

# Impact of Chronic Intermittent Hypoxia on Cognitive Function and Hippocampal Neurons in Mice: A Study of Inflammatory and Oxidative Stress Pathways

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**Purpose:** Chronic intermittent hypoxia (CIH) is considered one of the main pathophysiological mechanisms of obstructive sleep apnea (OSA). CIH can further lead to cognitive dysfunction by inducing processes such as neuroinflammation and oxidative stress. The hippocampus is primarily associated with cognitive functions such as learning and memory. This study aimed to explore the effects of CIH on cognitive function and hippocampal neurons in mice and to reveal its potential molecular mechanisms.

**Methods:** SPF-grade C57BL/6J mice (n=36) were selected as subjects and divided into control, mild CIH, and severe CIH groups (12 mice per group). Cognitive function was assessed using the Morris water maze test, and hippocampal neuron numbers and morphological changes were observed using HE staining and Nissl staining. Additionally, differential genes and pathways were revealed through RNA sequencing (RNA-seq) and bioinformatics analysis. We examined oxidative stress-related biochemical markers in the hippocampal tissue and used Western Blot to verify changes in the expression of potential key genes. Statistical analyses were performed using ANOVA and post hoc tests to ensure robust comparisons between groups.

**Results:** CIH mice exhibited significant cognitive impairment, including decreased learning and memory abilities. The severe CIH group had a longer escape latency compared to the mild CIH group ( $p < 0.001$ ) and the control group ( $p < 0.01$ ), while the mild CIH group took longer than the control group ( $p < 0.01$ ). In the probe test, the severe CIH group showed a significant decrease in platform crossings ( $p < 0.01$ ) and target quadrant dwell time ( $p < 0.05$ ), while the mild CIH group exhibited a reduction in target quadrant dwell time ( $p < 0.05$ ). Abnormal hippocampal neuron morphology was observed, with a significant reduction in hippocampal neurons ( $p < 0.05$ ). RNA-seq analysis revealed numerous differentially expressed genes, mainly enriched in biological processes such as inflammation and oxidative stress, as well as multiple signaling pathways. Specifically, downregulated *LepR*, *SIRT1*, and *Nrf2* genes were found to exacerbate oxidative stress and neuroinflammation, impairing neuronal integrity and cognitive function. Further validation showed increased oxidative stress levels in hippocampal tissue and downregulation of key gene expression. Western blot analysis confirmed significantly reduced expression of *LepR* ( $p < 0.01$ ), *SIRT1* ( $p < 0.001$ ), and *Nrf2* ( $p < 0.001$ ) in the severe CIH group.

**Conclusion:** While oxidative stress and inflammation are well-established mechanisms in CIH-induced cognitive impairment, our study provides novel insights by identifying the specific roles of *LepR*, *SIRT1*, and *Nrf2* in this process. The downregulation of these key genes suggests potential new targets for therapeutic intervention. Importantly, the differential expression patterns observed in varying degrees of hypoxia severity highlight the potential for tailored therapeutic strategies that modulate these pathways in response to the intensity of hypoxic exposure. These findings offer unique opportunities for developing targeted therapies aimed at mitigating CIH-related cognitive decline and neural damage. However, a key limitation of this study is the exclusive use of animal models, which may not fully replicate human pathophysiology. Further studies are needed to validate these findings in clinical settings and to explore the regulatory relationships between the key genes involved.

**Keywords:** obstructive sleep apnea, chronic intermittent hypoxia, cognitive impairment, hippocampal neurons, RNA sequencing, oxidative stress, inflammation

## Introduction

Obstructive sleep apnea (OSA) refers to the recurrent partial or complete obstruction of the upper airway during sleep, leading to impaired ventilation and disrupted sleep architecture, which in turn triggers a series of pathophysiological changes.<sup>1</sup> Studies have shown that OSA affects approximately 17% of women and 34% of men in the United States, with similar prevalence rates in other countries. The prevalence in children ranges from 1.2% to 5.7%, and it has been rising in recent years.<sup>2,3</sup> OSA can lead to cognitive impairments, such as attention deficits, memory loss, and reduced reasoning ability.<sup>4</sup> However, the underlying mechanisms of OSA-induced cognitive impairments remain unclear, creating a gap that limits the development of effective targeted treatments. Specifically, the precise roles of key molecular pathways in mediating neuronal damage under chronic intermittent hypoxia (CIH) conditions need further exploration. Recent studies have suggested that oxidative stress and inflammatory pathways, particularly those mediated by proteins such as SIRT1, Nrf2, and IL-6, play a critical role in OSA-related neuronal damage. These findings highlight the importance of integrating oxidative and inflammatory signaling into research on OSA-induced cognitive impairments.

Chronic intermittent hypoxia (CIH) is the primary pathophysiological feature of OSA. The hippocampus, a brain region closely related to cognitive function, is primarily involved in learning and memory.<sup>5</sup> The hippocampus is highly sensitive to hypoxic injury, and under CIH conditions, repeated cycles of hypoxia and reoxygenation may disrupt the balance between oxidative and antioxidant systems, leading to oxidative stress-related neuronal damage.<sup>6,7</sup> Studies have shown that signaling pathways such as MAPK/NF- $\kappa$ B and PERK/ATF4/CHOP are involved in CIH-induced cognitive impairment.<sup>8,9</sup> Specific genes, such as SIRT1 and Nrf2, play critical roles in regulating oxidative stress and inflammation in various hypoxic conditions.<sup>10,11</sup> SIRT1, a NAD<sup>+</sup>-dependent deacetylase, has been shown to mitigate oxidative damage and modulate inflammatory responses by deacetylating key transcription factors, including NF- $\kappa$ B and p53.<sup>12,13</sup> Recent studies in hypoxia models have demonstrated that SIRT1 activation can reduce neuronal apoptosis and enhance cellular antioxidant capacity, which may protect against CIH-induced hippocampal damage.<sup>14</sup> Similarly, Nrf2, a master regulator of the antioxidant response, governs the expression of detoxifying and antioxidant enzymes such as heme oxygenase-1 (HO-1) and NAD(P)H: quinone oxidoreductase 1 (NQO1), which are essential for maintaining redox homeostasis under oxidative stress.<sup>15,16</sup> Evidence from both animal and cellular models of hypoxia indicates that upregulation of Nrf2 can attenuate oxidative stress and inflammation, preserving neuronal function.<sup>17</sup> Despite these findings, the precise roles of SIRT1 and Nrf2 in CIH-induced hippocampal damage and cognitive impairment remain underexplored. Identifying their contributions to oxidative stress and inflammatory pathways in OSA could offer potential therapeutic targets for mitigating neuronal injury and improving cognitive outcomes.

RNA sequencing (RNA-seq) is a powerful transcriptomic analysis tool capable of performing genome-wide transcriptional analysis. One of its major advantages is its ability to identify new genes or detect differential gene expression on a large scale, which is crucial for uncovering the potential molecular pathways involved in CIH-induced hippocampal damage.<sup>18</sup> Given the complexity of molecular changes in the hippocampus under CIH conditions, RNA-seq is well-suited to provide comprehensive insights into potential mechanisms. However, a potential limitation of RNA-seq is the challenge of translating large-scale transcriptomic data into clinical applications, as the functional relevance of some differentially expressed genes may require further validation.<sup>19</sup> Western blot is an important method for validating findings at the protein level, but it also has limitations, such as sensitivity issues and the difficulty of directly linking protein expression with functional outcomes in CIH-related cognitive impairment.

Therefore, this study aims to analyze the hippocampal tissue of mice exposed to mild and severe CIH using RNA-seq, and to validate key molecular findings at the translational level using Western blot. The goal is to explore the molecular changes associated with CIH-induced hippocampal damage and further analyze the underlying molecular mechanisms, with the hope of identifying potential targets for future research. We hypothesize that CIH will lead to differential expression of genes related to oxidative stress and inflammation, particularly downregulation of key genes such as SIRT1, LepR, and Nrf2, thereby resulting in hippocampal damage and cognitive impairment. This hypothesis will be tested by examining the expression patterns of these genes and related signaling pathways in response to varying degrees of CIH.

## Material and Methods

### Animals

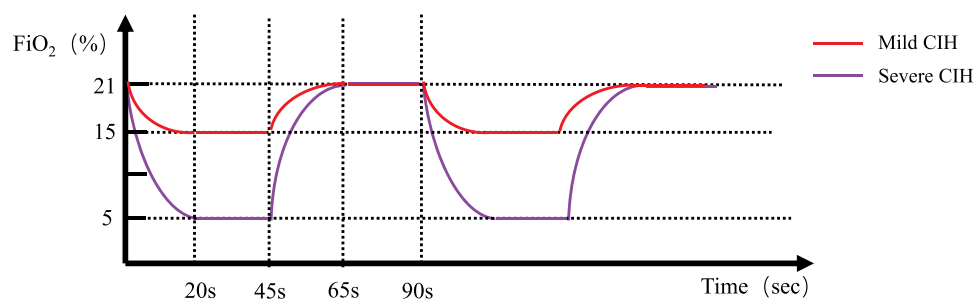
Thirty-six SPF-grade male C57BL/6J mice, aged 6–7 weeks (weighing 18–22 grams), were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (license number: SCXK Beijing 2021–0011). The C57BL/6J mouse strain was selected due to its well-documented use in cognitive and hypoxia-related studies, as well as its genetic uniformity and susceptibility to hypoxia-induced neural changes, which makes it ideal for studying the mechanisms underlying CIH. The animals were housed under SPF-standard conditions (temperature:  $21\pm1^{\circ}\text{C}$ ; humidity:  $50\pm10\%$ ) with a 12-hour light-dark cycle. All animals were kept in SPF isolation units and provided with adequate food and water. Animal experiments were conducted in accordance with the principles outlined in the “Guidelines for Laboratory Management and Use” and approved by the Ethics Committee of Beijing Children’s Hospital, Capital Medical University (No: 2020-k-93).

### Chronic Intermittent Hypoxia (CIH)

After a one-week adaptation period, mice (7–8 weeks old) were randomly divided into three groups, with 12 mice per group: control, mild CIH, and severe CIH. They were exposed to CIH conditions for 6 weeks using a gas control delivery system that alternated between oxygen and nitrogen to cycle between hypoxia and normoxia. The gas delivery system was validated before the experiment to ensure accurate oxygen and nitrogen cycling, and periodic calibration was conducted to maintain stable gas concentration levels. The cycling duration was 8 hours per day, with one CIH cycle defined as 90 seconds, consisting of a oxygen decline phase (20 seconds), hypoxic phase (25 seconds), oxygen rise phase (20 seconds), and normoxic phase (25 seconds). Throughout the experiment, an oxygen analyzer (Tawang Technology, S1007, Shanghai, China) continuously monitored oxygen and carbon dioxide concentrations. The specific oxygen profiles for each group were as follows: control group (21% O<sub>2</sub>, 25 seconds), mild CIH group (15% O<sub>2</sub>, 25 seconds), and severe CIH group (5% O<sub>2</sub>, 25 seconds) (Figure 1). This specific hypoxia model was selected because it closely mimics the intermittent oxygen levels found in obstructive sleep apnea (OSA) and has been extensively used in studies of cognitive dysfunction and neuroinflammation.

### Morris Water Maze (MWM)

Spatial memory testing of mice was conducted using the MWM apparatus. The maze consisted of a circular water tank with a diameter of 150 cm and height of 50 cm, filled with water and non-toxic white dye to enhance contrast, maintained at a temperature of 24–26°C. Visual cues were placed around the perimeter of the tank. The pool was divided into four quadrants, with Quadrant I containing a movable circular platform with a diameter of 15 cm submerged approximately 1 cm below the water surface. During the spatial acquisition training phase, each day at 10:00 AM, mice from the three groups were placed into the water maze from different quadrants sequentially for training. The mice were allowed a maximum of 60 seconds to swim or until they found the platform, after which they were allowed to stay on the platform for 5 seconds for learning. Escape latency to reach the platform was recorded using computer software. In the spatial probe trial phase, the platform was removed, and mice from the three groups were placed into the quadrant farthest from



**Figure 1** Schematic diagram of the CIH exposure protocol. The horizontal axis represents time, and the vertical axis represents oxygen concentration.

the former platform. The time spent by each group of mice in the target quadrant within 60 seconds, the number of platform crossings, and swimming speed were recorded. During the navigation and spatial exploration experiment phase, behavior data were acquired and analyzed, and trajectories were detected using the Tracking Master software (Fanbi Intelligent Technology, Shanghai, China).

## HE Staining

After behavioral testing, three mice from each group were anesthetized and then sequentially perfused with 0.9% saline and 4% paraformaldehyde. Following perfusion, the mice were euthanized by cervical dislocation, and brain tissues were collected. The tissues were placed in a pre-prepared fixative solution to denature and coagulate the proteins. Subsequently, dehydration and paraffin embedding were performed. Paraffin sections were prepared and processed according to the instructions of the HE staining kit (Solarbio, G1120, Beijing, China). The pathological results of neurons in the hippocampal CA1 and CA3 regions were observed using a light microscope.

## Nissl Staining

Paraffin sections were placed in Nissl staining solution (Solarbio, G1432, Beijing, China) and stained at 37°C for 5 minutes, followed by rinsing in 95% ethanol for 5 minutes and air-drying. Subsequently, the sections were washed twice in xylene for 5 minutes each. Finally, neutral mounting medium was used, and neuronal counts were performed using an optical microscope at  $\times 400$  magnification. Three random fields in the CA1 and CA3 regions of hippocampal tissue were selected from each slide, and neuronal counts were quantified using Image J software.

## RNA Sequencing

Three hippocampal tissue samples were randomly selected from each group. Total RNA was extracted using Trizol (Ambion, USA), and mRNA with polyA tails was enriched using Oligo(dT) magnetic beads. Subsequently, the enriched mRNA was fragmented using a Fragmentation Buffer with divalent cations. Fragmented mRNA was used as a template to synthesize the first strand of cDNA in the M-MuLV reverse transcriptase system with random oligonucleotides as primers. Then, using the RNaseH degradation method, the second strand of cDNA was synthesized in the presence of DNA polymerase I and dNTPs. The purified double-stranded cDNA was subjected to end repair, A-tailing, and ligation of sequencing adapters. cDNA fragments of approximately 370–420 bp in size were selected using AMPure XP beads and subjected to PCR amplification. The amplified products were further purified using AMPure XP beads to construct the final libraries. After library construction, the libraries were initially quantified using the Qubit 2.0 Fluorometer (Life Technologies, USA) and diluted to a concentration of 1.5 ng/ $\mu$ L. Subsequently, the insert fragment sizes of the libraries were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Once the insert sizes met expectations, the effective concentrations of the libraries (above 1.5 nM) were accurately measured using qRT-PCR to ensure library quality. Following quality control, different libraries were pooled based on their effective concentrations and desired data output requirements. Sequencing was performed using the Illumina NovaSeq 6000 (Illumina, USA) to generate paired-end reads of 150 bp each. During the data processing stage, the raw image data obtained from sequencing were converted into sequence data (reads) using CASAVA. The raw data were filtered to remove adapter sequences, reads containing N bases, and low-quality reads (reads with more than 50% of bases having a Qphred score  $\leq 5$ ). Subsequently, Q20, Q30, and GC content were calculated for the clean data. All subsequent data analyses were performed using high-quality clean data. For sequence alignment, reference genomes and gene model annotation files were downloaded from genome websites. An index for the reference genome was built using HISAT2 (v2.0.5), and the paired-end clean reads were aligned to the reference genome using HISAT2. Finally, featureCounts (1.5.0-p3) was used to calculate the number of reads mapped to each gene and the FPKM (fragments per kilobase of transcript per million mapped reads) values based on gene lengths.

## Differential Gene Analysis

Based on quantitative expression results, differential gene analysis between groups was conducted. DEGseq was used to identify differentially expressed genes (DEGs) between groups, with a threshold set at  $|\log_2\text{FC}| \geq 0.585$  and  $P < 0.05$ .

The intersection of DEGs between the control vs mild CIH group and the control vs severe CIH group was determined, including DEGs that were upregulated or downregulated with increasing severity of chronic intermittent hypoxia.

## GO and KEGG Analysis

To further understand the functions of differentially expressed genes (DEGs) and their molecular pathways, we performed GO annotation using the Blast2GO functional annotation tool and the GO database (<http://www.geneontology.org/>), as well as KEGG pathway annotation using the KEGG database (<http://www.genome.jp/kegg/>).

## Detection of Tissue Iron and Malondialdehyde(MDA) Content

Three mice were randomly selected from each group. After isolating the hippocampal tissue, 1 mL of extraction solution was added, and the tissue was thoroughly homogenized and centrifuged to extract the supernatant. The microplate reader was preheated for 30 minutes, with the wavelength set to 520 nm and distilled water used for zero calibration. Samples were added sequentially according to the kit instructions (Solarbio, BC4350, Beijing, China) to calculate the iron content in the hippocampal tissue.

The supernatant from the homogenized hippocampal tissue was used for MDA content measurement. Samples were added sequentially according to the kit instructions (Solarbio, BC0020, Beijing, China), and the absorbance of each sample was measured at 532 nm and 600 nm to calculate the MDA content. Potential variability in tissue homogenization efficiency was controlled by using a standardized homogenization procedure across all samples.

## Western Blot

Three mice were randomly selected from each group. Total protein was extracted from hippocampal tissues of each group of mice. Protein quantification was performed using a BCA protein quantification kit to standardize the concentration. The proteins were then mixed with protein loading buffer and heated to boiling to denature the proteins. Subsequently, SDS-PAGE electrophoresis, transfer to membranes, antibody incubation, and visualization were carried out. Finally, gel imaging analysis was performed using a gel imaging system to scan and measure the optical density. The primary antibodies included Anti-Leptin Receptor antibody (Abcam, #ab5593), Anti-SIRT1 antibody (Abcam, 1:2000, cat#ab12193), Anti-NRF2 antibody (Cell Signaling Technology, 1:1000, cat#12721), and Anti-GAPDH antibody (Abcam, 1:2500, cat#ab9485). The secondary antibodies included Goat Anti-Rabbit IgG H&L (HRP) (Abcam, 1:5000, cat#ab6721). The ratio of optical density between the target band and the internal reference protein band was used for statistical analysis.

## Statistical Analysis

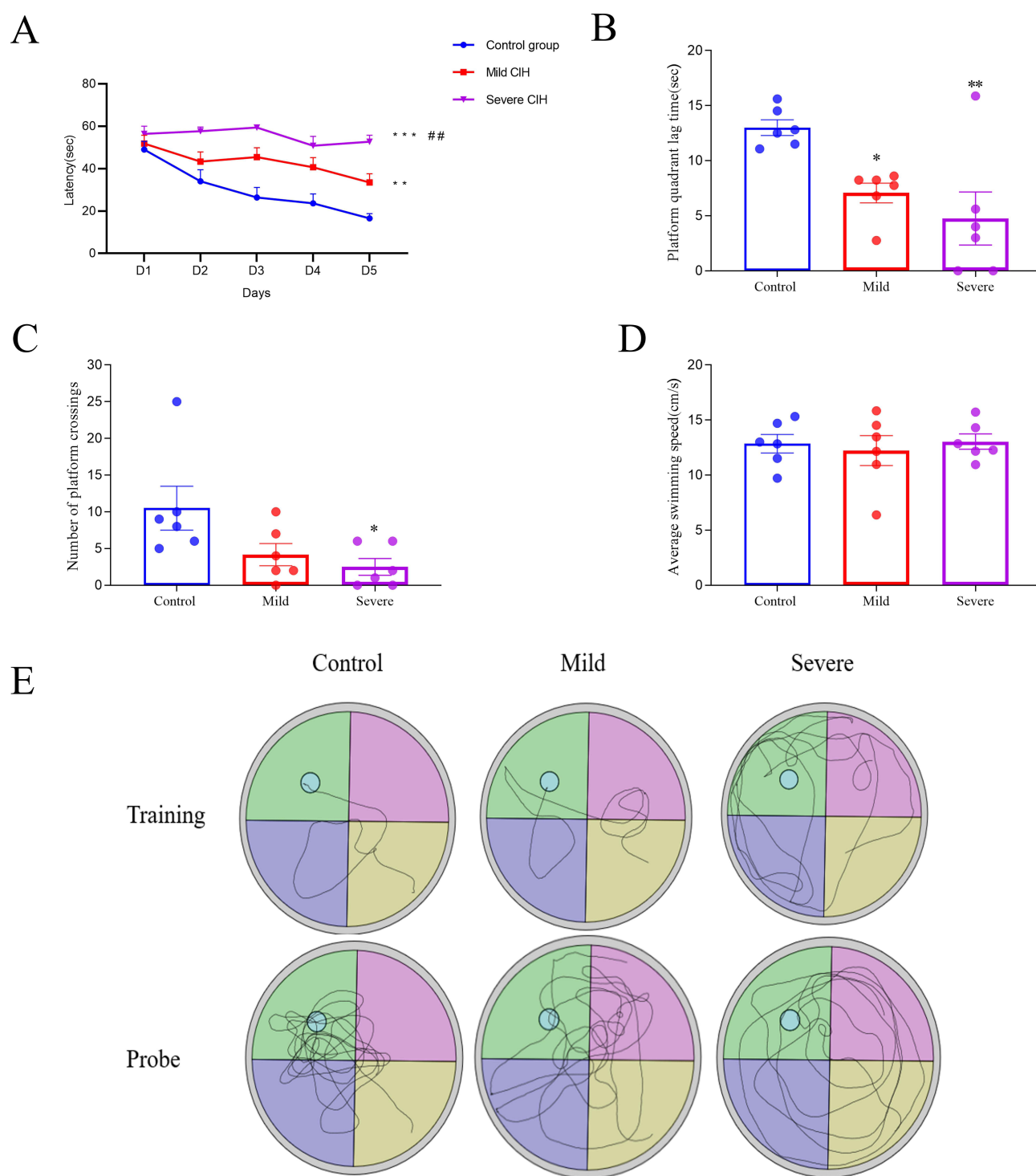
We used R software (version 4.2.2) and GraphPad (version 8.0.2) for statistical analysis. All data are presented as mean  $\pm$  standard error. One-way analysis of variance (ANOVA) was used to analyze statistical significance among multiple groups, and pairwise comparisons were conducted using two-tailed t-tests. To analyze the water maze data, two-way repeated measures ANOVA with Sidak's multiple comparisons test was performed ( $\alpha[PF]=1-(1-\alpha[PT])^C$ ). A p-value of less than 0.05 was considered statistically significant.

## Results

### Cognitive Impairment in CIH Mice

The Morris water maze (MWM) is a widely used method for assessing learning and memory in mice. Results showed that during the training phase, mice in the severe CIH group took significantly longer to find the platform compared to the mild CIH group and the control group ( $p < 0.001$ ;  $p < 0.01$ ), while mice in the mild CIH group took longer than the control group ( $p < 0.01$ ) (Figure 2A). In the testing phase, mice in the severe CIH group exhibited impaired spatial navigation, characterized by a decrease in platform crossings and reduced time spent in the target quadrant ( $p < 0.01$ ;  $p < 0.05$ ), whereas mice in the mild CIH group showed only reduced time spent in the target quadrant with no significant change in platform crossings ( $p < 0.05$ ) (Figure 2B, C and E). There were no significant differences in swimming speed among the three groups (Figure 2D). These data indicate that CIH results in cognitive impairment in mice.





**Figure 2** Morris water maze results. Data are presented as the mean  $\pm$  standard error ( $M \pm SE$ ) ( $n = 6$  mice per group). **(A)** Escape latency time. **(B)** Time spent in the target quadrant. **(C)** Number of platform crossings. **(D)** Average swimming speed. **(E)** Representative trajectory maps from the training and probe phases, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; compared to the Mild group, ### $p < 0.01$ .

## CIH Increases Neuronal Damage in Mice

HE staining of hippocampal tissues from each group of mice showed that neurons in the control group were neatly arranged with intact morphology. In contrast, hippocampal tissues from the severe CIH groups exhibited loosely arranged neurons with irregular sizes and blurred outlines. Nissl staining results revealed a reduction in the number of

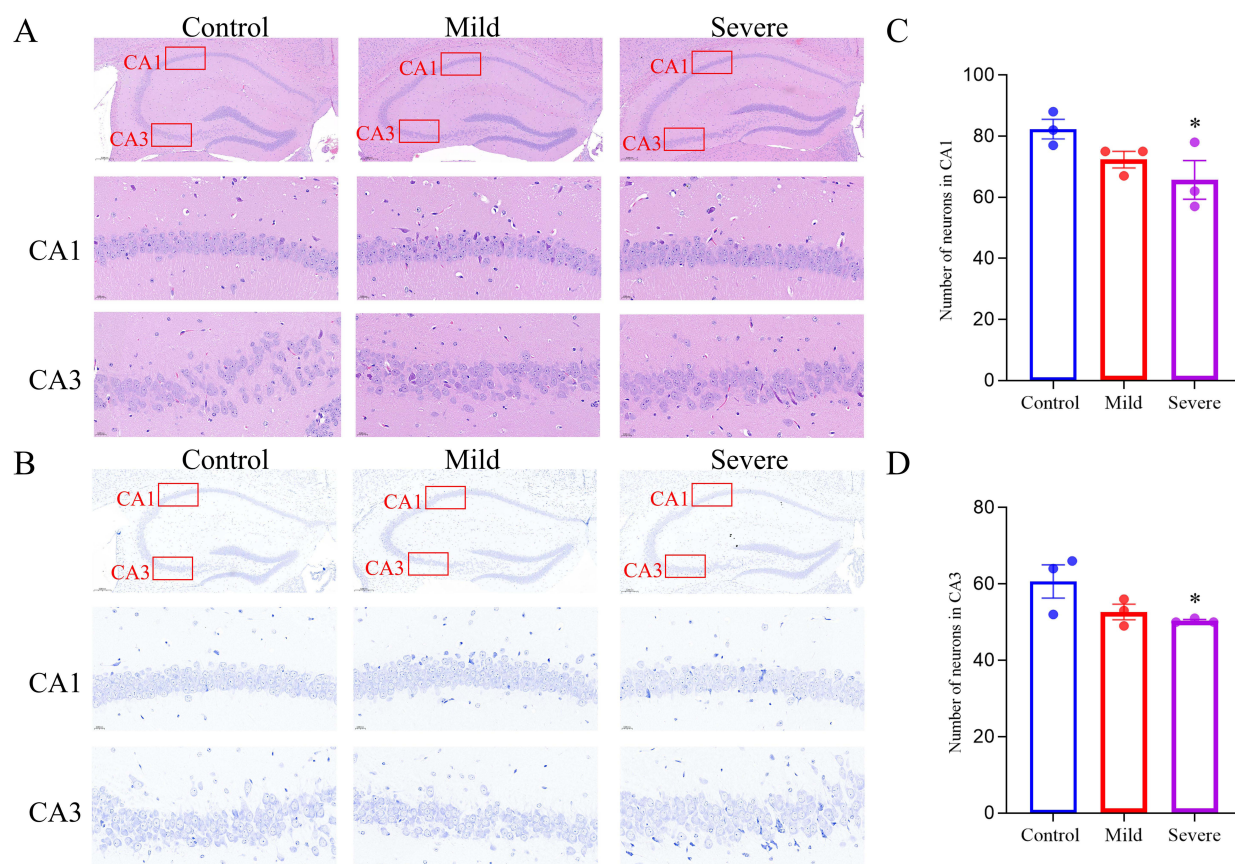
hippocampal neurons, loose arrangement, decreased Nissl body density, and lighter staining in the severe CIH groups ( $p < 0.05$ ). These findings indicate that CIH can lead to neuronal damage (Figure 3).

## Differential Expression in Hippocampal Tissues After Chronic Intermittent Hypoxia

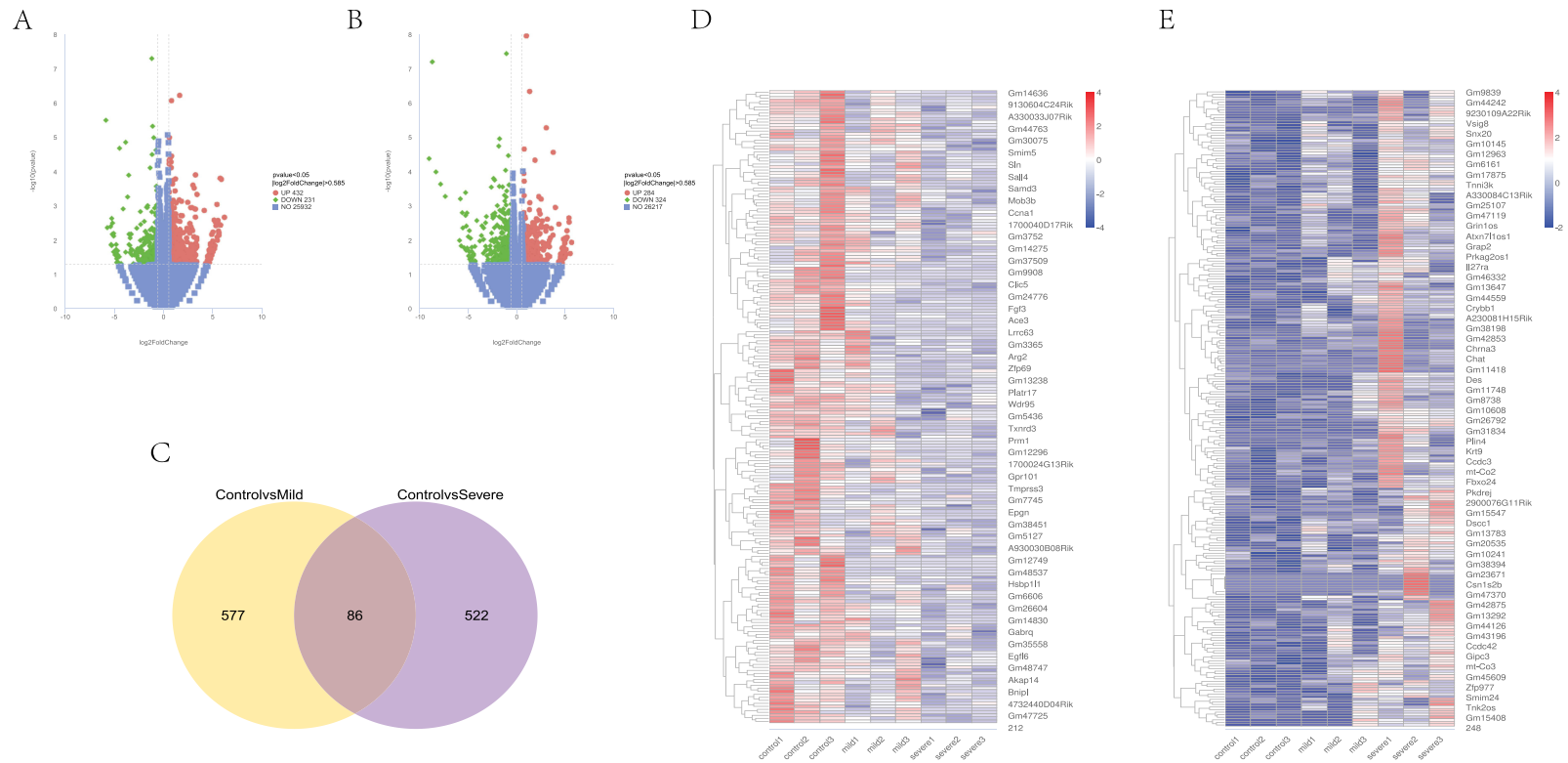
Compared to the control group, the mild CIH group exhibited a total of 663 differentially expressed genes, including 432 upregulated genes and 231 downregulated genes (Figure 4A, Supplementary Table 1). Similarly, the severe CIH group showed 608 differentially expressed genes compared to the control group, with 284 genes upregulated and 324 genes downregulated (Figure 4B and Supplementary Table 2). There were 86 common differentially expressed genes between the mild CIH group vs control group and the severe CIH group vs control group (Figure 4C, Supplementary Table 3). As the severity of hypoxia increased, 248 genes were upregulated and 211 genes were downregulated among the differentially expressed genes (Figure 4D and E).

## Functional Enrichment and Pathway Enrichment of Differential Genes Statistical Analysis

To gain deeper insights into the biological functions and pathways of differentially expressed genes (DEGs) in the hippocampal tissues of CIH mice, we conducted GO and KEGG enrichment analyses on the previously obtained sets of differential genes. The GO enrichment analysis revealed that, compared to the control group, DEGs in the mild CIH group were mainly involved in biological processes such as lymphocyte proliferation, regulation of leukocyte activation, and regulation of insulin secretion (Figure 5A). On the other hand, DEGs in the severe CIH group, compared to the control group, were primarily associated with biological processes including regulation of type 2 immune response, interleukin-1 production, and response to oxygen radical

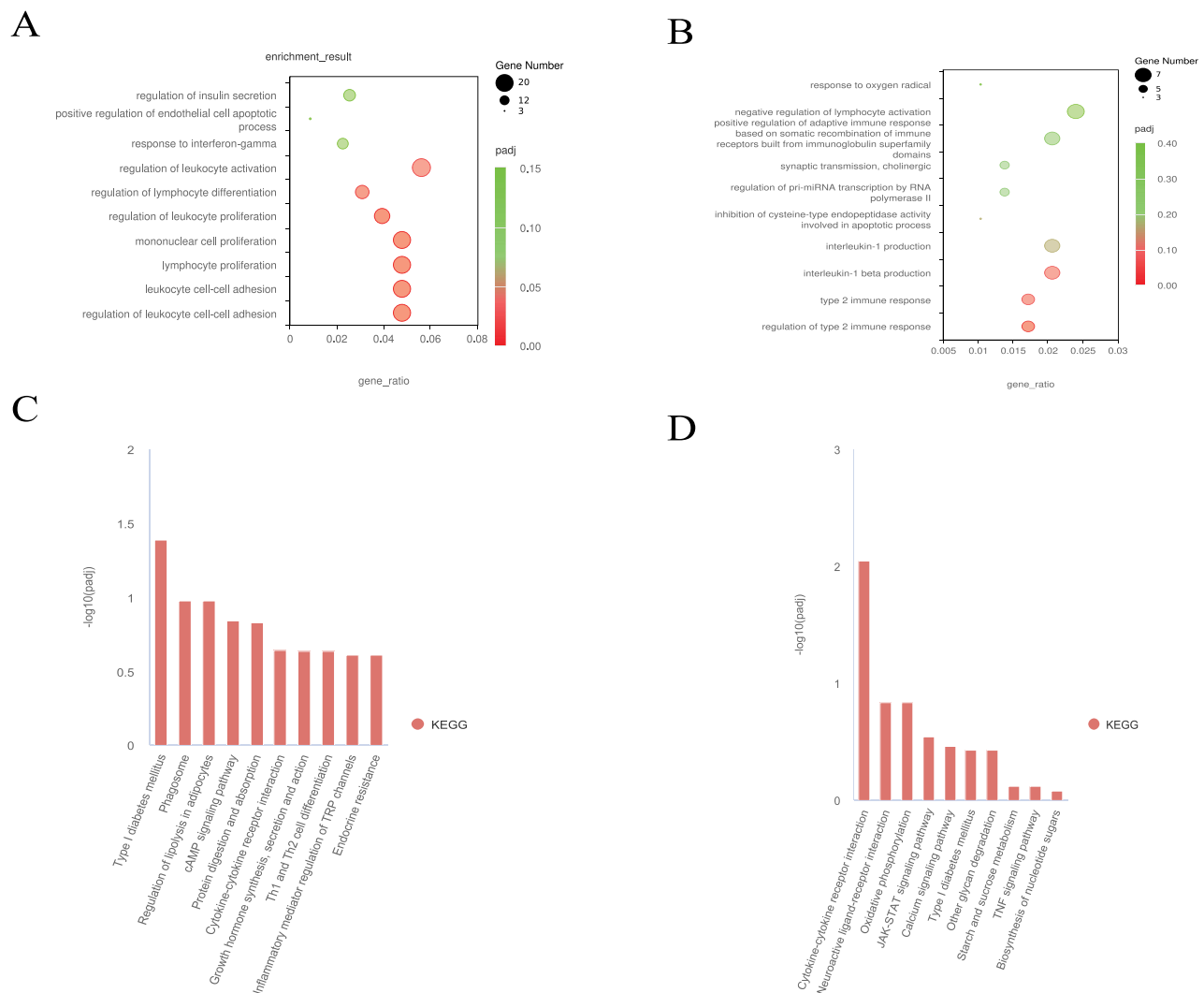


**Figure 3** Pathological Results. Data are presented as the mean  $\pm$  standard error ( $M \pm SE$ ) ( $n = 3$  mice per group). (A) Histological changes in hippocampal neurons assessed by HE staining. (B) Neuronal damage in the hippocampus assessed by Nissl staining. (C and D) Number of neurons in the CA1 and CA3 region ( $\times 400$  magnification field). Compared to the Control group, \* $p < 0.05$ .



**Figure 4** Differential Gene Analysis (n=3 mice per group). **(A)** Differentially expressed genes (DEGs) in the mild CIH group compared to the control group. **(B)** DEGs in the severe CIH group compared to the control group. **(C)** Venn diagram showing overlapping DEGs. **(D and E)** Genes upregulated and downregulated with increasing severity of hypoxia.





**Figure 5** Functional and pathway enrichment analysis of DEGs. (A and B) GO enrichment analysis of DEGs in the mild and severe CIH groups compared to the control group. (C and D) KEGG pathway enrichment analysis of DEGs in the mild and severe CIH groups compared to the control group.

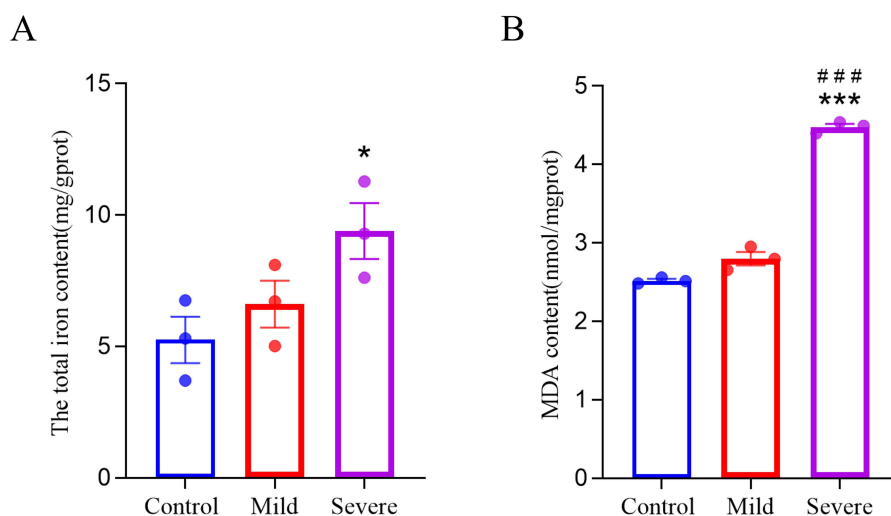
(Figure 5B). The KEGG enrichment analysis showed that DEGs in the mild CIH group, compared to the control group, were mainly related to biological pathways such as regulation of lipolysis in adipocytes, inflammatory mediator regulation of TRP channels, and endocrine resistance (Figure 5C). In contrast, DEGs in the severe CIH group, compared to the control group, were significantly enriched in pathways including the JAK-STAT signaling pathway, Type I diabetes mellitus, and TNF signaling pathway (Figure 5D). These results indicate that CIH primarily induces biological processes such as inflammation and oxidative stress in hippocampal tissues, involving pathways related to inflammation and insulin resistance.

## Increased Oxidative Stress in the Hippocampal Tissues of CIH Mice

To validate whether CIH leads to increased oxidative stress in hippocampal tissues, this study measured the total iron content and levels of lipid peroxidation product MDA in the hippocampal tissues. The results showed that both total iron content and MDA levels were significantly elevated in the severe CIH group ( $p < 0.05$ ;  $p < 0.001$ ) (Figure 6A and B). This indicates that CIH can increase oxidative stress levels in the hippocampal tissues of mice.

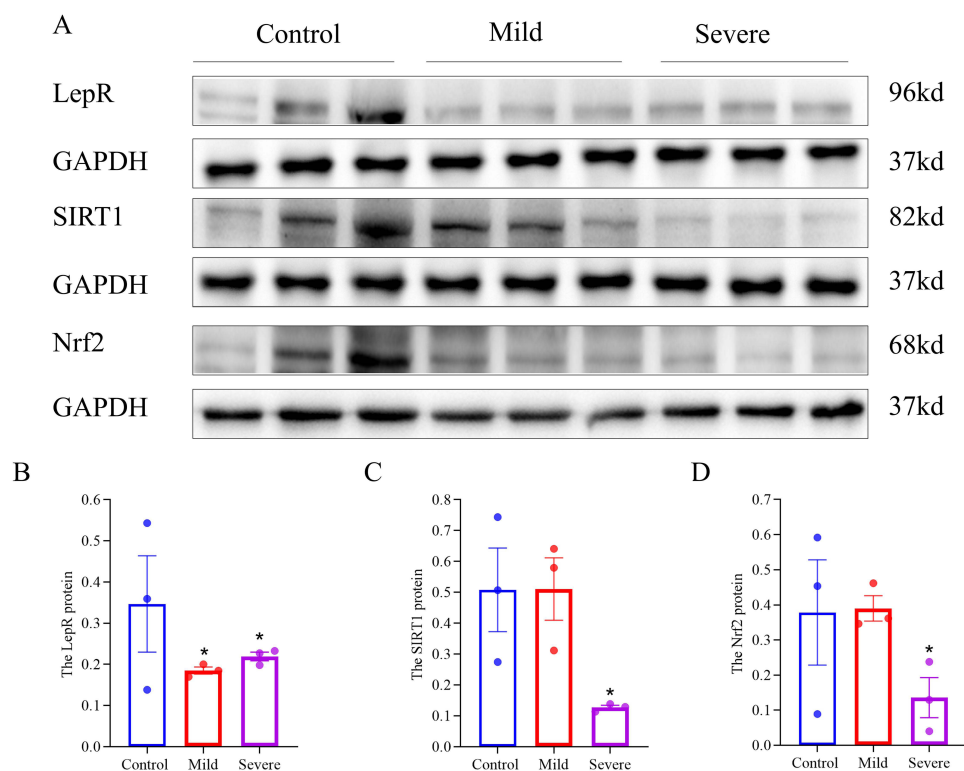
## Validation of Potential Key Genes Related to Cognitive Impairment in CIH Mice

Transcriptomic analysis of hippocampal tissues from CIH mice revealed decreased expression of *LepR* in both the mild and severe CIH groups, with a progressively downregulated trend as hypoxia severity increased. Additionally, the



**Figure 6** Oxidative stress levels in the hippocampus. Data are presented as the mean  $\pm$  standard error ( $M \pm SE$ ) ( $n = 3$  mice per group). **(A)** Total iron content in hippocampal tissues. **(B)** MDA levels in hippocampal tissues. Compared to the Control group, \* $p < 0.05$ , \*\*\* $p < 0.001$ ; compared to the Mild group, ### $p < 0.001$ .

differentially expressed genes were primarily enriched in biological processes such as inflammation and oxidative stress, involving pathways related to inflammation and insulin resistance. Within these pathways, genes like SIRT1 and Nrf2 are key regulators of inflammatory and oxidative stress transcription and may modulate LepR sensitivity. Therefore, this study validated the expression of LepR and proteins in the SIRT1 and Nrf2 pathways through Western blot analysis. The results showed significantly decreased expression of LepR in both the mild and severe CIH groups ( $p < 0.01$ ;  $p < 0.05$ ) (Figure 7A and B). Additionally, the expression of SIRT1 and Nrf2 was significantly downregulated in the severe CIH



**Figure 7** Validation of key genes via Western blot. Data are presented as the mean  $\pm$  standard error ( $M \pm SE$ ) ( $n = 3$  mice per group). **(A)** Representative Western blots of LepR, SIRT1, and Nrf2 in hippocampal tissues. **(B–D)** Quantification of protein expression levels for LepR, SIRT1, and Nrf2. Compared to the Control group, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; compared to the Mild group, ### $p < 0.001$ .

group ( $p < 0.001$ ;  $p < 0.001$ ) (Figure 7A, C and D). By analyzing the correlation between protein expression and behavioral results, we found a significant positive correlation between the time spent in the platform quadrant and the expression levels of SIRT1 and Nrf2 ( $p < 0.05$ ), while no significant correlation was found with LepR expression levels (Supplementary Figure 1A–C). Additionally, the correlation between the number of platform crossings in the MWM and the expression levels of LepR, SIRT1, and Nrf2 proteins was assessed. No significant correlation was observed between the number of platform crossings and the expression levels of any of the three proteins (Supplementary Figure 1D–1F).

## Discussion

The specific mechanisms by which OSA leads to cognitive impairment remain unclear; however, neuronal damage, particularly in the hippocampal region, may be a key pathophysiological feature of cognitive impairment in OSA patients.<sup>20,21</sup> The hippocampus is a critical brain region responsible for learning and memory in mammals, but it is particularly sensitive and vulnerable to damage under conditions of CIH.<sup>22</sup> This study aimed to investigate the effects of different degrees of CIH on mouse behavior and hippocampal pathology, and through analyzing changes in gene expression in the hippocampus, to elucidate potential molecular mechanisms underlying OSA-induced cognitive impairment.

OSA is a chronic condition characterized by frequent partial or complete upper airway obstruction during sleep, leading to intermittent pauses in breathing and intermittent hypoxia and hypercapnia.<sup>23</sup> Currently, organizations such as the Chinese Medical Association and the European Respiratory Society use an obstructive apnea-hypopnea index (OAH)  $>1$  event/hour in polysomnography as the diagnostic criterion for pediatric OSA, categorizing OSA severity based on OAH values.<sup>24,25</sup> Our previous clinical research has demonstrated that varying severities of OSA result in differing degrees of cognitive impairment.<sup>26</sup> One of the primary pathological features of OSA is CIH, and numerous studies indicate that CIH can impair cognitive function in mice. However, there is currently limited systematic investigation into the impact and mechanisms of different severity levels on cognitive function. Therefore, this study aimed to establish mouse models of varying degrees of CIH to simulate different severities of OSA in patients, with the goal of better elucidating the relationship between CIH mouse models and cognitive impairment.

The results of the Morris water maze experiment in this study demonstrated significant impairment in learning and memory abilities in mice exposed to CIH conditions. During the training trials, mice in the severe CIH group took significantly longer to find the platform, and although the performance of the mild CIH group was better than that of the severe CIH group, it was still significantly longer than the blank control group. Results from the probe trial further indicated decreased spatial learning abilities in the severe CIH group, characterized by reduced time spent in the target quadrant and fewer platform crossings, whereas the mild CIH group exhibited partial impairment in spatial learning and memory. These observations are consistent with previous literature reports and support the negative impact of CIH on cognitive function.<sup>27,28</sup> Under hypoxic conditions, the metabolism and function of neurons and brain cells are affected, leading to impaired higher cognitive functions such as learning and memory, suggesting changes in brain structure and function, particularly in memory-related brain regions such as the hippocampus.<sup>11,29</sup>

Further pathological exploration of mouse hippocampal tissue revealed pronounced morphological and structural changes in neurons in the CIH group, characterized by loose neuronal arrangement and reduced numbers. These observations suggest that CIH may lead to neuronal damage and pathological changes. Combined with existing research, this neuronal damage may be associated with various factors. Firstly, under hypoxic conditions, cellular oxidative stress levels may significantly increase, causing intracellular oxidative damage and generation of free radicals, thereby impairing neuronal structure and function. Secondly, the hypoxic environment may induce neuronal apoptosis, resulting in reduced neuronal numbers and loose arrangement.<sup>30,31</sup> Oxidative stress leads to lipid peroxidation, protein oxidation, and DNA damage, all of which compromise neuronal integrity and function. Additionally, inflammatory responses may also play an important role in neuronal damage caused by chronic intermittent hypoxia.<sup>32</sup> Under hypoxic conditions, immune cells and related cytokines may be activated, participating in neuroinflammatory responses, thereby altering the neuronal microenvironment and affecting normal neuronal function and survival.<sup>33,34</sup> Pro-inflammatory cytokines such as TNF- $\alpha$  and IL-6 are known to disrupt synaptic plasticity, which is critical for learning and memory, further contributing to cognitive deficits.

Through transcriptomic analysis, we identified differentially expressed genes in the hippocampal tissue of mice under mild and severe CIH conditions. These differentially expressed genes were primarily enriched in functional categories related to inflammation, oxidative stress, and other biological processes, suggesting that chronic intermittent hypoxia may influence neuronal function and cognition by regulating these biological processes. Further KEGG enrichment analysis revealed significant enrichment of multiple hypoxia-related signaling pathways in the hippocampal tissue of CIH mice. Particularly, neuroinflammation due to JAK-STAT signaling pathway and TNF signaling pathway is associated with cognitive impairment.<sup>35</sup> The JAK-STAT signaling pathway plays a crucial role in regulating cell proliferation, differentiation, and immune response, while the TNF signaling pathway is involved in modulating inflammatory responses and neuronal protection.<sup>36,37</sup> Hypoxia activates the JAK-STAT pathway, leading to the transcription of pro-inflammatory genes that exacerbate neuroinflammation. This inflammation disrupts synaptic communication and impairs neurogenesis, processes essential for maintaining cognitive function.

By screening for intersection genes between differentially expressed genes in the mild CIH vs control group, severe CIH vs control group, and genes showing gradual upregulation or downregulation with CIH severity, we identified LepR as a key gene. In the CIH mouse model, the expression level of LepR exhibited a gradual downregulation with increasing severity of CIH. Some studies have indicated that OSA patients with symptoms of attention deficit hyperactivity disorder (ADHD) exhibit increased leptin levels accompanied by visceral fat accumulation, suggesting the presence of leptin resistance.<sup>38</sup> Leptin receptors are distributed in brain regions such as the hippocampus, cortex, and cerebellum, and they reduce neuroinflammation and oxidative stress through multiple pathways to protect neurons.<sup>39,40</sup> LepR is thought to be involved in regulating neuronal growth, survival, and function, and its downregulation may increase neuronal sensitivity to oxidative stress.<sup>41</sup> In this study, the downregulation of LepR in CIH mice suggests that leptin signaling may be impaired, leading to reduced neuroprotective effects against oxidative stress and inflammation in the hippocampus. This suggests that LepR downregulation may directly contribute to cognitive deficits by reducing the hippocampal tissue's ability to counteract oxidative damage and neuroinflammation, both of which are key players in neuronal dysfunction.

The SIRT1/Nrf2 signaling pathway participates in metabolic regulation, energy regulation, and antioxidant stress, among other biological processes.<sup>42</sup> Studies have shown a negative correlation between leptin levels and SIRT1 expression.<sup>43</sup> SIRT1 activation promotes the expression of Nrf2, which regulates the antioxidant response, mitigating oxidative damage in neurons.<sup>44</sup> By inhibiting SIRT1 expression, downregulation of LepR may disrupt the SIRT1/Nrf2 axis, leading to decreased antioxidant capacity in the hippocampus and thus heightening oxidative stress, contributing to neurodegeneration and cognitive impairment. Therefore, LepR downregulation may contribute to cognitive impairment by inhibiting SIRT1/Nrf2 signaling, thereby reducing the brain's resilience to oxidative stress and promoting neuroinflammation. Additionally, this study observed increased levels of total iron content and oxidative stress markers like MDA in hippocampal tissue, suggesting possible leptin resistance and neuroinflammation. Therefore, further research is needed to validate whether CIH promotes hippocampal leptin resistance, leading to downregulation of the SIRT1/Nrf2 pathway, which subsequently induces neuroinflammation and contributes to cognitive impairment. Building on these mechanistic insights, the LepR/SIRT1/Nrf2 signaling pathway emerges as a promising therapeutic target for OSA-related cognitive impairments. Given the association of OSA with disrupted leptin signaling, interventions aimed at restoring LepR function or enhancing SIRT1/Nrf2 activity could provide neuroprotective effects, potentially mitigating cognitive deficits. Pharmacological strategies, such as antioxidants or inhibitors of inflammatory cytokines, specifically targeting oxidative stress and neuroinflammation, may offer substantial benefits. Furthermore, the early detection of leptin resistance or dysregulation in the leptin signaling pathway could serve as a valuable biomarker for cognitive dysfunction, enabling timely and targeted therapeutic interventions.

Further measurement of protein levels by Western blot revealed that the expression of LepR, SIRT1, and Nrf2 proteins in the hippocampal tissue of CIH mice was downregulated. These proteins play important roles in intracellular oxidative stress and inflammation regulation, and their downregulation may be closely associated with cognitive impairment. LepR is believed to be involved in regulating neuronal growth, survival, and function, and its downregulation may increase neuronal sensitivity to oxidative stress.<sup>45</sup> Additionally, the SIRT1/Nrf2 pathway is a crucial intracellular antioxidant stress pathway, and its downregulation may weaken the hippocampal tissue's defense against oxidative stress, thereby exacerbating neuronal damage and cognitive decline.<sup>44,46</sup>

It is important to note that this study has certain limitations. First, the use of a chronic intermittent hypoxia (CIH) mouse model may not fully replicate the complexity of OSA in humans, particularly regarding the interaction of CIH with other factors present in human patients. The extrapolation of these results to human OSA patients should be done cautiously. Second, the choice of a single mouse strain (C57BL/6J) limits the generalizability of the findings to other mouse strains or species. Different strains or species may exhibit distinct physiological and cognitive responses to CIH, so future studies should include multiple strains and species to enhance the generalizability of the results. Moreover, we only measured the relative protein levels of key genes by Western blot but did not explore the regulatory relationship between LepR and the SIRT1/Nrf2 pathway or its association with cognitive function in CIH mice by manipulating LepR expression. This limits our understanding of the potential causal link between these molecular pathways and cognitive impairment. Future studies should aim to perform mechanistic investigations by manipulating LepR expression, either through gene knockdown or overexpression models, to directly assess its role in SIRT1/Nrf2 regulation and neuronal health. Additionally, this study did not include longitudinal assessments of cognitive function over time, which could have provided insights into the progression of cognitive impairment and neuronal damage in CIH mice. The lack of neuroimaging data, such as MRI or PET scans, also limits our ability to assess brain structure and function changes in a non-invasive and time-sensitive manner. Including such data could enhance our understanding of the dynamics of neuronal injury. Therefore, future studies could conduct intervention experiments to further explore the impact of target genes on cognitive function in CIH mice.

## Conclusion

In summary, this study identified key molecular targets, particularly LepR and the SIRT1/Nrf2 signaling pathway, which may play crucial roles in the regulation of oxidative stress and inflammation in OSA-induced cognitive impairment. These findings provide potential molecular targets for developing therapeutic strategies aimed at mitigating oxidative stress and neuroinflammation. For example, targeting the upregulation of the SIRT1/Nrf2 pathway or restoring leptin receptor (LepR) function could serve as potential therapeutic interventions. Drugs that enhance SIRT1/Nrf2 pathway activity or reduce leptin resistance may offer promising avenues for intervention. Therapies aimed at directly modulating these pathways could be designed to enhance neuroprotection, reduce neuronal inflammation, and counteract oxidative damage, all of which are central to OSA-related cognitive decline. Future research could focus on elucidating the precise mechanisms by which these genes and pathways influence cognitive function, as well as developing drugs or intervention strategies that directly target these molecular pathways, offering new insights and approaches for preventing and treating cognitive impairment caused by OSA. However, it is important to acknowledge the limitations of this study, including the use of a single mouse strain and the lack of longitudinal and neuroimaging assessments, which limit the generalizability of the findings and the full understanding of CIH's long-term effects on cognition. Further validation of these findings in human studies is essential to confirm their clinical relevance. To advance these findings, future research could take concrete steps such as conducting gene manipulation studies to validate the therapeutic potential of modulating LepR or the SIRT1/Nrf2 pathway, for example, by overexpressing or silencing LepR. Clinical trials could also be conducted to test the efficacy of drugs targeting these pathways, which would be essential in translating these findings into practical therapeutic approaches. Such studies will help clarify the biological underpinnings of cognitive impairment in OSA and explore effective treatments.

## Data Sharing Statement

The datasets used during the current study are available from the corresponding author on reasonable request.

## Consent for Publication

This study did not include the human subjects.

## Ethics Approval

This study complies with the principles of the Declaration of Helsinki. The Ethics Committee of Beijing Children's Hospital of Capital Medical University approved the study (No: 2020-k-93).



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## Disclosure

The authors declare no conflicts of interest related to this study.

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