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RESEARCH ARTICLE

Anatomical distribution of histone H3 acetylation in human hippocampus

Bingyue Cao[a], Fengling Luo[b], Wenwen Zhang[b], Renwei Luo[c], Lile Cai[d], Yongchang Wei[a], Yanqing Wang[e], Pan Liu[a,e*]

[a] Department of Radiation and Medical Oncology, Hubei Cancer Clinical Study Center & Hubei Key Laboratory of Tumor Biological Behaviors, Zhongnan Hospital of Wuhan University, Wuhan 430072, China

[b] Department of Immunology, School of Basic Medical Science, Wuhan University, Wuhan 430071, China

[c] Department of Thoracic Surgery, Renmin Hospital of Wuhan University, Wuhan, 430060, China.

[d] Department of Orthopedics, Renmin Hospital of Wuhan University, Wuhan, 430060, China.

[e] Department of radiation, Changzhi People's hospital, Changzhi, 046000, China.

***Corresponding Author's E-mail**: Pan Liu, liupanzn@whu.edu.cn

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Running title: Histone H3 acetylation in human hippocampus

Abstract

In recent years, the role of histone acetylation in the brain has been studied from different aspects. However, the anatomical distribution of histone H3 acetylation in the human hippocampus and its potential relevance to triple synaptic circuits are unknown. As one of epigenetic remodeling ways, the modification of histones is involved in multiple aspects of neuronal function and development and is a key process in the onset and development of Alzheimer's disease (AD). In our study, we compared acetylation levels of histone H3 at different regions of the hippocampus in AD and non-AD patients. We found that histone H3 acetylation can be detected in the dentate gyrus (DG), CA4, CA3, CA2, CA1, and lower Toya regions of the human hippocampus. The highest degree of acetylation in the hippocampus is in DG, and the level of acetylation changes gradually and systematically along the triple synaptic circuit. Besides, there were no significant differences in histone acetylation between AD and non-AD groups.

Keywords: hippocampus, histone H3, acetylation, anatomical distribution.

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Introduction

The hippocampus (HIP) is an elongated crescent-shaped neural structure located in the medial temporal lobe of the brain. HIP is a Greek-derived name for its resemblance to seahorses (1). The coronal section of hippocampus

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including DG, CA4, CA3, CA2, CA1 and subiculum usually occurs in research involved in HIP (1). There is complex anatomical connectivity in hippocampus, which underlies the basis for neuronal circuits. The classic triple synaptic loop has received much attention from scientists and its function as well as working mechanisms were explored in multidimensional ways (2). The cortical inputs from the entorhinal cortex (EC) project to DG through the perforant path, then DG manages the information and projects to CA3 through the mossy fiber pathway. CA3 projects to CA1 through the Schaffer Collateral pathway. Feedback from CA1 projects to EC directly or through subiculum, and the classic triple synaptic loop is completed.

The exact functional role of the hippocampus is still a topic of much debate. The prevailing view is that the dorsal (or posterior) hippocampus is involved in memory and spatial navigation, while the ventral (or anterior) hippocampus mediates behaviors related to anxiety (3). Its function for memory and learning is associated with the formation of long-term episodic memories and spatial navigation (4). Learning contributes to neuronal engrams formation and memories are the representation of engram reactivation (5). There is functional heterogeneity within individual neuronal ensembles (6). Thomas Hainmueller and Marlene Bartos found that DG provides stable code for downstream hippocampal regions which undergo constant reassignment for neuronal engram formation (2).

Alzheimer's disease (AD) is the most common form of neurodegenerative dementia. As the population ages, its prevalence will increase substantially in the coming years. At the same time, the field of AD research has also grown exponentially over the past few decades (7). Epigenetic remodeling is a key process in the pathogenesis and development of AD, including DNA methylation, chromatin remodeling, and histone post-translational modification, involving multiple aspects of neuronal function and development (8). Histone acetylation, one of the epigenetic modulators regulating DNA replication and gene transcription, plays an important role in chromatin structure and function regulation (9). Nuclear acetyl-CoA causes cognitive degradation in AD patients by acetylating the synaptic histone (10). Histone acetylation is typically associated with active chromatin, and the acetylation of lysine residues lysine 64 (H3K64ac) and lysine 122 (H3K122ac) in the spherical domain of histone H3 marks a subset of active gene promoters and active enhancers (11). A pair of histones H2A, H2B, H3 and H4 consist of the structural base for nucleosome, Acetylation of histones opens DNA double helix structure or activates promoter which promotes gene expression (9,12). According to the positive correlation of acetylation levels with transcription rates (13–15), we infer that histone acetylation levels in specific brain regions may represent its corresponding gene transcription levels.

The N-terminal tail of histones extends outward from the nucleosome core and serves as a target site for various histone modifications, including acetylation and methylation. Histone acetylation is widely perceived to enhance transcription activity within chromatin (16). Histone acetylation changes are considered to be associated with memory formation (17) and memory impairment (18). Histone acetylation also acts as an important epigenetic regulation tool to regulate neurogenesis and neural plasticity (19,20). Histone acetylation can be influenced by many factors. Adolescent intermittent ethanol exposure was found to increase hippocampal histone deacetylase activity and decrease histone H3-K9 acetylation levels in the hippocampus (21). The metabolite acetyl-CoA which

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can be produced from alcohol metabolism has a direct link with brain histone acetylation, and the histone acetylation level influences learning and memory-related transcriptional activities (22). In cancer, acetyl-CoA promotes histone acetylation and then promotes the expression of some oncogenes, thus affecting the growth, proliferation and metastasis of tumors (23). Histone acetylation is related to the loss of cell homeostasis and the change in tissue function, which leads to the process of aging (24). Carla and her colleague found that acute stress dynamically regulates histone-3-lysine-27-acetylation which impacts glutamatergic gene expression in hippocampus (25). Other impact factors such as environmental exposure and prenatal stress also regulate histone H3 acetylation levels in the frontal cortex and hippocampus (26).

The function of histone acetylation in the brain has been researched for many years from different aspects. The triple synaptic loop is associated with memory impairment. Corticosterone and estradiol can cause specific cell structural changes in the hippocampus of the triple synaptic loop, thus causing hormone-dependent memory impairment (27). However, the normal anatomical distribution of histone H3 acetylation in the human hippocampus and its potential correlation with the triple synaptic loop are still elusive. In our study, we detected histone H3 acetylation in human hippocampus sub-regions including DG, CA4, CA3, CA2, CA1 and subiculum. The highest acetylation part of hippocampus is DG, and the acetylation level changes gradually in an organized way along the triple synaptic loop pathway.

We hypothesized that the acetylation level in the pathway of a triple synaptic loop may represent different levels of transcriptional intension, the higher acetylation level means more transcriptional activities and information dealing, supporting more complex neural activities.

Methods

Brain tissues

Human postmortem brain tissues were obtained from the Brain Bank of the Chinese Academy of Medical Sciences & Peking Union Medical College. The left side hippocampus was freshly frozen in -80℃ fridge, while the right side was fixed in 10% formaldehyde and then paraffin-embedded for later immunohistochemistry. The pathological diagnosis of human brain samples was conducted by “ABC” score according to a protocol recommended by the National Institute on Aging–Alzheimer’s Association. Samples with “Intermediate” or ‘‘High’’ AD neuropathologic change and clinical dementia symptoms were classified as AD, and samples with “low” or “none” AD neuropathologic change and no clinical dementia symptom were classified as negative control (NC) group.

Immunohistochemistry

Human brain paraffin sections were cut by rotary microtome at a thickness of 5 μm. We put sections in 60 ℃ oven to dewax, then, go through xylene two times and a decreasing concentration gradient of ethanol to hydrate. Citric acid repair liquid was used to perform antigen repair in the microwave oven. Add 3% H_2O_2 to the slides to eliminate the endogenous peroxidase. To exclude the impacts of nonspecific antigens, 10% goat serum was added. The slides

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were then incubated with primary antibodies at 4℃ overnight in the wet box. Following the upper step, Slides were incubated with biotinylated goat anti-rabbit secondary antibody. Diaminobenzidine (DAB) was used to conduct immunostaining and the dyeing degree was monitored under a microscope. Nuclear was counterstained with haematoxylin and dehydration was operated with a serial increasing concentration gradient of ethanol before immersing the slides into xylene. At last, slides were sealed by neutral gum under cover glass and ready for observation.

Western blot

Brain tissues were lysed by ultrasonic cell disruptor in Radio Immunoprecipitation Assay Lysis buffer (RIPA). 15% polyacrylamide gels were prepared for protein separation. Proteins were transferred to polyvinylidene fluoride membrane and blocked with 5% nonfat milk. Primary antibodies were incubated overnight at 4 ℃. Followed by incubation with anti-rabbit secondary antibodies and Beyo-Enhanced chemiluminescence (ECL) Plus kit was used to reveal protein bands.

Statistical analysis

To measure the relative expression of H3 acetylation, we used Image J software to detect the optical density (OD) of the immunostaining samples. The relative expression of the western blot band is also measured by Image J software, and the differences were compared by unpaired student's t-test.

Result

Histone H3 is acetylated at specific molecular sites in the human hippocampus

The 7 samples from the Brain Bank of the Chinese Academy of Medical Sciences & Peking Union Medical College were valued and classified into NC group and AD group by "ABC" score. (Table 1)

Table 1. The "ABC" score according to a protocol recommended by the National Institute on Aging–Alzheimer's Association values for 7 postmortem tissue Samples. PTB041, PTB158, PTB117 and PTB040 were assessed low or no AD neuropathologic change and no clinical dementia symptoms were classified into the NC group, while PTB129, PTB114 and PTB078 were assessed high AD neuropathologic change and clinical dementia symptoms were classified into AD group.

Sample Code	Gender	Age	Postmortem Tissue Sampling		ABC Score
			Beginning Time	Ending Time	
PTB041	**M**	**80**	**7.5**	**8.5**	**None**
PTB158	**M**	**86**	**5**	**7.5**	**None**
PTB117	**F**	**81**	**4**	**7**	**Low**
PTB040	**F**	**85**	**7**	**8**	**Low**
PTB129	**M**	**83**	**4.5**	**6**	**High**
PTB114	**F**	**80**	**13**	**14.5**	**High**

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PTB078	**F**	**86**	**6.33**	**9**	**High**

We pre-analyzed the immunohistochemical results of histone H3 acetylation at lysine 9 (H3K9), histone H3 acetylation at lysine 18 (H3K18), histone H3 acetylation at lysine 23 (H3K23) and histone H3 acetylation at lysine 27 (H3K27). In our research, histone H3 immunohistochemical staining was strongly positive (Fig.1A). Histone H3 acetylation at H3K18, histone H3 acetylation at lysine 36 (H3K36) and histone H3 acetylation at lysine 56 (H3K56) were negative in the immunohistochemistry or can only be dyed with a small part and the background is too strong, which means these sites are not suitable for the observation of our immunohistochemistry study. (Fig.1B) H3K9, H3K18, H3K23, and H3K27 were positive, which means these acetylated sites can be selected for our research work. (Fig.2＆Fig.3)

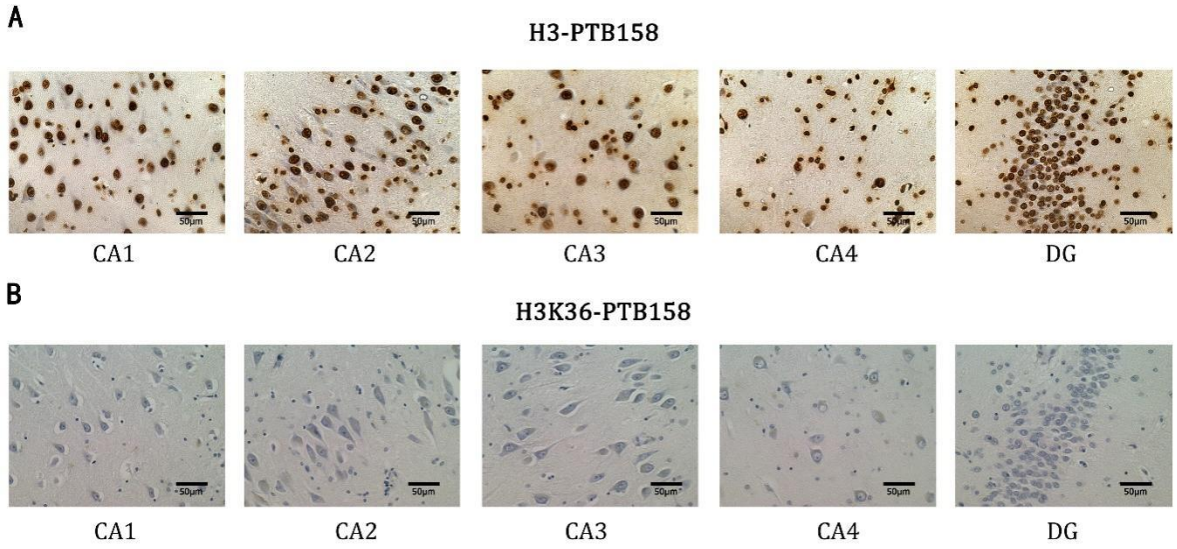

Fig.1. (A) Immunohistochemical staining of histone H3 in CA1, CA2, CA3, CA4 and DG of PTB158. (B)Immunohistochemical staining of histone H3 lysine36 in CA1, CA2, CA3, CA4 and DG of PTB158. All the pictures were taken at 100 × magnification.

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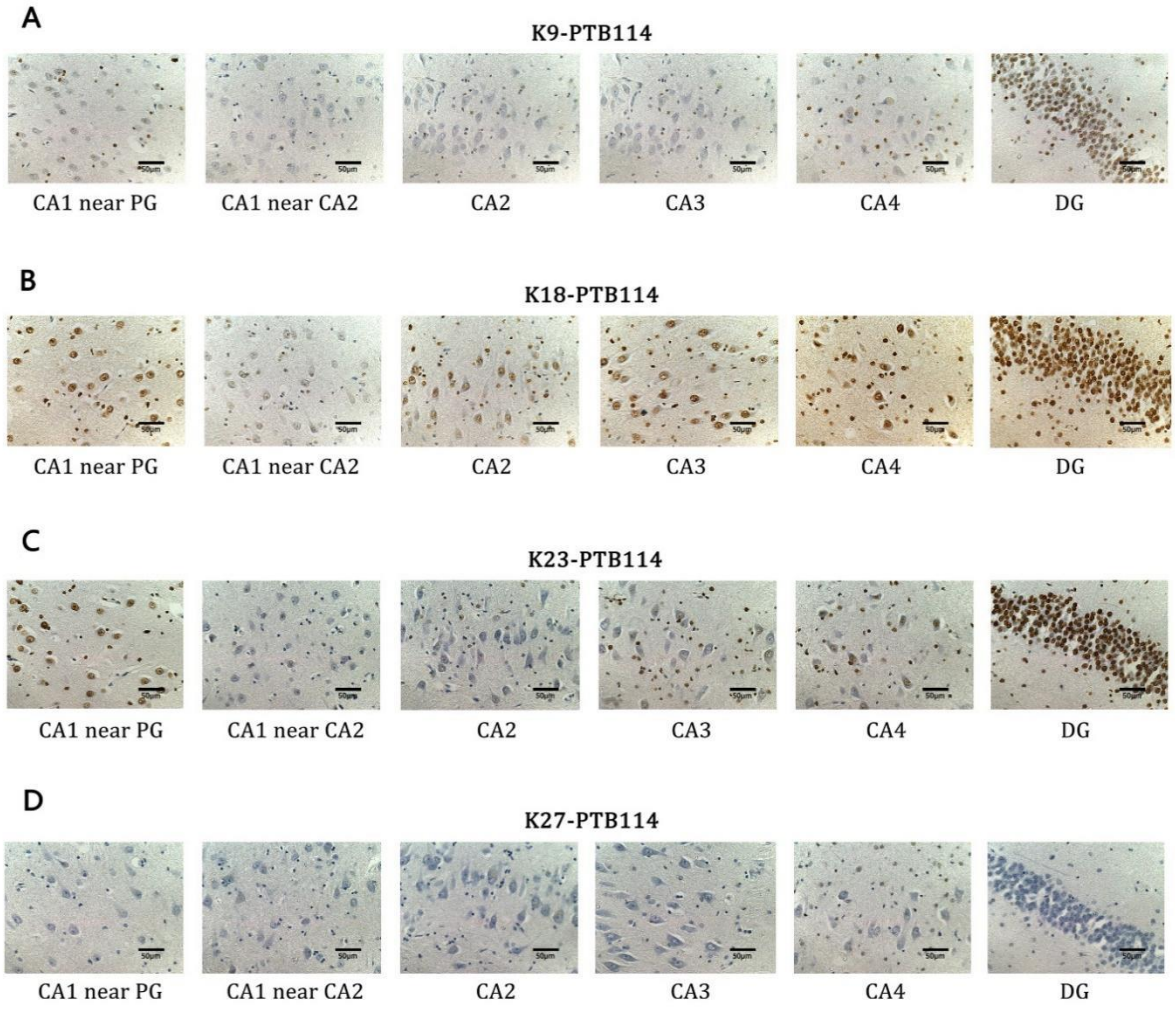

Fig.2. (A) The results of histone H3K9 immunohistochemical staining of postmortem brain tissues in different regions of the PTB114's hippocampus (CA1 near para hippocampal gyrus (PG), CA1 near CA2, CA2, CA3, CA4 and DG). (B) The results of histone H3K18 immunohistochemical staining of postmortem brain tissues in different regions of the PTB114's hippocampus (CA1 near PG, CA1 near CA2, CA2, CA3, CA4 and DG). (C) The results of histone H3K23 immunohistochemical staining of postmortem brain tissues in different regions of the PTB114's hippocampus (CA1 near PG, CA1 near CA2, CA2, CA3, CA4 and DG). (D) The results of histone H3K27 immunohistochemical staining of postmortem frozen brain tissues in different regions of the PTB11's hippocampus (CA1 near PG, CA1 near CA2, CA2, CA3, CA4 and DG). All the pictures were taken at 100 × magnification.

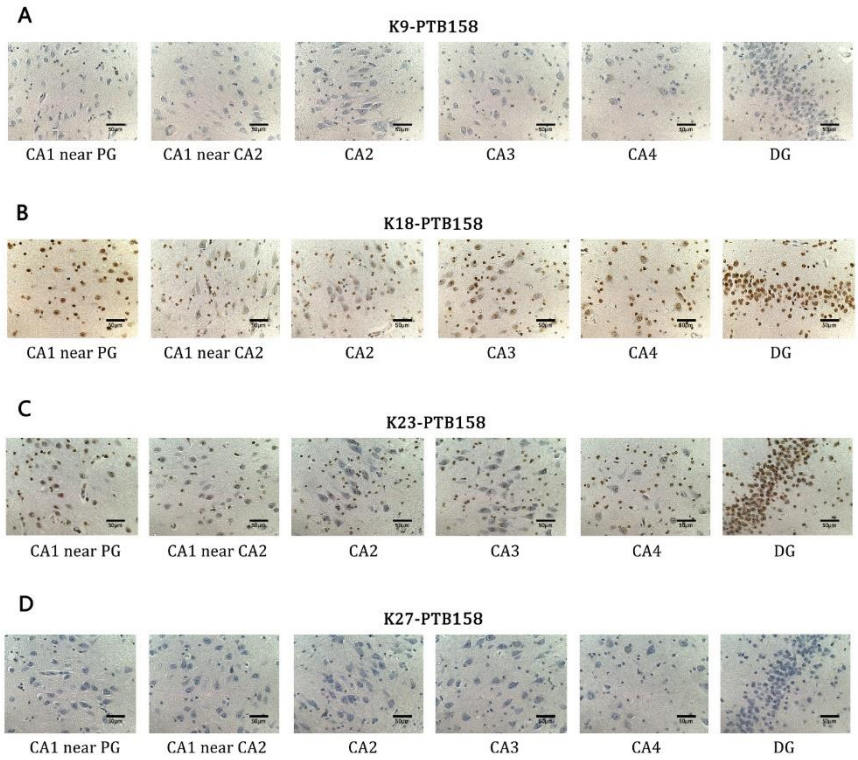

Fig.3. (A) The results of histone H3K9 immunohistochemical staining of postmortem brain tissues in different regions of the PTB158's hippocampus (CA1 near PG, CA1 near CA2, CA2, CA3, CA4 and DG). (B) The results of histone H3K18 immunohistochemical staining of postmortem brain tissues in different regions of the PTB158's hippocampus (CA1 near PG, CA1 near CA2, CA2, CA3, CA4 and DG). (C) The results of histone H3K23 immunohistochemical staining of postmortem frozen brain tissues in different regions of the PTB158's hippocampus (CA1 near PG, CA1 near CA2, CA2, CA3, CA4 and DG). (D) The results of histone H3K27 immunohistochemical staining of postmortem brain tissues in different regions of the PTB158's hippocampus (CA1 near PG, CA1 near CA2, CA2, CA3, CA4 and DG). All the pictures were taken at 100 × magnification.

The distributions of histone H3 acetylation in different sub-regions of hippocampus

The expression of histone H3 acetylation has anatomical differences in the human hippocampus. We evaluated the expression of H3K9, H3K18, H3K23, H3k27 in distinct anatomical areas of the human hippocampus by immunohistochemistry. In the human hippocampus, histone H3 lysine acetylation was at the highest level in DG and gradually decreased along CA4, CA3, CA2, and CA1. H3K9 and H3K18 were relatively expressed at the highest level in DG, and at a low level in CA4, CA3, CA2 and CA1, showing obvious differences. The expression level of H3K23 was highest in DG, gradually decreasing from CA4 to CA3, then to CA2, and finally to CA1.

H3K27 increased from DG to CA4 and decreased from CA4 to CA1, and its expression level was higher in DG and CA4 than in CA3, CA2 and CA1. (Fig.2; Fig.3; Fig.4)

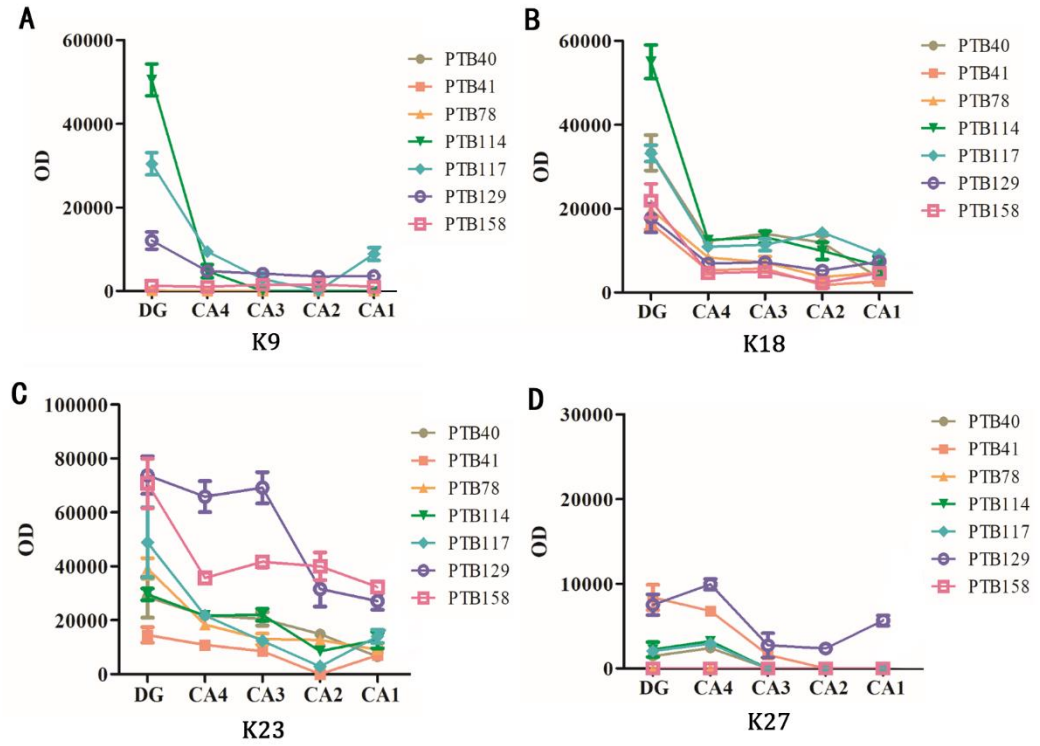

Fig 4. The results of immunohistochemical detection of different areas of human hippocampus in four different acetylation sites. The error bars represent mean ± standard error of the mean (SEM). (A) Expression level of H3K9 in different areas of human hippocampus was measured by immunohistochemistry. (B) Expression level of H3K18 in different areas of the human hippocampus was measured by immunohistochemistry. (C) Expression level of H3K23 in different areas of the human hippocampus was measured by immunohistochemistry. (D) The expression level of H3K27 in different areas of human hippocampus was measured by immunohistochemistry.

The distribution of histone H3 acetylation is consistent with a triple synaptic loop

It is well-known that there is a canonical triple synaptic pathway within the hippocampus, involving signal transducing from the EC to the DG, then to the CA3, and finally to the output node CA1 (28). (Fig.5) Further analysis of histone H3 acetylation expression revealed that the distribution of histone H3 acetylation decreased with triple synaptic loop, suggesting the distributions of histone H3 acetylation was consistent with triple synaptic loop. At the same time, we also found that histone H3 acetylation was unevenly distributed in CA1, and its expression was lower in CA1 near CA2 than in CA1 near PG. (Fig.5)

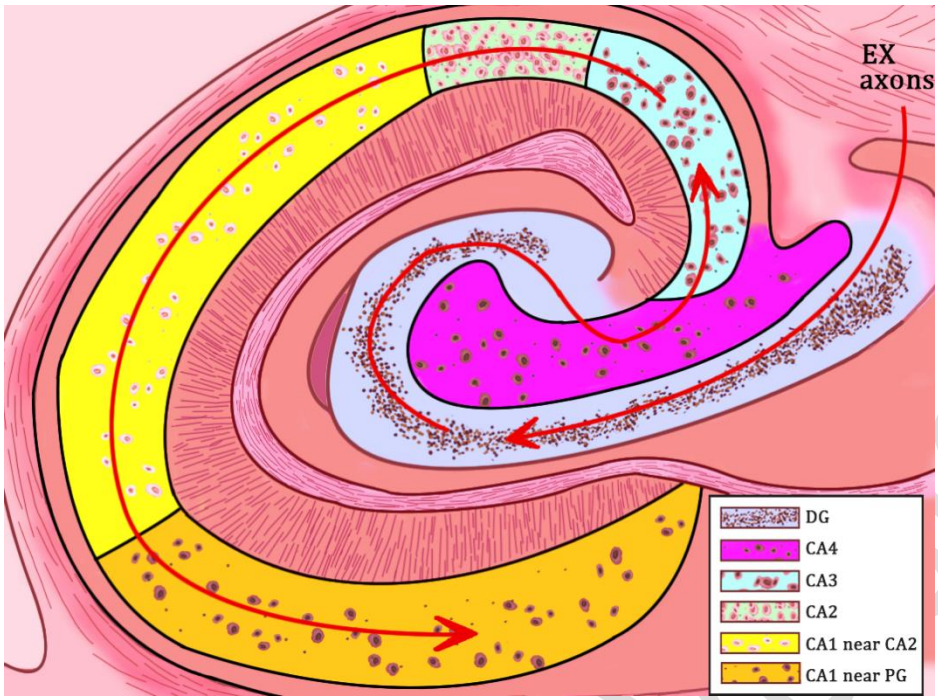

Fig.5. ***The distributions of histone H3 acetylation are consistent with a triple synaptic loop. The red lines depict the classic "triple synaptic loop", the arrow indicates the orientation of "triple synaptic loop". The highest acetylation part of hippocampus is DG. The least acetylation part of the hippocampus is CA1 near CA2. The acetylation level decreases gradually in an organized way along the triple synaptic loop pathway (from DG to CA4, CA3, CA2 and finally CA1 near CA2). Meanwhile, from CA1 near CA2 to CA1 near PG, the level of histone H3 acetylation rises.***

Suspicious difference in histone H3 acetylation between NC and AD hippocampus

We observed that there was little difference in the expression of histone H3 between the AD patients and the non-AD patients (Fig.6). However, there were seemingly some differences in acetylation levels at certain sites of the H3 protein between the AD patients and the non-AD patients. Through the western blot, we observed that the acetylation level of H3 protein in AD patients at K18 and k56 was significantly lower than that in non-AD patients, and the level of acetylation at K27 and K9 in AD patients was less than non-AD patients, and no obvious difference was discovered at K14, K23 and K36 sites (Fig.7). However, limited by the number of samples and individual differences, we could only assume by the trend of the results of western blot, and couldn't make a clear conclusion.

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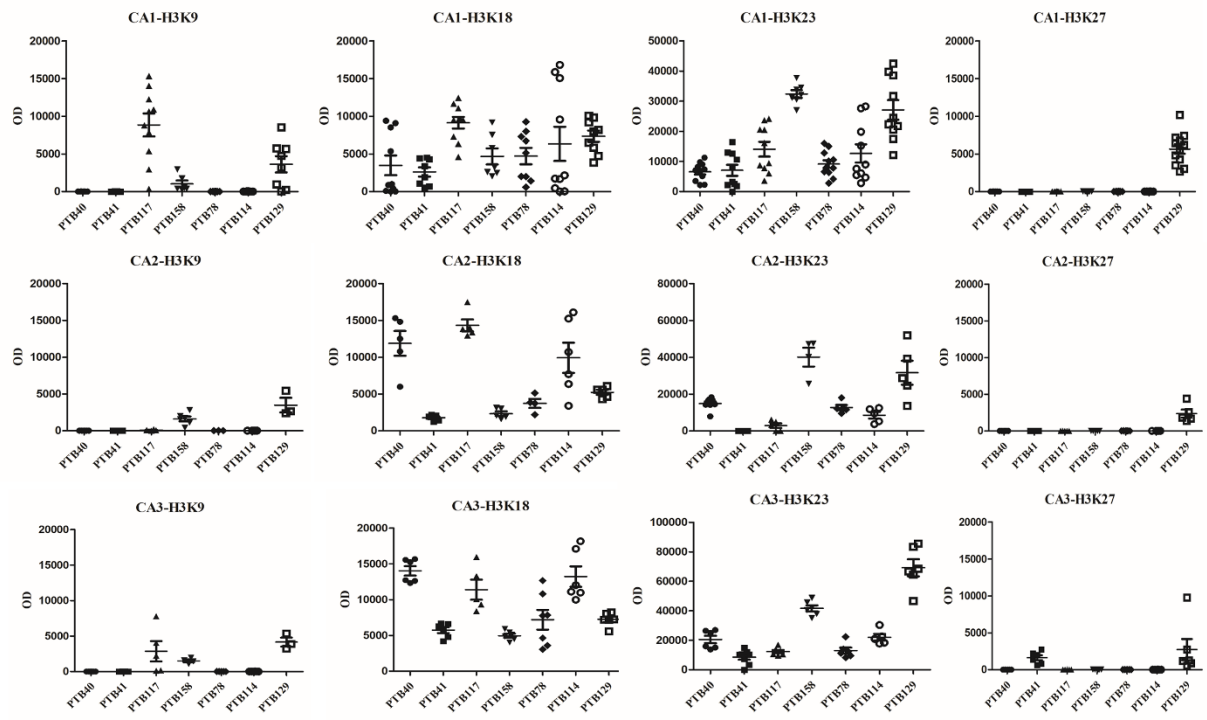

Fig.6. Immunohistochemical detection of histone H3 in seven different acetylation sites in different areas of human hippocampus. The error bars represent mean ± SEM.

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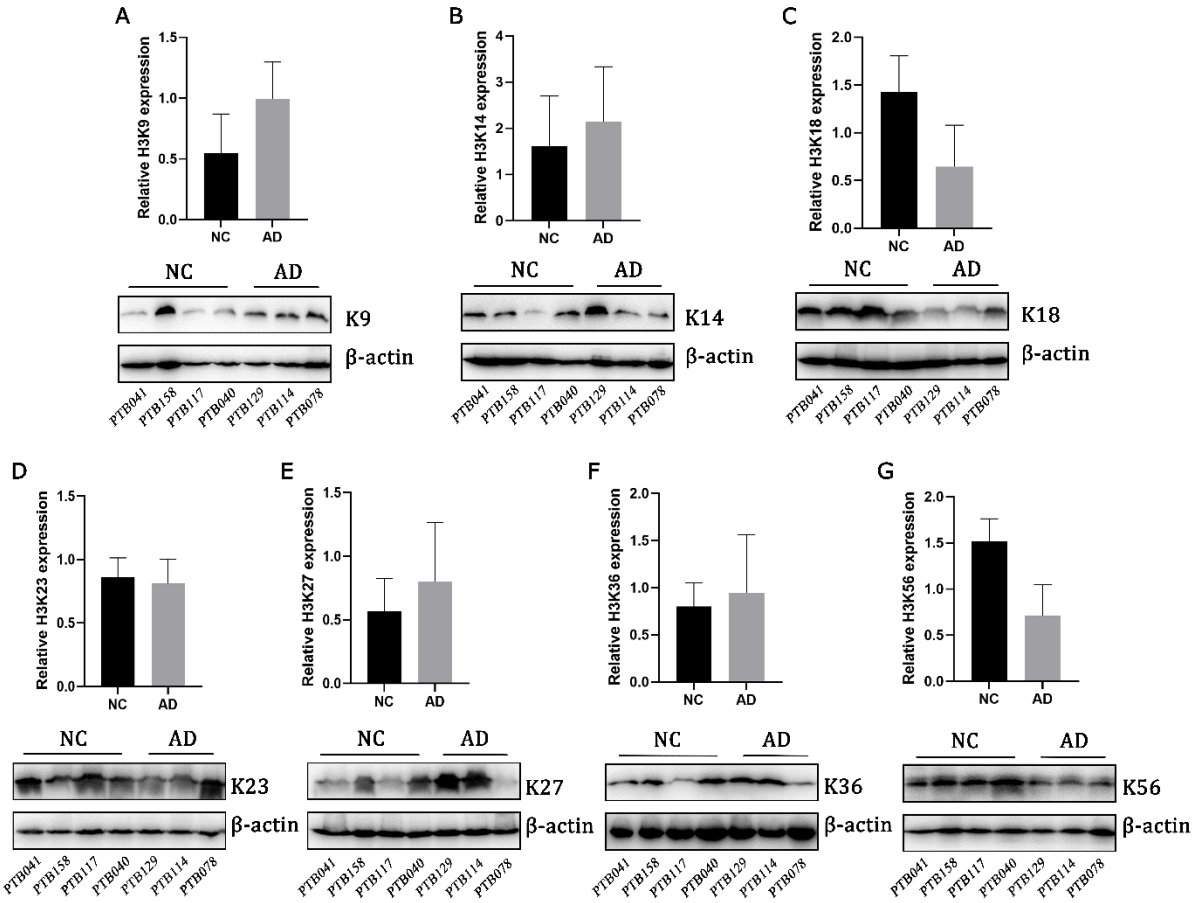

Fig.7. Expression of histone H3 in seven different acetylation sites between the NC group and AD group, including H3K9, H3K14, H3K18, H3K23, H3K27, H3K36 and H3K56. The error bars represent mean ± SEM. (A-G) From left to right are samples PTB041, PTB158, PTB117, PTB040, PTB129, PTB114 and PTB078, the first four samples are the NC group, and the last three samples are the AD group. The histogram is the relative amount of the gray value of the target protein and the internal reference protein.

Discussion

In this study, we uncovered the distribution of histone H3 acetylation in different areas of human hippocampus including DG, CA4, CA3, CA2, CA1 and subiculum. Our data shows that DG has the highest expression of histone H3 acetylation, and the acetylation level decreases gradually in an anatomical way along the classical pathway "triple synaptic loop". Our work suggests that the acetylation level is different in human hippocampus sub-regions, which may represent different levels of transcriptional intention. Besides, there seems no difference in histone H3 acetylation between NC and AD hippocampus. However, the results of the western blot show some suspicious differences in histone H3 acetylation at certain acetylated sites between the NC group and the AD group. However, due to the individualizations and limitations of the samples, as well as the possible degradation of proteins during

the post-mortem time, no further conclusions can be drawn.

Conclusion

The acetylation of histone H3 is anatomically different in the human hippocampal region, and its acetylation level gradually decreases along the classical pathway "triple synaptic ring", and the expression of histone H3 acetylation is the highest in DG. The difference in acetylation levels in DG, CA4, CA3, CA2, CA1 may represent different levels of transcriptional activities. In addition, there appeared to be no obvious difference in histone H3 acetylation between AD samples and non-AD samples.

Acknowledgments

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Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

List of Abbreviations

Full name	Abbreviation
Alzheimer's disease	**AD**
Negative control	**NC**
Parahippocampal gyrus	**PG**
Dentate gyrus	**DG**
Optical density	**OD**
Diaminobenzidine	**DAB**
Hippocampus	**HIP**
Cornu Ammonis	**CA**
Histone H3 lysine 9 acetylation	**H3K9**
Histone H3 lysine 14 acetylation	**H3K14**
Histone H3 lysine 18 acetylation	**H3K18**
Histone H3 lysine 23 acetylation	**H3K23**
Histone H3 lysine 27 acetylation	**H3K27**
Histone H3 lysine 36 acetylation	**H3K36**
Histone H3 lysine 56 acetylation	**H3K56**
Deoxyribo Nucleic Acid	**DNA**
Radio Immunoprecipitation Assay Lysis buffer	**RIPA**
Entorhinal cortex	**EC**
Enhanced chemiluminescence	**ECL**
Standard error of mean	**SEM**

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