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Cellular senescence as a driver of cognitive decline triggered by chronic unpredictable stress

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A B S T R A C T

When an individual is under stress, the undesired effect on the brain often exceeds expectations. Additionally, when stress persists for a long time, it can trigger serious health problems, particularly depression. Recent studies have revealed that depressed patients have a higher rate of brain aging than healthy subjects and that depression increases dementia risk later in life. However, it remains unknown which factors are involved in brain aging triggered by chronic stress. The most critical change during brain aging is the decline in cognitive function. In addition, cellular senescence is a stable state of cell cycle arrest that occurs because of damage and/or stress and is considered a sign of aging. We used the chronic unpredictable stress (CUS) model to mimic stressful life situations and found that, compared with nonstressed control mice, CUS-treated C57BL/6 mice exhibited depression-like behaviors and cognitive decline. Additionally, the protein expression of the senescence marker p16^{INK4a} was increased in the hippocampus, and senescence-associated β-galactosidase (SA-β-gal)-positive cells were found in the hippocampal dentate gyrus (DG) in CUS-treated mice. Furthermore, the levels of SA-β-gal or p16^{INK4a} were strongly correlated with the severity of memory impairment in CUS-treated mice, whereas clearing senescent cells using the pharmacological senolytic cocktail dasatinib plus quercetin (D + Q) alleviated CUS-induced cognitive deficits, suggesting that targeting senescent cells may be a promising candidate approach to study chronic stress-induced cognitive decline. Our findings open new avenues for stress-related research and provide new insight into the association of chronic stress-induced cellular senescence with cognitive deficits.

1. Introduction

Stress is not necessarily harmful. Mild stress can promote alertness, motivation, and readiness to respond to danger. However, excessive stress or chronic stress may increase the risk of health problems and lead to various mental diseases, particularly depression (de Kloet et al., 2005; Ross et al., 2017; Yang et al., 2015). Exposure to long-term unpredictable environmental stress is widely recognized as the main determinant of the risk and severity of mental illness and plays a major role in many etiological theories of major depressive disorder (Bale, 2005; Kendler et al., 1999; Yang et al., 2015). Chronic stress not only induces depression that may lead to the worst outcome, suicide, but may also affects how individuals feel and even change how they think, causing cognitive impairment (Lee et al., 2016; McEwen and Sapolsky, 1995; Sandi and Pinelo-Nava, 2007). Furthermore, previous studies have shown that individuals with chronic stress also have a higher risk of

memory loss (Peavy et al., 2009; Ribeiro et al., 2013) and an increased neurodegenerative risk, including Alzheimer's disease (Justice, 2018; Saeedi and Rashidy-Pour, 2021). A systematic review and meta-analysis of longitudinal studies revealed that depressed patients have a higher rate of brain aging than healthy subjects (John et al., 2019). Thus, exploring whether chronic stress could accelerate brain aging is valuable.

Brain aging is a complex process involving subtle changes in brain structure, chemistry, and brain function, particularly characterized by impaired memory and cognitive functions (Mattson and Arumugam, 2018). Emerging evidence has demonstrated that the hippocampus may play important roles in cognitive aging (Bettio et al., 2017; Nordin et al., 2018). It is involved in the formation, composition, and storage of new memories and the connection of certain memories and emotions with these memories (Bruel-Jungerman et al., 2007; Deng et al., 2010; Miller and Sahay, 2019). Taken together, these findings raise the possibility that the hippocampus has multifaceted roles in chronic stress-induced

Abbreviations

CUS	chronic unpredictable stress
SA-β-gal	senescence-associated β-galactosidase
DG	dentate gyrus
D + Q	dasatinib plus quercetin
SASP	senescence-associated secretory phenotype
GPT	glutamic pyruvic transaminase
GOT	glutamic-oxaloacetic transaminase
ALP	alkaline phosphatase
BUN	blood urea nitrogen
DI	discrimination index
PBS	phosphate-buffered saline;
FST	forced swim test

brain aging.

The chronic unpredictable stress (CUS) protocol comprises random, intermittent, and unpredictable exposure to various stressors for weeks and has been widely used to study the impact of stress exposure based on animals with different stresses. Animals subjected to certain stresses, such as being deprived of water and/or food, will lose their ability to respond to rewards (Katz, 1982; Monteiro et al., 2015). The CUS model is currently well-validated. It is the most commonly used, reliable, and effective rodent model of depression (Antoniuk et al., 2019) and causes numerous impairments in mental function and cognition (Yuen et al., 2012). Therefore, we used CUS-treated mice to model stressful life situations and investigate factors that affect chronic stress-triggered brain aging, particularly those associated with cognitive functions.

Cellular senescence was first defined by Hayflick and Moorhead in 1961, who found that human cells divide a finite number of times in culture (Hayflick and Moorhead, 1961), opening new avenues of biogerontology. Cellular senescence is a state of cell cycle arrest that occurs because of injury and/or stress and is considered a hallmark of aging (de Magalhaes and Passos, 2018). Scientists have discovered that senescent cells are widespread in human, primate, and rodent tissues (Ovadya et al., 2018; van Deursen, 2014). Additionally, senescent cells are a normal part of wound healing and damage repair. They secrete important signals, recruit immune cells and other cells to damaged tissues, and play a role in cleaning and rebuilding; conversely, the excessive accumulation of senescent cells may cause tissue disruption and/or degeneration that is associated with diseases, particularly Alzheimer's disease (Childs et al., 2017; van Deursen, 2014). However, whether senescent cells are causally related to chronic stress-related cognitive impairment and whether their removal is beneficial remains unclear.

Here, we demonstrate that CUS-treated mice exhibited higher expression of senescence markers, such as senescence-associated β-galactosidase (SA-β-gal) and p16^{INK4a}, in the brain, particularly in the hippocampus, than age- and sex-matched nonstressed (control) mice. The levels of SA-β-gal or p16^{INK4a} expression were strongly negatively correlated with memory performance in CUS-treated mice. Furthermore, CUS-treated mice receiving the senolytic cocktail dasatinib plus quercetin (D + Q) displayed decreased numbers of senescent cells, reduced serum senescence-associated secretory phenotype (SASP) factors, and improved cognitive functions compared with the outcomes observed for vehicle-treated mice. Importantly, the clearance of senescent cells by senolytics alleviated chronic stress-induced cognitive deficits. Our work suggests that targeting senescent cells in the brain may serve as a new therapeutic target to prevent and treat chronic stress-related memory loss.

2. Materials and methods

2.1. Animals

Eight-week-old male C57BL/6 mice were housed at 5 mice per cage under standard conditions (21 ± 2 °C and a 12:12 light/dark cycle) with accessible food and water. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Cheng Kung University.

2.2. Chronic unpredictable stress (CUS)

CUS is a one-month stress model as described previously (Abelaira et al., 2013; Krishnan and Nestler, 2011). CUS comprises 10 types of stressors, including food/water deprivation, removal of bedding, rapid light and dark changes, crowding, cage tilting, restraints, cold/hot cages, social defeat, sleep deprivation, and empty cages with water on the bottom. Experimental mice were randomly subjected to one form of stress every day. The body weight of the mice was measured and recorded before food/water deprivation every week. The sucrose preference test was conducted the next day.

2.3. Sucrose preference test

The sucrose preference test was used to indicate anhedonia as one of the symptoms of depression. The principle of this test was to assess the animals' interest in seeking a sweet rewarding drink as described previously (Gross and Pinhasov, 2016; Liu et al., 2018). Before beginning testing, the mice were pretrained on two bottles of 2% sucrose solution for 3 days. The mice were deprived of food and water overnight before the test. They were then given a bottle of 2% sucrose solution and a bottle of normal drinking water at the same time for 2 h. The weight of the bottles was recorded before and after the test, and the proportion of mice drinking sucrose solution was calculated. Reduced preference for a sweet solution in the sucrose preference test represented depression-like behavior.

2.4. Forced swim test

The forced swim test was used to assess behavioral despair in the mice as described previously (Castagné et al., 2011; Costa et al., 2013). The mice were placed in a cylinder with water for 10 min at approximately 22 °C. The mice were then forced to swim in a cylinder with no escape. The score involving active (swimming) or passive (immobility) behaviors was recorded. The time spent swimming and the time of immobility were calculated using the SMART digital tracking system. An increase in immobility time indicated behavioral despair.

2.5. Open field test

The open field test was used to assess the locomotor activity of mice as described previously (Huang et al., 2015; Seibenhener and Wooten, 2015). The experimental mice were placed in an open field box (46 × 46 × 46 cm) for 10 min; the box was cleaned between tests. The travel distance and velocity were recorded, and the data were analyzed using Noldus EthoVision XT video tracking software (Noldus Information Technology, Wageningen, The Netherlands).

2.6. Object location test

The object location test was used to assess the spatial memory of mice. The experimental procedure required 5 days as described previously (Murai et al., 2007; Vogel-Ciernia and Wood, 2014). The mice were placed in a 30 × 30 × 30-cm box for 10 min to habituate to the environment for 3 successive days. In the training phase on the fourth day, the experimental mice were placed in the box, which had two

identical objects on the same side, and the mice were allowed to explore the objects for 10 min. Twenty-four hours later, one of the objects was moved to the other side of the box, and the mice were placed in the same box and allowed to explore the objects for 10 min. The box was cleaned with 70% EtOH to remove olfactory cues between trials. The time that the mice spent exploring the objects was recorded and expressed as discrimination index (DI) values, which were calculated as follows: DI = (time$_{novel}$ - time$_{familiar}$)/(time$_{novel}$ + time$_{familiar}$).

2.7. Y maze test

The Y maze was used to assess the spatial memory of mice as described previously (Kraeuter et al., 2019; Yau et al., 2007). First, one side of the Y-shaped maze was closed, and then the mice were placed in the maze and allowed to explore the other two sides for 5 min. After 1 h, the side that had been closed was opened, and the mice were placed in the maze and allowed to explore the space for 5 min. The time spent exploring the novel arm (that was previously closed in the first trial) was recorded and measured as a percentage of the time spent exploring all arms during the first 2 min of the test trial.

2.8. Senescence-associated beta-galactosidase (SA-β-gal) activity

SA-β-gal activity was used to detect putative senescent cells as described previously (Cai et al., 2020; Piechota et al., 2016). The mice were sacrificed by perfusion, and the brains were removed and fixed with 4% PFA. After fixation with 4% PFA for one day, the cells were dehydrated with 30% sugar water three times. The thickness of each frozen brain slice was 20 μm. The frozen brain slices were first rinsed with phosphate-buffered saline (PBS) and incubated overnight in SA-β-gal solution (Cell Signaling; Cat: # 9860) at 37 °C. After staining, the SA-β-gal solution was removed, and the cells were overlaid with 70% glycerol and then stored at 4 °C. Images were captured at 10 × and 40 × using a light microscope, and the number of SA-β-gal-positive cells was quantified by manual counting.

2.9. Western blot analysis

The hippocampus of each mouse was dissected and sonicated using lysis buffer. The extracts were separated by SDS-PAGE and then transferred to a membrane. The membrane was then blocked with 3% bovine serum albumin and incubated with primary antibodies against p16^{INK4a} (Ab189034; Abcam) overnight at 4 °C, with actin (MAB1501; Millipore) as an internal control. The membrane was washed three times for 10 min and shaken with secondary antibodies for 1 h at room temperature. The protein levels were detected and quantitated using the UVP Chemiluminescent Darkroom System (UVP Inc., Upland, CA).

2.10. Senolytic drug treatment

After exposure to CUS, the mice were treated with senolytic drugs. CUS-treated mice were gavaged with dasatinib (5 mg/kg) (LC Laboratories; Cat: # D-3307) and quercetin (50 mg/kg) (Sigma; Cat: #Q4951) or vehicle (60% Phosal, 10% ethanol, and 30% PEG-400) for 5 days a week for 2 weeks (Ogrodnik et al., 2019; Sierra-Ramirez et al., 2020). Each mouse was given a dose of 100 μl. Behavioral tests were performed after senolytic drug treatment.

2.11. In vivo safety and toxicology analysis

The mice were sacrificed, and serum was collected. The serum samples were assessed using an automated dry chemistry analyzer (FUJI DRI-CHEM 4000i). DRI-CHEM SLIDE glutamic pyruvic transaminase (GPT)/ALT-P III, glutamic-oxaloacetic transaminase (GOT)/AST-P III, and alkaline phosphatase (ALP)-P III were used to evaluate liver function, and blood urea nitrogen (BUN)-P III was used to evaluate kidney function. Ten microliters of serum samples were added to the test slides, and the data were read using the analyzer.

2.12. Multiplex assay

After the mice were sacrificed, blood was collected and centrifuged at 2000×*g* for 5 min. The supernatant was carefully collected and stored at −80 °C. The serum samples were thawed before the assay and centrifuged at 12000 rpm for 15 min to collect approximately 40 μl of the supernatant. Multiplex assays were performed using a Mouse High Sensitivity T Cell Magnetic Bead Panel (EMD Millipore; Cat: # MHSTCMAG-70KPMX, USA). Twenty-five microliters of serum samples were added to the sample well, 50 μl of the standard was added to the standard well, and then 25 μl of premixed 18-plex beads was added to each well. The 96-well plate was incubated on a plate shaker overnight at 800 rpm at 2°C–8°C. Next, 25 μl of streptavidin-phycoerythrin was added to each well containing 25 μl of detection antibody and then was incubated at room temperature for 30 min for development. The well contents were gently removed, and 150 μl of sheath fluid was added to all the wells. The 96-well plate was read using a Luminex MAGPIX instrument system (Luminex® Corporation, Austin, TX), and the median fluorescent intensity (MFI) data were analyzed to calculate analyte concentrations in the serum samples.

2.13. Statistical analysis

All the data analyses were displayed using GraphPad Prism 8. Statistical significance of differences was measured with Student's *t*-test between two groups and analysis of variance (ANOVA) with Tukey's multiple comparison test for more than two groups. Linear correlations between two variables of interest were determined by calculating Pearson's correlation coefficients. The values were presented as means ± SEM, and statistical significance was defined as $p < 0.05$.

3. Results

3.1. Chronic unpredictable stress (CUS) induces depression-like behaviors in C57BL/6 mice

Previous studies revealed that the CUS model is the most popular method to induce depression-like phenotypes in animals (Abelaira et al., 2013; Krishnan and Nestler, 2011). In the paradigm of the CUS model, 8-week-old C57BL/6 mice were exposed to a series of mild unpredictable stressors for 28 consecutive days. Based on symptoms of depression, we first evaluated whether the depression model of CUS affected the interest in pleasure of C57BL/6 mice using the sucrose performance test. Accordingly, we measured the percentage of sucrose water intake before and after CUS treatment. In this assay, the mice were given sucrose water and plain water. Exhibiting a significant preference for sucrose water over plain represented pleasure. The percentage of sucrose intake was significantly lower in CUS-treated mice after 4 weeks of stress than in nonstressed (control) mice after the same period (Fig. 1A). Next, we used the forced swim test (FST) to assess whether CUS-treated mice displayed feelings of hopelessness and despair after CUS treatment. CUS-treated mice exhibited a characteristic immobile posture and increased levels of immobility, indicating increased behavioral despair (Fig. 1B). Additionally, a previous study showed that chronic stress-induced depression-like behaviors affect body weight. Thus, we measured the body weight of mice at different ages: 8, 9, 10, 11, and 12 weeks old. We found that 12-week-old CUS-treated mice exhibited significantly lower body weights than age- and sex-matched control mice (Fig. 1C). These data show that we have established a successful CUS protocol for observing depression-like behaviors in C57BL/6 mice.

A

B

C

D

E

F

G
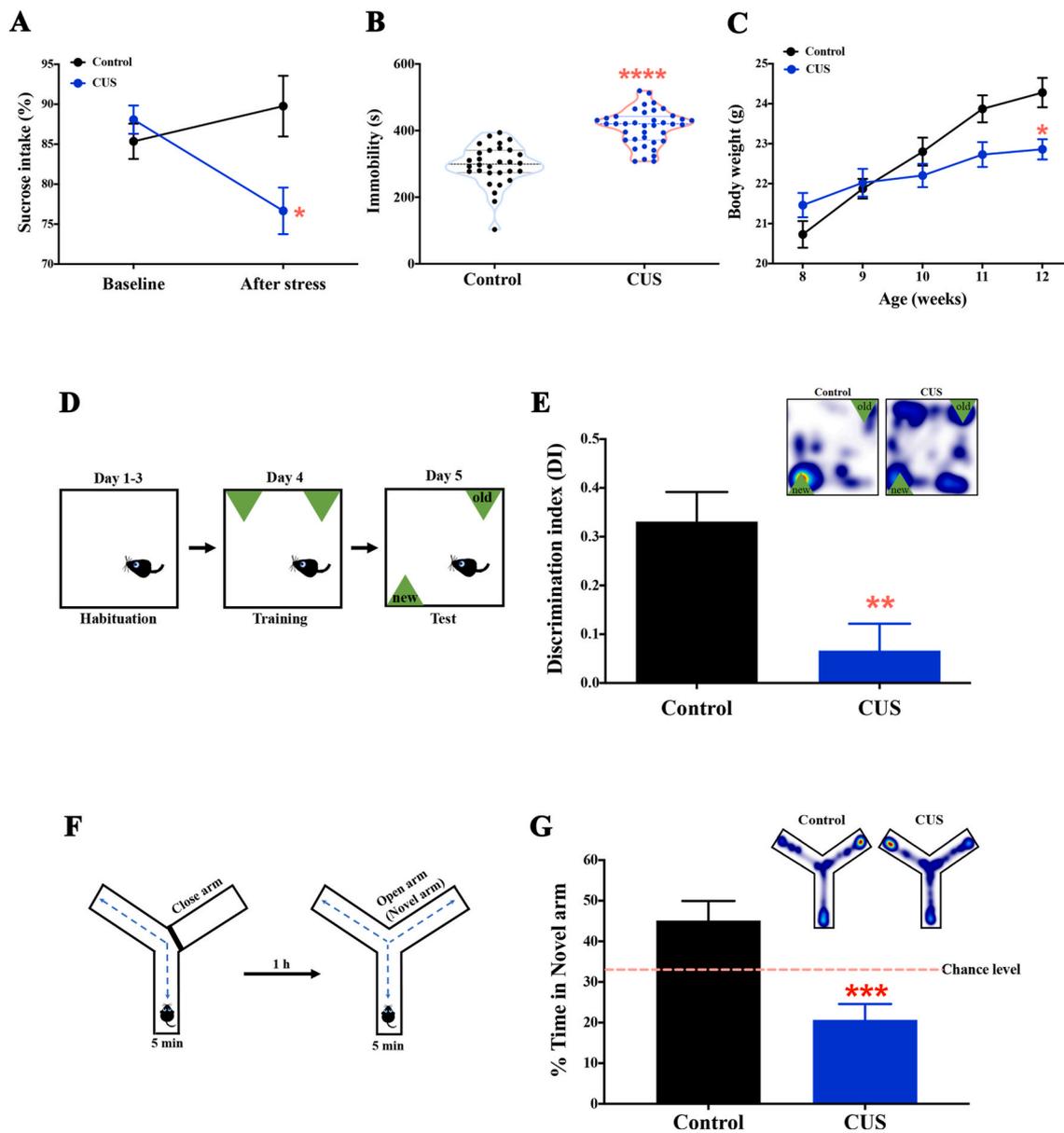
Fig. 1. CUS induces depression-like phenotypes and impairs spatial memory in C57BL/6 mice. (A) Sucrose preference was calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake in each cage. Five mice were housed in one cage (control groups: $n = 3$ cages; CUS groups: $n = 5$ cages). Two-way analysis of variance revealed a significant treatment (control vs. CUS-treated mice) × time interaction [$F_{(1,12)} = 8.065, p = 0.0149$]. *$p < 0.05$ vs. baseline of the CUS groups as determined by Tukey's *post hoc* analysis. (B) The total duration of immobility was measured using the forced swim test for 10 min (control groups: $n = 30$; CUS groups: $n = 39$) [two-tailed unpaired Student's *t*-test; $t_{(67)} = 8.292; p < 0.0001$]. ****$p < 0.0001$ vs. control groups. (C) The body weights of the control and CUS-treated mice were measured (control groups: $n = 14$; CUS groups: $n = 29$ at 8, 9, 10, 11, and 12 weeks). Two-way analysis of variance revealed a significant treatment × time interaction [$F_{(4,203)} = 3.153; p = 0.0153$]. *$p < 0.05$ vs. age- and sex-matched controls as determined by Bonferroni's *post hoc* analysis. (D) Experimental design for the object location test. In the training trial, the objects were placed in the same location. In the test trial, one object was moved and relocated in the opposite direction of another object. (E) A representative heat map and the discrimination index (DI) were used to assess hippocampal-related memory performance (control groups: $n = 30$; CUS groups: $n = 35$) [two-tailed unpaired Student's *t*-test; $t_{(63)} = 3.236; p = 0.0019$]. **$p < 0.01$ vs. controls. (F) Schematic drawings of the Y-maze and experimental procedures. (G) Representative heat map and CUS-induced spatial memory deficit in the modified Y-maze test (control groups: $n = 14$; CUS groups: $n = 20$) [two-tailed unpaired Student's *t*-test; $t_{(32)} = 3.965; p = 0.0004$]. ***$p < 0.001$ vs. controls. The data are represented as means ± SEM.

3.2. CUS-treated mice exhibit memory impairment

Emerging evidence suggests that prolonged stress can change the structure and function of the brain and even cause brain damage, leading to the impairment of multiple learning and memory processes (Beery and Kaufer, 2015; Delgado et al., 2018; Sandi and Haller, 2015). Previous studies revealed that the hippocampus, a brain area of the limbic system important for learning and memory, is particularly sensitive to chronic stress (Conrad, 2008; Elizalde et al., 2010). Prolonged

stress can reduce the hippocampal volume, inducing the shrinkage of dendrites and spines of hippocampal neurons and causing hippocampus-dependent memory loss (Kalman and Keay, 2017). Thus, we used the object location test to verify whether CUS treatment might affect hippocampal-associated memory, as shown in Fig. 1D. The discrimination index (DI) values were significantly lower in CUS-treated mice than in control nonstress-treated mice (Fig. 1E). Additionally, we used the modified Y maze test to measure mouse spatial memory (Fig. 1F). CUS-treated mice spent less time in the novel arm than control

mice (Fig. 1G). These findings suggest that CUS treatment impairs memory. Furthermore, to exclude the hypothesis that changes in immobility could be due to nonspecific effects of stress on locomotion, the open field test was conducted. The data in Fig. S1 show that there were no differences in locomotion, including traveled distance (Fig. S1B) and velocity (Fig. S1C), between the control and CUS-treated mice. These findings suggest that the cognitive deficits observed in CUS-treated mice are not caused by stress-induced locomotor deficits.

3.3. Senescence markers are increased in the hippocampi of CUS-treated mice

Emerging evidence indicates that depression may be associated with accelerated cellular aging (Diniz et al., 2019; Verhoeven et al., 2014). Thus, SA-β-gal staining, the most widely used marker to detect the presence of SA-β-gal activity, was used to evaluate whether CUS induced cellular senescence in the brain. β-galactosidase is a lysosomal hydrolase that is usually active at pH 4; however, in senescent cells, β-galactosidase is often active at pH 6. This change can be detected using simple biochemical analysis. Before analyzing the senescent cell experiments, the mice were confirmed to display depression-like behaviors and memory loss based on the behavioral results. The intensity of blue particles was significantly increased in the brain region of CUS-treated mice compared with that in control mice (Fig. 2A). Interestingly, we found that SA-β-gal-positive cells abundantly existed in the dentate gyrus (DG) of the hippocampus, particularly the hilus of the DG in CUS-treated mice, but not in the amygdala, the other emotion-related brain region (data not shown). Thus, we also measured p16^{INK4a} protein expression in the mouse hippocampus. The hippocampal p16^{INK4a} levels were 18.102% higher in CUS-treated mice than in control mice, suggesting that chronic stress induced cellular senescence in CUS-treated mice (Fig. 2B).

3.4. Increased SA-β-gal-positive cells and senescence marker p16^{INK4a} levels are associated with impaired memory performance

Recent evidence suggests that the accumulation of senescent cells may lead to neurodegenerative disorders (Di Malta et al., 2012), whereas eliminating senescent cells may help prevent cognitive decline

in mouse models (Baker and Petersen, 2018; Bussian et al., 2018). However, no prior study has reported a correlation between the number of senescent cells and/or levels of cellular senescence markers and the severity of cognitive deficits induced by CUS treatment. Therefore, we first plotted the quantity of SA-β-gal-positive cells vs the memory performance score in the same animal from control and CUS-treated mice. A strong correlation was found between the number of SA-β-gal-positive cells and DI values of the object location test (Fig. 3A). We also investigated a possible association between the number of SA-β-gal-positive cells and time spent in the novel arm of the Y maze test and found a negative linear relationship (Fig. 3B).

Additionally, we further confirmed the association between hippocampal expression of the cellular senescence marker p16^{INK4a} and memory performance in mice. Linear regression analysis revealed a significantly negative correlation between the DI values of the object location test (Fig. 3C) or the time spent in the novel arm of the Y maze test (Fig. 3D) and hippocampal p16^{INK4a} levels in control and CUS-treated mice. These results suggest that the number of SA-β-gal-positive cells and the hippocampal p16^{INK4a} content are strongly associated with spatial memory performance in mice.

3.5. Senolytic therapy alleviates cognitive deficits in CUS-treated mice

The above results demonstrated a negative correlation between the expression of cellular senescence markers and the extent of memory performance in mice. However, a causal relationship between CUS-induced cellular senescence and memory loss has not been investigated. In addition, previous studies indicated that senolytic therapies could selectively target senescent cells, suggesting that treatment with D + Q, a senolytic drug, can prevent cell damage, delay physical dysfunction and prolong the life of naturally aging mice (Xu et al., 2018). Therefore, we speculated whether the removal of senescent cells could alleviate CUS-induced cognitive decline using a pharmacological strategy. CUS-treated mice were gavaged with the senolytic drug cocktail D + Q for 5 days a week for 2 weeks (Zhu et al., 2015). To confirm whether D + Q treatment effectively reduced senescent cells, we used SA-β-gal staining and measured hippocampal p16^{INK4a} expression and found that the senolytic drug D + Q reduced the number of SA-β-gal-positive cells (Fig. 4A) and p16^{INK4a} expression (Fig. 4B) in
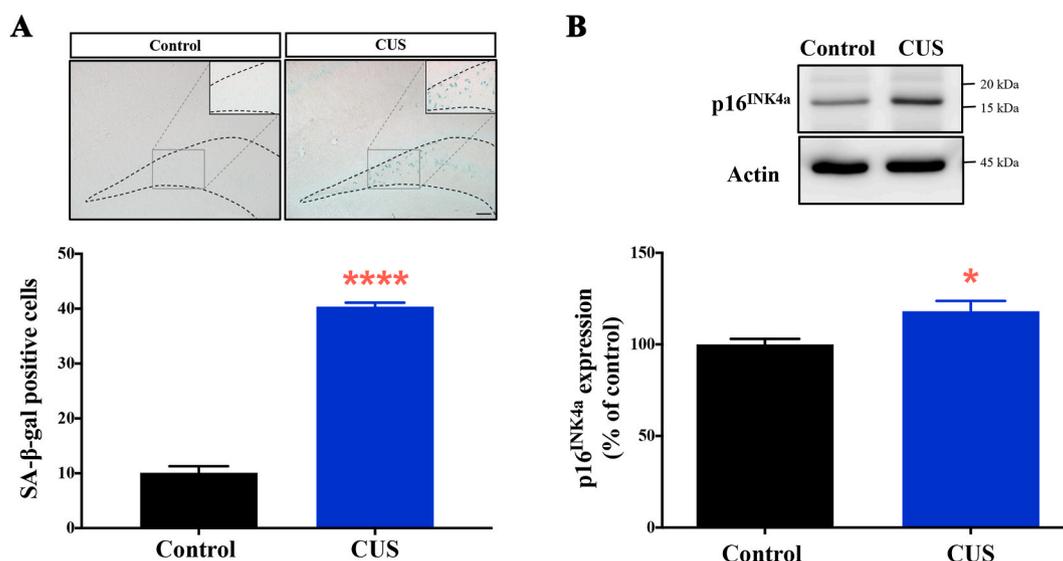
Fig. 2. Markers of senescence are increased in CUS-treated mice. (A) Representative images of the quantitation of SA-β-gal-positive cells, which were increased in the CUS-treated groups ($n = 4$ in each group). Original magnification, 10 × objective. Scale bar, 100 μm. The inserts show a higher magnification of the boxed area using a 40 × objective. Scale bar, 20 μm [two-tailed unpaired Student's *t*-test; $t_{(6)} = 21.70$; $p < 0.0001$]. ****$p < 0.0001$ vs. controls. **(B)** Western blot analysis of p16^{INK4a} expression in control and CUS-treated mice ($n = 6$ in each group) [two-tailed paired Student's *t*-test; $t_{(5)} = 2.668$; $p = 0.0444$]. *$p < 0.05$ vs. controls. The data are represented as means ± SEM.
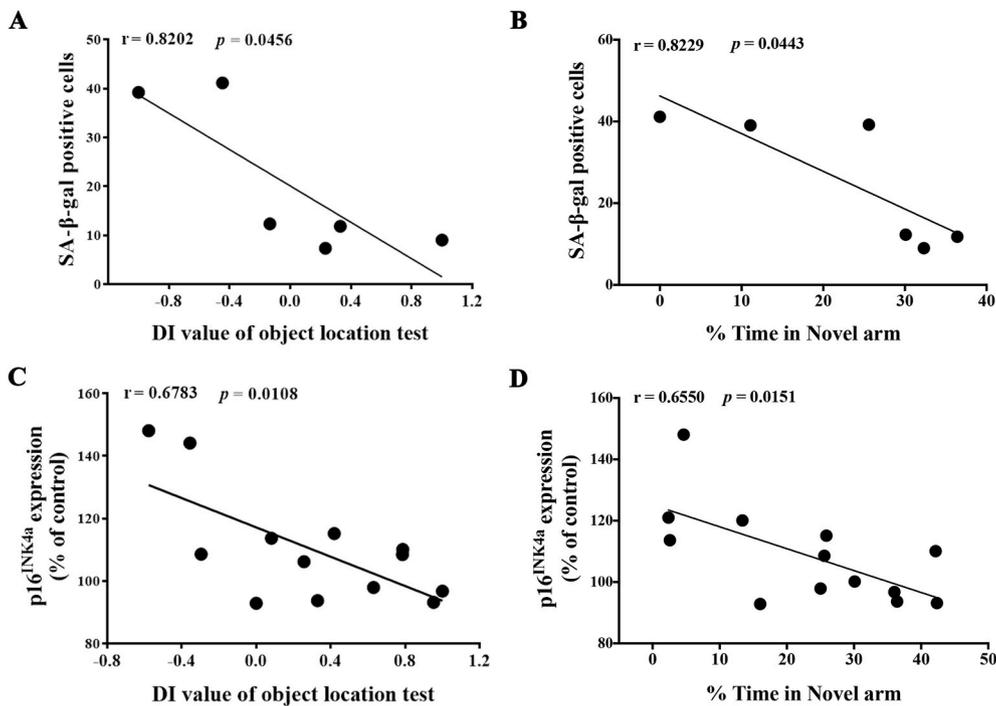
Fig. 3. Linear regression analysis between senescence markers and memory performance of mice shows a significant negative correlation. Quantitation of SA-β-gal-positive cells vs. the memory score plot of the object location test **(A)** or Y maze test **(B)** showed a negative linear relationship in mice (object location test: r = 0.8202, *p* = 0.0456, *n* = 6; Y maze test: r = 0.8229, *p* = 0.0443, *n* = 6). The percentage of hippocampal p16^{INK4a} levels vs. the memory score plot of the object location test **(C)** or Y maze test **(D)** showed a negative linear relationship in mice (object location test: r = 0.6783, *p* = 0.0108, *n* = 13; Y maze test: r = 0.6550, *p* = 0.0151, *n* = 13).

mouse hippocampi.

Furthermore, to eliminate the interference caused by locomotor deficiency during the memory tests, the open field test was utilized. No significant differences were found in the travel distance and velocity of control and CUS-treated mice either with or without D + Q administration (Fig. 5A). After analyzing locomotor activity, object location and Y maze tests were performed to observe whether the memory performance of mice was altered after treatment with the senolytic drug cocktail D + Q. CUS mice treated with D + Q had significantly improved spatial memory in the object location (Fig. 5B) and Y maze tests (Fig. 5C) compared with that of the vehicle-treated groups.

Furthermore, because dasatinib is an anticancer drug, we collected mouse serum to measure *in vivo* toxicology indices using an automated dry chemistry analyzer. Blood level analysis displayed no difference in glutamic-oxaloacetic transaminase (GOT) (Fig. S2A) and alkaline phosphatase (ALP) for liver function (Fig. S2C) or blood urea nitrogen (BUN) for kidney function (Fig. S2D). In particular, glutamic pyruvic transaminase (GPT) in serum level analysis was increased after CUS treatment but was decreased by D + Q treatment, and no significant difference was found between the control vehicle- and CUS D + Q-treated groups (Fig. S2B), indicating that the administration of dasatinib did not induce *in vivo* toxicology.

3.6. Senolytic therapy attenuates senescence-associated secretory phenotype (SASP) factors in CUS-treated mice

Recent findings suggested that the excessive accumulation of senescent cells could secrete various factors of the SASP, including many proinflammatory cytokines, chemokines, growth factors, and proteases, and excessive senescent cells could also use different strategies to avoid apoptosis, such as acting as zombie cells that resist death and alter the local and systemic tissue milieus (Campisi and d'Adda di Fagagna, 2007; Childs et al., 2014). To measure the levels of SASP factors, we analyzed serum from control and CUS-treated mice for many SASP factors using a mouse cytokine array/chemokine array. A representative heatmap (Fig. 6A) and accompanying results showed that CUS increased SASP factors, such as interleukin-1 β (IL-1β) (Fig. 6B), IL-6 (Fig. 6C), and IL-13 (Fig. 6D). Conversely, D + Q treatment reduced these factors

(Fig. 6B–D).

To explore whether SASP factors could affect memory, we analyzed the correlation between the expression of serum SASP levels and mouse memory performance using the object location test and Y maze test. Interestingly, we found a significant negative correlation between the serum IL-1β levels and DI values of the object location test (Fig. 7A) or the percentage of time spent in the novel arm in the Y maze test (Fig. 7B). The levels of IL-6 (Fig. 7C and D) and IL-13 (Fig. 7E and F) in serum also showed a negative correlation with memory scores based on the object location test and Y maze test. These findings suggest that senolytic approaches act systemically by reducing proinflammatory SASP factors to impact cognitive function.

4. Discussion

Stress is not all harmful, but chronic stress may have negative effects. Chronic stress is one of the major causes of depression (Richter-Levin and Xu, 2018), particularly when it is of moderate or severe intensity; hence, it can become a serious health problem. Depression is a common mental disorder and a concerning issue worldwide due to its high prevalence, high rate of recurrence, suicide rate, and economic burden (Keeney et al., 2006; Kessler et al., 2005). Additionally, it can affect anyone at any stage of life. The World Health Organization (WHO) recently reported that more than 300 million individuals of all ages have depression, estimating that 10%–15% of the general population will experience clinical depression in their lifetime. Mounting evidence suggests that unrelenting stress is involved in the development and progression of disease(de Kloet et al., 2005). Chronic stress has become a very serious social problem. Therefore, understanding how chronic stress causes physical and mental damage is important.

Furthermore, chronic stress affects emotional responses, thinking, and memory via changes in hormonal and immune system functions, leading to depression and even dementia, particularly Alzheimer's disease (Justice, 2018; Yaribeygi et al., 2017). Clinical studies also found that elderly subjects either in mid-life or elderly subjects prone to psychological distress are more likely to develop dementia than nonstressed individuals (Johansson et al., 2010; Wilson et al., 2005). Evidence suggests stress-related cognitive decline and dementia are thought to

A

B

(caption on next column)

result from the effects of prolonged elevations of cortisol (Peavy et al., 2007), which may lead to hippocampal atrophy and result in the loss of synapses within the hippocampus (McEwen, 2000; Tatomir et al., 2014). The hippocampus is critical for long-term memory and spatial navigation (Bannerman et al., 2014; Lynch, 2004), and its dysfunction is also correlated significantly with mild cognitive impairment and cognitive impairment in Alzheimer's patients (Jack et al., 2000; Morris et al., 2001).

We demonstrated that CUS-exposed eight-week-old male C57BL/6 mice exhibited depressive-like behaviors, such as the cessation of or a reduction in body weight gain and sucrose preference and an increase in immobility time in the FST, compared with the behaviors of age-matched nonstressed mice. Studies suggest that different ages have different responses to stress, and the effects are also different (Scott et al., 2013; Stawski et al., 2019). In the study, eight-week-old C57BL/6 mice were used because this age is equivalent to the young adult period of humans. Younger rodents are more likely to be induced by stress to produce depression-related behaviors than older rodents, while older rodents may show interfered stress perception because of increasing sensorial loss, such as sight or olfaction (Tikhonova et al., 2015; Zeng and Yang, 2015). In addition, previous studies on chronic stress have also often selected eight-week-old mice, which can be successfully induced to express stress-related behaviors, including depression-related behaviors, under chronic stress (Bollinger et al., 2020; Fang et al., 2021; Wang et al., 2010). Therefore, to eliminate conditions that may affect stress perception, we chose eight-week-old mice for the study.

Rodent studies have highlighted that gender differences exist in the response to stress. In the behavioral response, female mice were more sensitive to stress and prone to depression-related behaviors than male mice (Bangasser and Valentino, 2014; Franceschelli et al., 2014; Zuloaga et al., 2020). In human studies, statistics on the prevalence of neuropsychiatric diseases also found that women have higher rates of neuropsychiatric diseases than men (World Health Organization, 2017). In the response to stress-related hormones, female mice have a larger locus coeruleus, the brain region that produces norepinephrine (Pinos et al., 2001), and a reduction in the negative feedback loop on the hypothalamic-pituitary-adrenal (HPA) axis, leading to the secretion of corticosterone, was lower than that in male mice under stress (Novais et al., 2017; Palumbo et al., 2020; Zuloaga et al., 2020). These findings indicate that female mice are more likely to exhibit stress-related behaviors than male mice. However, most studies still use male mice for stress-related experiments. The main reason may be that the HPA axis can be regulated by the hypothalamus-pituitary-gonadal (HPG) axis, and the hormone levels secreted by males and females are different (Oyola and Handa, 2017). Therefore, differences may be evident between the sexes (Dalla et al., 2010; Toufexis et al., 2014). In particular, the natural hormonal changes that occur in females throughout their lifespan are accompanied by differences in the stress response. Female hormone secretion changes during the preovulation and late stages of ovulation, leading to HPA imbalance and stress-related hormones such as cortisol secretion imbalance (Glynn et al., 2013; Hamidovic et al.,

A

B

C
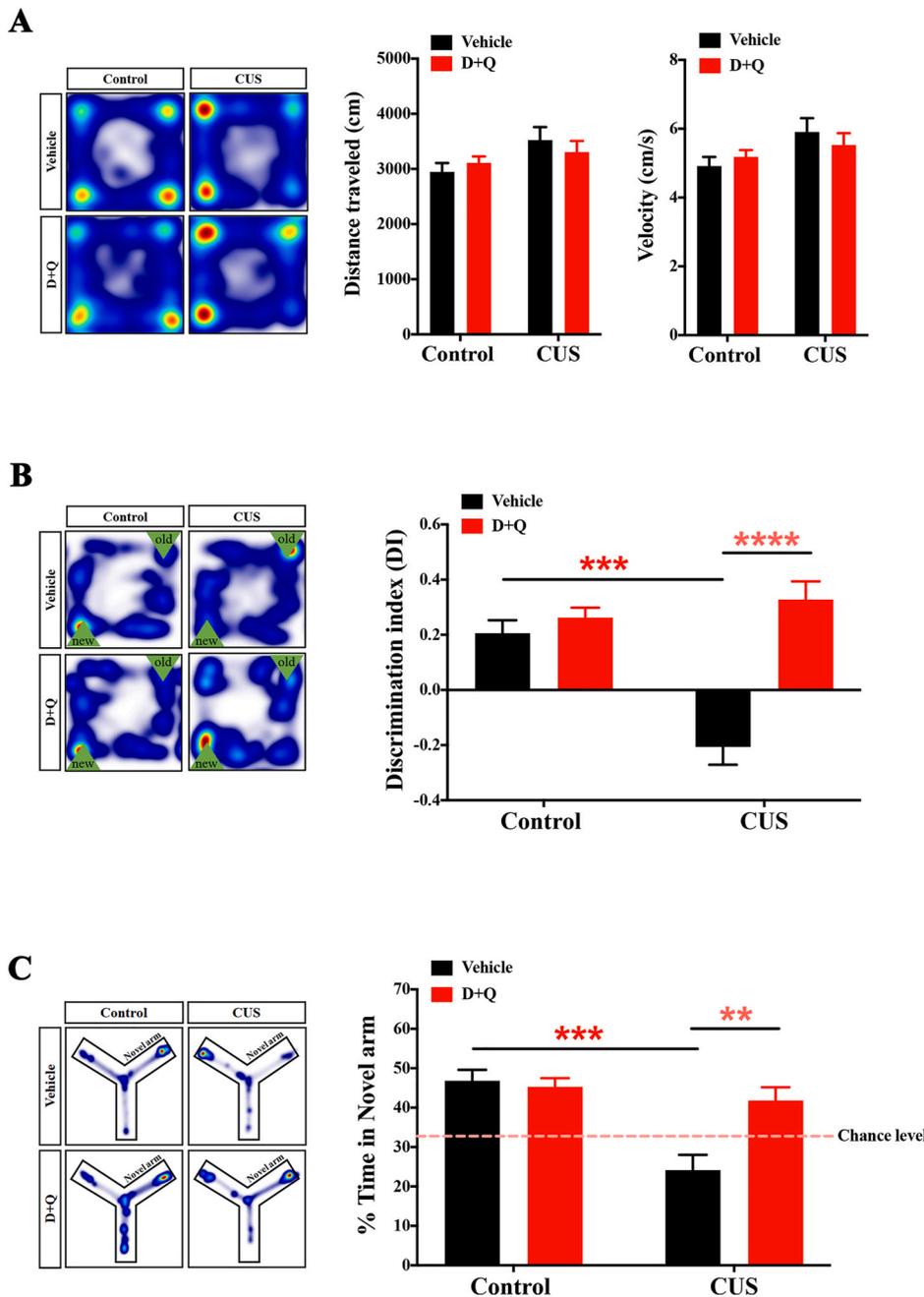
Fig. 5. Treatment with dasatinib plus quercetin rescues memory loss in CUS-treated mice. (A) The total distance traveled and velocity were not significantly different between vehicle- and D + Q-treated control and CUS mice. The data indicate an improvement in memory tests in CUS-treated mice following D + Q administration as determined by the object location (B) and Y maze tests (C) (control and CUS groups: $n = 9$ for the vehicle group; $n = 15$ for the D + Q group). Object location test: Two-way analysis of variance revealed a significant stress treatment × drug administration interaction [$F_{(1,44)} = 17.29$, $p = 0.0001$]. ***$p < 0.001$ and ****$p < 0.0001$ vs. respective controls as determined by Tukey's *post hoc* analysis. Y maze tests: Two-way analysis of variance revealed a significant stress treatment × drug administration interaction [$F_{(1,44)} = 9.097$, $p = 0.0042$]. **$p < 0.01$ and ***$p < 0.001$ vs. respective controls as determined by Tukey's *post hoc* analysis. The data are represented as means ± SEM.

2020). These changes may lead to uncertain experimental results. Thus, the use of male mice still accounts for most animal experiments.

Additionally, previous studies showed that the concentrations of glucose in the blood of mice increased after stress, which would affect the percentage of sucrose intake in CUS-treated mice. Therefore, we measured the glucose concentration in the serum of mice and no difference between the control and CUS-treated groups (Fig. S3). The data were consistent with previous findings showing that elevated blood levels after stress were dependent on the time of exposure to the stressor. Stress over 40 days could alter blood sugar levels (López et al., 2018), whereas the one-month CUS protocol that we used might not be sufficient to affect the concentrations of blood sugar (López et al., 2018; Raghav et al., 2019). Thus, our data indicated that the percentage of sucrose intake was significantly lower in CUS-treated mice after 4 weeks of stress than in nonstressed mice was not because they do not need sugar due to their blood sugar is very high.

Additionally, we found that CUS-exposed mice exhibited significantly lower memory performance than age- and sex-matched non-stressed mice. Previous studies have shown that chronic stress can change how individuals think, has a higher risk of memory loss, and can even increase one's neurodegenerative risk, including Alzheimer's disease (Justice, 2018; Ribeiro et al., 2013; Turner et al., 2015). In our study, although not all mice developed depression-like behaviors and cognitive decline after CUS treatment, 80% to 90% of CUS-treated mice exhibited significant depression-like behaviors and cognitive decline compared with the nonstressed control group. The small number of mice that did not express depression-like behaviors and cognitive decline after CUS treatment may be due to individual differences. Thus, before analyzing the senescent cell experiments, the mice were confirmed to display depression-like behaviors and memory loss based on the behavioral results. Additionally, we found a significant increase in hippocampal senescent cells in mice exhibiting depressive behavior and
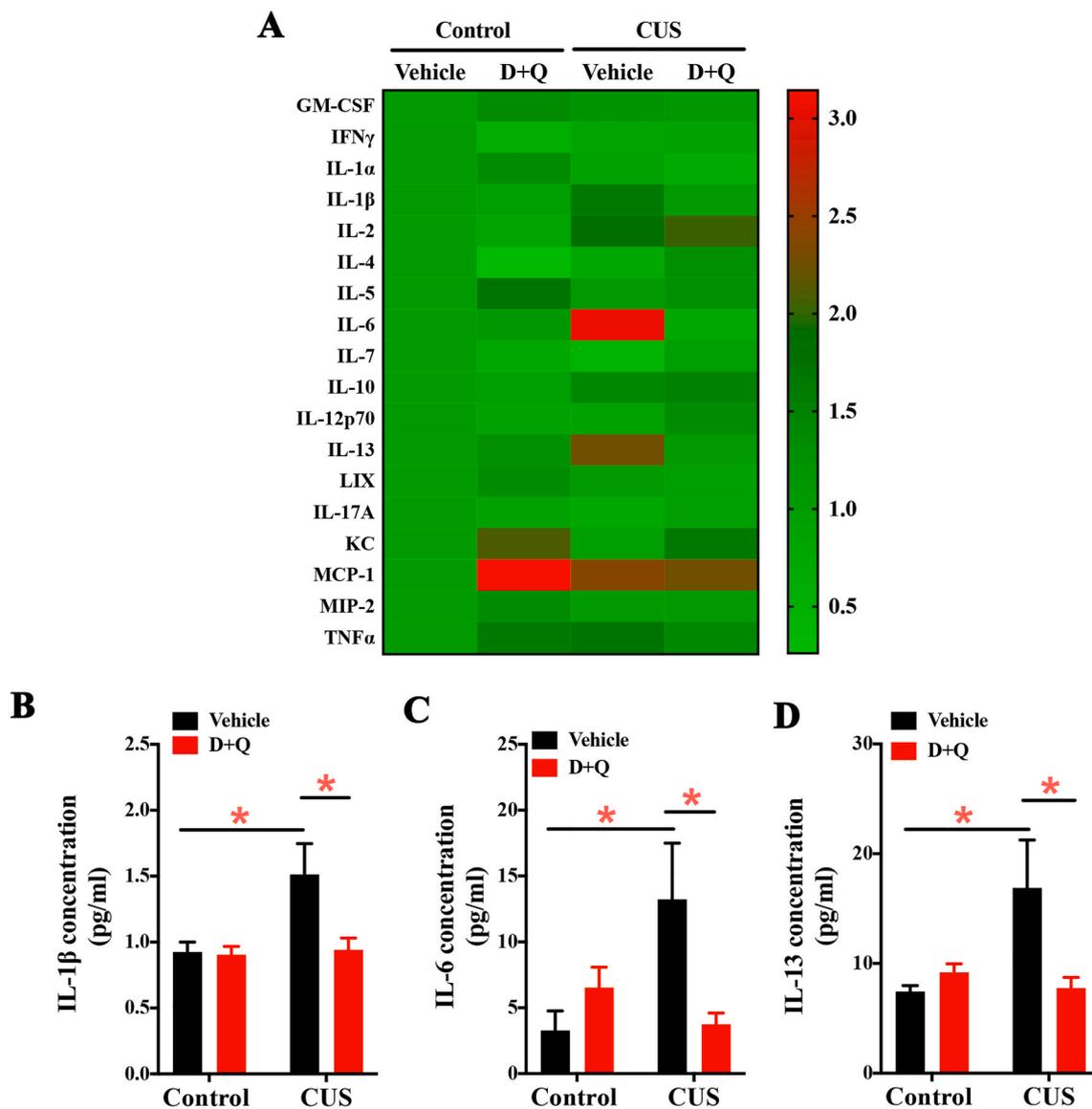
Fig. 6. D + Q treatment attenuates senescence-associated signatures in CUS-treated mice. (A) Representative heatmaps of multiplex assays indicated fold changes in cytokine/chemokine protein expression in serum from control and CUS mice treated with/without D + Q ($n = 11$ in each group). Concentrations of the SASP components **(B)** IL-1β, **(C)** IL-6, and **(D)** IL-13 in the serum of control and CUS mice treated with/without D + Q. Two-way analysis of variance revealed a significant stress treatment × drug administration interaction [IL-1β: $F_{(1,40)} = 4.222$, $p = 0.0465$; IL-6: $F_{(1,40)} = 6.891$, $p = 0.0122$; IL-13: $F_{(1,40)} = 5.642$, $p = 0.0224$]. *$p < 0.05$ vs. respective control as determined by Tukey's *post hoc* analysis. The data are represented as means ± SEM.

memory loss. We then made relevant comparisons and found that the memory performance of mice was negatively correlated with the number of senescent cells.

Additionally, compared with nonstressed control mice, CUS-treated mice exhibited increased protein expression of the hippocampal senescence marker p16[INK4a] and enhanced SA-β-gal activity in the DG. Thus, we investigated the effects of CUS-triggered cellular senescence on cognitive deficits. To investigate which cell types in the brain become senescent, we performed double immunofluorescence staining for SA-β-gal and cell-type-specific markers, including NeuN (neuronal marker), glial fibrillary acidic protein (GFAP; astrocyte marker), ionized calcium-binding adapter molecule 1 (Iba1; microglia marker), and Olig2 (oligodendrocyte marker), to quantify SA-β-gal-positive cells colocalizing with different cell types in the CUS-treated mouse brain. SA-β-gal-positive cells colocalized with the neuronal marker NeuN but not with GFAP, Iba1, or Olig2, indicating that CUS-triggered brain senescence is associated with neurons (Fig. S4).

Preclinical studies on age and cellular senescence-related diseases

show that D + Q treatment can significantly reduce senescent cells (Ellison-Hughes, 2020; Hickson et al., 2019). These new drugs are called senolytics. The combination of the two drugs that induce senescent cell apoptosis can delay, prevent, and alleviate multiple age-related phenotypes (von Kobbe, 2019; Zhang et al., 2019) or significantly reduce aging diseases in mice (von Kobbe, 2019). A recent study revealed that senolytic therapy could alleviate cognitive deficits in an Alzheimer's disease model (Zhang et al., 2019). The removal of senescent cells by inducing apoptosis is the most straightforward option, and several agents have been developed that can be used to eliminate senescent cells. Therefore, we speculate that approaches targeting senescent cells are needed and would constitute a novel therapy to treat chronic stress-triggered brain aging. Thus, senolytics were used to examine whether the elimination of senescent cells would rescue chronic stress-induced cognitive deficits in CUS-treated mice. We found that the senolytic cocktail D + Q alleviated cognitive deficits in CUS-treated mice. Additionally, to exclude the possibility of toxicity induced by the anticancer drug dasatinib, we collected mouse serum to measure *in vivo* toxicology indices using an
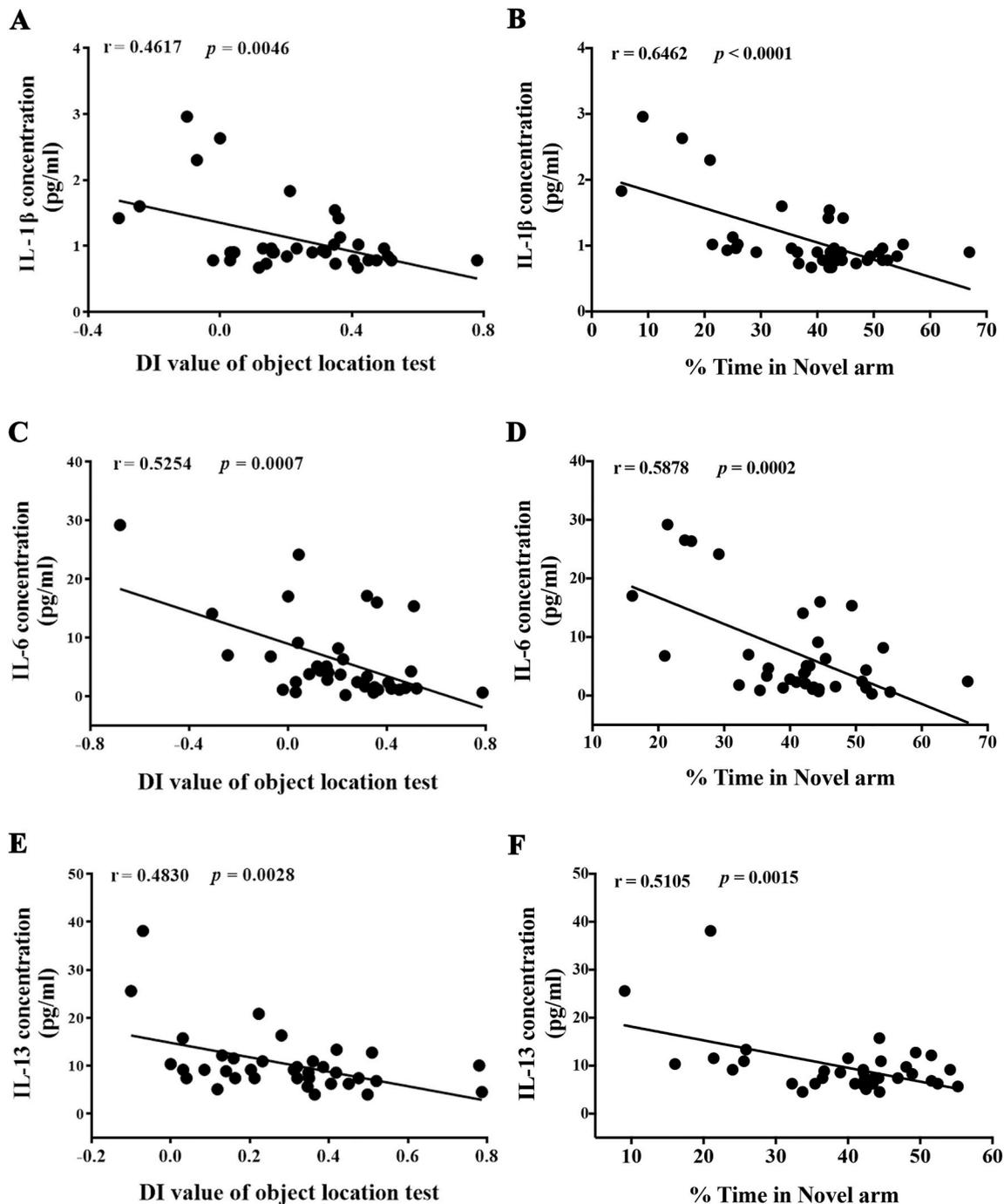
Fig. 7. SASP levels are correlated with the severity of memory impairment. (A) Correlation between the IL-1β levels and DI values using the object location test (r = 0.4617; p = 0.0046). **(B)** Correlation between the IL-1β levels and percentage of time spent in the novel arm using the Y maze test (r = 0.6462; p < 0.0001). **(C)** Correlation between the IL-6 levels and DI values using the object location test (r = 0.5254; p = 0.0007). **(D)** Correlation between the IL-6 levels and percentage of time spent in the novel arm using the Y maze test (r = 0.5878, p = 0.0002). **(E)** Correlation between the IL-13 levels and DI values using the object location test (r = 0.4830, p = 0.0028). **(F)** Correlation between the IL-13 levels and percentage of time spent in the novel arm using the Y maze test (r = 0.5105; p = 0.0015).

automated dry chemistry analyzer. Blood level analysis displayed no difference in GOP, ALP, or BUN values. Interestingly, we found that GPT levels increased after CUS treatment, but the increased levels returned to normal following D + Q treatment. This phenomenon was consistent with previous research revealing that chronic stress may affect liver function and lipid metabolism, including glutathione (GSH), malondialdehyde (MDA), and GPT (Bilal et al., 2017; Diaconu et al., 2011; Hasan et al., 2016).

Notably, senescent cells have different performance characteristics in each type of tissue. They secrete various SASP factors, including many proinflammatory cytokines, chemokines, growth factors, and proteases, and use different strategies to avoid apoptosis, such as acting as zombie cells that resist death and altering the local and systemic tissue milieus (Campisi and d'Adda di Fagagna, 2007; Childs et al., 2014). Additionally, recent studies have demonstrated that senescent cells can be detected in the mammalian brain (Chinta et al., 2015). Furthermore, systemic proinflammatory SASP factors can penetrate the blood-brain barrier and potentially have far-reaching effects on the brain. Thus, senescent cells could contribute to neurodegenerative processes by secreting proinflammatory SASP factors and/or destroying cells

required to maintain structural and functional neuron-glia interactions, which promote neurodegeneration (Chinta et al., 2015; Erickson and Banks, 2019; Maciel-Baron et al., 2018). Therefore, senescent cells and their SASP factors may constitute potential and important contributors to subsequent brain aging. Aging cells in the brain can be used as a new therapeutic target to prevent and treat chronic stress-related neuro-degeneration. However, how senescence is increased or regulated in CUS-treated mice remains unclear. Previous studies have indicated that TGF-β signaling is associated with aging-related diseases, including Alzheimer's disease, muscle wasting, and obesity. The downstream targets of TGF-β signaling include cell cycle regulation, ROS production, telomere regulation, and cytokine and chemokine regulation, all of which may lead to cellular senescence (Tominaga and Suzuki, 2019). Additionally, we screened the $p16^{INK4a}$ (*Cdkn2a*) promoter for potential binding sites of transcriptional regulators. Using transcription factor binding databases, we found a well-conserved recognition element for glucocorticoid receptor 1 (GR1, also known as NR3C1) in the proximal promoter region of *Cdkn2a*. Therefore, we will further investigate the underlying mechanism of cellular senescence and identify which factors cause senescence markers to increase in CUS-treated mice in the future.

In conclusion, our study revealed that **(1)** CUS could induce depression-like behaviors and memory impairments in mice, **(2)** cellular senescence was increased by CUS and had positively correlated with the severity of the memory deficit, **(3)** the clearance of senescent cells could rescue CUS-induced memory impairment, and **(4)** CUS could affect the SASP levels. Our proof-of-principle experiments help improve the present understanding of the impact of cellular senescence and demonstrate that therapeutic interventions to clear senescent cells or block their effects may open a new avenue to treat or delay chronic stress-related dysfunctions and improve human health.

CRediT authorship contribution statement

Yu-Fen Lin: Conceptualization, Formal analysis, Investigation, Data curation, Visualization. **Li-Yun Wang:** Formal analysis, Visualization. **Chi-Sheng Chen:** Formal analysis, Data curation. **Chia-Chun Li:** Formal analysis, Data curation. **Ya-Hsin Hsiao:** Conceptualization, Writing – original draft, Supervision, Writing – review & editing.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2021.100341.

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