Adiponectin gene therapy for Alzheimer disease in a mouse model: abridged secondary publication

RCL Ng, M Jian, LW Yick, M Bunting, JSC Kwan, SK Chung, KH Chan *

KEY MESSAGES

1. Liver-specific expression of trimeric adiponectin can cross the blood-brain barrier.
2. Overexpression of trimeric adiponectin can improve memory function.
3. Overexpression of trimer adiponectin enhances neuronal insulin sensitivity and reduces amyloid-β deposition.
4. Adiponectin suppresses Aβ-induced microglia activation and neuroinflammatory responses, exerting protective effects to neurons and synapses in an Alzheimer disease mouse model.
5. Adiponectin deficiency exacerbates microglia-mediated neuroinflammation in an Alzheimer disease mouse model.

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[1] RCL Ng, [1] M Jian, [1] LW Yick, [1] M Bunting, [1] JSC Kwan, [2] SK Chung, [1] KH Chan

LKS Faculty of Medicine, The University of Hong Kong:
[1] *Department of Medicine*
[2] *School of Biomedical Sciences*

* Principal applicant and corresponding author: koonho@hku.hk

Introduction

Alzheimer disease (AD) is the most common cause (>60% cases) of dementia in older adults. Pathological studies of brains from AD patients have revealed extracellular accumulation of senile plaques containing amyloid-β fibrils in the neocortex and hippocampus. Amyloid-β peptides are derived from the cleavage of amyloid precursor protein. Amyloid-β-mediated toxicity includes oxidative stress secondary to excessive production of reactive oxygen species, activation of microglia and astrocytes that secrete proinflammatory cytokines by activating nuclear factor-kappa B (NF-κB) pathway, and synaptic dysfunction and loss resulting in cognitive impairments.

Adiponectin (APN) is a serum adipokine secreted predominantly by adipocytes, which possesses insulin-sensitising, anti-inflammatory, and anti-oxidative properties. Low-molecular-weight APN trimers, hexamers, and possibly globular APN are believed to be able cross the blood-brain barrier. We have reported that chronic APN deficiency in aged mice results in cerebral insulin resistance associated with AD-like pathologies and cognitive impairments.[1] Insulin-sensitising and anti-inflammatory effects of APN should be beneficial for AD characterised by cerebral insulin resistance, neuroinflammation, and oxidative stress. We hypothesise that APN is protective against amyloid-β-mediated neurodegeneration in AD. In this project, we investigated the therapeutic effects of trimeric APN on amyloid-β-mediated pathologies in a 5xFAD mouse model, using an adeno-associated virus (AAV)-mediated delivery method.

Method

Mouse APN cDNA with FLAG epitope (Origene, USA) was subcloned into AAV vector (AAV2/8-eGFP) under control of apolipoprotein E promoter replacing eGFP sequence. Mutation in mouse APN gene C39S (cysteine to serine) ensured that only trimeric APN was produced by the transduced cells. The vector was packaged by triple transfection of HEK293 cells with either pAAV2/8-eGFP or pAAV2/8-APN-Flag, p5E18-VD2/8, and adenoviral helper plasmid pXX6. Vector particles were purified from cell lysate by polyethaleneglycol-ammonium sulphate method.

Female transgenic mice (5xFAD) carrying overexpress mutant human amyloid precursor protein and human PS1 harbouring were used as the animal model of AD. 5xFAD mice aged 4 months received AAV-APN gene therapy via intravenous injection (via tail vein) with purified AAV at a dosage of 1×10^{11} vector genomes. Four months after AAV injection. Behavioural tests including Morris water maze test and openfield test were performed to investigate the spatial learning and memory functions as well as anxiety levels (Fig. 1a). Each group contained at least 10 mice for behavioural test. Mice were then dissected, and the brain, plasma, and liver collected.

For immunohistochemistry, brain sections (frontal cortex) were studied for amyloid-β with antibodies against amyloid-β residues 17-24 (clone 4G8), microglia with Iba1 antibody, and astrocytic

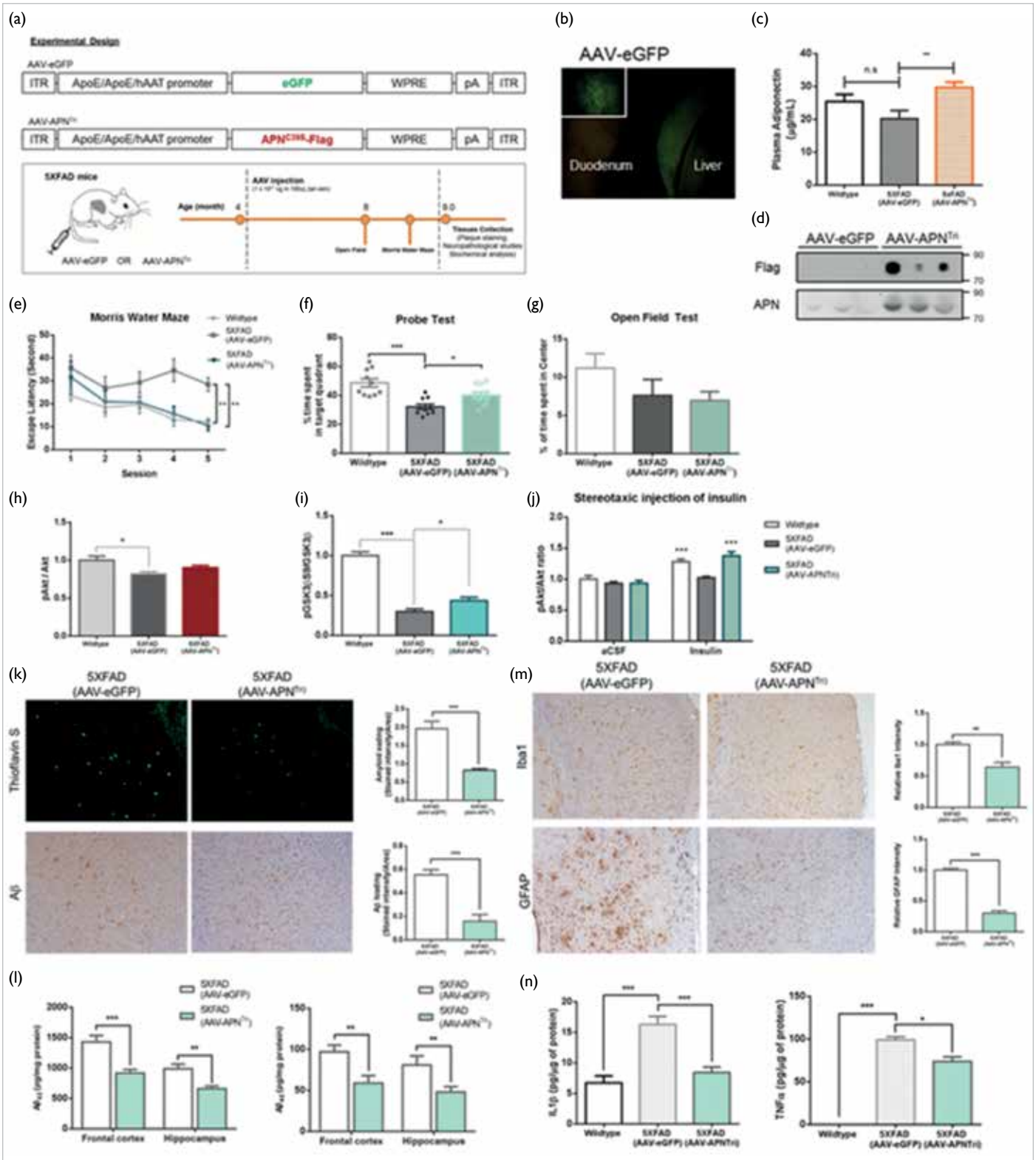

FIG 1. Overexpressing trimeric adiponectin by liver-specific adeno-associated virus (AAV) delivery improves memory functions and Alzheimer disease (AD) pathologies in an AD mouse model. (a) Schematic of experimental design. (b) Representative image showing eGFP expressed in the liver after AAV-eGFP delivery. (c) ELISA analysis of plasma adiponectin. (d) Western blot analysis of plasma trimeric adiponectin. (e) Escape latency of Morris water maze test. (f) Percentage of time spent in the target quadrant of the probe test in the Morris water maze. (g) Percentage of time spent in the centre region of the open field test. Densitometric analysis of Western blotting on the (h) pAkt/Akt and (i) pGSK3β/GSK3β in the hippocampus of the mice and on the (j) pAkt/Akt ratio after stereotaxic injection. (k) Immunostaining of amyloidβ (Aβ) using thioflavin S and anti-Aβ antibodies. (l) ELISA analysis of Aβ40 and Aβ42 in the frontal cortex and hippocampus. (m) Immunohistochemistry analysis of Iba1 and GFAP. (n) ELISA analysis of TNFα and IL1β Aβ42 in the frontal cortex and hippocampus.

activation with GFAP antibody. Thioflavin S fluorescent staining was performed to further confirm amyloid deposition. For immunoblotting, tissues lysates or cell lysates were studied by standard SDS-PAGE and western blotting for detecting proteins of interest using primary antibodies and secondary antibodies. Chemiluminescent signal was developed using Westernbright Quantum HRP substrate.

To study hippocampal insulin sensitivity, mice with AAV injections after 8 weeks were anaesthetised and subjected to intracerebral injection of insulin. Human insulin (0.02 IU/g bodyweight of mice) was injected to the right hippocampus by stereotaxic injection platform. The hippocampi were dissected 30 minutes after injection, and protein lysate collected for pAkt detection.

Murine BV2 microglia cell line and HT-22 hippocampal neuronal cells were used. BV2 cells and HT-22 cells were cultured in Dulbecco modified Eagle's medium with 10% foetal bovine serum and 1% penicillin/streptomycin. The cells were grown in a humidified incubator at 37°C with 5% CO_2. BV2 cells were pretreated with APN (10 μg/mL) or compound C (10 μM) for 2 hours and then treated with amyloid-β Oligomer (AβO) [10 μM] for 24 h in serum-free culture medium. To knockdown APN receptors in BV2 microglia, AdipoR1 and AdipoR2 siRNAs and non-targeting control siRNA were transfected to BV2 cells.

Cell viability were performed after co-culturing BV2 and HT22 cells using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide. The absorbance of the solution in each well was determined at 570 nm using a CLARIO star microplate reader. Amyloid-β40 and amyloid-β_{42} in the soluble brain lysates were measured using the human amyloid-β40 and human amyloid-β_{42}, respectively. IL1β and TNFα levels in the brain lysates were measured using ELISA. The assays were performed following supplier instructions. The optical density of each well at 450 nm was determined by a CLARIO star microplate reader.

Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software) or SPSS (Windows version 24; IBM Corp, Armonk [NY], US). For Morris water maze test, dataset was analysed by two-way ANOVA. Open field test was analysed by one-way ANOVA. In other experiments, between-group differences were determined with one-way ANOVA, followed by Bonferroni post hoc test. Alternatively, the mean significant difference between two groups was determined with two-tailed unpaired Student t test. Statistical significance was defined as $P<0.05$.

Results

GFP signal was detected in the liver but not in other tissues 1 month after injecting AAV-eGFP virus (Fig. 1b). ELISA analysis indicated that the plasma APN level was significantly increased in 5xFAD mice injected with AAV-APN^{Tri} APN 2 months after injection ($P<0.01$, Fig. 1c). Western blot analysis by anti-flag and anti-APN antibodies indicated that trimeric APN (~74 kDa) level was increased in plasma (Fig. 1d).

In the Morris water maze test, AAV-APN^{Tri}-treated 5xFAD mice showed shorter escape latency significantly compared with that of AAV-eGFP-treated 5xFAD mice, and the escape latency was comparable to that of the wildtype littermates (Fig. 1e). In the probe test, 5xFAD mice injected with AAV-APN^{Tri} spent significantly more time in the target quadrant than control 5xFAD mice (Fig. 1f). In the open field test, treatment with AAV-APN^{Tri} could not reduce the anxiety level in 5xFAD mice (Fig. 1g). These results demonstrated that overexpressing trimeric APN can improve memory and spatial learning performance but not psychiatric symptoms in the AD mouse model.

Western blot analysis demonstrated that AAV-APN^{Tri} treatment insignificantly increased hippocampal Akt phosphorylation and moderately increased GSK3β^{S9} phosphorylation (Figs. 1h & 1i). Phosphorylation of Akt in wildtype mice was increased after insulin injection, whereas that of AAV-eGFP-treated 5xFAD mice showed no significant change. However, the induction of Akt phosphorylation was rescued in 5xFAD with AAV-APN^{Tri} treatment indicating that trimeric APN restores insulin sensitivity in the hippocampus of 5xFAD mice (Fig. 1j).

Next, we examined the effect of AAV-APN^{Tri} on amyloid-β levels in the 5xFAD mice. AAV-APN^{Tri} displayed significant reduction of amyloid and amyloid-β deposition in thioflavin S staining (Fig. 1k) and immunohistochemistry analysis (Fig. 1m). ELISA analysis also supported that amyloid-β40 and amyloid-β42 peptides were significantly reduced in the frontal cortex and hippocampus of 5xFAD mice after treating with AAV-APN^{Tri} (Fig. 1l).

Iba1 levels in the frontal cortex of 5xFAD mice were reduced by >40% with AAV-APN^{Tri} treatment. Immunohistochemistry staining of GFAP in the frontal cortex showed AAV-APN^{Tri} treatment dramatically reduced GFAP levels by ~70% indicating reduction of astrogliosis. Reactivated microglia secretes proinflammatory cytokines in AD brains causing neuroinflammation. Both the levels of TNFα and IL1β increased in the cortex and hippocampus of 5xFAD mice after AAV-APN^{Tri} treatment (Fig. 1n). These results indicated that trimeric APN treatment could suppress the amyloid-β-mediate neuroinflammatory responses in the AD mouse model.

We then studied if APN directly inhibits microglia activation and the molecular mechanism of

suppressing the cytokines secretion under amyloid-β stimulation (Fig. 2). AdipoR1 and AdipoR2 were found in BV2 microglia cell line and microglia cell in vivo. Reverse transcriptional PCR and western blot analysis were performed to detect mRNA and proteins, respectively. AβO induced the release of TNFα and IL-1β from BV2 cells, whereas APN inhibited the release of TNFα and IL-1β. In addition, $pAMPK^{T172}$ level was decreased in AβO-treated BV2 cells. In contrast, pretreating APN rescued the reduction of $pAMPK^{T172}$ level upon AβO-treatment. NF-κB p65 level in nuclear extracts was markedly increased in AβO treated BV2 cells, whereas APN pretreatment significantly reduced nuclear NF-κB p65 level. These results were further supported by the addition of compound C, which blocked AMPK phosphorylation. Together, these data suggest that APN inhibits AβO-induced proinflammatory cytokines through AMPK- NF-κB signalling cascade in microglia.

AdipoR1 and AdipoR2 expressions were significantly inhibited by siRNA at the dose of 100 nM. The ability of APN to suppress proinflammatory cytokines TNFα and IL-1β secretion from AβO-treated BV2 cells was abolished by AdipoR1 siRNA-transfections, but not by AdipoR2 siRNA-transfection. These data indicate that APN suppressed AβO-induced microglial proinflammatory cytokines release through AdipoR1.

To further investigate if APN could protect against neuronal toxicity induced by AβO-activated microglia, we used a transwell system to co-culture BV2 cells and HT-22 cells. The viability of HT-22 hippocampal cells co-cultured with BV2 microglia cells treated with AβO and APN was significantly increased. The presence of compound C blocked the neuroprotective effect of APN. Altogether, these results further reinforce that APN protects neuronal survival by suppressing microglia activation through AMPK activation.

We found that lacking APN increased cortical and hippocampal TNFα and IL1β levels, and that APN deficiency increased microgliosis in the cortex and hippocampus with the increased levels of microglia associated TNFα and IL1β expression (Fig. 3). These results suggest that APN deficiency results in increased activation and neuroinflammatory response of microglia in AD.

Discussion

We demonstrated the therapeutic potential of overexpressing trimeric APN using the AAV delivery method. Trimeric APN expressed in the liver cells can raise the trimeric APN level in plasma. By crossing the blood-brain barrier, trimeric APN improves memory functions and reduce amyloid-β deposition. Most importantly, we demonstrated the insulin-sensitising and anti-inflammatory effects of trimeric APN to central nervous system. Trimeric APN showed potent effects to treat AD.

One study reported the therapeutic effects of osmotin, plant APN homologue, to treat AD in the amyloid transgenic mouse model. Similarly, daily injection of osmotin can reduce amyloid pathologies by inhibiting the level of β-secretase, an enzyme which generates amyloid-β. AD mice treated with osmotin also showed improved neuronal functions and restored dendritic spine density in the hippocampus and reversed cognitive impairments.[2,3] Application of osmotin and trimeric APN demonstrated great potential to treat AD by activating APN signalling and its downstream effectors.[4] We further provide evidence to support activating APN signalling in central nervous system can reduce neuroinflammatory responses. Our method also demonstrated better clinical application with single injection (with effects last for months), compared with daily osmotin injection.

Overexpression of neurotrophic factors, hormones, or growth factors demonstrated great promises to protect neurons. However, injection of recombinant proteins is costly. Most of them such as brain-derived neurotrophic factor and glia-derived neurotrophic factor are not blood-brain barrier permeable requiring intracranial injection of AAV to overexpress these factors.[5] Intracerebral injection is an invasive method and may lead to traumatic brain injury. Although these factors have therapeutic potential, the method is still not applicable to treat neurodegenerative disease owing to the invasive drawbacks. Liver-specific AAV transduction can overexpress target protein to treat neurodegenerative disease. This method can be applied to overexpress other growth factors such as FGF21, which can cross the blood-brain barrier and has been recognised as neuroprotective agent.

Further investigation should include injection of AAV-APNTri to transgenic Tau^{P301L} mice or 3xTg mice to determine the efficacy of APN in suppressing tau-mediated pathologies, and generation of AD patient-specific iPSC-derived neurons to determine the therapeutic efficacy of human trimeric APN to human neurons. Moreover, before clinical trials, we should generate $AAV\text{-}APN^{Tri}$ (carrying human APN sequence) vector and proceed to large production of $AAV\text{-}hAPN^{Tri}$ particles.

Conclusion

AAV-trimeric-APN can reverse memory impairments and improve neuropathology in AD mice. Trimeric APN reduces amyloid-β deposition but the mechanism has not been identified yet in this study. Most importantly, trimeric APN exerts neuroinflammatory effects by inhibiting microglia reactivation but not enhances neuronal insulin

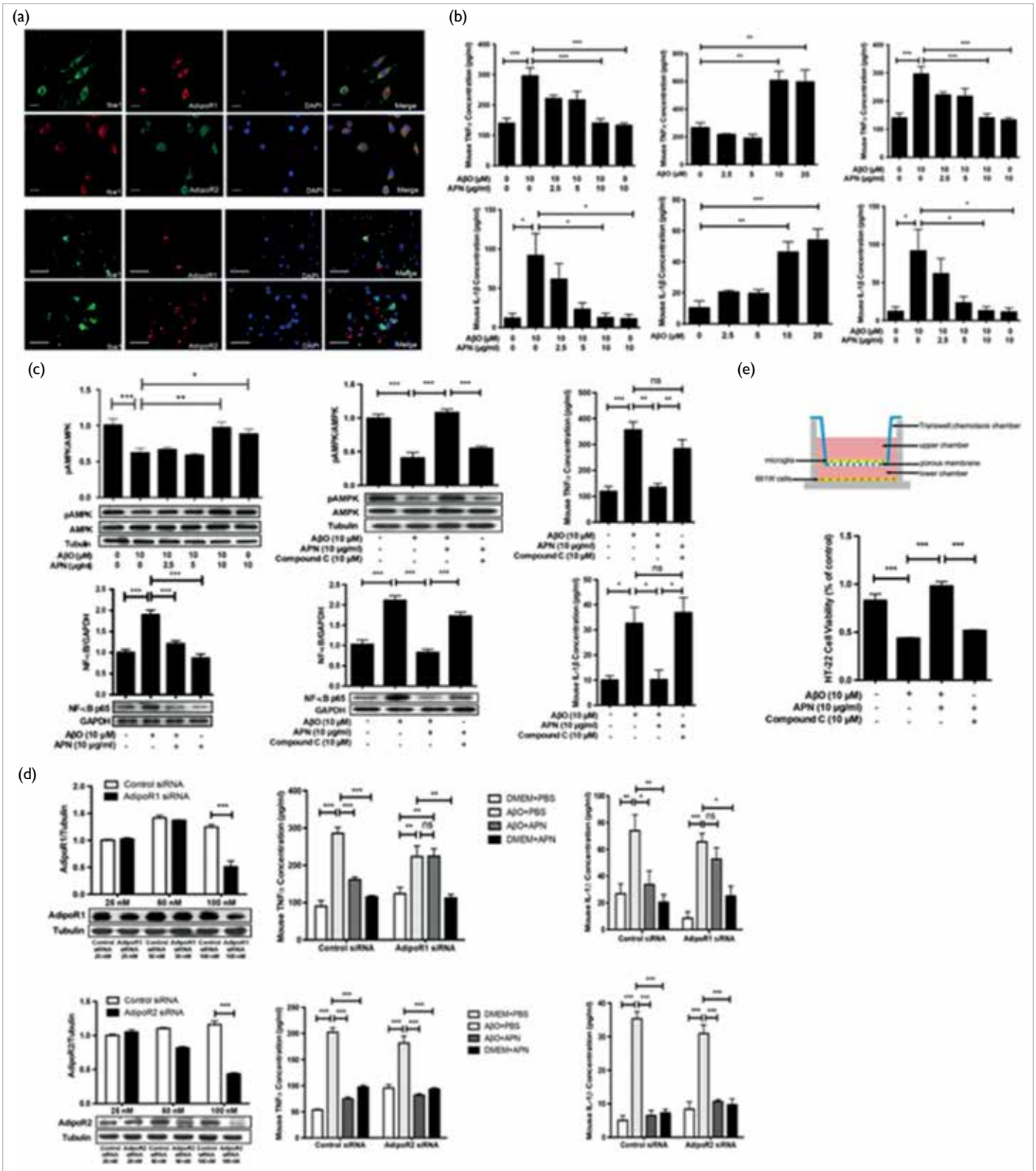

FIG 2. Adiponectin (APN) suppresses amyloid-β (Aβ)-induced microglia activation and proinflammatory cytokines secretion to protect neurons from neuroinflammatory toxicity through AdipoR1-AMPK-NF-κB signalling cascade. (a) Immunofluorescent staining of AdipoR1 and AdipoR2 in the BV2 cells and microglia in mice brain. (b) ELISA assay of TNFα and IL1β of medium after AβO stimulation with or without APN pretreatment. (c) Western blot analysis of pAMPK/AMPK and nuclear NF-κB levels in the AβO-exosed BV2 cells with or withour APN and compound C pretreatment. (d) ELISA assay of TNFα and IL1β of medium in the adipoR1- and adipoR2-knockdown BV2 cells exposed to AβO stimulation with or without APN preteatment. (e) MTT assay and survival analysis after co-culturing BV2 and HT22 cells.

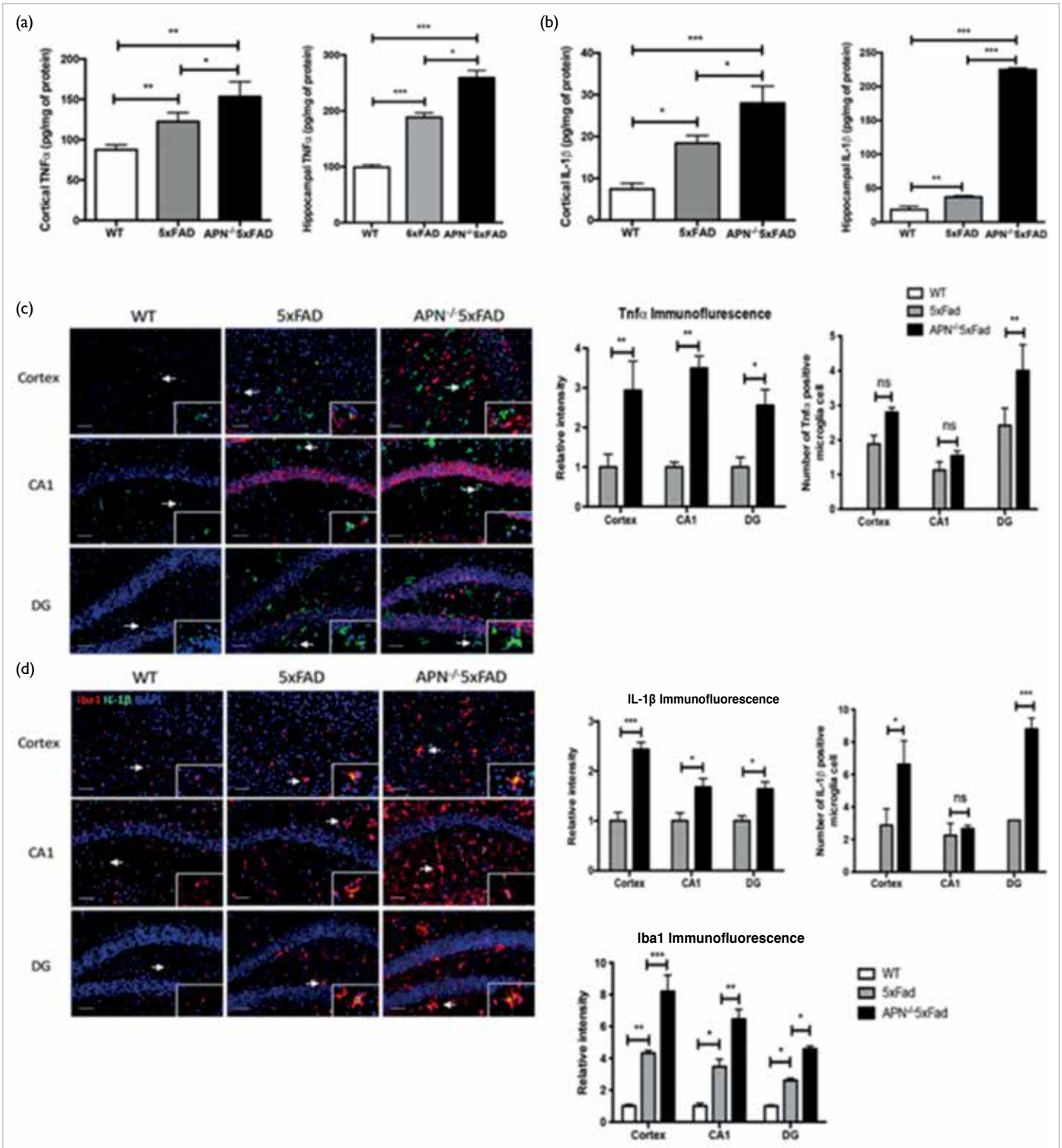

FIG 3. APN deficiency increases microgliosis and proinflammatory cytokines expression in the cortex and hippocampus in 5xFAD mice. (a & b) ELISA analysis of TNFα and IL1β levels in the cortex and hippocampus. (c & d) Double immunofluorescent staining of TNFα or IL1β and Iba1β in the cortex and hippocampus.

sensitivity. It suppresses microglial activation through AdipoR1/AMPK/NF-κB signalling. Reduction of APN in 5xFAD mice increases microglial activation along with dramatic elevation of TNFα and IL1β levels in both the cortex and hippocampus. These support the hypothesis that APN is essential to regulate microglia activation, and trimeric APN has potential therapeutic effects to treat AD.

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Disclosure

The results of this research have been previously published in:

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