Proceedings of the 3rd International Conference on Modern Medicine and Global Health
DOI: 10.54254/2753-8818/68/2025.20237

Netrin-1's Protective Effect on Aβ-Burdened BV2 Microglial Cells Enhanced Its Viability of Peptide Drugs for Alzheimer's Disease Treatment

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Abstract: The rising prevalence of Alzheimer's Disease (AD) has stimulated extensive research into novel therapeutic strategies. Current studies suggest peptide-based drugs are emerging as promising candidates targeting various aspects of AD pathophysiology. Microglia play a crucial role in AD due to their functions as the resident immune cells in the central nervous system, and are a prominent target for the development of peptide drugs. Netrin-1, a brain-derived extracellular factor, demonstrated therapeutic potential in AD by modulating microglial function. Here, we investigated the effects of netrin-1 on amyloid-beta (Aβ)-burden microglia using BV2 microglial cells as a model. Cell counting was performed to assess the cell viability under different concentrations of netrin-1. Immunofluorescence was employed to examine morphological changes associated with microglial activation. To examine the polarization towards M1 or M2 phenotypes, fluorescence detection was performed after amplification. ELISA quantified Aβ concentration to assess the protective effect of netrin-1. Microglial gene expression influenced by Aβ was identified in the Gene Expression Omnibus (GEO) database (GSE227221). Expression levels of netrin-1 receptors were analyzed to identify potential targets. Protein structures from the RCSB database were docked with netrin-1 using PyMOL to identify the amino acids on the binding interface that may stimulate the receptors and mitigate Aβ-induced damage. Specifically, a concentration of 100 ng/mL of netrin-1 optimally maintained cell viability in the presence of Aβ. Netrin-1 could lessen Aβ-induced microglial activation, especially M2 activation phenotype. ELISA data showed that netrin-1 treatment significantly improved the phagocytic abilities of Aβ-burden BV2 cells. Analysis of the GEO database revealed that the expressions of DCC, DSCAM, and UNC5a were significantly elevated following Aβ treatment in microglia. Eight potential polypeptides were identified as the potential targets for netrin-1 binding. In conclusion, netrin-1 mitigated the Aβ-induced damage in microglia through its protective effects. The eight identified peptides of netrin-1 may represent a significant advancement toward developing drugs targeting Aβ-related AD pathology. Netrin-1 based peptide drugs hold novel therapeutic promise for AD.

Keywords: Alzheimer's disease, peptide drugs, Netrin-1, Microglia activation, Amyloid-β.

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1. Introduction

Alzheimer's disease (AD) is the most common dementia, with its prevalence rising every year, projected to reach nearly 13 million cases by 2050. Currently, this disease affects approximately 50 million people worldwide, with nearly 10 million new cases diagnosed each year, and ranks as the fifth-leading cause of death among individuals aged 65 and older, highlighting its severity and the urgency for research and intervention [1]. The increasing burden of this neurodegenerative condition underscores the critical need for intensified research efforts aimed at understanding its complex mechanisms and developing effective treatments. The dominant theory in the pathology mechanism of AD is that it is caused by the wrong amyloid precursor protein (APP) cut leads to the formation of amyloid-β (Aβ). This process triggers oligomerization into neurotoxic plaques, ultimately resulting in neuron death [2]. The existing treatments for AD remain limited and primarily focus on alleviating symptoms rather than addressing the underlying causes. The therapeutic approach specifically targets the fundamental biological mechanisms that might provide a more effective avenue.

Upon pinpointing a disease-related target, such as the aggregation of Aβ, researchers proceed to generate compound libraries for subsequent screening [3]. Antibodies or polypeptides that are engineered to bind to precise regions, or epitopes, on the target protein have garnered significant attention due to their potential for fewer off-target effects compared to conventional small molecule drugs. Presently, there are three antibodies have been approved by the U.S. Food and Drug Administration (FDA) that are designed to target Aβ aggregates: aducanemab, lecanemab, and donanemab [4]; solanezumab, gantenerumab, and crenezumab, which were potential candidates for AD drugs targeting Aβ, were shown to be ineffective or even harmful for AD patients, thus were rejected [5,6]. These antibodies have demonstrated some efficacy in lowering the levels of cerebrospinal fluid (CSF) biomarkers and reducing Aβ accumulation in the brain, offering hope for novel therapeutic interventions in combating AD [7].

Netrin-1 is a secreted protein found in the extracellular matrix (ECM) and is recognized for its multifunctional roles, particularly in guiding axonal growth during neural development and modulating cell migration. Dysregulation of netrin-1 signaling has been implicated in various pathological conditions. The level of netrin-1 was found to be inversely related to seriousness of dementia [8]. It was also concluded in a postmortem study on late-onset AD patients that netrin-1 decreased the activity of β-secretase, which is a significant factor in apoptosis and neurodegeneration, demonstrating a protective effect [9]. In mouse models with Aβ 1-42 induced AD, levels of netrin-1 decreased in serum and CSF, leading to cognitive deficits [10]. Moreover, APP was shown to bind with netrin-1 in mice and modulate Aβ peptide production [11]. Specifically, it decreased Aβ amplification and, in turn, improved cognition (working memory, learning memory, and synaptic plasticity) by boosting long-term potentiation (LTP) [12,13]. Netrin-1 also enhanced spatial and object memory in rat models with increasing neuronal density [14]. Furthermore, netrin-1 had been shown to protect against Aβ toxicity, treat inflammation in human neuroblastoma cells, and inhibit cell death cascade induced by APP, increasing cell viability [15,16].

Netrin-1 has been shown to influence blood vessel formation. Peptides that can modulate netrin-1's angiogenic effects could be beneficial in treating conditions where abnormal blood vessel growth [17]. Peptides that mimic the neuroprotective effects of netrin-1 could potentially be developed to treat neurodegenerative disorders. By promoting neuronal survival and regeneration, such peptides might help slow disease progression or facilitate in recovery after nerve injury. Recently, Netrin-1 was reported to mediate microglia-neuron crosstalk and is involved in AD. Microglia is an important type of glial cell found in the central nervous system, making up about 10%-15% of glial cells. They become activated in response to the accumulation of Aβ plaques and neurofibrillary tangles, two hallmark features of AD. Activated microglia can exhibit both protective and detrimental effects.

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Understanding the dual role of microglia in AD is critical for developing therapeutic strategies that can modulate their activity to prevent or slow down disease progression. Targeting microglial functions has emerged as a promising approach in Alzheimer's research, aiming to harness their protective properties while mitigating their harmful effects.

Overall, netrin-1 is found to have a therapeutic effect on AD. And netrin-1 secreted by microglia is an important factor in AD pathology. However, the effect of netrin-1 on microglia activation in the AD brain is still unclear. This study aims to investigate the effects Aβ oligomers and netrin-1 has on BV2 microglial cell line, thus evaluating the potential therapeutic effect netrin-1 has on Aβ-burden microglia, and based on cell experiments and molecular docking, verify netrin-1's ability to enhance microglia's role in clearing Aβ, and derive netrin-1 peptides that might be helpful in eliminating Aβ in early-onset AD.

2. Methods

2.1. Cell model construction

2.1.1. Cell culture

BV2 microglial cells (derived from C57/BL6 mice, Guangzhou Yuanjing Biotechnology Co., LTD) were cultured in RPMI 1640 medium (VivaCell BIOSCIENCES) with 10% Fetal Bovine Serum (FBS) in culture dishes. The growth medium was incubated at 37°C with 5% CO_2 and high humidity. Cells were passaged every 48 hours, and after passaging a sample was used for cell counting using a hemocytometer to evaluate cell viability. When not in use, they are frozen and stored in a liquid nitrogen tank at -180°C. All procedures were performed in an uncontaminated cell culture hood.

2.1.2. Cell model construction and netrin-1 treatment

Experimental groups were divided on a 96-well plate, with control (culture medium), Aβ oligomer (2 μM), Aβ and various doses of netrin-1 (400, 200, 100, 50, 20, 10, 5 ng/mL, respectively). Each group occupied six wells, totaling 54 wells, each with 100 μL of solution, with the empty wells filled with saline solution.

2.2. Cell viability assay

Cell Counting Kit-8 (CCK-8) assay was used to evaluate cell viability, and the cells were incubated at 37°C for half an hour. 600 μL of CCK-8 reagent kit (Solarbio LIFE SCIENCES, previously stored at 4°C) was diluted with 5400 μL of culture medium. The previous 54 wells each had 100 μL of the mixture added after the old culture medium was removed. A microplate reader (BioTek SYNERGY H1) was used to measure absorbance at 450 nm.

2.3. Cell morphology

The morphology of cells for each group (control, Aβ, Aβ with 100 ng/mL netrin-1) was observed by phase-contrast microscopy. After that, immunofluorescence staining [18] was used to visualize the cell outlines. The culture medium was discarded, and phosphate-buffered saline (PBS) was added to wash the wells. 4% Paraformaldehyde (PFA) fixed proteins for 9 min and cleansing was done 3 times. 0.1% Triton X-100 (diluted from 10% using PBS) was added into wells and after 5 min, the wells were washed again. Blocking buffer was made with 3% of Bovine Serum Albumin (BSA), with Phalloidin staining (Beyotime, Actin-Tracker Green-488) and 4′,6-diamidino-2-phenylindole (DAPI) added. 200 μL of the buffer was added to each well. The plate was incubated at room temperature for 60 min. PBS with Tween-20 detergent (PBST) was used to rinse the wells, followed by a wash with

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PBS. A drop of 50% glycerol was added on each slide coverslips originally in the wells (on which cells were grown) were carefully extracted using tweezers, and the side with cells slowly lowered onto the slides. Two repeats from each group (control, Aβ, Aβ, and netrin-1) were examined using fluorescent microscopy (Olympus FLUOVIEW FV3000).

2.4. Detection of microglia activation using real-time PCR

Real-time PCR was conducted to quantify mRNA levels of target genes. RNA was extracted using the RNA Easy Fast Tissue/Cell Kit (TIANGEN, DP451) following the direct purification method. Briefly, after removing the culture medium, 10 μL of proteinase K and 350 μL of buffer RLA were added to each sample well. After transferring the solution to centrifuge tubes, it was centrifuged at 13400 g for 3 minutes. The supernatant was passed through gDNA eraser columns, and RNA was purified using RNase-free columns. Ethanol was added, and the solution was centrifuged again. Washing steps were performed with buffers RW3 and RW, followed by a final elution of RNA with 40 μL of RNase-free water. RNA concentration was measured using a spectrophotometer (NANODROP 2000c) at OD_{260}. The RNA was then reverse transcribed into cDNA using the FastKing gDNA Dispelling RT SuperMix (TIANGEN, KR118). Primers for TNF-α, IL-1β, CD86, iNOS, TGF-β1, IL-10, CD206, and ARG1, along with the reference gene GAPDH, were designed using NCBI resources. Reverse transcription was carried out at 42°C for 15 minutes, followed by enzyme inactivation at 95°C for 3 minutes.Real-time PCR was performed using SuperReal PreMix Plus (SYBR Green) (TIANGEN, FP205). Each reaction contained 10 μL of PreMix Plus, 0.6 μL of each primer, 2 μL of cDNA template, and 6.8 μL of RNase-free water. The PCR protocol included an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of 95°C for 10 seconds and 60-66°C for 20-32 seconds. The expression ratio was calculated using the 2^-ΔΔCt method."

2.5. Phagocytic ability detection using ELISA

Cells and medium were centrifuged at 4°C and 1000 g for 15 minutes, and the supernatant was stored at 4°C. An ELISA was performed using the Human Aβ1-42 ELISA kit (Solarbio LIFE SCIENCES). The kit components included a zymogen plate with primary antibody coating, standardized product, SR1 diluent, secondary antibody with its diluent, enzyme conjugate with its diluent, cleansing fluid, chromogenic substrate, terminating fluid, and sealing tape. Before the experiment, the kit and specimens were adjusted to room temperature for 30 minutes. Cleansing fluid was diluted from 25× to 1× with deionized water. The standardized product was diluted with 1 mL SR1 diluent from 1000 pg/mL, creating a gradient down to 15.63 pg/mL. A 500 μL diluent served as the 0 pg/mL control. Secondary antibodies and enzyme conjugate were diluted from 100× to 1× using SR2 and SR3 diluents, respectively. The ELISA protocol involved adding 100 μL of standardized product or specimen to each well, followed by incubation at 37°C for 90 minutes. After discarding the solution, 100 μL of secondary antibodies were added, and the plate was incubated at 37°C for 60 minutes. The plate was washed three times manually. Then, 100 μL of enzyme conjugate was added, and the plate was incubated for 30 minutes. The plate was washed five more times. Next, 90 μL of chromogenic substrate was added and incubated for 15 minutes. Finally, 50 μL of terminating fluid was added. Absorbance was measured at 450 nm and 630 nm using a microplate reader (BioTek SYNERGY H1). The Aβ concentration was calculated by subtracting the 630 nm values from the 450 nm values and using the standard curve equation.

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2.6. Microglial gene expression under the influence of Aβ provided by Gene Expression Omnibus (GEO) database

We chose the GSE227221 database from an array of options focusing on microglia in AD pathology, which used RNA-seq analysis to find differentially-expressed genes after different lengths of time after being treated with Aβ. The database used WT Induced pluripotent stem cells (iPSC)-induced microglia, and the level of expression of each gene was measured at the start without adding Aβ (0 h), 4 h, 24 h, and 96 h. We selected the genes controlling netrin-1 receptors, namely DCC, Neo1, DSCAM, UNC5a, UNC5b, UNC5c, and UNC5d. GO2R yielded sample values for each interested gene, which was used to plot line graphs to represent changes of gene expression with time.

2.7. Molecular docking to show receptor-ligand binding and provide potential polypeptide drugs

The RCSB Protein Data Bank (PDB) was accessed, and the relevant proteins (namely netrin-1, DCC, UNC5a, and DSCAM) were downloaded in PDB format and imported into PyMOL, where atoms other than the protein itself were discarded. The structures included human netrin-1 in complex with human DCC (in which only netrin-1 was used because the DCC was a short fragment) coded as 4URT, human DCC coded as 3LAF, human UNC5a Immunoglobin (Ig) domain coded as 4V2A, and human DSCAM Ig7-Ig9 coded as 6ZR7. Each receptor was imported into ClusPro 2.0 with netrin-1 and docked. The model scores were downloaded, and the best model for each interaction was imported into PyMOL. Pictures of the surfaces of ligand-receptor complexes were taken. Polar contacts to any atoms were found for each protein, and the interface between ligand and receptor (distance less than 5 Å between residues) was found and selected. These sequences on netrin-1 were recorded in order to yield polypeptides that may be used therapeutically, possibly protecting Aβ-burden microglia.

3. Results

3.1. Netrin-1 increased cell viability of Aβ-burden microglia with dose-dependent manner

In order to determine which concentration of netrin-1 was most beneficial to cell viability of BV2 microglia cell line, different concentrations (5, 10, 20, 50, 100, 200, 400 ng/mL) were tested, along with control and Aβ only, using CCK-8 at OD 450 nm. Each concentration was tested 6 times, the final results were obtained by averaging the 6 results. One-way ANOVA showed that treatment with 100 ng/mL of netrin-1 was most beneficial for cell viability of Aβ-burden microglia (none had shown statistical significance, p = 0.1473), and was used for later experiments (Figure. 1).

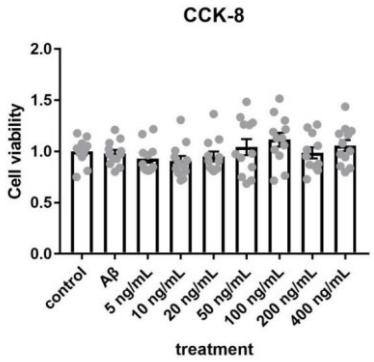

Figure 1: The optical density at 450 nm of groups of BV2 microglia cell line that underwent different treatments. 100 ng/mL of netrin-1 counteracted the detrimental effects of Aβ aggregates the most (p value=0.1473).

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3.2. Netrin-1 attenuated the activation of Aβ-burden microglia

The images showed that BV2 cells with nothing added were in the resting state, while adding Aβ induced activation. Adding netrin-1 slightly weakened activation (Figure. 2 up panel).

Phalloidin staining was used under fluorescent microscopy to examine the morphology of BV2 microglial cell line under different conditions. Contrary to human microglia, BV2 cells are spherical in resting state, and turn spindle-like when they are activated. The control group showed microglia mostly in the ramified state, with small and round shapes, while many microglia were starting to get activated and turn ameboid-like under Aβ, exhibiting long extensions on both ends. Adding 100 ng/mL of netrin-1 attenuated the activation of microglia (Figure. 2 lower panel). These findings suggested that netrin-1 can remedy the damage done by Aβ on microglial cell line, which is consistent with the results obtained from CCK-8. But the specific effects of netrin-1 on each microglial morphology, such as M1 and M2, remains to be found out.

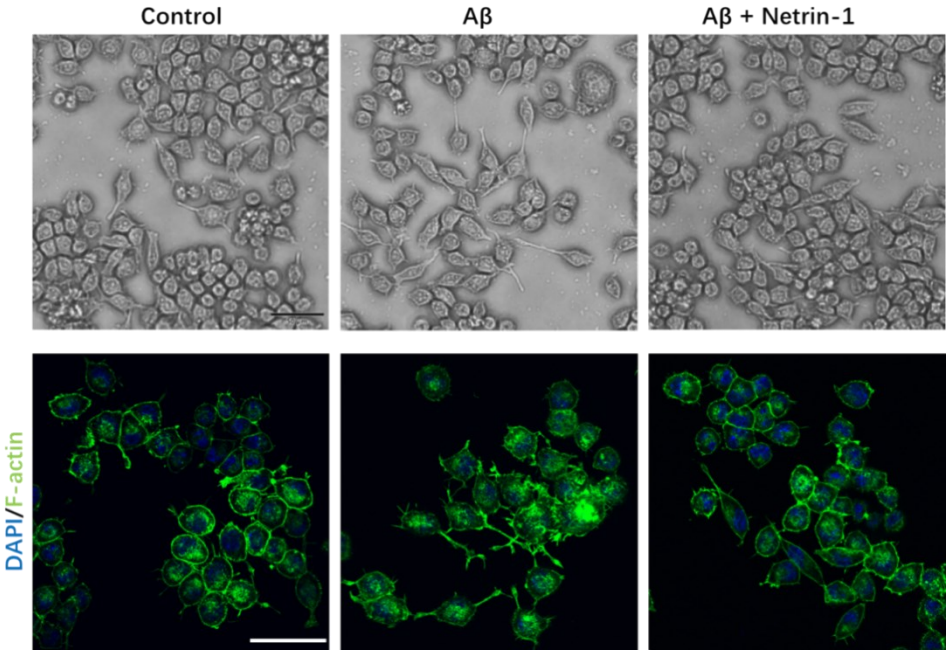

Figure 2: Microscopic images illustrate the cell morphology of microglia in the control group, Aβ group, and Aβ + 100 ng/mL netrin-1 group. The green dye stains the cytoskeleton, while the blue dye stains the nucleus. In the control group, most cells appear to be round. In the Aβ group, cells processes were obvious, and when netrin-1 was added, most cells return to the circular morphology. This shows that netrin-1 deadens the activation of Aβ-burden microglia. Scale bar = 50 μm.

3.3. Netrin-1 affected M2 activation more than M1 activation

It was already established that netrin-1 weakened the activation of microglia under the influence of Aβ, but the exact effects on each morphology (M1 and M2) remained unclear (Figure. 2). Microglial morphology is mainly divided into M1, the pro-inflammatory type that is characteristic in AD pathology, and M2, the anti-inflammatory type that protects the neural cells by phagocytosing unneeded waste products or harmful pathogens. In order to know the effects of netrin-1 on each of M1 and M2 activation in Aβ-burden microglia, Real-time PCR was used on 8 different genes, 4 expressed in M1 activation (TNF-α, IL1β, CD86, and iNOS), and 4 expressed in M2 activation (IL-10, TGF-β, CD206, and ARG1) (Figure. 3).

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In M1 activation, Aβ oligomers significantly increased the expression of TNF-α and CD86, while significantly reducing the mRNA levels of IL1β and iNOS. Adding netrin-1 significantly reduced the activation of TNF-α, while having no significant effect on the activation of the other three (Figure. 3A-D). Overall, Aβ may either elevate or decrease the level of expression of M1-related genes, thus enhancing or reducing pro-inflammatory effect, while netrin-1 may either attenuate M1 activation or have no significant effect, thus having a minor anti-inflammatory effect. In M2 activation, Aβ oligomers significantly increased the activation of TGF-β and ARG1, while having no significant effect on the activation of IL-10 and CD206. Adding netrin-1 significantly reduced the activation of all four genes (Figure. 3E-H). In summary, Aβ enhances the anti-inflammatory effects to some extent, while netrin-1 decreases the protective effects.

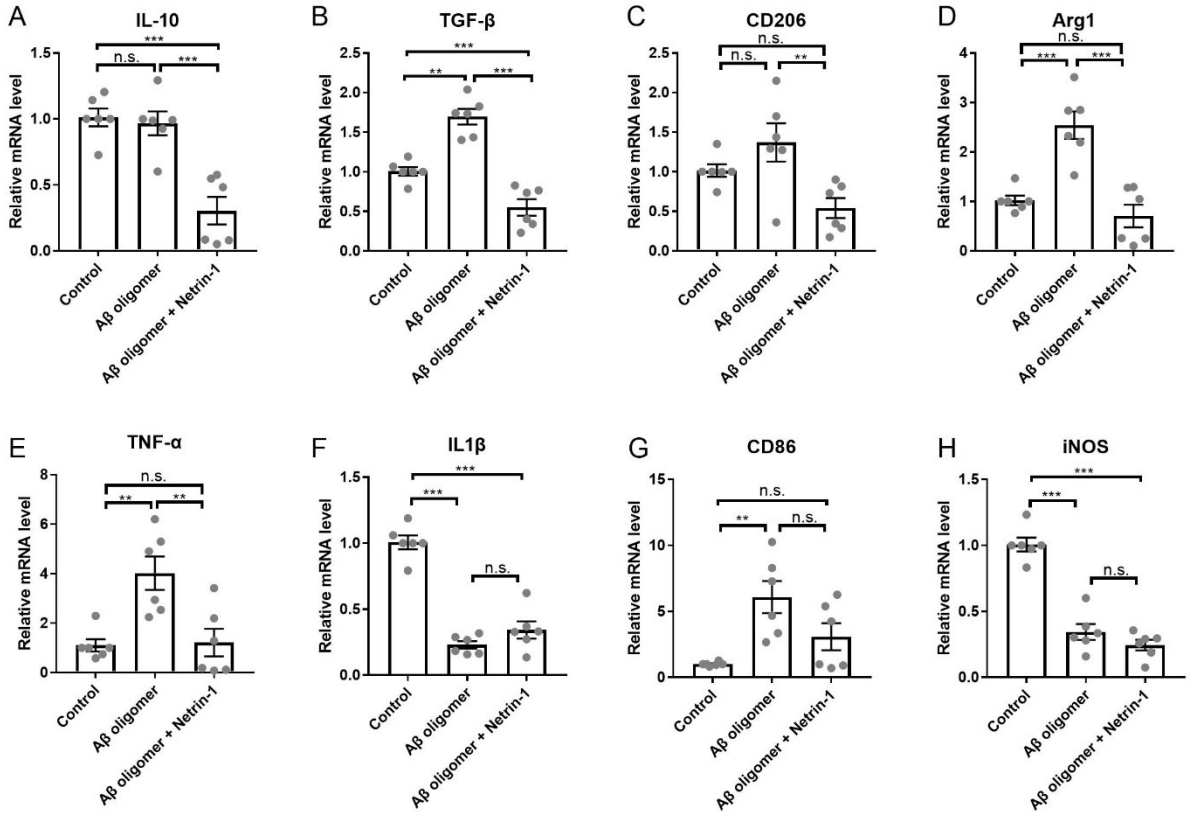

Figure 3: Relative mRNA levels were measured for the control group (culture medium), Aβ oligomer group (2 μM Aβ), and Aβ oligomer + netrin-1 group (100 ng/mL netrin-1). Netrin-1 had significant effects on only one gene expressed in the M1 state while having significant effects on all four genes expressed in the M2 state, therefore netrin-1 affects mostly M2 activation, significantly reducing activation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s. = non-significant.

3.4. Netrin-1 significantly increased phagocytic ability

Phagocytic ability is associated with M2 activation, and phagocytosis performed by microglia is important in clearing Aβ plaques and repairing the pathological damage. In order to know the effects of netrin-1 on phagocytic ability, ELISA was used to evaluate how much Aβ oligomers were left behind. The standard curve was defined by the equation $y=0.0065x+0.1606$, and $R^2=0.9823$ (Figure. 4A). Data suggests that treatment with netrin-1 significantly decreased the Aβ concentration in the medium, reducing concentration from approximately 100 ng/mL to about 60 ng/mL ($p<0.001$, ***)

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(Figure. 4B). This reduction suggests the increased phagocytic ability of BV2 cell line, therefore netrin-1 significantly increased protective abilities of BV2 cells, and may be a potential therapeutic for AD.

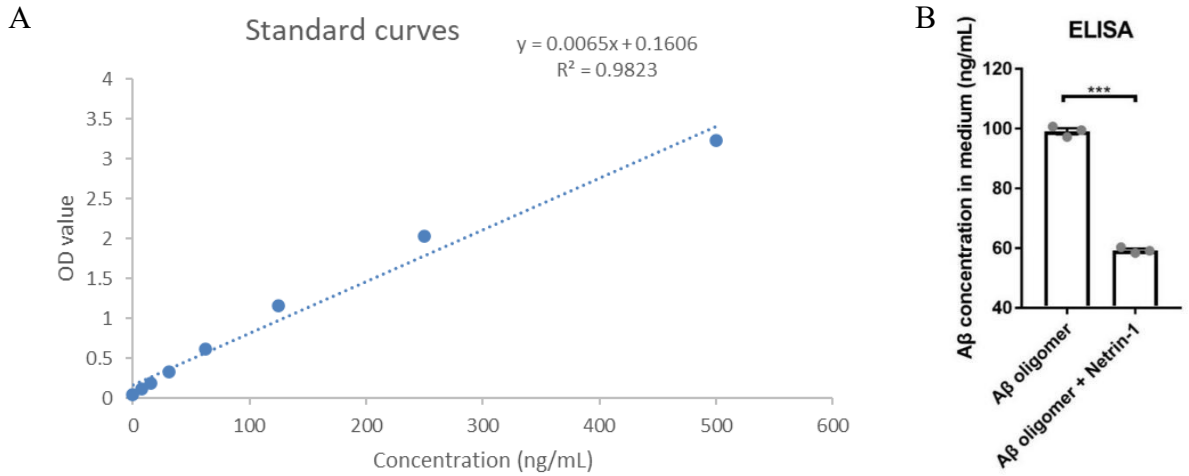

Figure 4: Aβ concentration in medium is an indicator of phagocytic ability, which was examined using ELISA for both oligomer and treatment groups. (A) Standard curve. (B) Aβ concentration in medium with and without netrin-1. Netrin-1 has a highly significant effect on enhancing the phagocytic abilities of Aβ-burden microglia.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s., non-significant.

3.5. Expression of each receptor gene showed large fluctuations with time

We already established that netrin-1 has a protective effect on Aβ-burden microglia, indicating the potential for developing a netrin-1-based polypeptide drug. Netrin-1 has many receptors, but which ones play a crucial role in promoting netrin-1's anti-inflammatory effect in microglia remain uncertain. Data concerning expression of genes DCC, Neo1, DSCAM, UNC5a, UNC5b, UNC5c, and UNC5d under different conditions (0h with no Aβ, 4h, 24h, and 96h after adding Aβ) were utilized in order to make comparisons (GSE227221) and determine which receptor(s) are functioning. A line graph was generated for each gene that plotted average expression value against time. The level of expression of DCC increased sharply from 4h to 24h, almost four-fold. DSCAM showed a major increase, by approximately 6 times, from 4h to 24h. The trend was also signficant rise for UNC5a from 24h to 96h, with a near 5.5-fold. The other four genes, namely, Neo1, UNC5b, and UNC5d, showed no perceptible rise, with UNC5c levels remaining stationary overall, and the other three showing noteworthy falls (Figure. 5). Overall, the results suggested that DCC, DSCAM, and UNC5a might be implicated in protecting microglia against Aβ. Molecular docking will be performed on these proteins to ascertain of their interaction with netrin-1.

DOI: 10.54254/2753-8818/68/2025.20237

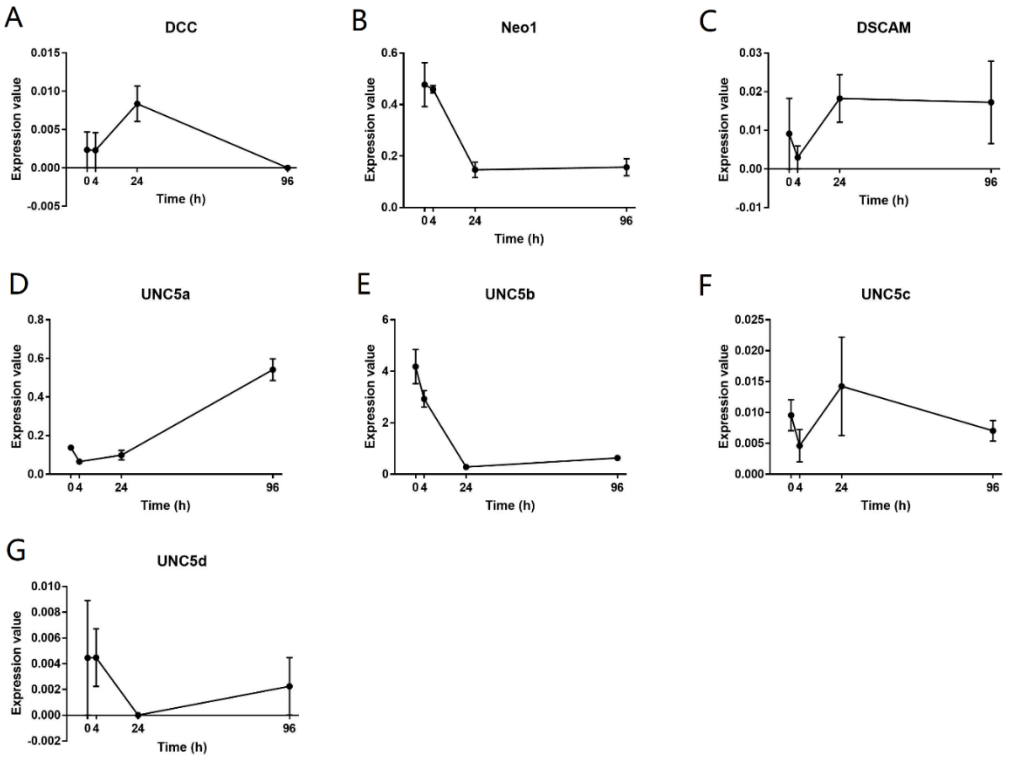

Figure 5: Line graphs relating expression of each gene and time to track changes. Raw data was obtained from GSE227221 in GEO database, from a study focused on using RNA-seq analysis to investigate microglial changes under progressing AD. DCC, DSCAM, and UNC5a showed significant rises 4h onward, indicating roles in netrin-1's anti-inflammatory effects.

3.6. Molecular docking suggested potential peptide drugs

Structures of receptors DCC, UNC5a, DSCAM, and ligand netrin-1 from RCSB PDB were docked in ClusPro and viewed in PyMOL. The model scores, all less than zero, proved that binding occurred for all three. The models with the highest scores was used. Pictures showing binding between netrin-1 (yellow) and UNC5a (red) (Figure. 6A), netrin-1 (yellow) and DSCAM (cyan) (Figure. 6C) and netrin-1 (yellow) and DCC (magenta) (Figure. 6B) were taken. The amino acids on the netrin-1 side of the binding interface were found and combined into strands of polypeptides that may be used to develop drugs that target each respective receptor and exert their anti-inflammatory functions (Table 1).

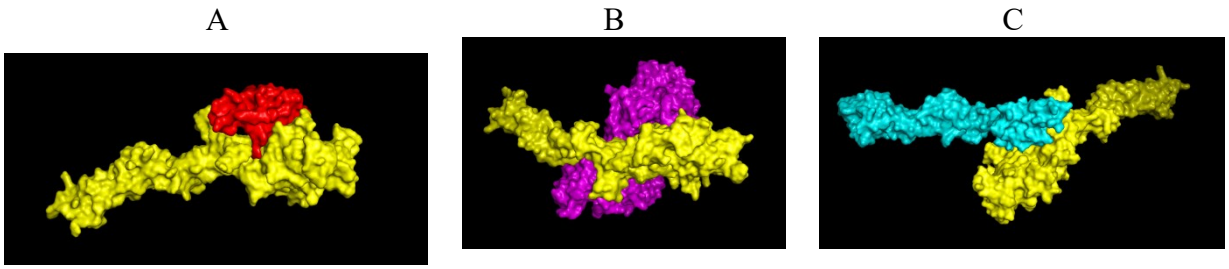

Figure 6: Binding between netrin-1 and UNC5a (A), netrin-1 and DCC (B) and netrin-1 and DSCAM (C), showing the surfaces of the proteins.

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Table 1: Netrin-1 peptide strands that can bind to each receptor (three for UNC5a, two for DCC, and three for DSCAM). Highlighted portions were binding sites.

Receptors	Peptide strands	Range	Length (a.a.)
UNC5a	DPCSDENGHPRRCIPDFVNAAFGK	39-62	24
	LNNPHNLTCWQSENYLQFPHNVTLTLSLG KKFEVTYVSLQ	111-150	40
	DLQVGGRCKCNGHAAR	278-293	16
DCC	PDPCSDENGHPR	38-49	12
	RCVRDRDDSLVCDCRHNTAGPECDRCKP FH	293-322	30
DSCAM	RRCIPDFVNAAF	49-60	12
	AFLTDLNNPHNLT	106-123	18
	LIAFSTLDGRPSAHDFD	217-233	17

3.7. Verification of peptides' binding with netrin-1 and their biological roles (Future plan)

Due to potential inaccuracies of molecular docking, GST-pull down is needed to justify the binding between netrin-1 peptides and receptors DCC, UNC5a, and DSCAM, and ascertain whether they played a role in protecting microglia under the stress of Aβ.

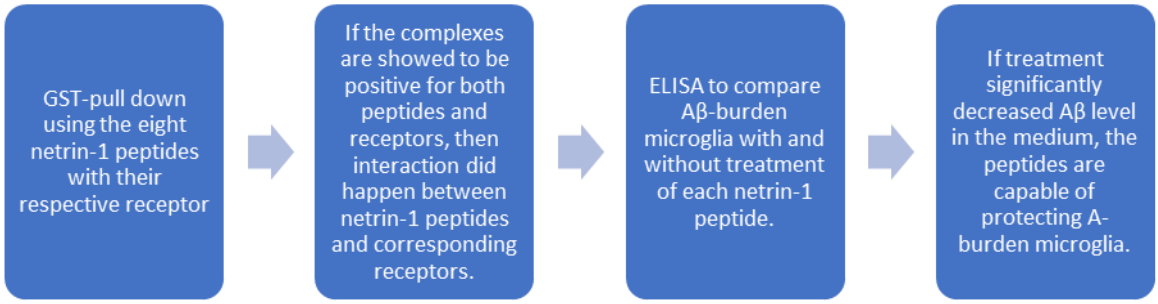

Figure 7: Flow chart showing steps to determine whether the peptides will bind with DCC, DSCAM, and UNC5a, and if biological roles of binding were to do with eliminating Aβ.

The method of GST-pull down is elaborated below: The netrin-1-based peptides sequence with a GST tag attached is inserted into a plasmid via Gibson Assembly that will be injected into a bacterial vector and cultured, which induces the netrin-1 peptides to be expressed, and then purified using affinity chromatography, making it bind to agarose beads. Unbound proteins are washed away, then the receptor proteins are mixed and incubated with netrin-1 peptides attached to GST and beads. Unbound proteins are washed away, and elution severs the links between GST and peptides, leaving ligand-receptor complexes, which are then analysed by SDS-PAGE, with four columns: marker, purified "bait" (peptides), agarose gel control, and purified protein-protein interaction [19]. If purified "bait" shows only netrin-1 peptide present, agarose gel with negatives for both, and final product positive for netrin-1 peptide and receptor, it can be concluded that interaction between receptor and netrin-1 peptide did occur.

In order to prove that netrin-1 binding with DCC, DSCAM, or UNC5a has a protective effect on Aβ-burden microglia, ELISA will be performed on untreated microglia under Aβ environment and those with synthesized peptides (mentioned above) added. If the treatment significantly decreases Aβ concentration in the medium, this peptide is able to reverse the damages done by Aβ oligomers, and may be of therapeutic use in treating AD.

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4. Discussion

Our research found out that an appropriate concentration of netrin-1 is effective at maintaining microglial cell viability under Aβ aggregates. Furthermore, netrin-1-treated cells were clearly less activated under Aβ stress. Netrin-1 selectively reduced Aβ-induced M1 activation, while significantly decreasing M2 activation raised by Aβ. Netrin-1 significantly improved the phagocytic abilities of Aβ-burden BV2 cells. Moreover, DCC, DSCAM, and UNC5a (receptors of netrin-1) expression significantly rose with the treatment of Aβ; docking of these proteins with netrin-1 yielded eight polypeptides that might be able to downplay the detrimental effects of Aβ on microglia. In summary, netrin-1 was found to have a protective effect on microglia under the stress of Aβ, and receptors DCC, DSCAM, and UNC5a potentially playing key roles in this process.

In addition to its well-known roles in axonal guidance and cell migration, netrin-1 also played a role in regulating inflammation and the immune response, suggesting ever-changing morphology and both protective and detrimental roles in different inflammatory diseases [20]. This establishes the relevance of microglia, which is also a key player in neuroinflammation. Microglia can be morphologically classified into ramified (or resting) microglia or ameboid (or activated) microglia. Following infection, inflammation, or injury, ramified microglia switches to ameboid type, specifically, M1 (pro-inflammatory), releasing more Tumour Necrosis Factor-α (TNF-α) and Interleukin 1-β (IL-1β) or M2 (anti-inflammatory), releasing more Nuclear Factor (NFs) and Arginase 1 (ARG1). M2 can switch to M1 under chronic and sustained stimulation [21]. When microglia detect damage, they elongate the process in that direction; while shrinking in the other, converting into phagocytes that do phagocytosis, engulfing pathogens, apoptotic neurons, and neuronal progenitors.

Our results suggests that adding Aβ will activate microglia, while netrin-1 attenuates the activation, almost making the long processes of Aβ-burden BV2 cells disappear. Aβ was found to either promote (TNF-α and CD86) or inhibit M1 activation (IL-1β and iNOS). Netrin-1 either had no effect on Aβ-burden BV2 cells (IL-1β and iNOS) or decreased the activation (TNF- α and CD86). For M2 activation, Aβ had either no effect on the activation (IL-10 and CD206), or increased the activation (TGF-β and Arg1). Netrin-1 significantly decreased the activation of all four genes (Figure. 3). Previous studies have shown that netrin-1 is capable of inducing the M2 state in microglia, decreasing apoptosis and thus protecting in vivo cell models of ischemic stroke [22]. The conclusion of their study was similar to our own, that netrin-1 may be of therapeutic use due to its anti-inflammatory effects.

Neuroinflammation mediated by microglia is important in AD progress. When patients enter the mild cognitive impairment stage, M2 microglia's protective, anti-inflammatory response is defective. As patients enter the clinical stage, M2 switches to M1, and its pro-inflammatory response is exaggerated, causing detrimental neuron loss [23]. In other words, inflammatory activity is enhanced while clearing mechanisms are compromised, leading to the accumulation of danger-associated molecular patterns (DAMPs), and neurodegeneration [24]. Our study found that netrin-1 inhibited microglial neuroinflammation (Figure. 3), and instead increased microglia's ability to phagocytoze Aβ (Figure. 4), suggesting that netrin-1 may be able to treat AD. Netrin-1 was previously found to suppress Aβ production in Alzheimer mouse models, and also ameliorate symptoms of AD [25]. Our study supplemented netrin-1's impact on AD in terms of microglia and neuroinflammation, and further ascertained its possibility to treat AD.

Netrin-1's structure greatly resembles the gamma-1 chain in laminin, consisting of domains VI, V, and C. There are many netrin receptors, including Deleted in Colorectal Cancer (DCC) and neogenin, UNC 5 proteins, and DS cell adhesion molecule (DSCAM) [26]. We found that under Aβ stress, netrin-1 activated DCC, DSCAM, and UNC5a. UNC5a is known to be a promoter of neuronal apoptosis [28], so it is unsurprising that it is expressed highly under Aβ pathology, which is known

DOI: 10.54254/2753-8818/68/2025.20237

to induce pro-inflammatory behavior of microglia, causing neuron death. Netrin-1 and DCC are also involved in melanoma, with high netrin-1 expression linked to worsening pathology, and DCC acting as a tumor death promoter [28]. Netrin-1's interaction with NEO1 also helps regulate hematopoietic stem cell dormancy, maintaining its function [29]. Under the context of Diabetic Kidney Disease (DKD), netrin-1 binding with UNC5b enhances angiogenesis effects and thus exacerbates kidney injury [30]. Netrin-1's myriad interactions with different receptors each plays an important role in disease (s) progression, indicating the importance of personal treatment and precision medicine.

Our study does have some limitations, however. The experiments were all done on the BV2 cell model, which resembles primary microglia in the human brain but there are some differences, like different morphologies. We only used 100 ng/mL of netrin-1 in experiments, but using other concentrations might be better in the mouse model. Therefore, future work should focus on perform experiments with animal models, varing the durations before performing experiments, and testing different dosages.

5. Conclusion

Our study discussed the potential therapeutic effects of Netrin-1, a brain-derived extracellular factor, on AD by modulating microglial function. We found that an appropriate concentration of Netrin-1 effectively maintains microglial cell viability under Aβ aggregates and reduced Aβ-induced M1 activation while significantly increasing M2 activation. Netrin-1 treatment also improved the phagocytic abilities of Aβ-burden BV2 cells. This study suggested that Netrin-1 may be of therapeutic use due to its anti-inflammatory effects and its ability to treat AD. We also identified potential targets for Netrin-1 binding, including DCC, DSCAM, and UNC5a, which may represent a significant advancement toward developing drugs targeting AB-related AD pathology. These peptide drugs are capable of specific binding, making personalized treatment possible. Overall, the study provided scientific evidence and technical roadmap for the development of Netrin-1-based peptide drugs for AD treatment.

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