

RESEARCH ARTICLE

Monomeric C-reactive protein induces the cellular pathology of Alzheimer's disease

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Abstract

Introduction: Human study shows that elevated C-reactive protein (CRP) in blood impacts apolipoprotein E (APOE) $\epsilon 4$, but not APOE $\epsilon 3$ or APOE $\epsilon 2$, genotype to increase the risk of Alzheimer's disease (AD). However, whether CRP is directly involved in cellular AD pathogenesis and in which type of neuronal cells of APOE $\epsilon 4$ carriers are unknown.

Methods: We aimed to use different primary neuronal cells and investigate if CRP induces cellular AD pathology depending on APOE genotypes. Here the different primary neuronal cells from the different APOE genotype knock-in mice cortex were isolated and used.

Results: Monomeric CRP (mCRP) increased amyloid beta production and, in parallel, induced tau phosphorylation in addition to their related proteins in the primary neurons in a pattern of APOE $\epsilon 4 > APOE \epsilon 3 > APOE \epsilon 2$ in a dose- and time-dependent manner. Consistently, mCRP induced the staining of other neurodegenerative biomarkers, including Fluoro-Jade B stain (FJB), TUNEL and Cleaved Caspase-3, in primary neurons in a similar pattern of APOE $\epsilon 4 > APOE \epsilon 3 > APOE \epsilon 2$. In contrast, pentameric CRP (pCRP) had a tendency to induce cellular AD pathology but did not reach statistical significance. On the other hand, it is intriguing that regardless of APOE genotype, mCRP did not influence the expressions of Iba-1 and CD68 in primary microglia or the expression of glial fibrillary acidic protein in primary astrocytes, and additionally mCRP did not affect the secretions of interleukin (IL)-1 α , IL-1 β , and tumor necrosis factor α from these cells.

Discussion: This is the first report to demonstrate that mCRP directly induces cellular AD pathogenesis in neurons in an APOE genotype-dependent pattern, suggesting that mCRP plays a role as a mediator involved in the APOE $\epsilon 4$ -related pathway for AD during chronic inflammation.

KEYWORDS

Alzheimer's disease, apolipoprotein E (APOE), astrocyte, microglia, monomeric C-reactive (mCRP), synapse, tauopathy

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Highlights

- Pentameric C-reactive protein (pCRP) can be dissociated irreversibly to form free subunits or monomeric CRP (mCRP) during and after the acute phase.
- mCRP increased amyloid beta production in the primary neurons in a pattern of apolipoprotein E (APOE) $\epsilon 4 > APOE \epsilon 3 > APOE \epsilon 2$ in a dose-dependent manner.
- mCRP induced the expression of phosphorylated tau in the primary neurons in a pattern of APOE $\epsilon 4 > APOE \epsilon 3 > APOE \epsilon 2$ in a dose- and time-dependent manner.
- mCRP plays an important mediator role in the APOE $\epsilon 4$ -related pathway of Alzheimer's disease risk.

1 | BACKGROUND

Apolipoprotein E (APOE) $\epsilon 4$ allele is the major genetic risk factor for late-onset Alzheimer's disease (AD),¹ but not all APOE $\epsilon 4$ carriers develop AD, even among those who reach > 90 years of age.² Our study used the data from the Framingham Heart Study (FHS) offspring cohort and found that chronically elevated C-reactive protein (CRP) impacts the risk of AD only in APOE $\epsilon 4$, but not in APOE $\epsilon 3$ and APOE $\epsilon 2$, carriers;³ another study showed that CRP is associated with the biomarkers of tauopathy in cerebrospinal fluid (CSF) only in APOE $\epsilon 4/\epsilon 4$ carriers.⁴ However, it is unclear whether CRP is directly involved in cellular AD pathogenesis in the presence of APOE $\epsilon 4$. As APOE $\epsilon 4$ leads to blood-brain barrier (BBB) dysfunction and leakage,⁵ it is possible that the leaked peripheral inflammatory factors like CRP directly act on neuronal cells in the brain.

CRP is widely known as the prototypic acute phase reactant, a hepatically produced protein which increases markedly in the blood within hours of any trauma or disease event that causes tissue damage.⁶⁻⁸ As CRP levels increase with age,^{9,10} there are two forms of CRP: (1) native CRP (pCRP), a pentameric homoprotein that is produced during active inflammatory disease¹¹ and (2) during and after the acute phase, pCRP can be dissociated irreversibly to form free subunits or monomeric CRP (mCRP), which has much lower aqueous solubility and damages tissues.¹² mCRP is shown to play a role in the pathogenesis of peripheral chronic diseases including cardiovascular diseases,¹³ age-related macular degeneration,¹⁴ and post-stroke inflammation.¹⁵

As the BBB prevents the passage of peripheral neurotoxic molecules into the brain,¹⁶ several studies suggest that the BBB is damaged in the AD brain.^{17,18} Thus, it is possible that the damaged BBB provides a mechanism for peripheral CRP proteins, especially mCRP dissociated and released during chronic peripheral inflammation, to enter into the brain during chronic peripheral inflammation and directly are involved in AD pathogenesis in APOE $\epsilon 4$ carriers. One study directly injected mCRP into the hippocampus region of an AD mouse model, 3XTg, and found it increased the AD pathology including tauopathy and amyloid pathology.¹⁹ Because 3XTg mice have underlying AD pathology in the

brain through a genetic engineering technique for generating transgenic mice of AD, it is still unclear if mCRP can directly induce AD pathogenesis or if it enhances the AD pathology formation in the presence of transgenic mice with amyloids and tauopathy. In this study, we used primary cultures from APOE genotype knock-in mice and treated them with mCRP versus pCRP to investigate if and which species of CRP can induce cellular AD pathology in the background of different APOE genotypes.

2 | MATERIAL AND METHODS

2.1 | Primary neuronal cultures

Primary mouse neuronal cultures were prepared from cortical tissue obtained from the brains of postnatal 0 to 2-day-old (P0-P2) wild-type (WT) and three APOE genotype mouse pups as described by Beaudoin et al.²⁰ with some modifications. Early postnatal mouse cortex was dissected, incubated in 0.25% trypsin (Gibco, #15090-046) with DNase I Solution (Stemcell, #7900; 1:100) for 15 minutes, and dispersed by trituration in Neurobasal medium (Invitrogen, #21103-049) with 2% B-27 supplement (Gibco, #17504-044), 2 mM L-Glutamine (Invitrogen, #25030-081), 0.3% glucose (Sigma-Aldrich, #G6152) and 5% FBS (fetal bovine serum; Gibco, #10438-026). Dissociated cells were plated onto 24-well plates precoated with 0.1 mg/mL poly-D-Lysine (Sigma-Aldrich, #P-9155) at a density of 1×10^4 cells/cm². Cells were maintained in Neurobasal medium with 2% B-27 supplement, 2 mM L-glutamine, 0.3% glucose, 100 U/mL penicillin/streptomycin (Gibco, #15140122) and incubated in 5% CO₂ at 37°C. The medium was changed twice a week. Experiments were carried out at 14 days in vitro (DIV).

In the experiments described herein, focused attention was placed on the two main CRP isoforms, pCRP or mCRP. pCRP is CRP measured in blood as a diagnostic index of inflammation. This CRP was purchased from Lee Bio. The mCRP isoform used was a recombinant protein which is described in detail in Potempa et al.²¹ to provide substantial evidence and validation that the recombinant mCRP protein is a useful,

RESEARCH IN CONTEXT

- 1. Systematic Review:** Our previous study found that elevated C-reactive protein (CRP) changes the risk of Alzheimer's disease (AD) only in apolipoprotein E (APOE) $\epsilon 4$ carriers. However, it is unknown whether CRP is directly involved in cellular AD pathogenesis when APOE $\epsilon 4$ is present.
- 2. Interpretation:** This is the first study to demonstrate that monomeric CRP directly induces cellular AD pathogenesis in an APOE genotype-dependent pattern using primary cultures from APOE genotype knock-in mice. The data obtained from this study suggest that CRP plays an important mediator role in the APOE $\epsilon 4$ -related pathway of AD risk.
- 3. Future directions:** Reducing CRP levels, especially in two APOE $\epsilon 4$ allele carriers, is needed to develop novel drug treatment of AD.

equivalent reagent for the biologically produced mCRP protein. On day 14 of culture, *E. coli* recombinant mCRP or human blood purified pCRP (LeeBio, #140-11A) was added in cell culture medium followed by cell incubation.

2.2 | Primary astrocyte and microglial cultures

Primary mouse astrocyte and microglial cultures were prepared from cortical tissue obtained from the brains of postnatal 0 to 2-day-old (P0–P2) WT and three APOE genotype mouse pups as described previously^{22,23} with some modifications. Briefly, early postnatal mouse cortex was dissected and mechanically dissociated. The cells were seeded into 75 cm² flasks in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12; Gibco, #11320033) supplemented with 10% FBS and 10% HS (horse serum; Gibco, #26050-088) and 100 U/mL penicillin/streptomycin and incubated in 5% CO₂ at 37°C.

For astrocyte cultures, the medium was changed twice a week. When astrocytes were confluent at 7 to 8 days, the mixed glial cultures were treated with 10 μ M cytosine-d-arabino-furanoside (Ara-C; Sigma Aldrich, #C1768) for 48 hours to prevent proliferation of other cell types. The astrocyte monolayer was then washed with phosphate-buffered saline (PBS) and split into two 75 cm² flasks in DMEM/F12 supplemented with 5% FBS and 5% HS and 100 U/mL penicillin/streptomycin. The cells were incubated in 5% CO₂ at 37°C and the medium was changed twice a week. After 12 to 14 days, the cells were reseeded onto 24-well plates at 2×10^5 cells/ and ready for treatment the next day.

For microglial cultures, the medium was changed weekly. After incubation for 14 to 16 days, microglia were isolated from the mixed glial

cultures by shaking at 180 rpm for 1 hour at 37°C. Detached cells were collected by centrifuging at 430 g for 6 minutes at 4°C, plated onto 24-well plates at 2×10^5 cells/cm², and incubated in 5% CO₂ at 37°C. Cultures were ready for treatment after 24 hours.

2.3 | Fluorescent immunocytochemistry

Primary mouse neurons, astrocytes, or microglia cultured in coverslips were washed using 1XPBS and fixed with 4% paraformaldehyde for 15 minutes. Primary cells were then permeabilized with 0.05% Triton X-100 and non-specific sites were blocked with 10% donkey serum for 1 hour at room temperature. Later, the cells were incubated individually with primary antibodies overnight. After washing, secondary antibodies were stained with conjugated Alexa Fluor 488, 594 (Thermo Fisher Scientific, #A21202, #A21208, #A21207; 1:1000) for 1 hour at room temperature. Cells were counterstained with DAPI (4', 6-diamidino-2-phenylindole, dihydrochloride) for nuclear staining. The stained cells were observed under fluorescence microscopy (Carl Zeiss, Germany). All of the images were taken in the same conditions and replicated three times.

Several primary antibodies were used. Human CRP antibody (R&D, AF1707-SP) was used to detect the binding of mCRP to neuronal membranes. Although this antibody probably binds to different forms of CRP, because we added mCRP in the culture, the results demonstrated the binding of mCRP to the primary cells. PHF1 antibody recognizes tau phosphorylation at Ser396 and Ser404 and was kindly provided by Dr. Benjamin Wolozin at Boston University. Anti-NeuN antibody (Abcam, #ab177487) was used for neurons with PHF1. Neuronal apoptosis was detected with anti-cleaved caspase-3 (Asp175) antibody (Cell Signaling, #9661). Presynapse was detected with anti-SYP antibody (Santa Cruz, #sc-17750). Postsynapse was detected with anti-PSD95 antibody (Cell Signaling, #3450T). Astrocytes were detected with anti-glial fibrillary acidic protein (GFAP) antibody (Fisher Scientific, #14-9892-82). Microglia was detected with anti-CD68 antibody (Bio-Rad Laboratories, #MCA1957GA).

The area and total intensity after adjusting for the threshold was analyzed by ImageJ software as described previously²⁴ and the ratio with the number of cell bodies was calculated.

2.4 | Enzyme-linked immunosorbent assays

Cell media from mCRP or pCRP treated neurons, astrocytes, or microglia was collected and centrifuged at 2800 g for 5 minutes at 4°C. For amyloid beta (A β) 1-42 measurement, cell media from mCRP or pCRP treated neurons was collected and the sandwich enzyme-linked immunosorbent assay (ELISA) to measure mouse A β 1-42 was performed according to the manufacturer's instructions (Thermo Fisher Scientific, #KMB3441). For interleukin (IL)-1 α , IL-1 β , and tumor necrosis factor alpha (TNF α) measurement, cell media from mCRP treated astrocytes or microglia was collected and the sandwich ELISA to measure mouse IL-1 α (R&D, #MLA00),

IL-1 β (R&D, #MLB00C), or TNF α (R&D, #MTA00B) was performed according to the manufacturer's instructions. Mean absorbance was recorded and concentrations were further calculated based on the standard.

2.5 | RNA isolation and real-time quantitative polymerase chain reaction

Total RNA was extracted from the tissues or cultured cells by RNeasy Mini Kit (Qiagen, #74104) according to the manufacturer's instructions. cDNA was synthesized by using iScript™ cDNA Synthesis Kit (Bio-Rad, #1708891). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, #4309155). Accumulation of fluorescent products were monitored by real-time PCR using 7900HT Fast Real-Time PCR System (Applied Biosystems). The mRNA levels were normalized to the mRNA levels of the housekeeping gene Gapdh and different experimental groups were compared using the delta Ct method.²⁵ The real-time PCR primer sequences used in this study are described in the following: Gapdh, 5'-GCAGTGGCAAAGTGGAGATT-3', 5'-TCTCCATGGTGGTGAAGACA-3'; APP, 5'-CAAGACACACACCACATCG-3', 5'-GTTAGTGCTCTTCTCGCTGC-3'; BACE1, 5'-CTGCCGTCAAGTCCATCAAG-3', 5'-CATAACAGTGCCCGTGGATG-3'; Cdk5, 5'-ACCCAGCTACAACATCCTTGG-3', 5'-CGCTGCACAGGGTTACACTT-3'; GSK3 β , 5'-CTAATGCTGGAGACCGTGA-3', 5'-CAAACGTGACCAGTGTGCT-3'; PS1, 5'-CGATGATGGTGGCTTCAGTG-3', 5'-CTTCTCCGGGTCTTCACT-3'.

2.6 | Western blot assay

Primary neurons were lysed in the RIPA (radioimmunoprecipitation assay) buffer (Thermo Fisher Scientific, PI89900) mixed with protease inhibitor cocktail (Thermo Fisher Scientific, 78444; 1:100). Protein concentration of each sample was measured by the BCA protein assay kit (Thermo Fisher Scientific, 2322). Protein (30 μ g) was electrophoresed on 4% to 20% Tris-Glycine Mini Gels (Thermo Fisher Scientific, XP04200BOX) and transferred to the PVDF transfer membrane (Thermo Fisher Scientific, PI88520). The membrane was blocked by 5% non-fat dry milk (Bio-Rad, 1706404) in Tris-buffered saline with Tween-20 (TBST; American Bioanalytical, Inc., AB14330-04000) and then incubated with the primary antibody diluted in the blocking buffer overnight at 4°C. Anti-APP (Cell Signaling, 2452S; 1:500), anti-BACE1 (Cell Signaling, 5606T; 1:500), anti-actin (Santa Cruz Biotech, sc-81178, 1:500), anti-GSK3 β (Abcam, ab93926; 1:1000), anti-phospho-GSK3 β (Ser9; Cell Signaling, 9336S; 1:500), anti-CDK5 (Cell Signaling, 2506T; 1:500) antibodies were used. After washing, the blots were incubated with m-IgG κ BP-HRP (anti-mouse IgG-HRP; Santa Cruz Biotech, sc-516102; 1:2000) or mouse anti-rabbit IgG-HRP (Santa Cruz Biotech, sc-2357; 1:2000) for 1 hour at room temperature. Immunoblots were visualized with the Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, 32106) accord-

ing to the manufacturer's instruction and imaged using a Bio-Rad imaging station. The control protein bands β -actin were used as the control in this experiment. For the quantification of the interested protein bands, a rectangular area was selected and the intensity of each band was determined from the area showing the most intense signal using ImageJ software. The signal intensities of all protein bands were normalized by the β -actin band and the ratio was calculated.

2.7 | Fluoro-Jade B (FJB) labeling

Fluoro-Jade B (FJB) labeling was performed as reported by Gu et al.²⁶ with some modifications. Cell culture medium containing 0.000025% FJB (Histo-Chem Inc., 1FJB30MG) was freshly prepared each time using a FJB stock solution (10 mg of FJB in 100 mL distilled water). The area and total intensity after adjusting for the threshold was analyzed by ImageJ software.

2.8 | Statistical analysis

All values are expressed as the mean \pm standard error. Statistical analysis was performed using GraphPad Prism 8 (version 8.1.0, GraphPad Software Inc.). One- or two-way analysis of variance (ANOVA) with Tukey's post hoc testing was used for multiple comparisons. For all cell experiments, there were three replications. In all cases, $P < .05$ was considered significant.

3 | RESULTS

3.1 | mCRP induces A β production of the primary neurons with APOE ϵ 4 genotype

After adding mCRP to the primary neuron cultures, we found that more of mCRP stuck/bound to primary neurons in an APOE genotype-dependent pattern APOE ϵ 4 > APOE ϵ 3 > APOE ϵ 2 (Figure 1A). Comparative quantitative analyses were performed (Figure 1B).

Because A β 42 is an early hallmark of AD pathology, we first investigated if mCRP has any effect on A β 42 production using the ELISA assay. mCRP induced A β 42 production in the media of primary neurons in a dose-dependent pattern and APOE genotype-dependent pattern, APOE ϵ 4 > APOE ϵ 3 > APOE ϵ 2 (Figure 2A). In contrast, pCRP showed a similar trend for inducing A β 42 production in the primary neurons but didn't reach statistical significance (Figure 2B). To further investigate, the RNA and protein expressions related to A β 42 production including amyloid precursor protein (APP) and β -secretase (BACE1)²⁷ were determined by using RT-qPCR and western blot experiments in these neurons. Figure 2C–2E revealed that mCRP increased the RNA and protein levels of APP and BACE1 in a dose-dependent manner and the intensity pattern of APOE ϵ 4 > APOE ϵ 3 > APOE ϵ 2 in primary cortical neurons. Interestingly, the effect of mCRP on the protein levels

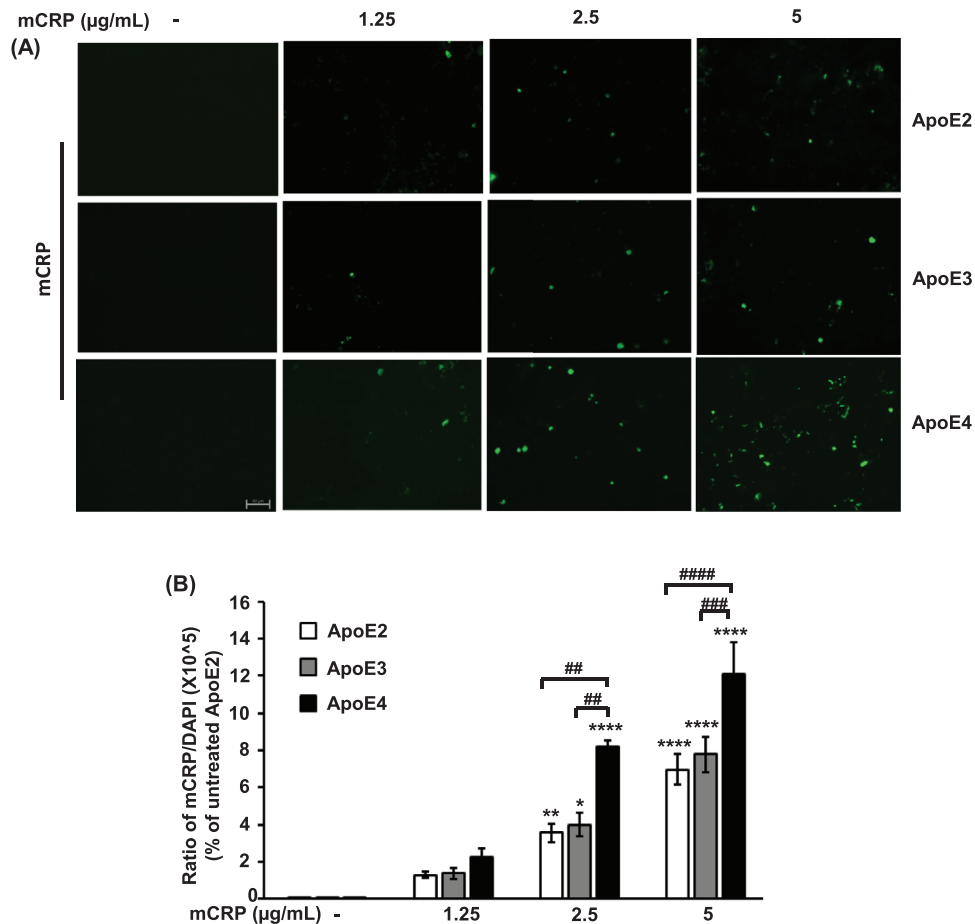


FIGURE 1 The binding of monomeric C-reactive proteins (mCRP) on primary cortical neurons. Postnatal day 0–2 cortical neurons from mice expressing different apolipoprotein E (APOE) genotypes were isolated and cultured for 14 days. The neurons were treated with medium only versus adding different concentrations of mCRP for 16 hours. A, The neurons were fixed, incubated with human CRP antibodies to detect the binding of mCRP to the cells. The representative images were shown (scale bar: 50 μm). B, The immunofluorescence staining of human CRP antibodies was normalized by 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) and quantitated. Values were the mean \pm standard error from all experiments. Compared to the untreated APOE ϵ 2 neurons, differences with statistical significance were shown with * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$. Compared to the same concentration of mCRP treated neurons, the differences were shown with statistical significance # $P < .05$, ## $P < .01$, ### $P < .001$, and #### $P < .0001$

of APP and BACE1 were more pronounced in APOE ϵ 2 neurons than APOE ϵ 4 neurons (Figure S1A and 1B in supporting information). In addition, the brain RNA levels of APP and BACE1 were also increased in mCRP-treated APOE ϵ 4 mice (Figure S2A, S2B in supporting information). There was no significant change for the expression of presenilin 1 (PS1) in these brain samples (Figure S2C).

3.2 | mCRP induces cellular tauopathy of the primary neurons with APOE ϵ 4 genotype

To test the effect of mCRP on cellular tauopathy, we added different concentrations of mCRP to the primary cortical neurons derived from WT, APOE ϵ 2, APOE ϵ 3, and APOE ϵ 4 mice (Figure 3A). mCRP induced cellular tau phosphorylation as detected by PHF1 antibody (Ser396/Ser404) immunostaining in a dose-dependent (from 1.25 to

5 $\mu\text{g}/\text{mL}$; Figure 3A,B) and time-dependent (5 $\mu\text{g}/\text{mL}$, 0–80 minutes) manner, with staining intensity patterns of APOE ϵ 4 > APOE ϵ 3 > APOE ϵ 2 (Figure 3C). In contrast, pCRP showed similar trends and pattern for inducing cellular tauopathy but did not reach statistical significance (Figure 3D,E).

To characterize if mCRP influences the key enzymes to phosphorylate tau protein, RNA and protein were extracted and the expressions of tau phosphorylation-related RNA and proteins, including cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3 beta (GSK3 β)²⁷ and Phospho-GSK3 β (Ser9), were determined by using RT-qPCR and western blot experiments. Figure 3F–3H revealed that mCRP increased the RNA and protein levels of CDK5 and GSK3 β in a dose-dependent manner, while decreasing the protein levels of GSK3 β . Figure S3 in supporting information also shows that the RNA levels of CDK5 and GSK3 β were increased in the brain of mCRP-treated APOE ϵ 4 mice (Figure S3A, S3B).

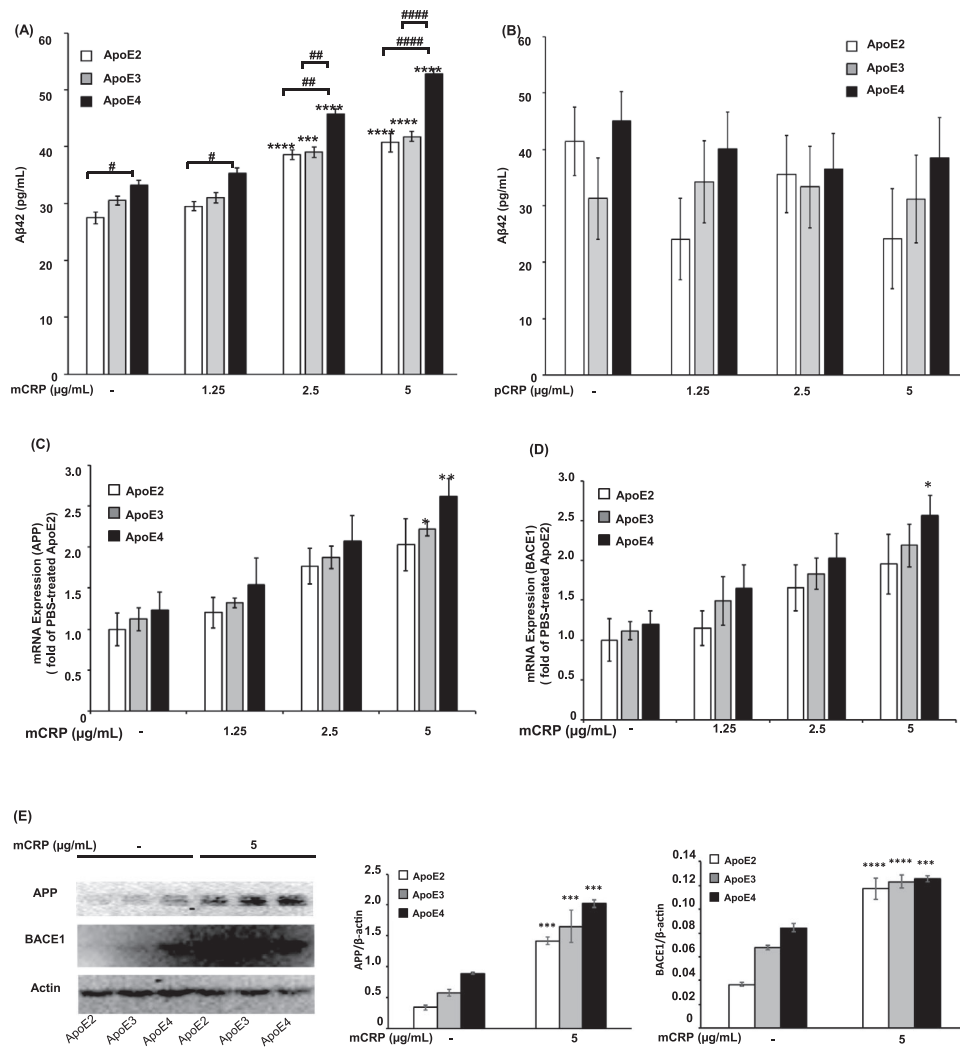


FIGURE 2 The effects of monomeric C-reactive proteins (mCRP) on the amyloid beta ($A\beta$)42 secretion in primary cortical neurons. Postnatal day 0 to 2 cortical neurons from mice expressing different apolipoprotein E (APOE) genotype were isolated and cultured for 14 days. The neurons were treated with medium only or adding different concentrations of mCRP or pentameric CRP (pCRP) for 16 hours. A, Primary neurons from different APOE mice were treated with control medium versus different concentrations of mCRP. Cell culture media were collected and the expression levels of $A\beta$ 42 were detected by enzyme-linked immunosorbent assay (ELISA). Values were the mean \pm standard error (SE) from all experiments by using ANOVA with with Tukey's post hoc testing. Within each genotype, compared to the untreated neurons, differences of $A\beta$ 42 level with each concentration of mCRP are shown with statistical significance * P < .05, ** P < .01, *** P < .001, and **** P < .0001. Within each concentration of mCRP, the comparisons of different APOE genotypes were conducted by using analysis of variance (ANOVA) with Tukey's post hoc testing. Statistical significance is shown with # P < .05, ## P < .01, ### P < .001, and #### P < .0001. B, Primary neurons from different APOE mice were treated with control medium versus different concentrations of pCRP. Cell culture media were collected and the expression levels of $A\beta$ 42 were detected by ELISA. Values were the mean \pm SE from all experiments by using ANOVA with with Tukey's post hoc testing. C, Real-time polymerase chain reaction (PCR) assays were conducted to reveal the expressions of amyloid precursor protein (APP) in neurons. Values are expressed relative to untreated condition in each genotype, which were set as 1. Values were the mean \pm SE from all experiments. Within each genotype, compared to the untreated neurons, differences with statistical significance were shown with * P < .05 and ** P < .01. D, Real-time PCR assays were conducted to reveal the expressions of β -secretase (BACE1) in neurons. Values are expressed relative to untreated condition in each genotype, which were set as 1. Values were the mean \pm SE from all experiments. Within each genotype, compared to the untreated neurons, differences with statistical significance were shown with * P < .05. E, Western blot assays for the expressions of APP and BACE1 in different primary APOE neurons treated with mCRP were performed and quantified after normalization against β -actin. The representative photographs are shown. Values were the mean \pm SE. Within each genotype, compared to the untreated neurons, differences with statistical significance were shown with *** P < .001

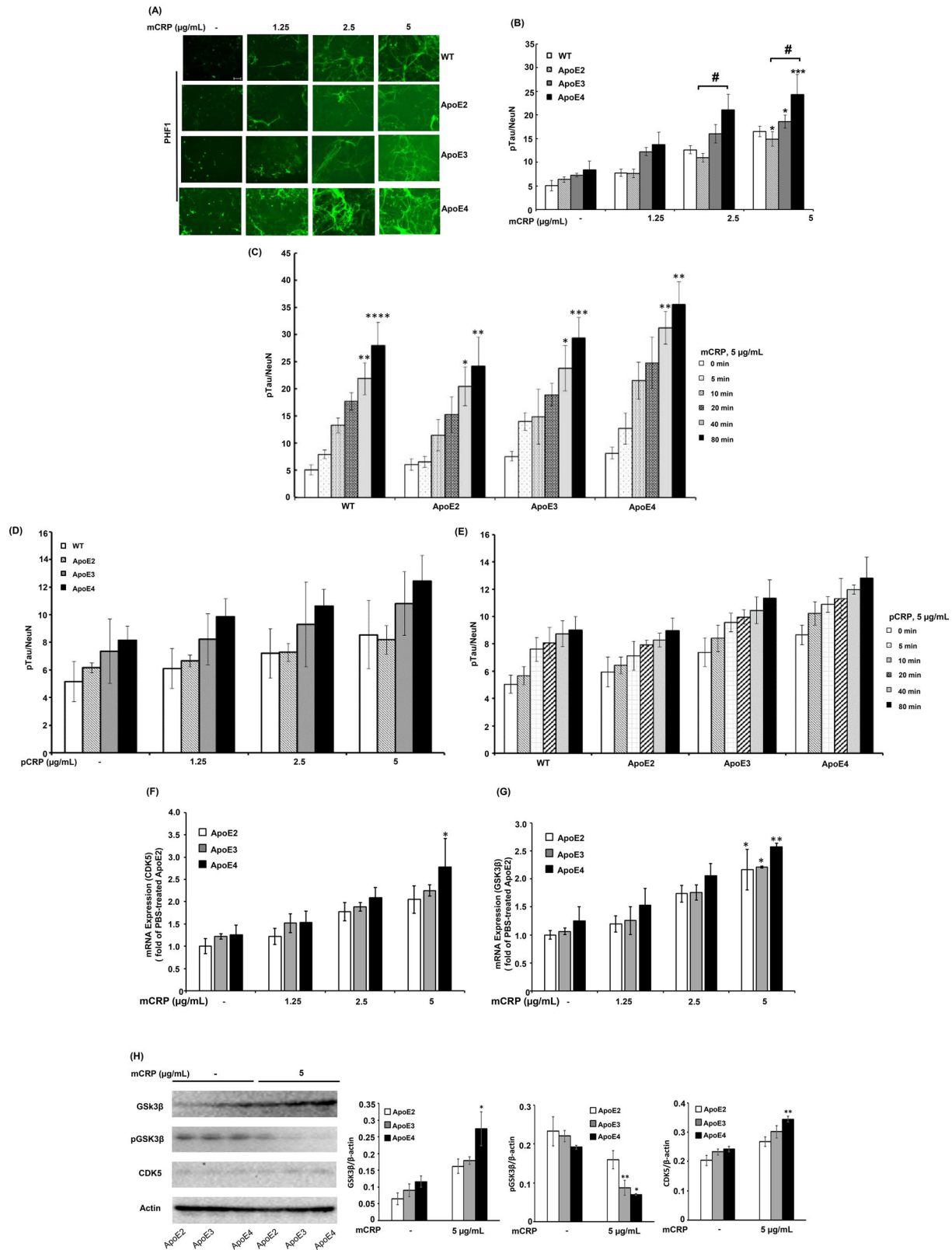


FIGURE 3 The effects of monomeric C-reactive proteins (mCRP) and pentameric CRP (pCRP) on the phosphorylated tau (p-tau) expression in primary cortical neurons. Postnatal day 0 to 2 cortical neurons from mice expressing different apolipoprotein E (APOE) genotype were isolated and cultured for 14 days. The neurons were treated with medium only or adding different concentrations of mCRP for different time. A, Neurons from wild-type (WT) mice or mice expressing different APOE genotypes were treated with control medium versus different concentrations of mCRP. The neurons were fixed, incubated with the p-tau antibody, PHF1, to detect cellular tauopathy. The representative images were shown (scale bar: 50 µm). B, The level of tau phosphorylation was normalized by NeuN and quantitated. Values were the mean ± standard error (SE) from all

3.3 | Dose-dependent effects of mCRP on other biomarkers of neurodegeneration in primary neurons based on APOE genotypes

Caspase-3, one of the members of caspase family, is responsible for the majority of proteolysis during apoptosis. Therefore, the detection of cleaved caspase-3 is considered a reliable marker for cellular apoptosis,²⁸ and we applied it to our experimental system. To determine whether mCRP also induce apoptosis of neurons, we performed cleaved caspase-3 staining by applying increasing concentration of mCRP to primary cortical neurons derived from APOE ϵ 2, APOE ϵ 3, and APOE ϵ 4. Figure 4A shows the representative imaging of cleaved caspase-3-positive neurons. Quantitative analysis demonstrated a significant increase of cleaved caspase-3-positive cells in 5 μ g/mL mCRP treated APOE ϵ 4 and APOE ϵ 3 neurons compared to cells grown in the absence of mCRP; in contrast, in APOE ϵ 2 neurons, there is no significant changes for the treatment of different mCRP concentrations (Figure 4B). As showed in Figure 4B, mCRP treatment (1.25–5 μ g/mL) demonstrated that mCRP had a dose-dependent effect to increase the level of cleaved caspase-3-positive cells with the intensity pattern of APOE ϵ 4 > APOE ϵ 3 > APOE ϵ 2.

Schmued et al. reported that FJB which is an anionic fluorochrome derived from fluorescein, sensitively and specifically stains degenerating neurons and can be used as neurodegenerative biomarker.²⁹ The histochemical application of FJB in staining degenerating neurons is simple and sensitive. We found that mCRP increased the number of FJB-positive neurons significantly (Figure 4C,D) in dose-dependent manner and the intensity pattern of APOE ϵ 4 > APOE ϵ 3 > APOE ϵ 2. The result suggests that mCRP induced neurodegeneration, especially in APOE ϵ 4 neurons.

We further investigated if mCRP has effects on neuron synapses using immunocytochemistry on the expressions of synaptophysin (SYP) and PSD95. In cortical neurons, we found that mCRP did not influence the expression of SYP regardless of the APOE genotype (Figure 4E). While in the absence of mCRP APOE ϵ 2 neurons had

the highest expression of PSD95 compared to APOE ϵ 3 and APOE ϵ 4 neurons, only APOE ϵ 2 neurons seemed to have tendency of a dose-dependent decrease of PSD95 expression responding to mCRP, but again did not reach significance (Figure 4F). These data indicate that mCRP probably did not have significant effects on neuronal synapses in our experimental system.

3.4 | The effects of mCRP on primary microglia

Microglia were isolated from mouse cortex and the primary cultures were incubated with mCRP with increasing concentrations (1.25 μ g/mL to 5 μ g/mL). The expressions of CD68 and Iba-1 in primary microglia were examined by immunostaining (Figure 5A–C). Surprisingly, despite that these concentrations of mCRP showed a dose-dependent influence on primary neurons described above, mCRP had no or minimum effects on the expression of CD68 and Iba-1 in primary microglia cells regardless of APOE genotype. Lipopolysaccharide (LPS), as a positive control for stimulating microglia, showed significantly increased level of Iba-1 and cellular shape of microglia (Figure S4 in supporting information). Additionally, activated microglia and astrocytes can produce and secrete a number of proinflammatory mediators, such as IL-1 α , IL-1 β , and TNF- α .³⁰ To further explore the effects of mCRP on the activation of microglia and astrocytes, the culture medium was collected to detect the expression levels of IL-1 α , IL-1 β , and TNF- α using ELISA assays. Consistent with the immunostaining results on Iba-1 and CD68 expressions, there were also no significant changes of IL-1 α , IL-1 β , and TNF- α in microglia treated with different concentrations of mCRP (Figure 5D–F).

3.5 | The effects of mCRP on primary astrocytes

Consistent with the microglia results, the expressions of GFAP were stained in primary astrocytes and we found that there were no differences after adding different concentrations of mCRP (Figure 6A,B).

experiments and compared using analysis of variance (ANOVA) with Tukey's post hoc testing. Within each genotype, differences with different concentrations of mCRP for each genotype were shown with statistical significance * P < .05, ** P < .01, and *** P < .001. Within the same concentration of mCRP treated neurons, the differences among different APOE neurons were compared with ANOVA with Tukey's post hoc testing. Statistical significance is shown # P < .05. C, The neurons were treated with control medium or 5 μ g/mL mCRP for different incubation time. The level of tau phosphorylation was normalized by NeuN and quantitated. Values were the mean \pm SE from all experiments by using ANOVA with Tukey's post hoc testing. Within each genotype, differences with statistical significance for each genotype were shown with * P < .05, ** P < .01, and *** P < .001. D, The neurons were treated with control medium versus different concentrations of pCRP. The level of tau phosphorylation was normalized by NeuN and quantitated. Values were the mean \pm SE from all experiments. E, The neurons were treated with control medium versus 5 μ g/mL pCRP for different incubation time. The level of tau phosphorylation was normalized by NeuN and quantitated. Values were the mean \pm SE from all experiments. F, Real-time polymerase chain reaction (PCR) assay was conducted to reveal the expressions of CDK5 in neurons. Values are expressed relative to untreated APOE ϵ 2 mouse primary neurons, which were set as 1. Values were the mean \pm SE from all experiments by using ANOVA with Tukey's post hoc testing. Within each genotype, differences with statistical significance were shown with * P < .05. G, Real-time PCR assay was conducted to reveal the expressions of GSK3 β in neurons. Values are expressed relative to untreated APOE ϵ 2 mouse primary neurons, which were set as 1. Values were the mean \pm SE from all experiments by using ANOVA with Tukey's post hoc testing. Within each genotype, differences with statistical significance were shown with * P < .05 and ** P < .01. H, Western blot assays for the expressions of GSK3 β , pGSK3 β , and CDK5 in neurons were performed and quantified after normalization against β -actin. The representative photographs are shown. Values were the mean \pm SE. Within each genotype, compared to the untreated neurons, differences with statistical significance were shown with * P < .05 and ** P < .01

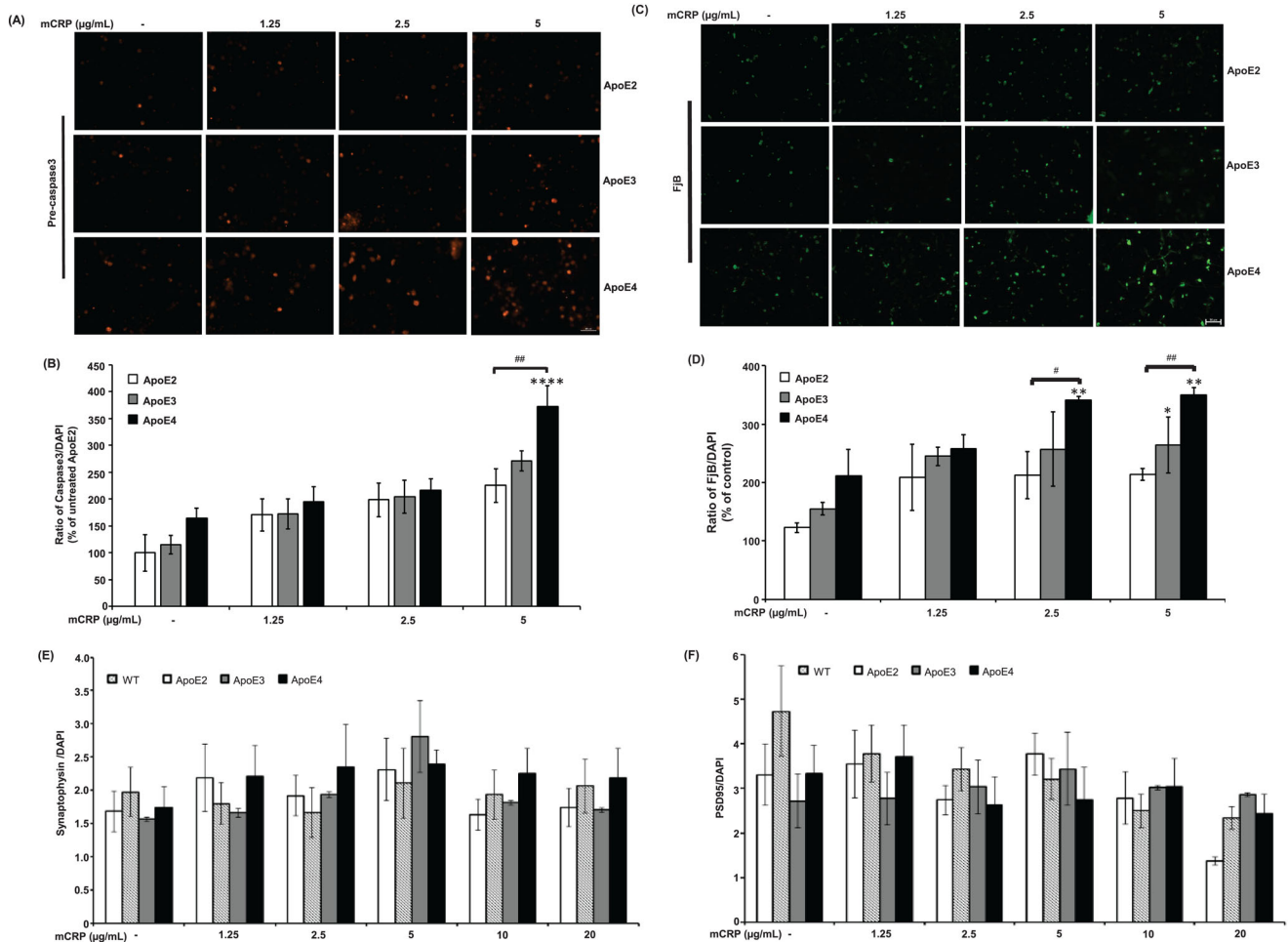


FIGURE 4 The impact of monomeric C-reactive proteins (mCRP) on the biomarkers of neurodegeneration in primary cortical neurons. Postnatal day 0 to 2 cortical neurons from mice expressing different apolipoprotein E (*APOE*) genotype were isolated and cultured for 14 days. The neurons were treated with medium only or adding different concentrations of mCRP for different time followed by immunostaining with different antibodies. The representative images were shown (scale bar: 50 µm). A, The neurons were fixed, incubated with the pre-caspase 3 antibodies to detect cellular apoptosis. B, The immunofluorescence staining of pre-caspase 3 antibodies was normalized by 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) and quantitated. Values were the mean ± standard error (SE) from all experiments by using analysis of variance (ANOVA) with Tukey's post hoc testing. Within each genotype, differences with statistical significance for each genotype were shown with * $P < .05$ and **** $P < .0001$. Compared to the same concentration of mCRP treated neurons, the differences were shown with statistical significance # $P < .05$. C, The neurons were fixed and stained with Fluoro-Jade B (FjB) to detect neuronal degeneration. The representative images were shown. D, The immunofluorescence staining of FjB was normalized by DAPI and quantitated. Values were the mean ± SE from all experiments by using ANOVA with Tukey's post hoc testing. Within each genotype, differences with statistical significance were shown with * $P < .05$ and ** $P < .01$. Compared to the same concentration of mCRP treated neurons, the differences were shown with statistical significance ## $P < .01$. E, The neurons were fixed and stained with synaptophysin (SYP) antibodies to detect synapse loss. The immunofluorescence staining of synaptophysin antibodies was normalized by DAPI and quantitated. Values were the mean ± SE from all experiments. F, The neurons were fixed and stained with PSD95 antibodies to detect synapse loss. The immunofluorescence staining of PSD95 antibodies was normalized by DAPI and quantitated. Values were the mean ± SE from all experiments.

mCRP had no or minimum effects on the expression of GFAP in primary astrocytes for each genotype. Again, LPS had significant effects to activate astrocytes and increased GFAP expression (Figure S5 in supporting information). There were also no significant changes of IL-1 α , IL-1 β , and TNF- α in the supernatant of astrocytes treated with different concentrations of mCRP (Figure 6C-E). Taken together, these results indicated that mCRP may have little to no direct effects on microglia and astrocytes, regardless of *APOE* genotype.

4 | DISCUSSION

This study extends our previous research^{3,4} by investigating if and how proinflammatory isoform of CRP, for example, mCRP,⁶⁻⁸ can contribute to the cellular pathology of AD. Results showed that mCRP induced A β 42 secretion and phosphorylated tau (p-tau) production in primary neurons in a dose-, time-, and *APOE* genotype (*APOE* ϵ 4 > *APOE* ϵ 3 > *APOE* ϵ 2)-dependent pattern. Of note, pCRP showed

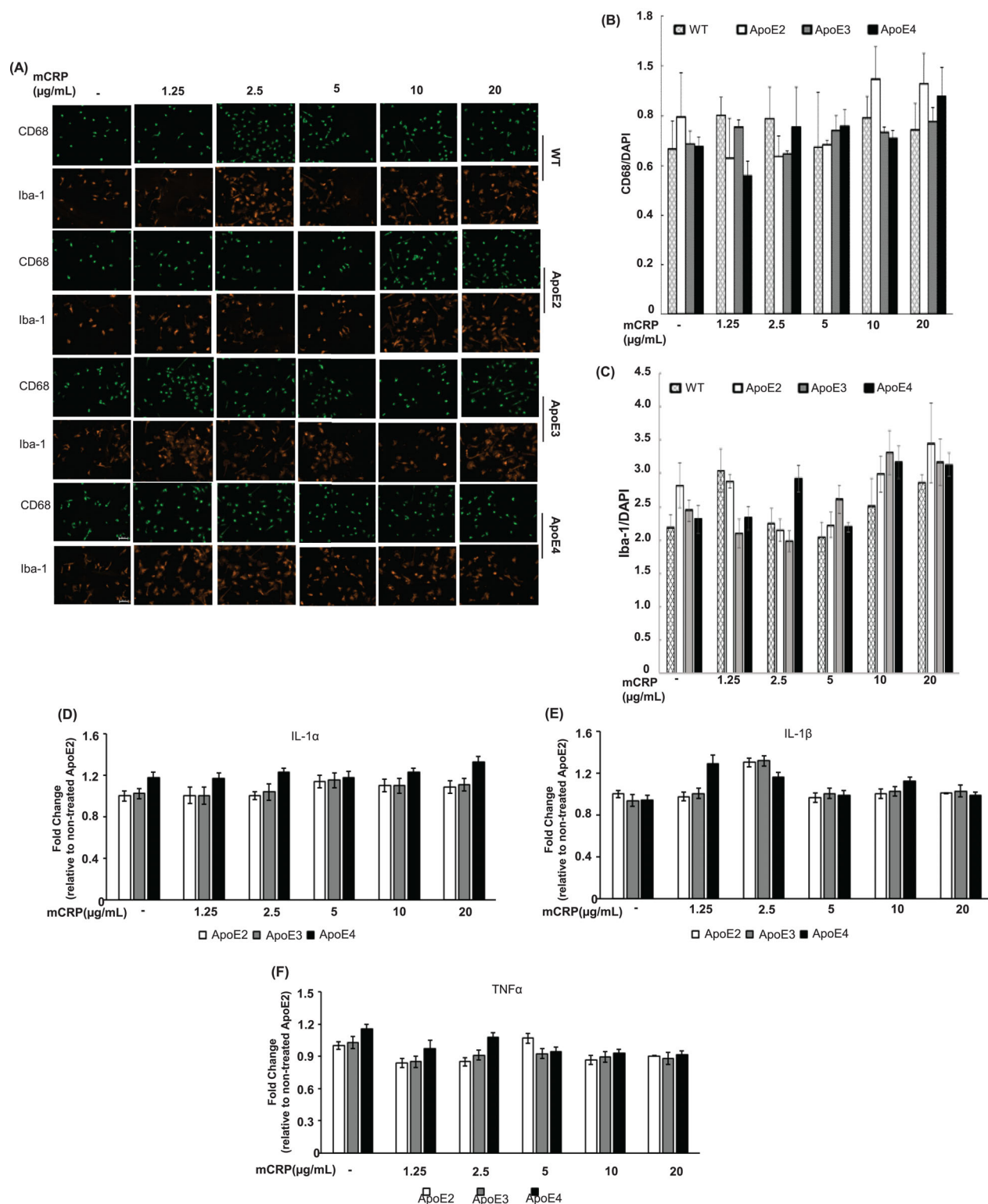


FIGURE 5 The effects of monomeric C-reactive proteins (mCRP) on microglia. Postnatal day 0 to 2 cortical microglia from wild-type (WT) mice or mice expressing different apolipoprotein E (*APOE*) genotype was treated with control medium or different concentrations of mCRP. A, The microglia were fixed, incubated with the CD68 and Iba-1 antibody. The representative images were shown (scale bar: 50 µm). B, The level of CD68 expression was normalized by 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) and quantitated. Values were the mean ± standard error (SE) from all experiments. C, The level of Iba-1 expression was normalized by DAPI and quantitated. Values were the mean ± SE from all experiments. D, Microglial cells culture medium was collected and the expression levels of interleukin (IL)-1α were detected by enzyme-linked immunosorbent assay (ELISA). Values were the mean ± SE from all experiments. E, Microglial cells culture medium was collected and the expression levels of IL-1β were detected by ELISA. Values were the mean ± SE from all experiments. F, Microglial cells culture medium was collected and the expression levels of tumor necrosis factor α were detected by ELISA. Values were the mean ± SE from all experiments

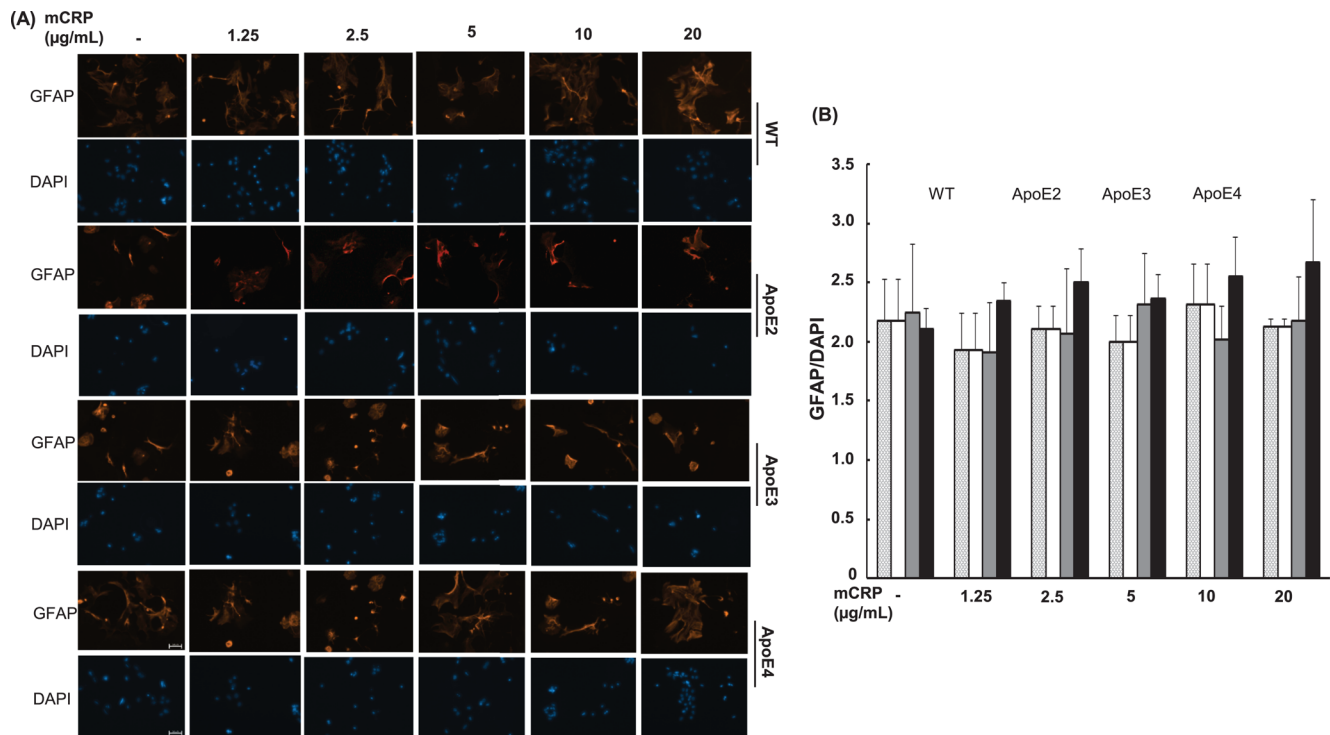


FIGURE 6 The effects of monomeric C-reactive proteins (mCRP) on astrocytes. Postnatal day 0 to 2 cortical astrocytes from wild-type (WT) mice or mice expressing different apolipoprotein E (APOE) genotype were treated with control medium versus different concentrations of mCRP. A, The astrocytes were fixed, incubated with the glial fibrillary acidic protein (GFAP) antibody. The representative images were shown (scale bar: 50 µm). B, The level of GFAP expression was normalized by 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) and quantitated. Values were the mean ± standard error (SE) from all experiments. C, Astrocytes culture medium was collected and the expression levels of interleukin (IL)-1 α were detected by enzyme-linked immunosorbent assay (ELISA). Values were the mean ± SE from all experiments. D, Astrocytes culture medium was collected and the expression levels of IL-1 β were detected by ELISA. Values were the mean ± SE from all experiments. E, Astrocytes culture medium was collected and the expression levels of tumor necrosis factor α were detected by ELISA. Values were the mean ± SE from all experiments

similar trends as mCRP but did not reach statistical significance. pCRP can and does spontaneously convert to mCRP under certain experimental conditions, such as cell culture incubations for as little as 4 hours,²¹ which may explain the non-significant results for pCRP. These cellular pathologies are consistent and parallel with the interactive effects of APOE ϵ 4 and CRP for AD risk³ and AD brain biomarkers in humans.⁴

Our study used in vitro primary neurons to demonstrate that APOE ϵ 4 neurons are more vulnerable than APOE ϵ 3 and APOE ϵ 2 neurons to be exposed to mCRP and develop cellular AD pathology including A β and tau phosphorylation (Figures 2, 3). This process may be through mCRP increasing the expressions of APP and BACE1 for A β production, CDK5 and GSK3 β for p-tau generation in the neurons. Paradoxically, the effect of mCRP on the protein levels of APP and BACE1 were more pronounced in APOE ϵ 2 genotype than APOE ϵ 4 genotype (Figure S1), but the effect of mCRP on the production of A β was more pronounced in APOE ϵ 4 than APOE ϵ 2 (Figure 2A). It is likely and consistent with other studies that the APOE isoform dependent-A β interaction after A β production mainly regulates A β level, aggregation, and clearance.³¹ While many studies demonstrate that amyloids and tauopathy induce neuroinflammation,³² our experiments demonstrate

that inflammatory factor, mCRP, can cause production of A β and p-tau in neurons depending on APOE genotypes. The data suggest that typical AD pathology, for example, A β or tau protein, and neuroinflammation enhance the formation of each other in AD pathogenesis. Most importantly, neurovascular dysfunction is shown in the AD brain.³³ Several studies have shown that APOE ϵ 4 leads to BBB disruption,⁵ which is shown to be a key step in late-onset AD pathogenesis,^{5,34} and could cause leakage of peripheral inflammatory factors like mCRP into the brain and become toxic to neurons. Clinical study shows that an increased CSF/serum ratio for albumin and immunoglobulin G in a subgroup of AD patients,³⁵ suggesting that peripheral inflammatory factors leaked into the AD brain due to some cerebrovascular pathology.³⁶

It is intriguing that unlike in neurons, mCRP had no direct effects on astrocytes (Figure 6), although astrocytes are shown to be closely associated/connected with the brain endothelia in BBB; the "default" condition, like peripheral inflammation, for endothelia are more leaky inducing influences on associated cells especially astrocytes.^{37,38,39} In addition, microglia cells also have interaction with the BBB to regulate neuroinflammation in the brain,⁴⁰ but also were not influenced by mCRP (Figure 5). It is probable that during peripheral inflammation,

blood mCRP could not directly influence astrocytes and microglia, but induced cerebrovascular inflammation of endothelia or pericytes to release some other proinflammatory factors, which will further influence astrocytes and microglia. Indeed, our recent research shows that peripheral intraperitoneal injection of mCRP into APOE knock-in mice induced significant neuroinflammation of astrocytes and microglia in the brain of APOE ϵ 4 mice.⁴¹ On the other hand, the receptor(s) for mCRP's activities in neurons is unknown. However, it is shown that CRP can bind to phosphocholine in cell membrane and bind to Fc γ RI and Fc γ RIIa in immune cells.⁴² Because neurons have enriched membrane phospholipids and do not have Fc γ RI and Fc γ RIIa receptors, it is likely that mCRP binds to phosphocholine or some unidentified receptor of neuron cell membrane to induce AD cellular pathology. On the contrary, microglia cells express Fc γ RI and Fc γ RIIa but do not respond to added mCRP for the activation.

Although our study did not show the correlation between mCRP and synaptic changes in the neurons (Figure 4E,F), we argue that during chronic inflammation mCRP induces A β and p-tau production, as well as causes neurodegenerative changes in APOE ϵ 4, but not in APOE ϵ 2 and minimally in APOE ϵ 3, neurons (Figures 2–4) is an early and accumulative step before synapsis impairment in AD pathogenesis. Our findings in this study are consistent and relevant with the findings in humans. After following for 20 years, we observed that chronically elevated CRP levels predicted accelerated global cognitive decline and AD incidence in APOE ϵ 4 carriers, but no such effect was observed among participants who were APOE ϵ 4 non-carriers.³ Using an Alzheimer's Disease Neuroimaging Initiative study, we additionally observed a positive association between baseline plasma CRP and trajectory levels of CSF total tau and p-tau biomarkers among those who had two APOE ϵ 4 alleles.⁴ Interestingly, this effect was reversed among those without an APOE ϵ 4 allele.

5 | CONCLUSION

Our results suggest that CRP may play an important mediator role in the APOE ϵ 4-related pathway for AD risk. Treatment of chronic low-grade inflammation to reduce CRP levels and block the dissociation of mCRP from pCRP, especially in APOE ϵ 4 carriers, may delay AD onset.

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the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

CONFLICTS OF INTEREST

The authors declare no biomedical financial interests or potential conflicts of interest. [Author disclosures](#) are available in the supporting information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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