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Association of Cdk5 and Soluble Oligomeric Species of β-amyloid Induced Tau Hyperphosphorylation

Shanshan Li[1#], Sumin Tian[2#], Lingzhi Sun[1], Zhihao Liang[1], Xiaohui Cheng[1], Han Wang[1], Yuxin Ma[1], Jing Liu[1], Guoying Li[1*], and Qing Mei Wang[3]

[1]*Department of Anatomy and Histology, School of Basic Medicine, Guangdong Pharmaceutical University, Guangzhou, Guangdong, China*
[2]*Department of Physiology, School of Basic Medicine, Guangdong Pharmaceutical University, Guangzhou, Guangdong, China*
[3]*Department of Physical Medicine and Rehabilitation, Spaulding Rehabilitation Hospital, Boston, MA, USA*
[#]*These authors contributed equally to this work.*

[*]**Corresponding author:** Dr. Guoying Li, Department of Anatomy and Histology, Basic Medical College, Guangdong Pharmaceutical University, Guangzhou, Guangdong, 510006, China, Tel: +86-020-39352232; Fax:+86-020-39352186; **E-mail:** gzlgying820@sina.com

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Abstract

Background, alzheimer's disease (AD) is a progressive neurodegenerative disease with deteriorating memory loss in the aged population. Currently, its exact pathogenesis remains elusive. Some studies have shown that soluble oligomeric Aβ (β-amyloid), inducing hyperphosphorylation of tau, may be the initial link to AD pathogenesis. However, it is poorly known how Aβ influences tau phosphorylation; Results, in this study, soluble oligomeric Aβ42 peptide was injected into the hippocampus of mice with saline as a control. Hematoxylin and eosin (HE) staining showed that Aβ42 was mainly deposited in the Cornu Ammonis area 1 (CA1). Within 7 to 21 days after the operation, the area of Aβ42 decreased gradually. Compared to the control, the expression of phosphorylated tau (p-tau) was significantly increased, suggesting that soluble Aβ oligomers activated phosphorylation of tau and increased total tau. Meanwhile, we found that elevated Cdk5, mainly in the CA1 area and subgranular zone (SGZ), correlates with increased phosphorylation of tau. Conclusions, thus, the results suggest that hyperphosphorylation of tau induced by soluble amyloid Aβ is associated with an increased level of Cdk5.

Keywords: Alzheimer's disease; β-amyloid; tau; Cdk5

Abbreviations

Aβ42: β-amyloid 42; Cdk5: Cyclin-dependent Kinase 5; DG: Dentate Gyrus; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; NS: Sterile Saline; HE: Hematoxylin and Eosin; NS: Sterile Saline

Background

AD is a progressive degenerative disease of the central nervous system in the aged or aging period [1-3]. The main pathologies of AD, mostly found in the hippocampus, cerebral cortex and subcortical tissue, are the accumulation of amyloid plaques outside the cells [4], neurofibrillary tangles in neurons [5], decreased synapses [6] and neuronal loss [7], etc.

Currently, the pathogenesis of AD has long been debated. Researchers have proposed numerous hypotheses, including cholinergic injury [8], Aβ neurotoxicity [9-14], hyperphosphorylation of tau [15-20], oxidative stress [21,22] and the mechanism of metal which induces oxidative neurotoxicity or amyloid aggregation [23,24]. Recent evidence suggests that AD was not attributed to a single causative factor, but due to a variety of concurrent effectors [25]. Furthermore, the combined effects of Aβ and tau might serve as a major etiological factor.

In the development of Aβ neurotoxicity, it was found that soluble oligomers of Aβ (i.e., amyloid beta derived diffusible ligands (ADDLs), nonfibrillar ligands derived from Aβ42) induced the strongest toxicity [26-29]. The presence of ADDLs leads to hyperphosphorylation of tau, and hyperphosphorylated tau then dissociates from the neural microtubules. As a result of the increased number of free tau, the stability of the cytoskeleton is disrupted. The abnormal change in the cytoskeleton generates large amounts of neurofibrillary tangles which are the other principal pathological change of AD. Moreover, Aβ is believed to catalyze hyperphosphorylation of tau through some intermediates like phosphorylated kinases [30, 31].

Tau, a microtubule-associated protein, mainly in axons, is located in the cell body in the early development of neurons, and then moves toward the axon after the maturation of neurons. Also, tau has the effect of stabilizing neurons. The association of neurofibrillary tangles (NFT) which is a major pathological feature of AD with tau is significant [32]. Recent clinical testing revealed that the total amount of tau and the content of p-tau in AD patients' blood and cerebrospinal fluid were substantially higher than those in patients with normal and non-neurological groups [33].

In addition, cyclin-dependent kinase 5 (Cdk5) can inhibit microtubule binding to tau which triggers tau hyperphosphorylation. Recent evidence has also confirmed that primary cultured hippocampal neurons co-incubated with Aβ stimulate tau phosphorylation and cause unusual activation of Cdk5, altering the distribution of tau and the levels of phosphorylated tau in neurons [34].

Above all, owing to the complicated pathogenesis of AD, this study will emphasize the effect of Aβ to tau phosphorylation. Researchers have revealed that Aβ plays a significant role in tau phosphorylation *in vitro*; however, it didn't directly describe *in vivo*. In this study, our data suggest that ADDL treatment induces hyperphosphorylation of tau via an increase of Cdk5.

Methods

Aβ preparation

Aβ42 (soluble Aβ oligomers) were purchased from AnaSpec (San Jose, CA). 500 μg Aβ42 was dissolved in 100 μl 1% NH_4OH to make a 5 μg/μl stock solution, which was placed in aliquots at -20°C. After adding NS, a working solution was diluted to a concentration of 2 μg/μl and incubated at 4°C for 24 hours, obtaining the oligomeric species of Aβ.

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Aβ intrahippocampal injection

One hundred nine-week-old male BALB/c-wild type mice (weight 20-28 g) were obtained from the Experimental Animal Center of Guangzhou University of Traditional Chinese Medicine. All animal procedures performed were approved by the Animal Ethics Review Committee and confirmed the guidelines for the Care and Use of Laboratory animals of Guangdong Pharmaceutical University. These mice should be bred in a Specific Pathogen Free (SPF) environment of separate ventilated cages.

The mice were randomly assigned to a normal group (n=20), a NS-injected group (n=40) and an Aβ42-injected group (n=40). For stereotactic injection, mice were anesthetized with 0.4% sodium pentobarbital in a dosage of 0.2 mL/10 g. All mice in the Aβ42-injected group were injected was into the CA1 area in the hippocampus bilaterally with 2 μl Aβ42 peptide (2 μg/μl) while mice in the NS-injected received 2 μl NS, and mice in the normal group did not receive any surgery or treatment. The coordinates from bregma used for the CA1 region were as follows: anteroposterior, -2.3 mm; mediolateral, ±1.8 mm; dorsoventral, -2.0 mm. The injection was performed by using a 25 ml Hamilton syringe driven by a minipump (KDS Model 310 Plus, KD Scientific, Holliston, MA) at 0.8 μl/min. After injection, the needle was kept in the area for another 2 min, and then slowly retreated to prevent spill. Following 7, 14 and 21 days post-injection, the mice was anesthetized and brains were dissected immediately. The right hemisphere was fixed in 4% paraformaldehyde for 48 hours for histological detection. The left hippocampus was snap-frozen in a pre-cooling vial in liquid nitrogen and finally stored at -80°C for western blot analysis and transcription polymerase chain reaction (RT-PCR) testing.

Histological staining

Paraffin-embedded tissue was sectioned on microtome and coronal slices with a thickness of 6 μm were collected. Serial sections (six sections per hippocampus) at an interval of 60 μm between -1.8 mm and -3.2 mm from the bregma were chosen. Mounted sections were washed with distilled water and stained with alum hematoxylin followed by differentiating with 0.3% acid alcohol. Then rinsed in distilled water, sections were stained with eosin for 2 min and dehydrated for mounting.

Immunohistochemical staining

After dewaxing with water, sections were incubated with 3% H_2O_2 at room temperature (RT) for 15 min, placed in a blocking solution (10% BSA in 0.3% Triton X-100/0.1M PBS pH 7.4) at RT for 30 min, and then binding to the unlabeled primary antibodies at 4°C overnight occurred. The following primary antibodies and working condition were used: rabbit polyclonal anti-Aβ42 (ab10148, Abcam, Cambridge, UK, 1:250), rabbit polyclonal anti-Cdk5 (ab151233, Abcam, Cambridge, UK, 1:100) for immunofluorescence, and rabbit polyclonal anti-p-tau (ab10891, Abcam, Cambridge, UK, 1:300) for immunofluorescence. On the second day, after washing three times, sections were incubated with secondary antibodies: biotinylated goat anti-rabbit (ab128978, Abcam, Cambridge, UK, 1:1000) at RT for 1 hour. Negative controls were incubated without primary antibody. Immunostaining was achieved by using the avidin-biotin complex (Vectastain: Vector Laboratories, Burlingame, CA) and diaminobenzidine plus Nickel (DAB Kit: Vector Laboratories) as the chromogen.

For density of Aβ42, Cdk5 and p-tau, randomly selected areas within the hippocampal CA1 and DG regions, were imaged using Olympus BX51 light microscopy (Olympus, Tokyo, Japan).

Western boltting analysis

Protein extraction from the hippocampus was separated by RIPA Lysis Buffer (Beyotime, Shanghai, China) (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail (sodium orthovanadate, sodium fluoride, EDTA, leupeptin), and 1 mM phenylmethylsulfonyl fluoride). Quantitative analysis of the protein was determined with a BCA protein assay kit (Beyotime, Shanghai, China). The protein samples were separated on 12% SDS-PAGE gels, and electronically transferred to a PVDF membrane at 0.8 mA/cm^2 for 1.5 hours. Then, the membrane was rinsed with 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.4) with 0.05% Tween20(TBS-T) for 1 hour at RT, and incubated with primary antibodies: rabbit polyclonal to anti-p-tau(sc-101813, Santa Cruz Biotechnology, 1:500), rabbit polyclonal anti-Cdk5 (sc-173, Santa Cruz Biotechnology, 1:500) and mouse monoclonal anti β-Actin (Santa Cruz Biotechnology, 1: 1000) at 4°C overnight. After being washed with TBS-T, the membrane was incubated with HRP-conjugated IgG (13688-1-AP, Proteintech, Chicago, IL, 1:1000) for 1 hour at RT, and washed with TBS-T again. Blots developed with enhanced chemiluminescence (ECL, Thermo Scientific, Waltham, MA, USA). Analysis of densitometric quantitation was performed with Image J Software (NIH, Bethesda, MD, USA) and standardized with β-Actin. The above samples were run in triplicate.

RT-PCR analysis

Tissue extracts were harvested and rinsed with phosphate-buffered saline (PBS) at corresponding time points and total RNA in the treated sections was extracted according to the total RNA extracting kit (AM1830 , Life Technologies). A solution was added consisting of 2 μL (5 mmol/μL) dNTP, 1 μL oligo(dT), 1 μL (200 U/μL) reverse transcriptase (m-mulv), pH 8.3 RT buffer (250 mmol/L Tris-HCl, 250 mmol/L KCl, 20 mmol/L $MgCl_2$, 50 mmol DTT) and deionized water. The total sample volume was 20 μL. Samples were incubated at 42°C for 1 hour and the reaction was stopped by heating at 70°C for 10 min. Reverse transcriptase was used to synthesize the first strand cDNA from an equal amount of the RNA sample following the manufacturer's instructions. About 40 cycles of the PCR reaction were used to cover the linear range of the PCR amplification. The band densities were scanned with a densitometer (Bio-Rad, United States).

Statistical analysis

Results were analyzed with SPSS 16.0 software (IBM, Armork, NY, USA), with significance at $P < 0.05$. Quantitative data were documented as the mean ± SD. Fisher's exact tests were performed on categorical data to verify the difference of degenerating neurons, Aβ aggregation, tau hyperphosphorylation and Cdk5 expressions between Aβ42-injected and NS-injected group, respectively. When comparing Aβ42-injected and NS-injected group, a two-tailed t-test was conducted for protein levels of tau and Cdk5 in the hippocampus. The Kruskal-wallis one-way test was used to test tau mRNA level among to the normal group, the NS-injected group and the Aβ42-injected group.

Results

Aβ42-injection caused neuronal degeneration

To determine whether Aβ42 are sufficient to trigger hippocampal neurodegeneration, we analyzed histological alternations of morphological structure using HE staining after treatment with Aβ42 after 7 days. In the normal group (Figure 1A), HE staining showed that the neurons were in alignment (Figure 1B). In the sterile saline (NS)-injected group (Figure 1C), the surgery-induced injury was limited to the zone of the needle track (Figure 1D). However, neurodegeneration was significantly shown in the area where Aβ42 was injected (Figure 1E). In addition, Aβ42-injected animals have degenerating neurons in the Cornu Ammonis area 2 (CA2) (Figure 1F). This result demonstrated that Aβ42 triggered neurotoxicity of neurodegeneration in hippocampal pyramidal neurons.

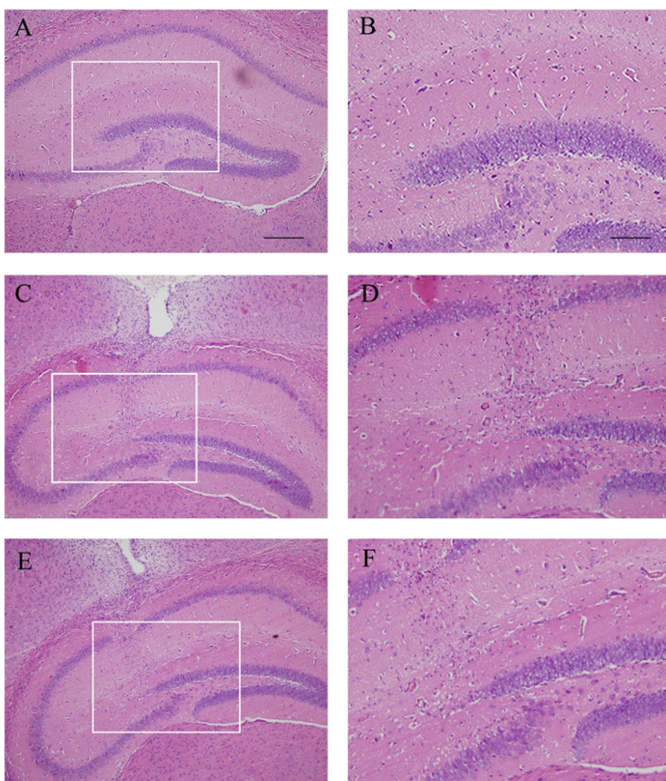

Figure 1: Histological changes in the hippocampus. (*A*) HE staining in normal hippocampus of BALB/c mice. (*C*) HE staining in NS-injected hippocampus. (*E*) HE staining in Aβ42-injected hippocampus. (*B, D, F*), respectively, represent the region of high magnification in (*A, C, E*). (*F*) HE staining showed neuronal loss at the injection site surrounding Aβ peptide. Scale bar=200 μm in (*A, C, E*) and 50 μm in (*B, D, F*).

Deposition of soluble Aβ oligomers in the hippocampus

After injecting Aβ42 into the hippocampus, Aβ42 were deposited and metabolized. The level of Aβ42 in the hippocampus was measured using anti-Aβ antibody. When observing the expression of Aβ for 7 days, 14 days and 21 days, we found that Aβ accumulation was much higher on day 7 than on the other days (data not shown). Therefore, the mice after Aβ injection for 7 days were selected. In the NS-injected group (Figure 2A), Aβ expression was barely observed at the injection site (Figure 2B). In contrast, Aβ expression was significantly expressed in SGZ of dentate gyrus (DG), which showed in approximately 94% of 40 Aβ-injected animals (Figures 2C and 2D) versus none of 40 NS-injected animals (P<0.0001, Fisher's exact test).

Aβ42-injection induced tau hyperphosphorylation

Our results above showed that Aβ42 primarily deposited in DG. We asked whether injecting Aβ42 into the hippocampus enhanced tau phosphorylation. We assessed the level of phosphorylated tau after injected with Aβ42 (Figure 3D) using immunofluorescence. Compared to NS-injection (Figures 3A-3C), tau hyperphosphorylation was observed in 35 of the 40 Aβ42-injected animals in both CA1 (Figure 3E) and DG (Figure 3F) versus 8 of the 40 NS-injected animals (P<0.0001, Fisher's exact test). Thus, Aβ42 accelerated hyperphosphorylation of tau.

Aβ42-injection activated Cdk5 when inducing tau hyperphosphorylation

Cdk5, a member of the cyclin-dependent protein kinase (CDK) family, can lead to neurological dysfunction and trigger neurodegenerative diseases when it is abnormal. Also, increased Cdk5 may hasten tau

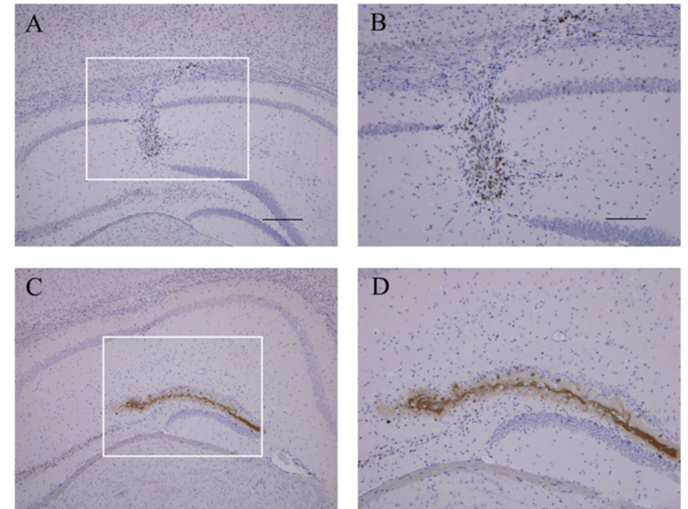

Figure 2: Immunostaining of Aβ in the hippocampus. (A) NS-injected group. (*B, D*), respectively, represent the region of high magnification in (*A, C*). (B) Higher magnification of negative Aβ aggregation at the injection site from NS-injected hippocampus. (C) Aβ42-injected group. (D) Aβ aggregation in SGZ of DG from Aβ42-injected hippocampus. Scale bar=200 μm in (A, C) and 100 μm in (B, D).

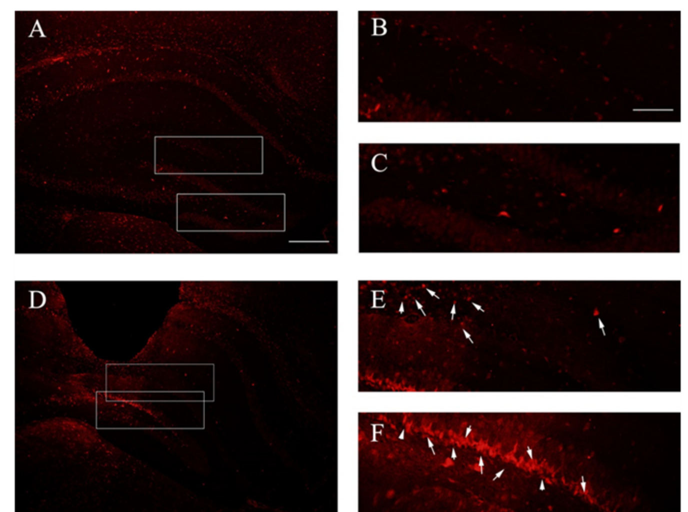

Figure 3: Immunofluorescence for expression of hyperphosphorylated tau in hippocampus. (*A*) Tau staining in control group (BALB / c + NS). (*B, C*) represent the region of high magnification in (*A*). (*B*) Negative tau expression located in CA1. (*C*) Negative tau expression located in DG. (*D*) Tau staining in Aβ42-injected group (BALB / c + Aβ42). (*E, F*), respectively, represent the region of high magnification in (*D*). (*E*) p-tau located in CA1. (*F*) p-tau located in DG. Scale bar=100 μm in (*A, D*) and 50 μm in (*B, C, E, F*).

phosphorylation [35]. We tested Cdk5 expression in primary cortical neurons within Aβ42-injected within Aβ42-injected and NS-injected groups in the hippocampus by immunofluorescence (Figure 4). We observed Cdk5 in 32 of 40 (P<0.05, Fisher's exact test) Aβ42-injected animals (Figure 4D) both in the CA1 (Figure 4E) and DG (Figure 4F), respectively, versus 8 of 40 NS-injected animals (Figures 4A-4C, P <0.0001, Fisher's exact test). In previous studies, they indicated that Cdk5 kinase activity in phosphorylating tau dramatically increased in the presence of p25 compared to p35 *in vitro* [36]. And the enhancement of Cdk5/p25 triggered tau hyperphosphorylation [37]. It can be speculated that Aβ42 may promote tau phosphorylation by activating Cdk5, which results in hyperphosphorylation of tau in AD pathology.

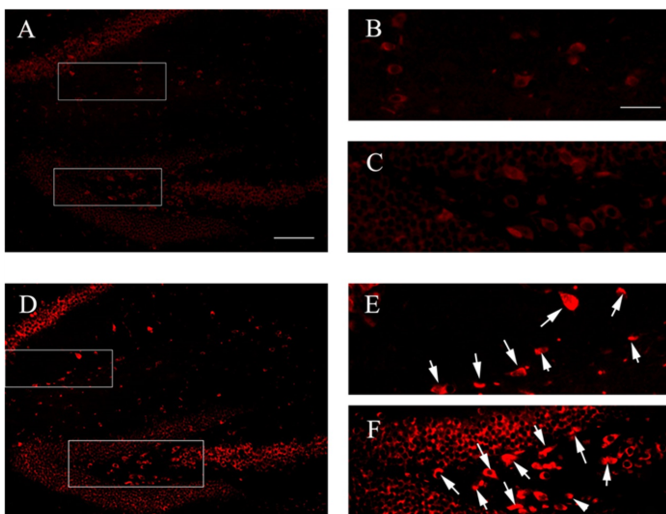

Figure 4: Immunofluorescence for expression of Cdk5 in the hippocampus. (*A*) Cdk5 staining in control group (BALB / c + NS). (*B, C*) represent the region of high magnification in (*A*). (*B*) Negative Cdk5 expression located in CA1. (*C*) Negative Cdk5 expression located in DG. (*D*) Cdk5 staining in Aβ42-injected group (BALB / c + Aβ42). (*E, F*), respectively, represent the region of high magnification in (*D*). (*E*) Cdk5 located in CA1. (*F*) Cdk5 located in DG. Scale bar=100 μm in (*A, D*) and 50 μm in (*B, C, E, F*).

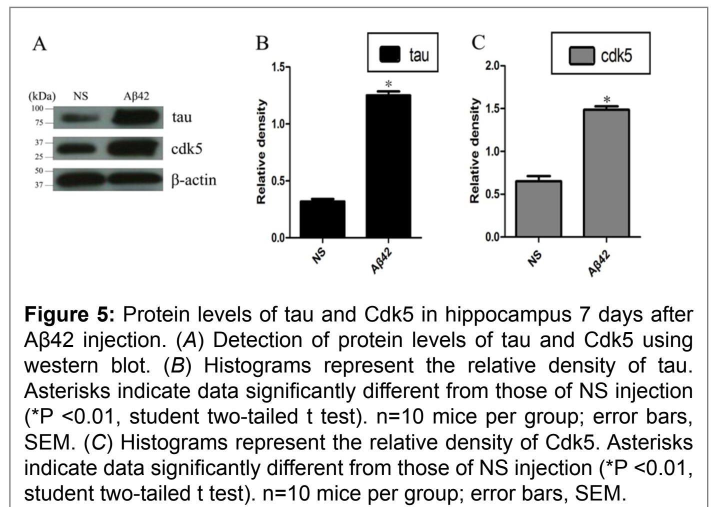

Figure 5: Protein levels of tau and Cdk5 in hippocampus 7 days after Aβ42 injection. (*A*) Detection of protein levels of tau and Cdk5 using western blot. (*B*) Histograms represent the relative density of tau. Asterisks indicate data significantly different from those of NS injection (*P <0.01, student two-tailed t test). n=10 mice per group; error bars, SEM. (*C*) Histograms represent the relative density of Cdk5. Asterisks indicate data significantly different from those of NS injection (*P <0.01, student two-tailed t test). n=10 mice per group; error bars, SEM.

Sample	CT value of Tau	CT value of GAPDH	ΔCT	ΔΔCT	Relative Quantity ($2^{-\Delta\Delta CT}$)
Control	16.91	6.14	10.77	0	1
NS-injection	16.99	6.15	10.84	0.07	0.952637998
Aβ42-injection	20.37	6.05	14.32	3.45	0.091505355*

*P < 0.05 compared to control group, Kruskal-Wallis test

Table 1: The cycle threshold values (CT) of real-time Q-RT-PCR

Aβ42-injection in hippocampus showed increase of Cdk5 and hyperphosphorylation of tau

To further confirm the mechanism of whether Aβ may activate the enzyme of phosphorylated tau-Cdk5, which led to hyperphosphorylation of tau, we assessed both the phosphorylation state of tau proteins (p-tau) and the level of Cdk5 in the hippocampus, which were assayed by quantitative western blotting with epitope-specific antibodies after being cultured for 7 days (Figure 5A). The analysis of gray value in western blot displayed a ~3.9-fold higher in p-tau protein in the hippocampus 7 days after Aβ42-injection (Figure 5B), indicating that an increased p-tau level might be related to Aβ42-induced neurodegeneration. Meanwhile, we found ~2.3-fold difference of Cdk5 enzyme between the Aβ42-injection and NS-injection (Figure 5C). Thus, Cdk5 catalyzed tau hyperphosphorylation, consistent with a positive role in AD.

Total protein tau in Q-RT-PCR validation

Hyperphosphorylation of tau in neurons results in tau dissociating from neural microtubules and becoming free tau, which increases the amount of total protein tau in neurons. To test and verify whether total value of tau was regulated by the Aβ oligomeric, the melt and amplification plots were carried out via Q-RT-PCR. Tau mRNA 7 days after Aβ42 injection was significantly higher than that of the control and saline-injected groups (P<0.05, Kruskal-Wallis test).

The delta cycle threshold (CT) value of tau from Aβ42-injection was 10.32, while that of the control and saline-injected groups was about 11 (Table 1, P<0.05, Kruskal-Wallis test). The results of relative quantity among three groups, suggesting that expression of tau 7 days after Aβ42 injection significantly increased, while the others were not statistically different (P<0.05, Kruskal-Wallis test).

Discussion

Finding the specific relationship between Aβ and tau, in this study, further enriches AD etiology. Here, using synthetic oligomer Aβ42 injected into mouse hippocampi, we observed this single factor was sufficient to cause neurodegeneration in the hippocampal cortex and induce hyperphosphorylation of tau in the CA1 and SGZ of DG. Meanwhile, we showed that Cdk5 was activated by Aβ42, suggesting that this new target spot might intervene in the occurrence of AD.

Given that Aβ promoting tau hyperphosphorylation has long been proposed, one is unable to specify which direct role of Aβ is responsible for this process, as free-state, oligomeric peptides or amyloid plaques. Moreover, some studies have performed that oligomeric Aβ has a higher toxicity and is an important factor in the process of AD [23,24,38,39]. Also, any AD animal models show cognitive impairment, especially AD transgenic mice, those with different genetic backgrounds, mutable sites of tau, and a variety of different features of amyloid deposition or abnormal behaviors, are likely to lead to inconsistencies in neurogenesis. For example, some study found out the expression of Aβ42 was aggravated in the brain tissues of neonatal AD transgenic mice using sevoflurane anesthesia compared to neonatal naive mice. Therefore, our study selected BALB / c-wild type male mice as experimental subjects [40]. After an injection of Aβ42 into mice, tau moves toward the dendrites, resulting in some changes in the physiological characteristics of neurons. Tau in the dendrites of neurons can inhibit the transport of organelles, reduce the supply of ATP, and induce synaptic joint recession. Compared to normal neurons, the processus spinosus of neurons injected with Aβ42 decreased by approximately 75%. In the meantime, owing to the redistribution of tau, the number of neural microtubules also declines significantly. Therefore, Aβ42 affect neuronal function by inducing the redistribution of tau in dendrites, which induces neuronal apoptosis [41,42].

Furthermore, under normal circumstances, only 2-3 phosphate groups are in modification of tau, so tau can keep a balance between phosphorylation and dephosphorylation, which regulates the cytoskeleton. Conversely, linking to 9-10 phosphate groups, the co-expression of Aβ42 and tau leads to enhanced hyperphosphorylation of tau, and then the aggregation of tau leads to the formation of neurotrophic factors (NFTs) in pathological situations. Owing to loss of its biological function, hyperphosphorylated tau is insufficient to promote microtubule

accumulation and maintain a stable cytoskeleton. After dissociating from the microtubules, hyperphosphorylated tau competitively binds to the normal tau, resulting in increased free tau but reduced normal physiological functioning of tau [43,44]. It was confirmed that this form of Aβ induced tau hyperphosphorylation in hippocampal neurons, and then caused rupture of the microtubule cytoskeleton as well as neuron degeneration [45]. Therefore, we hypothesized that soluble oligomeric forms of Aβ may play the principal role in triggering cytoskeletal changes as well as neuronal toxicity.

Our observation on the expression of Aβ42 effects on tau hyperphosphorylation has relevance to increasing activity of Cdk5. To our knowledge, the presence of non-cyclin protein p35 within the cerebral cortex is a Cdk5 activator, but the limitations of its distribution restrict activation of Cdk5 [35]. When subjected to Aβ42 stimulation, p35 is cleaved into p25 and the other fragment toward calpain activity. p25, whose half-life is 5-10 times longer than p35, completely activates Cdk5. p25-Cdk5 complex lacks the membrane-anchoring signal motif after cleavage, leading to its change of position within the cell [46-48]. On one hand, Cdk5 can directly trigger phosphorylation of tau protein [49], but on the other, Cdk5 can play a role in tau by regulating the relevant kinase or phosphatase [50]. Therefore, Cdk5 has a large effect on abnormal phosphorylation of tau related to the occurrence and development of AD.

We still found several limitations in this study. The primary limitation was the inability to prove whether Aβ triggered p-tau or p-tau triggered Aβ through the reduction to absurdity. We could not evaluate the change of Cdk5 or p-tau when it was lack of Aβ. Further limitation was different behavior types of analysis; however, the data was obtained prospectively.

Taken together, our study confirms that Cdk5 is involved in the disease process of neurodegenerative diseases, whose long overexpression can result in cell death. Aβ induced Cdk5 activity increased by p25, which further triggers the hyperphosphorylation of tau, leading to neuronal degeneration and cell death [51,52]. Recent studies have proven the developmental and metabolic disorder of Aβ as well as accelerated tau hyperphosphorylation, in addition to significantly increasing expression level of Cdk5, in the pathogenesis of AD [53]. Rather, such associations among the above three are firmly suggestive.

Conclusions

In this study, we demonstrated that Aβ, a source of AD, whose metabolic abnormalities lead to excessive accumulation of toxic soluble oligomeric forms of Aβ in the brain, is likely to contribute to the profound influence in pathological features of AD, by raising Cdk5 expression and tau hyperphosphorylation.

Acknowledgments

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Authors' contributions

Ms. Shanshan Li takes charge in the preparation of animal models and the experiments of neuronal histochemistry. Ms. Sumin Tian is charge of western blot and PCR. Ms. Lingzhi Sun assists Ms. Shanshan Li and Ms. Sumin Tian in all the experiments. Mr. Zhihao Liang, Ms. Xiaohui Cheng and Mr. Han Wang give assistance for preparation of animal models and postopreative care for animals, Ms.Yuxin Ma and Dr. Jing Liu provide guidance on immunohischemistry, fluorescence chemistry and etc. Dr. Guoying Li is responsible for the design and implementation of this subject. Dr. Qing Mei Wang gives advice to the design.

Competing Interests

All authors declare that they have no competing interests.

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