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Original Article

Autophagy involving age-related cognitive behavior and hippocampus injury is modulated by different caloric intake in mice

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Abstract: Recent studies indicated that different caloric intake may influence neuronal function. Excessive caloric intake associated with accelerated aging of the brain and increased the risk of neurodegenerative disorders. And low caloric intake (caloric restriction, CR) could delay aging, and protect the central nervous system from neurodegenerative disorders. The underlying mechanisms remain poorly understood. In this study, thirty six-week-old male C57/BL male mice were randomly divided into three different dietary groups: normal control (NC) group (fed standard diet), CR group (fed low-caloric diet) and high-calorie (HC) group (fed high-caloric diet). After 10 months, spatial memory ability was determined by Morris water maze. Pathological changes of the hippocampus cells were detected with HE and Nissl staining. The expression of proteins involved in autophagy in the hippocampus was determined by immunofluorescence and Western blot. The result of Morris water maze showed that the learning and memory capacity significantly increased in the CR group, and significantly decreased in the HC group. HE and Nissl staining showed cells damaged obviously in the HC group. The expression of mTOR and p62 was increased in the HC group, and decreased in the CR group. The expression of Beclin1, LC3 and cathepsin B was decreased in the HC group, and increased in the CR group. Our findings demonstrate that long-term high caloric intake is a risk factor that can significantly contribute to the development of neurological disease via suppressing autophagy, and CR may prevent age-related learning ability impairment via activating autophagy in mice.

Keywords: Caloric restriction, high-caloric intake, autophagy, cognitive behavior, obesity

Introduction

Obesity is a pandemic and a serious global health concern. It is a risk factor for multiple conditions, causing multiple chronic metabolic disturbances including insulin resistance, diabetes mellitus, dyslipidaemia and hypertension, which are further associated with impaired cognition [1, 2]. Obesity has been associated with changes in brain structure, cognitive deficits, dementia and Alzheimer's disease (AD) [3, 4]. Excessive caloric intake will easily lead to obesity, so long-term consumption of a high-caloric diet is a risk factor that can significantly contribute to the development of neurological disease [5]. Epidemiologic studies suggest that the prevalence of AD is greater in countries with higher intake of high-caloric diets and lower in those that consume low-caloric diets [6-8]. Long-term low-caloric intake (caloric restriction, CR) without malnutrition has been considered to have beneficial effects on human health, including retarding the progression of many age-associated molecular, physiological, and pathological processes which occur in tissues with high oxidative demand, such as kidney, heart and brain [9-12]. CR can protect the central nervous system from neurodegenerative disorders, such as AD, Parkinson's disease, and Huntington's disease [13]. Some potential mechanisms have been proposed to explain the metabolic effects of high or low caloric intake on brain function, but the molecular mechanisms remain poorly understood.

Autophagy (from Greek for "self-eating") is an evolutionarily conserved process in eukaryotic organisms, defined as a catabolic pathway involving the degradation of cellular components via the lysosomal machinery [14]. Autophagy is an intracellular recycling pathway that functions during basal conditions but can be induced under stress such as starvation, hypoxia, or cell injury [15, 16]. Defects in the activation of autophagy are involved in the pathogenesis of AD [17, 18]. mTOR, a serine/threonine protein kinase, has been recognized as a important negative regulator of autophagy [19]. Decreases in mTOR activity lead to increased autophagy. Several lines of evidence show that reduced mTOR signaling by rapamycin treatment enhances autophagic degradation of aggregate proteins, and can effectively treat age-related neurodegenerative diseases [20-22].

In the present study, we subjected 6-week-old C57/BL6 to diets containing different levels of calories to investigate changes in cognition and hippocampal neurons. Then, we examined the level of mTOR, Beclin1, LC3 (microtubule-associated protein 1-light chain 3), p62 and cathepsin B to investigate the relationship between caloric intake and autophagy in brain, so as to provide a scientific basis for disease prevention through consumption of a reasonable caloric intake.

Materials and methods

Animals and diet

Thirty 6-week-old C57/BL6 male mice (Academy of Military Medical Sciences, Beijing, China), weighed 18.2-22.7 g, were housed in individual cages, exposed to 12 h light/dark cycles at 22 ± 2°C. All animal experiments were approved by the institutional Animal Care and Ethics Committee of Xuan Wu Hospital, Capital Medical University in Beijing, China.

After one week ad libitum fed, the mice were weighted matched and randomized to one of three dietary groups (n = 10 in each group): normal control (NC) group (fed standard diet, total calorie 4.0 kcal/g), CR group (fed low-caloric diet, total calorie 2.8 kcal/g) and high-calorie (HC) group (fed high-caloric diet, total calorie 5.2 kcal/g). These custom diets were formulated by the Experimental Animal Center of Academy of Military Medical Sciences. The low-caloric diet was composed of 58% stock diet, 34% dietary fiber, and 8% isolated soy protein, and the high-caloric diet was composed of 63% stock diet, 19% lard compound, 10% sucrose, and 8% isolated soy protein. The energy ratio of the feed of NC group, CR group and HC group is 1:0.7:1.3 [23]. Mice were fed for 10 months and weighed monthly.

Behavioral testing

The MWM test protocol consisted of a period of 5 days of hidden platform trial and a probe trial that was conducted on day 6. The probe trial was formed by removing the platform and allowing each mouse to swim freely for 60 s.

Tissue processing

After the behavioral test, mice (n = 5 per group) were anesthetized and perfused transcardially with 4% paraformaldehyde. Brains were then extracted, embedded in paraffin, then cut into 4 μm-thick coronal sections for Hematoxylin & Eosin (HE) staining, Nissl staining and immunofluorescence analysis. In addition, remnant animals (n = 5 per group) were decapitated under anesthesia. Brains were immediately taken out, and hippocampal tissues were isolated for Western blot analysis.

Hematoxylin and eosin staining, Nissl staining and immunofluorescence

After the regular deparaffinize and rehydrate, the sections were processed for immunofluorescence, and stained with HE and Nissl. Immunofluorescence was performed using previously described techniques [24] with following primary antibodies: rabbit anti-mTOR (1:500, Abcam), mouse anti-Beclin1 (1:300, Santa Cruz), rabbit anti-LC3 (1:500, Sigma), rabbit anti-p62 (1:300, Santa Cruz), and rabbit anti- cathepsin B (CatB, 1:100, Santa Cruz).

Western blot

The protocol of Western blot was the same as before [25]. The primary antibodies used in this study were rabbit anti-mTOR (1:1000, Abcam), mouse anti-Beclin1 (1:1000, Santa Cruz), rabbit anti-LC3 (1:1000, Sigma), rabbit anti-p62 (1:500, Santa Cruz), and rabbit anti-CatB (1:200, Santa Cruz).

Table 1. Change in body weight of each group (g)

Group	N	0 month	1 month	2 month	3 month	4 month	5 month	6 month	7 month	8 month	9 month	10 month
NC	10	20.04 ± 2.27	21.59 ± 1.86	20.58 ± 1.36	22.66 ± 2.69	23.86 ± 2.86	24.49 ± 3.14	26.24 ± 3.91	25.97 ± 4.83	26.48 ± 3.81	25.89 ± 4.90	26.86 ± 4.76
CR	10	20.52 ± 2.37	20.54 ± 1.97	18.57± 1.87$^{\Delta}$	20.17 ± 2.70	21.82 ± 2.87	22.97± 2.72	23.84 ± 2.67	22.94 ± 3.17	21.50 ± 3.07$^{\Delta}$	20.97± 2.71$^{\Delta}$	21.92 ± 2.16$^{\Delta}$
HC	10	20.33 ± 2.09	22.62 ± 2.03	22.88 ± 2.32$^{\Delta}$	27.19 ± 6.51$^{\Delta}$	31.79 ± 6.51$^{\Delta}$	33.06 ± 7.28$^{\Delta}$	36.76 ± 8.21$^{\Delta}$	35.12 ± 6.01$^{\Delta}$	35.89 ± 5.38$^{\Delta}$	35.60 ± 8.52$^{\Delta}$	34.71 ± 6.92$^{\Delta}$

Results are expressed as mean ± SD, $^{\Delta}P < 0.05$ vs. NC group.

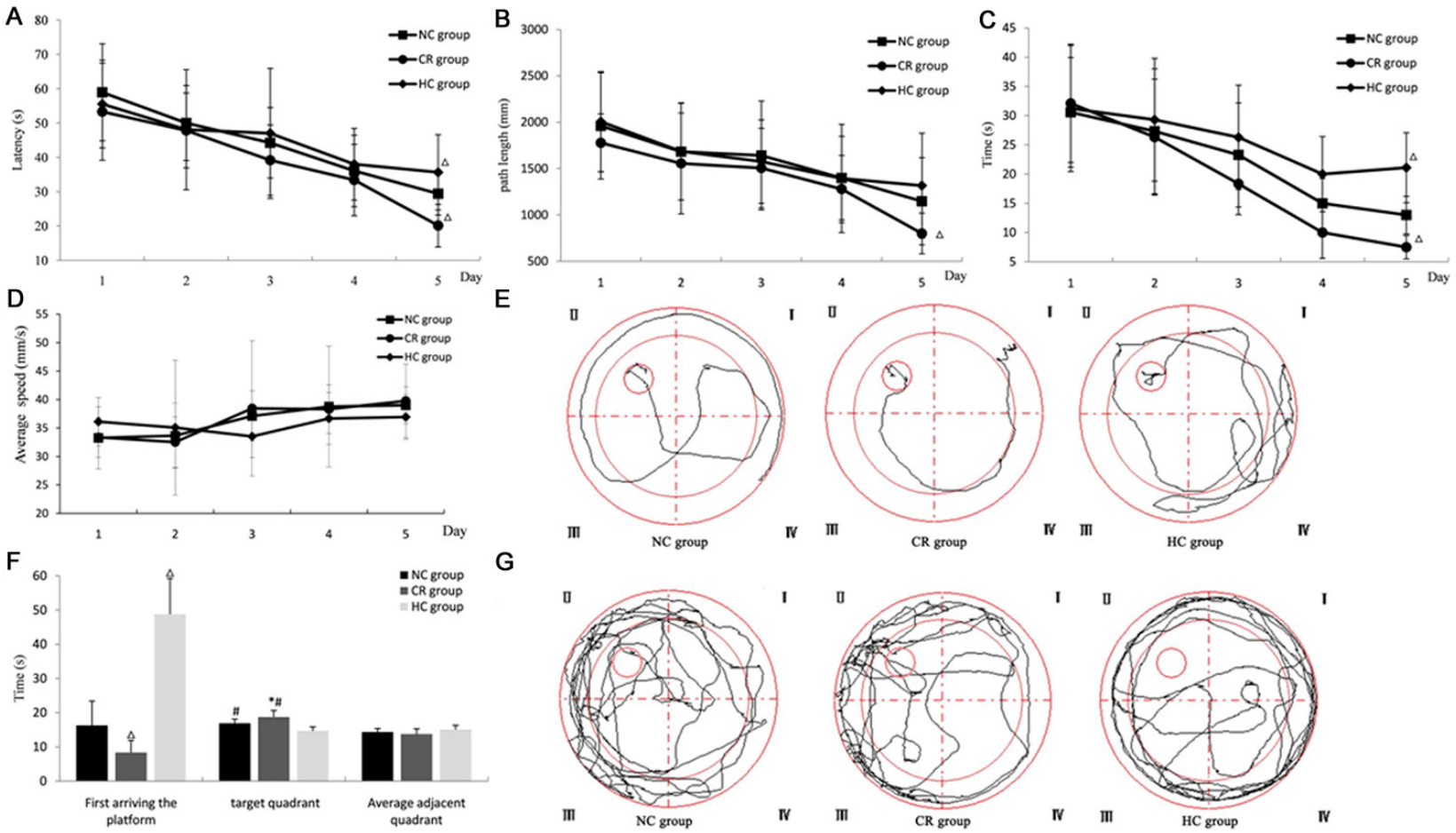

Figure 1. Changes in learning and memory in mice fed a standard diet (NC group), a low-calorie diet (CR group) or a high-calorie diet (HC group) as assessed using the Morris Water Maze. A. Latency to find platform. B. Path length before mounting the platform. C. Thigmotaxis Latency to platform. D. Swimming speed. E. Typical swimming patterns in the last hidden platform trial. F. Time taken to first reach the original platform area and spent in the four quadrants. G. Typical swimming patterns in the space exploring test. n = 10 per group, $^{\Delta}P < 0.05$ *vs.* the NC group, $^{*}P < 0.05$ *vs.* the HC group, $^{\#}P < 0.05$ *vs.* time spent in the adjacent quadrant.

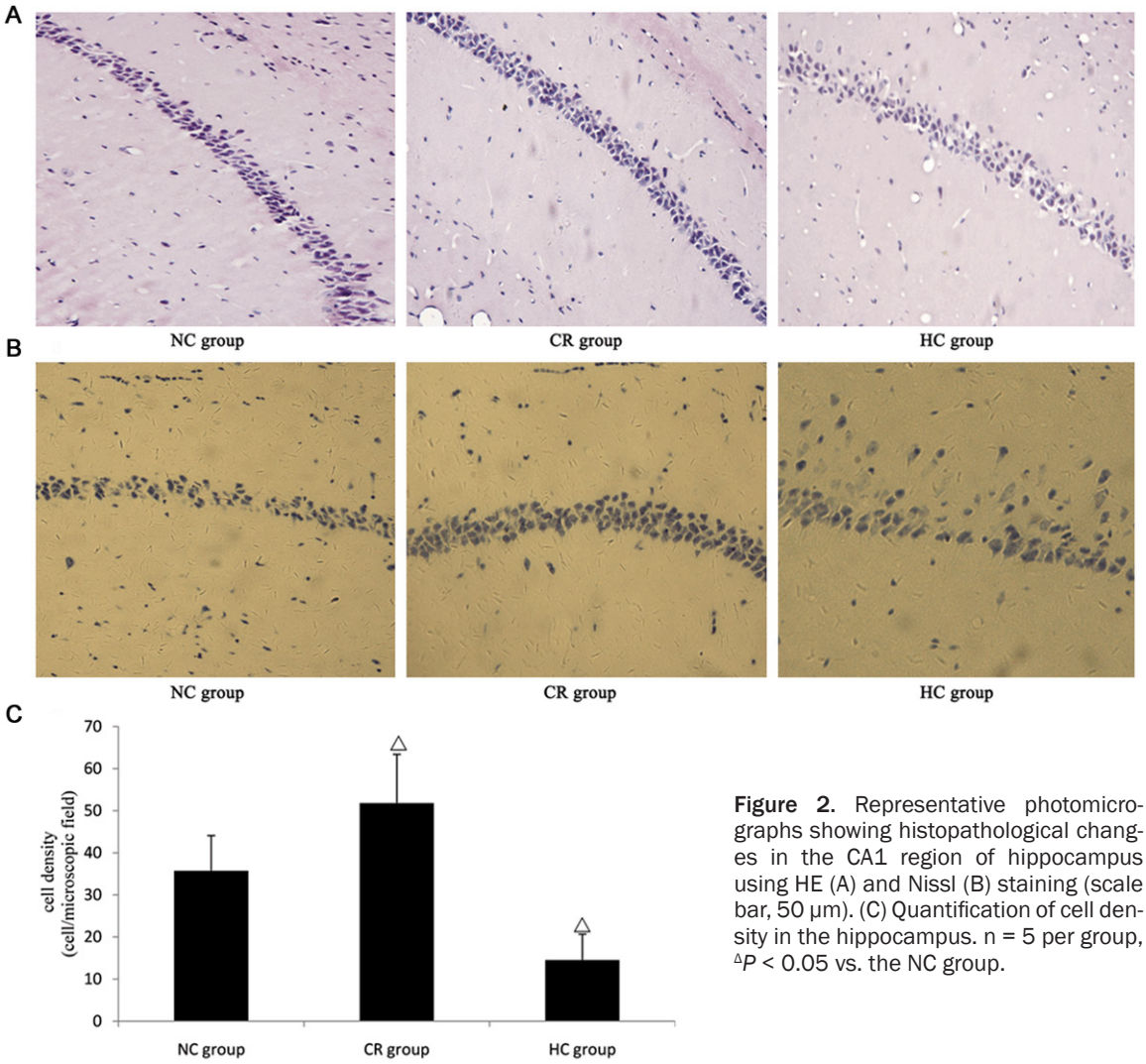

Figure 2. Representative photomicrographs showing histopathological changes in the CA1 region of hippocampus using HE (A) and Nissl (B) staining (scale bar, 50 μm). (C) Quantification of cell density in the hippocampus. n = 5 per group, $^{\Delta}P < 0.05$ vs. the NC group.

Statistical analysis

The data were expressed as the mean ± SD. The statistical significance was determined using one-way analysis of variance (ANOVA), except for comparisons among the three groups for animal body weight, escape latency, path length, swimming speed and thigmotaxis which were analyzed with repeated measures two-way ANOVA. The values were considered significant when $P < 0.05$.

Results

Changes in body weight

After 2 months, mice had greatly diverged in the body weight. Body weight of mice in the HC group was significantly greater than the control group at 2 months ($P < 0.05$; **Table 1**). The mice in the HC group displayed obese phenotype. In contrast, at 2, 8, 9 and 10 months of treatment, body weight in the CR group was significantly increased slower than the NC group ($P < 0.05$; **Table 1**). Mice in CR group were thin and sensitive to food, so they foraged actively.

Changes in learning and memory

Mice were trained for 5 days to find the hidden platform. All mice learned the task as indicated by latency, thigmotaxis (time spent in the zone within 10 cm of the wall of the pool), and path length measures. As shown in **Figure 1A-C** and **1E**, the latency and thigmotaxis ware significantly longer in mice in the HC group compared to mice in the NC group ($P < 0.05$). On Day 5 trials, the latency, thigmotaxis and path length

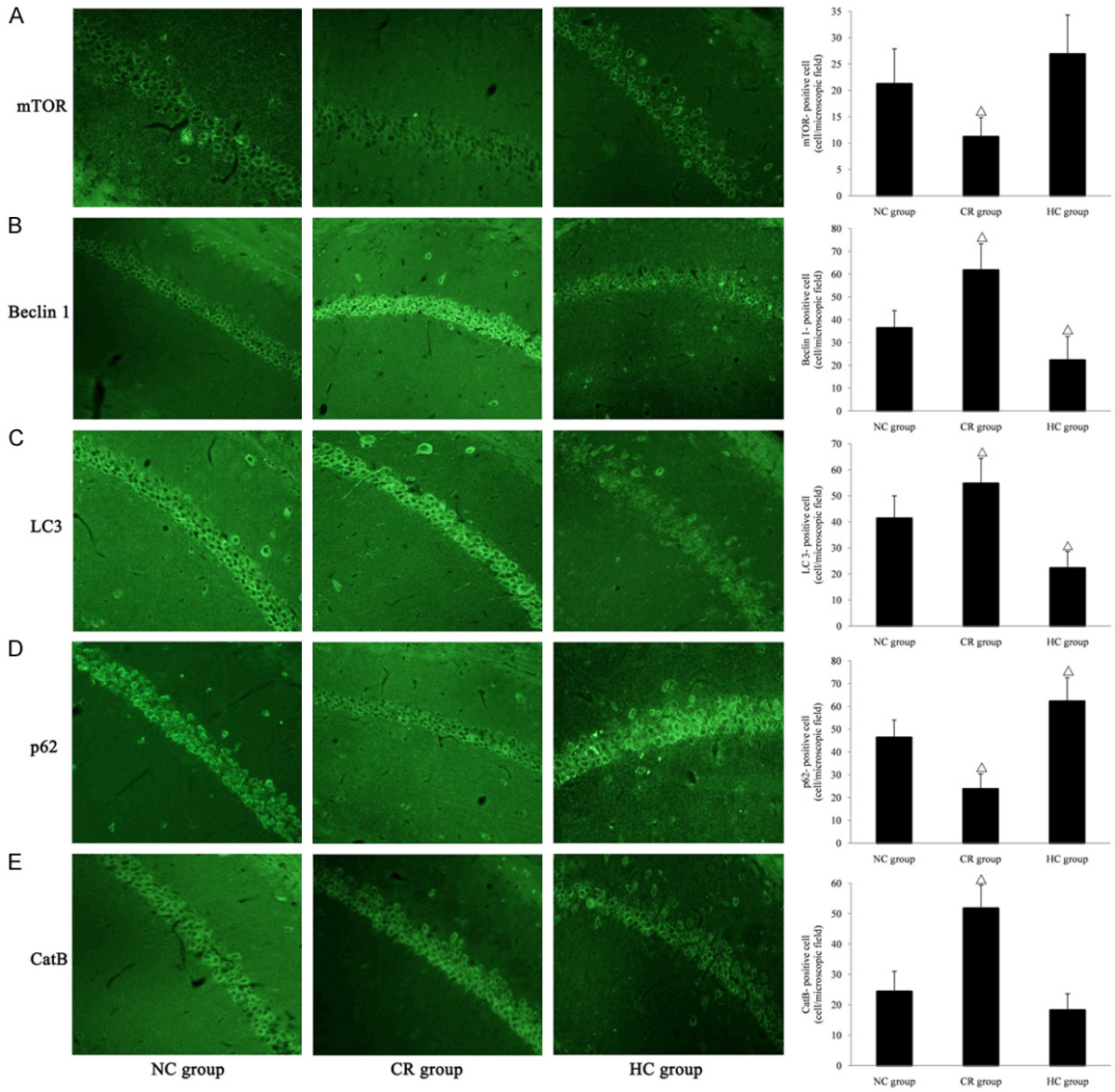

Figure 3. Representative photomicrographs (scale bar, 50 μm) and quantification of the numbers of mTOR-positive cells (A), Beclin 1-positive cells (B), LC3B positive-cells (C), p62 positive-cells (D) and CatB positive-cells (E) in the CA3 region of hippocampus. n = 5 per group, $^{\Delta}P < 0.05$ *vs.* the NC group.

of mice in the CR group were shorter than that of mice in the NC group.

There was no significant difference in mean swimming speed among three groups ($P > 0.05$; **Figure 1D**).

As presented in **Figure 1F** and **1G**, Compared with mice in the NC group, the time mice taken to first arrive the original platform area reduced in the CR group ($P < 0.05$) and significantly increased in mice in the HC group ($P < 0.05$). And the time spent in the target quadrant was significantly longer than that spent in the adjacent quadrant for mice in the NC and CR groups ($P < 0.05$). However, the time spent in the four quadrants was no significant difference ($P > 0.05$) in the HC group. Compared with the HC group, the time spent in the target quadrant significantly increased in the CR group ($P < 0.05$).

Histochemical changes in hippocampal neuron

The hippocampus, in particular the CA1 region, is crucial for spatial learning and memory performance [26, 27]. HE and Nissl staining were used to detect histomorphological changes of

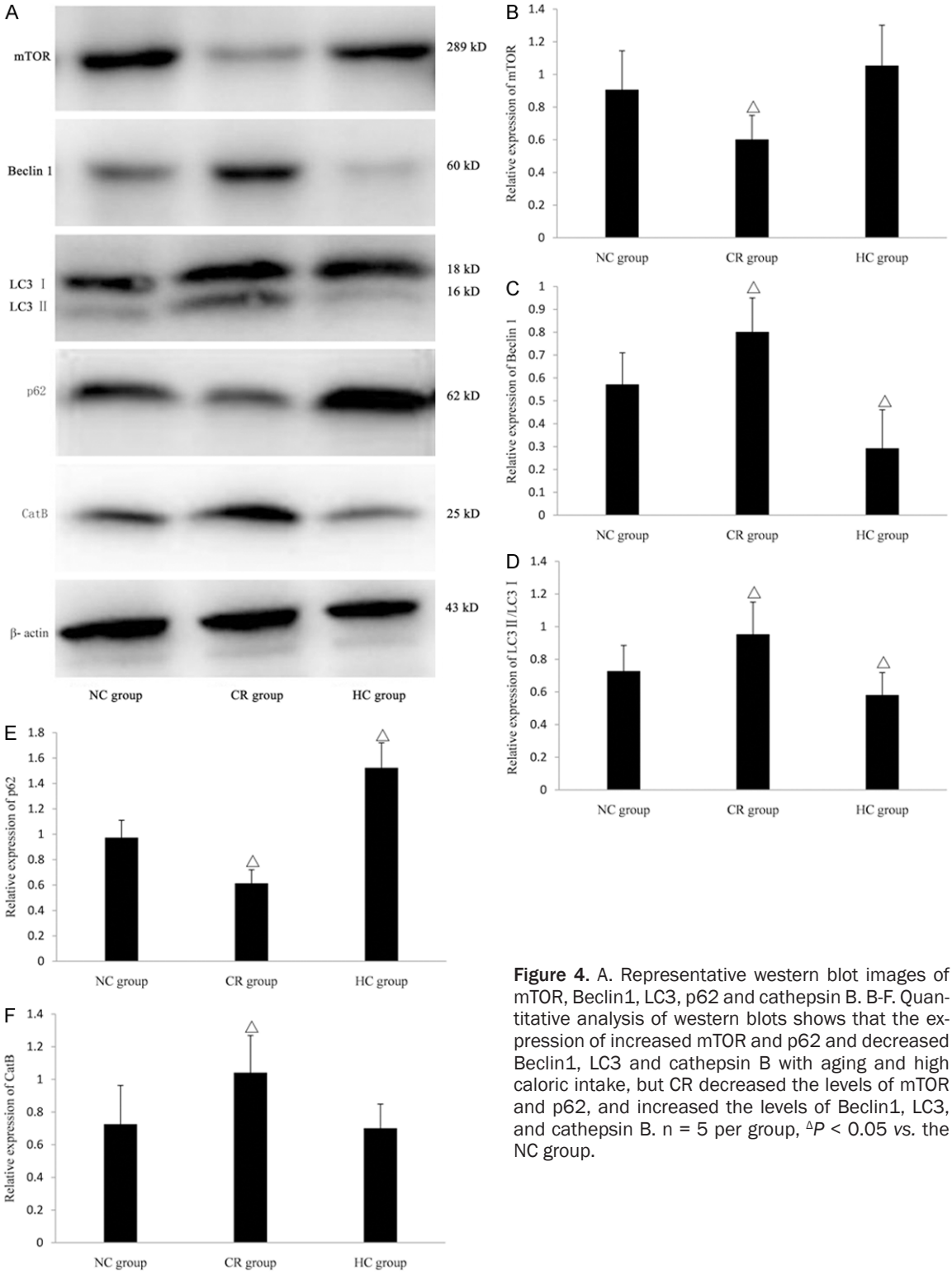

Figure 4. A. Representative western blot images of mTOR, Beclin1, LC3, p62 and cathepsin B. B-F. Quantitative analysis of western blots shows that the expression of increased mTOR and p62 and decreased Beclin1, LC3 and cathepsin B with aging and high caloric intake, but CR decreased the levels of mTOR and p62, and increased the levels of Beclin1, LC3, and cathepsin B. n = 5 per group, $^{\Delta}P < 0.05$ *vs.* the NC group.

the neurons in the CA1 region of hippocampus. Representative photomicrographs of HE and Nissl staining results were shown in **Figure 2A** and **2B**. In the NC group and CR group, CA1 pyramidal neurons were normal. In the HC group, typical neuropathological changes were found, including neuron loss, nucleus shrinkaged or disappearance of Nissl bodies decreased. Compared with the NC group, cell density was significantly decreased in the HC group ($P < 0.05$), and significantly increased in the CR group ($P < 0.05$; **Figure 2C**).

Changes of mTOR, Beclin1, LC3, p62 and cathepsin B protein expression in the hippocampus

The mTOR was recognized as the most important negative regulator of autophagy [19]. As shown in **Figure 3A**, there were a different number of mTOR positive cells in the CA1 region of hippocampus of each group. Compared with the NC group, the number of mTOR positive cells significantly significantly decreased in the CR group ($P < 0.05$). A similar pattern of result was seen in the western blot analysis (**Figure 4A** and **4B**).

Beclin1 had a positive role in the regulation of autophagy and LC3 played a critical role in the formation of early autophagic vacuole membranes. As shown in **Figure 3B** and **3C**, there were a different number of Beclin1 and LC3 positive cells in the CA1 region of hippocampus of each group. Compared with the NC group, the number of Beclin1 and LC3 positive cells was significantly lower in the HC group ($P < 0.05$), and significantly higher in the CR group ($P < 0.05$). A similar pattern of results was seen in the western blot analysis (**Figure 4A**, **4C** and **4D**). p62 regulated the formation of protein aggregates and was the substrate of autophagy [28, 29]. Consistent with the former results, hippocampus from mice in the HC group showed increased p62 levels, which were restrained by CR (**Figures 3D**, **4A** and **4E**).

To confirm these findings, we further used an assay based on levels of CatB, a lysosomal cysteine protease of the papain superfamily, primarily involved in the degradation or processing of lysosomal proteins. As shown in **Figure 3E**, Compared with the NC group, the number of CatB positive cells was significantly higher in the CR group ($P < 0.05$). A similar pattern of results was seen in the western blot analysis (**Figure 4A** and **4F**).

Discussion

Different caloric intake may be an important way to accelerate or slow the neurodegenerative disorder related to age. Long-term high-caloric intake leads to an increasing incidence of obesity. As illustrated in **Table 1**, the HC group showed approximately 29% higher body weight gain than the NC group, and reached the obesity standard [30]. Most experimental work has shown that obesity and consumption of a high-caloric diet affect the brain and is linked with structural abnormalities, such as reduced brain and hippocampal volume, atrophy, and white matter lesions. And obesity may cause cognitive deficits. The MWM is currently the most effective and reliable method to detect cognitive deficits in rodents and so was used in this study. Our results indicate that high-caloric intake impairs cognitive behavior in the HC group, and the parameters of the CR group are better than the NC and HC groups. It is believed that the number of the neurons with normal morphology in the hippocampus, especially CA1, is correlated with spatial learning and memory ability. Nissl bodies are used as a morphologic marker to detect neuronal activity. The HE and Nissl staining of the hippocampus can reveal that CA1 neurons and Nissl bodies obvious loss in the HC group, and a larger number of CA1 neurons, containing Nissl bodies, maintained structure intactly and arranged regularly and tightly in the CR group. Overall, long-term consumption of a high-fat diet could exhibit cognitive decline, and deteriorations in the number of neurons and neuronal activity in the hippocampus. But CR could delay aging, including the aging of hippocampal neurons, and protect hippocampal LTP, so as to maintain a better learning and memory [31, 32].

Autophagy is essential for maintaining protein homeostasis and healthy neurons. Once autophagy is initiated, cytoplasmic materials become enclosed in a double-membrane vesicle, which subsequently fuses with a lysosome. This leads to the degradation of damaged or unwanted components and recycling of the components for use in energy production and other biosynthetic reactions. As a result, it is generally thought that autophagy and mTOR-regulated autophagy pathways are at least partly responsible for aging and a range of age-related neurodegenerative disorders [33, 34]. Beclin1, LC3 and p62 are used to evaluate autophagy activity. Moreover, enzymes (such as CatB) in the lysosomes of eukaryotic cells are involved in autophagy. The results presented in our study show that the level of mTOR and p62 is significantly up-regulated, and the level of Beclin1, LC3 and CatB protein exhibit a significant decline in hippocampal neurons of HC group and NC group. The results reinforce that autophagy deficits in the hippocampus of mice at least part of the main mechanisms behind

normal brain aging and contribute to age-related cognitive decline. However, our data showed the age-related increase of mTOR and p62, and decline of Beclin1, LC3 and CatB in the hippocampus that were ameliorated by CR treatment. Abundant evidence shows that CR increases life span in several model organisms, ranging from yeast to mice and even primates. And earlier research have elucidated that CR ameliorates the age-related cognitive deficits, consistent with our recent findings [35, 36]. CR has been shown to efficiently stimulate autophagy in vivo and in vitro [37]. We find that CR deactivated mTOR signaling pathway, up-regulated expression of Beclin1 and LC3, and down-regulated expression of p62 in the hippocampus. The promotion of mTOR activity that successfully declines and sustains autophagic degradation with aging in the hippocampus by CR treatment may be involved in CR slowing aging, delaying age-dependent cognitive dysfunction and preventing age-related neurodegenerative disorders [38, 39]. CR has been shown to suppress mTOR complex expression and deactivate mTOR by deactivating the PI-3K/AKT pathway [40, 41]. In part, CR deactivates mTOR pathway by activating AMPK and SIRT1 [42]. However the mechanisms by which CR delays age-related cognitive deficits need to be further researched. There are more emerging experiments and molecular themes focus on CR, autophagy and age-related cognitive deficits. And all the studies are very important to further understanding and prevention age-related neurodegenerative disorders.

All these data demonstrate that high-caloric intake and CR have opposite effects. High-caloric intake is a risk factor that can significantly contribute to the development of neurological disease. Conversely, CR has been shown to reduce symptom progression of neurological disorders, suggesting that appropriate low-caloric intake is important to prevent or reduce the prevalence of neurological diseases in the developing countries. The results may be helpful for us to better understand the mechanisms behind normal brain aging and age-associated neurodegeneration.

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Disclosure of conflict of interest

None.

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