BDNF and NGF are pivotal neurotrophic factors in organisms, which exert important nutritional effects on the brain neurons. Specifically, they can promote the development and growth of the brain neurons, facilitate the synapse formation in the brain and maintain the normal physiological functions of neurons. According to research [13,14], BDNF and NGF are able to stimulate the repair and remyelination of neurons after brain injury, thus playing a vital neuroprotective role. As one of the crucial signaling pathways in the body, the cAMP/CREB signaling pathway have important functions in the development and generation of neurons, formation of synapses and maintenance of neurological functions. Studies have revealed that the high expression of cAMP in the signaling pathway can prominently promote the phosphorylation of its downstream CREB and play an essential regulatory role by increasing p-CREB [15-17]. Based on further research, BDNF and NGF are downstream effectors of the cAMP/CREB signaling pathway, and the activation of this signaling pathway exerts pivotal neuroprotective effects through enhancing the expressions of BDNF and NGF. It was found in this research that the protein expression of p-CREB, a key molecule of the cAMP/CREB signaling pathway, was decreased clearly in the brain tissues of rats with brain injury, suggesting that the phosphorylation level of CREB is lowered, and the cAMP/CREB signaling pathway is repressed. Meanwhile, the content of BDNF and NGF was reduced. Therefore, it is inferred that the cAMP/CREB signaling pathway is inhibited in the pathological process of brain injury, thereby affecting the expressions of BDNF and NGF, which may be an important pathologi-
Penehyclidine hydrochloride in rats with brain injury

It has been confirmed that penehyclidine hydrochloride, a novel selective anti-cholinergic drug, has favorable effects on microcirculation improvement, capillary dilatation and brain tissue protection. Studies have uncovered that penehyclidine hydrochloride is capable of alleviating and preventing brain injury to some extent, as well as ameliorating cognitive impairment following brain injury [18,19]. The results of this research also verified that penehyclidine hydrochloride could improve neurological deficit and memory impairment (cognitive impairment) in rats with brain injury. Besides, it facilitated the phosphorylation of CREB in the cAMP/CREB signaling pathway and increased the expressions of BDNF and NGF that have neuroprotective effects.

5. Conclusions

It can be seen that penehyclidine hydrochloride stimulates the expressions of BDNF and NGF by up-regulating the cAMP/CREB signaling pathway, thus exerting neuroprotective effects and improving the cognitive function of rats with brain injury.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of Fudan University Animal Center.

Informed Consent

The authors declare not used any patients in this research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors’ contributions

Yuting Wang, Wen Zhao: Conceptualization, methodology, writing original draft preparation. Shuhui Zhu: Investigation, software, statistical analysis. Bin Du: Reviewing and editing, funding acquisition, supervision. All authors read and approved the final manuscript.

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