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NTN-1 attenuates amyloid-β-mediated microglial neuroinflammation and memory impairment via the NF-κB pathway and NLRP3 inflammasome in a rat model of Alzheimer's disease

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Introduction: Neuroinflammation driven by microglial activation represents a pivotal pathological mechanism underlying brain injury in Alzheimer's disease (AD), with NLRP3 inflammasome activation being a hallmark feature of this process. Netrin-1 (NTN-1) was recently shown to have potent anti-inflammatory and anti-apoptotic properties in a range of inflammatory diseases; however, its potential effect on neuroinflammation in AD treatment has not been well examined. Accordingly, this study aimed to investigate the effects of NTN-1 on cognitive impairment and to explore the anti-inflammatory properties related to the NLRP3 inflammasome and NF- κ B signaling in A β 1-42-induced rat models.

Methods: We assessed the effects of NTN-1 on neurobehavioral function, microglial activation and neuroinflammation mechanisms in A β 1-42-treated rats using the Morris water maze test and Western blotting.

Results: Our results indicated that microinjections of NTN-1 attenuated A β 1-42-induced memory and cognitive dysfunction and significantly inhibited microglial proliferation and NLRP3 inflammasome activation in the hippocampus and cortex of AD rats. Additionally, NTN-1 effectively prevented proinflammatory factor (IL1 β and IL18) release and NF- κ B signaling upstream activation.

Discussion: Overall, the results of the present study indicated that exogenous NTN-1 treatment prevented neuroinflammation and cognitive deficits by inhibiting microglial activation, which is possibly mediated by the NF- κ B signaling pathway and NLRP3 inflammasome activation in A β 1-42-simulated rat models. NTN-1 emerges as a promising therapeutic candidate for mitigating microglia-mediated neuropathology in AD through its anti-inflammatory properties.

KEYWORDS

netrin-1, microglia, neuroinflammation, NLRP3 inflammasome, NF- κ B, Alzheimer's disease (AD), apoptosis-associated speck like protein (ASC), amyloid- β (A β)

Introduction

The most prevalent form of dementia, Alzheimer's disease (AD), is characterized by amyloid- β (A β) accumulating in oligomers and senile plaques in the central nervous system (CNS) (Lane et al., 2018). Chronic deposition of A β peptides within the CNS, particularly in microglia, triggers neuroimmune cascades that critically contribute to AD pathogenesis through sustained neuroinflammation (Webers et al., 2020). Activated microglia proliferate and secrete proinflammatory cytokines, including the key cytokines interleukin-1 β (IL-1 β), interleukin-18 (IL-18) and tumor necrosis factor (TNF)- α , which eventually cause neuroimmune damage, A β plaque accumulation in neurons, neurotoxicity, progressive behavioral abnormalities and loss of memory symptoms in AD (Hansen et al., 2018; Nanjundaiah et al., 2021).

The transcription and production of the inactive precursor IL-1 β is mediated by the activation of nuclear factor- κB (NFκB), one of the classical signaling pathways in the microglial immune response (Jiang et al., 2015; Li et al., 2019). Moreover, pro-IL-1ß and pro-IL-18 are processed into biologically active forms to play a role in neuroinflammation by protease caspase-1, a member of the NLRP3 inflammasome (Halle et al., 2008; Kelley et al., 2019). Recently, the NLRP3 inflammasome has been established to be a novel inflammasome signaling pathway in response to various stimuli such as infection, cell damage or Aβ fibrils (Thawkar and Kaur, 2019). The inflammasome comprises the cytoplasmic receptor NLRP3, apoptosis-associated speck-like protein (ASC) and caspase-1 downstream (Spel and Martinon, 2020). Activation of the NLRP3 inflammasome requires a twostep process: NF-KB-mediated NLRP3 transcription and subunit assembly of the NLRP3 inflammasome by upstream activating molecules (Bauernfeind et al., 2009; Dun and Parkinson, 2017). It has been demonstrated that cleaved caspase-1 expression is strongly higher in human AD brains, and NLRP3 or caspase-1 gene knockout AD mice have less memory impairment and enhanced Aßclearance (Heneka et al., 2013). NLRP3 is also reported to be active and produce IL-1ß along with microglial activating and neurotoxic factors aggregating in Aβ-driven models (Heneka et al., 2013; Tschopp and Schroder, 2010). These results suggest that the NF-KB-NLRP3 inflammasome pathway mediates neuroimmune damage and amyloid-beta production and is proposed as an alternative strategy in the treatment of AD.

Netrin-1 (NTN-1), an extracellular protein that has been shown to regulate cell migration, guide axonal growth and exert antiinflammatory functions in the CNS (Lou et al., 2020; Mulero et al., 2017). Several studies have observed that altering the expression of NTN-1 has beneficial effects in tumors, autoimmune disorders, ischemic stroke and Parkinson's disease (El-Gamal et al., 2020; He et al., 2018; Moon et al., 2006; Tadagavadi et al., 2010). Spilman PR et al. found that overexpression of NTN-1 in transgenic AD mice reduced both AB1-42 and AB1-40 and improved cognition (Spilman et al., 2016). Intrahippocampal fissure-injected NTN-1 prevented memory impairment in an AB1-42-related rat model (Zamani et al., 2019). In our previous study, we also found that the expression of NTN-1 was decreased in both the cerebrospinal fluid (CSF) and serum of Aβ-induced AD rats (Sun et al., 2019). It was demonstrated that the expression of NTN-1 is associated with amyloid-beta production, which might be a key factor in A\beta regulation (Lourenço et al., 2009). NTN-1 strongly exerts an anti-inflammatory properties by suppressing NF-KB pathway activation in endothelial cells and subarachnoid hemorrhage rats, and it also plays a critical role in preventing NFκB and caspase-3/7 activation and ROS damage by microinjection in AD rats and SH-SY5Y cells (Lin et al., 2018; Xie et al., 2018; Zamani et al., 2020, 2019). Moreover, it was reported that NTN-1 reduced IL-1 β and IL-12 β and increased PPAR γ expression in cultured astrocytes after brain injury (He et al., 2018). Despite this evidence, the detailed molecular mechanism of NTN-1 in AB1-42-simulated neuroinflammation regulation and neuroprotection in AD was previously unknown. Herein, we focused on exploring the molecular mechanisms of NTN-1 in the anti-inflammatory and neuroprotective effects of A\beta1-42-induced AD rats.

Materials and methods

Animals

Adult male Sprague–Dawley rats (300~350 g) from the center of experimental animals at Harbin Medical University of China were housed under standard laboratory conditions (T: $25 \pm 1^{\circ}$ C, a 12-h light/dark cycle). Before the experiment, rats were kept for 7 days to adapt to their environment and had free access to food and water available *ad libitum*. All animal experimental procedures were performed strictly in accordance with the Animal Ethics Committee of Harbin Medical University.

A β 1-42 and NTN-1 treatment preparation

The rat A β 1-42 peptide (SCP0038, Sigma) was dissolved in sterilized saline to achieve the desired concentration (5.5 μ g/ μ L). It was equipped in several microtubes and stored at -20° C. Before use, the peptide was incubated for 1 week at 37°C (Zhang et al., 2013). NTN-1 (R&D) powder was dissolved in sterilized saline, vortexed to a final concentration of 160 ng/ μ L and stored at -20° C before use (Zamani et al., 2019).

Rat microinjection and surgery

The animals were stratified into the following groups: wild-type group(WT), sterilized saline operated group (sham), A β 1-42 group (A β), Netrin-1 treatment A β 1-42 group (A β +NTN-1) and vehicle control A β 1-42 group (A β +PBS), with similar numbers of male rats included (15 rats per group). Age-matched adult male animals of

Abbreviations: AD, Alzheimer's disease; ASC, apoptosis-associated speck like protein; A β , amyloid- β ; CSF, cerebrospinal fluid; CNS, central nervous system; IL-1 β , interleukin-1 β ; IL-18, interleukin-18; I κ B α , IkappaBalpha; NF- κ B, nuclear factor- κ B; NTN-1, netrin-1; NLRP3, pyrin domain-containing protein 3; ROS, reactive oxygen species; SEM, standard error of the mean; TNF, tumor necrosis factor; WT, wild-type; APP, amyloid precursor protein; sAPP α , secreted amyloid precursor protein-alpha.



similar weights were anesthetized with sodium pentobarbital (40 mg/kg) by intraperitoneal injection. Rats were placed on a heating blanket to keep the body temperature, the head was fixed in a stereotaxic frame, and microinjection was performed as previously described (Zamani et al., 2019). Briefly, 4 µL Aβ1-42 sterilized saline were slowly injected (0.5 µL/min) into CA1 region of each hippocampus using a Hamilton syringe at a position: -3.5 mm lateral to bregma, $\pm 2 \text{ mm}$ from midline, and -2.8 mm deep from the surface of the skull over a period of 120 s. For NTN-1 group, 5 µL NTN-1 buffer was slowly injected into each hippocampal fissure $(0.5 \,\mu\text{L/min})$ using the same syringe 30 min later at the following position (AP: -4.7 mm, laterality: $\pm 3.4 \text{ mm}$, and DV: -3.4 mm). For sham and vehicle control groups, equal volumes of sterile saline or PBS were injected into the respective areas instead according to the coordinates that mentioned earlier, followed by a 14-day rest to allow for recovery (Figure 1).

Morris water maze task

Spatial learning and memory were assessed on Day 16 postsurgery by the Morris water maze (MWM) task as previously described (Ai et al., 2013). Briefly, a 2.0 m diameter black circular pool was filled with plain water ($24 \pm 1^{\circ}$ C) and divided into four quadrants. A transparent platform (20 cm diameter) was submerged 2 cm under the water surface and placed in the first quadrant. Prior to testing, the rats received three learning trials

per day for 5 consecutive days to adapt to the hidden platform. For each trial, the rats started from a random quadrant (not the target quadrant) to swim freely for 60 s and then rested for 30 s on the 6th day. An online DigBehav-Morris water maze video analysis system (Mobile Datum Software Technology) was used to record the escape latencies, the total time spent in target quadrants and the number of platform crossings of rats in this test.

SDS-PAGE and immunoblotting

Molecular studies began on the 22nd day postsurgery. Hippocampus (bilateral) and cortex of brain were removed in each group, respectively, homogenized and extracted in cold RIPA buffer (Beyotime, China) containing protease and phosphatase inhibitor cocktail (Roche) for 30 min, then centrifuged at 13,000 g for 15 min at 4°C. Equal amounts of protein samples were separated by SDS-PAGE and transferred onto nitrocellulose filters (Merck Millipore Ltd., USA), followed by blocking with 5% skim milk. The membranes were incubated with the following primary antibodies: anti-netrin-1(ab126729, Abcam, 1:500), anti-NLRP3 (ab263899, Abcam, 1:1,000), anti-caspase-1 (A18646, Abclone, 1:500), anti-ASC (sc-514414, Santa, 1:1,000), anti-IL-1β (ab254360, Abcam, 1:1,000), anti-IL-18 (ab71495, Abcam, 1:5,000), anti-Aβ (Millipore, 1:1,000), anti-β-actin (ab8226, Abcam, 1:1,000), anti-p65 (8242, CST, 1:1,000), anti-p-p65 (3033, CST, 1:1,000), anti-Ikba (4814, CST, 1:1,000), anti-pIkba (2859, CST, 1:1,000), anti-Iba-1 (ab178847, Abcam, 1:1,000). After washing with PBST, secondary antibodies from Li-COR Biotechnology (1:10,000) were added to the samples. Immunoreactivity was detected using an Odyssey CLx imager (Licor, Bad Homburg, Germany), and pictures were analyzed using Image Studio (Licor, Bad Homburg, Germany).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits were used for determining quantitatively cerebrospinal fluid (CSF) sAPPa (27419, IBL, Japan) and A β 1-42 (27721, IBL, Japan) concentrations of rats, according to the protocols provided by the manufacturer. The CSF was collected from the rat by foramen magnum puncture. Briefly, 30 μ L of each sample and its control were added to the 96-well microtiter plate which precoated with capture antibody and incubated overnight at 4°C. Plates were washed for 5 times and incubated for 30 min with detection antibody at room temperature, followed by added chromogen solution for 30 min. Optical densities were all measured at 450 nm.

Statistical analysis

Statistical analyses were presented as GraphPad Prism 5.0 (GraphPad Software, La Jolla, USA). P<0.05 was considered

statistically significant. Data are presented as the mean \pm standard deviation. Differences between two paired groups was evaluated with *t* tests and two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* analysis were assessed among the multiple groups. GraphPad Prism 5.0 was used to construct graphs.

Results

NTN-1 ameliorated memory and cognitive impairment in $a\beta$ 1-42-induced AD rats

The behavioral tests of rat models were measured in our experiment, as shown in Figure 2, there was no significant difference between wild-type and sham groups, however, the A β 1-42 and A β 1-42+PBS rats had a decreased level in the total time spending in target quadrants and the number of crossing times of platform, further more, the escape latencies were elevated for AD rats. Spatial learning and memory was significantly improved in A β + NTN-1 rats, along with an increased degree of total time spent in the target quadrants (*P* < 0.001) and the number of platform crossings (P<0.01) and a decreased level of escape latency (*P* < 0.05) compared with the vehicle group of AD rats (Figures 2A–C). Our results were consistent with previous studies showing that NTN-1 administration produced a clear elevation in memory and cognitive ability in A β 1-42-induced AD rats (Zamani et al., 2019).



FIGURE 2

NTN-1 rescues impaired water maze learning in A β 1-42-induced AD rats (n=10 per group). (A) Escape latencies in water maze phase. (B) The number of times of platform in the probe test. (C) The total time spent in the target quadrant in the test. (D) Representative swimming paths in the training phase. The data are presented as the mean±SD. *P < 0.05, ***P < 0.001 vs. WT, #P < 0.05, ##P < 0.01 vs. the A β 1-42 group. NTN-1, Netrin-1; WT, Wild-type.



The NLRP3 inflammasome and microglia were activated in $a\beta$ 1-42-induced AD rat hippocampal tissues

Microglia are promptly activated by vast amounts of stimuli, such as Aß peptide. Activated microglial cells generate neuroinflammation and NLRP3 inflammasome activation and secrete a variety of proinflammatory factors, resulting in neuronal damage and psychological symptoms of AD (Jin et al., 2019). The hippocampus and cerebral cortex are densely populated areas at the junction of gray matter and white matter in the brain, with abundant neurons and microglia involved in cognition and memory. Therefore, we chose to take samples from these areas for detecting the immune activity response of microglia cells in AD. In our previous experiments utilizing an A\beta1-42-induced rat model, neurotoxicity was significantly more pronounced in the AD14d group compared to the AD7d group, indicating a temporal correlation between disease progression and the extent of neuronal damage/repair (Sun et al., 2019). To maximize observable injury and enhance NTN-1 treatment in vivo efficacy, tissue sampling was conducted at 22 days post-intervention. In our study, the levels of the NLRP3 inflammasome in the hippocampal tissues of wild-type and A\beta1-42 rats were tested at 22 postsurgery by Western blotting. As shown in Figure 3, compared to the WT group, the protein expression of NLRP3/ASC/caspase-1 was significantly increased in AD rat brains. Additionally, the expression of proinflammatory

factors downstream (IL1 β and IL18) was also upregulated in A β 1-42 rats (P < 0.01). We also measured Iba-1 protein levels (a microglial marker) to detect activated microglia, which were increased in the AD group (P < 0.05). Similar results were also observed between sham group and AD group (data not shown). These results confirmed previous studies that microglial inflammation and the activation of NLRP3 inflammasomes result in neuropathological progression in AD, and NTN-1 might be correlated with these processes.

NTN-1 inhibited the activation of the NLRP3 inflammasome in a β 1-42-induced AD rats

Recent studies have reported that NTN-1 exhibits an antiinflammatory effect in various diseases. To investigate the effects of NTN-1 on the A β 1-42-induced NLRP3 inflammasome and microglial activation, age-matched adult male rats were separated into four groups: WT group, A β 1-42 group,vehicle control group (A β 1-42+PBS) and A β 1-42+NTN-1 group, and A β 1-42 or NTN-1 microinjection was measured as previously described. The hippocampal and cortical tissues were dissected to measure the protein expression of NLRP3 inflammasomes by Western blot analysis. In our results, compared with that in the WT group, the NLRP3 inflammasome was activated in the A β 1-42 group



(P < 0.001), and NTN-1 significantly prevented this increase in hippocampal protein expression ([P < 0.001, Figures 4A– G)]. Moreover, the expression of IL1 β and IL18 was diminished by NTN-1 compared to the A β 1-42 group (P < 0.01), and similar results were observed in the cortical tissues of each group (Figure 5). These results indicated that NTN-1 reduced the activation of microglia and NLRP3 inflammasomes in both hippocampal and cortical tissues of A β 1-42-induced AD rats, which might exert neuroprotective effects.

NTN-1 reduced the levels of the NF- κ b signaling pathway in a β 1-42-stimulated rats

The transcription factor NF- κ B upregulates the expression of NLRP3, which is a critical event for NLRP3 inflammasome activation (Kelley et al., 2019). To further investigate the role of NTN-1 in NLRP3 inflammasome regulation, the protein levels of the NF- κ B signaling pathway were tested in AD rats. Our results demonstrated that A β 1-42 stimulated the phosphorylation of I κ B α and NF- κ B p65 expression compared with the WT group (P < 0.05), and NTN-1 treatment significantly inhibited A β induced activation of the NF- κ B pathway in rat cortex (Figures 6, 7, P < 0.01)). Collectively, these findings suggested that NTN-1 most likely inhibits the activation of NLRP3 inflammasomes through the NF- κ B signaling pathway to reduce microgliamediated neuroinflammation in A β 1-42 rats.

NTN-1 prevented an increase in aβ protein and improved the microglial microenvironment in AD rat hippocampal tissues

Aβ1-42, dysregulated amyloidogenic pathway in amyloid precursor protein (APP) processing, is thought to be the key component of amyloid plaques and is shown to be neurotoxic in AD pathogenesis (Iwatsubo et al., 1994). It has been shown that the alternative cleavage product of APP, secreted amyloid precursor protein-alpha (sAPPa) is reduced in AD. Notably, sAPPa species has been found to exhibit many neuroprotective activities, which is important for neuron survival under pathological conditions such as AD (Bailey et al., 2011). Contrary to sAPPa, higher levels of Aβ, especially soluble A β 25–35 and A β 1–42, could lead to aggregation states with enhanced toxicity. Pathologic AB inhibits FL APP cleavage and decreases sAPPa production, led to the pathogenic process (Spilman et al., 2016). Soluble Aβ oligomers can stimulate chronic microglial proliferation and activation, which initiate the inflammatory cascade in AD (Jin and Yamashita, 2016). It has been shown that activated microglia can release proinflammatory



FIGURE 5

NTN-1 inhibited NLRP3 inflammasome activation in cortical tissues of A β 1-42-stimulated rats. Western blotting **(A)** and quantification **(B–G)** of the levels of NLRP3, ASC, caspase-1, IL-1 β , IL-1 β , IL-1 β , and NTN-1 proteins were performed in the wild-type group, A β 1-42 group, A β 1-42 +NTN-1 group and A β 1-42+PBS group. Protein levels were normalized to β -actin. Data are expressed as the mean \pm SD (n =4), *p < 0.05, **p < 0.01, ***p < 0.001 compared with the wild-type group, #P<0.05, ##P<0.01, ###P<0.001 compared with the A β 1-42+NTN-1 group.



The NF- κ B signaling pathway was activated in the cortex of A β 1-42-stimulated rats. Western blot analysis of the protein levels of p-p65, p65, p-l κ B α and κ B α in wild-type and A β 1-42-stimulated rats (A, n=4 per group). The protein levels were quantified and normalized to the level of β -actin (B–E), *P<0.05, **P<0.01 compared with the wild-type group. Data are recorded as the mean \pm SD for three independent experiments. I κ B α , IkappaBalpha; NF- κ B, Nuclear factor- κ B; A β , Amyloid- β ; NTN-1, Netrin-1; WT, Wild-type.

cytokines, thereby reducing microglial phagocytosis, enhancing A β aggregation and inhibits FL APP cleavage (Pan et al., 2011). Therefore, we next evaluated the effects of NTN-1 on sAPP α

and A β 1-42 production in A β stimulated rats, levels of sAPP α and A β 1-42 in rat CSF were determined by ELISA. As shown in Figure 8A we found A β microinjection decreased CSF sAPP α



FIGURE 7

NTN-1 inhibited NF- κ B activation in the cortex of A β 1-42-stimulated rats (n=4 per group). The protein levels of p-p65, p65, p-l κ B α and l κ B α were measured by Western blot (A), and the expression of protein was normalized to β -actin using ImageJ software (B–E). **P<0.01, ***P<0.001 indicates that the expression was significantly different compared to the wild-type group, ##P<0.01, ###P<0.001 compared with the A β 1-42+NTN-1 group. Data are recorded as the mean \pm SD for three independent experiments.



compared with WT and sham groups (P < 0.001), and NTN-1 significantly increased it (P < 0.05). In Figure 8B, ELISA analysis also showed that CSF A β 1-42 production was elevated in AD rat (P < 0.001), NTN-1 treatment reversed the increase of A β 1-42 (P

< 0.05), which might suggest NTN-1 regulate APP processing and inhibits A β amplification.

Microglial cells play central role in degrading Aβlevel through proliferation, polarization and phagocytosis, abnormal



Aß accumulations active microglial M1 phenotypic and induce CNS inflammatory damage. Teter et al. demonstrated that Aß stimulation induced microglial proliferation, activation, and phagocytic encapsulation of Aß aggregates in human brain sections. Activated microglia predominantly localize to plaque cores, while peripheral regions exhibit diminished microglial density (Teter et al., 2019). However, chronic Aβ accumulation overwhelms microglial clearance capacity, leading to aberrant activation, polarization, and subsequent neuroinflammatory damage. To further verify these results, we tested the Iba-1(microglial marker) and AB protein levels in the hippocampus of AD rats and wildtype littermates. Microglial proliferation and AB accumulations were observed in the AD group, and NTN-1 treatment inhibited microglial activation and Aß aggregation in the AD group (P < 0.01, Figures 9A-C). These findings imply a role of NTN-1 in affecting Aβ plaque formation in AD rats. In addition, we explore the effects of NTN-1 on the microglial M1/M2 polarization in AB stimulated rats, we examined the expressions of M1 and M2 phenotypic markers in the hippocampus of NTN-1 treated AD rats, the expressions of M1 phenotypic marker (iNOS) was significantly elevated in the AD group, and the expressions of M2 phenotypic marker (Arg1) was reduced compared with the WT group. Consistently, NTN-1 treatment remarkably reduced the expressions of M1 marker (P < 0.05) and increased the expressions of M2 marker (P < 0.01), compared with the AD group (Figures 9D-F). Taken together, these results of protein expression indicate that NTN-1 treatment induces microglia from M1 to M2 polarization in the hippocampus of A β 1-42-induced AD rats, and improves the M2-mediated neuronal microenvironment.

Discussion

Reactive microglial polarization is mechanistically linked to AD progression, driving neuroinflammatory cascades that culminate in synaptic dysfunction and cognitive decline. As resident phagocytes in the central nervous system, microglia exhibit two states during activation: the M1 state and M2 state. The M1 state is classically activated to produce proinflammatory cytokines; in contrast, the M2 state releases anti-inflammatory factors, ingesting debris and repairing CNS damage. Abnormal Aß protein may indirectly induce CNS inflammatory damage by reacting with M1 microglia. It has been reported that without microglia present, high concentrations of abnormal AB protein cannot cause neuronal damage in AD (Giulian, 1999). NTN-1 acts as a new anti-inflammatory factor in the CNS to regulate immunity, angiogenesis and neuronal survival (Shabani et al., 2017). In a rat model of subarachnoid hemorrhage, administration of NTN-1 suppressed microglial activation and brain edema (Xie et al., 2018). The absence of cytotoxic effects was observed in rat cardiomyocytes treated with Netrin-1 alone, as evidenced by nonsignificant reductions in TUNEL positivity and LDH release, while demonstrated significantly enhanced survival of neonatal cardiomyocytes (Durrani et al., 2012). Brain slices from non-PDAPP transgenic mice were cultured with NTN-1, and there was no obvious A β 1–40 and A β 1–42 net productions appeared, thus it reflected that NTN-1 was safety and efficacy (Lourenço et al., 2009). another research showed that netrin-1 increased sAPPα levels in primary hippocampal mouse neurons without Aβ1-40 compared with wildtype. These studies indicate netrin-1 may exert an ameliorate sAPPa self-increasing effect in AD therapeutic (Spilman et al., 2016). Relevant studies gave evidence that NTN-1 could pass through the ventricle wall into brain tissue to generate long-term efficacy after acute injection, and support undertaking of subsequent chronic pump delivery studies (Spilman et al., 2016). This regulatory mechanism positions NTN-1 as a potential modulator of amyloidogenic pathways in neurodegenerative contexts. In our study, we observed that NTN-1 significantly improved learning and memory behavior in the water maze task, which was consistent with a previous study, and significantly inhibited microglial proliferation and activation in AB1-42-induced AD rats, indicating that exogenous NTN-1 treatment attenuated Aβ-induced neurological impairments in memory and learning, which may be due to M1 microglial activation in AD rats. Schwann cells were identified as the predominant source to secrete NTN-1 in the adult rat sciatic nerve. Meanwhile, NTN-1 treatment also induced Schwann cells proliferation through Unc5b receptor (Lee et al., 2007). Therefore, based on the above evidence, we speculate that exogenous injection of NTN-1 may promote myelinating oligodendrocytes proliferation and increase endogenous NTN-1 secretion in CNS. Another study suggested that NTN-1 promoted rat RSC96 Schwann cell proliferation and migration at 100 ng/mL concentration, but suppressed migration at the concentration of 500 ng/mL, this biphasic response suggested the concentration-dependent receptor signaling modulation of NTN-1. We observed that NTN-1 administration ameliorated memory and cognitive ability in A\beta1-42-induced AD rats at a concentration of 160 ng/µL, but effective concentration range of NTN-1 in the central nervous system remains further investigation (Lv et al., 2015).

Previous investigators revealed that NLRP3 inflammasome activation plays a critical role in Alzheimer's disease. Abnormal microglial activation simulated by $A\beta$ is fundamental for activation of the NLRP3 inflammasome and subsequent proinflammatory cytokine IL-1β/IL-18 release in the brains of AD patients (Saresella et al., 2016). Furthermore, it was suggested that microglia tended to be in an M2 state and resulted in decreased deposition of Aβ in an NLRP3-/- APP/PS1 mouse model (Heneka et al., 2013). It is well documented that treatment with NTN-1 also regulates neuroinflammation and reduces blood-brain barrier disruption and disease severity (Podjaski et al., 2015). However, it remains unknown whether NTN-1 contributes to NLRP3 inflammasome activation in AD. Neurons and myelinating oligodendrocytes serve as primary cellular sources of NTN-1 synthesis and secretion within the adult CNS. In our study, there was a low level of NTN-1 in the brains of A\beta1-42-simulated AD rats. In contrast, NLRP3/ASC/caspase-1 pathway proteins were highly expressed, and the proinflammatory cytokines IL-1ß and IL-18 were expressed in both hippocampal and cortical tissues, which was effectively inhibited by administration of NTN-1 by microinjection. The results indicated the impact of the NLRP3/ASC/caspase-1 pathway on AD. NTN-1 inhibited the abnormal activation of the NLRP3 inflammasome and the release of proinflammatory cytokines induced by the A\beta1-42 protein. Interestingly, the expression of NTN-1 was also diminished in the untreated A\beta1-42 group, which was consistent with our previous studies in both serum and cerebrospinal fluid compartments of AD rat and patients (Sun et al., 2019; Ju et al., 2022). Recent evidence documents significant NTN-1 downregulation in diverse pathologies, including autoimmune disorders and chronic CNS injuries (Bruikman et al., 2020). Notably, elevated A β concentrations promote pathological aggregation states characterized by heightened neurotoxic potential. Taken together, the decrease in NTN-1 levels may be related to the reduced in cell proliferation and secretion after nerve injury. The observed inverse correlation between NTN-1 and AB burden suggests that amyloid plaque progression may inhibit NTN-1 metabolic, which at least partially contributes to its accumulation in the AD brain.

To further identify the molecular mechanism related to NLRP3 inflammasome regulation in AD rats by NTN-1, we measured the relative protein expression of the NF-κB pathway by Western blot. NF-κB is a master transcription factor that generates NLRP3 and inflammatory molecules. During inflammation stimulation, activated IKK rapidly induces the phosphorylation and degradation of IκBα, followed by NF-κB p65 subunit phosphorylation, which enhances nuclear translocation and regulates the transcription of target genes, including IL-1β, IL-18, IL-6 and NLRP3 (Chang et al., 2015; Chen et al., 2016). A number of works have proposed a strong correlation between the NF-kB pathway and AD (Thawkar and Kaur, 2019). Our data were consistent with previous studies showing that the NF-kB pathway was activated by A β 1-42 stimulation and that treatment with NTN-1 significantly inhibited AB1-42-induced activation of the NF-KB pathway in the brains of AD rats. It has been reported that NTN-1 signals are essential to receptors expressing in cells to survive in the extracellular environment.Unc5b, one of the receptors of NTN-1, has been widely investigated in microglia, endothelial cells, and neurons. Specific knockdown of Unc5b significantly reverses the anti-inflammation effect of NTN-1, which suppresses microglia activation (Lin et al., 2018; Xie et al., 2018). Lin et al. find that NTN-1 can bind to its receptor Unc5b and prevent NF-κB signaling in endothelial cells via an anti-inflammatory properties (Lin et al., 2018). A study has shown that connecting the NTN-1 to UNC5B via the PI3K signaling pathway exerts potent anti-apoptotic effects, significantly enhancing neuronal survival under pathological conditions (Tang et al., 2008). Interestingly, NTN-1 demonstrates cytoprotective capabilities in vascular endothelia, counteracting hyperglycemia-induced injury and angiogenic dysfunction through PI3K/AKT/eNOS pathway activation (Xing et al., 2017), this signaling cascade enhances eNOS enzymatic activity and protein expression, subsequently suppressing nuclear NF-KB translocation via eNOS/NO/NF-KB pathway (Yu et al., 2018). Based on current evidence, we hypothesize that NTN-1 lowered the $A\beta1\text{-}42$ induced NLRP3 inflammasome-mediated inflammatory response and microglia activation, through modulating the PI3K/eNOS/NFkB signaling pathway in the hippocampal and cortical tissues of AD rats, which might bind to its receptor Unc5b. However, due to the multiplicity of NTN-1 receptor subtypes and binding sites, distinct functional roles may arise from different interaction sites. Multiple

signaling pathways might participate in the inhibitory effects of NTN-1 on NF-kB activation, future investigation of NTN-1 and its specific receptor in regulating A β 1-42 induced neuroinflammation injury need further investigation.

M2 microglia form a protective barrier to prevent the accumulation of Aß plaques; however, chronic toxicity and aging conditions render M1 microglia activated in the AD brain, which causes a prolonged production of neuroinflammatory responses correlated with increased AB pathology in AD patients and transgenic models (Zuroff et al., 2017). It had been reported that there was an increase level of AB 1-42 in the CA3 hippocampal region of AD-like mice model evaluating by Immunohistochemistry Garcez et al., 2019). In addition, Zamani et al. used Congo red staining technique to qualitative assessment the effect of NTN-1 on Aβ aggregation in the AD rat hippocampal sections, the accumulated A^β plaques are visible and significantly decreased in A β +NTN-1 group compared with A β group (Zamani et al., 2019). In our results, NTN-1 was found to decrease CSF Aß level, increase sAPPa production, FL APP cleavage and induce microglia from M1 to M2 polarization, thereby preventing Aβ aggregates in AD rats and might be a result of alterations in neuroinflammatory impairment. Future studies will incorporate multi-timepoint assessments in transgenic AD models to evaluate dynamic changes in downstream biomarkers, thereby enhancing data clarity and robustness.

This study still has some shortcomings, our current studies focus on male rats but limit the applicability of the findings to female rats, the possible changes that may occur between different genders in the pathological process of AD are different. Early-stage A β pathology models (8~10-month-old adults) are selected in this study, as AD is an age-related disease, late disease models (18~22-month-old rodents) should be valued and applied in subsequent research. This experiment only applies a single drug dose (160 ng/µL) based on prior efficacy/safety data in CNS models (Zamani et al., 2019). In future studies, dose gradients should be added to establish therapeutic windows, with preliminary data showing concentration-dependent NLRP3 suppression. Single NTN-1 group without A\beta1-42 injection is lacked which help distinguish between neuroprotective and general cognitive-enhancing effects of NTN-1. Although Morris Water Maze was prioritized for its sensitivity to hippocampal-dependent spatial memory, multimodal behavioral tests would provide a more comprehensive evaluation of cognitive function. Multiple inflammatory pathways are involved in the pathological process of Alzheimer's disease, while our hypothesis-driven approach centered on NLRP3/NF-KB due to their established AD relevance, other potential inflammatory pathways, such as the JAK-STAT pathway, may be implicated in AD progression need to be further explored in the future.

Conclusions

In conclusion, our findings suggest the beneficial effect of NTN-1 on neuroprotective and cognitive improvement in A β 1-42-induced AD rats. In addition, the involvement of NF- κ B-NLRP3 inflammasome pathway inhibition by NTN-1 might lead to a new

agent for the underlying pathophysiology that is responsible for antagonizing and alleviating Alzheimer's disease.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Animal Ethics Committee of Harbin Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

TW: Writing – original draft, Writing – review & editing. YLi: Writing – original draft. YLu: Writing – original draft. LC: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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