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Original Research

DHA Ameliorates Alzheimer's Disease by Attenuating Microglial Pyroptosis via Regulation of the HOXA9-NLRP3 Pathway

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Abstract

Background: Alzheimer's disease (AD) involves a progressive deterioration of cognitive abilities, memory loss, and persistent brain inflammation. Emerging evidence indicates that pyroptosis mediated by the NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome, contributes significantly to AD development. Docosahexaenoic acid (DHA) has demonstrated neuroprotective properties; however, the precise mechanisms by which it modulates pyroptosis in AD have yet to remained incompletely elucidated. **Objective**: To explore the role of DHA in modulating microglial pyroptosis via the HOXA9-NLRP3 pathway in an AD model. **Methods**: Effects of DHA on Aβ25–35-induced pyroptosis were assessed in human microglial clone 3 (HMC3) human microglial cells using CCK-8, western blotting, immunofluorescence, and Enzyme-linked Immunosorbent Assay (ELISA) assays. The role of homeobox A9 (HOXA9) in pyroptosis regulation was evaluated through overexpression and knockdown experiments. Dual-luciferase reporter assays together with chromatin immunoprecipitation (ChIP) were used to verify the interaction of HOXA9 to NLRP3 promoter. Amyloid precursor protein / Presenilin-1 double-transgenic (APP/PS1) transgenic AD mice underwent DHA treatment *in vivo*, and cognitive performance was assessed using the Morris water maze paradigm. Expression of HOXA9, NLRP3, and pyroptosis-related proteins were analyzed by Quantitative Real-time Reverse Transcription PCR (qRT-PCR), Western blotting, and immunofluorescence. **Results**: DHA treatment significantly reduced Aβ25–35-induced microglial pyroptosis, as indicated by decreased levels of p30-Gasdermin D (GSDMD), cleaved-caspase-1, IL-1β, and IL-18. HOXA9 overexpression reversed the protective effects of DHA, whereas NLRP3 inhibition by MCC950 enhanced DHA inhibition of pyroptosis. Dual-luciferase and ChIP assays confirmed that HOXA9 directly regulates NLRP3 transcription. In APP/PS1 mice, DHA administration enhanced cognitive performance while simultaneously decreasing the expression of pyroptosis-related markers and inflammatory mediators in brain. Inhibition of NLRP3 signaling by MCC950 further strengthened the neuroprotective actions of DHA. **Conclusion**: DHA ameliorates AD-related cognitive decline and reduces microglial pyroptosis through suppressing the HOXA9-NLRP3 axis. These results offer novel insights into the molecular basis of DHA-mediated neuroprotection and highlight potential therapeutic targets for AD.

Keywords: Alzheimer's Disease; docosahexaenoic acid; pyroptosis; homeobox genes; NLR family pyrin domain-containing 3 protein; microglia; neuroinflammation

1. Introduction

Alzheimer's disease (AD) represents a progressive neurodegenerative condition with a substantial global disease burden, placing a significant strain on healthcare resources [1,2]. It is marked by a gradual decline in cognitive ability, memory loss, and behavioral changes, which arise from multifaceted pathological mechanisms, including β-amyloid (Aβ) deposition and chronic neuroinflammation [3–5]. An increasing body of evidence indicates that neuroinflammation plays a central role in the advancement of the disease. In particular, microglial activation has now considered a critical driver in AD [6,7]. Activated microglia can adopt distinct programmed cell death modalities. Among those, pyroptosis is a highly inflammatory process mediated by the gasdermin protein family, most notably Gasdermin D (GSDMD) [8,9].

Pyroptosis is characterized by specific features, including cellular edema, plasma membrane disruption, and the secretion of inflammatory factors [10,11]. The process amplifies the inflammatory cascades and potentially exacerbates neuronal injury and progressive cognitive impairment in AD [10]. The NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome is crucial for triggering pyroptosis, as it activates caspase-1 and promotes the processing and activation of inflammatory factors [12].

Increasing studies support the notion that interventions aimed at neuroinflammation and pyroptosis could offer promising therapeutic avenues for AD treatment. In this context, docosahexaenoic acid (DHA), a polyunsaturated omega-3 fatty acid abundant in neural tissues, has gained attention for its potential neuroprotective properties [13,14]. DHA influences a variety of cellular functions, including

the regulation of inflammation, oxidative stress, and synaptic activity [15].

Multiple studies have shown that DHA supplementation can reduce Aβ deposition, mitigate tau pathology, and enhance cognitive function in AD mice models [16]. Moreover, epidemiological studies have indicated a strong association between DHA intake and AD progression [17]. However, the precise molecular mechanisms underlying DHA's protective effects in AD, particularly its potential impact on microglial pyroptosis, remain unclear.

Recent findings have revealed notable links between transcription factors and inflammasome activity. Homeobox A9 (HOXA9)has been involved in diverse inflammatory pathways [18]. Although the involvement of HOXA9 in AD has not been thoroughly investigated, its involvement in regulating inflammatory pathways in other conditions suggests a potential link to AD pathogenesis [19]. Notably, bioinformatic analyses predict that HOXA9 may directly bind to the NLRP3 promoter, suggesting a role in the transcriptional regulation of this inflammasome component. This observation indicates the intriguing possibility that HOXA9 may act as a molecular switch in the activation of microglial pyroptosis in AD.

Based on these findings, we hypothesized that DHA exerts neuroprotective effects in AD by modulating the HOXA9-NLRP3 axis, thereby attenuating microglial pyroptosis. Specifically, we propose that DHA suppresses HOXA9 expression, thereby attenuating NLRP3 transcriptional activity and subsequently suppressing inflammasome assembly and pyroptotic cell death. By elucidating this novel mechanism, we aim to provide the potential molecular basis for DHA's neuroprotective roles and identify potential therapeutic targets for AD.

2. Methods

2.1 Cell Culture and Plasmid Transfection

Human microglial HMC3 cells (ATCC, Manassas, VA, USA) were maintained under standard conditions, authenticated by STR profiling, and confirmed as mycoplasma-free. Pyroptosis was induced with 25 µM Aβ25–35 for 24 h. DHA was applied at concentrations of 5, 10, and 25 µM. Cells were transfected with HOXA9 plasmids and treated with 1 µM MCC950 (Sigma-Aldrich, St. Louis, MO, USA) to inhibit NLRP3, as detailed in the **Supplementary Methods** [20,21].

2.2 Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from cultured HMC3 cells and mouse brain tissue using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). cDNA synthesis was performed using a commercial reverse transcription kit (Takara, Japan). RT-qPCR was conducted with SYBR Green chemistry, and gene expression levels were normalized. Primer sequences, thermal cycling conditions, and reagent details are provided in the **Supplementary Methods** [22,23].

2.3 Western Blot Analysis

Total proteins from cells and brain tissues were isolated using radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Equal protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride membranes. Following blocking, membranes were probed with primary antibodies targeting HOXA9 (PA5-102516; Invitrogen, Carlsbad, CA, USA), NLRP3 (MA5-32255, Invitrogen), apoptosis-associated speck-like protein containing a caspase-recruitment domain (PA5-50915, Invitrogen), p30-gasdermin D (ab215203; 1:1000; Abcam, Cambridge, UK), cleaved-caspase-1 (PA5-38099, Invitrogen), interleukin (IL)-1β (MA5-23691, Invitrogen), IL-18 (PA5-79479, Invitrogen), and GAPDH (MA5-15738; 1:1000; Invitrogen), and then incubated with horseradish peroxidase (HRP)-linked secondary antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence and analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA). Full antibody details and protocols are available in the **Supplementary Methods**.

2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

Pro-inflammatory cytokines (TNF-α, IL-1β, IL-18, and IL-6) in cell culture supernatants and brain tissue homogenates were quantified using ELISA kits (BioLegend, San Diego, CA, USA).

2.5 Cell Counting Kit-8 (CCK-8) Assay

Cell viability was evaluated using the CCK-8 assay. HMC3 cells were seeded into 96-well plates at 5×10^3 cells per well and treated with varying concentrations of DHA. After 24 h, 10 µL of CCK-8 reagent (Dojindo, Kumamoto, Japan) was added to each well, and absorbance was measured at 450 nm after 2 h of incubation. Viability was expressed as a percentage relative to the control.

2.6 Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed using the EZ-ChIP™ Kit (Millipore, Burlington, MA, USA) to investigate HOXA9 binding to the NLRP3 promoter. Cells were cross-linked with formaldehyde, lysed, and sonicated to form shear chromatin. The sheared chromatin was immunoprecipitated using anti-HOXA9 antibodies, followed by DNA purification and qPCR analysis to detect enrichment at the NLRP3 promoter site.

2.7 Dual-Luciferase Reporter Assay

HEK-293T cells were co-transfected with NLRP3 promoter constructs (wild-type or mutant) and either HOXA9 overexpression or control plasmids by Lipofec-

tamine 3000 (Thermo Fisher Scientific). Cells were harvested 48 h post-transfection, and luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Relative luciferase activity was calculated by normalizing firefly luciferase signals to Renilla luciferase signals.

2.8 Immunofluorescence Staining

Immunofluorescence analysis was performed to detect p30-GSDMD levels in both mouse brain tissue sections and cultured HMC3 microglial cells. Following fixation, permeabilization, and blocking, samples were incubated with primary antibodies and subsequently with fluorescence-conjugated secondary antibodies. Nuclear staining was performed using DAPI, and images were obtained by confocal microscopy (Leica, Wetzlar, Germany).

2.9 Animal Experiments

Male amyloid precursor protein / Presenilin-1 double-transgenic (APP/PS1) transgenic mice were randomly assigned to control and treatment groups. Mice received daily oral DHA or vehicle for 8 weeks, with some groups additionally receiving intracerebroventricular HOXA9-overexpressing adenovirus or the NLRP3 inhibitor MCC950 [24]. Mice were anesthetized with isoflurane (induction at 4% for 3 minutes, then maintenance at 2% in oxygen for approximately 30 minutes) until loss of pedal reflex, with body temperature maintained on a heating pad. At the experimental endpoint, animals were euthanized by intraperitoneal injection of sodium pentobarbital (200 mg/kg). After deep anesthesia was confirmed, transcardial perfusion with phosphate-buffered saline and 4% paraformaldehyde was performed for histological analysis, or PBS alone for molecular assays. Death was verified by cessation of heartbeat and respiration. Cognitive function was assessed using the Morris water maze test, and brain tissues were analyzed by RT-qPCR, Western blotting, and immunofluorescence.

2.10 Statistical Analysis

Data were presented as mean $\pm$ standard deviation and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. For behavioral data, repeated-measures ANOVA was used. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1 DHA Attenuates Aβ25–35-Induced Microglial Pyroptosis by Inhibiting NLRP3 Inflammasome Activation

To assess the influence of DHA on Aβ25–35-induced pyroptosis in HMC3 microglial cells, we first assessed the cell viability. CCK-8 data revealed that DHA concentrations from 0 to 25 µM had no significant toxic effects on HMC3 cells (Fig. 1A). Accordingly, we used 0–25 µM DHA for subsequent experiments. Compared to the control group, Aβ25–35 treatment markedly reduced cell viability, while DHA treatment (0–25 µM) dose-dependently increased cell viability (Fig. 1B). Immunofluorescence staining revealed that Aβ25–35 significantly increased p30-GSDMD fluorescence intensity compared to control, while DHA (0–25 µM) dose-dependently decreased p30-GSDMD fluorescence intensity (Fig. 1C). Western blot data suggested that Aβ25–35 exposure upregulated pyroptosis-associated proteins (p30-GSDMD, cleaved-caspase-1, IL-1β, and IL-18) and inflammasome factors (NLRP3 and ASC), whereas DHA administration led to a gradual reduction in their expression (Fig. 1D). ELISA results demonstrated that Aβ25–35 significantly increased TNF-α, IL-1β, IL-18, and IL-6 levels, while DHA treatment (0–25 µM) dose-dependently decreased these inflammatory factors (Fig. 1E). These results indicate that DHA effectively suppresses NLRP3 activation and decreases Aβ25–35-induced microglial pyroptosis.

3.2 DHA Inhibits Aβ25–35-Induced Microglial Pyroptosis via HOXA9 Suppression

We explored the involvement of HOXA9 in the protective effects of DHA against Aβ25–35-induced pyroptosis. Treatment with Aβ25–35 resulted in a pronounced enhancement in HOXA9 mRNA and protein expression, while DHA supplementation suppressed this upregulation. Overexpression of HOXA9 (oe-HOXA9) in DHA-treated cells further elevated HOXA9 expression compared with the negative control (oe-NC) (Fig. 2A,B). Cell viability assays revealed that Aβ25–35 significantly reduced cell viability, which was rescued by DHA treatment. However, HOXA9 overexpression partially reversed the protective effects of DHA (Fig. 2C). Immunofluorescence staining showed that Aβ25–35 increased p30-GSDMD fluorescence intensity, indicating enhanced pyroptosis. DHA treatment reduced this effect; however, HOXA9 overexpression counteracted DHA's protective action (Fig. 2D). Western blotting indicated that Aβ25–35 dramatically enhanced the expression of pyroptosis-related proteins and inflammasome components. DHA treatment decreased the levels of these proteins; however, HOXA9 overexpression partially reversed this effect (Fig. 2E). ELISA measurements showed that Aβ25–35 increased TNF-α, IL-1β, IL-18, and IL-6 expression but attenuated using DHA. Consistently, HOXA9 overexpression partially negated the inflammation-suppressive abilities of DHA (Fig. 2F). These results suggest that DHA inhibits Aβ25–35-induced microglial pyroptosis, at least in part, through suppression of HOXA9.

3.3 DHA Suppresses NLRP3 Activation Through HOXA9 Inhibition

To elucidate the cellular pathways regarding DHA's involvement in NLRP3, we used bioinformatics analysis,

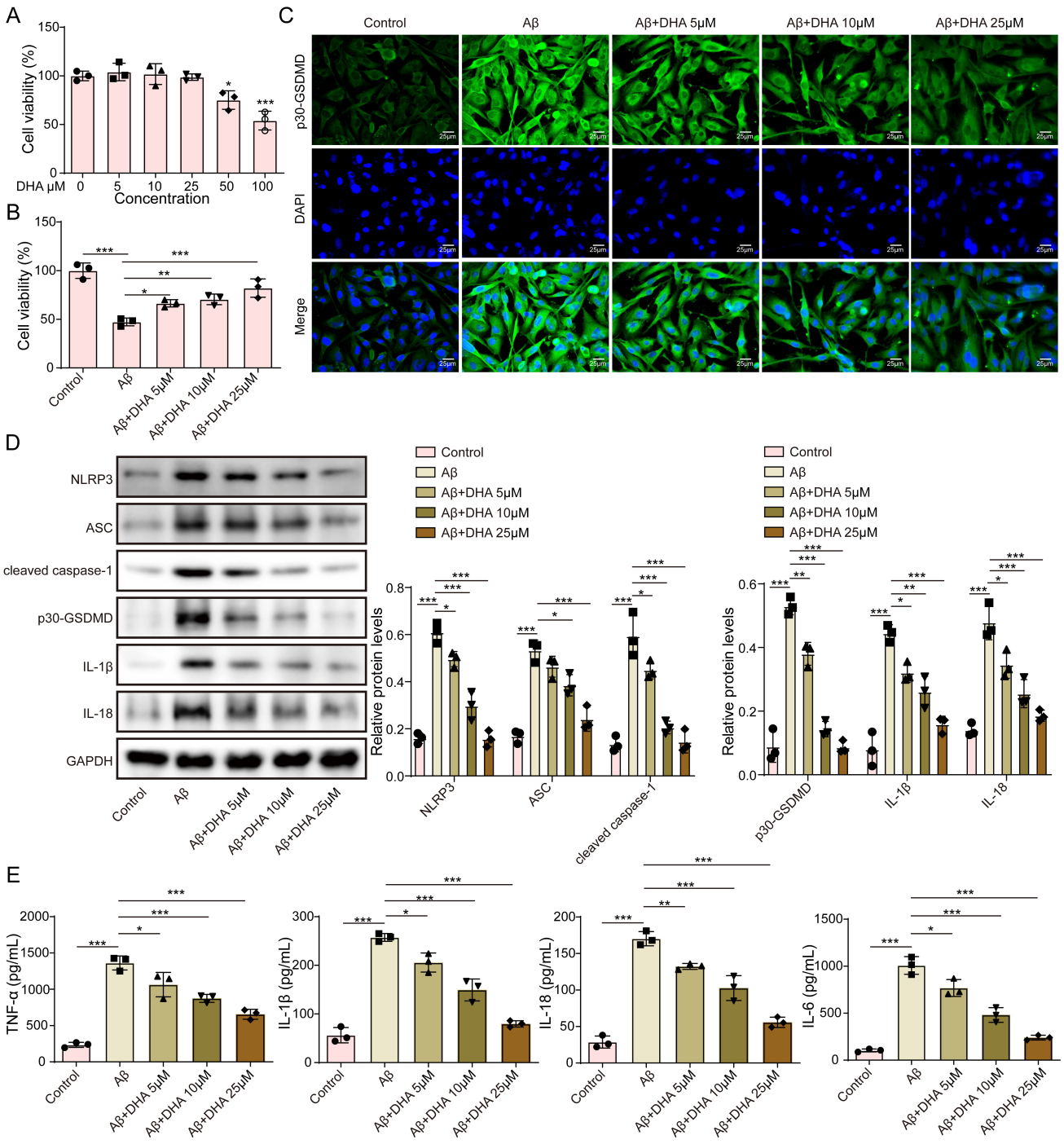

Fig. 1. Docosahexaenoic acid (DHA) inhibits NLRP3 inflammasome activation and reduces Aβ-induced microglial pyroptosis. (A) Cell viability of HMC3 cells treated with various DHA concentrations (0–25 μM) for 24 h, measured by CCK-8 assay. (B) Cell viability of HMC3 cells exposed to Aβ with or without DHA treatment (0–25 μM) for 24 h, measured by CCK-8 assay. (C) Representative immunofluorescence images and quantification of p30-GSDMD in HMC3 cells treated with Aβ and various DHA concentrations (0–25 μM). Scale bar: 25 μm. (D) Western blot analysis of pyroptosis-related proteins (p30-GSDMD, cleaved-caspase-1, IL-1β, IL-18) and inflammasome components (NLRP3, ASC) in treated HMC3 cells. (E) ELISA measurements of pro-inflammatory cytokines (TNF-α, IL-1β, IL-18, IL-6) in culture supernatants of treated HMC3 cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Three independent experiments with three technical replicates were performed for each experiment.

which predicted a HOXA9 binding site within the NLRP3 promoter region (Fig. 3A). Dual-luciferase assays indicated that knockdown of HOXA9 (sh-HOXA9) significantly decreased the transcriptional activity of the wild-type NLRP3

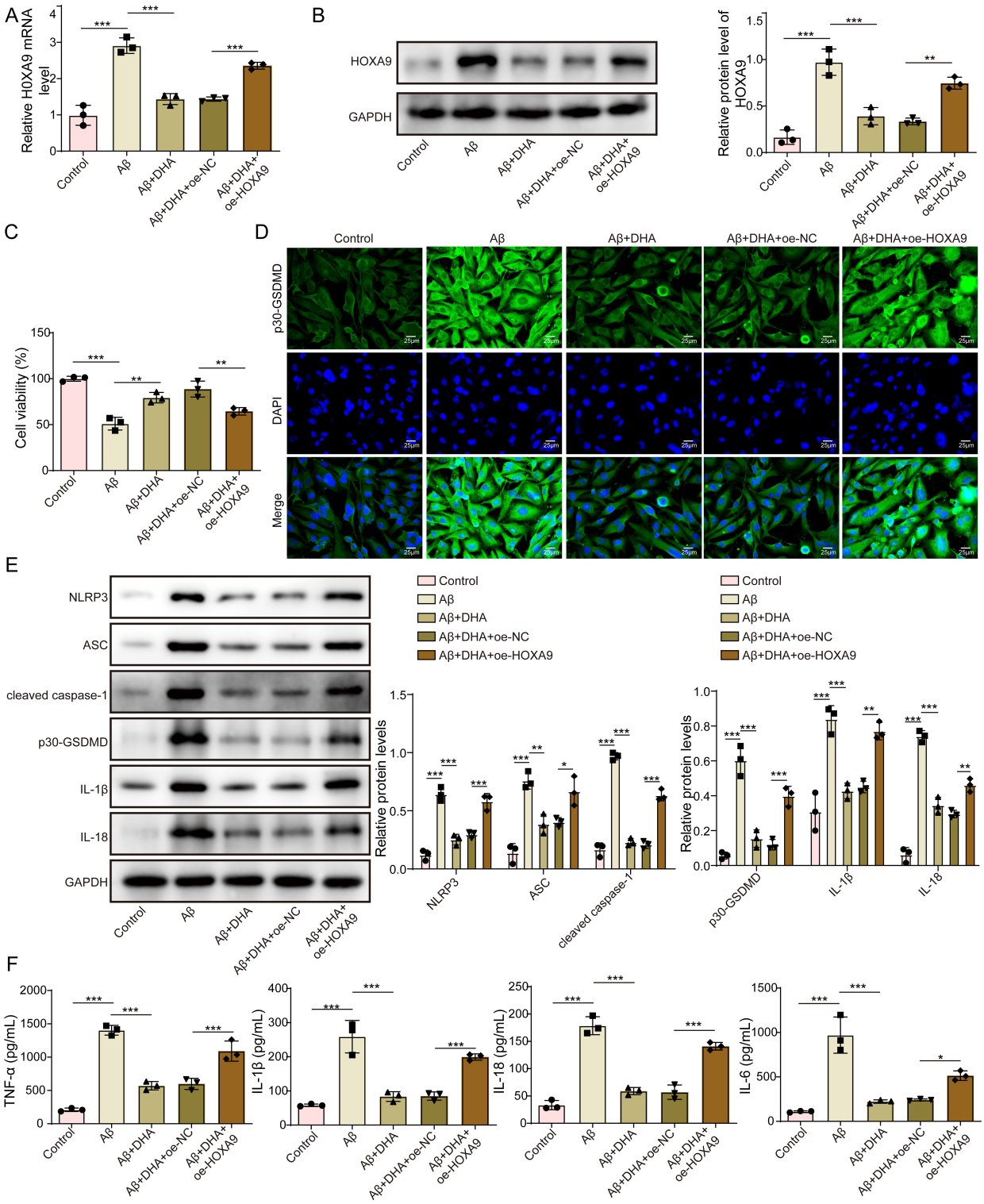

Fig. 2. Docosahexaenoic acid (DHA) inhibits Aβ-induced microglial pyroptosis via HOXA9 suppression. (A) qRT-PCR analysis of HOXA9 mRNA expression in HMC3 cells under different treatments. (B) Western blot analysis of HOXA9 protein levels. (C) Cell viability assessed using CCK-8 assay. (D) Representative immunofluorescence images and quantification of p30-GSDMD. Scale bar = 25 µm. (E) Western blot analysis of pyroptosis-related proteins (p30-GSDMD, cleaved-caspase-1, IL-1β, IL-18) and inflammasome components (NLRP3, ASC). (F) ELISA measurements of pro-inflammatory cytokines (TNF-α, IL-1β, IL-18, IL-6) in the culture supernatants. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Three independent experiments with three technical replicates were performed for each experiment.

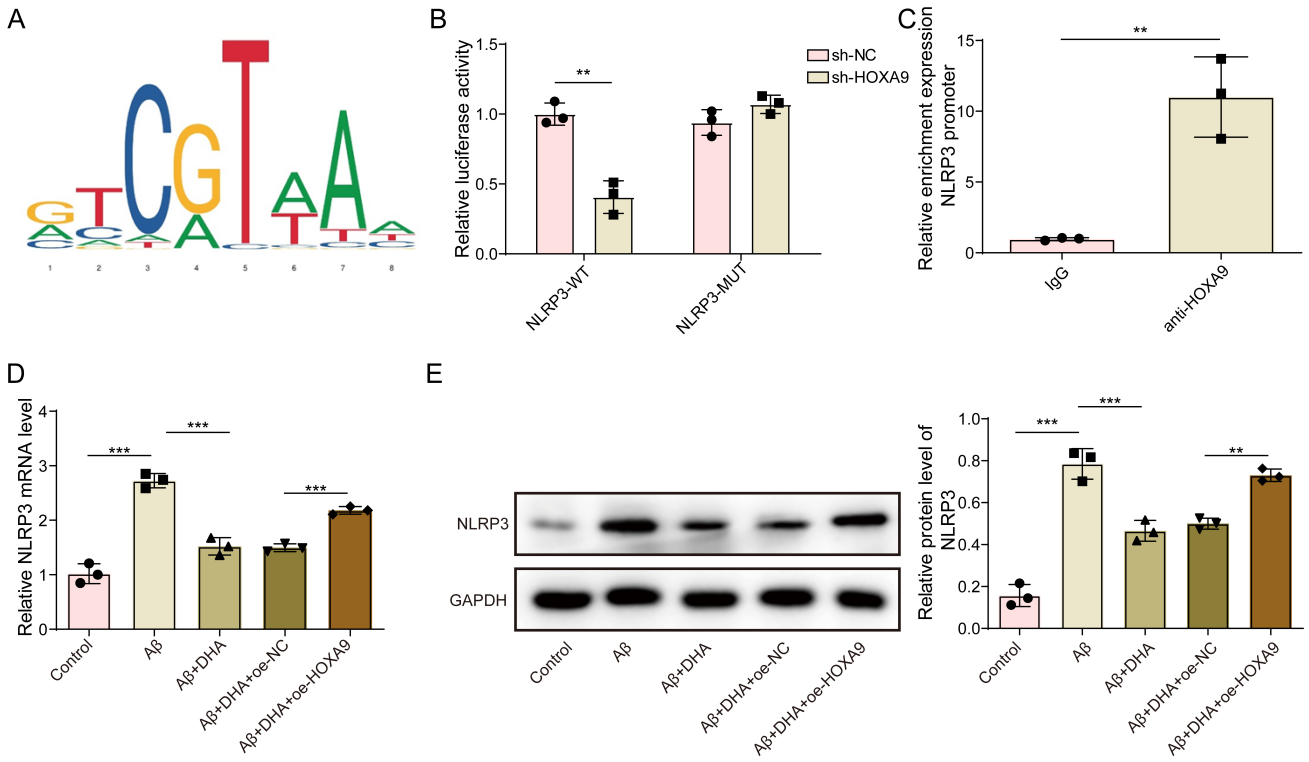

Fig. 3. Docosahexaenoic acid (DHA) suppresses NLRP3 activation through HOXA9 inhibition. (A) JASPAR prediction of HOXA9 binding site in the NLRP3 promoter region. (B) Dual-luciferase reporter assay results showing the effect of HOXA9 knockdown on NLRP3 promoter activity in HEK-293T cells. (C) ChIP assay results demonstrating HOXA9 enrichment at the NLRP3 promoter in HEK-293T cells. (D) qRT-PCR analysis of NLRP3 mRNA levels under DHA treatment and HOXA9 overexpression conditions. (E) Western blot analysis of NLRP3 protein levels under DHA treatment and HOXA9 overexpression conditions. $^{**}p < 0.01$, $^{***}p < 0.001$. Three independent experiments with three technical replicates were performed for each experiment.

promoter without affecting the mutant construct the mutant promoter (Fig. 3B). This finding suggests that HOXA9 directly regulates NLRP3 transcription via its predicted binding sites. ChIP assays further indicated enrichment of anti-HOXA9 at the NLRP3 promoter sequence in HEK-293T cells (Fig. 3C), providing strong evidence for the direct binding of HOXA9 to the NLRP3 promoter. To evaluate the functional impact of this interaction, we examined NLRP3 expression levels under various conditions. RT-qPCR data revealed that DHA treatment dramatically suppressed NLRP3 mRNA levels, while HOXA9 overexpression promoted NLRP3 mRNA expression (Fig. 3D). Western blot analysis was consistent with these results, demonstrating reduced NLRP3 protein expression following DHA treatment and increased expression with HOXA9 overexpression (Fig. 3E). Taken together, these data demonstrate that DHA suppresses NLRP3 activation by inhibiting HOXA9, which directly regulates NLRP3 transcription.

3.4 DHA Inhibits Cellular Pyroptosis by Regulating NLRP3 Transcriptional Activation via HOXA9

Further experiments were conducted to clarify how DHA influences NLRP3 transcriptional activation via HOXA9 in microglial cells. Three experimental groups were compared: Aβ25–35+DHA+oe-NC, Aβ25–35+DHA+oe-HOXA9, and Aβ25–35+DHA+oe-HOXA9+NLRP3 inhibitors (MCC950). Cell viability was significantly reduced by HOXA9 overexpression but partially restored by MCC950 treatment (Fig. 4A). Immunofluorescence analysis of p30-GSDMD, a key pyroptosis marker, suggested a dramatic enhancement in fluorescence intensity in oe-HOXA9 group. However, this effect was markedly attenuated by MCC950 treatment (Fig. 4B). Western blot analysis indicated that HOXA9 overexpression increased pyroptosis-related proteins and inflammasome components, whereas MCC950 reduced their expression in HOXA9-overexpressing cells (Fig. 4C). ELISA assays revealed that HOXA9 overexpression elevated TNF-α, IL-1β, IL-18, and IL-6, but MCC950 treatment decreased these cytokine levels (Fig. 4D). Collectively, these results demonstrate that DHA inhibits cellular pyroptosis by regulating NLRP3 transcriptional activation through HOXA9. The ability of the NLRP3 inhibitor MCC950 to reverse the effects of HOXA9 overexpression further confirms the pivotal involvement of the HOXA9-NLRP3 axis.

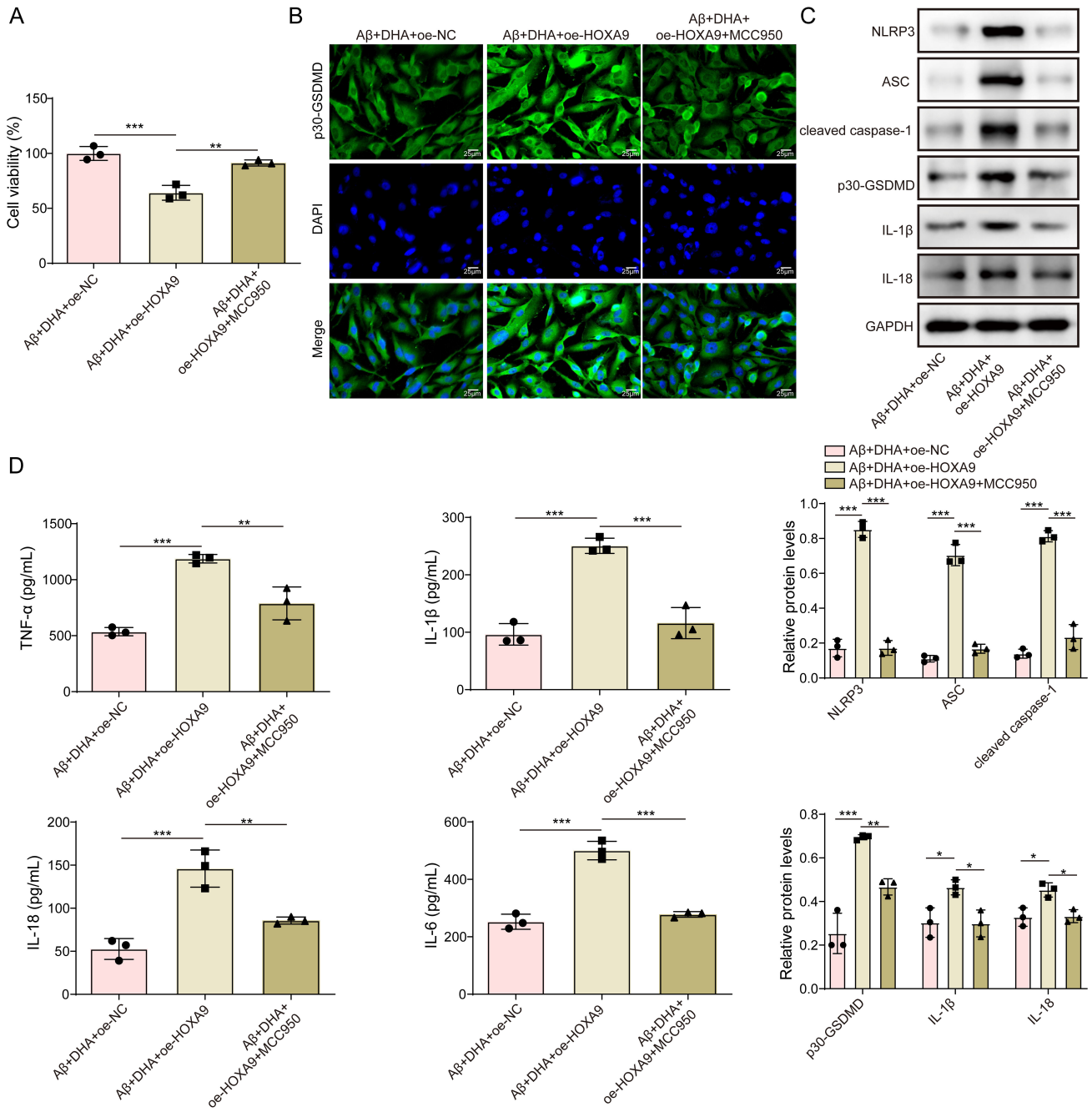

Fig. 4. Docosahexaenoic acid (DHA) inhibits cellular pyroptosis by regulating NLRP3 transcriptional activation via HOXA9. (A) Cell viability of HMC3 cells under different treatments, assessed using the CCK-8 assay. (B) Representative immunofluorescence images and quantification of p30-GSDMD. Scale bar = 25 µm. (C) Western blot analysis of pyroptosis-related proteins (p30-GSDMD, cleaved-caspase-1, IL-1β, IL-18) and inflammasome components (NLRP3, ASC). (D) ELISA measurements of pro-inflammatory cytokines (TNF-α, IL-1β, IL-18, IL-6) in culture supernatants. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Three independent experiments with three technical replicates were performed for each experiment.

3.5 DHA Improves Learning and Memory Abilities and Reduces Pyroptosis Levels in AD Mice via the HOXA9-NLRP3 Axis

Learning and memory functions of APP/PS1 transgenic mice were assessed using the Morris water maze. The AD model group exhibited a significantly prolonged escape latency, fewer platform crossings, and reduced time spent in the target quadrant. DHA improved these cognitive impairments, as evidenced by a shortened escape latency, increased platform crossings, and prolonged target quadrant time. However, overexpression of HOXA9 (DHA+oe-HOXA9 group) partially reversed the cognitive benefits conferred by DHA, resulting in increased escape latency, reduced platform crossings, and decreased tar-

get quadrant time compared with the DHA+oe-NC group. Notably, cotreatment with the NLRP3 inhibitor MCC950 (DHA+oe-HOXA9+MCC950 group) mitigated the adverse effects of HOXA9 overexpression and restored cognitive performance to levels (Fig. 5A).

The AD model group showed significant upregulation of HOXA9 and NLRP3 expression. Correspondingly, the levels of pyroptosis-related proteins were markedly increased in AD models. DHA treatment dramatically reduced the expression of these proteins, indicating its protective effect against neuroinflammation and pyroptosis. In contrast, overexpression of HOXA9 in DHA-treated mice (DHA+oe-HOXA9 group) resulted in a dramatically increase in HOXA9 and NLRP3 levels, along with higher levels of pyroptosis markers, compared with the DHA+oe-NC group. Co-administration of MCC950 effectively counteracted these effects, resulting in reduced expression of HOXA9, NLRP3, and associated pyroptosis markers in the DHA+oe-HOXA9+MCC950 group (Fig. 5B,C).

The levels of these cytokines were markedly higher in the AD model group, indicating a pronounced inflammatory response. DHA treatment markedly reduced TNF-α, IL-1β, IL-18, and IL-6 levels, suggesting its anti-inflammatory potential. Conversely, HOXA9 overexpression in the DHA-treated group (DHA+oe-HOXA9) enhanced the expression of these cytokines. Importantly, the addition of MCC950 in the DHA+oe-HOXA9+MCC950 group effectively reduced the TNF-α, IL-1β, IL-18, and IL-6 levels, further supporting the role of the HOXA9-NLRP3 axis in mediating inflammation in AD (Fig. 5D).

Immunofluorescence staining of p30-GSDMD was performed to visualize and quantify pyroptosis in brain tissues. The results were consistent with those of the biochemical analyses; the AD model group showed elevated p30-GSDMD levels, indicating increased pyroptosis. DHA treatment reduced p30-GSDMD staining, whereas HOXA9 overexpression in DHA-treated mice (DHA+oe-HOXA9) increased p30-GSDMD levels. Co-treatment with MCC950 in the DHA+oe-HOXA9+MCC950 group significantly diminished p30-GSDMD staining, corroborating the involvement of the HOXA9-NLRP3 axis in pyroptosis regulation in AD (Fig. 5E). These results emphasize the importance of the HOXA9-NLRP3 axis in mediating the neuroprotective effects of DHA against AD-associated cognitive decline and neuroinflammation.

4. Discussion

Numerous studies have underscored neuroinflammation as a key contributor to AD development. particularly regarding the impact of NLRP3 inflammasome activation on neurodegeneration [25–27]. Consistent with these observations, our data demonstrate that DHA administration leads to decreased levels of NLRP3 and related downstream molecules such as p30-GSDMD, cleaved-caspase-1, IL-1β, and IL-18. The data corroborate earlier reports indicating that omega-3 fatty acids can regulate inflammation in neurodegenerative diseases [28,29]. These results further extend previous findings by demonstrating the role of the HOXA9 transcription factor in regulating NLRP3 expression, which has not been extensively explored in the context of AD.

The innovative aspect of our research lies in identifying HOXA9 plays a central role in regulating NLRP3 inflammasome signaling. Although much of the existing literature has focused on NF-κB in the regulation of inflammasome activation [30], this study suggests that HOXA9 could act as an upstream modulator, directly binding to the NLRP3 while influencing the promoter activity. These findings provide new mechanistic insights into how DHA mediates its anti-inflammatory and neuroprotective actions in AD.

Our *in vivo* experiments further confirmed that DHA improved cognitive performance in AD mice, as demonstrated by superior outcomes in the Morris water maze. Improved cognitive performance was associated with decreased levels of pyroptosis-associated proteins and inflammatory factors in brain tissues, reinforcing the link between DHA's anti-pyroptotic effects and its cognitive benefits. This study is consistent with earlier studies suggesting that DHA can alleviate cognitive deficits in AD models, likely through effects on synaptic activity and neuroinflammatory processes [31,32]. However, our study extends beyond these findings by providing a mechanistic explanation for how DHA influences pyroptosis through the HOXA9-NLRP3 axis.

Notwithstanding these promising findings, this study has certain limitations that warrant consideration. Although we have demonstrated HOXA9 involvement in NLRP3-mediated pyroptosis *in vitro* and *in vivo*, the exact molecular interactions between HOXA9 and other transcriptional regulators remain unclear. Moreover, although the APP/PS1 transgenic mouse is a common model for AD research, it does not fully replicate the multifaceted nature of human AD. Future studies should explore the effects of DHA in additional AD models, including those incorporating tau pathology or other AD-related features. Furthermore, our study primarily focused on the role of the HOXA9-NLRP3 axis in microglia. Given the complexity of AD, it is likely that other cells including astrocytes and neurons also lead to the observed effects of DHA. Future research should investigate the broader impact of DHA on different cell types within the AD brain and explore whether similar regulatory mechanisms exist in these cells. Another limitation was the utilization of relatively young APP/PS1 mice (8 weeks old). At this age, Aβ deposition is just beginning, allowing us to explore the preventive ability of DHA at an early stage of pathology. However, this model does not completely recapitulate the complex aging environment in which Alzheimer's disease typically develops in humans. Future studies will therefore examine the effects of DHA in

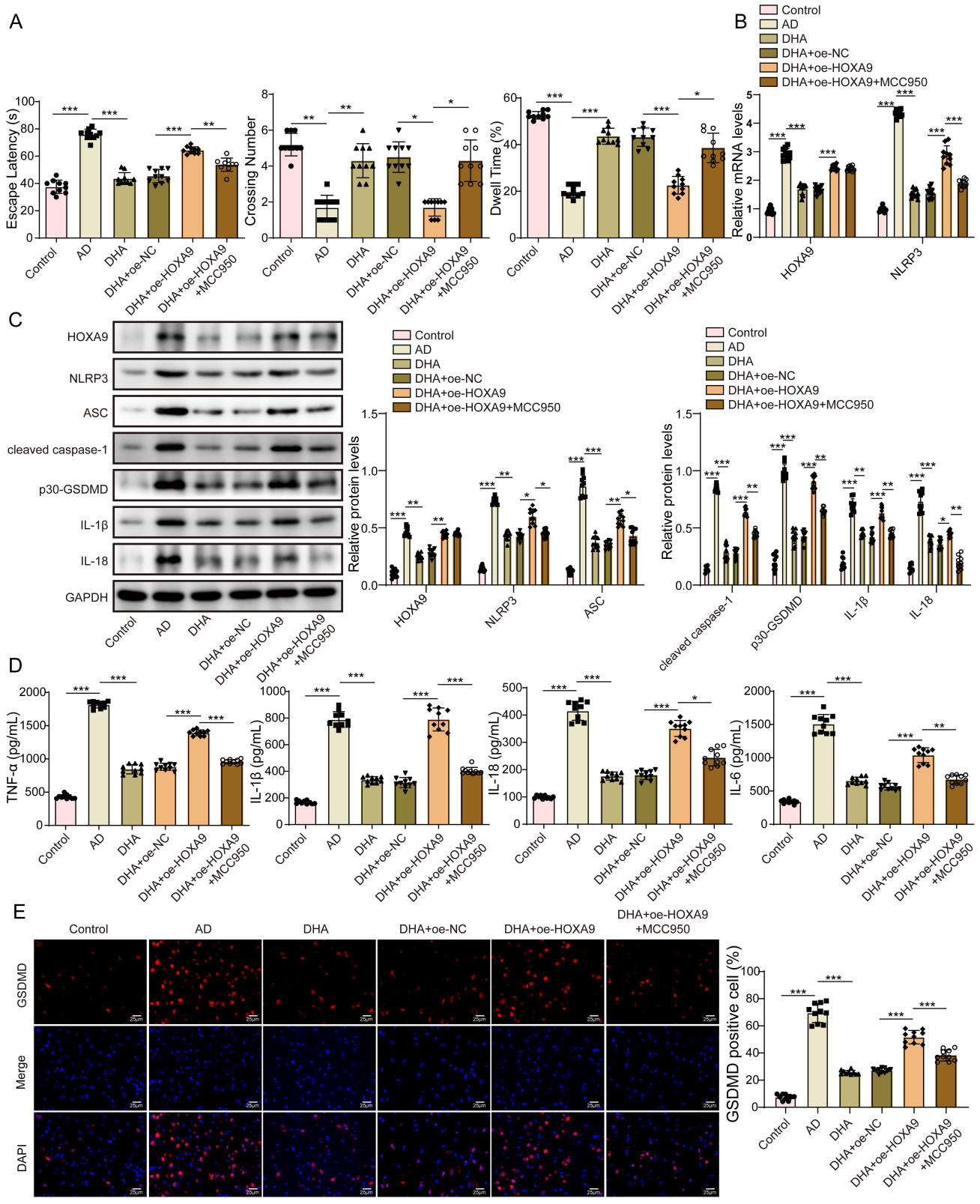

Fig. 5. Docosahexaenoic acid (DHA) improves learning and memory abilities and reduces pyroptosis levels in Alzheimer's disease mice via the HOXA9-NLRP3 Axis (n = 10 mice in each group). (A) Morris water maze performance of APP/PS1 mice across different treatment groups. Escape latency, platform crossings, and time spent in the target quadrant were measured. (B,C) qRT-PCR and Western blot analyses of HOXA9 and NLRP3 expression levels in the brain tissues of the different groups. (D) ELISA results showing the levels of inflammatory cytokines (TNF-α, IL-1β, IL-18, IL-6) in brain tissues. (E) Immunofluorescence staining of p30-GSDMD in brain tissues, indicating pyroptosis levels. Scale bar = 25 µm. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

older APP/PS1 mice (8–10 months of age), when pathology is more advanced, to better evaluate its therapeutic efficacy and translational relevance.

In this study, we demonstrated that DHA dramatically attenuates Aβ25–35-induced microglial pyroptosis through modulating the HOXA9-NLRP3 axis, leading to improved cognitive function in APP/PS1 AD mice. These results expand the existing evidence base by demonstrating that DHA mediates neuroprotection via diverse molecular mechanisms, notably through inhibition of neuroinflammation and pyroptosis. Our study offers new mechanisms into how DHA ameliorates AD-related cognitive decline, highlighting the importance of HOXA9-NLRP3 axis in pyroptosis. These findings advance our understanding of DHA's neuroprotective effects and suggest potential therapeutic targets for AD treatment. Future research should aim to further elucidate the complex regulatory networks involving HOXA9 and explore the clinical value of targeting this axis in AD and other neurodegenerative diseases.

5. Conclusion

DHA significantly alleviates AD-related cognitive impairment and neuroinflammation by suppressing microglial pyroptosis. The study demonstrates that DHA inhibits the HOXA9–NLRP3 axis, thereby reducing inflammasome activation and downstream pyroptotic signaling. These findings highlight this regulatory pathway as a potential therapeutic target and support the value of DHA as a promising intervention strategy for AD.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

JLX, XHH, and CLW designed the research study. JLX and QJ performed the experiments. CLW conducted data analysis. JLX drafted the manuscript. JPP participated in the study design, performed experiments, analyzed data, prepared figures and tables, searched references, and was the corresponding author. All authors contributed to editorial revisions, read, and approved the final manuscript. All authors have participated sufficiently in the work to take responsibility for its content and agreed to be accountable for all aspects of the study.

Ethics Approval and Consent to Participate

All animal procedures were conducted in accordance with the Guide for the Institutional Animal Care and Use and approved by the Institutional Animal Care and Use Committee of Gannan Health Vocational College (Ethics Number: 20240056). Author confirms all animal experiments were in accordance with the ARRIVE guidelines and the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Acknowledgment

Not Applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, the authors used ChatGPT (developed by OpenAI) to assist with spelling and grammar checking. After employing this tool, the authors carefully reviewed and edited the content to ensure its accuracy and clarity. The authors take full responsibility for the content of this publication and explicitly acknowledge that the scientific interpretation, data analysis, and conclusions were conceived and verified independently by the authors. The use of AI was limited to language refinement and did not influence the scientific results or integrity of the study.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL46572.

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