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Autophagy does not lead to the asymmetrical hippocampal injury in chronic stress

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HIGHLIGHTS

- Chronic stress leads to hippocampal neuron loss.
- The injury is asymmetrical.
- Autophagy signaling is involved in neuronal loss.
- Autophagy is however symmetrical.

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ABSTRACT

Chronic stress results in hippocampal injury, and impairs learning and memory ability of animals. However the cellular mechanisms underlying cell death within hippocampus remain elusive. The present employed the rat model of chronic unpredicted mild stress (CUMS) and examined the cellular mechanism responsible for learning and memory impairments. The results showed that in correlation to the decreased ability in novelty cognition and reverse learning, CUMS led to loss of CA3 neurons in hippocampus, especially in the right hippocampus. Interestingly, autophagy contributed to the cell loss but was asymmetrical on both sides. This suggested that CUMS resulted in asymmetrical hippocampal injuries, which is not fully determined by autophagy.

1. Introduction

Brain asymmetry includes anatomical asymmetry and functional lateralization [1]. The asymmetry based neural coding has multiple advantages, such as searching for food and predator with different eyes simultaneously. Hippocampus exhibits clear L–R asymmetry, ranging from gene expression to roles in spatial navigation [2]. For instance, the number of neurons in the right hippocampus is less than that in the left side, together with differences of expression in hundreds of genes [3,4]. Moreover, the right hippocampus is believed for global spatial information coding, and the left hippocampus for local spatial navigation [5].

In diseased conditions such as neurodegeneration (e.g., Alzheimer's disease/AD) and epilepsy, hippocampal volume asymmetry is as well reported, possibly due to the neuronal atrophy during disease [6–8]. Chronic stress impairs general brain physiological processes and functions, ranging from sensory function, cognition, emotion, to immune function, self-repair and aging [9–13]. However, it is unknown that if environmental factors such as stress impact the L/R hippocampus differentially. This presented for the first time reported asymmetrical L/R hippocampus cell loss, and the potential involvement of autophagy in the process.

2. Materials and methods

2.1. Animals

24 male Sprague–Dawley rats were purchased from Animal Experiment Center, Chinese Academy of Sciences, and raised under temperature 22 ± 2 °C, humidity 50–60%, 12/12 h L/D cycle, and free access to

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Table 1
Open field test results (M ± SD, n = 12).

Group	Before stress			After stress		
	Locomotion (cm)	Center cross	Vertical exploration	Locomotion (cm)	Center cross	Vertical exploration
Control	2493.6 ± 444.4	13.4 ± 5.2	23.5 ± 9.1	2146.4 ± 755.6	15.4 ± 8.1	21.1 ± 15.3
Stress	2449.7 ± 232.9	12.3 ± 3.8	23.0 ± 9.4	1047.8 ± 503.1	3.3 ± 2.3	8.4 ± 3.7
t	0.303	0.583	0.132	4.192	4.959	2.800
P	0.765	0.566	0.896	0.000**	0.000**	0.016*

* p < 0.05, ** p < 0.01.

Table 2
Y maze results (M ± SD, n = 12).

Group	Time spent (%)			Cross numbers (%)			Total cross
	Novel arm	Start arm	Other arm	Novel arm	Start arm	Other arm	
Control	52.42 ± 12.93	22.21 ± 9.78**	25.37 ± 11.50**	42.24 ± 7.24	30.14 ± 5.05**	27.62 ± 7.52**	22.42 ± 7.13
Stress	38.43 ± 13.31	31.48 ± 13.32	30.10 ± 11.39	36.63 ± 7.69	31.68 ± 9.47	31.69 ± 9.37	23.83 ± 5.49

Note:
** Suggests for P < 0.01 in comparison to the novel arm.

food/water. All animals were subjected for 10 days of adaptation period before being randomly assigned into either chronic stress group or control group (12 in each group). The control group animals were housed as 3 rats per cage, while the chronic stress group rats were housed alone, and subjected to chronic unpredictable mild stress (CUMS).

The study has been approved by the Ethic committee of Animal Experiments in Zhejiang Sci-tech University and all procedures followed the guidelines to animal experiments in Zhejiang Sci-tech University.

2.2. Procedures for CUMS

CUMS procedures included: food deprivation (24 h), water deprivation (24 h), tail pinch (1 min), food shock (1.0 mA for 10 s, 30 times with interval of 1 min), ice water swimming (4 °C, 5 min), wet bedding (24 h), and reversed light/dark cycle. The seven types of stress were randomly presented to animals with each per day.

2.3. Morris water maze

The water maze was in diameter 1.2 m and height 0.5 m, with a 10 cm-diameter hidden platform at 2 cm under the water surface. The maze is divided into four quadrants for data analyses. In the training phase, the animals were trained four times a day with 30 s interval each. The maximum time length of one training trial is 60 s. In the testing phase for reverse learning, the platform was placed in a different quadrant (quadrant 4) than the training trials (quadrant 2), and the swimming traces of the animals were recorded. The time latency to find the platform, the average distance to the platform during swimming phase, and the time spent in quadrant 4 were analyzed.

2.4. Y maze

Y maze is composed of three arms (start arm, novel arm, other arm) of 35 cm × 16 cm × 16 cm (length × width × height) with 120° between each arm. In the learning phase the animal was allowed to move freely in the start arm and the other arm for 10 min. Then the animals were placed back to home cage, and allowed to explore the three arms simultaneously after 90 min for 5 min. The differences of time spent in each arm were recorded and analyzed by Noldus software (Noldus co, Netherlands).

2.5. Immunohistochemistry

The animals were sacrificed and the brains were harvested for paraffin embedding, sectioning and immunostaining (or HE staining). Briefly, the sections were firstly incubated with primary antibody mice-anti Rat LC3 (Abcam, 1:200) overnight at 4°, then secondary antibody goat-anti-mice IgG conjugated for Envision kit (1:400, Boshide, Wuhan, China). The images were taken under a Zeiss fluorescence microscope and analyzed by Image-Pro plus software (Media cybernetics, US). For stereological analyses, six sections of hippocampus were chosen from each side (Bregma −3.3, −3.6, −3.9, −4.2, −4.5, −4.8 mm). The boundary of hippocampus was defined as previously described [14].

2.6. Statistics

The data were presented as mean ± SD, and analyzed with SPSS 13.0 software (Chicago, US). The differences between two groups were compared by independent sample t test. P < 0.05 was considered as statistically significant.

3. Results

3.1. Behavioral responses to CUMS

5 weeks of CUMS leads to behavioral changes in several tests that we performed. In open field test, the locomotor activities of CUMS animals were significantly lower than the control group (t22 = 4.192, P < 0.01), accompanying the decreases in times of central crosses (t22 = 4.959, P < 0.01) and the vertical exploration (t22 = 2.800, P < 0.05), which suggested for the depression-like behaviors (Table 1).

In the Y maze, the control group animals spent more time I novel arm than start arm (t22 = 6.456, P < 0.01) and the other arm (t22 = 5.414, P < 0.01); while CUMS animals showed no such preference. Similar results were found in the number of crosses in each arm. The control group animals exhibited increased number of crosses in novel arm,

Table 3
Water maze reverse learning results (M ± SD, n = 12).

		Control group	Stress group
Average distance to platform (cm)		34.16 ± 4.39	40.41 ± 5.97**
Time spent in each quadrant (%)	Quadrant 1 (%)	19.80 ± 3.55	21.60 ± 5.30
	Quadrant 2 (%)	19.87 ± 4.43	20.20 ± 4.96
	Quadrant 3 (%)	20.70 ± 4.58	23.38 ± 4.53
	Quadrant 4 (%)	29.63 ± 5.29	24.82 ± 4.73*
Latency to platform (s)	Trail 1	33.63 ± 22.48	50.50 ± 16.83*
	Trail 2	4.75 ± 2.52	21.18 ± 23.82*
	Trail 3	5.80 ± 3.70	11.60 ± 15.44
	Trail 4	7.97 ± 4.85	5.85 ± 3.53

Note:
** Suggests for P < 0.01, and * for P < 0.05 in comparison to the control group.

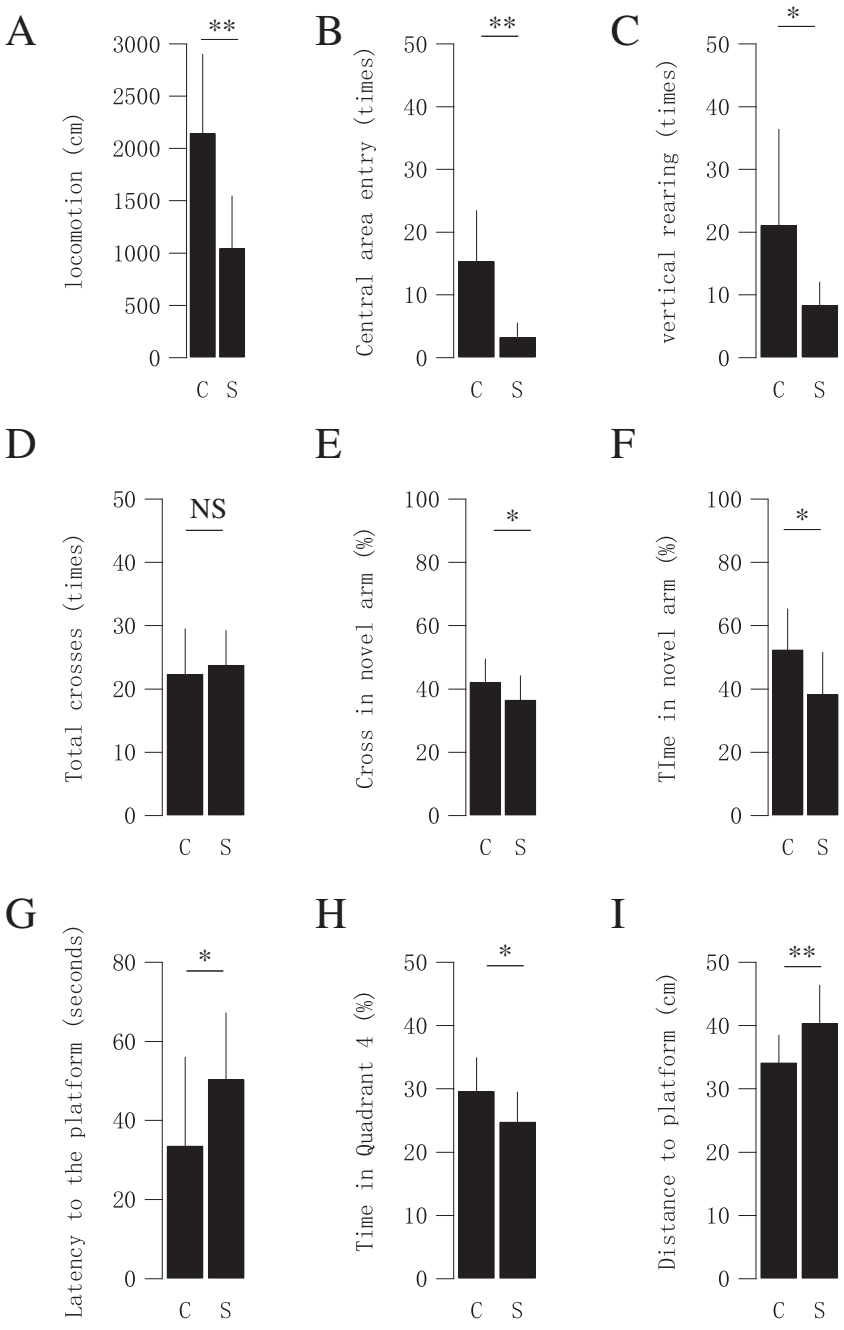

Fig. 1. CUMS leads to behavioral changes in rats, including open field test (OFT) (1A–C), Y Maze (1D–F), and reversal learning (1G–I) in Morris water maze. 1A: The locomotion in open field was decreased in CUMS group (S) in compared to control animals (C). 1B: The central entry in open field was decreased in CUMS group (S) in compared to control animals (C). 1C: The vertical exploratory activity in open field was decreased in CUMS group (S) in compared to control animals (C). 1D: The total locomotion in Y maze (after habituation) was similar in CUMS group (S) and control animals (C). 1E: The entry to novel arm was decreased in CUMS group (S) in compared to control animals (C). 1F: The percentage of time spent in novel arm was decreased in CUMS group (S) in compared to control animals (C). 1G: The latency to find the new platform (reversal learning) was increased in CUMS group (S) in compared to control animals (C). 1H: The time spent in quadrant 4 was decreased in CUMS group (S) in compared to control animals (C). 1I: The average distance of animal to the new platform (reversal learning) was increased in CUMS group (S) in compared to control animals (C). Note: NS for not significant; ** for $P < 0.01$; * for $P < 0.05$.

Table 4
Asymmetrical neuronal loss in CUMS animals revealed by number of neurons in hippocampus (n = 12).

Group	CA1		CA3		DG	
	Left	Right	Left	Right	Left	Right
Control	32.9 ± 2.8	32.8 ± 5.9	27.2 ± 8.0	29.1 ± 5.2	100.8 ± 24.2	102.5 ± 19.0
Stress	25.9 ± 3.8[a]	28.1 ± 4.4[a]	25.7 ± 4.3	22.5 ± 3.9[a,b]	94.6 ± 22.9	98.4 ± 25.7

Note:
[a] Suggests for $P < 0.01$ between control and stress group.
[b] Suggests for $P < 0.01$ between left and right side.

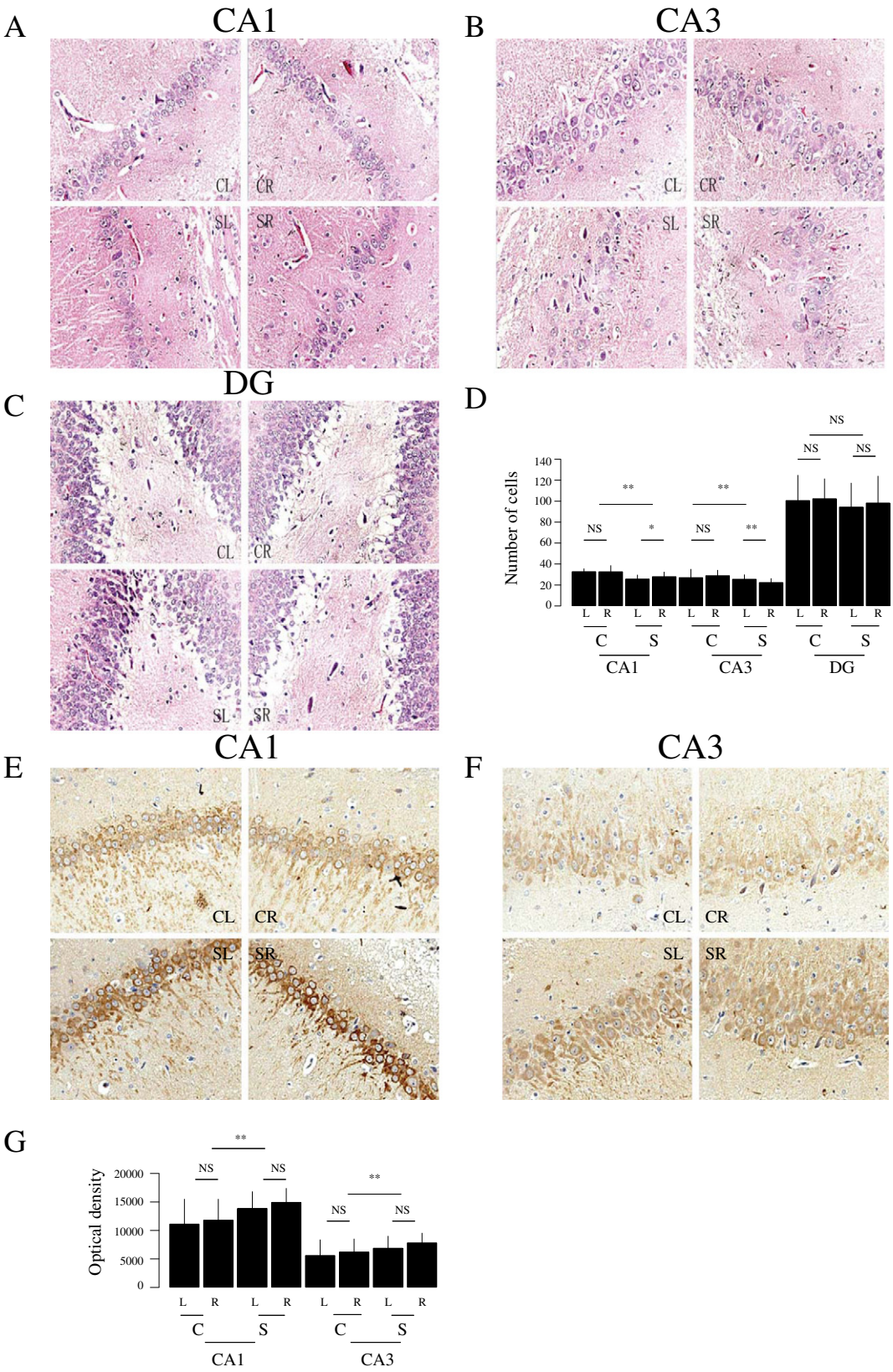

Fig. 2. CUMS results in asymmetrical hippocampal injury in CA1 and CA3 (2A–D), while autophagy signaling is symmetrically distributed (2E–G). 2A: Example pictures of histological examination of CA1 region from hippocampus in both control and CUMS animals (HE staining). 2B: Example pictures of histological examination of CA3 region from hippocampus in both control and CUMS animals (HE staining). 2C: Example pictures of histological examination of DG region from hippocampus in both control and CUMS animals (HE staining). 2D: Bar graph showing comparisons of neuron numbers in different groups. 2E: Example pictures of immunohistochemical examination of autophagy signaling at CA1 region from hippocampus in both control and CUMS animals (LC3 staining). 2F: Example pictures of immunohistochemical examination of autophagy signaling at CA3 region from hippocampus in both control and CUMS animals (LC3 staining). 2G: Bar graph showing comparisons of LC3 staining optic intensity in different groups. Note: NS for not significant; ** for $P < 0.01$; * for $P < 0.05$. CL for left side of control animal; CR for right side of control animal; SL for left side of CUMS animal; SR for right side of CUMS animal.

Table 5
Symmetrical distribution of autophagy signaling in CUMS animals revealed by LC3 optic density (n = 12).

Group	CA1		CA3	
	Left	Right	Left	Right
Control	11,159.7 ± 4366.9	11,852.6 ± 3674.4	5639.9 ± 2757.7	6264.5 ± 2281.7
Stress	13,914.5 ± 2935.8[a]	14,965.9 ± 2461.5[b]	6920.1 ± 2119.5[a]	7867.01 ± 1698.0[a]

Note:
[a] Suggests for $P < 0.01$.
[b] Suggests for $P < 0.05$ between control and stress group.

when compared to the start arm ($t22 = 4.750$, $P < 0.01$) and the other arm ($t22 = 4.853$, $P < 0.01$); however there was no such differences in CUMS animals (Table 2).

In the reversal learning of Morris water maze test, the CUMS group rats exhibited significantly decreased time spent in the new quadrant (quadrant 4) ($t22 = 2.351$, $P < 0.05$) as well as increased distances to the hidden platform ($t22 = 2.919$, $P < 0.01$), exhibiting deficits in reversal learning ability (Table 3).

3.2. Asymmetrical neuronal loss in CUMS animals

Histological examination of hippocampus sections revealed mild changes in soma size, cellular organization and cell numbers in both CA1 and CA3 sub-regions. In DG there were slight changes in the cellular morphology, but not in cell numbers.

For CA1 subregion, in CUMS group, the number of neurons decreased in both the left ($t58 = 8.088$, $P < 0.01$) and right sides ($t58 = 3550$, $P < 0.01$) significantly, when compared to the control group (Fig. 1).

In CA3 subregion, in CUMS group, the number of neurons decreased in the right side ($t58 = 5.509$, $P < 0.01$) when compared to the control group. Interestingly, the number of neurons in the right side CA3 is also significantly smaller to the left CA3 ($t58 = 3.025$, $P < 0.01$), suggesting for asymmetrical neuronal loss in CUMS (Table 4).

3.3. Symmetrical distribution of autophagy signaling in CUMS animals

Autophagy can contribute to the neuronal loss under CUMS. With immunohistochemical staining of LC3 on series of hippocampus sections, we quantified the expression level of LC3 in different brain regions by optic density. We found that in comparison to control group, the CUMS animals exhibited significantly increased expression of LC3 in both CA1 and CA3 regions ($P < 0.01$), suggesting that autophagy contributed to the neuronal injury following chronic stress (Fig. 2).

Interestingly, we did not observe significant difference of LC3 expression between left and right CA1/CA3 in CUMS animals ($P > 0.05$), suggesting that the autophagy distribution is symmetrical in both sides. Notably there is a slight trend of increased autophagy in the right side of CA1 region, in comparison to the left side, yet non-significant ($P = 0.06$) (Table 5).

4. Discussion

Chronic stress impairs brain function and general physiology in multiple manners [15], and may exacerbate aging processes [9–11,16,17]. In the present study, we found that chronic stress impairs Y maze performance and reversal learning in Morris maze, both of which are hippocampus-dependent tasks [15]. Notably, in novel environment (open field), the CUMS animals exhibited locomotion decrease; while the total activities in Y maze were not impaired — this is potentially due to the fact that the animals were allowed to be familiar with the maze in the first period. The results suggested that CUMS induced impairments in learning of novel spatial information [15], and dampens the brain plasticity that is critical for reversal spatial learning.

Hippocampal asymmetry has been known for many decades, and evidences were reported on patients with epilepsy, schizophrenia, and Alzheimer's disease, including both brain imaging and post-mortem histological studies [2]. Hippocampal asymmetry is also reported on healthy individuals and animals, yet with controversies. The present study did not find significant changes in cell numbers between left and right CA1 as well as CA3. However, chronic stress triggered clear asymmetry of bilateral hippocampus, potentially due to the differential levels of neuronal loss. The study is therefore, to our knowledge, the first report linking chronic stress to pathological hippocampus asymmetry. It will be interesting to extend the analyses into the proteomics level [3], in order to dissect if the left and right sides of the hippocampus show differential vulnerability to corticosterone increase, for example.

The mechanisms underlying chronic stress triggered neuronal loss remain elusive. We examined the extent of autophagy cell death by performing LC3 staining in CA1 and CA3 regions. We found significant increases in LC3 expression in CUMS group, in comparison to control groups. This would strongly argue for the participation of autophagy in neuronal loss in animals with chronic stress. However the autophagy protein expression was not asymmetrical bilaterally, implying that there might be additional mechanisms contributing to the pathological hippocampus asymmetry underlying chronic stress.

In conclusion, chronic stress impairs the hippocampus in an asymmetrical manner, which is not fully determined in the autophagy pathway.

Author contributions

GH, YZ, XY, and TY designed the experiment; YZ and XY performed the experiment; GH, YZ, XY, and TY analyzed the results and wrote the manuscript together; all authors have read and approved the submitted version of this manuscript.

Conflicts of interest

None declared.

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