The Role of Potassium Channels in Chronic Stress-Induced Brain Injury

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Summary

Chronic stress-induced brain injury (CSBI) is the organic damage of brain tissue caused by long-term psychological and environmental stress. However, there is no effective drug for the treatment of CSBI. The present study aimed to investigate possible mechanisms of CSBI and to explore related therapeutic targets. A rat model of CSBI was established by combining chronic restraint and cold water immersion. Our CSBI model was validated via Nissl staining, Western blotting, and behavioral tests. RNA sequencing (RNA-seq) was used to identify differentially expressed genes (DEGs) within brain tissue during CSBI. Both Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses were performed to determine signaling pathways associated with CSBI-induced DEGs. Agonists/antagonists were used to validate the pharmacodynamics of potential therapeutic targets. A combination of chronic restraint and cold water immersion successfully induced a rat model of CSBI, as indicated by various markers of brain injury and cell apoptosis that were verified via Nissl staining, Western blotting, and behavioral tests. RNA-seq analysis identified 1,131 DEGs in CSBI rats. Of these DEGs, 553 genes were up-regulated and 778 genes were down-regulated. GO and KEGG pathway analyses revealed that significant DEGs were predominantly related to membrane-bound ion channels, among which the potassium channel function was found to be significantly affected. Pharmacological experiments revealed that retigabine, a voltage-gated potassium
channel opener, demonstrated a protective effect in CSBI rats. Taken together, our findings suggest that potassium channel function is disrupted in CSBI, and that potassium channel regulators may function as anti-CSBI drugs.

**Keywords:** chronic stress; brain injury; potassium channels; RNA-seq; bioinformatics; retigabine
1 Introduction

The stress response is a collective term that includes neural, endocrine, and other physiological responses to environmental, psychological, and/or homeostatic stressors.\(^1,\ 2\) When stimulated by stressors such as hunger, disease, and fear, multiple coordinated systems are activated—such as the hypothalamic pituitary adrenal (HPA) axis\(^3\) and the sympathetic nervous system (SNS)\(^4\)—which can promote cognition, immune function, metabolism, and homeostasis of the internal environment. However, long-term stress can reverse the beneficial and protective effects of acute stress, leading to serious diseases.\(^5\)

Many neuropsychiatric disorders are associated with chronic stress. Chronic stress is known to be one of the important causes of depression. Stressful events such as economic difficulties, unemployment, and diseases contribute to depression in adults; similarly, children are affected by severe stressors such as violence and abuse during childhood, which are known to also greatly increase the incidence of depression.\(^6\) In addition, chronic stress often increases the risk of diseases such as hypertension and diabetes. In a study of 500 diabetic patients, Madu and colleagues found that stress levels experienced by diabetic patients were significantly higher than those of healthy controls.\(^7\) Another study has shown that chronic stress can result in hypertension in rats.\(^8\) Chronic stress also affects the immune system and cardiovascular function.\(^9,\ 10\)
The brain plays a commanding role in stress responses throughout the body; it is the most sensitive organ to stress responses. Prolonged or intense stress can hinder the function of neurons in various regions of the brain. For instance, in rats, it has been shown that hippocampal neurogenesis is not affected by acute restraint stress, but it is inhibited after three weeks of chronic restraint stress, during which a concomitant increase in the number of immature neurons is also observed.\textsuperscript{11}) Similarly, another study showed that 21 days of chronic restraint stress resulted in the atrophy of apical dendrites of CA3 pyramidal neurons, as well as an increased expression of apoptosis-related proteins in the prefrontal cortex.\textsuperscript{12,13}) In addition, repeated restraint stress can lead to neuronal remodeling and changes in synaptic transmission within the amygdala.\textsuperscript{14}) Collectively, these findings indicate that chronic stress reduces neuron survival, and induces brain injury.

Chronic stress-induced brain injury (CSBI) is a type of brain injury caused by chronic stress; it includes anxiety- and depressive-like behaviors, and a reduction of survival neurons. If CSBI is not effectively treated, more serious psychiatric disorders will eventually develop. Therefore, it is essential to discover and develop drugs for the treatment of CSBI, which may also prevent the occurrence of chronic stress-triggered diseases.

This study aims to investigate the possible mechanisms of CSBI, and to discover potential therapeutic drugs. A rat model of CSBI was established via a combination of
chronic restraint stress and cold water immersion, which were used, respectively, to simulate psychological and environmental stressors. Furthermore, Nissl staining, Western blotting, and behavioral testing, including the open field test, forced swimming test, and sucrose preference test, were used to validate the establishment of brain injury in our CSBI model. Subsequently, RNA sequencing was used to detect differentially expressed genes (DEGs) within brain tissue after chronic stress. Finally, bioinformatic analysis was employed to assess interactions between DEGs and possible mechanisms that induce CSBI in order to identify single or multiple drug targets for treating stress responses.

2 Materials and Methods

2.1 Animals and experimental groups

Specific pathogen-free (SPF) female Sprague-Dawley (SD) rats (n=66) at 10–11 weeks of age (180–220 g) were obtained from the Medical Laboratory Animal Center (Guangzhou, China). Rats were housed with a constant light/dark cycle of 12/12 h (lights on at 7 a.m. and lights off at 7 p.m.), under controlled temperature (20–24°C) and humidity (45–65%). Rats received nesting material and free access to standard food and tap water in their home cage. Every effort was made to avoid or reduce pain or discomfort to the study animals, and to minimize the number of animals used in this study. Experiments began after a week of adaptive rearing. All animal experiments were approved by the University Committee on the Use and Care of Animals at Guangdong Pharmaceutical University (Guangzhou, China).

Thirty of these rats were randomly divided into either the normal control group (n=15) or the model group (n=15, chronic stress treatment) during the establishment
of the CSBI model. In addition, 36 rats were randomly divided into three groups during the drug-intervention experiments, which included normal control (n=12), model (n=12, chronic stress treatment), and retigabine (n=12, retigabine and chronic stress treatment) groups.

2.2 Chronic stress procedure

A chronic stress procedure was performed as previously described. In brief, all rats, except for the normal controls, were restrained in homemade holders (plastic, cylindrical holders with holes, 18 cm × 5 cm). Then, every sixth restrained rat was placed into a tank (30 cm × 18 cm × 20 cm), which was filled with water (temperature: 20 ± 1°C, depth: 15 cm). The chronic stress procedure lasted for 8 h, from 9 a.m. to 5 p.m., each day for 21 days. All rats were deprived of food and water during chronic stress, but were then provided food/water ad libitum after each session.

2.3 Drugs and treatments

Retigabine (98 % purity) was purchased from Maclean Biochemical Technology Co., Ltd. (Shanghai, China). Retigabine was prepared in a normal saline solution containing 1% Tween80. The rats in the retigabine treatment group were intraperitoneally (i.p.) administered retigabine (4 mg / kg) at 1 h before the chronic stress procedure was initiated each day.

2.4 Tissue collection

After 21 days of chronic stress, rats were anesthetized with ether and sacrificed via decapitation. The brain tissue from each rat was harvested and washed with normal saline. Next, the hippocampus from each group of rats (n=6 each) was separated from the brain tissue and fixed with 4% paraformaldehyde for 24 h, after which it was used for Nissl staining. The hippocampus from each group of rats (n=6 each) was snap frozen in liquid nitrogen for subsequent RNA sequencing (RNA-seq), quantitative real-time PCR (qRT-PCR), and Western blotting.
2.5 Library construction and RNA sequencing

Hippocampus total RNA from the normal control and model groups (n=3 per group) was extracted using TRIzol reagent (Takara, Japan), following the manufacturer's protocol. In addition, the purity and concentration of total RNA were screened by measuring the absorbances at 230, 260, and 280 nm. RNA integrity was assessed using an Agilent 4200 TapeStation (Agilent Technologies, USA). Next, mRNA was obtained by oligo (dT) magnetic beads. Fragmentation buffer was added to the beads with mRNA, which induced random breaks in the mRNA strands. Subsequently, RNase H and DNA polymerase I were used to synthesize second-strand cDNA. After purification, the cDNA was eluted with an elution buffer (EB) for end repair and poly (A) addition. Finally, AMPure XP beads (Beckman Coulter, USA) were used to screen 200 bp of cDNA for PCR amplification and purification of PCR products, after which a sequencing library was obtained. The cDNA library was sequenced on an Illumina sequencing platform (NovaSeq 6000).

2.6 Differentially expressed genes (DEGs) and functional enrichment analysis

DEGs between the normal control and model groups were determined by DESeq2. The false discovery rate (FDR) was controlled using the Benjamini and Hochberg algorithm. Genes with \(| \log_2(\text{Fold Change}) | > 1 \) and an FDR<0.05 were considered DEGs. Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed via clusterProfiler. Finally, all GO terms and KEGG pathways were considered based on an FDR<0.05.

2.7 Nissl staining

Brain hippocampus tissues were fixed in 4% paraformaldehyde, dehydrated by gradient alcohol, and made transparent by xylene. Next, these hippocampal tissues were embedded in paraffin wax and cut into 4μm sections. The sections were deparaffinized with xylene and gradient alcohol, after which they were treated with
Nissl staining solution for 5 min. Finally, the sections were mounted onto coverslips with neutral gum.

2.8 Open field test (OFT)

Open field testing was performed 1 day after the chronic stress procedure. Rats were placed into the center of an open-field apparatus (100 cm x 100 cm x 40 cm) with a black inner wall. The rats were allowed to move freely for 5 minutes. The total distance travelled, central square duration, and rearing (number of times standing on hind legs with nose in the air for exploration) was digitally extracted and analyzed via SuperMaze (Shanghai, China). After each rat finished the test, the open-field apparatus was thoroughly cleaned with 75% ethanol.

2.9 Sucrose preference test (SPT)

Rats were housed individually in standard polycarbonate cages. This test lasted for three days. On the first day, all the rats were given two bottles of water for 24h. On the second day, rats were given a bottle of water and a bottle of 2% sucrose solution for another 24h. On the third day, all rats were deprived of water and food for 24 hours. Afterwards, all rats were given a bottle of water and a bottle of 2% sucrose solution. The rats drank freely, and any remaining liquid was weighed after 1 hour. Similarly, preferences were calculated as percentages \[\left(\frac{\text{sucrose consumption}}{\text{sucrose + water consumption}}\right) \times 100\].

2.10 Western blotting

Hippocampus sections were homogenized in RIPA lysis buffer (Beyotime,
China), centrifuged at 12,000 rpm for 30 min at 4°C, and protein concentrations were then measured via a BCA Assay Kit (Thermo Scientific, USA). An equal amount of protein from each sample was subjected to 12% SDS-page and then transferred to a polyvinylidene-fluoride membrane via electroblotting. Next, the membranes were blocked with 5% skim milk at room temperature for 2 h. After washing three times with tris-buffered saline and Tween 20 (TBST), the membranes were incubated with antibodies against Bax (2772S, CST, USA), Bcl-2 (26593-1-AP, Proteintech, China), cleaved-caspase 3 (96664S, CST, USA), and tubulin overnight at 4°C. After washing three times with TBST, membranes were incubated with HRP-conjugated anti-rabbit antibodies (SA00001-2, Proteintech, China) or anti-mouse IgG antibodies (SA00001-1, Proteintech, China) for 1 h at room temperature. Protein bands were detected by ECL reagent (Biosharp, China), and were captured by a chemiluminescence system. Finally, ImageJ was used to quantify protein bands.

2.11 Quantitative real-time PCR (qRT-PCR)

The hippocampus total RNA from normal control and model groups (same batch as RNA-seq) was extracted using TRIzol reagent (Takara, Japan), and the purity and concentration of total RNA was detected by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). Next, 1 μg of total RNA and a PrimerScript RT Kit (Takara, Japan) were used to synthesize cDNA. Then, qRT-PCR was performed with SYBR Premix Taq TM II (Takara, Japan) and a Roche LightCycler 480 (Roche, Switzerland). All primer sequences used are listed in Table 1. All genes were normalized to GAPDH or β-actin as an internal control. Relative expression levels were calculated via the $2^{-\Delta\Delta Ct}$ method.19)

2.12 Statistical analysis

All data are expressed as the mean ± SEM. Unpaired Student’s t tests with two-tailed distributions were used to analyze statistical differences between two
groups. Differences among multiple groups were analyzed by one-way analysis of variance (ANOVA). All statistical analysis was performed in SPSS 22.0 or GraphPad Prism 7.0. Differences were considered statistically significant at a p value of <0.05.

3 Results

3.1 Chronic restraint stress with cold water immersion induces depression-like behavior in rats

The open field test (OPF) and sucrose preference test (SPT) were performed to measure whether the chronic restraint stress with cold water immersion (the model group) induced anxiety- and depression-like behaviors.

The OPF results are shown in Fig. 1A - C. The total distance travelled of rats in the model group was significantly lower than those in the control group (Fig 1A, p < 0.001). In addition, compared with the control group, central square duration and rearing were both decreased in the model group (Fig 1B, p < 0.001, Fig 1C, p < 0.001).

In SPT, sucrose preference in the model group was significantly decreased compared with that in the control group (Fig 1D, p<0.001).

The results of the behavior testing indicate that chronic restraint stress with cold water immersion induced anxiety- and depression-like behaviors.

3.2 Chronic restraint stress with cold water immersion induces brain injury in rats

Nissl staining showed that in the control group hippocampal neurons were neatly arranged with clear nuclei. Nissl bodies were indigo blue and were evenly distributed. In contrast, in the CSBI model group, the number of Nissl bodies was comparatively reduced. In addition, the morphologies of some neurons were shrunken, and their nuclei were unclear (Fig 2A). The number of survival neurons in the model group were significantly decreased compared with the control group (Fig 2B, C, p < 0.001). Western-blot analysis of total protein within the hippocampus revealed that, compared with those in the control group, the expression levels of cleaved-caspase 3 (p < 0.05)
and Bax (p < 0.05) were increased in the model group, while Bcl2 (p < 0.05) was decreased (Fig 1D, E). Collectively, these results suggest that chronic restraint stress with cold water immersion induced brain injury in rats. Therefore, our paradigm successfully established a rat model of CSBI.

3.3 Differentially expressed genes (DEGs) between brain tissues in control and CSBI model rats

In order to investigate the mechanism of CSBI, we next characterized the brain transcriptomic profiles between control and CSBI model rats via RNA-seq. Genes were considered to be differentially expressed based on a $|\text{log}_2 \text{Fold Change}| > 1$ and an FDR < 0.05. A total of 1,131 DEGs were identified via RNA-seq, of which 553 were up-regulated and 778 were down-regulated in the model group, compared to those in the control group (Fig 3A). Then, 10 randomly selected genes (Cftr, Camk2n1, Mef2c, Satb2, Olr59, Kcng4, Ngfr, Zic1, Calb2, Kenj16, and Clic6) were verified by qRT-PCR. The results showed that Cftr, Camk2n1, Mef2c, Satb2, and Olr59 were significantly up-regulated (Fig 3B), whereas Ngfr, Zic1, Calb2, Kenj16, and Clic6 were significantly down-regulated (Fig 3C) in the model group compared to those in the control group, which was consistent with the results of RNA-seq.

3.4 GO annotation and KEGG pathway analysis

GO (gene ontology) annotations—consisting of biological processes (BP), cellular components (CC), and molecular functions (MF)—represent an internationally standardized classification system of gene function for describing the characteristics of genes and their products in any organism. Additionally, the international gene database KEGG (Kyoto Encyclopedia of Genes and Genomes) integrates genomics, transcriptomics, and metabolomics. Using KEGG pathway analysis, associations among DEGs can be determined.

To investigate potential mechanisms of CSBI, we performed GO annotation and
KEGG pathway analysis of DEGs between the control and CSBI model rats. The top-five GO terms of each category with an FDR < 0.05 are shown in Fig 4A. Biological processes involved in CSBI-induced DEGs included regulation of transmembrane ion transport, regulation of transmembrane transport, regulation of membrane potential, cellular potassium-ion transport, and transmembrane potassium-ion transport. Furthermore, the cellular components involved in CSBI-induced DEGs included ion channel complexes, transmembrane transporter complexes, transporter complexes, proteinaceous extracellular matrices, and cation channel complexes. Lastly, the molecular functions involved in CSBI-induced DEGs included ion channel activity, voltage-gated ion channel activity, potassium channel activity, potassium-ion transmembrane transporter activity, and voltage-gated potassium channel activity.

The KEGG enriched pathways are shown in Fig 4B. According to the standard of FDR < 0.05, the top five pathways were those involving neuroactive ligand-receptor interactions, Cushing syndrome, complement and coagulation cascades, calcium signaling pathways, and the cAMP signaling pathway.

Taken together, GO annotation and KEGG pathway analysis revealed that membrane-bound ion-channel function was altered following CSBI. Therefore, changes in membrane-bound ion channels may have contributed to CSBI in rats.

3.5 Potassium channels are affected by chronic stress

GO annotation and KEGG pathway analysis suggested that CSBI was related to changes in membrane-bound ion channels. Further analysis revealed that 46 DEGs were related to potassium channels, calcium channels, sodium channels, and chloride channels expressed in neuronal membranes (Table 2). Among these, 29 genes were up-regulated and 17 genes were down-regulated in the model group compared to those in the control group.
Potassium channel genes accounted for the largest proportion of CSBI-induced DEGs, accounting for 50% of all DEGs corresponding to ion channels (Fig 5B). In total, there were 23 differentially expressed genes related to potassium channels, of which 19 were up-regulated and four were down-regulated in the model group compared to those in the control group (Fig 5A). According to their structures and functions, the differentially expressed potassium channel genes were divided into four categories: voltage-gated potassium channels, inward-rectifying potassium channels, two-pore-domain potassium channels, and sodium-activated potassium channels. First, there were 13 DEGs related to voltage-gated potassium channels, accounting for 58.52% of all DEGs corresponding to potassium channels (Fig 5C), of which KcnA3, Kcnab3, Kcnf1, Kcnh3, Kcnh4, Kcnh5, Kcnh7, Kcnip3, KcnS1, and Kcnv1 were up-regulated, whereas KcnG2, KcnG4, and KcnE5 were down-regulated in the model group compared to those in the control group. Second, there were two DEGs related to inward-rectifying potassium channels, such that Kcnj4 was up-regulated and Kcnj16 was down-regulated in the model group compared to those in the control group. Third, seven DEGs were related to two-pore-domain potassium channels—including Kcnk1, Kcnk2, Kcnk3, Kcnk4, Kcnk9, Kcnk12, and Kcnk15—all of which were up-regulated in the model group compared to those in the control group. Finally, Kcnt1 was the only DEG that was a sodium-activated potassium channel, which was up-regulated in the model group compared to that in the control group.

Voltage-gated potassium channels are the primary channels that regulate potassium flux across the plasma membrane. Inadequate opening of voltage-gated potassium channels may affect repolarization. The proteins encoded by Kcnf1, Kcnv1, KcnS1 and KcnE5 are regulatory subunits of voltage-gated potassium channels, and up-regulation or down-regulation of these subunits is known to decrease the activity of potassium channels.21, 22, 23, 24) We found that Kcnf1, Kcnv1, and KcnS1 were
significantly up-regulated, and \textit{Kcne5} was down-regulated (Fig 5D) in the model group compared to those in the control group, which suggests that chronic stress may hinder the opening of voltage-gated potassium channels.

\textbf{3.6 Retigabine, a voltage-gated potassium channel opener, provides neuroprotection from chronic stress}

Our RNA-seq results suggested that reduced activity of voltage-gated potassium channels may contribute to CSBI. Therefore, we next tested the efficacy of retigabine, a voltage-gated potassium channel opener, in ameliorating anxiety- and depression-like behavior following chronic restraint stress with cold water immersion.

The results of OFT found that chronic restraint stress with cold water immersion reduced the spontaneous activity of rats in the model group. However, rats in the retigabine group exhibited an amelioration after treatment. Compared with those in the control group, the total distance travelled of model group decreased significantly (Fig 6A, p < 0.001), while central square duration and rearing decreased (Fig 6B, C, p < 0.001). Rats in retigabine group shown significant amelioration compared with the model group. The total distance travelled (Fig 6A, p < 0.01), central square duration (Fig 6B, p < 0.001) and rearing (Fig 6C, p < 0.001) in CSBI model mice increased after retigabine treatment.

The results of SPT show that the sucrose preference of rats in the model group was lower than that in the control group (Fig 6D, p < 0.01). By contrast, rats in the retigabine group after treatment shown increased sucrose preference (Fig 6D, p < 0.01.)

Subsequently, we tested the efficacy of retigabine in ameliorating brain damage in rats following chronic stress. Nissl staining showed that, compared with those in the model group, rats treated with retigabine had neatly arranged neurons and an increased number of Nissl bodies in the hippocampus (Fig 7A). The number of
survival neurons of the CA1 and CA3 areas in the model group were decreased significantly, compared with control (Fig 7B, C, p < 0.001). In contrast to model group, the number of survival neurons of the CA1 and CA3 area in the retigabine group were increased (Fig 7B, C, p < 0.001). Western blotting revealed that the expression levels of Bax (p<0.05) and cleaved-caspase3 (p<0.01) in the brain tissue of rats treated with retigabine were decreased, while Bcl-2 (p<0.01) was increased (Fig 7D, E) compared with those in the model group.

Collectively, these results demonstrate that the voltage-gated potassium channel opener, retigabine, significantly ameliorates brain injury induced by chronic stress in rats. Hence, our findings suggest that voltage-gated potassium channel blockage may contribute to CSBI, and thus represents a therapeutic target.

4 Discussion

Chronic stress can damage tissues and organs; this kind of injury is positively correlated to the duration and intensity of stress, and is negatively correlated to one’s anti-stress ability. The nervous system, especially the brain, acts as a regulatory center for various types of stress. Continuous and repeated chronic stress not only induces brain injury, but also adversely affects the repair of damaged tissues, leading to impaired brain function. In the present study, we performed RNA-seq analysis in a rat model of CSBI and found that the function of neuronal membrane-bound ion channels was affected by chronic stress. Among them, mRNA levels of voltage-gated potassium channel genes were significantly altered following chronic stress, suggesting that the opening and closing of voltage-gated potassium channels is closely related to stress-induced brain injury.

4.1 Rat model of CSBI

Chronic restraint is a well-established model of chronic stress that emulates the neuroendocrine responses of humans under stress and induces depression- and
anxiety-like behaviors. In the present study, cold water immersion combined with chronic restraint was used to establish a rat model of CSBI. The behavior tests, including the open field test (OPF) and sucrose preference test (SPT), are usually used to investigate anxiety- and depression-like behaviors in rats. After 21 days of chronic restraint stress and water immersion, rats in the model group showed a reduction in spontaneous activities and exploration behaviors in OPF. In SPT, the sucrose preference of rats in the model group decreased. The behavior test suggested that after 21 days of chronic stress, rats in the CSBI model exhibited anxiety- and depression-like behaviors. Additionally, Nissl staining showed that the model group had a reduced number of Nissl bodies and survival neurons in the hippocampus. These results were similar to those of brain injury caused by hydraulic shock. In addition, cleaved-caspase 3, Bcl2, and Bax are markers that reflect neuronal apoptosis. Our present results, obtained by Western blotting, were consistent with those of previous studies. As compared to levels in the control group, the expression levels of cleaved-caspase 3 and Bax in the hippocampus of the model group mice were increased, while the expression of bcl2 was decreased.

Anxiety- and depression-like behaviors are associated with neuronal injury within a special brain region. Animal models of depression research have revealed that depression-like behavior is accompanied by neuronal apoptosis in the hippocampus, with reduced levels of Bel-2 and increased levels of caspase-3. However, inhibition of apoptosis in the hippocampus can significantly ameliorate depression-like behavior in animal models. On the other hand, increasing neuronal apoptosis in the hippocampus is associated with anxiety-like behavior, while inhibition of apoptosis in the hippocampus can alleviate abnormal behavior in rats. Thus, CSBI was shown to be responsible for anxiety- and depression-like behavior in rats.
After 21 days of chronic restraint stress and water immersion, we successfully established a rat model of CSBI that resulted in anxiety- and depression-like behaviors and neuronal apoptosis.

4.2 Role of potassium channels in chronic stress-induced brain injury

Our GO annotation and KEGG pathway analysis showed that changes in ion channel function were the most significant under chronic stress, suggesting that ion channel dysfunction contributed to CSBI in our rat model. Ion channels regulate the excitability of neurons by regulating ion transport across the plasma membrane.33) Na⁺, K⁺, Ca²⁺, and Cl⁻ are the main ions that regulate neuronal excitation and inhibition. Activation of sodium channels induces Na⁺ influx, which contributes to triggering action potentials.34) Similarly, activation of calcium channels triggers action potentials via Ca²⁺ influx, and also plays an important role in the secretion and reuptake of neurotransmitters.35) In addition, K⁺ and Cl⁻ are the main ions that contribute to repolarization, hyperpolarization, and maintaining resting membrane potential.36, 37)

In the present study, among the CSBI-induced DEGs corresponding to ion channels following RNA-seq analysis, potassium channels represented the largest proportion of this group. According to their structures and functions, potassium channels can be divided into four types: inward-rectified K⁺ channels (Kir), two-pore domain K⁺ channels (K₂P), calcium-activated K⁺ channels (KCa), and voltage-gated K⁺ channels (Kv).38)

Our present results show that voltage-gated potassium channel (Kv) genes represented the largest proportion of DEGs corresponding to potassium channels. High densities of voltage-gated potassium channels are located on neuronal plasma membranes, which contribute to repolarization, regulation of action potentials, and maintenance of resting membrane potential.39) Voltage-gated potassium channels are closely related to neuronal excitability, and their dysfunction can induce various
neuropsychiatric diseases. For example, benign familial neonatal convulsions are caused by a mutation in KCNQ3 or KCNQ2. Furthermore, a mutation in KCNAI yields type-I episodic ataxia. In addition, blockade of voltage-gated potassium channels can induce apoptosis.

Among the DEGs corresponding to potassium channels in our present study, Kcnf1 (Kv5.1), Kcnsl (Kv9.1), Kcnvl (Kv8.1) and Kcne5 belong to electrically silent voltage-gated potassium channel subunits (KvS). In general, potassium channels are homotetramers formed by a combination of four identical functional subunits. However, electrically silent voltage-gated potassium channel subunits are unable to form homotetramers. Instead, they form heterotetramers with other functional subunits to regulate currents and inactivation times of potassium channels. These genes have been demonstrated to regulate voltage-gated potassium channels. Hugnot and colleagues found that Kv8.1 inhibits K+ currents by forming heterotetramers with Kv2. Similarly, Kramer et al. reported that the co-expression of Kv2.1 and Kv5.1 in Xenopus oocytes results in the inhibition of K+ currents. Furthermore, the co-expression of Kv9.1 and Kv2 channels suppresses K+ currents. Overexpression of KCNE5 in HEK cells can enhance Kv7.4 currents and increase the Kv7.4 time of deactivation.

Collectively, these findings suggest that under the influence of chronic stress, retardation of voltage-gated potassium channels may contribute to CSBI. Therefore, voltage-gated potassium channel openers targeting the central nervous system may result in anti-stress effects.

4.3 Anti-stress effect of retigabine, a potassium channel opener

After analysis of DEGs, we speculated that the blockage of KCNB (Kv2) or KCNQ (Kv7) is related to the CSBI. Since there is no specific opener for KCNB, we chose a KCNQ opener for the central nervous system for our pharmacological
experiments. The Kv7 family consists of Kv7.1, Kv7.2, Kv7.3, Kv7.4, and Kv7.5. Kv7.2–Kv7.5 are mainly expressed in the central nervous system.\(^\text{46}\) Retigabine shows anti-epileptic effects in multiple epileptic models by activating \(\text{KCNQ2–5} (\text{Kv7.2–7.5})\) channels and enhancing \(K^+\) currents.\(^\text{47}\) For this reason, retigabine has been approved by the FDA for the treatment of epilepsy. In addition, it has been reported that retigabine protects brain tissue from damage by activating voltage-gated potassium channels and enhancing \(K^+\) currents. Furthermore, retigabine can ameliorate traumatic brain injury via enhancing potassium channel activity.\(^\text{48}\) Retigabine also exhibits a protective effect on focal cerebral ischemic injury and sarin-induced brain injury.\(^\text{49,50}\)

In our present study, RNA-seq analysis suggested that KCNQ (Kv7) in the brain was blocked following CSBI. Therefore, we assessed whether the potassium channel opener, retigabine, could ameliorate brain injury in rats following chronic stress.

Nissl staining, OFT, SPT and Western blotting showed that rats treated with retigabine exhibited neuroprotection from chronic stress, such that brain tissue damage was significantly reduced compared with that of the model group. The protection mechanism of retigabine will be the focus of our future study. Chronic stress can induce neuronal hyperexcitability,\(^\text{51,52}\) and neuronal hyperexcitability is one cause of neuronal apoptosis. Retigabine is a specific opener for KCNQ — it is able to increase the number of KCNQ channels. On the other hand, retigabine can also enhance the magnitude and duration of KCNQ channel action and shift the voltage dependence of KCNQ activation, leading to a more rapid and prolonged ability to depolarize stimuli or neuronal hyperexcitability.\(^\text{47}\) Thus, the protective mechanism of retigabine may be related to the rapid and prolonged opening of KCNQ, which acts against chronic stress induced neuronal hyperexcitability. Reduced potassium channel activity may contribute to chronic stress-induced brain injury, thus voltage-gated potassium channel openers may serve as a therapeutic approach for CSBI.
In summary, the etiology of CSBI remains unclear. Multiple environmental, psychological, and homeostatic factors contribute to CSBI. In our present study, RNA-seq analysis suggested that high expression levels of \textit{Kcnf1}, \textit{Kcns1}, \textit{Kcnv1}, and \textit{Kcne5} may lead to the dysfunction of voltage-gated potassium channels and contribute to CSBI. We found that retigabine, a voltage-gated potassium channel opener, exhibited neuroprotection against the effects of chronic stress. Therefore, voltage-gated potassium channels represent potential drug targets for the treatment of chronic stress.

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**Conflict of Interest:** The authors declare no conflict of interest.
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Table 1 Primers used for real-time PCR.

| Gene   | Forward (5’-3’)         | Reverse (5’-3’)          |
|--------|-------------------------|--------------------------|
| Kcnj16 | CTGCACCTCGGACACCAACAC | GGCTGGTCCTCCTGAGGTTCCT   |
| Clic6  | CTGCCCATTTCTCACAGCGTCTC| CGGTTCAGGTCACCGTTGTC     |
| Calb2  | TCACAGGATTGTGGTAGGG   | GCACCTGTGTGGACTCGGAAGC   |
| Ngfr   | CCAACGGTCAAACGGAGCATC | AGAGGTCAGAAAGCAAGGG      |
| Zic1   | CACAGTCTCTTGCTTTCGGG  | GCCGCACTCCTGCCCATTGAC    |
| Olr59  | TTCATCAGCCACCTTCAATGC | GGCTCGTCTGTCTCCTACG      |
| Cftr   | CGGCACCTCCAGCAAGCAGAGA| TCCAGCACCCAGCAGACTGAGC   |
| Camk2n1| CGGAGGTCGTCCCTACGG   | GCAGGCGGCAGAAGATC        |
| Mef2c  | TGCTGTGCGACTGTGAGATTGC| CTCGTGCCTGCCTGTGACTC     |
| Satb2  | GTCCAGCGCCAGCCAAGTTTCAG| CCATCCAACTGCTCCACCACAC  |
| Gapdh  | ACAGCAACAGGGTGAGGTGAC | TGGAGGTCAGCGAATT         |
| Gene  | Gene ID | log₂FC | FDR     | Style | Gene description                              |
|-------|---------|--------|---------|-------|-----------------------------------------------|
| Kcnf1 | 298908  | 2.926649 | 3.59E-31 | up    | Potassium Voltage-Gated Channel Modifier Subfamily F Member 1 |
| Kcnh5 | 171146  | 2.561279 | 7.11E-16 | up    | Potassium Voltage-Gated Channel Subfamily H Member 5 |
| Kcnl1 | 117023  | 2.087972 | 6.20E-10 | up    | Potassium Voltage-Gated Channel Modifier Subfamily S Member 1 |
| Kcnk4 | 116489  | 1.816018 | 1.17E-12 | up    | Potassium Two Pore Domain Channel Subfamily K Member 4 |
| Kcnip3 | 65199 | 1.708096 | 1.79E-12 | up    | Potassium Voltage-Gated Channel Interacting Protein 3 |
| Kcnj4 | 116649  | 1.664317 | 2.36E-11 | up    | Potassium Two Pore Domain Channel Subfamily J Member 4 |
| Kcnk15 | 156873 | 1.451746 | 0.000231 | up    | Potassium Two Pore Domain Channel Subfamily K Member 15 |
| Kcnh7 | 170739  | 1.27681  | 4.04E-06 | up    | Potassium Voltage-Gated Channel Subfamily H Member 7 |
| Kcnh3 | 27150   | 1.235379 | 6.39E-07 | up    | Potassium Voltage-Gated |
| Gene  | Accession | log2FoldChange | False Discovery Rate | Status |
|-------|-----------|----------------|----------------------|--------|
| Kcnab3 | 58981     | 1.198436       | 2.78E-05             | up     |
| Kent1  | 60444     | 1.140749       | 4.71E-06             | up     |
| Kcnk2  | 170899    | 1.123004       | 3.71E-05             | up     |
| Kcna3  | 29731     | 1.070987       | 0.001353             | up     |
| Kcnk1  | 59324     | 1.063034       | 4.48E-05             | up     |
| Kcnk3  | 29553     | 1.016777       | 0.003001             | up     |
| Kcng4  | 307900    | −1.16476       | 0.000138             | down   |
| Kcng2  | 307234    | −1.38431       | 7.55E-07             | down   |
| Kcne5  | 681190    | −1.71856       | 0.015829             | down   |
| Kcnj16 | 29719     | −2.06396       | 8.18E-11             | down   |
| Cacng3 | 140724    | 1.218907       | 1.02E-06             | up     |
| Cacnb4 | 58942     | 1.12627        | 6.95E-06             | up     |
| Cacna1s| 682930    | 2.299475       | 0.003707             | up     |

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| Gene   | Accession | log2 Fold Change | p-value | Direction |
|--------|-----------|------------------|---------|-----------|
| Cacng8 | 140729    | -1.06891         | 3.01E-05| down      |
| Cacng5 | 140726    | -1.6671          | 2.09E-09| down      |
| Trpv6  | 114246    | 1.154701         | 2.38E-05| up        |
| Trpc5  | 140933    | -1.07393         | 9.13E-05| down      |
| Trpv3  | 497948    | -1.54345         | 4.87E-05| down      |
| Clic5  | 94272     | 1.517053         | 1.20E-05| up        |
| Clic6  | 304081    | -7.29608         | 6.69E-31| down      |
| Clic4  | 83718     | -1.67147         | 1.49E-10| down      |
| Clcn2  | 29232     | -1.27821         | 0.010229| down      |
| CFTR   | 24255     | 4.902666         | 4.58E-25| up        |
| Gabrd  | 29689     | 1.239462         | 2.77E-06| up        |
| Gabra3 | 24947     | 1.326115         | 1.81E-07| up        |
| Gabre  | 65191     | -4.22253         | 3.62E-10| down      |
| Gabra5 | 29707     | -1.24863         | 2.89E-07| down      |
| Trpm4  | 171143    | 1.359117         | 2.71E-05| up        |

**Gene Descriptions:**
- Calcium Voltage-Gated Channel Auxiliary Subunit Gamma 3
- Calcium Voltage-Gated Channel Auxiliary Subunit Gamma 5
- Transient Receptor Potential Cation Channel Subfamily V Member 6
- Transient Receptor Potential Cation Channel Subfamily C Member 5
- Transient Receptor Potential Cation Channel Subfamily V Member 3
- Chloride Intracellular Channel 5
- Chloride Intracellular Channel 6
- Chloride Intracellular Channel 4
- Chloride Voltage-Gated Channel 2
- Cf Transmembrane Conductance Regulator Gamma-Aminobutyric Acid Type A Receptor Delta Subunit
- Gamma-Aminobutyric Acid Type A Receptor Alpha3 Subunit
- Gamma-Aminobutyric Acid Type A Receptor Epsilon Subunit
- Gamma-Aminobutyric Acid Type A Receptor Alpha5 Subunit
- Transient Receptor Potential Cation Channel Subfamily
| Gene   | Score | log Fold Change | p-value  | Status |
|--------|-------|----------------|----------|--------|
| Scn3b  | 245956| -1.06637       | 1.34E-05 | down   |
|        |       | Sodium Voltage-Gated Channel Beta Subunit 3 |
| Scn7a  | 64155 | -1.45892       | 0.029075 | down   |
|        |       | Sodium Voltage-Gated Channel Alpha Subunit 7 |
| Scn4a  | 25722 | -1.82435       | 0.000371 | down   |
|        |       | Sodium Voltage-Gated Channel Alpha Subunit 4 |
| Scn5a  | 25665 | -2.94601       | 4.53E-17 | down   |
|        |       | Sodium Voltage-Gated Channel Alpha Subunit 5 |
Fig 1 Chronic restraint stress with cold water immersion induces depression-like behavior in rats. A Total distance travelled of rats in OFT, B Central square duration of rats in OFT, C Rearing of rats in OFT, D Sucrose preference of rats in SPT. All values are expressed as means ± SEMs. *p<0.05, **p<0.01, and ***p<0.001 model vs control.
**Fig2** Chronic stress-induced brain injury. A Representative images of Nissl-stained hippocampal CA1 and CA3 regions (magnification ×20, scale bar=25 μm), B The number of survival neurons of CA1 area in hippocampus, C The number of survival neurons of CA3 area in hippocampus, D Representative Western blots for cleaved-caspase 3, Bcl-2, and Bax expression, E Protein expression levels of cleaved-caspase 3, Bax, and Bcl-2. All values are expressed as means ± SEMs. *p<0.05, **p<0.01, and ***p<0.001 model vs control. (Color figure can be accessed in the online version.)
Fig 3 Differentially expressed genes in brain tissue following chronic stress. A Differentially expressed genes (DEGs) are shown in a volcano plot, B, C qRT-PCR verification of DEGs characterized by RNA-seq analysis. All values are expressed as means ± SEMs. *p<0.05 and **p<0.01 model vs control. (Color figure can be accessed in the online version.)
Fig 4 GO and KEGG analysis of differentially expressed genes following chronic stress. A GO terms related to biological processes (BP), cellular components (CC), and molecular functions (MF), B KEGG enriched pathways following CSBI. (Color figure can be accessed in the online version.)
Fig 5 Differentially expressed genes corresponding to potassium channels following chronic stress. A Heatmap of differentially expressed genes corresponding to potassium channels following chronic stress, B Proportions of sodium, potassium, calcium, and chloride channels, C Proportions of voltage-gated potassium channels, inward-rectifying potassium channels, two-pore-domain potassium channels, and...
sodium-activated potassium channels, D FDR values of voltage-gated potassium channels. (Color figure can be accessed in the online version.)
**Figure 6**

The potassium channel opener, retigabine, ameliorates depression-like behavior induced by chronic stress.  

A Total distance travelled of rats in OFT,  

B Central square duration of rats in OFT,  

C rearing of rats in OFT,  

D sucrose preference.
of rats in SPT. All values are expressed as means ± SEMs. *p<0.05, **p<0.01, and ***p<0.001 model vs control.
Fig 7 The potassium channel opener, retigabine, provides neuroprotection from chronic stress. A Representative images of Nissl-stained hippocampal CA1 and CA3
regions (magnification ×20, scale bar=25 μm), B The number of survival neurons of CA1 area of hippocampus, C The number of survival neurons of CA3 area of hippocampus, D Representative Western blots for cleaved-caspase 3, Bcl-2, and Bax expression, E Protein expression levels of cleaved-caspase3, Bax, and Bcl-2. All values are expressed as means ± SEMs. *p<0.05, **p<0.01, and ***p<0.001 model vs control; #p<0.05 and ##p<0.01 model vs retigabine. (Color figure can be accessed in the online version.)