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Short Communication

The Wnt/β-catenin signaling pathway affects the distribution of cytoskeletal proteins in Aβ treated PC12 cells

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Alzheimer's disease is pathologically characterized by the presence of senile plaques and neurofibrillary tangles in the central nervous system. Amyloid β-protein is toxic to neurons and induces phosphorylation of Tau protein, which accumulates in paired helical filaments and leads to the formation of neurofibrillary tangles. This study is focused on the Wnt/β-catenin pathway influence on Tau phosphorylation and the distribution of microtubules and neurofilaments in adrenal pheochromocytoma cells. It was found that neurofilament heavy polypeptide and microtubule-associated protein-2 aggregated after treatment with Aβ_{1-42}. Treatment with Wnt5a reduced this aggregation, while Dickkopf-1 treatment promoted microtubule and neurofilament aggregation. Furthermore, Tau phosphorylation at Ser396, Ser422, and Ser199 was significantly reduced after Wnt5a treatment, whereas Dickkopf-1 increased the level of phosphorylation. These results suggest that the Wnt/β-catenin pathway influences the distribution of microtubules and neurofilaments, possibly by modulating the phosphorylation of Tau protein in adrenal pheochromocytoma cells.

Keywords

Alzheimer's disease; amyloid β-protein; Wnt/β-catenin signaling pathway; Tau phosphorylation

1. Introduction

Alzheimer's disease (AD) is a familiar and irreversible neurodegenerative disease characterized by personality changes, memory loss which can lead to dementia (Puzzo et al., 2015). The main histopathological AD-associated changes include senile plaques taked shape the deposition of extracellular Amyloid β-protein (Aβ) which induces phosphorylation of Tau protein that leads to intracellular filament entanglement (Liu et al., 2014). The misfolding and hyper-phosphorylation of Tau protein are together with the neural loss and severe cognitive impairment observed in the pathogenesis of AD (Gomez-Isla et al., 1997). It is estimated that by 2050, the global prevalence rate of AD will be four times that of 2006, such that one out of 85 people will suffer from AD (Zhang et al., 2018). Despite numerous clinical trials and experimental studies, the underlying mechanisms of AD are still not clear.

The Wnt/beta-catenin signaling pathway, which (plays a pivotal role) in various bio-processes consisting of adult tissue homeostasis and embryonic form development, is the most studied and hence best understood, developmental pathway (Zhang et al., 2018). Wnt signaling can be splited into two categories: the quintessential Wnt/β-catenin signaling pathway and the non-canonical pathways, which include the planar cell polarity and Wnt/β-calcium signaling pathways (Choi et al., 2012). The Wnt/β-calcium signaling pathway also haves an important role in the central nervous system (CNS) (Budnik and Salinas, 2011; Shimogori et al., 2004) and it is haved a hand in the pathology of several neurological confusions and cancers (De Ferrari and Moon, 2006; Logan and Nusse, 2004). It is reported that dislocation of the Wnt/β-calcium signaling pathway is associated with AD (Hooper et al., 2008), however, whether there is a relationship between this signaling pathway and the phosphorylation of Tau protein is unclear. Here, the act of the Wnt/β-catenin pathway on the cell toxicity of Aβ of the adrenal pheochromocytoma (PC12) cell line was studied, and it was found that the Wnt/β-catenin pathway regulates the Aβ_{1-42} induced hyper-phosphorylation of Tau protein. These findings support the hypothesis that the Wnt/β-catenin pathway on the cell toxicity of Aβ of the adrenal pheochromocytoma (PC12) cell line was studied, and it was found that the Wnt/β-catenin signaling pathway regulates the Aβ_{1-42} induced hyper-phosphorylation of Tau protein. These findings sustain the hypothesis that the Wnt/β-catenin signaling pathway is had a hand in the pathological processes underlying AD and provides a lurking therapeutic target for AD treatment.

2. Materials and methods

2.1 Drugs and reagents

Aβ_{1-42} (Cat# SCP0038) and Dickkopf-1 (DKK1, Cat# SRP3258) were purchased from Sigma-Aldrich. Rabbit anti-microtubule associated protein-2 (anti-MAP-2, Cat# 4542) and mouse anti-neurofilament heavy polypeptide (anti-NF-H, Cat# 2836) were obtained from cell signaling technology. Wnt5a (Cat#ab204627), rabbit anti-Tau (phospho S422), rabbit anti-Tau (phospho S396), rabbit anti-Tau (phospho S199, Cat #ab79415, Cat #ab109390 and Cat# ab81268, respectively) and rabbit anti-GAPDH (Cat# ab181602) were purchased from Abcam. Pierce™ BCA Protein Assay Kit (Cat# 23227) was bought from ThermoFisher. All other reagents were obtained from Sigma-Aldrich.

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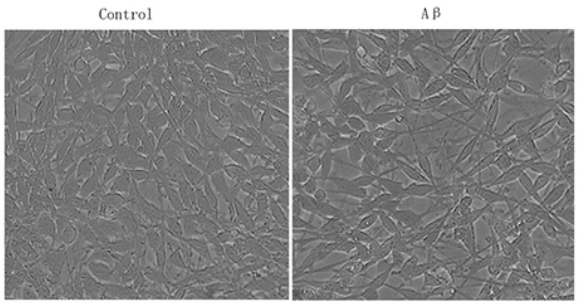

Figure 1. Toxicity of $A\beta_{1-42}$on PC12 cells. Images were taken after three days sub-culture using an inverted microscope. Cells in the $A\beta$ group grew more slowly when compared to the control group.

2.2 Cell culture

PC12 Cells were purchased from Chinese Academy of Medical Sciences. Cells were grained at a density of 1×10^5/ml in Dulbecco's Modified Eagle Medium provided with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin and cultured at 37 °C with 5% CO_2. To observe the affect of the Wnt/β-catenin pathway on the cell toxicity of $A\beta$ of the PC12 cell line, cells were gave 40 μM $A\beta_{1-42}$ for 24 hours; then with Wnt5a (30 ng/ml) or DKK1 (100 ng/ml) two hours before immunochemistry and Western blotting experiments.

2.3 Immunostaining

PC12 cells were seeded onto slides and cultured for two days. After drug treatment, the slides were rinsed three times with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 minutes at room temperature. The cells were then washed with PBS and blocked with 10% FBS, 0.01% Triton for one hour. Cells were then incubated with antibodies against NF-H or MAP-2 (1 : 1000) overnight at 4 °C. After three washes with PBS, cells were incubated with Alexa fluor 594-conjugated goat anti-rabbit or anti-mouse antibody, (1 : 3000, Invitrogen, USA) for one hour, in the dark, at room temperature. Slides were then washed three times in PBS and incubated with DAPI (1 : 1000) at 37 °C in the dark for 20 minutes to stain the cell nuclei. NF-H and MAP-2 were observed by a laser scanning microscope.

2.4 Western blotting

The protein abundance was detected by Western blotting. Briefly, treated and untreated PC12 cells were harvested and washed three times with PBS and then lysed in ice-cold RIPA buffer containing a phosphatase and protease inhibitor cocktail. Cell lysates were centrifuged at 12,000 rpm at 4 °C for 15 minutes, and the supernatants were collected and stored until analysis. The protein concentration was measured using a BCA protein quantification kit. Total proteins in the cell lysates (10 μg/condition) were separated by 10% SDS-PAGE and transferred to polyvinyl difluoride membranes (Millipore, USA) using standard procedures. The 5% (w/v) BSA was used to seal the membrane at room temperature for 2 hours in Tris buffer brine containing 0.1% Tween-20 (TBS-T), Then, the antibody was incubated overnight with the antibody dilution solution at 4 °C. After washing with tbst, the membranes were incubated with secondary horseradish peroxide binding antibodies (1 : 5000; cwbio, China) for 1 hour at room temperature. Detection of protein bands by ECL reagent and analysis by FluroChem^{TM} SP software.

2.5 Statistical analysis

The SPSS 22.0 software was used for statistical analysis. The data are expressed as the mean value of the average ($\pm$ standard error) (S.E.M.). The differences among groups were evaluated by one-way ANOVA, and then the post-event LSD test with the same hypothetical variance was performed. Only $P < 0.05$ was considered to have statistical significance.

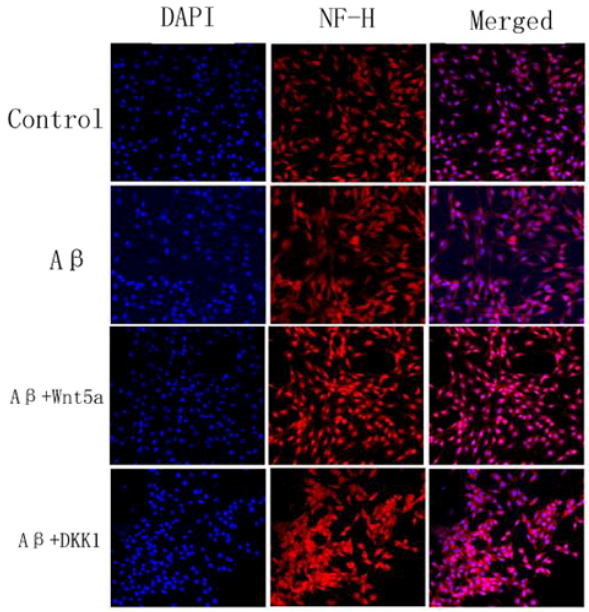

Figure 2. Influence of the Wnt/β-catenin signaling pathway on NF-H location in PC12 cells. Images show staining with DAPI (blue, left), NF-H (red, middle) and the co-localization of both in control (right), $A\beta$, $A\beta$ + Wnt5a or $A\beta$ + DKK1 treatment groups.

3. Results

3.1 Toxicity of $A\beta_{1-42}$ on PC12 cells

To determine the cell toxicity of $A\beta$ of the PC12 cell line and interrogate its biological features, PC12 cells were sub-cultured and treated with 40 μM $A\beta_{1-42}$ for 24 hours. Cell morphology and growth characteristics were observed using an inverted microscope (Fig. 1). Cells grew in a spindle shape and were evenly dispersed in the control group. After $A\beta_{1-42}$ treatment, cells kept the spindle shape but grew more slowly.

3.2 Wnt/β-catenin signaling pathway affects NF-H MAP-2 locations on PC12 cells

Immunochemistry was employed to examine the effect of the Wnt/β-catenin signaling pathway on the location of neurofilaments and microtubules on PC12 cells. As shown in Fig. 2 and Fig. 3 , treatment with $A\beta_{1-42}$ affected the cytoskeleton stability, resulting in microtubules and neurofilaments aggregating and condensing. Wnt5a is the activator of the Wnt/β-catenin signaling pathway, and after treatment with it, NF-H and MAP-2 aggregation were reduced (Fig. 2). DKK1 is the Wnt/β-catenin signaling pathway inhibitor, and when used to treat NF-H and MAP-2, it promoted their aggregation (Fig. 3).

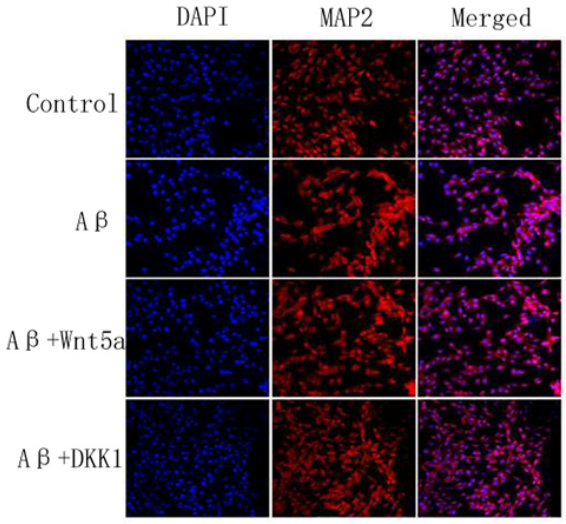

Figure 3. Influence of the Wnt/β-catenin signaling pathway on NF-H location in PC12 cells. Images show staining with DAPI (blue, left), MAP-2 (red, middle) and the co-localization of both in control (right), Aβ, Aβ + Wnt5a or Aβ + DKK1 treatment groups.

3.3 Wnt/β-catenin signaling pathway influences p-Tau abundance in PC12 cells

To examine whether the Wnt/β-catenin signaling pathway modulates the phosphorylation of Tau protein, PC12 cells were pretreated with Aβ_{1-42}, Aβ_{1-42} + Wnt5a, and Aβ_{1-42} + DKK1 respectively. After 24 hours of treatment with Wnt5a, the expression of Tau protein phosphorylated at Ser396, Ser422, and Ser199 were significantly reduced, whereas an increase was seen after treatment with DKK1 (Fig. 4).

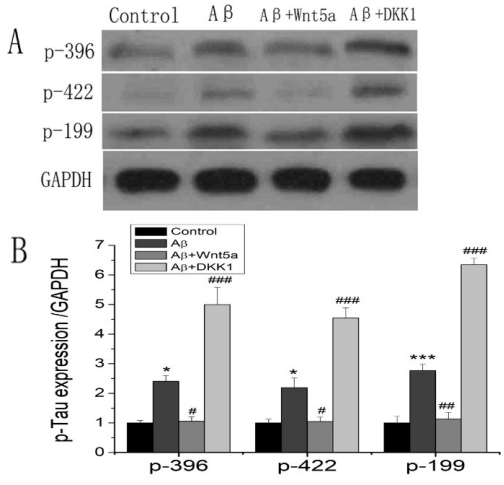

Figure 4. Influence of the Wnt/β-catenin signaling pathway on *p*-Tau abundance in PC12 cells. A. Western blot showing expression of p-396, p-422 and p-199 Tau protein. B. Quantification of p-396, p-422 and p-199 Tau protein expression by Western-blotting (n = 3). Data are presented as mean ± SEM. $^{*}P < 0.05$ vs. Control; $^{***}P < 0.001$ vs. Control. $^{\#}P < 0.05$ vs. Aβ; $^{\#\#}P < 0.01$ vs. Aβ; $^{\#\#\#}P < 0.001$ vs. Aβ.

4. Discussion

In this study, immunochemical results have shown that microtubules and neurofilaments tend to aggregate in PC12 cells. This aggregation was reduced by Wnt5a and increased by DKK1 treatment. Protein levels of *p*-Tau in the cell lysate were reduced by Wnt5a and increased by DKK1 treatment. Results show that the Wnt/β-catenin pathway influences the cellular hallmarks of AD.

Aβ is neurotoxic to the CNS, and the level of neurotoxicity is determined by the rates and levels of Aβ self-association (Feng and Zhang, 2004). Aβ_{1-42} was used for creating the toxicity in the adrenal pheochromocytoma (PC12) cell line (Pike et al., 1993; Yankner et al., 1990) by the high level of self-association (Feng and Zhang, 2004; Jarrett et al., 1993; Snyder et al., 1994). It has been reported that Aβ accumulation is a distinct morphological hallmark of AD, and it is the most-used AD drug target (Borlikova et al., 2013). Moreover, *p*-Tau aggregation may induce sequential brain lesions, and Aβ is proposed to be the activator (Maxwell et al., 2018).

Notch1 overexpression has been shown in AD cortex, and altered Notch signaling is related to enhanced Aβ expression and neurodegeneration in AD (Berezovska et al., 1999; Galeano et al., 2018). Moreover, Nortch signaling and Wnt signaling are closely related (Kaemmerer et al., 2019; Ye et al., 2019). Previous studies have also reported that activation of Wnt signaling function may reverse the Aβ-dependent neurotoxicity (De Ferrari et al., 2003). Hyperphosphorylated Tau accumulates in paired helical filaments (PHF) and leads to the formation of neurofibrillary tangles as well as neuronal degeneration in AD (Alonso et al., 1996). Several studies point to a possible role for Wnt in AD (Cisternas et al., 2019; Hu et al., 2019; Huang et al., 2018).

PHF shares epitopes with MAP-2 and other neurofilament associated proteins (Kosik et al., 1986). Furthermore, previous studies report that Wnt3a could regulate the growth and directionality of microtubules, resulting in microtubule remodeling (Purro et al., 2008). In this study, it has been shown that aggregation of microtubules and neurofilaments in PC12 cells can be observed following the use of MAP-2 and NF-H antibodies to separately label MAP-2 and NF-H proteins. Wnt5a treatment reduced NF-H and MAP-2 aggregation, while DKK1 treatment increased their aggregation. Microtubule-associated protein Tau is abnormally phosphorylated in AD (Grundke-Iqbal et al., 1986) and the dephosphorylation of *p*-Tau prevents formation of neurofibrillary tangles (Alonso et al., 1996). It was further found the phosphorylation of Tau at Ser396, Ser422 and Ser199 were significantly higher in the Aβ group than for normal controls. These results are consistent with those from previous studies and are indicative of a role for the Wnt signaling pathway in AD.

5. Conclusion

This study shows that the Wnt/β-catenin pathway can modulate the intracellular distribution of microtubules and neurofilaments, as well as the levels of phosphorylated Tau protein in PC12 cells. This demonstrates that the pathway is a promising target for AD research. The model system employed to show this result will likely be useful in future studies of the role of the Wnt/β-catenin pathway in AD.

Acknowledgment

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Conflicts of interest

The authors have no conflicts of interest to declare.

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