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WNT5A Signaling Contributes to A β -Induced Neuroinflammation and Neurotoxicity

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Abstract

Neurodegeneration is a pathological hallmark of Alzheimer's disease (AD), but the underlying molecular mechanism remains elusive. Here, we present evidence that reveals a crucial role of Wnt5a signaling in this process. We showed that Wnt5a and its receptor Frizzled-5 (Fz5) were up-regulated in the AD mouse brain, and that beta-amyloid peptide (A β), a major constituent of amyloid plaques, stimulated Wnt5a and Fz5 expression in primary cortical cultures; these observations indicate that Wnt5a signaling could be aberrantly activated during AD pathogenesis. In support of such a possibility, we observed that inhibition of Wnt5a signaling attenuated while activation of Wnt5a signaling enhanced A β -evoked neurotoxicity, suggesting a role of Wnt5a signaling in AD-related neurodegeneration. Furthermore, we also demonstrated that A β -induced neurotoxicity depends on inflammatory processes, and that activation of Wnt5a signaling elicited the expression of proinflammatory cytokines IL-1 β and TNF- α whereas inhibition of Wnt5a signaling attenuated the A β -induced expression of the cytokines in cortical cultures. Our findings collectively suggest that aberrantly up-regulated Wnt5a signaling is a crucial pathological step that contributes to AD-related neurodegeneration by regulating neuroinflammation.

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Introduction

Beta-Amyloid (A β) peptide is a dominant candidate of the causative agents for Alzheimer's disease (AD) [1,2]. According to the widely-held amyloid hypothesis of AD, A β initiates an array of molecular and cellular cascades that eventually lead to progressive neuronal dysfunction and degeneration [1,2]. However, mechanistic molecular processes that link A β and neurodegeneration remain to be firmly established.

Chronic neuroinflammation associated with persistent glial activation is a major disease process evoked by A β and intimately associated with the progress of AD pathologies [3,4]. Previous studies suggest that neuroinflammation contributes to the development of neurodegenerative hallmarks in AD brains, including A β plaques [4] and tau tangles [5,6,7]. AD therapeutic approaches that target neuroinflammation are under development [8,9,10,11]. AD neuroinflammation is likely triggered by A β -mediated activation of microglia and astrocytes [3,4,12,13,14]. It was reported that A β induces the expression of cytokines (including IL-1 β , TNF- α , IL-6, and IL-8) in cultured astrocytes and microglia [15,16,17,18]. Mounting evidence suggests that A β may activate glial cells via specific sensor receptors such as toll-like receptors (TLR), receptors for advanced glycoxidation end-products (RAGE) and NOD-like receptors (NLR) [4]. Despite the significant understandings on the

induction of AD neuroinflammation, the downstream molecular processes that are elicited by A β and regulate the inflammation remain poorly understood.

Wnts are secreted signaling proteins that play important roles in neural development and plasticity [19,20,21,22]. Multiple lines of evidence indicate a critical role of Wnt signaling in AD [22]. β -catenin, a key downstream effector protein in the canonical Wnt signaling pathway, interacts with and is regulated by presenilin [23,24,25]. Glycogen synthase kinase (GSK)-3, a central serine/threonine kinase in the canonical Wnt signaling pathway, plays a critical role in the regulation of A β production [26] and aggregation [27] and in tau phosphorylation [28]. Genetic studies revealed that LRP6 polymorphisms are causally linked to AD [29]. In AD brains, canonical Wnt signaling is impaired [30], and DKK1, an antagonist of Wnt signaling, is upregulated [31,32]. Importantly, A β was reported to inhibit Wnt signaling by directly binding to the Frizzled receptors [33]. The impairment of canonical Wnt signaling is likely etiologically significant, because forced up-regulation of the canonical Wnt signaling pathway has rescuing effects on the development of AD-related phenotypes in both neuron cultures and animal models [27,30,34,35]. In contrast to the canonical pathway, the involvement of non-canonical Wnt signaling pathways in the regulation of AD pathogenesis is less clear. A recent study indicates that Wnt5a-activated non-canonical Wnt signaling antagonizes A β synaptotoxicity [36].

In this paper, we report an important role of Wnt5a signaling in the regulation of A β -evoked neurotoxicity and neuroinflammation. We observed that (1) Wnt5a/CaMKII signaling is up-regulated at the early stages of AD development in an APP^{swe}/PSEN1 Δ E9 transgenic mouse model, (2) A β activates Wnt5a signaling in primary cortical cultures, (3) A β -induced Wnt5a up-regulation is a critical molecular step leading to the development of A β neurotoxicity in cultures, (4) Wnt5a stimulates inflammatory processes, and (5) Wnt5a is critical for A β -induced inflammatory response. Our results suggest that abnormally up-regulated non-canonical Wnt5a signaling may regulate chronic neuroinflammation in AD brains.

Materials and Methods

Preparation of A β 42 peptides

Recombinant human A β (1–42) (Chemicon) was used to prepare monomers (A β -mon), oligomers (A β -olig) and fibrils (A β -fib), as described [37]. For monomer preparation, 5 mM A β 42 in DMSO was diluted directly with cell culture media. For oligomer preparation, 5 mM A β 42 in DMSO was diluted to 100 μ M in ice-cold D-MEM/F-12 (Invitrogen), vortexed for 30", centrifuged (10,000 g; room temperature) for 1', and incubated at 4°C for 24 h. For fibril preparation, 10 mM HCl was added to the A β 42 solution (5 mM in DMSO) to bring A β to a final concentration of 100 μ M, vortexed for 30", centrifuged (10,000 g, room temperature) for 1', and incubated with shaking at 37°C for 24 h. Products from such preparations are mixtures that contain A β monomers and the intended aggregates [37]. The quality of the preparations was confirmed by Western blotting analysis with the 6E10 (Covance; 1:5000), and the molecular mass was estimated by rainbow pre-stained protein markers (GE Healthcare).

Animals and hippocampus dissection

All procedures were approved by Animal Care and Use Committees of the University of Texas Medical Branch. Male APP (amyloid precursor protein)/presenilin-1 double transgenic (APP^{swe}/PSEN1 Δ E9, 2 \times Tg) mice [38] and wild-type littermates at 3.5 months of age were anesthetized and then decapitated. The hippocampi were rapidly collected on ice for Western blotting, immunohistochemistry, or fluorescent immunostaining.

Neuron cultures

C57BL E18 cortical cultures were prepared, as described [39]. The cells were plated on poly-D-lysine (30,000–70,000; Sigma) -

coated dishes at a density of 1.5×10^5 cells/cm². Immunostaining with cell-type markers indicate that the cultures are mixtures of neuron (~60%) and glial cells (~40%). Cultures at 12–14 DIV were used in this study. All experimental treatments were carried out by adding the administrated agents into the freshly changed culture media. Only morphologically healthy neuronal cultures were used for drug treatments.

Western blotting and antibodies

Western blotting was performed as described [40]. The intensity of non-saturated bands on Western blots was quantified by densitometry analysis with NIH ImageJ. GAPDH or β -actin were used as loading controls. Rabbit-anti-APP antibody was from Sigma-Aldrich (1:3000); rabbit-anti-Wnt5a (1:1000) and rabbit-anti-Fz5 (1:1000) antibodies were from Abcam; rabbit-anti-p- α CaMKII(Thr286) (1:1000), rabbit-anti-NIK (1:1000), and rabbit-anti-I κ B- α (1:1000) antibodies were from Cell Signaling Technology; and mouse-anti-GAPDH (1:1000) and rabbit-anti- β -actin (1:1000) antibodies were from Santa Cruz Biotechnology.

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed on 5 μ m-thick paraffin-embedded sections. The sections were deparaffined, dehydrated and treated with 1% H₂O₂ as described [41]. Antigen retrieval was carried out using citrate buffer (0.01 M; pH 6.0). The sections were then incubated with the primary antibody recognizing A β (1–40/42) (Millipore; 1:100) overnight at 4°C followed by incubation with biotinylated goat anti-rabbit IgG. After treatment with streptavidin-HRP and staining with DAB, the sections were stained with hematoxylin and mounted. Indirect fluorescence immunostaining was performed, as described [42], with rabbit anti-Wnt5a (abcam; 5 μ g/ml) and rabbit anti-Fz5 (abcam; 1 μ g/ml) primary antibodies and Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch; 1:200).

Measurement of secreted cytokines

Cortical cultures (12–14 DIV) were treated with Wnt5a (200 ng/ml), and media were collected at 0.5, 1, 2, 6, 12 and 24 h after Wnt5a application. Tumor necrosis factor alpha (TNF- α) and interleukin 1 beta (IL-1 β) in collected media were quantified using Mouse TNF- α or IL-1 β Quantikine ELISA Kits (R&D Systems), respectively.

MTT assay and trypan blue staining

The MTT assay and trypan blue staining were performed as described in [43]. Three independent experiments were performed

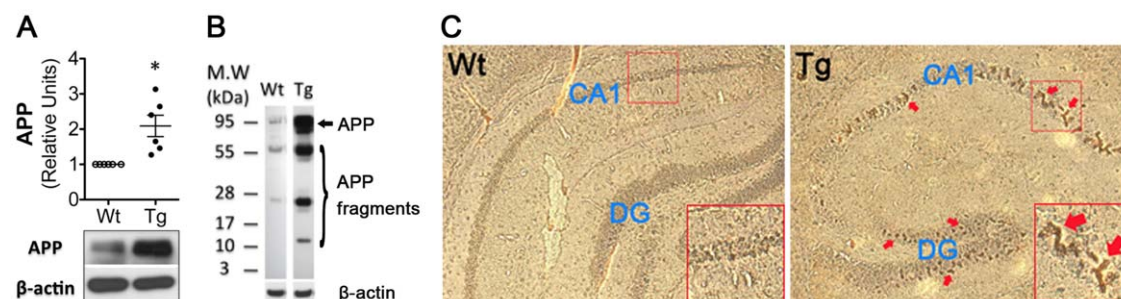


Figure 1. A β increases significantly in the hippocampus of the APP^{swe}/PSEN1 Δ E9 (2 \times Tg) mouse at the age of 3.5 months. A, APP in the wild-type and 2 \times Tg hippocampus (3.5-months). **B,** APP processing in the hippocampus revealed by immunoblotting with the monoclonal antibody 6E10 (recognizing residues 1–16 of A β). **C,** Immunohistochemical staining of the hippocampus with 6E10. Note the clustered A β signals (arrows) in the cell body layer of the 2 \times Tg hippocampus. Similar results were obtained from 6 pairs of wild-type and 2 \times Tg mice. doi:10.1371/journal.pone.0022920.g001

with each experiment including 4–6 replicates for each treatment. Reagents: 3% hydrogen peroxide solution (Sigma-Aldrich); recombinant mouse Wnt-5a (R&D Systems); anti-mouse Wnt-5a antibody (R&D Systems); anti-Fz5 antibody (against the extracellular cysteine-rich domain of human Fz5) (R&D Systems); Foxy5 peptide (Inbiolabs); Box5 peptide (Inbiolabs); recombinant mouse IL-10 (R&D Systems); and activated protein C (APC) (Sigma-Aldrich).

Statistical analysis

Data were expressed as the means \pm SEM. Statistical analysis was performed with the Prism software (GraphPad). We used the Student's two-tailed *t*-test for statistical comparisons between any two groups, and one-way ANOVA analyses with a Bonferroni post-hoc test for comparisons between multiple groups.

Results

Increased expression of key proteins in the non-canonical Wnt signaling pathway in the hippocampus of AD mouse models

Previous studies revealed down-regulation of canonical Wnt signaling in the brain of APP^{swe}/PSEN1 Δ E9 AD mouse models [27]. Consistent with these findings, we observed decreased expression of proteins in the canonical pathway, including Wnt3a, Fz1 and β -catenin (data not shown). In contrast, in preliminary studies, we also observed up-regulated expression of several proteins implicated in the non-canonical Wnt signaling pathway, including Wnt5a and its receptor Fz5, at various postnatal stage (3.5–9.5 months). At 3.5 months of age, APP transgene was clearly expressed (Fig. 1A). APP processing expected to generate A β was evident in the transgenic hippocampi (Fig. 1B). In addition, immunohistochemistry staining with an antibody that recognized A β revealed clustered signals in the cell body layer (Fig. 1C), which may include A β that starts accumulating. Because memory deficits have not manifested at this stage [44], we reasoned that the up-regulation of non-canonical Wnt signaling is potentially an early molecular abnormality during AD pathogenesis. Therefore, we performed more detailed analysis on the non-canonical Wnt signaling in mice at 3.5 months of age. Western blotting analysis showed that, compared with wild-type controls, Wnt5a protein increased 1.5 fold in the hippocampus of the 2 \times Tg mouse ($p < 0.01$; Fig. 2A). Similarly, Fz5 protein was also significantly up-regulated in the 2 \times Tg hippocampus (1.6 fold; $p < 0.01$; Fig. 2B). Because activation of non-canonical Wnt signaling can cause α CaMKII phosphorylation [45], we next determined the protein level of phosphorylated α CaMKII (pT286- α CaMKII). pT286- α CaMKII was increased 2.6 fold ($p < 0.05$; Fig. 2C). Fluorescent immunostaining showed that, in comparison with control, the increase of Wnt5a (Fig. 2D) and Fz5 (Fig. 2E) occurred in all hippocampal fields, including CA1, CA2/CA3 and dentate gyrus (DG), of the 2 \times Tg mouse, which resembled the spatial pattern of A β plaques (Fig. 1C). These data together suggest that non-canonical Wnt signaling is up-regulated in the 2 \times Tg AD hippocampus.

Rapid up-regulation of non-canonical Wnt signaling by A β oligomers in primary neuron culture

We next sought to understand the mechanism by which non-canonical Wnt signaling is activated in the AD mouse brain. Because A β is a key pathological agent for AD, we hypothesized that it causes the up-regulated expression of Wnt5a and other relevant proteins measured in 2 \times Tg mice. To test this hypothesis,

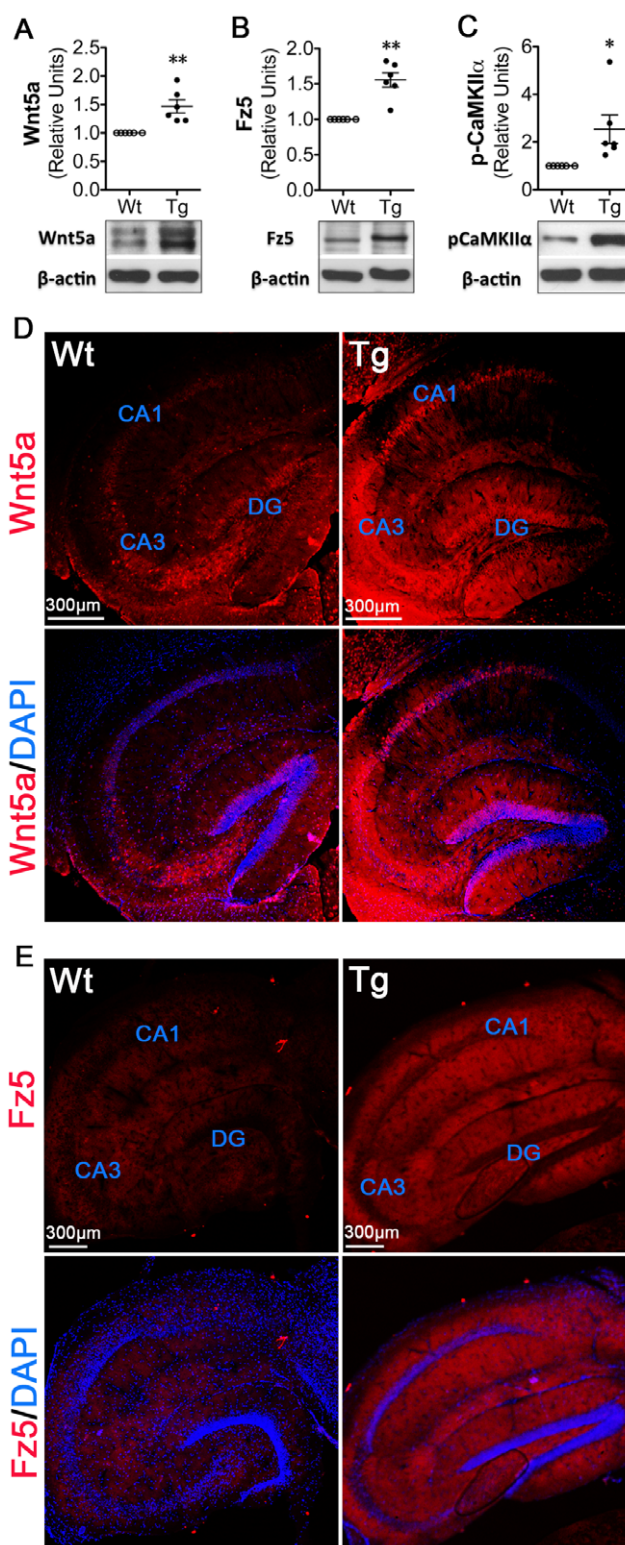


Figure 2. Wnt5a/CaMKII signaling is up-regulated in the hippocampus of the 2 \times Tg AD mouse model. A, B, and C. Protein levels of Wnt5a (A), Fz5 (B) and pT286- α CaMKII (C) in the wild-type and 2 \times Tg hippocampus. In summary graphs of quantitative data, the levels of target proteins were normalized with the β -actin loading control and expressed as relative units to the wild-type controls. Data presented in graphs are means \pm SEM from six pairs of mice. * $p < 0.05$. D and E. Distribution of Wnt5a (D) and Fz5 (E) protein in the wild-type and 2 \times Tg hippocampus. DAPI staining was performed to visualize the cell bodies. doi:10.1371/journal.pone.0022920.g002

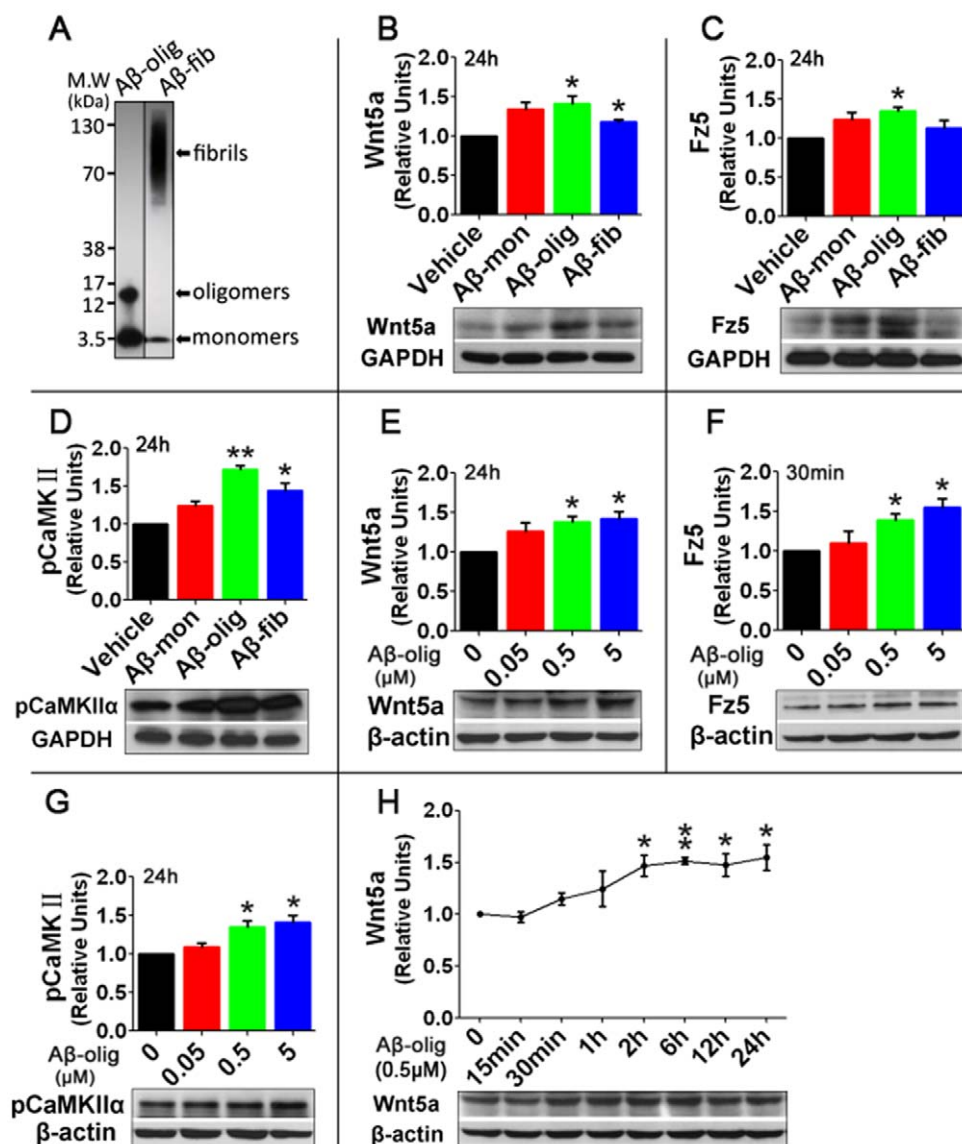


Figure 3. Up-regulation of proteins in the non-canonical Wnt signaling pathway by A β peptide in cortical neuron cultures. **A.** Western blot analysis of the preparations of A β (1–42) oligomers and fibrils. A β preparations were separated on SDS–PAGE (4–12% NuPAGE bis-Tris gel), and probed with the monoclonal antibody 6E10. **B, C, and D.** Protein levels of Wnt5a (**B**), Fz5 (**C**) and pT286- α CaMKII (**D**) in cortical cultures treated with 500 nM of monomeric, oligomeric, and fibrillar A β peptide for 24 hrs. **E, F, and G.** Concentration-dependent effects of A β oligomer on the protein levels of Wnt5a (**E**), Fz5 (**F**) and pT286- α CaMKII (**G**). Although the dose effect of A β on Wnt5a was most clearly observed at 24 hours post A β administration, the effect on Fz5 could be observed at 30 min. **H.** Time course of the A β oligomer effects (500 nM) on Wnt5a. In summary graphs of quantitative data, the levels of target proteins were normalized against GAPDH or β -actin and expressed as relative units to the vehicle-treated controls. Data in summary graphs are means \pm SEM from three independent experiments. * $p < 0.05$; ** $p < 0.01$. doi:10.1371/journal.pone.0022920.g003

we determined the effect of A β on the expression of these proteins in mouse cortical cultures (12–14 DIV). A wide range of A β concentrations (100 nM - 15 μ M) have been used in previous *in vitro* studies. We chose to use a relatively low A β concentration (500 nM) to better reflect the fact that the proteins are up-regulated at early AD stages when A β begins to accumulate (Fig. 1C). Prior works have shown that 500 nM A β is efficient to initiate some of the earliest and reversible AD-relevant pathologies [46,47]. More recent studies have indicated that different forms of A β (monomer, oligomer and fibril) vary in their AD-related toxicity, with the oligomer being considered the most toxic form [37,48]. We prepared the oligomers and fibrils of A β (1–42) (Fig. 3A) and compared the effect of A β monomers, oligomers and

fibrils on Wnt5a, Fz5 and pT286- α CaMKII protein expression in cortical cultures. As shown in Fig. 3B–D, A β oligomer treatment for 24 h caused significant increases in Wnt5a (1.4 fold; $p < 0.05$), Fz5 (1.4 fold; $p < 0.01$) and pT286- α CaMKII (1.7 fold; $p < 0.01$). Under the same experimental conditions, preparations of monomers and fibrils caused changes with lower magnitudes (Fig. 3B–D). Next, we investigated the dose effects of the oligomer. As shown in Fig. 3E–G, the oligomer increased expression of Wnt5a, Fz5, and pT286- α CaMKII proteins in a concentration-dependent manner (50 nM–5 μ M). Although Wnt5a and pCaMKII displayed a clear dose-effect at 24 hours after treatment, Fz5 showed this effect at 30 min. In addition, 500 nM of A β oligomer caused a time-dependent increase in Wnt5a (Fig. 3H).

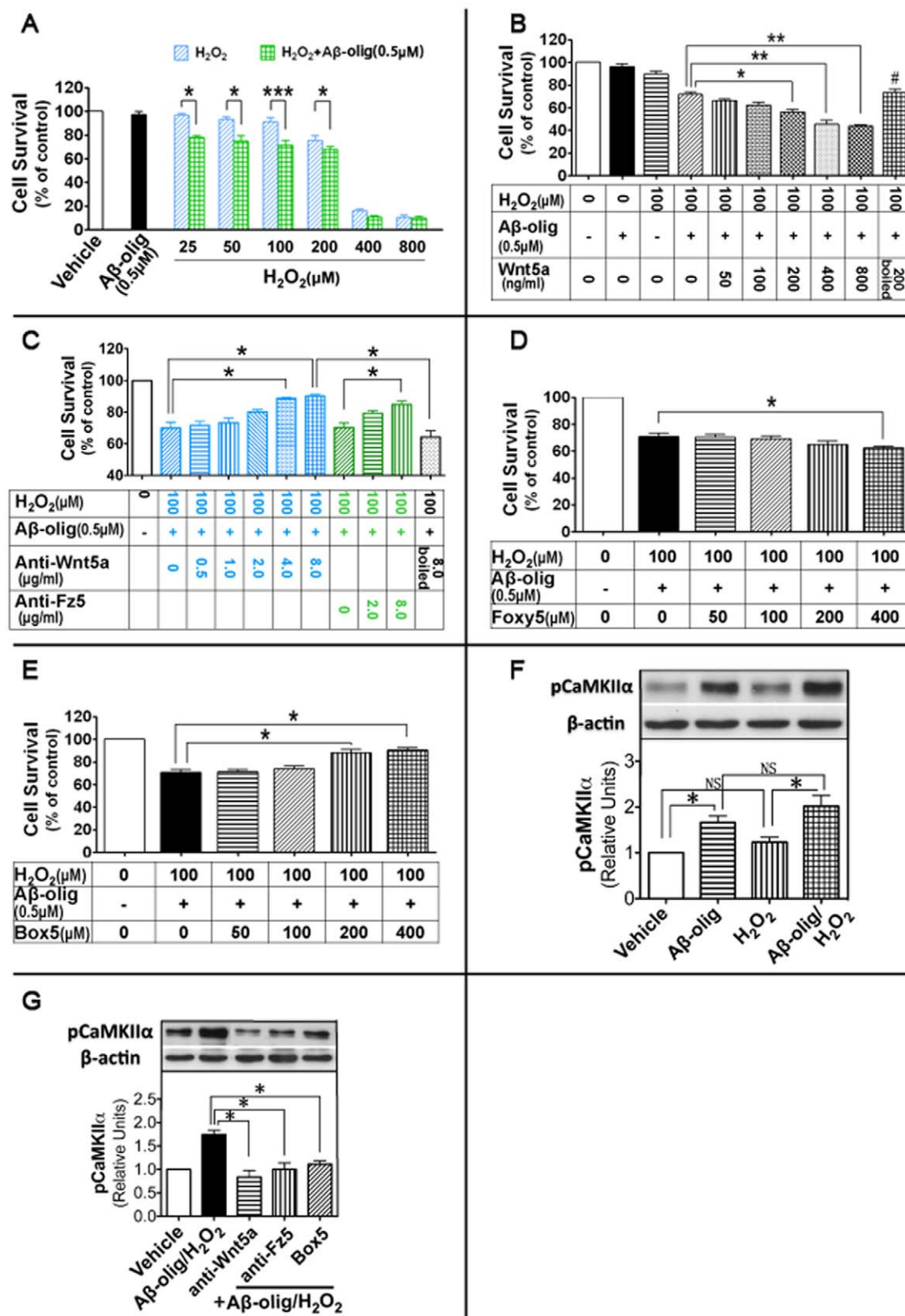


Figure 4. Wnt5a signaling regulates Aβ neurotoxicity. The MTT assay was used to measure cell viability in cortical cultures at 24 hrs after indicated treatments. **A.** Cell survival rates in cultures challenged with Aβ oligomers, H₂O₂, or a combination of Aβ oligomers and H₂O₂. **B.** Cultures challenged with Aβ (500 nM) and H₂O₂ (100 μM) in the presence of Wnt5a at different concentrations. **C.** Cultures challenged with Aβ and H₂O₂ in the presence of anti-Wnt5a or anti-Fz5 antibodies. **D.** Effects of Foxy5 on the neurotoxicity of Aβ/H₂O₂. **E.** Effects of Box5 on the neurotoxicity of Aβ/H₂O₂. Data are expressed as mean ± SEM (n≥12 from three independent experiments). * *p*<0.05; ** *p*<0.01; *** *p*<0.001. # *p*<0.05 (vs. the group of

Wnt5a (200 ng/ml)+A β /H₂O₂). **F.** Protein levels of pT286- α CaMKII in cortical cultures treated with A β (500 nM), H₂O₂ (100 μ M), or the combination of A β and H₂O₂. **G.** Protein levels of pT286- α CaMKII in cortical cultures treated with anti-Wnt5a (4 μ g/ml), anti-Fz5 (4 μ g/ml) antibodies or Box5 (200 μ M), in the presence of A β (500 nM) and H₂O₂ (100 μ M). * $p < 0.05$; NS, not significant. doi:10.1371/journal.pone.0022920.g004

These results demonstrated that the non-canonical Wnt5a signaling is up-regulated by A β peptide, especially the oligomers.

Suppression of Wnt5a signaling alleviates A β neurotoxicity

The observations of the upregulation of the Wnt5a signaling in early AD mouse brains and by A β in cortical cultures suggest a potential role of Wnt5a signaling in AD pathogenesis. We next sought to directly test this idea by determining the significance of A β -mediated Wnt5a up-regulation on A β cytotoxicity in cortical cultures. Previous work revealed concentration-dependent neurotoxicity of A β peptides [37,49,50], with high concentrations of A β (>5.0 μ M) causing neuron death in cultures and low concentrations of A β (0.1–1.0 μ M) sensitizing neurons to become vulnerable to further stresses [50]. To mimic the early AD brain condition when the non-canonical Wnt signaling proteins are aberrantly upregulated, in these experiments we used a relatively low concentration (500 nM) of A β that can upregulate Wnt5a and Fz5 (Fig. 3). A β oligomer at this concentration did not cause cell death, as measured by MTT assays (Fig. 4A). We also used trypan blue staining to confirm this observation (Fig. S1). This finding is consistent with the idea that in the early stages of AD pathogenesis A β may not cause neuron death. Next, we wanted to investigate if A β at this concentration causes the cells to become more vulnerable to detrimental stresses that are relevant to AD pathogenesis. To this end, we tested the effect of A β on the sensitivity of the cultures to hydrogen peroxide (H₂O₂) stresses. Hydrogen peroxide was chosen because it is generated during the very early stages of aggregation of the amyloid peptides [51] and is critically involved in AD pathogenesis [49,52]. At low concentrations (25–100 μ M), hydrogen peroxide did not cause cell death by itself; however, when co-administrated with A β oligomers (500 nM) it caused marked cell death (Fig. 4A, Fig. S1). These results indicate that neurons exposed to 500 nM A β are more vulnerable to other detrimental factors. We also showed that H₂O₂ neither stimulated α CaMKII phosphorylation by itself nor significantly enhanced the A β -induced phosphorylation (Fig. 4F), indicating that H₂O₂ did not activate the non-canonical pathway.

To determine the potential role of Wnt5a up-regulation in A β -induced sensitization to H₂O₂ stress, we neutralized Wnt5a in

media with specific anti-Wnt5a antibody. As shown in Fig. 4C, the antibody (0.5–8.0 μ g/ml) displayed a rescue effect on cell death in a concentration-dependent manner, which reached statistical significance after 4.0 μ g/ml. Recent studies identified a modified Wnt5a-derived hexapeptide (Box5) that can specifically antagonize Wnt5a in malignant melanoma cells [53]. Similar to the anti-Wnt5a antibody, Box5 also had a rescue effect on A β /H₂O₂-induced cell death in a concentration-dependent manner (Fig. 4E). Because Fz-5 is a Wnt5a receptor [54], we further tested the effect of anti-Fz5 antibody (against the extracellular cysteine-rich domain). Consistent with the findings with anti-Wnt5a antibody and Box5, the anti-Fz5 antibody also had a rescue effect (Fig. 4C). We further tested the effect of, anti-Wnt5a, anti-Fz5 antibodies and Box5 on the phosphorylation of α CaMKII, a molecular marker of the activation of the non-canonical Wnt/Ca²⁺ pathway. The results showed that all these reagents significantly blocked A β /H₂O₂-induced up-regulation of p- α CaMKII (Fig. 4G), and thus provide an independent molecular confirmation of the activity of anti-Wnt5a, anti-Fz5 antibodies and Box5. These results together strongly suggest that A β -induced Wnt5a up-regulation at least partially mediates A β neurotoxicity.

Exogenous Wnt5a potentiates A β neurotoxicity

Because A β -induced Wnt5a up-regulation is critical for A β neurotoxicity (Fig. 4C and E), we next investigated if exogenous Wnt5a can potentiate A β /H₂O₂-induced cell injury. To this end, we tested the effect of recombinant Wnt5a on A β /H₂O₂ neurotoxicity. As shown in Fig. 4B, compared with A β /H₂O₂-treated cultures, addition of purified recombinant Wnt5a protein (50–800 ng/ml) increased cell death in a concentration-dependent manner. This potentiation activity of Wnt5a was completely abolished when the protein was heat-inactivated (Fig. 4B). This observation indicates that the biochemical activity of Wnt5a rather than the mere presence of Wnt5a peptide is essential for the potentiation. In addition, Foxy5, a Wnt5a-derived hexapeptide (the same peptide as Box5 but differently modified) that can mimic Wnt5a-induced activities in malignant melanoma and in breast cancer cells [55], was also able to potentiate the neurotoxicity of A β /H₂O₂ (Fig. 4D). These results indicate that Wnt5a is sufficient to elicit neurotoxicity.

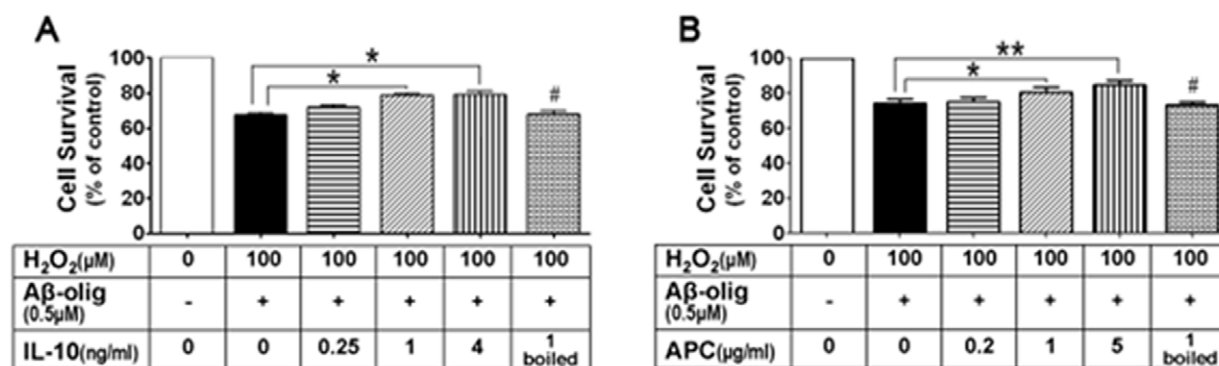


Figure 5. Anti-inflammatory factors alleviate A β neurotoxicity. **A.** Effects of IL-10 on cell viability of cortical cultures challenged with A β /H₂O₂ for 24 hrs. **B.** Effects of APC. Data (means \pm SEM) are from three independent experiments. * $p < 0.05$; ** $p < 0.01$; # $p < 0.05$ (vs. the group treated with active IL-10 (1 ng/ml) or APC (1 μ g/ml) in the presence of A β /H₂O₂). doi:10.1371/journal.pone.0022920.g005

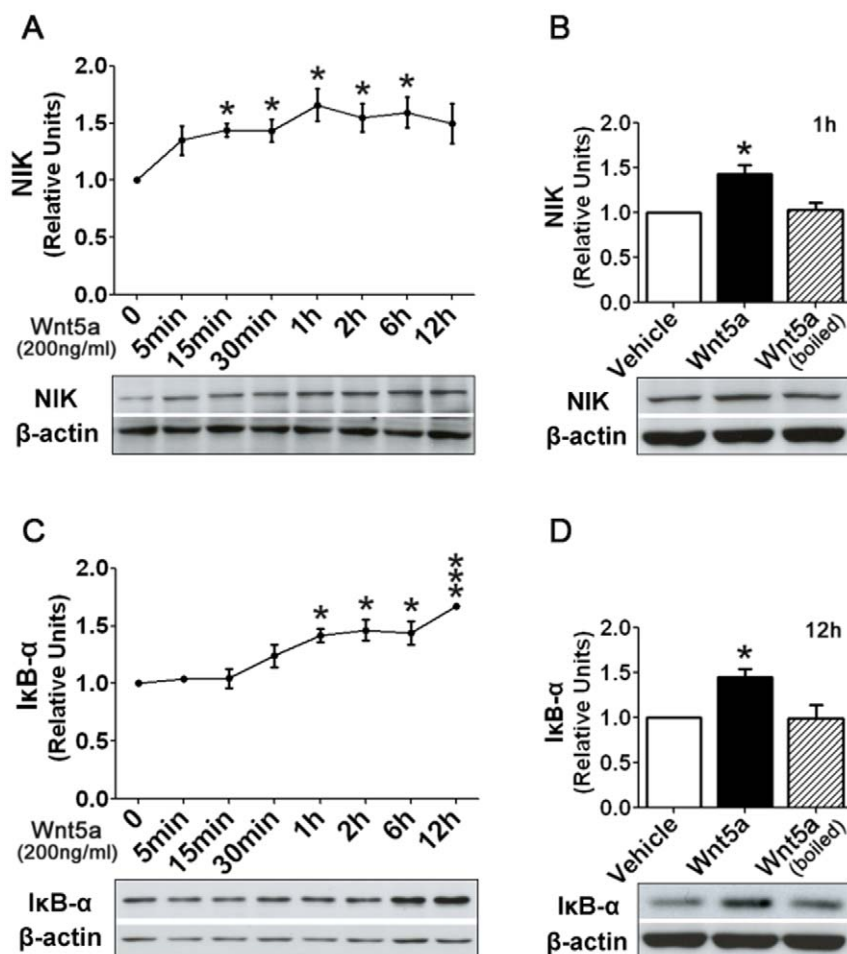


Figure 6. Wnt5a activates molecular pathways critical for inflammatory response. **A.** The time course of NIK protein dynamics after Wnt5a (200 ng/ml) treatment. **B.** Heat-inactivated Wnt5a cannot induce NIK. **C.** The time course of I κ B- α protein dynamics after Wnt5a (200 ng/ml) treatment. **D.** Heat-inactivated Wnt5a cannot induce I κ B- α protein. * $p < 0.05$; *** $p < 0.001$ (vs. 0 h in **A** and **C**, or vs. vehicle in **B** and **D**). doi:10.1371/journal.pone.0022920.g006

Anti-inflammation factors attenuate A β neurotoxicity

The results described above indicate that up-regulated Wnt5a may mediate A β neurotoxicity. We then sought to understand the mechanism by which Wnt5a contributes to A β neurotoxicity. Because recent studies revealed a critical role of Wnt5a in the regulation of inflammatory responses in peripheral systems, we were interested in determining if Wnt5a contributes to A β neurotoxicity by regulating inflammation. To this end, we first investigated the contribution of neuroinflammation to the A β -induced sensitization of cortical cultures to H₂O₂ stress. We found that IL-10, a prototypic anti-inflammatory cytokine, caused concentration-dependent rescue effects on A β toxicity (Fig. 5). IL-10 started to rescue A β /H₂O₂-induced cell death at 0.25 ng/ml and reached a plateau effect at 1 ng/ml (Fig. 5A). Heat-inactivated IL-10 did not have any rescuing effect (Fig. 5A). To further confirm the involvement of inflammation in the A β toxicity, we determined the effect of activated protein C (APC), another well established anti-inflammatory protein. The results showed that APC displayed a similar rescue effect as IL-10 (Fig. 5B). These results indicate that neuroinflammatory response likely contributes to A β neurotoxicity.

Wnt5a regulates proteins that involve in inflammatory response in cortical cultures

Recent studies reveal an important role of Wnt5a in control of inflammatory response in non-neuronal peripheral tissues [56,57,58], but its involvement in regulation of inflammatory response in nervous systems has not been reported. To investigate the potential contribution Wnt5a signaling to neuroinflammation, we examined the effect of Wnt5a on the protein expression of NF- κ B-inducing kinase (NIK), a key positive regulator of inflammation that controls NF- κ B activity by promoting the processing of p100, the inactive NF- κ B2 precursor, to produce the functional p52 subunit [59]. We observed that NIK protein began to increase 5 min after Wnt5a administration (200 ng/ml), and this increase continued gradually, peaked at 60 min, and stayed at a higher level afterward (Fig. 6A). Heat-inactivated Wnt5a did not induce a NIK increase (Fig. 6B), indicating that Wnt5a protein activates inflammatory pathways in cortical cultures. We also investigated the effect of Wnt5a on the expression of I κ B- α protein, an inhibitory regulator of inflammation response by sequestering NF- κ B in the cytosol [60]. Interestingly, Wnt5a also induced a gradual increase of I κ B- α (Fig. 6C). In contrast to the NIK increase, the I κ B- α up-regulation did not begin until 30–60 min after Wnt5a

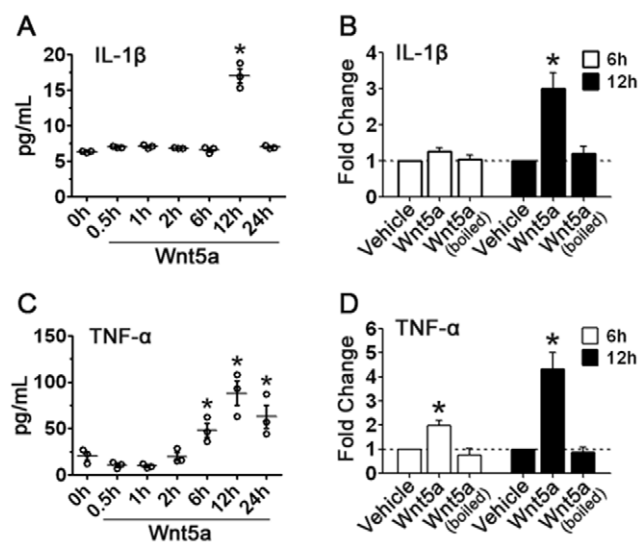


Figure 7. Wnt5a evokes cytokine release. **A.** The temporal profiles of IL-1 β in media of Wnt5a-treated cortical cultures measured by ELISA. **B.** Heat-inactivation abolished the activity of Wnt5a in eliciting IL-1 β release. **C.** The temporal profiles of TNF- α in media after Wnt5a treatment. **D.** Effects of heat-inactivation on the Wnt5a activity in eliciting TNF- α release. * $p < 0.05$ (vs. 0 h in **A** and **C**, or vs. vehicle in **B** and **D**).

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treatment but continued for the rest of the time that measurements were recorded (Fig. 6C). It is possible that the lag in up-regulation of I κ B- α functions to resolve the Wnt5a-initiated inflammatory response.

Inflammatory response culminates with the secretion of an array of inflammatory cytokines. If Wnt5a indeed induces the inflammatory response, specific inflammatory cytokines must be secreted after Wnt5a administration. Thus, we used ELISA to measure IL-1 β and TNF- α in culture media after Wnt5a treatment for 0.5, 1, 2, 6, 12, or 24 hrs. We observed that Wnt5a stimulation caused a 2.7-fold increase in IL-1 β at 12 h, while no change was detected at other time points (Fig. 7A). On the other hand, TNF- α increased significantly at 6 h (2.3 fold), 12 h (4.3 fold) and 24 h (3.1 fold) (Fig. 7C). However, this effect of Wnt5a was completely abolished when the protein was heat-inactivated (Fig. 7B and D). These results together strongly suggest that Wnt5a can elicit the inflammatory response in neuron cultures.

Blockage of Wnt5a signaling impairs A β -induced up-regulation of proinflammatory cytokines

Because A β evoked inflammation-mediated neurotoxicity, we next investigated the effect of Wnt5a signaling inhibition on the A β -elicited inflammatory response. As shown in Fig. 8A, A β oligomers (500 nM) significantly increased IL-1 β release (3.4 fold; $p < 0.05$). Box5 peptide (200 μ M), anti-Wnt5a (2 μ g/ml) or anti-Fz5 (2 μ g/ml) antibody suppressed the increase of IL-1 β induced by A β , by 58%, 59% and 55%, respectively (all 3 with $p < 0.05$). In contrast, Foxy5 (200 μ M), an agonist of Wnt5a, increased IL-1 β release (1.5 fold, compared with A β alone; $p < 0.05$; Fig. 8A). Box5, anti-Wnt5a and anti-Fz5 antibodies caused similar effects on A β -induced TNF- α release (Fig. 8B). These results together demonstrate that the suppression of Wnt5a signaling inhibits A β -evoked inflammatory responses in cortical neuron cultures.

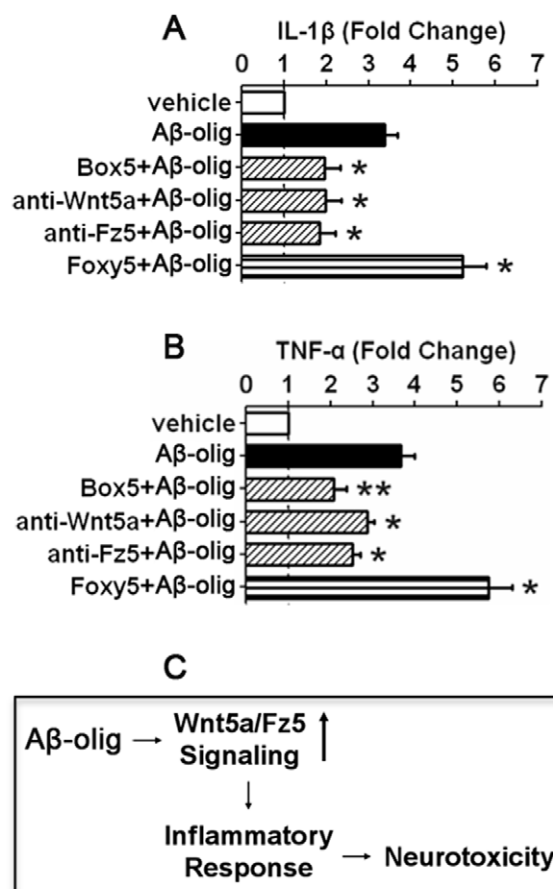


Figure 8. Wnt5a is critical for A β -elicited cytokine release. **A.** Inhibition of Wnt5a/Fz5 signaling impaired A β -evoked IL-1 β increase in media. **B.** Inhibition of Wnt5a/Fz5 signaling impaired A β -evoked TNF- α increase in media. ELISA assay of IL-1 β (**A**) and TNF- α (**B**) secreted in cortical cultures treated for 12 hrs with A β (500 nM) or A β and Box5 (200 μ M), anti-Wnt5a (2 μ g/ml) or anti-Fz5 (2 μ g/ml) antibody. Foxy5, a Wnt5a agonist, potentiates the cytokine release. * $p < 0.05$; ** $p < 0.01$ (vs. vehicle). **C.** A working model for the role of Wnt5a signaling in AD pathogenesis.

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Discussion

In this study, we found that non-canonical Wnt5a signaling is up-regulated in mouse brains prior to AD phenotypes and by A β peptide in cortical neuron cultures. The up-regulated Wnt5a signaling contributes to the inflammation-dependent A β neurotoxicity in cultures. We also found that Wnt5a up-regulates inflammation regulatory proteins and proinflammatory cytokines and that Wnt5a is required for the A β -induced proinflammatory cytokines. These observations collectively suggest the following working model (Fig. 8C): accumulation of A β in the brain aberrantly up-regulates Wnt5a signaling, which in turn evokes an inflammatory response that causes neurodegeneration or cell death in AD brains.

The observed up-regulation of Wnt5a signaling is probably an early etiologically relevant event during AD development. Both Wnt5a and Fz5 proteins significantly increase in the APP^{swe}/PSEN1 Δ E9 hippocampus at the age of 3.5 months (Fig. 2). Previous studies showed that this AD mouse model started to accumulate A β plaques after 4 months of age [61] and did not develop cognitive impairments until 5–7 months of age [44]. Thus,

the observed Wnt5a and Fz5 up-regulation at 3.5 months of age is likely prior to the development of major AD phenotypes. This notion is consistent with the finding that a relatively low concentration of A β is able to up-regulate Wnt5a and Fz5 (Fig. 3), suggesting that Wnt5a signaling is a potential target for slowing or blocking early AD pathogenesis.

Converging lines of evidence support a critical role of the down-regulation of the canonical Wnt/ β -catenin pathway in AD pathogenesis. In contrast, the involvement of non-canonical Wnt signaling is less clear. Our findings reveal an early up-regulation of Wnt5a signaling in the hippocampus of 2 \times Tg AD mice. Etiological significance of this dysregulation is suggested by the observation that Wnt5a signaling is necessary for A β to fully induce neurotoxicity in cortical cultures. Previous studies demonstrated that down-regulation of canonical signaling contributed to A β neurotoxicity [27,30,34,62]. It is possible that A β causes parallel up-regulation of the non-canonical Wnt signaling and down-regulation of the canonical signaling to initiate neurotoxicity cascades. The Wnt canonical and non-canonical pathways often antagonize one another [63]. Thus, another possible scenario is that A β may directly down-regulate the canonical pathway, as suggested by a recent study [33], which consequently causes the up-regulation of the non-canonical pathway.

Our results reveal a neurotoxic activity of Wnt5a signaling, and this Wnt5a activity contributes to A β toxicity in neuron cultures. Cerpa et al. recently reported that acute administration of exogenous Wnt5a (500 nM) prevented A β -induced synaptotoxicity within 40 min after A β oligomer application [36]. Their results indicate a synapto-protective activity of Wnt5a signaling soon after A β exposure. Because A β -up-regulated Wnt5a does not occur by 1 hour after A β treatment in cultures (Fig. 3H) and 500 nM A β itself does not induce obvious cell death in this period (Fig. 4A and S1), we reason that basal Wnt5a has a synapto-protective activity. On the other hand, sustained up-regulation of Wnt5a, which occurs at 2 hours after A β treatment (Fig. 3H), probably potentiates neurotoxicity.

We further found that activation of Wnt5a signaling stimulates the expression of proinflammatory cytokine in cortical cultures

(Figs. 6 and 7). This finding indicates that up-regulation of Wnt5a may mediate A β -induced neuroinflammation in AD brains (Fig. 8C). Because the A β -elicited inflammatory response (Fig. 8A, 8B) and alleviated A β -induced neurotoxicity (Fig. 4) was impaired by the anti-Wnt5a antibody and Box5, A β likely induces Wnt5a secretion, although the kinetics of the secretion is currently unknown. In peripheral non-neuronal systems, Wnt5a is implicated in inflammation of multiple chronic disorders, including rheumatoid arthritis [64], sepsis [56], atherosclerosis [65], melanoma [66], and psoriasis [67]. Our results provide the initial evidence for a critical role of Wnt5a signaling in the regulation of inflammatory responses in CNS disorders. Because the primary cortical cultures used in this study contain neurons and glia (including microglia and astrocytes), we currently do not know the specific type of glial cells through which Wnt5a evokes the observed inflammatory responses. In a recent study, Halleskog et al. reported that Wnt3a stimulated the expression of proinflammatory cytokines in microglia [68]. It would be interesting to know if Wnt5a regulates neuroinflammation by stimulating the same or different types of glia. Nonetheless, the findings on Wnt5a and Wnt3a indicate that proteins in the Wnt family may orchestrate neuroinflammatory response during AD pathogenesis.

Supporting Information

Figure S1 Cell survival rates revealed by trypan blue staining. Cortical cultures at 24 hrs after indicated treatments were used. Dying cells were stained due to the increase of membrane permeability to the dye. (TIF)

Author Contributions

Conceived and designed experiments: S-JT. Performed the experiments: BL LZ XY. Analyzed the data: BL LZ S-JT. Contributed reagents/materials/analysis tools: LZ MH TA. Wrote the manuscript: BL S-JT.

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