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NT-3-Expressing BMSCs Treatment: Promoting Remyelination to Reduce Aβ Amyloid Production

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Abstract. Alzheimer's disease (AD) is a progressive neurodegenerative disease whose core pathology includes amyloid-β (Aβ) plaques. The current studies have proved that myelin dysfunction is the upstream factor of Aβ deposition, which provides a new target for the prevention and treatment of AD. Nevertheless, no effective therapeutic strategy currently exists to halt or repair myelin damage. Bone marrow mesenchymal stem cells (BMSCs) are recognized as highly promising for adult stem cell therapy in neurological conditions, given their accessibility, rapid expansion, genetic adaptability, and feasibility for autologous grafting. Neurotrophin-3 (NT-3) is critically involved in the growth, differentiation, and maturation of oligodendrocyte lineages and promotes oligodendrocyte progenitor cell (OPC) maturation and myelination, highlighting its therapeutic potential. This study attempts to explore whether the use of NT-3-modified BMSC to promote myelin regeneration can reduce Aβ production and aims to find potential therapeutic approaches to improve the efficacy of treating degenerative disorders of the nervous system, with Alzheimer's disease as a prime example.

Keywords: Aβ Amyloid, NT-3, Bone Marrow Mesenchymal Stem Cells, Myelin Regeneration, Alzheimer's Disease

1. Introduction

1.1. Pathogenesis of Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease primarily affecting the elderly, characterized by a gradual decline in memory, cognition, and the ability to perform daily activities. The pathology of AD is complex and multifactorial, including both genetic and environmental factors.

One of the key pathological features of AD is amyloid-β (Aβ) plaques [1]. The formation of Aβ involves several critical steps and mechanisms. Initially, Aβ derives from the amyloid precursor protein (APP), which is a membrane-bound glycoprotein. APP undergoes proteolytic cleavage by β and γ secretases. The β-secretase (BACE1) and γ-secretase cut APP sequentially, resulting in the

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production of Aβ peptides, primarily Aβ40 and Aβ42. These Aβ peptides, especially Aβ42, tend to aggregate due to their hydrophobic nature [2]. The aggregation starts with the formation of small oligomers. Its soluble oligomers (e.g., dimers, trimers) are the main neurotoxic forms and can cause neuronal damage by disrupting calcium homeostasis, affecting synaptic plasticity, and interacting with multiple receptors (e.g., NMDA, LRP1). The oligomers further aggregate into protofibrils and eventually form amyloid fibrils. The fibrils accumulate to form amyloid plaques.

The accumulation of beta plaques is considered an early and initiating factor in the onset of alzheimer's disease, causing subsequent pathological changes. The neurotoxicity of Aβ aggregates is related to oxidative stress, neuroinflammation and disruption of calcium homeostasis, leading to synaptic dysfunction and neuronal cell death. In addition, the accumulation of Aβ triggers tau hyperphosphylation, leading to the formation of neurofibrile entranslations (NFT), which is closely related to neuronal loss and cognitive impairment observed in AD. In Eimer et al. 's report, amyloid beta (Aβ) can be used as an innate immune protein to capture herpes viruses (such as HSV1, HHV6A, HHV6B) by fibrosis to protect the brain from infections [1]. However, this process accelerates the deposition of beta-amyloid, which can facilitate the progression of AD.

1.2. Myelin dysfunction in AD

This suggests that myelin dysfunction selectively increases the cutting efficiency of the app and accelerates the deposition of the Aβ plate. In the research paper "Myelin dysfunction drives amyloid-β deposition in models of Alzheimer's disease" by Depp et al., the team demonstrates that myelin dysfunction promotes Aβ plaque formation and disrupts microglial function. These results suggest that age-related myelin damage may contribute to a risk of alzheimer's disease.

3D imaging of the hippocampus shows high density amyloid deposition in the alveolar region of mice with myelin development abnormality, demonstrating the spatial relationship between myelin integrity and accumulation of Aβ [3]. Mechanistically, APP cleavage—requiring BACE1 and γ-secretase generates Aβ plaques. 3D imaging of the hippocampus revealed high density amyloid deposition in the alveolar region of mice with myelin development abnormality, demonstrating the spatial relationship between myelin integrity and a β accumulation [3]. From A mechanical point of view, cutting by APP requires the production of Aβ plates by BACE1 and γ -secretase. Western blot data showed that BACE1 and its C99 product were elevated in myelin dysfunctional mice, while total app remained unchanged [3]. This suggests that myelin dysfunction selectively increases the cleavage efficiency of the app and accelerates the deposition of Aβ plates.

Further evidence from 2D immunostaining confirms increased Aβ plaque density alongside IBA1+ microglial accumulation. Microglia, which usually clear Aβ, exhibit aberrant responses due to myelin damage [3]. Specifically, cholesterol release from damaged myelin diverts microglia toward the repair of myelin and the metabolism of lipids instead of the clearance of Aβ plaques. The ApoE upregulation in microglia regulates cholesterol metabolism, but this upregulation leads to the stabilization of Aβ plaque, resulting in the accumulation of amyloid [3]. At the same time, the down-regulation of the Cst7 gene, essential for lysosomal function, results in the loss of microglia's ability to eliminate excess beta plaques [3]. Overexposure and overexposure genes in microglia stabilize Aβ plaque and aggravate disease.

Critically, whether through direct APP processing or microglial dysregulation, myelin dysfunction initiates a self-reinforcing toxic cycle. The integrity of myelin must therefore be seen as a key regulator of the formation and stabilization of a β in AD [3].

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1.3. Application of myelin treatment

Myelin is essential for the proper functioning of the nervous system by insulating axons and facilitating rapid electrical impulse transmission. In recent years, research has increasingly focused on the important role of myelin lesions in the pathogenesis of melody disease.

Dombrowski's team has discovered a new regeneration pathway in which regulatory T cells, Tregs, a subset of immune cells, can contribute directly to the remyelination of the central nervous system. In addition to its known immunosuppressive effects, Tregs secretes CCN3, a signaling protein that leads to oligodendrocyte maturation and accelerates myelin repair [4]. In experimental models, increased Treg activity or administration of CCN3 significantly improves myelin regeneration, suggesting potential therapeutic strategies for multiple sclerosis (MS) and other demyelin diseases [4].

Peripheral nerve damage is classified by Seddonand Sunderland. Schwann cells play a key role in repair. Regeneration includes myelin sheath regeneration, axonal regeneration and lateral branch germination, but recovery is slow [5]. Diagnosis typically relies on electrodiagnostic testing (EDX) after injury [5]. Current therapeutic strategies include pharmacological approaches, electrical stimulation to accelerate axonal regeneration and experimental stem cell therapy.. In addition, low intensity laser therapy and epigenetic regulation have shown a potential to promote regeneration [5]. For the future, combination treatments and further clinical trials are needed to optimize recovery results.

Liu’s team investigated the architecture and repair process of the myelin sheath in peripheral nerve injury repair and discovered that the double bilayer is the structural unit of myelin. The core feature of their method of myelin regeneration is the compaction process, which includes the shortening of the spacing between the two cell membranes within the membrane layers and the tight bonding of adjacent bilayers to form a major dense line (MDL) [6]. The level of myelin basic protein (MBP) expression showed a positive association with the formation of MDL, and the attachment of lipophilin particles to the myelin sheath was essential to the formation of dense myelin. In addition, the abnormal nerve fiber structure observed in autologous nerve grafting shows no relation to MBP and lipoproteins [6].

Given that myelin regeneration is possible and will potentially limit Aβ plaque accumulation, targeting remyelination may emerge as a therapeutic strategy to alleviate AD symptoms or even prevent disease progression. This study proposes to investigate whether NT-3-expressing bone marrow stromal cells (BMSCs) can promote remyelination, thereby reducing Aβ production and enhancing its clearance.

1.4. NT-3-expressing bone marrow mesenchymal stem cells

Neurotrophin factor 3 (nt-3) contributes critically to oligodendrocyte proliferation, differentiation, and maturation. In vitro and in vivo studies have shown that nt-3 induces OPC proliferation and survival.In addition, nt-3 promotes maturation and myelination of OPC, highlighting its therapeutic potential. On the other hand, mesenchymal bone marrow stem cells (MTC) are considered to be one of the most promising candidates for adult stem cells in the treatment of neurological diseases, as a result of their easy availability, quick growth, genetic flexibility, and promise for autologous application. Mesenchymal bone marrow stem cells also have immunomodulatory, neuroprotective and cell replacement properties that make them a universal tool for nerve repair.

The repair mechanisms of nt-3 and bone marrow mesenchymal stem cells have been demonstrated in studies involving nt-3-modified mesenchymal stem cells implanted in ethyl

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bromide (EB) -induced demyelinated spinal cord. These studies have significantly improved motor function and restored electrophysiological properties. These advnt-3-msc implants promote the endogenous regeneration of myelin by cells directly involved in myelination. In addition, nt-3 stimulates the transformation of endogenous OPCs into oligodendrocytes, which promotes myelin repair, while the MSCs themselves can differentiate into oligodendrocyte phenotypes such as NG2+ and APC+ cells.

Although this study focuses mainly on demyelination models such as ms, an indirect association with AD was also found. For example, demyelination leads to axonal damage and neurodegeneration, a common feature of ms and AD. In addition, failure of myelin sheath regeneration is a sign of neurodegenerative diseases, including AD associated white matter lesions. Thus, although direct application to AD is not the main objective, the role of nt-3 in the survival and myelination of oligodendrocytes could theoretically resolve the myelin dysfunction observed in AD.

2. Methods

2.1. Isolation of bone marrow mesenchymal stem cells

Bone marrow mesenchymal stem cells (BMSCs) were isolated according to a previously established protocol. In brief, cells were taken from the fevers of 5 to 10 day old sd male rats and inoculated into 50 mL culture flask at a density of 1 x 10^7/mL. 10% bovine foetal serum was added to the modified low sugar Eagle Dulbecco medium and 100 U/mL penicillin and 100 U/mL streptomycin. The crops are stored at 37°C at 5% CO2. After 48 hours, the medium is refreshed and the non-sticky cells are rejected. Following the convergence of mesenchymal bone marrow stem cells to be bonded to the wall, subcultures were performed with 0.25% trypsin and 0.02% edta in a fractional ratio of 1:3. The cells between passages 3 and 6 were selected for subsequent experiments. The overall procedure is shown in Figure 1.

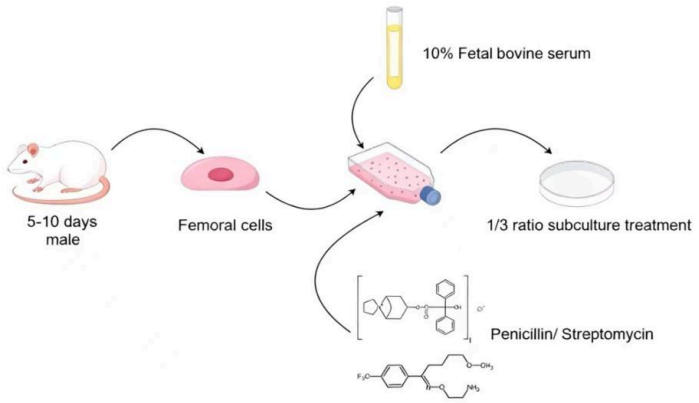


Figure 1. The process of preparing BMSCs

2.2. Construction of rAAV vector and production of recombinant AAV

The purpose of this method is to produce and purify recombinant AAV vectors carrying the human NT-3 gene for subsequent transduction experiments [7]. The full-length human NT-3 cDNA was cloned into pAAV/RES-hrGFP, and the construct (pAAV-NT-3-RES-hrGFP) was verified by restriction digestion [7]. Paav-rc, pHelper and recombinant/control (paav-hrgfp) plasmids were co-transfected with calcium phosphate ina ratio of 1:1:1 [7]. 72 hours after transfection, the cells are

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cleaved by an ultrasonic cycle of freezing and thawing [7]. The viral particles were purified by ammonium sulfate precipitation, and the dot blot assays determined the titers [7].

2.3. AAV-mediated NT-3 gene transduction of BMSCs from the mouse embryonic cortex

The aim of this method is to evaluate the transduction efficiency and expression levels of NT-3 gene in neural stem cells (BMSCs) using different doses of AAV-NT-3, with AAV-GFP serving as the control [7]. BMSCs were isolated from E14.5 mouse cortex and cultured in serum-free DMEM/F12 containing N2, EGF, and bFGF. Neurospheres were passaged weekly [7]. Before transduction, cells were dissociated and seeded at 2×10^5 cells/ml. Different volumes (10, 20, 50 μl) of AAV-NT-3 or control AAV-GFP were diluted to 200 μl and applied to the cells. After 24 hours, 1 ml fresh medium was added designated as 1 DPI [7]. GFP expression was assessed by fluorescence microscopy and FACS at 3 and 5 DPI [7].

2.4. Mouse strains and husbandry

Adult male and female sprague-dawley rats (n=30), 220-280 g, aged 9 to 10 weeks were used [3,8]. Animal experiments are in compliance with the Public Health Service Guide for the Care and Use of Laboratory Animals [3,8].

The mice are raised under a 12-h dark–12-h light cycle and have access to food and drink freely throughout the experiment [3,8]. The mice are divided into three groups and performed the following operations: no treatment, Cnp- (myelin dysfunction)/NT-3, and Cnp-/O. The numbers of male and female mice in each group are equal [3,8].

2.5. Injection

The purpose of this step is to regenerate and restore the function of the myelin sheath. The bone marrow mesenchymal stem cells (BMSCs) can differentiate into OPCs. OPCs can migrate and gather at the dysfunctional myelin [9]. The transduction of neurotrophin-3 (NT-3) into BMSCs could promote BMSC proliferation and neuronal differentiation efficiently without changing the stemness of BMSCs, which plays a role in strengthening the efficacy [10]. In addition, NT-3 has been proven to induce OPC proliferation and survival [8].

One week after the establishment of the experimental model, mice were anesthetized with 1% pentobarbital sodium (50 mg/kg, intraperitoneal injection) [9]. A midline skin incision was made over the skull, and a burr hole was drilled at the target coordinates using a dental drill [9]. A 10-μl microsyringe was loaded with the BMSC-NT-3 cell suspension and slowly inserted into the brain to a depth of 2.0 mm below the dura [11]. After injection, the burr hole was sealed with bone wax, and the skin incision was sutured with 4-0 silk thread [9]. Mice were placed on a heating pad until full recovery from anesthesia, then returned to their cages with free access to food and water [9].

2.6. Assessment of stemness, proliferation, and differentiation of MBSCs following AAV transduction

AAV (severe infections = 1 × 10 fed vg/cell transduction MBSCs in vitro 48 h, then 5-ethyl-2 deoxidation galactose-1-phosphate esu, 10 pg/M) in order to monitor cell proliferation 24 h [10]. Approximately 5 x 10 marked MBSCs were injected stereotaxically into the toothed gypsum of the mouse hippocampus (coordinates: AP = -2.0 mm, ML =± 1.5 mm, DV = -2.0 mm relative to bregma) [10]. Sham-operated mice received an equal volume of PBS [10].

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2.7. Determination of NT-3 expression

The ELISA kit is used to detect the expression of NT-3 protein in the medium, in accordance with GB/T 38480-2020 specifications [10]. The serum-free medium was replaced 24 h before sample collection, and three re-well supernatants (100 μL/well) were taken from each group at 3, 7, 14, and 28 DPI, centrifuged at 4°C (1200×g, 5 min), and stored at 80°C [10].

Follow the kit instructions: add the standard and sample to the plate and incubate at 37°C for 2 h; after washing the plate, biotin antibody was added to incubate for 1 h, and then the plate is washed and incubated with HRP-labeled streptavidin for 30 minutes [10]. TMB color development was terminated with 2M H_2SO_4 after 15 minutes, and the OD value was measured at 450 nm [10].

Multiskan FC microplate reader readings, standard curve method calculated concentration, results expressed in x±s, experiment repeated 3 times [10].

2.8. Tissue processing

Following the recording behavior test and evoked potential, the rats in all three groups (WT, Cnp-/NT-3, and Cnp-/O) were perfused with 4% paraformaldehyde, and the rostral and caudal regions were removed [3]. Half the process is observed under the electron microscope, and the remaining sample is left pure for immunofluorescence or morphological analysis [8].

2.9. Electron Microscopy (EM)

Brain samples were frozen under high pressure, chemically fixed with tannic acid, osmium tetroxide, and uranyl acetate, then sliced into thin sections [3]. Using High-Pressure Freezing Electron Microscopy to compare myelin tissue between WT and Cnp- mice to ensure the progression of regeneration [3,12].

2.10. Immunofluorescence and laser scanning microscopy imaging

After the tissue fixation and sectioning, prepare an antibody and staining for Aβ amyloid plaque and β-tubulin III. The imaging parameter will illustrate the Aβ amyloid plaque clearance and recovery of myelin.β-tubulin III and Nestin will show the regeneration of the neuron in the myelin and the absorption of the NT-3 gene.

Immobilized hemibrains and spinal cord were treated using a modified iDISCO protocol that included dehydration with methanol, bleaching with hydrogen peroxide and penetration with Triton x-100, DMSO and glycine [3]. The tissues are then stained with Congo red to detect amyloid or immunomarked plaques with primary and secondary resistance for several days [3]. Finally, the samples are dehydrated, dehydrated in methanol-dichloromethane and removed in ethyl cinnamate for imaging, all steps being performed in constant rotation at the specified temperature [3].

NSCs, neurons and astrocytes were identified by immunocytochemistry using primary antigens specific to nestin, β-tubulin III and GFAP and incubated with secondary antigens coupled to fitc and DAPI counter-staining [10]. All procedures followed an established laboratory protocol, using an Olympus fluorescence microscope for imaging, and PBS instead of a primary antigen as a negative control.

laser scanning microscopy (LSM) imaging was performed on immunolabeled and cleared tissue samples following the completion of all staining and transparency treatment steps [3].

The imaging should most directly visualize the treatment progress. The specific Immunocytochemical protocol can reference the original methods in the reference [3,10].

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2.11. Western blot

Perform a standard Western blot procedure comparing experimental and control groups [3,10]. The protein was isolated by SDS-PAGE, transferred to the membrane and incubated with specific primary and secondary resistance to detect the target protein. The signal is then visualized using a chemiluminescence enhanced detection system (ECL).

The first set, the target protein and antibody, should target BACE1 and C99. The result showed the Aβ amyloid plaque production rate and accumulation before and after the treatment [3].

Other proteins confirmed to need to go through Western Blot are Nestin and β-tubulin III. The results will indicate whether NT-3 BMSCs effectively promote myelin repair and regeneration [10]. All those Western Blot results should be reproducible.

2.12. Imaging of whole tissues, analysis, and visualization

Aβ plaques were first analyzed in the brain and spinal cord of mice after removal using a thin layer microscope [3]. The images obtained were sutured using the Vision4D or Imaris software, then manually anremarked on the Allen brain atlas on the three key king - isocortex, hippocampus and alveolo - and the 2D follow-up was extrapolated to 3D [3]. The quantitative analysis includes number of plates, total volume, average size, density and percentage of volume coverage [3]. Using three-dimensional representations to show the Aβ amyloid plaque accumulation area and density of the brain.

2.13. Behavior test

Behavioural tests, including the y-maze for assessment of working memory, the high positive maze (EPM) for anxiety behaviours, and a grip test for motor coordination [3]. In all experiments, age - and sex-matched mice were used and placed under standard housing conditions.

The Y-maze test was employed to examine spontaneous alternation behavior as a measure of working memory. Wild type (WT) mice with intact myelin function and standard behaviour were used as a positive control. The experimental group was subjected to 3 y maze trials using recording time and trajectory as a base. Following baseline testing, the experimental cohort was randomized into two groups: one group received treatment (Cnp-/ nt-3) and the other group was used as a negative control (Cnp-/O). After treatment, all mice were re-tested in a y-maze to compare exploration modes and alternations rates.

The same experimental design was applied to the elevated plus maze test and clasping test, where we quantified time spent in open arms as an indicator of anxiety-like behavior—all experimentation with experimenters blinded to treatment groups to eliminate bias.

2.14. Electrophysiological detection: evoked potential (CAPs)

Measures the axonal conduction velocity of the corpus callosum. The purpose of this experiment is to test whether conduction velocity is restored after myelin repair shown in Figure 2. Anesthetized mice undergo sciatic nerve exposure with proximal/distal electrode placement. Electrical stimulation (pulses of 0.1 ms, 1Hz) excites the cmap recorded by the twin muscle [11]. Measurements include response latency (time from stimulation to attack), peak to peak amplitude, and calculated nerve conduction velocity (distance/ latency difference between stimuli) [11]. Data acquisition was performed using an amplified/filtered signal and analyzed by unidirectional analysis of variance and Tukey post mortem test ($p< 0.05$ significance threshold) [11].

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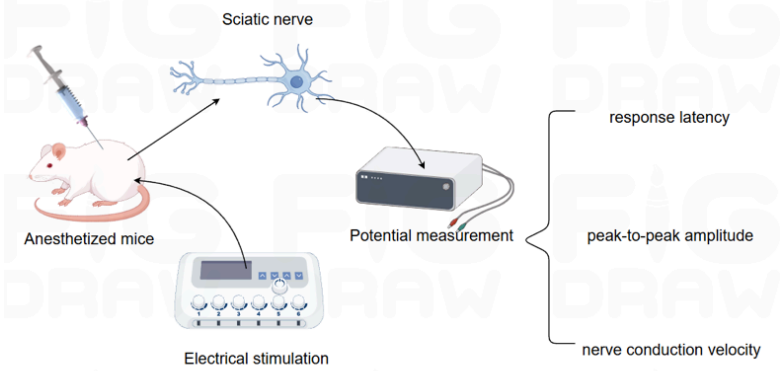


Figure 2. Electrophysiological detection process

3. Result

If the hypothesis that NT-3 BMSC treatment successfully reduces amyloid production is validated, this approach may potentially alleviate symptoms of both MS and AD. Expression of NT-3 in BMSC should promote OPC proliferation and facilitate myelin restoration, just as illustrated in Figure 3a. Western blot analysis will provide molecular evidence regarding whether myelin regeneration influences Aβ amyloid production or enhances microglia-mediated clearance. EM and LSM result shown in Figure 3b enabled the direct visualization of Aβ amyloid plaque deposition and the OPC populations.

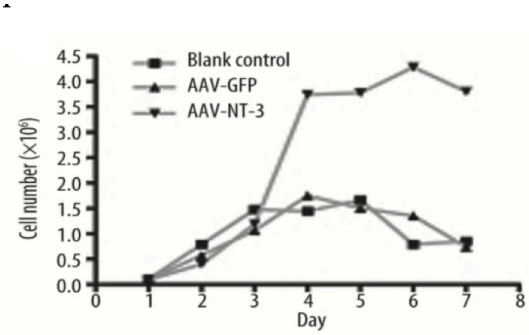


Figure 3a

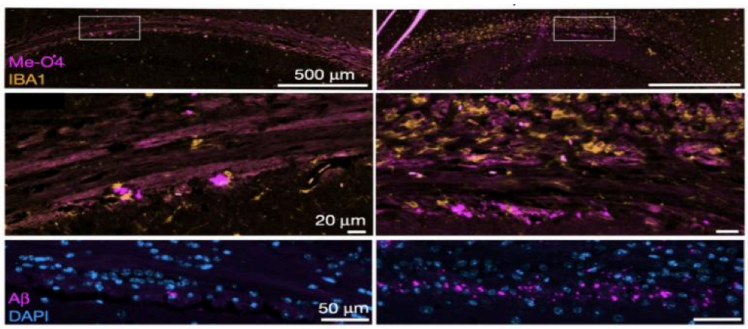


Figure 3b

Figure 3. a: NT-3 cell successfully expressing, promoting oligodendrocyte progenitor cell growth and proliferation [10] b: LSM imaging after Immunofluorescence, IBA-1 shows the microglia, and Me-O4 shows Aβ plaques. The expected result is the left-side image, which represents Cnp-/O mice, showing Aβ plaque deposits and myelin destruction, leading to microglial accumulation. The right is Cnp-/NT-3 has fewer Aβ plaques and fully functional myelin [3]

To assess functional recovery, behavioural and electrophysiological analyses will be used. The restoration of myelin integrity is expected to be directly reflected in behavioural performance as Figure 4 and we expect significant differences in monitoring time and performance of the Cnp-/O and Cnp-/ nt-3 mice. In addition, electrophysiological evaluation (Figure 2. Electrophysiological detection) will be conducted to determine if the newly formed myelin sheaths exhibit normal

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physiological function, as measured by nerve conduction velocity. These complementary methods will together demonstrate the progression of functional recovery.

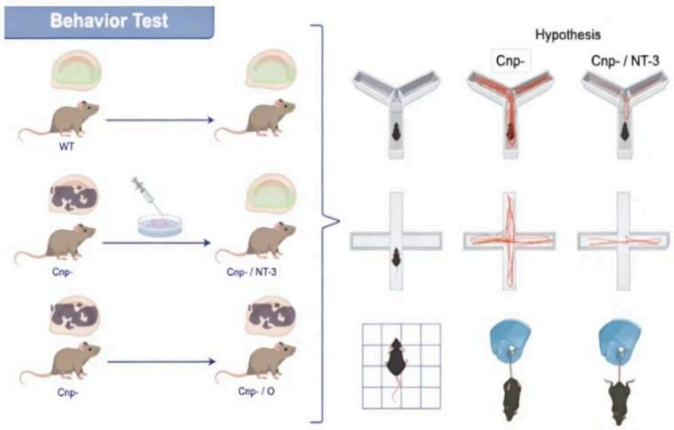


Figure 4. The behavior test will directly demonstrate how myline regeneration eases the pathological symptoms. The right side of the image shows the comparison between the negative control mice and Cnp-/NT-3 mice; the red line illustrates the movement track. After the treatment, the mice are expected to show improved recovery in working memory and reduced anxiety behavior

A novel aspect of this study is the computational conversion of 2D LSM images into 3D reconstructions. As shown in Figure 5, these 3D models effectively illustrate the distribution of Aβ amyloid plaques and the migration of OPCs, confirming the expected efficacy of the treatment.

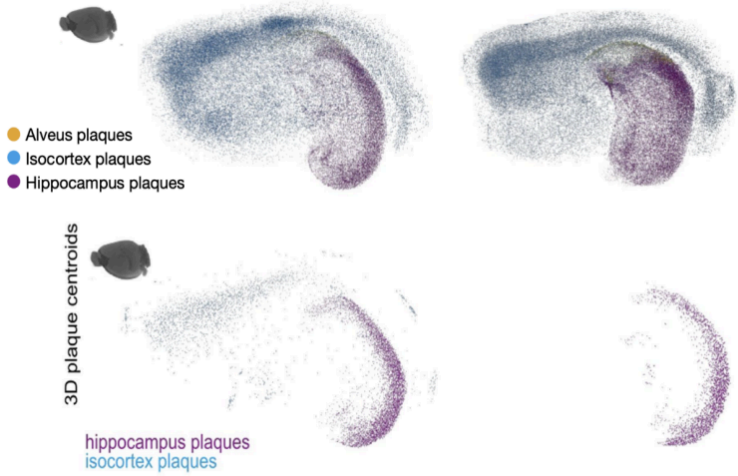


Figure 5. Using computational software to analyze LSM to form a 3D image of the specific protein distribution, a particular method is mentioned in Method 2.12

The Accepted hypothesis should show that NT-3, when successfully expressed in BMSC, promotes OPC maturation and migration to repair damage to myelin. The helath myline did not disrupt the balance and attention of migorilia, promoting plaque clearance and limiting plaque production caused by the dysfunctional myline.

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If NT-3 BMSC treatment fails to promote remyelination or ameliorate MS- or AD-related phenotypes, alternative or more precise methodological approaches may need to be considered. It is also possible that the accumulation of Aβ plates remains constant despite the observed myelin sheath regeneration, suggesting that there is no direct correlation between these two pathological characteristics. Although this possibility is considered unlikely due to previously established relationships, supported by the available literature, such a finding would require a reassessment of the mechanical link between myelin repair and amyloid pathology.

4. Discussion

The experimental approach requires further refinement and modification during operation. A primary limitation is the reliance on outdated literature, as there are few recent publications or studies in this specific research area. Nonetheless, this direction remains promising and warrants further elaboration and investigation.

A practical problem is the method of transplanting the nt-3 gene into mesenchymal stem cells of the bone marrow and its transport in the hippocampus. The current protocol, cited in the available literature, has been applied only in practice to organotype cultures of sepharm sections, which may limit its translational relevance to in vivo models.

Another limitation pertains to the model's association with Alzheimer's disease (AD). The model is designed either to promote recovery from myelin dysfunction or to reduce Aβ amyloid production and enhance its clearance. Although Aβ amyloid is considered a pathological factor in AD, the rationale presupposes that myelin repair should limit Aβ accumulation and alleviate symptoms. However, since Aβ amyloid is likely only one of several contributing factors in AD, no direct causal relationship has been established between myelin treatment and the overall severity of AD symptoms.

Acknowledgment

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We, Jialu Shi, Qiongdan Zhang, and Qiyun Fu, have contributed equally to the design of the experimental model and the preparation of this proposal. All three authors have participated extensively in the conceptualization and writing process, and will be listed as co-authors in subsequent publications.

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