Alterations of myelin morphology and oligodendrocyte development in early stage of Alzheimer's disease mouse model

Yu Wu^{1#}, Yuanlin Ma^{1#}, Zhuo Liu^{1, 2#}, Qi Geng¹, Zexin Chen¹, Yan Zhang^{1*}

1. State Key Laboratory of Membrane Biology, College of Life Sciences, PKU-IDG/McGovern Institute for Brain Research, Peking University, Beijing, 100871, China
2. College of Forensic Science, People's Public Security University of China, Beijing, China

*Corresponding author: Yan Zhang, Ph.D.

[#]These authors contributed equally.

Mailing Address:

College of Life Sciences, Room 239

Peking University

Beijing, 100871, China

Tel: 86-10-62754880

Fax: 86-10-62751526

E-mail: yanzhang@pku.edu.cn

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Y.W., Y.M., Z.L., Q.G and Z.C. performed all the experiments and analyzed the data. Y.Z. conceptualized the study, performed analyses and drafted the manuscript.

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Abstract

Alzheimer's disease (AD) is the most common cause to dementia and predicted to influence about 35 million people by the end of 2050. In this study, we discover alterations of myelin morphology in hippocampus tissues of 2-month-old APP/PS1 mouse. Myelin sheath is thicker and internodal distance is shorter in APP/PS1 mouse. Oligodendrocytes, differentiated from oligodendrocytes progenitor cells (OPCs), are responsible for formation and maintenance of myelin sheath in central nervous system (CNS). Our current results demonstrate that the oligodendrocytes development is disordered in 2-month-old APP/PS1 mouse. Neuregulin-1 type III, which is critical for both oligodendrocytes development and CNS myelination, is found up-regulated in hippocampus tissues of APP/PS1 mouse by western blots. Furthermore, we find active-caspase-6 can cleave neuregulin-1 type III at the cytoplasmic region. Given together, this study indicates the alterations of myelin morphology and oligodendrocytes development in 2-month-old APP/PS1 mouse, which might be highly associated with neuregulin-1 type III and active-caspase-6.

1. Introduction

Alzheimer's disease (AD), the most common cause to dementia in people aged over 65 years old, is predicted to influence about 35 million people by the end of 2050 [12]. Previous studies have demonstrated that compared with age-matched controls, white matter areas of AD patients are atrophied by using magnetic resonance imaging (MRI). White matters atrophy is also known as the other kind of pathological feature for AD besides amyloid plaques and neurofibrillary tangles [11, 14, 16].

In central nervous system (CNS), mature oligodendrocytes differentiated from oligodendrocytes progenitor cells (OPCs) are responsible for the formation of myelin sheath [17]. Increasing evidences have proven the role of oligodendrocytes in the cellular phase of Alzheimer's disease [3, 7]. Myelin, providing discontinuous insulation along axons, is a critical player in saltatory impulse conduction and very important for physiological function [13]. In human, myelin development starts from

the very beginning of infant and will last to the second decade of life [2, 8]. Given the critical role of myelin, disruption of myelin developments may lead to inefficient interneuronal communication [8].

Neuregulin-1 type III, encoded by *NRG1*, is well known for the necessary role in peripheral nervous system (PNS) myelination. Previous studies have shown that neuregulin-1 type III is non-essential, but do play an important role in CNS myelination [4, 15]. Oligodendrocytes are responsible for CNS myelin formation and maintenance, and the developments of oligodendrocytes is tightly linked to neuregulin-1 from axons [1, 5, 10]. The former study has reported that plenty of hypermyelinated axons were found in the white matter of the mouse overexpressing cDNAs encoding neuregulin-1 type III, which suggests that neuregulin-1 type III might be associated with CNS myelination with unclear mechanisms [4].

The APP/PS1 mice is well known as the Alzheimer's disease mouse model and does not show behavior defects until at the age of 6 months. Myelin disruptions and demyelinations are found in 6-month-old and older APP/PS1 mouse, which is quite different with the phenotypes found in 2-month-old APP/PS1 mouse. Our present results indicate the alterations of myelin morphology and oligodendrocytes developments in hippocampus tissues of 2-month-old APP/PS1 mouse before occurrence of behavior defects and most of other pathological features. According to furthermore results, we propose the critical contributions of neuregulin-1 type III and active-caspase-6 to the alterations in APP/PS1 mouse.

2. Materials and Methods

2.1 Mice

APP/PS1 (APP^{swe} / PS1^{ΔE9}) mice were purchased from Model Animal Research Center of Nanjing University and maintained in compliance with guidelines of Peking University Animal Care and Use. Genotyping was followed the methods described by Jackson Labs. All animal experiments were performed under the guidance of Peking University Animal Care and Use Committee.

2.2 Immunofluorescence

To fix the brain tissues, both WT and APP/PS1 mice were anaesthetized and perfused with 4% paraformaldehyde (PFA) diluted in phosphate buffered saline (PBS). 35 μ m thick brain slides generated by routine methods were blocked with 5% albumin from bovine serum (BSA/PBS) and incubated with primary antibodies. Cells cultures were fixed with 4% PFA/PBS. After blocked with 5% BSA/PBS, cells cultures were incubated with primary antibodies. The following primary antibodies were used: Caspr (75-001, NeuroMab, 1:1000), MBP (40390, Abcam, 1:1000), Caspase-6 (BS7006, Bioworld, 1:100). After washed with PBS, brain slides and cells cultures were incubated with secondary antibodies for 1 h at RT. Secondary antibodies conjugated with Alex-Fluor-Dye were all purchased from Life Technology and diluted at 1:500. DAPI (Sigma) was used to stain the nuclei. We processed three pairs of APP/PS1 mice and WT controls and analyzed at least 103 myelinated axons from ten brain slices from each group.

2.3 Immunoblots

Hippocampus tissues were identified and then homogenized and extracted in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100) with proteinase inhibitor (Roche) and phosphatase inhibitor (Roche). After denatured at 100 °C for 5 min, proteins were separated by 10% or 12% SDS-PAGE. Following primary antibodies were used to incubate with membranes: neuregulin-1 type III (BS1173, Bioworld, 1:500), Mbp (7349, Abcam, 1:2000), NG2 (5320, Millipore, 1:2000), CNPase (BS3461, Bioworld, 1:1000), GAPDH (BE0034, EASYBIO, 1:5000). Secondary antibodies conjugated with HRP were all purchased from EASYBIO and diluted at 1:5000. HRP were then detected by Chemiluminescent.

2.4 Electron microscopy

Mice were anaesthetized and perfused with 4% PFA and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Hippocampus tissues were identified from both WT and APP/PS1 mice and post-fixed with 2% OsO₄ for 2 h. 4% uranyl acetate was used to stain membranes. After dehydrated by a graded series of alcohol, corpus callosum was infiltrated in propylene oxide and Spurr's resin and then embedded in Spurr's resin. Ultrastructure images were obtained by transmission electron microscope (Tecnai G2 20 Twin, FEI) and analyzed by Image J. We measured at least 40 myelinated axons from each group (n=3).

2.5 In vitro Cleavage assay

Cytoplasmic region of neuregulin-1 type III (shown as FL) was expressed and purified as described before [6]. FL neuregulin-1 type III was then diluted to 0.5 $\mu\text{g}/\mu\text{l}$ by the same buffer used in desalting column (20 mM Tris-HCl pH 7.5 and 200 mM NaCl) and incubated with 5 ng/ μl active-caspase-6 at 37 °C. Every single minute, part of protein solutions was taken out and denatured at 100 °C for 5 min until 1 h was passed. Protein samples with different incubation time were analyzed by 15% SDS-PAGE.

2.6 Statistical analysis

Data were analyzed by GraphPad Prism 6 and paired two-tailed student's *t* tests were used to declare statistical significance.

3. Results

3.1 Alterations of myelin morphology in hippocampus tissues of 2-month-old APP/PS1 mouse

Myelin basic protein (MBP), a major constituent of mature oligodendrocytes and myelin sheath, was found up-regulated in hippocampus tissues of APP/PS1 mice at the age of 2-month-old compared with age-matched WT mice (Fig. 1A). To investigate whether higher level of MBP in APP/PS1 mice contributed to abnormal myelination, we applied transmission electron microscope to study the ultrastructure of myelin sheath. We found that the ultrastructure of axon-myelin units was regular, but thickness of myelin sheath was much thicker in hippocampus tissues of APP/PS1 mice when compared with age-matched WT mice, which was caused by additional membrane wraps (Fig. 1B). Diameters of axons (d_{axon}) and fibers (axon and myelin) (d_{fiber}) were quantified and the g ratio ($d_{\text{axon}}/d_{\text{fiber}}$) was introduced to indicate relative thickness of myelin sheath. Different from WT (n=41) whose average g ratio was 0.84, APP/PS1 (n=45) were hypermyelinated with an average g ratio of 0.75 ($p<0.001$) (Fig. 1C). Not only thickness of myelin, but also internodal length, the distance between consecutive nodes in APP/PS1 mice were quite different from WT. 35 μm brain slices of WT and APP/PS1 mice were immunostained with anti-Caspr (paranodes marker) and anti-MBP (myelin sheath marker), so that the internodal distance could be labeled

and quantified (Fig. 1D). Compared with WT (n=103), internodal lengths in APP/PS1 mice (n=127) decreased dramatically (Fig. 1E).

3.2 Alterations of oligodendrocytes development in hippocampus tissues of 2-month-old APP/PS1 mouse

Mature oligodendrocytes, which could wrap axons to form myelin, were differentiated from oligodendrocytes progenitor cells (OPCs). Chondroitin sulfate proteoglycan (NG2) was expressed in OPC specifically and always used as marker of OPCs. 2', 3'-Cyclic nucleotide-3'-phosphodiesterase (CNPase) was applied to label pre-oligodendrocytes which was differentiated from OPCs and would further differentiated into mature oligodendrocytes. Our results indicated that NG2 and CNPase were both up-regulated in hippocampus tissues of 2-month-old APP/PS1 mice compared with their WT littermate (Fig. 2). Based on the results, we assumed that oligodendrocytes development in 2-month-old APP/PS1 mouse might be disordered.

3.3 Upregulation of neuregulin-1 type III in APP/PS1 mouse

Neuregulin-1 type III was well known for the essential role in PNS myelination, but not that necessary in CNS myelination. Previous studies had reported that overexpression of neuregulin-1 type III induced hypermyelination in CNS with unknown mechanisms [4]. In this study, hippocampus tissues were identified from 2-month-old WT and APP/PS1 mice and then treated with lysis buffer to extract proteins. Western blots analysis of the protein lysates demonstrated a strikingly higher level of neuregulin-1 type III in APP/PS1 mice (Fig. 3A). Analysis results of bioinformatics showed that caspase-6 cleavage site was conserved throughout six kinds of neuregulin-1 isoforms (data not shown). To verify neuregulin-1 type III could be cleaved by caspase-6, in vitro cleavage assays were performed. Cytoplasmic region of neuregulin-1 type III (shown as FL), expressed and purified as discussed before [6], was diluted and incubated with purified active caspase-6 at 37 °C. Incubation was lasted for 60 min, and within this time some of the protein solutions was taken out and denatured at 100 °C to stop the reaction every single minute. The proteins solutions with different incubation time were analyzed by 15% SDS-PAGE. Cytoplasmic region of neuregulin-1 type III (shown as FL) was decreasing with time passing by and finally disappeared at the end of incubation, while N-terminal

fragments was increasing (Fig. 3B). The results of in vitro cleavage assays demonstrated that caspase-6 could cleave neuregulin-1 type III into two fragments. Furthermore, we explored locations of caspase-6 and neuregulin1 type III in HEK 293 cells. The cDNA encoding neuregulin-1 type III tagged with GFP was transfected into HEK 293 cells. By using confocal microscope, we found neuregulin-1 type III (green) was localized at cell membrane. HEK 293 cells over-expressed neuregulin-1 type III were immunostained with an anti-active-caspase-6 antibody or anti-pro-caspase-6 antibody. Confocal microscope images showed that active caspase-6 (red) was co-localized with neuregulin-1 type III at the cell membrane, however pro-caspase-6 (red) were widespread in cytoplasm (Fig. 3C). Based on the results of in vitro cleavage assays and in vivo immunocytochemistry assays, we could assume that neuregulin-1 type III could be cleaved by active caspase-6. According to previous studies, neuregulin-1 type III could be cleaved by β -site of amyloid precursor protein cleaving enzyme 1 (BACE 1) [10] and BACE 1 was known to play a key role in CNS myelination [9]. To investigate whether the alteration of BACE 1 contributed to the hypermyelination in 2-month-old APP/PS1 mouse, we detected the level of BACE 1 in APP/PS1 mouse and the WT littermate. BACE 1 level showed no significant differences between APP/PS1 and WT mouse by western blots (Fig. 3D), which indicated that BACE 1 might be not responsible for the hypermyelinaion.

4. Discussion

In the present study, we found alterations of myelin morphology in hippocampus tissues of 2-month-old APP/PS1 mouse. Myelin sheath was thicker and internodal distance was shorter in APP/PS1 mouse compared with age-matched WT mouse (Fig. 1). In central nervous system, mature oligodendrocytes were responsible for myelin formation and maintenance. Our results indicated upregulations of NG2 and CNPase, specifically expressed in oligodendrocytes lineage cells, which suggested that in APP/PS1 mouse, the developments of oligodendrocytes might be disordered (Fig. 2). Neuregulin-1 type III, playing an important role in oligodendrocytes developments and myelination, was found up-regulated in 2-month-old APP/PS1 mouse (Fig. 3A). We also found active-caspase-6 could regulate level of neuregulin-1 type III by cleaving cytoplasmic region of neuregulin-1 type III (Fig. 3B-C). Given together, we discovered alterations of myelin morphology and oligodendrocytes development in 2-month-old APP/PS1 mouse, and proposed that the alteration might be tightly linked to

neuregulin-1 type III and active-caspase-6 with unclear mechanism. The previous study showed that there were no obvious developmental abnormalities in conditional *Nrg1* null mice [4], based on which we speculated that down-regulated the level of neuregulin-1 type III by inhibitor or siRNA might not rescue the abnormalities in APP/PS1 mouse. We need to do further study in future in order to figure out the mechanisms.

In central nervous system, myelin sheath was formed by membrane of oligodendrocytes and important for the conduction of action potential along the axons. Both thickness and length of myelin sheath were critical factors to evaluate myelination processes and physiological functions [13]. Thicker myelin sheath and shorter internodal lengths might lead to disordered conduction of saltatory impulse in early stage (2-month-old) APP/PS1 mouse, and then further contributed to abnormality of cognition function.

Neuregulin-1 type III from axons was reported to be a critical regulator of oligodendrocytes development and myelin formation [1, 10], but how neuregulin-1 type III regulated the processes remained unclear yet. Our results demonstrated level of neuregulin-1 type III was increased in APP/PS1 mouse. Enlighten by former study [4], we proposed that up-regulated neuregulin-1 type III could be tightly linked to the hypermyelination found in hippocampus tissues of 2-month-old APP/PS1 mouse. Active-caspase-6 could cleave neuregulin-1 type III at the cytoplasmic region and further regulate the level of neuregulin-1 type III. In our study, we detected levels of caspase-6 in 2-month-old APP/PS1 mice and their WT littermates. According to the Western blots results (data not shown), we found that neither the level of full-length caspase-6 nor the level of active-caspase-6 was altered in APP/PS1 mice. We assumed that the level of active-caspase-6 could be changed locally in APP/PS1 mouse without affecting the total level. And there was also a possibility that less active-caspase-6 was recruited to cleave neuregulin-1 type III, which leads to elevation of neuregulin-1 type III. In future study, we need to figure out the unclear mechanisms.

White matters were disrupted in the brains of AD patients, which was proved by plenty of postmortem studies. In this study, we explored the myelin morphology and

oligodendrocytes developments in 2-month-old APP/PS1 mouse, while the mouse did not show significant defects on memory and other behaviors.

Given together, Alzheimer's disease was well known as a kind of aging-related disease, however our results indicated that developmental disorder of myelination could be found in early stage of APP/PS1 mouse, which proposed a possible association between myelination development and pathogenesis of AD.

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Figure legends

Figure 1. Myelin morphology was altered in hippocampus tissues of 2-month-old APP/PS1 mice. (A) Western blots analysis of protein extracts prepared from hippocampus tissues of 2-month-old WT and APP/PS1 mouse (top). MBP level was up-regulated in APP/PS1 mouse compared with WT control (bottom). Data represent mean $\pm$ SE, ** $p < 0.01$. (B) Transmission electron microscopy identified hypermyelinated axons with regular axon-myelin units in hippocampus tissues of 2-month-old APP/PS1 mouse. Scale bar: 200 nm. (C) The g ratio ($d_{\text{axon}} / d_{\text{fiber}}$), as a function of relative myelin sheath, was dramatically decreased in APP/PS1 mouse as compared with WT. Data represent mean $\pm$ SE (n=41 for WT group, n=45 for APP/PS1 group), $p < 0.001$ compared with WT. (D) 35 μm brain slides were immunostained with anti-MBP and anti-Caspr, and representative confocal images were shown. Scale bar: 20 μm . (E) Quantification revealed a dramatically reduction of internodal lengths (distances between consecutive nodes) in APP/PS1 groups. Data represent mean $\pm$ SE (n=103 for WT group, n=127 for APP/PS1 group), *** $p < 0.001$.

Figure 2. Oligodendrocytes development was disordered in 2-month-old APP/PS1 mouse. Western blots analysis of protein extracts prepared from hippocampus tissues of 2-month-old WT and APP/PS1 mouse. NG2 was used as specific marker for OPC and CNPase for pre-oligodendrocytes. Quantification indicated a dramatically increase of NG2 and CNPase in 2-month-old APP/PS1 mouse. Data represent mean $\pm$ SE, * $p < 0.05$.

Figure 3. Neuregulin-1 type III was up-regulated in hippocampus tissues of 2-month-old APP/PS1 mouse and under the regulation of active-caspase-6. (A) Western blots analysis of protein extracts prepared from hippocampus tissues of 2-month-old WT and APP/PS1 mouse (top). Quantification indicated a significantly increase of neuregulin-1 type III in APP/PS1 mouse (bottom). Data represent mean $\pm$ SE, * $p < 0.05$. (B) Purified cytoplasmic region of neuregulin-1 type III (shown as FL) was diluted and incubated with purified active-caspase-6 at 37 °C. Every single minute, part of mixed protein solutions was taken out and denatured at 100 °C for 5

min until 60 min was passed. The proteins solutions with different incubation time were further analyzed by 15% SDS-PAGE. Level of FL neuregulin-1 type III was decreasing over time and finally disappeared at the end of incubation, while level of N-terminal fragments was increasing with time passing by. (C) HEK 293 cells, transfected with cDNA encoding neuregulin-1 type III tagged with GFP, were immunostained with anti-active-caspase-6 antibody or anti-pro-caspase-6 antibody. Confocal microscope images showed that active-caspase-6 (red) was co-localized with neuregulin-1 type III at the cell membrane (top), however pro-caspase-6 (red) were widespread in cytoplasm (bottom). (D) Western blots analysis of protein extracts prepared from hippocampus tissues of 2-month-old WT and APP/PS1 mouse (top). Quantification indicated that levels of BACE 1 were similar in APP/PS1 and WT mice.

Figure 1

Fig. 2

Fig. 3