# 2025年臺灣國際科學展覽會
# 優勝作品專輯

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參展科別　醫學與健康科學

作品名稱　Non-Invasive Vagus Nerve Stimulation as a Novel Therapy for Alzheimer’s Disease by Enhancing the Brain Clearance System(非侵入性迷走神經刺激術作為阿茲海默症的新療法—透過增強大腦清除系統)

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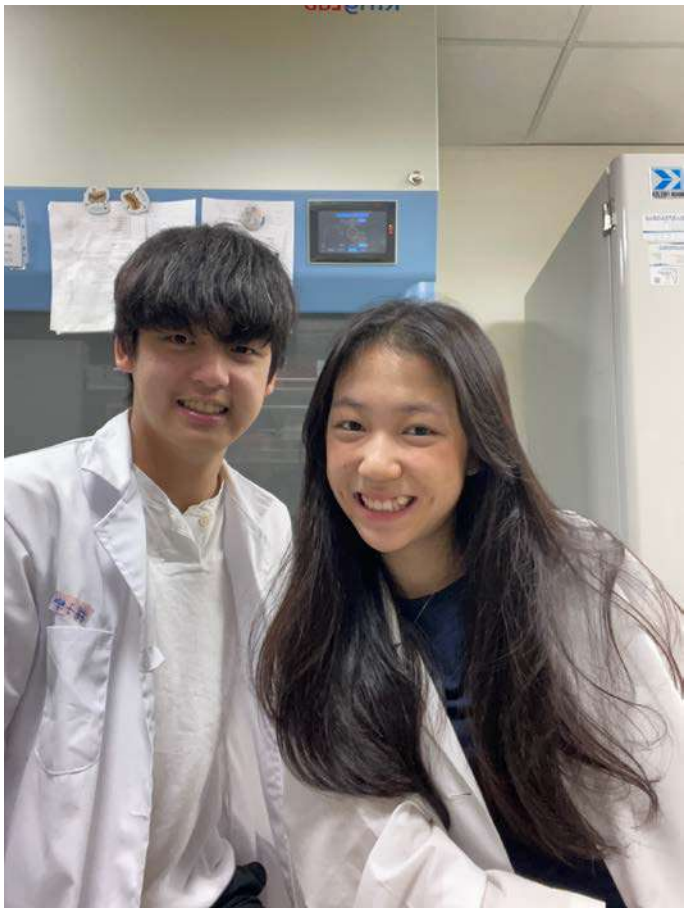
關鍵詞　nVNS、Glymphatic system、Alzheimer’s disease

# 作者簡介



我們是楊子樂和蘇怡安，目前就讀康橋國際學校秀崗校區。自國中起，我們就對科學實驗充滿熱忱，並對神經退化疾病抱有濃厚的興趣。很幸運地，我們有機會進入陽明交大的臨醫所——陳世彬老師的實驗室，深入學習和探索這一領域。

在這段旅程中，感謝教授、老師和學長姐們的耐心指導和陪伴，讓我們不斷進步與成長。

# 2025年臺灣國際科學展覽會

# 研究報告

區別：

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編號：
（編號由國立臺灣科學教育館統一填列）

## Abstract

Alzheimer's disease (AD) is ranked as the leading cause of dementia, affecting more than 55 million people worldwide. However, AD's current treatments remain limited and are often ineffective. The accumulation of amyloid-beta (Aβ) plaques in the brain has long been recognized as the pathological hallmark of AD. Previous studies have revealed the pivotal role of the brain clearance system in AD pathology as its dysfunction leads to the accumulation of toxic metabolites. Non-invasive vagus nerve stimulation (nVNS) was recently discovered to enhance cerebrospinal fluid flow, but its potential role and exact mechanism in the treatment of neurodegenerative diseases, such as AD, remain unknown. Therefore, our study aimed to use nVNS to target the enhancement of the brain clearance system as a novel therapy to halt AD progression. To investigate the effects of nVNS on the disease progression, the Aβ-induced AD mouse model was used. Transcranial macroscopic imaging and immunohistochemistry were applied to evaluate glymphatic function, and a novel object recognition test was employed to evaluate cognitive function. Our results showed that nVNS enhanced the function of the glymphatic system, which may be due to the upregulation of the water channels aquaporin-4 in the brain clearance system. Consistent with this finding, the cognitive function of mice was improved after nVNS as compared to sham operation. Our findings demonstrate for the first time that nVNS improves dementia symptoms induced by AD pathology and halts AD progression by enhancing the brain clearance system, showing the feasibility of nVNS as a potential therapy for AD.

## 摘要

阿茲海默症(AD)是導致失智症的主因, 影響全球數千萬人。然而, AD目前的藥物大多昂貴且療效有限。目前已知腦內β類澱粉蛋白(Aβ)斑塊為AD的病理特徵, 且大腦清除系統被認為對AD的治療具有重要性。先前研究發現非侵入性迷走神經刺激術(nVNS)增加腦脊髓液循環, 但在神經退化疾病中的機制和應用尚不明確。本研究旨在探討nVNS增強大腦清除系統來作為AD新療法之成效, 使用Aβ誘導之AD小鼠模型, 利用巨視顯微鏡和免疫組織化學染色評估其膠淋巴系統功能, 並以新奇事物測試評估認知功能。本研究發現於AD小鼠中, 給予nVNS使大腦清除系統之水通道蛋白-4顯著增加、促進膠淋巴系統, 進而改善認知功能。本研究首次發現nVNS可通過增強大腦清除系統功能, 進而改善AD病理引起的失智症狀, 支持nVNS作為AD新療法的可行性。

## A. 前言 Foreword

**Purpose of research**

As the global population continues to age, the prevalence of neurodegenerative diseases such as Alzheimer's disease (AD) has also increased dramatically, posing a major health concern due to its irreversible neuronal loss, cognitive function impairment, and eventual death in its final stage. AD is responsible for 60% to 80% of dementia cases, and by 2050, it is projected that over 100 million individuals will be affected (DeTure & Dickson, 2019; Matthews, 2016). Significant progress has been made in understanding the underlying mechanism of AD pathology, characterizing AD by its abnormal deposition of pathological proteins, such as amyloid beta (Aβ), leading to gradual and progressive neurodegeneration. However, despite these advances, the number of AD patients is excessively increasing each year as current treatments yield limited outcomes. Therefore, there is an urgent need to develop novel therapeutic strategies capable of halting AD progression.

Recently, research has highlighted the crucial role of the brain clearance system in the removal of metabolic wastes from the brain, including Aβ. Our novel approach, non-invasive vagus nerve stimulation (nVNS), has shown the potential to improve this clearance system. Thereby, nVNS may prevent Aβ accumulation, which offers non-invasive therapeutic potentials to target the underlying pathology of AD. We believe that this novel approach will mark a critical step toward a more effective treatment for AD.

## B. 研究背景 Research background

### B1: Overview of Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia. According to the Taiwan Alzheimer's Disease Association (TADA), in 2022, 1.37% of the Taiwanese population was diagnosed with dementia. With the expected demographic shift toward an aging global population, by 2041, the percentage is projected to double and rise to roughly 3% of the total population, as depicted in Figure 1 (Taiwan Alzheimer's Disease Association., 2023).

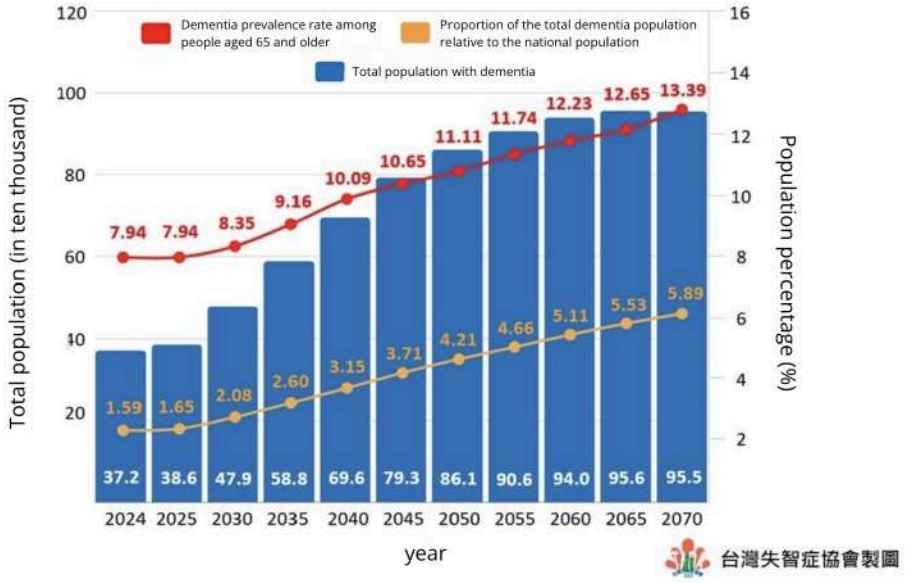


Figure 1. Projected prevalence and population of dementia patients in Taiwan from 2024 to 2070. The red line demonstrates the dementia prevalence rate among elders (age 65 or older), while the yellow line shows the proportion of the total dementia population relative to the national population (Taiwan Alzheimer's Disease Association, 2023).

Currently, more studies continue to uncover the complex brain changes in AD patients. AD progresses through several stages, as shown in Figure 2, each characterized by increasing cognitive impairment. Pathophysiological changes in the brain begin years before the symptoms appear. In the early stage of AD, patients may have no noticeable cognitive deficits, but abnormal accumulations of amyloid beta (Aβ) species and hyperphosphorylated tau protein are already present. The initial damage occurs in the hippocampus and entorhinal cortex, they play a crucial role in neurogenesis and memory formation (Rao et al., 2022). Subsequently, as more neurons die, other parts of the brain are affected and begin to shrink, including the medial prefrontal cortex (mPFC), where reasoning and higher cognitive skills are performed (Jobson et al., 2021; Choudhury et al., 2021). Such damages eventually lead to dementia behaviors and impaired neuronal activities. At the final stage, the damage is widespread: Brain tissue, including gray and white matter, will severely shrink by size as Aβ amyloid plaque forms and lead to neurotoxicity (Breijyeh&Karaman, 2020). Individuals may lose their ability to respond to the environment and require round-to-clock care. Ultimately, the loss of brain function becomes life-threatening.

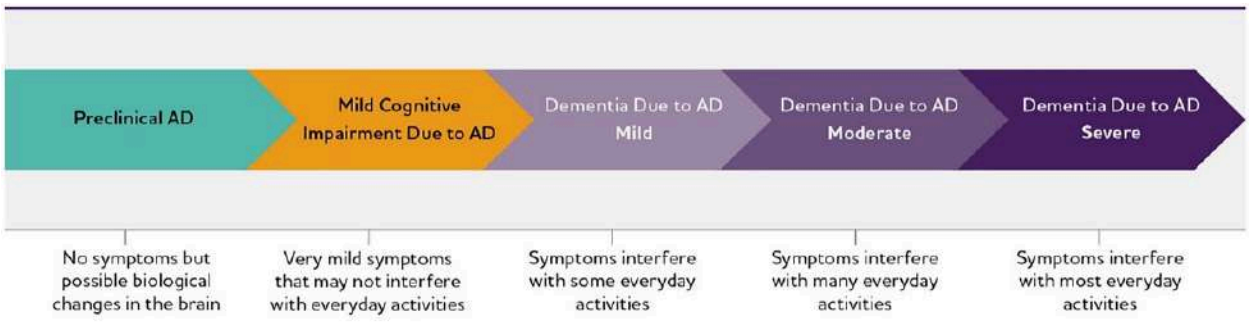


Figure 2. Alzheimer's Disease progression. This figure depicts the AD continuum. While the arrows are equal in size, the graph is not drawn to scale and the components of AD are not equal in duration (Gaugler et al., 2024).

Despite being identified for more than a century, AD continues to pose challenges for effective treatments, with recent anti-AD therapies yielding limited effects. Recently, there are three anti-amyloid antibodies approved by the U.S. Food and Drug Administration (FDA) to specifically target AD pathology. While these anti-AD drugs including Aducanumab, Lecanemab, and Donanemab have demonstrated efficacy in treating AD by targeting the toxicity of Aβ plaques, they can only be applied to patients with very early stages. In addition, their adverse clinical responses suggest limited efficacy accompanied by significant side effects and complications. Notably, these include severe complications such as brain edema or hemorrhage (Ameen et al., 2024). Moreover, all three drugs are not affordable for the general population. Taking Lecanemab as an example, in 2023, the annual treatment cost per patient was 26,500 USD, which is around 850,000 NTD (Jönsson et al., 2023). However, the median annual total salary for all employees in Taiwan is 16,000 USD, which is around 510,000 NTD (DGBAS, 2023). This disparity underscores the economic burden these treatments place on patients and healthcare systems while the drugs are simply not affordable to most of the patients. Hence, we propose an affordable novel therapeutic approach that targets stimulating the brain's internal clearance system to facilitate rapid and safe Aβ clearance to halt AD progression.

**B2 The Glymphatic System and Water-Channel Protein Aquaporin-4 (AQP4)**

The brain is the most complex organ in the human body, allowing for advanced cognitive abilities, emotions, and control of bodily functions. In this manner, the exchange of excess fluid, interstitial solutes, and wastes is crucial to maintain homeostasis. In 2012, *Iliff* et al. showed a noticeable cerebrospinal fluid (CSF) and interstitial fluid (ISF) inter-exchange as a new form of

brain waste clearance system (Iliff et al., 2012). This exchange is driven by convective influx of CSF along the periarterial space. In particular, in the subarachnoid space, CSF is driven by arterial pulsations into the Virchow-Robin spaces that surround the arteries. Subsequently, CSF is mixed with ISF in the brain parenchyma with the aid of AQP4 water channel protein expressed in astrocytes (Hablitz & Nedergaard, 2021). Then, CSF-ISF is drained out to the meningeal lymphatic vessels to be circulated out to cervical lymphatics, ultimately deep cervical lymph nodes (dCLN), and superficial cervical lymph nodes (sCLN). This fluid exchange mechanism facilitates the removal of extracellular molecules and wastes from the inner brain, namely Aβ, tau, etc. Following these findings, this brain clearance system is now named the glymphatic system due to its relation to glia and the ultimate resemblance to the meningeal and peripheral lymphatic system (Silva et al., 2021).

The aquaporin (AQP) family are water channel proteins characterized by six transmembrane pore-forming helices (Meli et al., 2018). The AQP family consists of over 150 diverse forms, and are found ubiquitously in animal cells, particularly mammals such as humans and mice. Generally, as a membrane protein, AQP facilitates cellular transport via diffusion and plays a pivotal role in maintaining homeostasis. In particular, a form of the AQP family, AQP4, is the amplest in the brain. It is known to play an important role in the clearance network of the glymphatic system. In the normal brain, AQP4 is abundant around the astrocytic endfeet. This allows AQP4 to contact the perivascular spaces and the brain parenchyma, in which AQP4 facilitates the CSF influx into the parenchyma and efflux out wastes. AQP4 also connects the astrocyte cytoplasm with ISF, which promotes the movement of the fluids (Nakada et al., 2017).

These, as a whole, facilitate interstitial movement and CSF-ISF exchange, ultimately fostering the glymphatic system.

The loss of AQP4 expression is linked to neurodegenerative diseases, notably AD, from recent studies. *Illif* et al. observed Aβ aggregation, loss of synaptic plasticity, as well as neuroinflammation in AQP4 knockout mice, resulting in cognitive deficit (Illif et al., 2012). In addition, significant AQP4 loss in APP/PS1 AD transgenic mice is observed to exacerbate AD phenotype (Pedersen et al., 2023). Recently, various studies have further linked AQP4, the glymphatic system, and AD together; however, the exact mechanism remains unclear. Based on current findings, we hypothesize that loss of AQP4 impairs the glymphatic system, which leads to aggregated Aβ plaques and results in dementia symptoms. Therefore, we speculate that the increase in AQP4 expression enhances the glymphatic system's efficiency, which improves the clearance of Aβ and halts AD progression.

**B3 Applications of vagus nerve stimulation (VNS)**

The Vagus nerve, composed of 20% efferent fibers and 80% afferent fibers, plays a key role in maintaining metabolic homeostasis via its pathways. The right and left vagus nerve travel through the neck, and continue through the upper chest, lower chest, diaphragm, and abdominal cavity. The most important role of the vagus nerve is afferent, which brings information from outer organs to the brain. In particular, nucleus tractus solitarii (NTS) receives input from these sensory afferent fibers, which send fibers that connect to various brain regions (Howland, 2014).

Conventionally, VNS involves invasive procedures with the placement of an electrode on the vagus nerve to deliver low-frequency, intermittent electrical pulses. Such stimulation minimizes the risks of potential adverse effects to other body structures as the vagus nerve is mostly composed of afferent fibers, which travel from the stimulation site to the brain, rather than the body. However, despite that invasive VNS have been approved by FDA for treatments of various diseases, such as refractory epilepsy, its application has been limited due to its surgical risks and high procedural cost (Kong et al., 2018). Thus, non-invasive VNS (nVNS) becomes a promising, inexpensive, and safer therapeutic alternative. Although its exact mechanism is not fully understood, it has been shown to reduce inflammation in both the body and brain (Bonaz et al., 2016). It has also been proven to stimulate the same brain loci as the invasive vagus nerve stimulation VNS (Badran et al., 2018). In recent years, FDA has approved nVNS for acute and preventive treatments of migraine and cluster headaches.

Other than the diseases mentioned, current studies also reveal the potential of nVNS to treat cognitive disorders. In 2022, *Choi et al.* discovered that non-invasive auricular VNS enhances CSF circulation, particularly in the perivascular space, promoting the clearance of toxic metabolites, which further suggests its potential role in improving glymphatic function. They also observed that such stimulation may improve cerebrovascular disorders, particularly vascular cognitive disorders (Choi et al., 2022). However, its application to neurodegenerative diseases such as AD still needs to be discovered. On the other hand, instead of auricular VNS, we would like to delve into the effects of non-invasive cervical VNS. The underlying reason is that group Aβ of the type II sensory fiber, one of the specific nerve fibers nVNS targeted, is far more present in the cervical vagus nerve compared to the auricular branch of the vagus nerve (Hilz,

2022). Hence, we believe it may have higher stimulation efficiency and subsequent therapeutic benefits. Based on these findings, we hypothesize that non-invasive cervical VNS, one of the nVNS, may facilitate glymphatic function and further ameliorate symptoms of AD.

## Hypothesis

As shown in Figure 3, we hypothesize that nVNS stimulation may lead to the upregulation of AQP4 expression in the hippocampus (CA1) and entorhinal cortex (EC). This increased expression may facilitate CSF flow, which enhances the glymphatic function. Ultimately, the enhanced glymphatic function may clear out the Aβ accumulation in the brain parenchyma and improve cognitive deficits and dementia induced by AD.

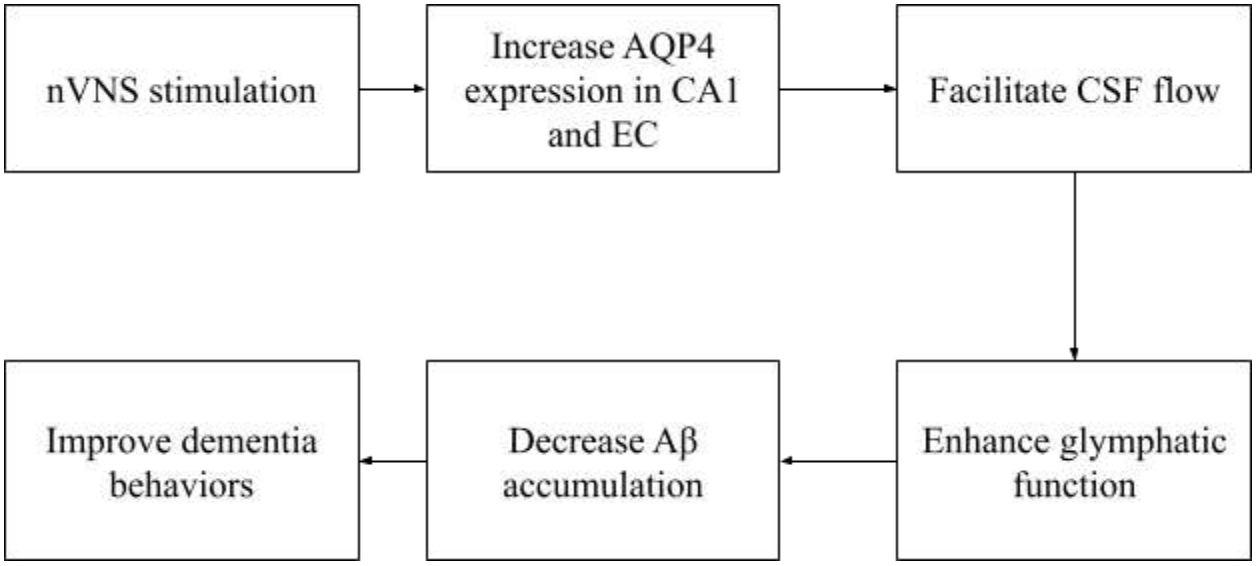


Figure 3. Schematic representation of our hypothesis.

## AD Mice Model

In this study, the AD mice model was induced by intracerebroventricularly (i.c.v.) injecting the Aβ peptides. The accumulation of Aβ plaque in the brain has long been recognized as the

hallmark and central event in the pathogenesis of AD. This choice of our animal model is based on the strong evidence that Aβ accumulation correlates with cognitive decline and neurodegenerative changes characteristic of AD. The amyloid plaques disrupt neural connections, promote inflammation, and trigger neuronal death, which ultimately leads to the cognitive deficits observed in AD. As demonstrated by various researchers, the injection of Aβ1-42 peptides in healthy mice induces cognitive impairments seen in human AD patients (Kim et al., 2016; McAllister et al., 2020). Thus, we believe that the Aβ-injected mice comprise the behavioral and physiological features to mimic human AD conditions.

## C. 研究過程與方法 Method and materials

### 1. Ethics

3Rs (Replacement, Reduction, Refinement) were implemented to minimize the harm to the animals and make sure that the outcomes are scientifically significant and that the quality of the science is high. All experiments align with the guidelines for the Care and Use of Laboratory Animals proposed by the Council of Agriculture, Executive Yuan, and are approved by the Institutional Animal Care and Use Committee.

### 2. Animals

A total of 12 C57BL/6 mice (male, 8 weeks old) were used in this study. Animals were purchased from the National Laboratory Animal Center (NLAC), in Taiwan. Mice were housed in laboratory animal rooms, with thermostatic control at 22°C (21°C-23°C), 40–70% humidity, and 12-hour light/dark cycle (All experiments are performed in the light cycle), and were allowed access to standard animal chow and water *ad libitum*. Mice were randomly assigned to either the chronic nVNS treatment group or the sham-operated group.

### 3. Intracerebroventricular (i.c.v.) injection of beta-amyloid protein

For preparation, Aβ1-42 peptide (Sigma–Aldrich, A9810) was made as a stock solution at a concentration of 1 mg/ml in sterile saline. Four days prior to use, Aβ 1-42 protein fragments were incubated at 37° C to create Aβ 1-42 peptides. Aliquots were then stored at -20° C. To inject the Aβ1-42 peptides, as demonstrated in Figure 4, we located the lateral ventricle at the following coordinates from bregma: Anteroposterior (AP)= -0.1mm, mediolateral (ML)=-1mm, dorsoventral (DV)= -3mm. Subsequently, after 4% gas isoflurane anesthesia, 3 μl Aβ 1-42 oligomers (total 400 pmol/mouse) was injected (i.c.v.) through a microsyringe (Hamilton #1700)

with a rate of 1 µl/min. Lastly, the microsyringe was slowly withdrawn to prevent the reflux of liquid (Amin et al., 2017).

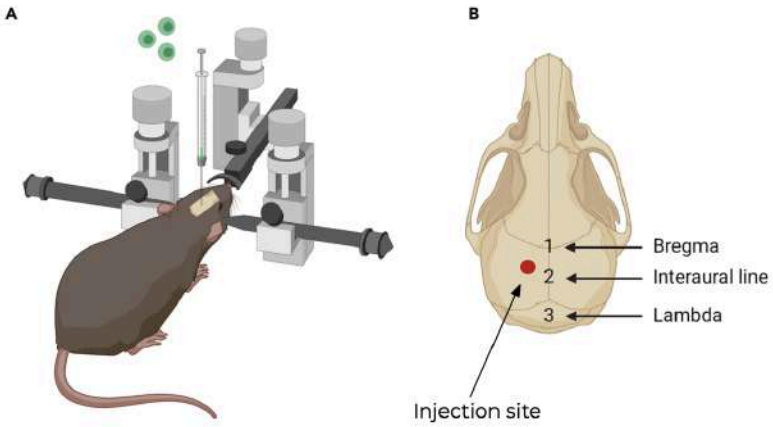


Figure 4. Representation diagram of the injection site when intracerebroventricularly injecting Aβ peptides.

**4. Non-Invasive Vagus Nerve Stimulation (nVNS)**

Mice were anesthetized with 4% isoflurane gas and placed on a heat pad to maintain rectal temperature at 37°C. The ventral neck region was shaved and disinfected. A custom-made gammaCore nVNS device (a 5-kHz sine wave for 1 millisecond repeating at a rate of 25 Hz) was used to deliver electrical stimulation by placing 2 disk electrodes with conductive gel on the shaved skin covering the right cervical vagus nerve. nVNS's parameter is 24.4 V, for a duration of 2 minutes, with a total of 2 stimuli, 5 minute rest in between (Fig. 5).

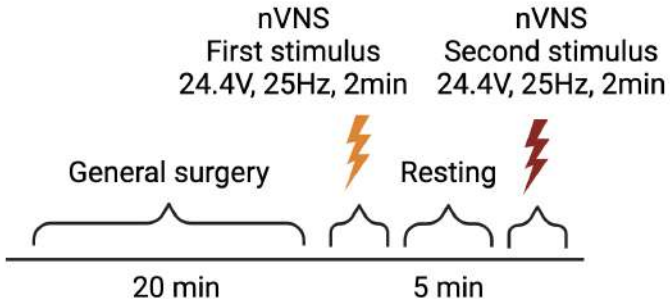

Figure 5. Schematic diagram of nVNS treatment. The nVNS treatment was conducted during the light cycle, particularly between 2PM to 4PM.

### 5. Transcranial Macroscopic Imaging

Transcranial macroscopic imaging was used to directly visualize the transport efficiency of the glymphatic system noninvasively (Leica M205 FA) (Fig. 6). After anesthesia, fluorescent tracer BSA 647 (75 kDa) was introduced into the CSF using intra cisterna-magna injection. Subsequently, real-time in vivo imaging of the glymphatic system was conducted for a duration of 60 minutes post-injection. MetaMorph Offline software was used for quantification of the tracer's intensity.

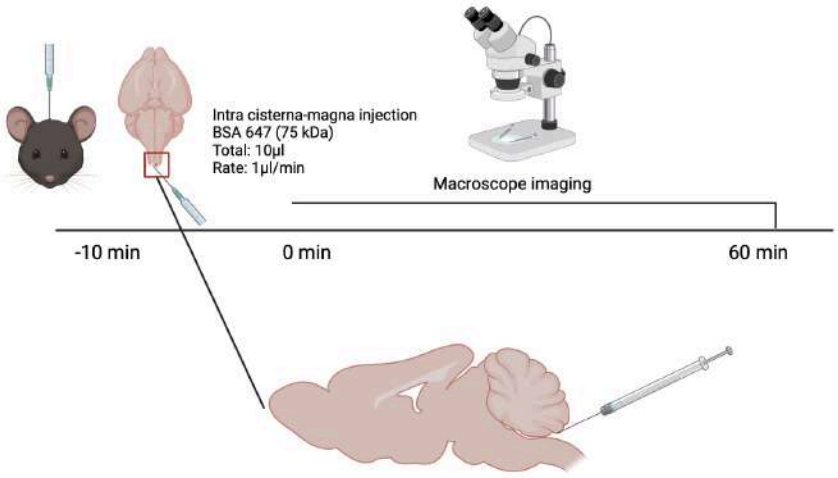


Figure 6. Schematic diagram of transcranial macroscope imaging.

### 6. Immunohistochemistry

Immunohistochemistry staining was performed on the hippocampus and entorhinal cortex. Mice were anesthetized with 4% isoflurane, then perfused with 0.9% normal saline, followed by 4% paraformaldehyde in 0.1 mol/l PBS. The brain samples were further post-fixed in 4% paraformaldehyde at 4°C overnight, and immersed in 20 and 30% sucrose for 2 days at 4°C. Tissues were cut into 20 µm frozen sections using cryostat (CryoStar NX70, ThermoFisher

Scientific). The examined brain sections for AQP4 and C-Fos are shown in Figures 7 and 8, respectively. Then, tissue sections were quenched in $H_2O_2$, and blocked in normal goat serum to prevent non-specific antibody binding. Sections were incubated in Anti-Aquaporin-4, C-terminus (1:500, AB3594-200ULa, Merck) primary antibody overnight at 4°C. Samples were washed three times with 1X PBS for 5 min in an orbital shaker, then incubated in Goat Anti-Rabbit IgG Antibody (H+L), Biotinylated secondary antibody (BA-1000-1.5, Vector Laboratories) for primary antibody targeting for 1 hour. Post-secondary antibody incubation, samples were washed with 1X PBS, then placed on glass slides, and used DPX mountant for histology (06522-100ML, Sigma-Aldrich).

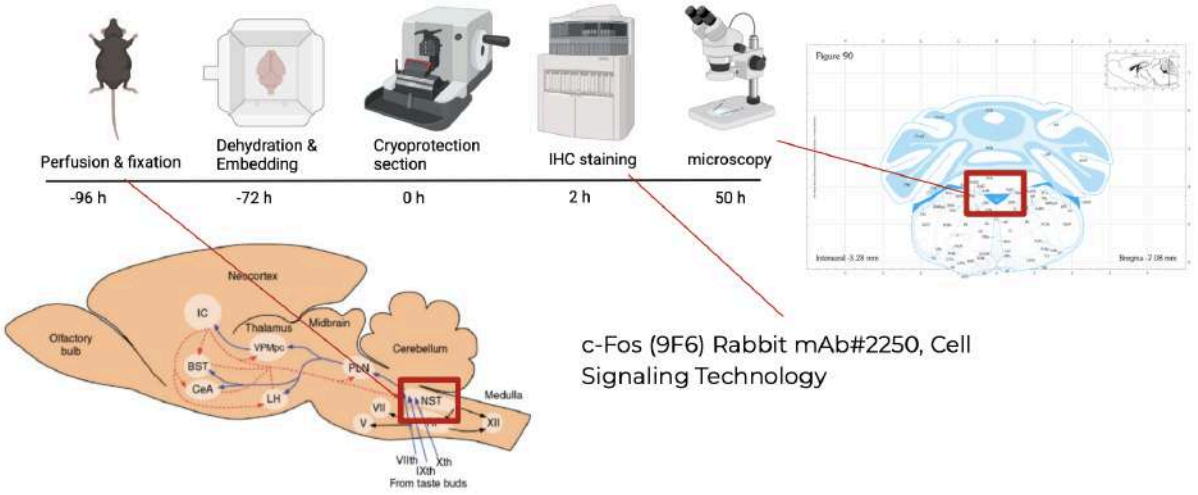


Figure 7. Schematic diagram of immunohistochemistry for c-Fos at NTS. The brain section of NTS that was examined is boxed.

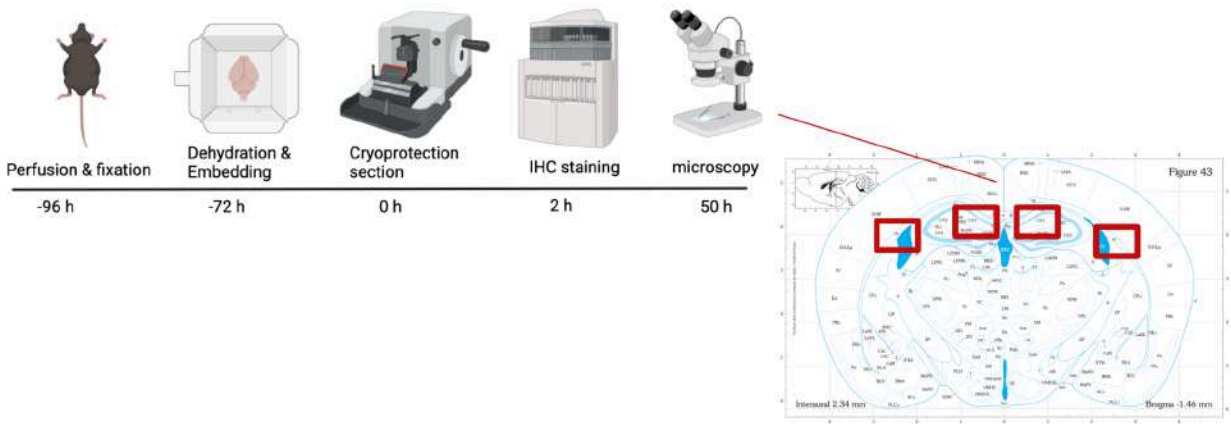


Figure 8. Schematic diagram of immunohistochemistry for AQP4 at hippocampus (CA1). Brain sections that were examined are boxed, which are the entorhinal cortex (EC) and hippocampus (CA1).

**7. Novel Object Recognition test (NOR)**

NOR was used to assess the memory performance in mouse models via the discrimination index (DI), calculated from the difference in time spent exploring novel and familiar objects divided by the total time spent exploring both objects. One and a half hours prior to the experiment, mice were brought to the behavioral access room for familiarization with space. Then, for habituation, mice were introduced to a NOR box (50x50x50 cm) to explore freely without any object for 10 minutes, then rested for 1.5 hours. After rest, mice were placed in the same box containing two identical objects for 10 minutes, and then allowed to rest for 1.5 hours. Lastly, mice were placed back in the same box with an identical copy of the previously encountered object along with a novel object for 10 minutes. Mice that did not explore any object for a minimum of 20 seconds

will be excluded from the analysis (Lee et al., 2021).

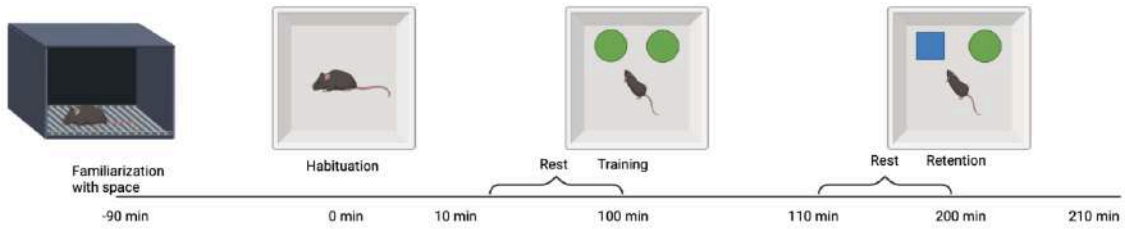


Figure 9. Schematic diagram of novel object recognition test

## 8. Statistical Analysis

All statistical analyses were performed using GraphPad Prism 9. For immunohistochemistry, images were taken using MShot microscope imaging software and analyzed by ImageJ, followed by unpaired t-test analysis. For novel object recognition, data was acquired via Ethovision XT 17, followed by paired t-test analysis.

## D. 研究結果 Results

### 1. Non-Invasive vagus nerve stimulation leads to c-Fos activation in the NTS

We investigated whether the vagal afferents were successfully activated by our nVNS. Past studies have shown that stimuli from the vagus nerve pass through NTS when entering the central nervous system from medulla oblongata, and such stimuli increase the immune response of c-Fos, a neuronal activation marker, in NTS. Thereby, the activation of NTS is attributed to nVNS. After chronic nVNS treatment or sham operation, we conducted immunohistochemistry of c-Fos in the NTS, then measured the total c-Fos positive cells expressed in NTS for every sample. In the chronic nVNS group, c-Fos immune response was observed in all regions of NTS (Fig. 10A). In the sham-operated group, a very limited immune response with low intensity was observed (Fig. 10B). Statistical analysis shows a significant ($p$<0.01) increase in c-Fos expression in the chronic nVNS group compared to the sham-operated group (Fig. 11). These results suggest that after nVNS treatment, the vagus nerve successfully delivered the activating signals to the brain.

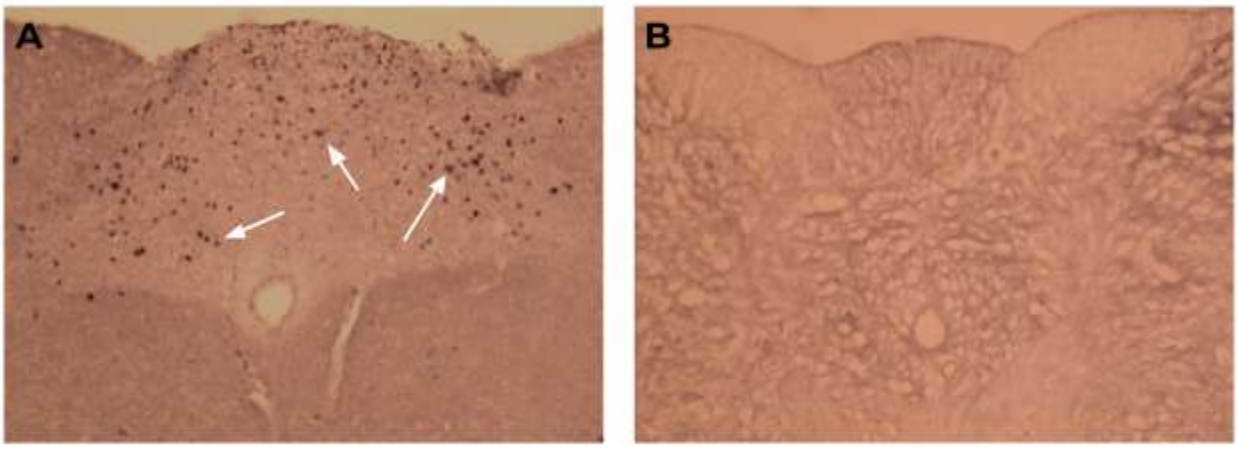


Figure 10. Representative histological images of c-Fos specific immunohistochemistry staining in the NTS of AD mice. Once the vagus nerve was stimulated, c-Fos expression (arrows) was

upregulated. (**A**) Chronic nVNS group (**B**) Sham-operated group.

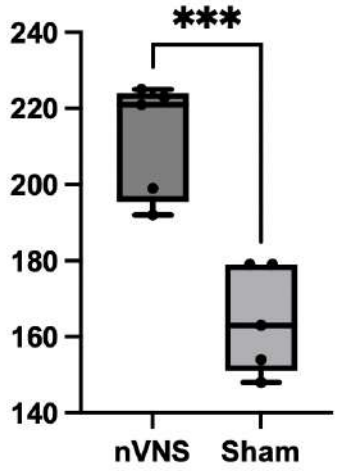


Figure 11. Box plot comparisons for count of c-Fos positive cells in NTS, n=5 for each group, Unpaired t-test, ***$p$<0.001.

**2. Non-Invasive vagus nerve stimulation stimulation enhances glymphatic transport**

We then investigated whether nVNS stimulation enhanced CSF circulation and glymphatic flow in the parenchyma. CSF circulation is highly associated with the glymphatic function. After intra-cisterna-magna injection of the BSA 647 tracer, in vivo transcranial macroscopic imaging was performed to track and visualize the spread of CSF. In the chronic nVNS group, a higher flow speed is shown compared to the sham-operated group. We observed that in the chronic nVNS group, CSF tracer started to flow in 10 minutes, while the sham-operated group showed CSF tracer after 20 minutes or more. After 60 minutes, nVNS groups are shown to drain the tracer more obviously with more branches (Fig. 12). Thus, compared to the sham-operated group, the chronic nVNS group promoted CSF flow deeper into the glymphatic system. For further evaluation of the CSF dynamics, we calculated the mean pixel intensity (MPI) for BSA 647 tracer. CSF tracer signal in AD mice after nVNS treatment is observed to have a higher intensity and faster flow speed compared to the sham-operated group (Fig. 13). Therefore, we

conclude that chronic nVNS treatment enhanced CSF flow, increased glymphatic influx, and enhanced glymphatic transport in the brain.

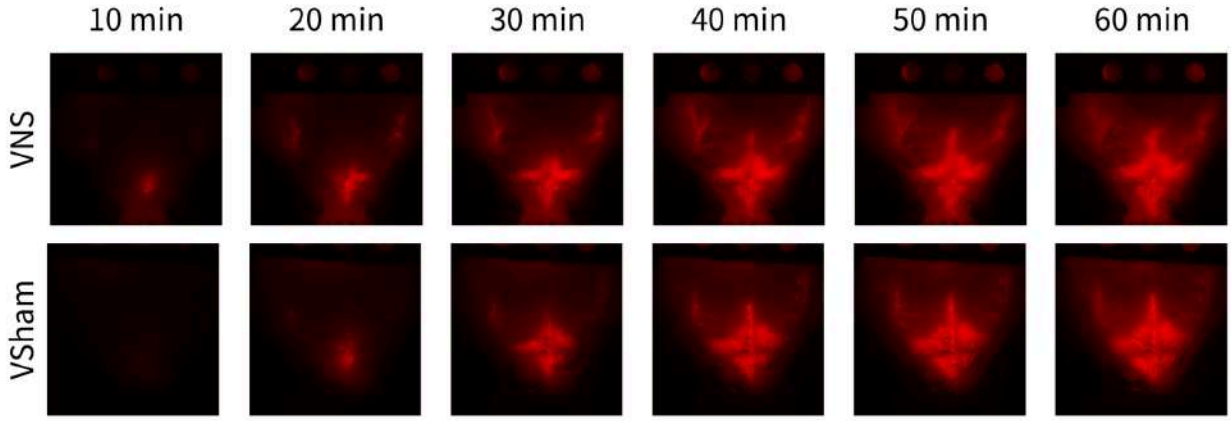


Figure 12. Transcranial macroscopic imaging of BSA 647 (75 kDa) tracer distribution in the glymphatic system.

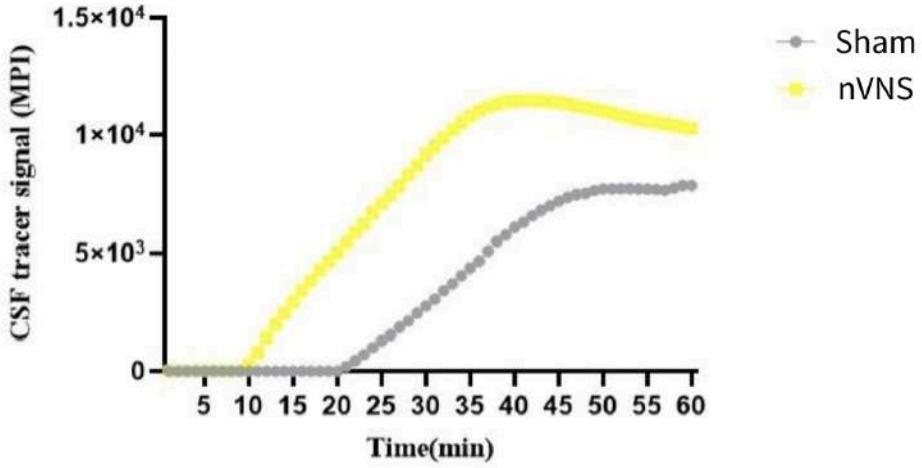


Figure 13. CSF tracer signal for different time frames in the nVNS group and sham-operated group.

3. **nVNS stimulation enhances AQP4 expression in the hippocampus and entorhinal cortex**

To determine whether nVNS enhances the glymphatic system by increasing expression of AQP4,

we performed immunohistochemistry (IHC) in brain regions, hippocampus and entorhinal cortex, which are known to be most affected by AD. The process of brain clearance is dependent on AQP4 water-channel protein, as AQP4 contacts the perivascular spaces and the brain parenchyma and facilitates the CSF influx into parenchyma and efflux out wastes. Therefore, to determine the efficiency of transportation of wastes, evaluating the expression of AQP4 is essential.

**Hippocampus**

The hippocampus is closely associated with AD's progression. Starting at the very mild stage of AD, Aβ plaque and deposition form specifically in the hippocampus. Then, as AD progresses, the decreased efficiency of elimination of these extracellular accumulation of plaques results in cognitive impairment and dementia. When comparing the chronic nVNS group and sham-operated group, a significant increase ($p<0.01$) in AQP4 positive cell area was detected, thereby suggesting that AQP4 has been upregulated by nVNS treatment, as shown in Figure 14 and 15. For the corresponding histological observation, AQP4 positive cells were shown in "vessel-like" structures. The chronic nVNS group showed more abundant AQP4 immunopositivity compared to the sham-operated group.

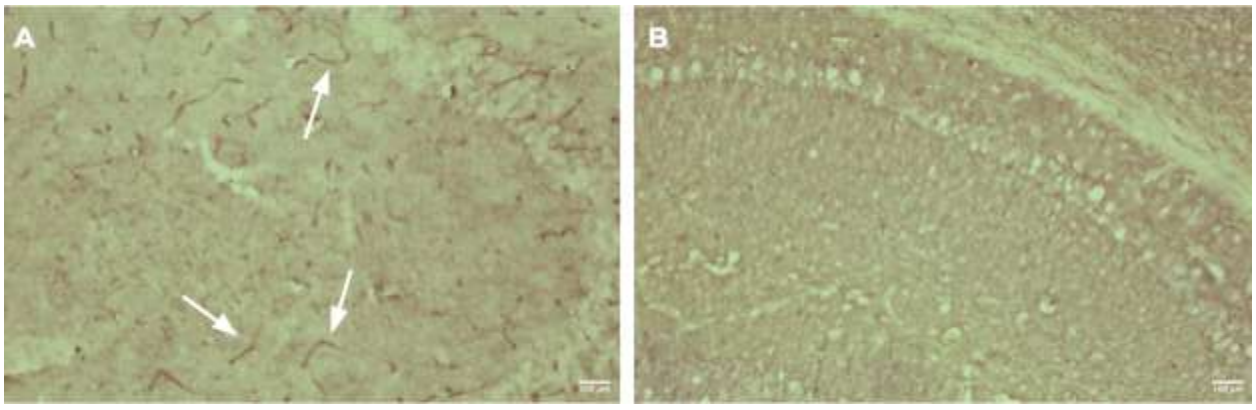

Figure 14. Immunohistochemistry of AQP4 at hippocampus (scale bar=100μm). Histological photomicrography (100X) of the hippocampus (CA1) showing AQP4 expression (arrows) of (**A**) chronic nVNS group (**B**) Sham-operated group.

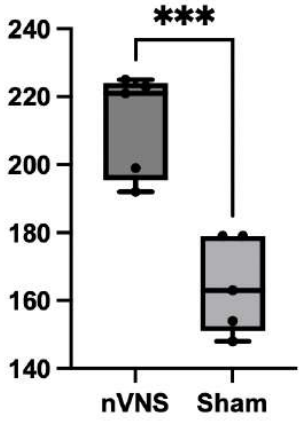


Figure 15. Box plot comparisons for AQP4 positive cell area in hippocampus, n=4 for each group, Unpaired t-test, $**p<0.01$.

**Entorhinal Cortex**

Past anatomical studies in both humans and mice showed abnormal accumulation of Aβ is likely to originate in the entorhinal cortex. In preclinical stages of AD, Aβ plaque is already present, while in moderate and severe stages, excess wastes in the entorhinal cortex lead to structural atrophy. Prominent AQP4 positive cell expression was observed in the chronic nVNS group. In contrast, AQP4 was expressed at a low level without nVNS treatment (Fig. 16). In addition, there was a significant ($p<0.0001$) increased immunohistochemical expression of AQP4 in the chronic nVNS group (Fig. 17).

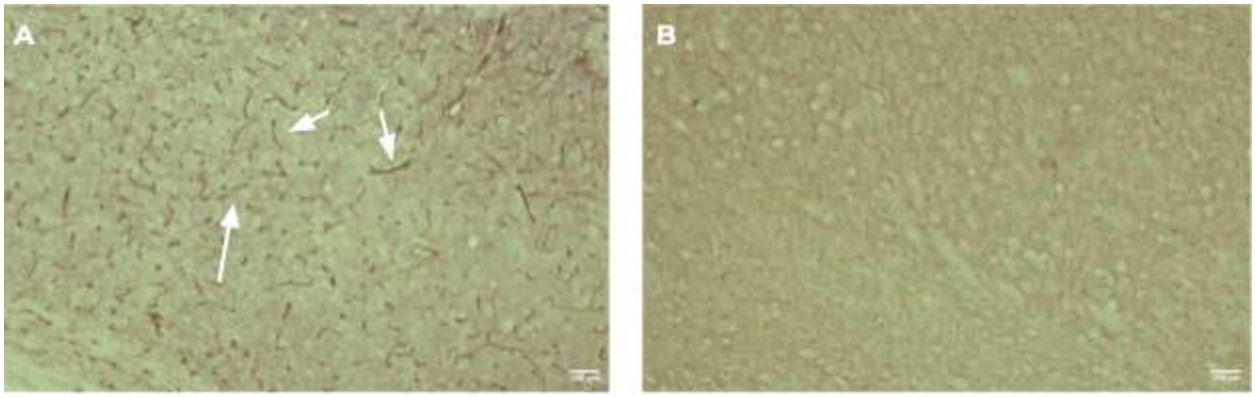


Figure 16. Immunohistochemistry of AQP4 at the entorhinal cortex (scale bar=100μm). Histological photomicrography (100X) of the entorhinal cortex (EC) showing AQP4 expression (arrows) of (**A**) chronic nVNS group (**B**) Sham-operated group.

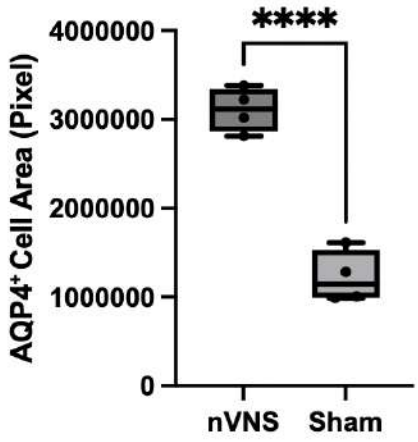


Figure 17. Box plot comparisons for AQP4 positive cell area in entorhinal cortex, n=4 for each group, Unpaired t-test, ****$p$<0.0001.

**4. Non-Invasive vagus nerve stimulation reduces dementia behavior of AD mice**

To further investigate nVNS's direct correlation with AD progression, we employed NOR test in order to examine AD mice's change in short-term cognitive and memory functions. The first test was conducted three days following the i.c.v. injection, serving as the pre-treatment evaluation.

After seven days of nVNS treatments, the mice were tested again to evaluate post-treatment effects. The discrimination index shows a significant increase after chronic nVNS treatments (Fig. 18A), while there is no significant difference shown in the sham-operated group (Fig. 18B). This suggests that nVNS improves short-term cognitive function and demonstrates its potential to ultimately halt the progression of AD.

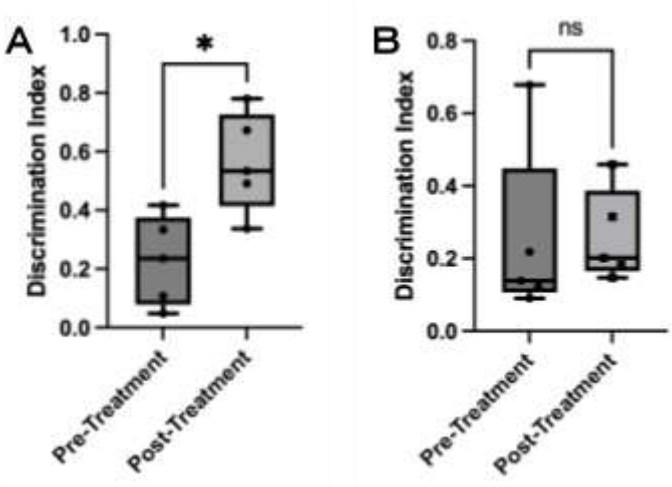


Figure 18. Box plot comparison of discrimination index for pre-treatment and post-treatment in AD mice, paired t-test, n=5 for each group, **(A)** Chronic nVNS group, *$p$<0.05 **(B)** Sham-operated group, $p$>0.05 (ns).

## E. 討論 Discussion

The current disease-targeting treatments of AD are only applicable to preclinical and mild stages and are at high cost, which lack accessibility to most patients. Our study provides evidence that nVNS could facilitate glymphatic function and improve cognitive performance in mouse models of AD, suggesting the potential of nVNS as a novel therapeutic approach for AD.

Despite the ongoing research on nVNS, its underlying mechanism and implications on AD remains largely unexplored. Our research provided new insight into nVNS-induced enhancement of CSF flow and the glymphatic system. Previous research has connected AQP4 and the glymphatic system, but most studies only focus on the cortex around the meninges. However, the correlation between AQP4 in brain areas associated with AD is unknown. Our results showed an upregulation of AQP4 in brain regions that is heavily affected by AD after nVNS treatment. This finding supports the observation that nVNS enhances glymphatic flow, and suggests that nVNS activates glymphatic pathways, addressing one of the most fundamental issues in AD pathology, which is the clearance of toxic wastes. In this study, we demonstrated for the first time that nVNS improves cognitive function induced by AD pathology through glymphatic clearance. Specifically, the increased discrimination index in novel object recognition tests, along with the increased glymphatic flow, demonstrates nVNS's ability to halt AD pathology. Beyond Alzheimer's disease, future research may delve into nVNS's potential in other neurodegenerative diseases, such as Parkinson's Disease. As a whole, these findings constitute an advancement in the current understanding of nVNS' potential in neurodegenerative diseases and open up future research directions.

Compared to traditional vagus nerve stimulation, nVNS omits the trauma caused by incision. The only side effect of nVNS is related to the conduction of transcutaneous electrical current, as it causes redness and skin irritation (Bonaz, 2023). In alignment with current studies, we did not identify any noticeable complications when giving nVNS treatment. However, further investigation that establishes the exact mechanism of nVNS in the brain and excludes the possibilities of side effects will strengthen the clinical value of this strategy. As with all preclinical studies, the translation of these findings to human AD patients requires a more careful and comprehensive evaluation with human trials to ensure the safety of such novel therapy. Future research should also explore the potential synergistic effects of combining nVNS with existing AD drugs or other approaches that target the glymphatic system.

Furthermore, our study found that nVNS activated NTS as shown by the increased expression of c-Fos, thereby halting AD progression. Past studies have reported another possible pathway for nVNS to moderate Aβ-induced toxicity, demonstrating its potential to affect AD progression. As vagal afferents projecting to the NTS release the fast excitatory neurotransmitter glutamate, brain-derived neurotrophic factor (BDNF) expression also increases accordingly (Martin & Finsterwald, 2011; Hermes et al., 2014). Moreover, *Arancibia et al.* demonstrate that BDNF exhibits neuroprotective effects against Aβ-induced toxicity, both in vitro and in vivo using rat models, reversing the damage caused by Aβ (Arancibia et al., 2008). In addition, via the NTS, vagal afferents form primarily asymmetric synapses with neuronal targets that are suggestive of excitatory neurotransmission, and the nerve terminal of the tractus solitarius releases glutamate (Hermes et al., 2014; Ragozzino et al., 2020). Glutamate binds to N-methyl-D-aspartic acid (NMDA) or non-NMDA receptors and mediates synaptic transmission within the brain, then

activation of NMDA and AMPA results in the opening of ion channel pores including the aquaporin family, which aligns with our results that nVNS upregulates AQP4 expression.

In summary, the findings we presented represent an exciting opportunity for a novel therapy that enhances the brain's internal clearance system for improving AD's dementia symptoms. Unlike existing anti-AD drugs, which are often limited to early stages of AD and come with substantial side effects and cost, nVNS has the potential to be applied to various stages of AD and might be more accessible to patients. For the first time, our findings show that nVNS enhances the brain clearance system, improves dementia symptoms induced by AD pathology and halts AD progression, showing the feasibility of nVNS as a potential therapy for AD.

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# 【評語】090022

1. 創新性與重要性:

本研究探討非侵入性迷走神經刺激（nVNS）對阿茲海默症（AD）的潛在治療作用，特別聚焦於其對腦淋巴系統功能的影響，具有重要的創新性和臨床意義。AD 是一種嚴重影響生活質量的神經退行性疾病，目前尚無有效的治療方法。研究發現 nVNS 能上調水通道蛋白 4（AQP4）並增強腦淋巴系統功能，為 AD 的治療提供了新的思路。這種非藥物、非侵入性的治療方法不僅可能改善 AD 患者的認知功能，還可能通過增強腦部清除系統來延緩疾病進展，這對於開發新的 AD 治療策略具有重要意義。

2. 優點:

該研究研究採用了多角度的實驗方法，包括分子生物學技術和行為學實驗，全面評估了 nVNS 對 AD 的影響，以及對 AQP4 表達和腦淋巴系統功能的影響，這為理解 nVNS 的作用機制提供了重要線索。此外，研究使用了 AD 動物模型，增加了結果的可靠性和臨床相關性。最後，研究結果顯示 nVNS 在改善腦清除系統和認知功能

方面具有潛力，這一發現為開發非侵入性 AD 治療策略提供了重要依據。

3. 待改進的部分:

雖然研究觀察到 nVNS 上調了 AQP4 並增強了腦淋巴系統功能，但具體的分子機制仍未被完全闡明。建議進一步研究 nVNS 如何精確影響神經元網絡或其他細胞類型（如星形膠質細胞）。其次，研究未考慮將 nVNS 與現有藥物治療或其他干預手段（如飲食或運動）聯合使用的可能性，這可能進一步增強療效。在 AD 模型方面，研究需要進一步確認所誘發的 AD 小鼠是否出現類澱粉斑塊和神經元纖維纏結等 AD 的典型病理特徵。此外，研究應明確所誘發的 AD 小鼠模型對應人類 AD 的哪一階段（早期、中期或晚期），並探討 nVNS 對不同階段 AD 的治療效果。這些改進將有助於更全面地評估 nVNS 作為 AD 治療策略的潛力，為其臨床應用提供更堅實的科學基礎。