Biochemical and Biophysical Research Communications 454 (2014) 196–201

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

NDP52 associates with phosphorylated tau in brains of an Alzheimer disease mouse model

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ARTICLE INFO

Article history:
Received 11 September 2014
Available online 18 October 2014

Keywords:
Alzheimer's disease
Autophagy
Autophagy receptor
LC3
NDP52
Tau

ABSTRACT

We previously showed that NDP52 (also known as calcoco2) plays a role as an autophagic receptor for phosphorylated tau facilitating its clearance via autophagy. Here, we examined the expression and association of NDP52 with autophagy-regulated gene (ATG) proteins including LC3, as well as phosphorylated tau and amyloid-beta (Aβ) in brains of an AD mouse model. NDP52 was expressed not only in neurons, but also in microglia and astrocytes. NDP52 co-localized with ATGs and phosphorylated tau as expected since it functions as an autophagy receptor for phosphorylated tau in brain. Compared to wild-type mice, the number of autophagic vesicles (AVs) containing NDP52 in both cortex and hippocampal regions was significantly greater in AD model mice. Moreover, the protein levels of NDP52 and phosphorylated tau together with LC3-II were also significantly increased in AD model mice, reflecting autophagy impairment in the AD mouse model. By contrast, a significant change in p62/SQSTM1 level was not observed in this AD mouse model. NDP52 was also associated with intracellular Aβ, but not with the extracellular Aβ of amyloid plaques. We conclude that NDP52 is a key autophagy receptor for phosphorylated tau in brain. Further our data provide clear evidence for autophagy impairment in brains of AD mouse model, and thus strategies that result in enhancement of autophagic flux in AD are likely to be beneficial.

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1. Introduction

Autophagy is an intracellular pathway for bulk protein degradation, as well as the removal of damaged organelles and infectious microorganisms by lysosomes. The autophagic process starts with the formation of a cup-shaped membrane, called phagophore, which enwraps cytoplasmic components to form double-membrane vesicles, autophagosomes. Autophagosomes eventually fuse with lysosomes to generate autolysosomes, where proteins, organelles and invading microbes are degraded. The autophagic process involves numerous autophagy-regulated gene (ATG) products including ATG6 (beclin-1), ATG7 and ATG8 (LC-3) [1,2]. In addition to the bulk removal of cellular components, the process of selective autophagy, which involves autophagy adaptors that target specific cargo for lysosomal degradation by interaction with LC3-II, likely plays a significant role in maintaining protein homeostasis [3,4]. Autophagic cargo receptors include proteins such as p62/sequestosome1 (SQSTM1), neighbor of BRCA1 gene 1 (NBR1), optineurin and nuclear dot protein 52 (NDP52) [3,4].

Alzheimer's disease (AD) is the most prevalent older age onset neurodegenerative disease. In brains of AD patients, the extracellular deposition of amyloid plaques and intracellular accumulation of neurofibrillary tangles (NFTs) are two major pathological hallmarks. Amyloid plaques are composed of amyloid-beta (Aβ) which is derived from the proteolysis of amyloid precursor protein (APP) via sequential cleavages by β- and γ-secretase [5,6]. NFTs are aggregates of hyperphosphorylated tau [7,8]. Although these pathological features are unlikely the toxic entities, oligomeric forms of Aβ and tau that precede the formation of either the plaque or tangle, respectively, have been shown to compromise neuronal viability and therefore probably contribute to disease pathogenesis [9,10]. One possible contributing factor to the accumulation of these disease relevant oligomers is inefficient clearance processes.

Abbreviations: ATG, autophagy-regulated gene; AVs, autophagic vesicles; AD, Alzheimer's disease; Aβ, amyloid-beta; APP, amyloid precursor protein; NFTs, neurofibrillary tangles; NDP52, nuclear dot protein 52; p62/SQSTM1, p62/sequestosome1; NBR1, neighbor of BRCA1 gene 1; GFAP, glial fibrillary acidic protein; Nrf2, nuclear factor erythroid 2-related factor 2.

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http://dx.doi.org/10.1016/j.bbrc.2014.10.066
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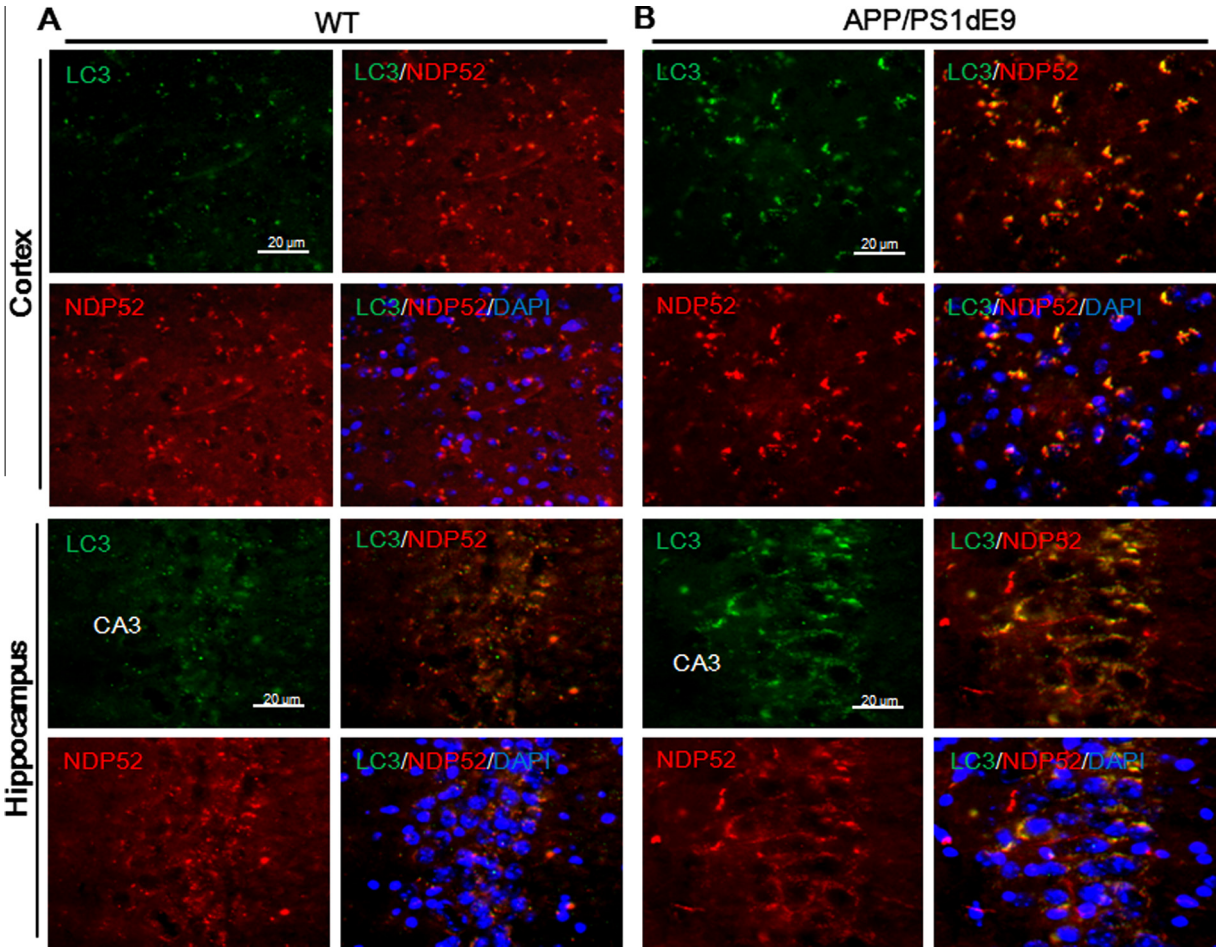

Fig. 1. NDP52 co-localizes with LC3 in mouse brains. Brain slices obtained from wild-type (A) and APPsw/PS1dE9 (B) mice 9 months old were immunohistochemically stained using mouse anti-NDP52 and rabbit anti-LC3 antibodies.

In AD model mouse and AD brains there is evidence that the autophagy is compromised as accumulations of autophagosomes and late autophagic vesicles (AVs) have been observed in dystrophic and degenerating neuritis [11–13]. Moreover, AVs were suggested as intracellular Aβ-generating compartments [13,14], thus contributing to the worsening of AD pathology. There is also compelling evidence that tau is cleared by autophagy [15–17]. A recent study suggested that autophagy receptor protein NDP52 plays a crucial role in the degradation of phosphorylated tau in neurons by targeting it to the autophagy pathway [15]. Given this finding, there is a need to understand how NDP52 associates with ATG proteins and phosphorylated tau *in vivo*.

Here, we examined the expression and localization of NDP52 in brains of wild type mice as well as in the APPsw/PS1dE9 AD mouse model, which is a widely used and well-characterized mouse model [18–20]. In this study we found that NDP52 co-localized with both phosphorylated tau and intracellular Aβ, as well as with ATG proteins, indicating that it likely plays a role in not only the autophagic clearance of phosphorylated tau, but also of Aβ.

2. Materials and methods

2.1. Antibodies

Anti-beclin-1, ATG7 and LC3 antibodies were obtained from Cell Signaling Technology. Anti-NDP52 mouse (H00010241-B01P) and rabbit (9036) antibodies were purchased from Novus Biologicals and Cell Signaling Technology, respectively. Polyclonal tau (A0024) antibody was obtained from Dako, and anti-p62/SQSTM1 (BML-PW9860) antibody was from Biomol. PHF1 (phospho-Ser396/404) antibody was kindly provided by Dr. P. Davies. Anti-NeuN and $A\beta_{1-40}$ antibodies were purchased from Millipore. Anti-CD11b and GFAP antibodies were purchased from AbD Serotec and Sigma, respectively. Anti-Aβ 1-16 (6E10) antibody was obtained from Covance. Anti-β-actin antibody was purchased from Millipore.

2.2. Animals

Wild-type (C57BL/6J) and AD transgenic model (APPsw/PS1dE9) mice used in this study were described previously [19], which were obtained from the Jackson Laboratory. All studies were conducted with a protocol approved by the local Institutional Animal Care Use Committee in compliance with Korea National Institute of Health guidelines for the care and use of experimental animals.

2.3. Immunoblotting

Mouse hippocampal tissues were homogenized in modified RIPA buffer (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EGTA, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) containing 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and 10 μg/ml each of aprotinin, leupeptin and pepstatin. Proteins were extracted on ice with periodic vortexing for 30–40 min, and homogenates were cleared by

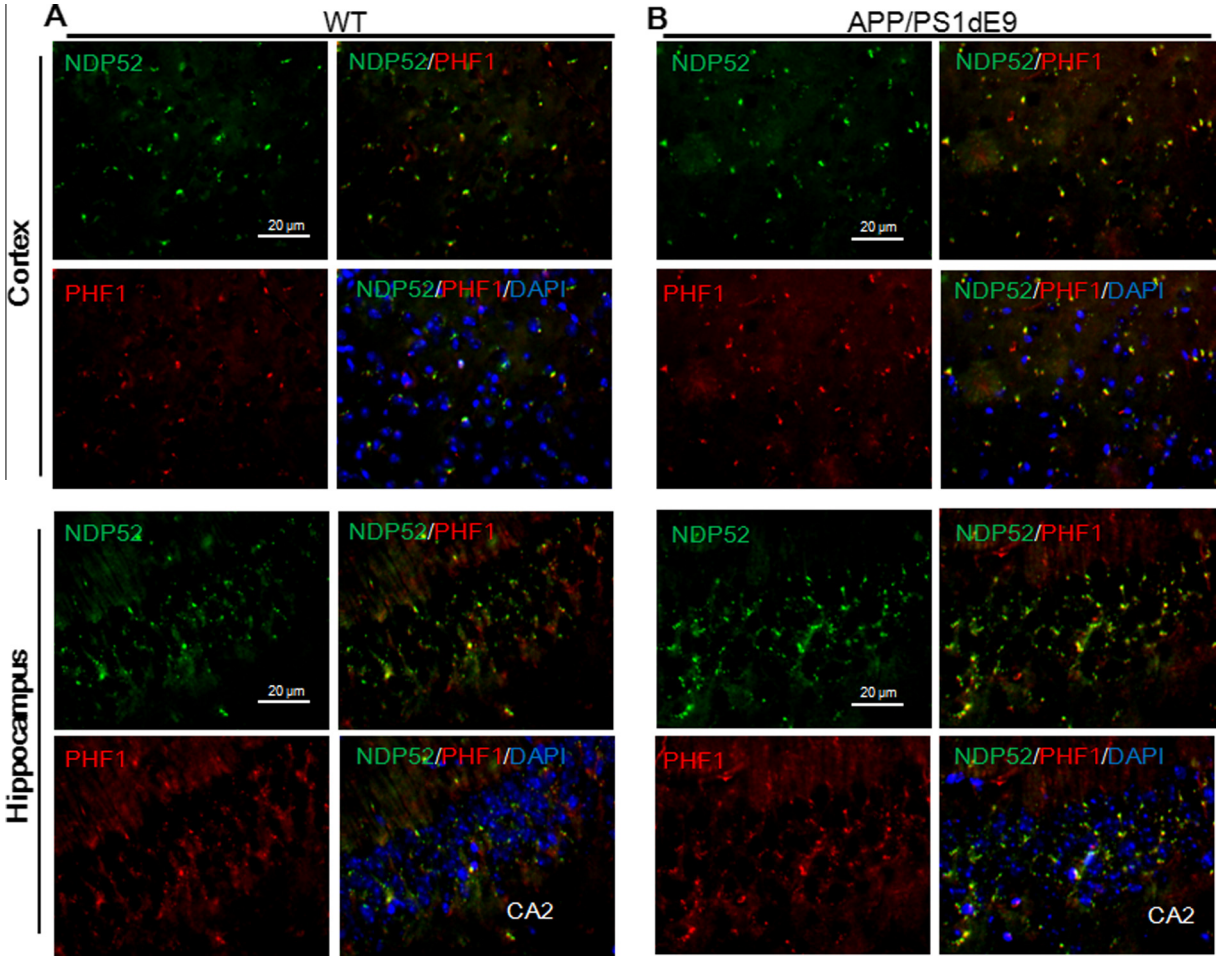

Fig. 2. NDP52 co-localizes with phosphorylated tau in mouse brains. Brain slices obtained from wild-type (A) and APPsw/PS1dE9 (B) mice 9 months old were immunohistochemically stained using rabbit anti-NDP52 and mouse monoclonal PHF1 antibodies.

centrifugation at 10,000×*g* for 10 min at 4 °C, and the supernatants were used for immunoblotting following boiling in 1× SDS-sample loading buffer for 5 min. For analysis protein samples (50 μg) were separated on 4–12% gradient SDS–polyacrylamide gels (Invitrogen) followed by transfer to nitrocellulose membranes (GE Healthcare) and immunoblotting with the indicated antibodies. Blots were developed with chemiluminescence (GE Healthcare). All protein concentrations were determined using the BCA method (Sigma).

2.4. Immunohistochemical staining

Brain slices (16 μm) prepared from mouse brains following fixing with 4% paraformaldehyde were washed three times using PBS to remove cryoprotectant, and blocked in a PBS blocking solution (3% BSA, 0.3% Triton-X-100 in PBS) for 1 h. The slices were treated with the appropriate primary antibodies diluted in the blocking solution at 4 °C overnight. The antibodies used were as follows: rabbit anti-LC3 (1:200), anti-ATG7 (1:200), anti-beclin-1 (1:200) or anti-$A\beta_{1\text{-}40}$ antibody (1:200) along with mouse anti-NDP52 (1:200) antibody; mouse anti-PHF1 (1:500) or anti-GFAP (1:250) antibody together with rabbit anti-NDP52 (1:200) antibody; rat anti-CD11b (1:100) antibody together with rabbit anti-NDP52 (1:200) antibody. Then, the slices were incubated with donkey anti-rabbit (anti-rat for CD11b) Alexa488 and goat anti-mouse Alexa568 conjugated antibodies (1:500) for 1 h at room temperature. The coverslips were mounted on the glass slides with ProLong® gold antifade reagent (Invitrogen, P36935) following three washes with TBS. Images were photographed using an epifluorescence microscope (Carl Zeiss).

3. Results

3.1. NDP52 is expressed in glial cells as well as neurons

NDP52 is expressed in neurons, and present in the perinuclear regions of neurons [15]. To examine whether NDP52 is expressed in microglia or astrocytes in addition to neurons, we co-immunostained brain slices with the NDP52 antibody and either with an antibody to CD11b, a marker protein of microglia or glial fibrillary acidic protein (GFAP), a specific protein for astrocytes. As seen in Supplementary Fig. S1, NDP52 was not only seen in neurons immunostained with NeuN, a neuronal marker protein, but also observed in the perinuclear region of microglia as well as astrocytes *in vivo*.

3.2. NDP52 co-localizes with ATG proteins including LC3 in mouse brains

To examine the expression and localization of NDP52 in brain *in vivo*, we immunostained brain slices with an NDP52 specific antibody along with specific antibodies for autophagy-regulated gene (ATG) proteins such as LC3, beclin-1 and ATG7. As shown in

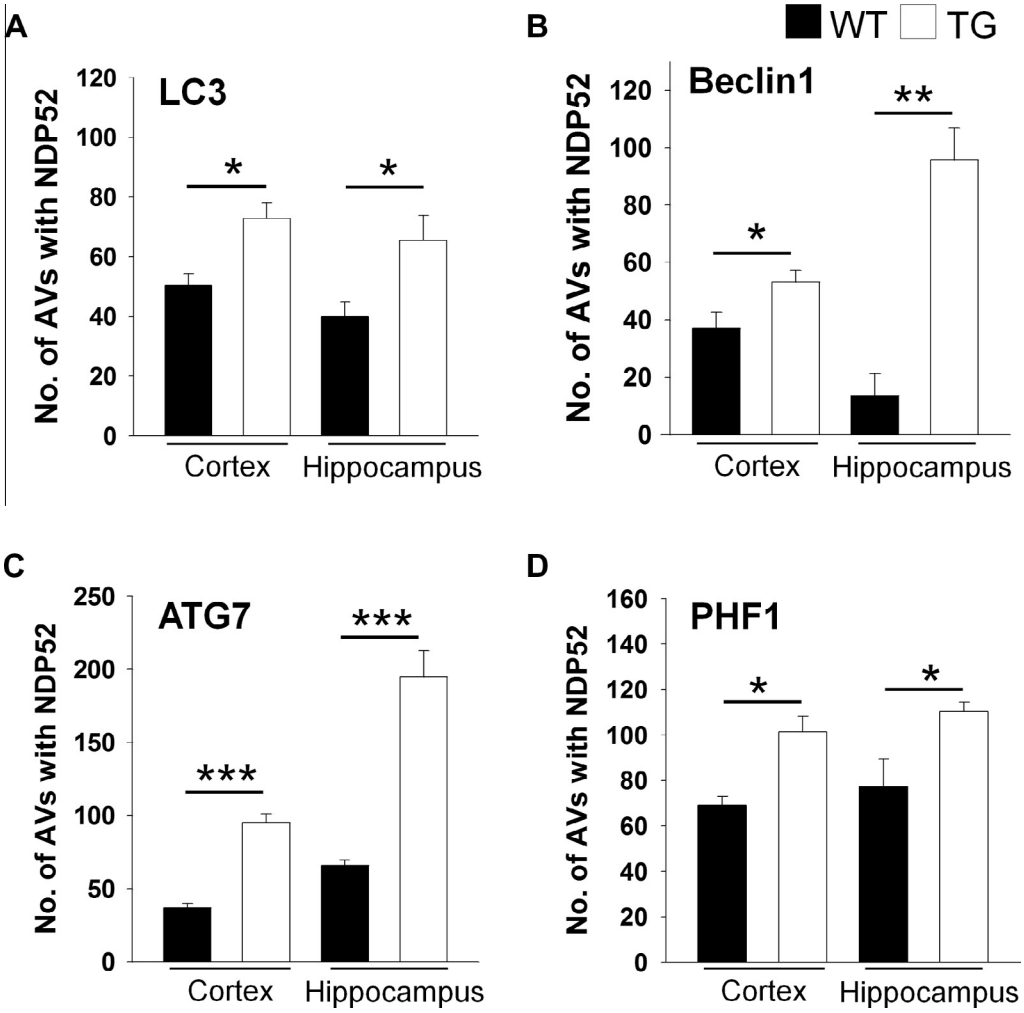

Fig. 3. Autophagic vesicles increase in AD model mice compared to wild-type mice. Mouse brain slices prepared from either wild-type (WT) or AD model mice (TG, APPsw/PS1dE9) were immunostained using anti-NDP52 together with anti-LC3, anti-beclin-1, anti-ATG7 or PHF1 antibody. The number of autophagic vesicles containing in NDP52 together with LC3 (A), beclin-1 (B), ATG7 (C) or PHF1 (D) in cortex and hippocampal regions was counted in about 10 areas randomly chosen. Data shown are mean ± s.e.m. and were analyzed using Student's *t* test. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Fig. 1 and Supplementary Figs. S2 and S3, immunohistochemical signals corresponding to NDP52 were easily observed in all the brain tissues, demonstrating that NDP52 is expressed in both cortex and hippocampal regions of mice. Moreover, all the red fluorescence corresponding to endogenous NDP52 was co-localized with green fluorescence to LC3, beclin-1 or ATG7 in brain tissues, supporting the finding that it functions as an autophagy receptor in brain [15].

3.3. NDP52 co-localizes with phosphorylated tau in mouse brains

In APPsw/PS1dE9 mice, the levels of phosphorylated tau protein have been reported to be increased compared to wild-type mice of the same age [18,20]. To examine if NDP52 is also associated with phosphorylated tau, we co-stained brain slices with an NDP52 antibody along with the PHF1 antibody that recognizes tau phosphorylated at Ser396/Ser404. As shown in Fig. 2, the green fluorescent signal for NDP52 overlaps with the red signal of PHF1 in most neurons, indicating that NDP52 co-localizes with phosphorylated tau in brain. These results support previous findings which indicate that NDP52 acts as an autophagy receptor for phosphorylated tau in brain [15].

3.4. Autophagic vesicles increase in AD model mice compared to wild-type mice

The number of autophagic vesicles (AVs) showing yellow color, which is an overlapped signal between NDP52 and ATG proteins or phosphorylated tau, significantly increased in APPsw/PS1dE9 mice compared to wild-type mice (Fig. 3). Given that the accumulation of autophagosomes occurs in brains of AD model mice [11–13], it is speculated that autophagy impairment resulted in the increase in the number of AVs containing NDP52 in brain of AD model mice.

3.5. NDP52 increases along with the augment of phosphorylated tau in APPsw/PS1dE9 mice

To further investigate whether NDP52 and ATG proteins were accumulated in brains of AD model mice, we analyzed hippocampal tissues by immunoblotting using corresponding specific antibodies. As expected, the level of LC3-II was increased in APPsw/PS1dE9 mice compared to in wild-type mice; by contrast, however, the levels of beclin-1 and ATG7 were slightly decreased compared to wild-type (Fig. 4). Of note, a significant increase in the level of NDP52 together with phosphorylated tau was observed in APPsw/PS1dE9

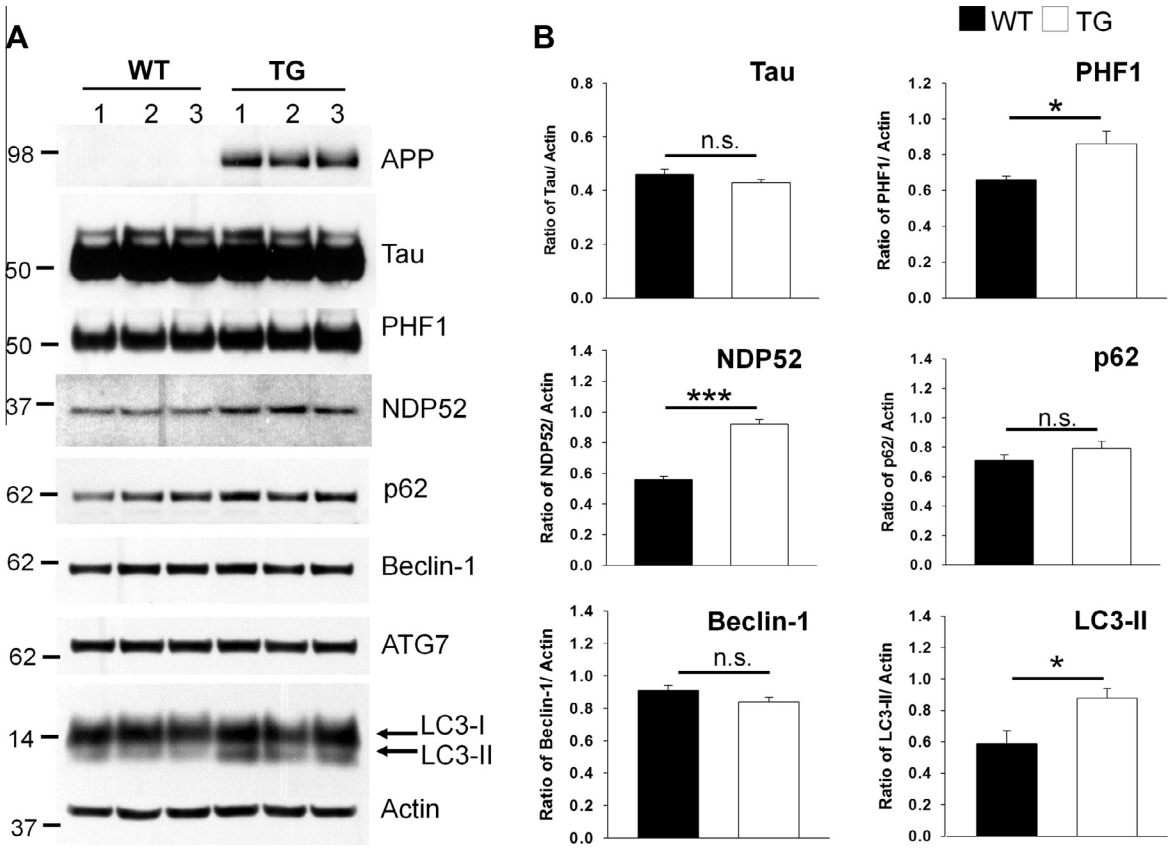

Fig. 4. NDP52 increases along with the augment of phosphorylated tau in APPsw/PS1dE9 mice. (A) Hippocampal tissues obtained from wild-type (WT, $n = 5$) and AD model (TG, APPsw/PS1dE9, $n = 5$) mice 17 months old were immunoblotted with a polyclonal antibody to total tau (Tau), PHF1, NDP52, p62/SQSTM1, beclin-1, ATG7, LC3, or 6E10 antibody. The relative molecular masses (kD) are indicated to the left of each blot. (B) Bar graphs represent the relative optical density of total tau (Tau), PHF1, NDP52, p62/SQSTM1, beclin-1 and LC3-II in the hippocampal tissues. APP, amyloid precursor protein; n.s. means data are not significant. Data shown are mean ± s.e.m. and were analyzed using Student's t test. (*, $p < 0.05$; ***, $p < 0.001$).

mice compared to wild-type mice, while no significant changes in the levels of p62/SQSTM1 were observed in the APPsw/PS1dE9 mice (Fig. 4), thus suggesting that NDP52 plays a key role in selective autophagy in brain.

3.6. NDP52 co-localizes with intracellular Aβ peptide in mouse brains

To examine if NDP52 is also associated with Aβ peptides in APPsw/PS1dE9 mice, we co-stained brain slices with the NDP52 antibody and an $A\beta_{1\text{-}40}$ peptide specific antibody. As shown in Supplementary Fig. S4, green fluorescence corresponding to $A\beta_{1\text{-}40}$ peptide shows co-localization with red fluorescence corresponding to NDP52, indicating that NDP52 may be associating with $A\beta_{1\text{-}40}$ peptide in brain. Given that AVs can be intracellular Aβ-generating compartments [13,14], the result indicates that AVs with NDP52 could be a source for Aβ production, or NDP52 might play a role in the clearance of intracellular Aβ peptides present in neurons.

We next examined if NDP52 was expressed in microglia or neurons around amyloid plaques. NDP52 was not directly associated with amyloid plaques, indicating that it is not secreted or released from cells (Supplementary Fig. S5A). Microglia around amyloid plaques express NDP52 (Supplementary Fig. S5B). It is speculated that microglia may localize around amyloid plaques to remove secreted Aβ peptides via their engulfment and digestion. However, it remains to be elucidated whether NDP52 is involved in the degradation following the engulfment of Aβ peptides. Also, all neurons with immunosignals for tau in the vicinity of the plaques showed fluorescence corresponding to NDP52 (Supplementary Fig. S5C), again supporting a role for NDP52 as an autophagic receptor for tau.

4. Discussion

Autophagy is essential for neuronal homeostasis, and its dysfunction has been directly linked to neurodegenerative diseases including AD [21]. Growing evidence demonstrates that autophagosomes and late AVs are accumulated in the brains of AD model mice and AD patients, which is likely to be a result of autophagy impairment [11–13]. In line with those findings, the number of autophagic vesicles (AVs) containing NDP52 significantly increased in APPsw/PS1dE9 mice compared to wild-type mice (Fig. 3). Given that AVs accumulated in the dystrophic neurons of AD patient brains are an active site for generating Aβ peptides [13,14], our results suggest that strategies which enhance autophagic flux, and not just autophagosome formation, are needed to attenuate pathogenic processes in AD.

NDP52 was originally identified as an autophagy receptor for bacteria that had invaded peripheral tissues and been ubiquitinylated, thus directing them to the autophagy pathway for degradation [22,23]. Recently, we showed that NDP52 plays a role as an autophagy receptor for phosphorylated tau facilitating its clearance [15]. Most neurons with a fluorescence signal for phosphorylated tau showed a co-localizing fluorescence signal corresponding to NDP52, indicating that they are associated and supporting the proposed role of NDP52 as an autophagy receptor for phosphorylated tau (Fig. 2). As previously described in other studies [18,20], the amount of phosphorylated tau is increased in brains

of APPsw/PS1dE9 mice compared to age-matched wild-type mice, which occurred together with an increase of NDP52 and LC3-II (Fig. 4). In contrast, the levels of p62/SQSTM1 in APPsw/PS1dE9 mice were not significantly different from those in wild-type mice (Fig. 4). These data may suggest that NDP52 plays a more important role in autophagy in brain than previously thought.

In brain NDP52 is in microglia and astrocytes as well as in neurons (Supplementary Fig. S1). Microglia are present around amyloid plaques in brain, and also have NDP52 in their perinuclear region (Supplementary Fig. S5B). In addition, NDP52 co-localized with intracellular Aβ (Supplementary Fig. S4). Even though it remains to be investigated, it is plausible that NDP52 might act as an autophagic receptor for intracellular Aβ in addition to phosphorylated tau.

The expression of NDP52 is very dependent on nuclear factor erythroid 2-related factor 2 (Nrf2) activation in neurons, and its reduced expression is directly related to the accumulation of sarkosyl-insoluble tau in brains of AD patients [15]. In previous studies, the hippocampal expression of Nrf2 or its activation with Nrf2 activators such as sulforaphane in AD model mice significantly improved learning and memory retention compared to untreated mice [24,25]. In addition, sulforaphane can increase autophagic flux in neuronal cells [26]. Thus, NDP52 up-regulation and enhancement of autophagy flux using an appropriate Nrf2 activator could have the potential of ameliorating disease progression in AD.

Competing interests

The authors declare no competing interest.

Acknowledgments

We thank Dr. Peter Davies (Albert Einstein College of Medicine) for providing PHF1 antibody for this study. This study was supported by a grant of Korea National Institute of Health Intramural Research/Korea Ministry of Health and Welfare (4845-302-210-13).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.066.

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