

# Homocysteine-Potentiated Keap1 Promotes Neuronal Senescence via Inhibiting Ubiquitination of $\beta$ -Catenin

**Yao Zhang**

Liyuan Hospital of Tongji Medical College of Huazhong University of Science and Technology

**Jiazhao Xie**

Huazhong University of Science and Technology Tongji Medical College

**Yan-li Jiang**

Liyuan Hospital of Tongji Medical College of Huazhong University of Science and Technology

**Shao-juan Yang**

Liyuan Hospital of Tongji Medical College of Huazhong University of Science and Technology

**Hui Wei**

Huazhong University of Science and Technology Tongji Medical College

**Ying Yang** (✉ [yingyang@mail.hust.edu.cn](mailto:yingyang@mail.hust.edu.cn))

Huazhong University of Science and Technology Tongji Medical College <https://orcid.org/0000-0002-9479-4766>

**Jian-zhi Wang**

Huazhong University of Science and Technology Tongji Medical College

---

## Research article

**Keywords:** Homocysteine, Keap1,  $\beta$ -catenin, methylation, senescence

**Posted Date:** January 3rd, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-1120464/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

## Background

Elevated serum homocysteine (Hcy) is an independent risk factor of Alzheimer's disease (AD). It has been reported that Hcy dramatically accelerates the aging of endothelial progenitor cells or endothelial cells. However, whether and how Hcy produces neuronal senescence is largely unknown.

## Methods

Mouse neuroblastoma 2a (N2a) cells were treated with Hcy, and senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) staining was applied to assay senescence. Senescent markers and related proteins were examined by western blot, quantitative Polymerase Chain Reaction (qPCR), immunofluorescence staining. Methylation of promoter was assayed by bisulfite sequencing PCR (BSP). Immunoprecipitation (IP) was applied to examine association between proteins. Rats were injected with homocysteine and examined neuronal senescence.

## Results

In this study, we observed that Hcy significantly promoted the senescence of N2a cells with elevated  $\beta$ -catenin and Kelch like ECH-associated protein 1 (Keap1). Intriguingly, Hcy increased the interaction between Keap1 and Wilms tumor gene on X chromosome (WTX), but decreased  $\beta$ -catenin-WTX interaction simultaneously. Mechanistically, Hcy attenuated the methylation level of Keap1 promoter's CpG island and activated the transcription of Keap1. While, slow degradation rate rather than transcriptional activation contributed to the high level of  $\beta$ -catenin. Hcy-increased Keap1 competed with  $\beta$ -catenin to bind to WTX. Knockdown of  $\beta$ -catenin and Keap1 both attenuated Hcy-induced senescence of N2a cells. Hcy-induced rats model also showed neuronal senescence in cortex along with elevated senescent markers.

## Conclusions

Our data highlight a crucial role of Keap1- $\beta$ -catenin pathway in Hcy-induced neuronal-like senescence and provide a promising target for AD treatment.

## Introduction

Homocysteine (Hcy) is an intermediate product of cysteine homologue and methionine metabolism and is considered a high risk factor for AD (Miller, 1999). Previous reports have shown that the AD-like animal model established by tail vein injection of Hcy almost completely characterizes the pathological characteristics of AD (Jiang et al., 2015). The animal model of Hcy reproduces the pathological features

of AD, such as Tau hyperphosphorylation,  $\beta$ -amyloid plaque ( $A\beta$ ) accumulation and neuron loss (Zhang et al., 2008; Zhang et al., 2009). In addition, it has been reported that the increase Hcy level in blood leading to hyperhomocysteinemia is related to vascular aging and accelerating the aging process, which also contributes to the progression and development of AD (Ostrakhovitch et al., 2019; Zhang et al., 2015).

Aging is a high risk for AD. The traditional view of cellular senescence is a phenotype characterized by a durable arrest of cell cycle and a collection of stress granules (Moreno-Blas et al., 2019). As a result, studies on brain cellular senescence mostly focused on glial cells (Chinta et al., 2015). Recent studies focus on cortical and Purkinje neurons found several features of senescence, such as senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) activity,  $\gamma$ H2AX and macro-H2A foci, and lipofuscin accumulation all exist in a p21<sup>CIP1/WAF1</sup>-dependent manner (Kang et al., 2015). According to this criterion, it is found that human neurons will also become senescence, because *p16/Cdkn2a* is expressed in the prefrontal cortex pyramidal neurons in the brain of people over 77 years of age (Jurk et al., 2012). Long-term cultured rat primary cortical neurons shown the characteristics of cellular senescence before glial cells, and developed a functional senescence-related secretory phenotype, which can induce premature paracrine in mouse embryonic fibroblasts senescence (Ishikawa et al., 2020). While senescent cells in peripheral tissues induced by Hcy have been the focus of numerous reports, their participation in or contribution to cognitive decline with aging or neurodegenerative diseases remains largely unknown. Particularly, Hcy could accelerate telomere attrition by increasing telomere loss per replication in vascular endothelial cells (Shin et al., 2016; Zhang et al., 2015), upregulation of p16, p21, and p53 (Curro et al., 2014), activation of  $\beta$ -galactosidase activity (Walsh et al., 2007), all of which are typical pathological characteristics of cellular senescence. Until now, most reports focus on homocysteine-induced cellular senescence in non-neuronal cells such as endothelial cells (McCully, 2018; Sun et al., 2019). In view of these facts, it is of great research value to study whether Hcy can induce neuronal senescence and its mechanism.

In general, the cellular Wnt/ $\beta$ -catenin signaling pathway is essential for regulating proliferation, cell cycle, apoptosis and axis polarity induction (Reya et al., 2005). Once activated by Wnt, the accumulated  $\beta$ -catenin is transferred to the nucleus, binds to transcription factors and changes the expression of downstream target genes, which will promote neuron survival and neurogenesis, and enhance synaptic plasticity (Jia et al., 2019). Previous studies indicated that the loss of function of Wnt/ $\beta$ -catenin signaling component promoted the occurrence and progression of AD-like symptoms, and recovery is a promising strategy to ameliorate these symptoms (De Ferrari et al., 2014; Jia et al., 2019). However, recent studies have revealed another function of  $\beta$ -catenin, indicating that abnormally activated  $\beta$ -catenin is related to the dysfunction of distal lung epithelial cells and lead to accelerated aging (Liu et al., 2007). Moreover, another study showed that intervention of the association of GSK3 $\beta$  reduced the phosphorylation level and degradation rate of  $\beta$ -catenin, and the accumulated  $\beta$ -catenin further induced neuronal senescence (Chow et al., 2019). In addition to GSK3 $\beta$ , Wilms tumor gene on X chromosome (WTX) is another factor inducing  $\beta$ -catenin's ubiquitination (Major et al., 2007). Besides, another study found that as blood Hcy

levels increased,  $\beta$ -catenin accumulated in the nucleus of the vascular endothelium (Beard et al., 2012). Since it is predicted that the senescence marker p53/p21 mediates the senescence of normal  $\beta$ -catenin cells, it is reasonable to propose that Hcy promotes cell senescence by activating  $\beta$ -catenin (Zhang et al., 2013).

In present study, we first investigated SA- $\beta$ -gal activity, p16, p21 and p53 in mouse neuroblastoma N2a cells after induction by Hcy. Then  $\beta$ -catenin and related proteins were evaluated. Furthermore, WTX and Keap1 were investigated to reveal underlined mechanism of how  $\beta$ -catenin was activated.

## Materials And Methods

### Reagents

BAC Assay (BL521A) and enhanced chemiluminescent substrate (BL523A) were purchased from Biosharp (Beijing, China). Fetal bovine serum (FBS, 10099141), and Dulbecco's modified Eagle medium (DMEM, 11965092) were purchased from Gibco (ThermoFisher, USA). Homocysteine (HY-W040821), MG132 (HY-13259) and cycloheximide (HY-12320) were purchased from MedChemExpress (USA). Senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) staining colorimetric kit was purchased from Beyotime (C0602, China). The following antibodies were used in this study:  $\beta$ -catenin (A19657), p16 (A11651), p21 (A19094) and p53 (A19585) rabbit polyclonal antibody from Abclonal (1:1000, MA, USA). Keap1 (10503-2-AP) rabbit polyclonal antibody, GAPDH (60004-1-Ig),  $\beta$ -actin (60008-1-Ig) mouse monoclonal antibody, CoraLite488 goat-anti-mouse (SA00013-1) or rabbit (SA00013-2) IgG, CoraLite 594 goat-anti-mouse (SA00013-3) or rabbit (SA00013-4) IgG were purchased from Proteintech (1:1000, Chicago, USA). GSK3 $\beta$  (ab93926), GSK3 $\beta$  pS9 (S9) (ab107166) polyclonal antibodies were purchased from Abcam (1:1000, Cambridge, UK). WTX (AP6553c) polyclonal antibody was purchased from Abcepta (dilution 1:500, CA, USA). Ubiquitin (3936) and acetylated-Lysine (9441) monoclonal antibody were products of CST (1:1000, USA).

### Establishment of homocysteine rat model

Two months' old male SD rats were purchased from the animal center of Tongji Medical College, Huazhong University of Science and Technology. All rats were kept at 23 $\pm$ 2 $^{\circ}$ C on daily 12-h light–dark cycles with ad libitum access to food and water. For establishment of rats' model, homocysteine (400  $\mu$ g/kg/day) was injected via vena caudalis for 14 days.

### Cell culture and transfection.

Mouse neuroblastoma 2a (N2a) cells were purchased from China Center for Type Culture Collection (Wuhan, China), and cultured in DMEM/10% fetal bovine serum. For Hcy treatment, N2a cells are planted on the culture plate for 24 hours, and then Hcy is added and cultured for another 24-72 hours. For siRNA transfection, Keap1 or  $\beta$ -catenin siRNA (AUGCT, China) were added and incubated for 24 h according to the manufacturer's instructions, followed by Hcy treatment. siRNA sequences are: Keap1 (sense)

AUAUCUACAUGCACUUCGG; Keap1 (antisense) CCGAAGUGCA UCUAGAUAU.  $\beta$ -catenin (sense) CACGCAAGAGCAAGTAGCTGATATT;  $\beta$ -catenin (antisense) AATATCAGCTACTTGCTCTTGCGTG.

### **Senescence-associated $\beta$ -galactosidase assay with immunostaining.**

Senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) staining was performed according to the manufacturer's protocol. In brief, 10,000 N2a cells seeded on 15-mm glass coverslips were washed with PBS and fixed with 4% formaldehyde for 15 min at room temperature. Fixed cells were washed twice with PBS before incubation in SA- $\beta$ -gal staining solution at 37 °C for 16 h. After incubation, samples were washed twice with PBS, and the numbers of blue SA- $\beta$ -gal-positive cells were quantified using a microscope. For staining of tissue samples, frozen specimens sectioned at 30- $\mu$ m thick were briefly fixed in 4% formaldehyde for 5 min, then staining was performed using the same procedures as for the cultured cells. The exact timing was varied according to the thickness of the section and prior treatment of the tissue.

Co-immunostaining was performed after the SA- $\beta$ -gal staining procedure. In brief, cells glass coverslips were washed twice in PBS after the blue color was developed, then they were permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. After that, blocking with 0.5% BSA in PBS was performed for 1 h, and primary antibodies (Keap1 or  $\beta$ -catenin) incubation in 0.5% BSA was allowed for overnight at 4°C. Once this incubation step was completed, samples were washed three times with PBS (3 min each), and incubated with secondary antibodies (Alexa Fluor 488 goat-anti-mouse or rabbit IgG (1:1000), and Alexa Fluor 594 goat anti-rabbit or mouse IgG (1:1000) in PBS with BSA) for 60 min at room temperature in 0.5% BSA. The washing step was then repeated, followed by nuclei staining with 1  $\mu$ g/ml 4,6-diamidino-2-phenylindole (DAPI) solution for 10 min. Samples were washed twice in PBS, mounted and observed under microscope. The coverslips were then rinsed and mounted onto glass slides with glycerol. Cells slice were examined using immunofluorescence microscopy (SV120, Olympus, Japan).

### **Protein degradation assay**

For assay the degradation rate of  $\beta$ -catenin, N2a cells were added 100  $\mu$ M MG132 and 100  $\mu$ M cycloheximide after 72 hours' treatment with 100  $\mu$ M Hcy, and cells were collected every hour until 5 hours. All cell samples were harvested and stored at -80°C for further analyzed by western blot.

### **Western blot and immunoprecipitation**

Protein extracts were generated from rat brains or cell cultures, resolved by SDS-PAGE, and transferred onto PVDF membranes. After being blocked by 5% nonfat milk in TBS with 0.1% Tween-20, the membrane was incubated with the primary antibodies in 0.5% BSA overnight at 4°C, followed by peroxidase-conjugated goat anti-rabbit or mouse IgG (1:1000; Beyotime, China) for 60 min at room temperature. The immunoreactive bands were detected using the Enhanced Chemiluminescent Substrate in luminometer (ChemiScope 6000, Clinx, China). Band intensity was measured using ImageJ (NIH, USA).

To analyze ubiquitinated  $\beta$  catenin, we performed immunoprecipitation (IP) experiments using N2a cells lysates. Specified antibody ( $\beta$  catenin) and protein G agarose were incubated with the N2a cells lysates (100 $\mu$ g) overnight at 4°C. The resins were washed three times with PBS. After elution by 2 $\times$ loading buffer, and boiled at 95°C for 10 min, the bound proteins were analyzed by western blotting using  $\beta$  catenin and ubiquitin antibodies.

### **Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

Total RNA was isolated from the primary astrocyte using TRIzol Reagent (15596018 ThermoFisher Scientific, USA) according to the manufacturer's manual. Reverse transcription was carried out to synthesize cDNA using Hifair® 1st Strand cDNA Synthesis Kit (11121ES60, Yeasen, China) and qRT-PCR assays were performed using SYBR mix (11203ES03, Yeasen, China). Primer sequences for each gene are synthesized by AUGTC (Beijing, China) and listed as followed: p16 forward primer: AACTCTTTCGGTCGTACCCC, reward primer: GCGTGCTTGAGCTGAAGCTA. p21 forward primer: CCTGGTGATGTCCGACCTG; reward primer: CCATGAGCGCATCGCAATC. P53 forward primer: CTCTCCCCCGCAAAGA AAAA, reward prime: CGGAACATCTCGAAGCGTTTA;  $\beta$ -catenin forward primer: ATGGAGCCGGACAGAAAAGC, reward primer: CTTGCCACTCAGGGAAGGA. Keap1 forward primer: GATGGGCAGGACCAGTTGAA, reward primer: CCGAGGACGTAGATCTTGCC. The specificity of the PCR product was confirmed by analyzing the melting curve. All PCRs were performed in Triplicate. Results were normalized to mRNA expression in primary astrocytes of sham-operated control.

### **Bisulfite sequencing PCR (BSP) assay**

The Keap1 promoter in N2a cell was determined to range from - 2000 to - 70 bp by the Transcriptional Regulatory Element Database from Cold Spring Harbor (<http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=promInfo&pid=19717>). The Keap1 promoter in mouse was searched in the Transcriptional Regulator Element Database (accession number 46672, NM 009741). The CpG island in the promoter (- 300 to 0 bp) was detected using the UCSC Genome Browser, and the methylation status was analyzed using BSP. Primers for BSP were designed through MethPrimer (<http://www.urogene.org/methprimer/>), and then were blasted and confirmed using meth BLAST. Keap1's primer as followed, F primer: AATTTTAGGGGATATTGTAT AGTTTA; R primer: ATTCCAACCCTTCTACAAATAC. A Genomic DNA Extraction kit (Takara, Japan) was used to extract DNA from the cells. The bisulfite conversion of DNA was performed with an EpiTect Bisulfite kit (Qiagen), PCR was performed on the bisulfate-modification samples, and PCR products were cloned into plasmids, and the inserted sequences were sequencing analysis.

### **Statistical Analysis**

Results were expressed as means  $\pm$ SEM. Unpaired t test was used to assess statistical significance between two groups. With respect to multiple comparisons involving 3 or more groups, statistical significance was assessed by one-way ANOVA followed by post hoc test (Bonferroni's method). Statistics were computed with Graphpad Prism 6 (GraphPad Software).  $p < 0.05$  was considered as statistically significant.

# Results

## Hcy promoted N2a cells senescence in a time-dependent way.

Cell senescence was characterized by enhancement of  $\beta$ -galactosidase activity, upregulation of p16, p21 and p53, which are regarded as marker of senescence. To verify whether Hcy promote neuronal cell senescence, we treated with N2a cells using Hcy. In order to avoid its toxicity, the effect of different concentrations of Hcy (0 to 200  $\mu$ M) on cell viability was tested. According to CCK8 result (Figure 1 A), Hcy didn't produce toxicity to N2a cells until 200  $\mu$ M ( $p$  value =0.036), so we chose a concentration of 100  $\mu$ M Hcy in the follow-up study. Next, in order to find the appropriate time for inducing cell senescence, N2a cells was cultured with Hcy or DMSO and collected at different time point. As shown by the number of SA- $\beta$ -gal positive staining cells, we observed that Hcy promoted the senescence of N2a cells in a time-dependent manner (0 vs 48 h,  $p$  value=0.0088; 0 vs 72 h,  $p$  value =0.001) (Figure1 B and D), and 72 h of induction was considered the ideal time. In addition, after 72 h of Hcy treatment, Western blot and qPCR were used to detect cell senescence markers p16, p21, and p53 to confirm the senescence-promoting effect of Hcy. Compared with the normal control, the protein level of senescence markers p16 ( $p$  value=0.047), p21 ( $p$  value=0.019) and p53 ( $p$  value=0.0112) in Hcy-treated N2a cells increased significantly (Figure 1 C and E). In consistent with protein level, Hcy also enhance mRNA level of p16 ( $p$  value=0.0336), p21 ( $p$  value=0.047) and p53 ( $p$  value=0.0164), indicating that Hcy can effectively enhance the senescence of N2a cells.

## Hcy induced cell senescence by enhancing protein level of $\beta$ -catenin

Previous reports indicated that non-degraded  $\beta$ -catenin is both necessary and sufficient to drive the neuronal senescent-like phenotype (Chow et al., 2019). And hyperhomocysteinemia could potentiate Wnt/ $\beta$ -catenin signaling in initiating cardiogenesis(Han et al., 2009). Then we assayed the expression of  $\beta$ -catenin in N2a cells after Hcy induction. Upregulation of  $\beta$ -catenin was observed after induction of Hcy ( $p$  value=0.001), without alternation of  $\beta$ -catenin' mRNA level (Figure2 A, B and C). Next, degradation rate of  $\beta$ -catenin was assayed after Hcy's treatment. After incubation of Hcy, MG132 and cycloheximide were added to avoid *de novo* expression. Compared to control, degradation rate was more slowly in the condition of Hcy treatment (3 h,  $p$  value=0.034; 4 h,  $p$  value=0.021; 5 h,  $p$  value=0.012) (Figure 2 D, E). To further verify the ubiquitination level,  $\beta$ -catenin was immunoprecipitated by its specific antibody and examined using pan-ubiquitination antibody. As expected, the ubiquitination level of  $\beta$ -catenin was markedly reduced after Hcy's induction ( $p$  value=0.0167) (Figure 2 F and G). Moreover, positive staining of SA- $\beta$ -gal ( $p$  value=0.0001) (Figure 2 I) further confirmed increasing  $\beta$ -catenin (Figure 2 J) ( $p$  value=0.001) co-located with senescent N2a cells, indicating augment of  $\beta$ -catenin was positively related with senescent N2a cells. To further verify the relationship between  $\beta$ -catenin and senescence, knockdown of  $\beta$ -catenin using siRNA (Ctrl vs siRNA,  $p$  value=0.007; Ctrl vs Hcy,  $p$  value=0.013; Hcy vs Hcy+siRNA,  $p$  value=0.023) (Figure 2 K and L) reduced positive SA- $\beta$ -gal staining of N2a cells by induction of Hcy (Ctrl vs siRNA,  $p$  value=0.43; Ctrl vs Hcy,  $p$  value=0.001; Hcy vs Hcy+siRNA,  $p$

value=0.006) (Figure 2 M and N). These evidences suggested accumulation of  $\beta$ -catenin mediated N2a cellular senescence.

### **Keap1 inhibited $\beta$ -catenin's binding to WTX**

As reported by previous studies,  $\beta$ -catenin could be ubiquitinated by GSK3 $\beta$  and WTX (Chow et al., 2019; Major et al., 2007). Next, we examined both activity of GSK3 $\beta$  and expression of WTX in N2a cells after induction of Hcy. Unexpectedly, both activity of GSK3 $\beta$ , indicated by GSK3 $\beta$  pS9 (S9), and expression of WTX didn't change significantly after induction of Hcy (Figure 3 A and B). However, another WTX binding protein Keap1 was observed upregulated greatly after induction of Hcy ( $p$  value=0.001) (Figure 3 A and B). This result led us to the hypothesis that the increase in Keap1 expression promoted an increase in its binding to WTX, thereby reducing the binding of  $\beta$ -catenin. Further analysis by IP revealed that after induction of Hcy, the association between WTX and Keap1 increased ( $p$  value=0.0167) along with decreased binding between WTX and  $\beta$ -catenin ( $p$  value=0.0196) (Figure 3 C, D and E), suggesting the increased binding of Keap1 to WTX is the cause of the decreased binding of  $\beta$ -catenin. Moreover, mRNA of Keap1 ( $p$  value=0.0163) (Figure 3 F) was enhanced after induction of Hcy further confirmed upregulation of Keap1. These results suggested Keap1 contributed to upregulation of  $\beta$ -catenin.

### **Hcy induced hypomethylation of Keap1's promoter and expression**

Previous studies discovered Hcy induced demethylation of DNA, especially the promoter sequence (Zhang et al., 2013), thus promoted mRNA expression of targeted gene and resulted in increased protein expression. Moreover, it's observed that protein and mRNA level of Keap1 was enhanced significantly after induction of Hcy compared to control. Next, DNA methyltransferase 1 (DNMT1), whose main function is the transfer of methyl groups, was tested by western blot after induction of Hcy. Compared to control, Hcy treatment significantly downregulated the expression of DNMT1 in N2a cells ( $p$  value =0.0475) (Figure 4 A and B). Then we used the BSP method to analyze the methylation level of the CpG island enrichment region of the Keap1 promoter, and observed that Keap1's CpG island methylation was reduced compared to the control ( $p$  value =0.0254) (Figure 4 C and D). Co-localization using immunofluorescence-labeled Keap1 and positive SA- $\beta$ -gal staining found that the expression of Keap1 in positive SA- $\beta$ -gal staining cells was significantly increased ( $p$  value=0.0023) (Figure 4 E and F), indicating that  $\beta$ -galactosidase activity was higher when enhancing Keap1's expression ( $p$  value=0.0043) (Figure 4 E and G). And  $\beta$ -catenin was also observed upregulated and co-located with Keap1 and SA- $\beta$ -gal staining (Figure 4 E). At last, to confirm the role Keap1 played in mediating Hcy-induced cell senescence, we knockdown the expression of Keap1 by siRNA before induction of Hcy (Ctrl vs siRNA,  $p$  value=0.003; Ctrl vs Hcy,  $p$  value=0.036; Hcy vs Hcy+siRNA,  $p$  value=0.042) (Figure 4 H and J), and observed reducing positive number of SA- $\beta$ -gal staining N2a cell compared to control (Ctrl vs siRNA,  $p$  value=0.286; Ctrl vs Hcy,  $p$  value=0.0024; Hcy vs Hcy+siRNA,  $p$  value=0.0042) (Figure 4 J and K). The above results indicate that Hcy promoted the expression of Keap1 and cell senescence through demethylation of Keap1's promoter.

### **Keap1 and $\beta$ -catenin were both enhanced in hippocampus of Hcy-induced rats**



To verify the above alternations in animal model, SD rats were given 400 µg/kg/day via tail intravenous injection for 14 days. Then rat's brain was sectioned and co-labelled with SA-β-gal staining. Microscopic image revealed that positive number of SA-β-gal staining increased after stimulation of Hcy in neuron of hippocampus compared to rats just received normal saline ( $p$  value=0.0003) (Figure 5 A and B). Furthermore, protein level of senescent markers p16 ( $p$  value=0.011), p21 ( $p$  value=0.029) and p53 ( $p$  value=0.01) were upregulated by the administration of Hcy (Figure 5 C, D) in the homogenate of brain, along with increased Keap1 ( $p$  value=0.015) and β-catenin ( $p$  value=0.023). In consistent with above results, mRNA level of p16 ( $p$  value=0.006), p21 ( $p$  value=0.0107), p53 ( $p$  value=0.0028) and Keap1 ( $p$  value=0.025) in Hcy rats' brain also enhanced greatly compared to control rats (Figure 5 E), providing evidences that senescence was occurred in neurons. Besides, neuron loss was observed in region of CA1 ( $p$  value=0.021) and CA3 ( $p$  value=0.016) by Nissl staining in brain of rats compared to normal (Figure 5 E and F). Based on above results, we proposed that neuron loss was probable due to senescence of neuron.

## Discussion

Organ aging and age-related diseases are the main contributors that induce the accumulation of senescent cells in the brain of humans and animals (Baker et al., 2018). In this study, we observed that Hcy induces neuronal senescence in both *in vivo* and *in vitro* models, as evidenced by an increase in SA-β-gal positive staining cells and senescence markers. We next observed that β-catenin, an upstream of senescent markers, increased after Hcy treatment and co-localized with SA-β-gal-stained positive cells, whereas knockdown attenuated SA-β-gal-stained positive cells. The increase in β-catenin is due to decreased binding to WTX, resulting in increased protein accumulation caused by increased de-ubiquitination. On the other hand, we observed that Keap1, another protein that binds to WTX, was increased after Hcy treatment. IP assay showed that WTX binds to more Keap1 after Hcy treatment, while reducing β-catenin binding. Mechanistically, we found that Hcy induced a decrease in DNMT1 activity, leading to an increase in the level of Keap1 promoter CpG island demethylation and mRNA transcriptional activation, leading to an increase in Keap1 protein expression. Rat models confirmed that Hcy promoted cortical neuronal senescence as well as increases in senescent markers, Keap1 and β-catenin, which are thought to be contributing to neuronal loss. Our results uncover a novel function of Keap1 in neuronal senescence and highlight the Keap1/β-catenin pathway may be promising target for the treatment of AD.

In our previous study, elevated serum Hcy replicated many AD-like pathologies, such as neuron loss, synaptic impairment and hyperphosphorylated tau in neuron (Zhang et al., 2019), suggesting Hcy impaired neuron in many aspects. On the other hand, Hcy has been reported to induce cellular senescence, i.e. reducing telomerase activity and AKT phosphorylation (Zhu et al., 2006), enhancing SA-β-gal activity (Xu et al., 2000) and DNA hypomethylation (Zhang et al., 2015) in endothelial progenitor cells or endothelial cells, and upregulation of p21 in hepatic HepG2 (Yu et al., 2013) and p53 in neuron (Kruman et al., 2000), suggesting Hcy is an important causality in promoting cellular senescence. In addition, neurons with neurofibrillary tangles in the brains of AD patients exhibit high expression of the senescent marker Cdkn2 mRNA (Musi et al., 2018). In light of this evidence, we propose the hypothesis that

neurons develop a tendency toward senescence upon stimulation with Hcy. In this study, we observed that Hcy induced N2a cells toward senescence, as evidenced by that enhancing activity of  $\beta$ -galactosidase as well as senescent marker p16, p21 and p53. Meanwhile, Hcy treated rats also showed positive SA- $\beta$ -gal staining in cortex neuron and enhanced senescent marker p16, p21 and p53 in hippocampus. These results lead us to believe that Hcy promotes not only somatic cell senescence, but also has the same effect on neurons, so that the elucidation of the mechanism of Hcy-promoted neuronal senescence providing new target for the prevention and treatment of AD.

Next, we explored how Hcy promotes the upregulation of these senescent markers. Aberrant activation of  $\beta$ -catenin has been reported linked to accelerated aging(Liu et al., 2007). It's reported that aberrant  $\beta$ -catenin activity could induce a parallel p53/p21-mediated senescence pathway(Chow et al., 2019). Besides,  $\beta$ -catenin translocated and increased nuclear localization in response to elevated homocysteine in vascular endothelial cadherin (Beard et al., 2012), suggesting a close between  $\beta$ -catenin and Hcy. Thus, we examined protein level of  $\beta$ -catenin in N2a cells after induction of Hcy. As expected, we observed that Hcy induces elevated  $\beta$ -catenin protein levels as determined by Western blot. Immunofluorescence further showed that SA- $\beta$ -gal-stained positive cells co-located with high expression of  $\beta$ -catenin, whereas knockdown of  $\beta$ -catenin attenuated SA- $\beta$ -gal-stained positive cells. However, mRNA of  $\beta$ -catenin didn't change by treatment of Hcy, suggesting it was not directly regulated by Hcy, as Hcy could induced hypomethylation of cytosines in cytosine-guanine dinucleotide (CpG) islands in promotor regions, thus promote gene's expression (Kato et al., 2009). Next, we observed that accumulation of  $\beta$ -catenin was resulted from slowdown of its degradation. It is reported that GSK3 $\beta$  could form a multimeric complex with  $\beta$ -catenin that induces N-terminal phosphorylation of  $\beta$ -catenin, leading to its ubiquitin/proteasome-mediated degradation (Ji et al., 2015). However, we did not observe an increase in GSK3 $\beta$  activity, indicting the presence of another protein that deubiquitinates  $\beta$ -catenin. According to published literatures, WTX could bind to  $\beta$ -catenin and its E3 ubiquitin ligase aptamer  $\beta$ -transducin repeat-containing protein ( $\beta$ -TrCP), promoting  $\beta$ -catenin degradation via ubiquitination (Major et al., 2007). Contrary to expectations, we found no significant change in WTX protein level after induction of Hcy. However, it has been discovered that WTX could interact with another ubiquitin ligase adaptor Keap1, which functions to regulate the ubiquitination of the transcription factor NRF2. After WTX's competing for binding to Keap1, less NRF2 was deubiquitinated and accumulated (Hast et al., 2013). Then we examined protein level of Keap1 in N2a cells after Hcy's treatment. Intriguingly, Keap1's protein level was observed to be elevated by Hcy, along with increasing mRNA level. As determined by immunoprecipitation assays, WTX showed increased binding to Keap1 and less binding to  $\beta$ -catenin. Immunofluorescence staining also confirmed that Hcy promoted co-localization of Keap1 with SA- $\beta$ -gal staining positive cells, while knockdown of Keap1 expression attenuated the Hcy-induced SA- $\beta$ -gal staining positive cells, suggesting Keap1 mediated  $\beta$ -catenin's accumulation by competitive combination to WTX.

It has been studied that methylation levels of gene *KEAP1* CpGs at various promoter and intragenic locations showed a significant inverse correlation with the transcript levels(Fabrizio et al., 2020). In addition, genetic experiments have defined the importance of the DNA methyltransferase DNMT1 for the maintenance of CpG island methylation in cells(Robert et al., 2003). In consistent with previous study, we

found Hcy decreased the protein levels of DNMT1 compared to controls, along with the decreased CpG methylation of *KEAP1* as measured by BSP, suggesting that the rise in Keap1 protein level is due to the elevation of CpG demethylation caused by the Hcy-induced decrease in DNMT1 activity, which in turn activates the transcription and translation of mRNA.

However, most of the experiments in this study were performed on *in vitro* N2a cells, and studies at the animal level as well as on transgenic animals are lacking. Whether removal of Hcy-induced senescent cells can reverse the learning memory impairment has also not been performed, which may be the next step to be focused on.

## Conclusion

In summary, we found that Hcy enhanced upregulation Keap1 via demethylation its CpG island in promoter, resulting in activation of mRNA transcription and protein expression. More Keap1 accumulates with reduced  $\beta$ -catenin binding due to WTX binding, which in turn activates senescence markers p16, p21 and p53 with SA- $\beta$ -gal activation, ultimately promoting cellular senescence. The discovery of this Keap1/ $\beta$ -catenin pathway provides a new target for the prevention and treatment of AD.

## Abbreviations

AD

Alzheimer's disease

Hcy

Homocysteine

GSK-3 $\beta$

Glycogen synthase kinase 3

Keap1

Kelch like ECH-associated protein 1

WTX

Wilms tumor gene on X chromosome

A $\beta$

$\beta$ -amyloid plaque

SA- $\beta$ -gal

senescence-associated  $\beta$ -galactosidase

PCR

Polymerase Chain Reaction

mRNA

message RNA

IP

Immunoprecipitation

DNMT1

DNA methylation transferase 1  
CA  
Cornu Ammonis  
BSP  
bisulfite sequencing PCR.

## **Declarations**

### **Ethics approval and consent to participate**

All animal experiments were approved by the Animal Care and Use Committee of Huazhong University of Science and Technology. All animal experiments were performed according to the “Policies on the Use of Animals and Humans in Neuroscience Research” revised and approved by the Society for Neuroscience in 1995, and the Guidelines for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People’s Republic of China, and the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology approved the study protocol.

### **Consent for publication**

Not applicable

### **Availability of data and material**

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request

### **Competing interest**

The authors declare that they have no competing interests.

### **Funding**

This work was supported by the National Key R&D Program of China, National Basic Research Development Program of the Ministry of Science and Technology of China (2016YFC1305800), the National Natural Science Foundation of China (9194920041 and 31730035, and 91632305).

### **Authors’ contributions**

YZ and JZX performed experiment western blot, Nissl staining, cell culture, SA-β-gal staining and immunofluorescence staining, data analysis and manuscript writing, YLJ, SJY and HW helped in cell culture and performed western blot and qPCR, JZW modified manuscript. and YY designed experiment and modified manuscript. All authors read and approved the final manuscript.

### **Acknowledgments**

Not applicable

## Authors' information

<sup>2</sup>Department of Pathophysiology, School of Basic Medicine, Key Laboratory of Education Ministry of China/Hubei Province for Neurological Disorders, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China. <sup>3</sup>Endocrine Department of Liyuan Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430077, China. <sup>4</sup>Co-innovation Center of Neuroregeneration, Nantong University, Nantong 226000, China.

## References

1. Baker DJ, Petersen RC. Cellular senescence in brain aging and neurodegenerative diseases: evidence and perspectives. *J Clin Invest.* 2018; doi:10.1172/JCI95145
2. Beard RS, Jr., Reynolds JJ, Bearden SE. Metabotropic glutamate receptor 5 mediates phosphorylation of vascular endothelial cadherin and nuclear localization of beta-catenin in response to homocysteine. *Vascul Pharmacol.* 2012; doi:10.1016/j.vph.2012.01.004
3. Chinta SJ, Woods G, Rane A, Demaria M, Campisi J, Andersen JK. Cellular senescence and the aging brain. *Exp Gerontol.* 2015; doi:10.1016/j.exger.2014.09.018
4. Chow HM, Shi M, Cheng A, Gao Y, Chen G, Song X et al. Age-related hyperinsulinemia leads to insulin resistance in neurons and cell-cycle-induced senescence. *Nat Neurosci.* 2019; doi:10.1038/s41593-019-0505-1
5. Curro M, Gugliandolo A, Gangemi C, Risitano R, Ientile R, Caccamo D. Toxic effects of mildly elevated homocysteine concentrations in neuronal-like cells. *Neurochem Res.* 2014; doi:10.1007/s11064-014-1338-7
6. De Ferrari GV, Avila ME, Medina MA, Perez-Palma E, Bustos BI, Alarcon MA. Wnt/beta-catenin signaling in Alzheimer's disease. *CNS Neurol Disord Drug Targets.* 2014; doi:10.2174/1871527312666131223113900
7. Fabrizio FP, Mazza T, Castellana S, Sparaneo A, Muscarella LA. Epigenetic Scanning of KEAP1 CpG Sites Uncovers New Molecular-Driven Patterns in Lung Adeno and Squamous Cell Carcinomas. *Antioxidants (Basel).* 2020; doi:10.3390/antiox9090904
8. Han M, Serrano MC, Lastra-Vicente R, Brinez P, Acharya G, Huhta JC et al. Folate rescues lithium-, homocysteine- and Wnt3A-induced vertebrate cardiac anomalies. *Dis Model Mech.* 2009; doi:10.1242/dmm.001438
9. Hast BE, Goldfarb D, Mulvaney KM, Hast MA, Siesser PF, Yan F et al. Proteomic analysis of ubiquitin ligase KEAP1 reveals associated proteins that inhibit NRF2 ubiquitination. *Cancer Res.* 2013; doi:10.1158/0008-5472.CAN-12-4400
10. Ishikawa S, Ishikawa F. Proteostasis failure and cellular senescence in long-term cultured postmitotic rat neurons. *Aging Cell.* 2020; doi:10.1111/accel.13071

11. Ji XK, Xie YK, Zhong JQ, Xu QG, Zeng QQ, Wang Y et al. GSK-3beta suppresses the proliferation of rat hepatic oval cells through modulating Wnt/beta-catenin signaling pathway. *Acta Pharmacol Sin.* 2015; doi:10.1038/aps.2014.150
12. Jia L, Pina-Crespo J, Li Y. Restoring Wnt/beta-catenin signaling is a promising therapeutic strategy for Alzheimer's disease. *Mol Brain.* 2019; doi:10.1186/s13041-019-0525-5
13. Jiang X, Chai GS, Wang ZH, Hu Y, Li XG, Ma ZW et al. CaMKII-dependent dendrite ramification and spine generation promote spatial training-induced memory improvement in a rat model of sporadic Alzheimer's disease. *Neurobiol Aging.* 2015; doi:10.1016/j.neurobiolaging.2014.10.018
14. Jurk D, Wang C, Miwa S, Maddick M, Korolchuk V, Tsolou A et al. Postmitotic neurons develop a p21-dependent senescence-like phenotype driven by a DNA damage response. *Aging Cell.* 2012; doi:10.1111/j.1474-9726.2012.00870.x
15. Kang C, Xu Q, Martin TD, Li MZ, Demaria M, Aron L et al. The DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA4. *Science.* 2015; doi:10.1126/science.aaa5612
16. Kato K, Maesawa C, Itabashi T, Fujisawa K, Otsuka K, Kanno S et al. DNA hypomethylation at the CpG island is involved in aberrant expression of the L1 cell adhesion molecule gene in colorectal cancer. *Int J Oncol.* 2009; doi:10.3892/ijo\_00000358
17. Kruman, II, Culmsee C, Chan SL, Kruman Y, Guo Z, Penix L et al. Homocysteine elicits a DNA damage response in neurons that promotes apoptosis and hypersensitivity to excitotoxicity. *J Neurosci.* 2000;
18. Liu H, Fergusson MM, Castilho RM, Liu J, Cao L, Chen J et al. Augmented Wnt signaling in a mammalian model of accelerated aging. *Science.* 2007; doi:10.1126/science.1143578
19. Major MB, Camp ND, Berndt JD, Yi X, Goldenberg SJ, Hubbert C et al. Wilms tumor suppressor WTX negatively regulates WNT/beta-catenin signaling. *Science.* 2007; doi:10.1126/science/1141515
20. McCully KS. Review: Chemical Pathology of Homocysteine VI. Aging, Cellular Senescence, and Mitochondrial Dysfunction. *Ann Clin Lab Sci.* 2018;
21. Miller JW. Homocysteine and Alzheimer's disease. *Nutr Rev.* 1999; doi:10.1111/j.1753-4887.1999.tb06936.x
22. Moreno-Blas D, Gorostieta-Salas E, Pommer-Alba A, Mucino-Hernandez G, Geronimo-Olvera C, Maciel-Baron LA et al. Cortical neurons develop a senescence-like phenotype promoted by dysfunctional autophagy. *Aging (Albany NY).* 2019; doi:10.18632/aging.102181
23. Musi N, Valentine JM, Sickora KR, Baeuerle E, Thompson CS, Shen Q et al. Tau protein aggregation is associated with cellular senescence in the brain. *Aging Cell.* 2018; doi:10.1111/accel.12840
24. Ostrakhovitch EA, Tabibzadeh S. Homocysteine and age-associated disorders. *Ageing Res Rev.* 2019; doi:10.1016/j.arr.2018.10.010
25. Reya T, Clevers H. Wnt signalling in stem cells and cancer. *Nature.* 2005; doi:10.1038/nature03319
26. Robert MF, Morin S, Beaulieu N, Gauthier F, Chute IC, Barsalou A et al. DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nat Genet.* 2003;

doi:10.1038/ng1068

27. Shin C, Baik I. Leukocyte Telomere Length is Associated With Serum Vitamin B12 and Homocysteine Levels in Older Adults With the Presence of Systemic Inflammation. *Clin Nutr Res.* 2016; doi:10.7762/cnr.2016.5.1.7
28. Sun T, Ghosh AK, Eren M, Miyata T, Vaughan DE. PAI-1 contributes to homocysteine-induced cellular senescence. *Cell Signal.* 2019; doi:10.1016/j.cellsig.2019.109394
29. Walsh R, Martin E, Darvesh S. A versatile equation to describe reversible enzyme inhibition and activation kinetics: modeling beta-galactosidase and butyrylcholinesterase. *Biochim Biophys Acta.* 2007; doi:10.1016/j.bbagen.2007.01.001
30. Xu D, Neville R, Finkel T. Homocysteine accelerates endothelial cell senescence. *FEBS Lett.* 2000; doi:10.1016/s0014-5793(00)01278-3
31. Yu X, Lv J, Zhu Y, Duan L, Ma L. Homocysteine inhibits hepatocyte proliferation via endoplasmic reticulum stress. *PLoS One.* 2013; doi:10.1371/journal.pone.0054265
32. Zhang CE, Tian Q, Wei W, Peng JH, Liu GP, Zhou XW et al. Homocysteine induces tau phosphorylation by inactivating protein phosphatase 2A in rat hippocampus. *Neurobiol Aging.* 2008; doi:10.1016/j.neurobiolaging.2007.04.015
33. Zhang CE, Wei W, Liu YH, Peng JH, Tian Q, Liu GP et al. Hyperhomocysteinemia increases beta-amyloid by enhancing expression of gamma-secretase and phosphorylation of amyloid precursor protein in rat brain. *Am J Pathol.* 2009; doi:10.2353/ajpath.2009.081036
34. Zhang D, Sun X, Liu J, Xie X, Cui W, Zhu Y. Homocysteine accelerates senescence of endothelial cells via DNA hypomethylation of human telomerase reverse transcriptase. *Arterioscler Thromb Vasc Biol.* 2015; doi:10.1161/ATVBAHA.114.303899
35. Zhang D, Wen X, Wu W, Xu E, Zhang Y, Cui W. Homocysteine-related hTERT DNA demethylation contributes to shortened leukocyte telomere length in atherosclerosis. *Atherosclerosis.* 2013; doi:10.1016/j.atherosclerosis.2013.08.029
36. Zhang DY, Pan Y, Zhang C, Yan BX, Yu SS, Wu DL et al. Wnt/beta-catenin signaling induces the aging of mesenchymal stem cells through promoting the ROS production. *Mol Cell Biochem.* 2013; doi:10.1007/s11010-012-1498-1
37. Zhang Y, Xie JZ, Xu XY, Hu J, Xu T, Jin S et al. Liraglutide Ameliorates Hyperhomocysteinemia-Induced Alzheimer-Like Pathology and Memory Deficits in Rats via Multi-molecular Targeting. *Neurosci Bull.* 2019; doi:10.1007/s12264-018-00336-7
38. Zhu JH, Chen JZ, Wang XX, Xie XD, Sun J, Zhang FR. Homocysteine accelerates senescence and reduces proliferation of endothelial progenitor cells. *J Mol Cell Cardiol.* 2006; doi:10.1016/j.yjmcc.2006.01.011

## Figures

## Figure 1

Hcy promoted senescence of N2a cell in a time-dependent manner. (A) CCK8 assayed cell viability after treatment with different concentration of Hcy (0, 25, 50, 100 and 200  $\mu\text{M}$ ) for 24 h. (B) After treatment with Hcy (100  $\mu\text{M}$ ), N2a cells were stained using SA- $\beta$ -gal kit at different time point (0, 24, 48 and 72 h). Bar=50 $\mu\text{m}$ . (C) After treatment with Hcy for 72 h, N2a cells were collected and analyzed with p16, p21 and p53 antibodies using western blot. (D) Statistics histogram of positive SA- $\beta$ -gal staining cell to total cells. \*\*  $p < 0.01$ , vs 0 h; \*\*\*  $p < 0.001$ , vs 0 h. (E) Statistics histogram of relative intensity of p16, p21, p53 to GAPDH. \*  $p < 0.05$ , vs Ctrl. (F) Statistics histogram of qPCR analyzed mRNA of p16, p21 and p53 in N2a cells after Hcy treatment. \*  $p < 0.05$ , vs Ctrl; \*\*  $p < 0.01$ , vs Ctrl.

## Figure 2

Hcy inhibited  $\beta$ -catenin's degradation via de-ubiquitination. (A) N2a cell were treated with Hcy and analyzed using  $\beta$ -catenin antibodies. (B) Statistics histogram of the relative level of  $\beta$ -catenin to GAPDH. \*  $p < 0.05$ , vs Ctrl. (C) Statistics histogram of mRNA of  $\beta$ -catenin analyzed by qPCR in N2a cell after treated with Hcy. \*  $p < 0.05$ , vs Hcy. (D) After N2a cell treated with Hcy, MG132 (100  $\mu\text{M}$ ) and cycloheximide (100  $\mu\text{M}$ ) were added into medium, and cells were harvested at different time point (0, 1, 2, 3, 4 and 5 hour) after treatment and detected using  $\beta$ -catenin antibody. (E) Statistics histogram of the relative level of  $\beta$ -catenin to  $\beta$ -actin. \*  $p < 0.05$ , vs Ctrl. (F) After induction with Hcy,  $\beta$ -catenin in N2a cells was first immunoprecipitated using specific antibody, then examined using pan-ubiquitination antibody. (G) Statistics histogram of the relative level of Ubi to  $\beta$ -catenin. \*  $p < 0.05$ , vs Ctrl. (H) After treated with Hcy for 72 h, N2a cells were first stained using SA- $\beta$ -gal, then examined with Keap1 and  $\beta$ -catenin using immunofluorescence. Bar=20 $\mu\text{m}$ . (I) Statistics histogram of positive SA- $\beta$ -gal staining cell to total cells. \*\*\*  $p < 0.001$ , vs Ctrl. (J) Statistics histogram of relative fluorescence intensity of  $\beta$ -catenin. \*\*  $p < 0.01$ , vs Ctrl. (K) siRNA of  $\beta$ -catenin was transfected to N2a for 24 hours and examined us western blot using  $\beta$ -catenin antibody. (L) Statistics histogram of the relative level of  $\beta$ -catenin to GAPDH. \*  $p < 0.05$ , vs Hcy; #  $p < 0.05$ , vs Ctrl. (M) siRNA of  $\beta$ -catenin was transfected to N2a for 24 hours then treated with Hcy for another 72 hours, and stained using SA- $\beta$ -gal. Bar=20 $\mu\text{m}$ . (N) Statistics histogram of positive SA- $\beta$ -gal staining cell to total cells. \*\*  $p < 0.01$ , vs Hcy; #  $p < 0.05$ , vs Ctrl.

## Figure 3

Keap1 inhibited  $\beta$ -catenin's binding to WTX. (A) After induction of Hcy for 72 hours, N2a cells were harvested and examined by western blot using antibodies of Keap1, GSK-3 $\beta$  pS9 (S9), GSK-3 $\beta$ , WTX. (B) Statistics histogram of the western blot result showing the relative level of Keap1, WTX to GAPDH and ratio of S9 to GSK-3 $\beta$ . \*\*  $p < 0.01$ , vs Ctrl. (C) After induction of Hcy for 72 hours, WTX antibody was used to immunoprecipitation in N2a cells extracts and examined using  $\beta$ -catenin and Keap1 antibody. (D)



Statistics histogram of the western blot result showing the relative level of  $\beta$ -catenin to WTX. \*  $p < 0.05$ , vs Ctrl. (E) Statistics histogram of the western blot result showing the relative level of Keap1 to WTX. \*  $p < 0.05$ , vs Ctrl. (G) Statistics histogram of the qPCR result showing the relative mRNA level of Keap1 after induction of Hcy in N2a cells \*  $p < 0.05$ , vs Ctrl.

#### Figure 4

Keap1's promoter was demethylated by Hcy. (A) Western blot analyzed using DNMT1 antibody after induction of Hcy in N2a cells. (B) Statistics histogram of the western blot result showing the relative level of DNMT1 to GAPDH. \*  $p < 0.05$ , vs Ctrl. (C) BSP examined methylation level of Keap1's promoter after induction of Hcy in N2a cells. (B) Statistics histogram of methylation of Keap1. \*  $p < 0.05$ , vs Ctrl. (E) Co-labelled Keap1's immunofluorescence and SA- $\beta$ -gal staining after induction of Hcy in N2a cells. Bar=20 $\mu$ m. (F) Statistics histogram of positive SA- $\beta$ -gal staining to total cell. \*\*  $p < 0.01$ , vs Ctrl. (G) Statistics histogram of relative fluorescence intensity of Keap1. \*\*  $p < 0.01$ , vs Ctrl. (H) Western blot analyzed N2a cell using Keap1 antibody after Hcy induction and siRNA treatment. (I) Statistics histogram of the western blot result showing the relative level of Keap1 to GAPDH. \*  $p < 0.05$ , vs Hcy. #  $p < 0.05$ , vs Ctrl. (J) SA- $\beta$ -gal staining of N2a after transfection of siRNA that targeted Keap1 for 24 hours and treated with Hcy for another 72 hours. (K) Statistics histogram of positive SA- $\beta$ -gal staining to total cell. Bar=20 $\mu$ m. \*\*  $p < 0.01$ , vs Hcy. #  $p < 0.05$ , vs Ctrl.

#### Figure 5

Hcy promoted neuron senescence and loss in rats' model. SD rats were injected normal saline (N.S.) or Hcy (400  $\mu$ g/kg/day) for two weeks, and brains were sectioned and examined with SA- $\beta$ -gal staining. (A) Positive staining cell in cortex of rats after SA- $\beta$ -gal staining. Bar=20 $\mu$ m. (B) Statistics histogram of qPCR analyzed mRNA expression of p16, p21, p53, Keap1 and  $\beta$ -catenin in rat brains. \*  $p < 0.05$ , vs N.S.. (C) Western blot analyzed protein expression of p16, p21, p53, Keap1 and  $\beta$ -catenin in rat brains. (D) Statistics histogram of relative intensity of p16, p21, p53, Keap1,  $\beta$ -catenin to GAPDH. \*  $p < 0.05$ , vs N.S.. (E) The representative images of Nissl staining of rats' brain. Bar=50 $\mu$ m. (F) Statistics histogram of Nissl staining in CA1 and CA3. \*  $p < 0.05$ , vs N.S.