Thermal cycling stimulation via nasal inhalation attenuates $A\beta_{25\text{-}35}$-induced cognitive deficits in C57BL/6 mice

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ABSTRACT

Alzheimer's disease (AD) continues to pose a significant public health challenge, with current treatments demonstrating limited effectiveness, partly due to the difficulty of delivering therapeutics across the blood–brain barrier (BBB). The nose-to-brain (N-2-B) pathway offers a promising alternative, enabling pharmacological agents to circumvent the BBB. However, to date, no drugs have been successfully implemented in clinical settings for the treatment of AD via this route, underscoring the necessity for additional research in this area. Mild stress is thought to activate intrinsic protective mechanisms against neurodegeneration, but traditional methods of inducing stress often lack both specificity and practicality. To address this, we propose inhaling mildly heated air as thermal stimulation, leveraging the N-2-B pathway to elicit mild stress and stimulate the brain. Furthermore, this study employs our thermal cycling hyperthermia (TC-HT) concept, adapted as thermal cycling stimulation via nasal inhalation (TCSNI), which delivers cyclic stimulation to sustain pathway activity while minimizing thermal damage. In this study, we evaluated the health and olfactory effects of TCSNI in C57BL/6 mice, observing no adverse impact. In experimental groups administered with β-amyloid (Aβ), TCSNI treatment resulted in significant enhancements in cognitive performance as evidenced by Y-Maze and novel object recognition (NOR) assessments, suggesting an improvement in cognitive function. Protein analyses conducted on the hippocampus of the mice indicated a reduction in Aβ accumulation, alongside increased expression of heat-shock protein 70 (HSP70) and insulin-degrading enzyme (IDE) expression, as well as elevated levels of phosphorylated Akt (p-Akt). These results suggest that N-2-B-delivered TCSNI effectively modulates protein expression and enhances cognitive function, highlighting its potential for further exploration in AD treatment.

Keywords: Alzheimer's disease; β-amyloid; hyperthermia; nasal inhalation; mild stress

INTRODUCTION

Alzheimer's disease (AD) is one of the leading causes of dementia and poses an increasing threat to human health [1]. The abnormal deposition of β-amyloid (Aβ) in the brain is believed to contribute significantly to the oxidative stress and damage that underlie the development of AD [2-4]. Additionally, Aβ aggregation is associated with synaptic dysfunction and neuronal loss, contributing to cognitive decline in AD patients [5,6]. Therefore, Aβ has emerged as a primary target for AD therapy [5-7]. In recent years, drugs developed based on the Aβ hypothesis have gained FDA approval, namely aducanumab and lecanemab [8,9]. However, these drug treatments are costly and associated with the risk of brain edema and abnormal bleeding [10,11], which pose challenges to their widespread adoption. While medication is the main therapeutic approach, the blood-brain barrier (BBB) constrain advancements in new drug development and application [12,13], highlighting the need for further exploration of alternative therapies. Integrating mild stress-based non-pharmacological interventions into healthcare strategies offers a promising avenue for promoting health [14-18]. How to design optimal and well-controlled mild stress stimuli has attracted considerable interest due to their ability to stimulate brain cells, enhance cognitive function, and potentially extend lifespan, thereby providing beneficial effects in the context of neurodegenerative diseases [19-21]. These benefits are largely mediated through the principle of hormesis, wherein exposure to low levels of stressors induces adaptive cellular responses that enhance resilience against neurodegenerative processes [22-25]. Studies indicated that mild stress may upregulate heat shock proteins (HSPs), which are associated with reduced Aβ aggregation [26,27]. Exercise and sauna are widely recognized as physiological stimuli capable of inducing mild stress in humans [19-21,28,29]. However, despite their general health benefits, these long-term approaches are time-intensive and lack conclusive evidence of curative effects [30-33]. In view of this, exploring novel and convenient alternative approaches is valuable.

In order to address this issue, investigating other viable solutions is of utmost importance. The nose-to-brain (N-2-B) pathway offers an alternative approach. Unlike traditional drug delivery methods that rely on penetration enhancers to facilitate transport across the BBB, the N-2-B pathway enables drug delivery via nasal inhalation, thereby circumventing this limitation [34,35]. Drugs such as donepezil, rivastigmine, and natural plant extracts like quercetin and curcumin have also been tested in N-2-B-related research [36,37]. Additionally, insulin inhalation has garnered significant attention in N-2-B-related studies [36,38]. In parallel, aromatherapy is also considered an effective complementary or alternative therapy that may offer benefits in treating dementia [39-41]. Studies indicate that inhaled aromatherapy may mitigate

dementia-related symptoms through olfactory stimulation and modulation of brain regions governing emotion and cognition [42-44]. Unfortunately, despite extensive research on various drugs and bioactive compounds, no medication has yet been successfully applied through this route for the clinical treatment of AD [36], suggesting that further research into the N-2-B pathway is necessary.

While pharmacological treatments and complementary therapies such as aromatherapy have shown progress, their impact remains constrained. Building upon the concept of olfactory stimulation and beyond conventional pharmacological strategies, we propose, for the first time, the novel application of inhaling mildly heated air as a form of thermal stimulation via the N-2-B pathway to activate the brain and induce mild stress. Thermal stimulation has been regarded as having benefits in various medical fields for a long time [45-47]. Appropriate hyperthermia (HT) can enhance the expression of heat shock proteins (HSPs), particularly heat shock protein 70 (HSP70), and is considered beneficial for improving AD [48-50]. In addition to the HSP family, studies have also indicated that HT can increase the expression of insulin-degrading enzyme (IDE), which is believed to reduce Aβ accumulation [51,52]. Furthermore, thermal stress has been shown to activate Akt signaling [53,54], and the neuroprotective effects of phosphorylated Akt (p-Akt) in AD have also been discussed [54-57]. As a thermal stimulation practice for preventing dementia, sauna has garnered considerable interest in recent years [30,31,48]. However, while sauna use has been associated with potential cognitive benefits, its therapeutic effects are still inconclusive [30,31]. Besides, accessibility issues and safety considerations, particularly for elderly individuals with cardiovascular risk factors, may limit its practicality [58,59]. In contrast to systemic heating, this study proposes a novel strategy using the inhalation of slightly heated air to induce mild stress, with the aim of modulating protein expression and explore its potential benefits for cognitive function. Furthermore, to minimize the potential risks associated with continuous heat treatment, our team adapted the previously developed concept of thermal cycling hyperthermia (TC-HT) into the novel method, termed thermal cycling stimulation via nasal inhalation (TCSNI). Through repeated cycles of heated and ambient-temperature air delivery, TCSNI may mitigate excessive stress and promote sustained activation of HSP-related signaling pathways.

In the present study, we aimed to investigate the potential effects of inhaling slightly heated air as a novel form of mild thermal stimulation via nasal inhalation. We compared the effects of TCSNI with continuous hyperthermia stimulation via nasal inhalation (HTSNI), focusing on their respective impacts on olfactory function in C57BL/6 mice. Furthermore, we examined the potential of TCSNI to enhance cognitive performance, as assessed by Y-Maze and novel object recognition (NOR)

tests [60-63], in a mouse model of cognitive impairment induced by intracerebroventricular (i.c.v.) injection of $A\beta_{25-35}$ [64]. In addition, we investigated heat- and stress-responsive proteins related to neurodegeneration, including HSP70, IDE, and phosphorylated Akt (p-Akt). These proteins are believed to contribute to antioxidant defense, support cell survival, and facilitate the clearance of Aβ accumulation [48-57]. This study aims to provide new insights into the therapeutic potential of nasal inhalation-based thermal stimulation and its role in preventing or alleviating cognitive decline, thereby laying the groundwork for future applications in neurodegenerative disease management.

RESULTS

Effects of HTSNI and TCSNI on olfactory function in mice

The aim of this study is to investigate a novel hyperthermic stimulation applied through the nasal cavity. Before conducting cognitive tests in the AD mice model, we first examined safety in mice by administering HTSNI and TCSNI and assessing whether these thermal stimulation methods induced any adverse effects, as shown in Figure 1A. Since both HTSNI and TCSNI are forms of hyperthermic stimulation applied to the nasal cavity, the olfactory function of mice was examined after treatments. The buried food test and odor sensitivity test are common methods for assessing the olfactory function of mice [65,66]. In the experiments, we found that after treatment of TCSNI, the latency to find the food (15.2±3.1 s) did not differ significantly from that of the control group (12.2±2.1 s) in the buried food test. However, in the HTSNI group, the latency was significantly increased (60.8±5.1 s) compared with both the control and TCSNI groups. In addition, in the odor sensitivity test, the time spent sniffing odor stimuli did not differ significantly between the control and TCSNI groups across the range of odor dilutions. Notably, at the 10^{-3} dilution, the HTSNI group exhibited significantly shorter sniffing time (1.2±0.6 s) compared to the control (5.8±1.5 s) and TCSNI (3.3±1.2 s) groups. Furthermore, at the 10^{-2} dilution, the HTSNI group spent significantly less time sniffing (0.9±0.7 s) than both the control (12.3±1.8 s) and TCSNI (5.8±0.9 s) groups, as shown in Figure 1B. These findings suggest that mice in the HTSNI group had impaired olfactory sensitivity or reduced ability to detect and respond to odor cues at concentrations that remained effective in the control and TCSNI groups. In view of this, we applied TCSNI in the subsequent examinations to assess whether it could enhance recognition in the AD mouse model.

Figure 1. Effects of HTSNI and TCSNI on olfactory detection in mice. Olfactory performance was assessed using the buried food test and olfactory habituation test, as

described in the Materials and Methods section. (A) Buried food test: latency to locate the buried food. (B) Olfactory habituation test: response time to odor stimuli. Data are shown as mean ± standard error of the mean. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Significance levels between indicated groups are denoted as ***P < 0.001, while non-significant differences are indicated as ns.

TCSNI attenuates Aβ-Induced cognitive impairments in mice

Since memory loss is a hallmark of AD [67], studies using mouse models commonly employ Y-maze and NOR tests to assess cognitive deficits [60-63]. Spontaneous alternation in the Y-maze is a well-established measure of short-term and spatial working memory [60,61], whereas the NOR test is a widely used paradigm for assessing both short-term and long-term recognition memory [62,63]. In this study, we utilized these tests to evaluate the effects of TCSNI on Aβ-injected mice. As shown in Figure 2A, there was no significant difference in the number of arm entries in the Y-Maze test among all groups, suggesting that i.c.v. injection of phosphate-buffered saline (PBS) or Aβ, and TCSNI treatment did not influence the locomotor activity of mice. Moreover, the spontaneous alternation index was significantly reduced in the Aβ group compared to the control group, which declined from 62.1±3.5% to 49.8±2.3%. Notably, TCSNI treatment elevated the spontaneous alternation index, suggesting an improvement in Aβ-induced deficits in short-term and spatial working memory, as shown in Figure 2B. In addition, findings from the short-term and long-term NOR tests support the effectiveness of TCSNI in ameliorating Aβ-induced recognition memory impairment. As shown in Figure 2C, the discrimination index in the short-term NOR tests significantly decreased in the Aβ group compared to the control group, dropping from 62.2±7.5% to 8±11.5%, whereas TCSNI treatment significantly improved the discrimination index (59.2±6.6%). Furthermore, in the long-term NOR test, Aβ-induced memory impairment was more pronounced, with the discrimination index declining from 49.8±6.5% to 2.2±10.5%, which was restored to 46.2±5.5% following TCSNI treatment. Collectively, these findings indicate that TCSNI treatment effectively alleviates Aβ-induced cognitive deficits, as demonstrated in the Y-Maze and NOR tests.

Figure 2. Effect of TCSNI applications on mice's behavioral performances. The recovery effects of TCSNI treatment on Aβ-induced recognition impairments were assessed via (A) the total number of total arm entries and (B) the spontaneous alternation index in the Y-maze test, and (C) the discrimination index with intertrial period of 1 h and (D) 24 h in the NOR maze test. Data are shown as mean ± standard

error of the mean. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Significance levels between indicated groups are denoted as ***P < 0.001, while non-significant differences are indicated as ns.

TCSNI downregulates Aβ accumulation and elevates HSP70, IDE, and p-Akt levels in the mouse hippocampus

With Aβ being a major focus in AD treatment strategies [5-7], this study first evaluated its accumulation in the hippocampus of mice. As shown in Figure 3A, a 1.98-fold increase in Aβ level was observed in the Aβ-injected group compared to the control group. Notably, TCSNI treatment significantly reduced the hippocampal Aβ level, decreasing it from 1.98-fold to 1.12-fold that of the control group, demonstrating a strong inhibitory effect. Furthermore, HSP70, a key stress response protein, functions as a molecular chaperone to prevent protein misfolding and has also been reported to influence the Aβ aggregation process [68-70]. In vivo studies showed that Aβ administration may lead to a slight elevation in HSP70 levels without reaching statistical significance [71,72]. In this study, HSP70 was also investigated, and the results showed that hippocampal HSP70 levels in the Aβ-injected group were not significant compared to those in the control group, whereas TCSNI treatment significantly upregulated HSP70 levels, reaching 1.22-fold of the control group (Figure 3B). On the other hand, IDE has long been recognized for its contribution to Aβ clearance, and its altered activity or expression may lead to Aβ accumulation and increased AD risk [51,73,74]. As shown in Figure 3C, Aβ injection reduced hippocampal IDE levels in mice to 0.85-fold of the control. In this study, we found that TCSNI treatment reversed this decline, significantly increasing IDE expression to 1.52-fold of the control group. Furthermore, it is known that Akt serves a crucial function in neuronal survival signaling [75,76], and a decline in Akt activation has been observed in AD models [55-57,77,78]. In addition, Akt responds to stress and thermal stimuli [53,54,79], drawing our attention. Therefore, in this study, we examined the phosphorylation ratio of Akt. As illustrated in Figure 3D, injection of Aβ led to a marked downregulation of the p-Akt/t-Akt ratio (0.65-fold), while TCSNI significantly upregulated the ratio to 1.53-fold compared to the control. These findings suggest that Aβ removal might be associated with the upregulation of HSP70, IDE, and p-Akt/t-Akt in the hippocampi of mice.

Figure 3. Effect of TCSNI applications on the expression levels of Aβ, p-Akt, HSP70, and IDE in hippocampi. Representative western blots and the quantifications of (A) Aβ, (B) HSP70, (C) IDE, and (D) p-Akt are presented for

evaluating anti-Aβ effect in hippocampi. The protein levels of Aβ, HSP70, and IDE were normalized to β-actin, and p-Akt was normalized to t-Akt. Each relative expression level was compared with the control and represented as fold relative to the control. Data are shown as mean ± standard error of the mean. Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test. Significance levels between indicated groups are denoted as *P < 0.05, **P < 0.01, and ***P < 0.001, while non-significant differences are indicated as ns.

DISCUSSION

To date, AD continues to pose a major medical challenge for humanity. The BBB presents a significant obstacle to the targeted delivery of drugs to the brain for treating AD [12,13]. Alternatively, the N-2-B pathway has been recognized as the ideal route to bypass the BBB, and studies have indicated its potential to offer therapeutic effects for brain disorders or brain cancer [34-36]. In addition, aromatherapy is also regarded as a complementary or alternative therapy that may provide benefits for dementia treatment [41]. Despite the growing interest in the N-2-B pathway as a means to bypass the BBB, no medication has yet been clinically approved via this route for the treatment of AD [36]. Given the absence of a cure for AD and the limited efficacy of current pharmacological treatments, attention has shifted towards exploring alternative approaches [80-82]. Literature indicates that mild stress stimuli enhance the functional capacity of organisms and exert positive biological effects on cells [20,83-85]. Moreover, non-pharmacological interventions, such as exercise and sauna, which induce mild stress, are believed to enhance human health, promoting lifespan extension and cognitive improvement [28,29,84,85]. Among them, whole-body heating sauna has gained great attention and sauna-like conditions were evaluated [28,30,31,86]. Nevertheless, due to drawbacks such as being time-consuming, practical challenges, and the lack of a curative effect, it is crucial to explore more effective and safe ways to induce desired mild stress.

In the current study, we introduced a novel approach of using mildly heated air inhalation as a thermal stimulus to induce mild stress. Unlike whole-body heating methods, this approach delivers warm air specifically to the nasal cavity. Nonetheless, an evaluation of this approach is necessary to determine its safety implications before further testing. A previous study indicated that mice experience thermal collapse under excessive heat conditions [87]. In contrast to the aforementioned condition where the entire body is exposed to a high-temperature environment, this study employs localized nasal heating via gas application. Furthermore, the TC-HT method developed in our previous studies, has demonstrated efficacy in both in vitro and in vivo models with minimal side effects, primarily due to its intermittent stimulation

intervals, which mitigate excessive damage [52,54]. Accordingly, we evaluated the olfactory function in mice subjected to HTSNI and TCSNI. As shown in Figure 1, after administration of HTSNI, the mice showed impaired olfactory function, as assessed by the buried food test and the olfactory discrimination test, whereas the TCSNI-treated group maintained olfactory function comparable to the untreated control group. These findings suggest that TCSNI is a safer approach compared to HTSNI.

As memory loss is a defining feature of AD [67], research involving mouse models often utilizes Y-maze and NOR tests to examine memory deficits [60-63]. Given that TCSNI preserves olfactory function in mice, it was chosen as the thermal stimulation method for behavioral tests in cognitive impairment mice to reduce potential sensory disturbances, particularly considering the olfactory decline observed with HTSNI. This study investigated the alleviating effect of TCSNI on Aβ-induced memory impairment and demonstrated that TCSNI significantly enhanced the spontaneous index and the ability to recognize novel objects, as shown in Figure 2. These results indicate that TCSNI holds significant potential for improving memory deficits and cognitive dysfunction in vivo in a safety manner.

Although traditional continuous HT stimulation hold potential in inducing positive mild stress, literature indicated that unappropriated HT show no benefit [84,85,88], and may contribute to an increased accumulation of reactive oxygen species (ROS), which could exacerbate oxidative stress and lead to cellular damage [88-90]. In our previous in vitro study, HT lacked the ability to reduce ROS and exhibited less protective effects than TC-HT against hydrogen peroxide- and Aβ-induced cytotoxicity [54]. In addition, the other previous study using thermofoil demonstrated that continuous HT application was less effective than TC-HT in improving memory impairment in vivo [52]. These studies indicate that replacing continuous heating with cyclical thermal stimulation may elicit unique biological responses [52,54]. The underlying mechanism may be related to the activation and saturation of stimulated protein pathways. For example, in olfactory field, long-term and continuous exposure may lead to olfactory adaptation or receptors desensitization [91-94], which might affect the outcome of aromatherapy. Therefore, combining different scents or using intermittent exposure is an important strategy to sustain receptor stimulation and therapeutic efficacy [95,96]. In thermal stimulation research, the phenomenon of thermal adaptation has also been investigated and is considered one of the key factors contributing to the decreased effectiveness of HT [97,98]. Compared to HT, TC-HT applies periodic temperature fluctuations that might help prevent signaling desensitization and better sustain heat-induced protein responses. In addition, with an improved cellular safety profile, TC-HT methods potentially

enhance both the efficacy and feasibility of thermal stimulation, making them a preferable alternative methodology to traditional HT [52,54]. In this study, while HTSNI was found to impair olfactory function, TCSNI did not cause such impairment and was shown to ameliorate deficits in memory and cognition (Figures 1 and 2).

Given that the clearance of toxic Aβ deposits is a significant marker in AD therapy [99,100], we investigated Aβ expression levels in the hippocampus of mice from a molecular perspective. As depicted in Figure 3A, TCSNI application significantly reduced Aβ expression levels in the hippocampus. Furthermore, several proteins with potential roles in Aβ clearance were investigated. Among these, HSP70, a key heat shock protein activated by thermal stimulation, has gained significant attention for its role in suppressing AD progression [68-70]. As HSP70 primarily functions to prevent protein misfolding, it has also been reported to influence the Aβ aggregation process [68-70]. The study found that TCSNI application significantly enhanced HSP70 expression in the hippocampus of mice, as shown in Figure 3B. In addition, HSP70 has been suggested to upregulate the expression of IDE, a well-known Aβ-degrading enzyme, thereby facilitating Aβ clearance [68]. Studies have shown that IDE is induced by stress and identified it as a protein with heat shock-like characteristics [51,52,101]. Additionally, IDE has been found to regulate extracellular Aβ levels via proteolysis and mitigate its neurotoxic effects [102,103]. Research suggests that reduced IDE activity and expression may be associated with Aβ accumulation and an increased risk of AD [51,73,74], and thus upregulating IDE levels has been proposed as a potential therapeutic approach for AD [74]. In line with these studies, our study finds that TCSNI treatment significantly restores the Aβ-induced reduction in IDE in the hippocampus of mice, as shown in Figure 3C. Furthermore, in addition to IDE and HSP70, Akt is another key protein of interest in our study. Akt plays a pivotal role in mediating neuron survival signaling, growth, and synaptic plasticity, which are critical for brain development and function [75,76]. It was found that a decline in Akt activation occurred in AD models, while the upregulation of Akt activation conferred protective effects in both in vitro and in vivo models [55-57,77,78]. It is noteworthy that Akt is also recognized as a protein that responds to stress and thermal stimulation [53,54,79]. In addition, research indicated that Akt could mediate HSP70 expression, thereby providing neuroprotection [78]. Furthermore, study suggested that Akt signaling pathway may improve cognitive impairment in AD by promoting the expression of IDE and the degradation of Aβ [104]. In this work, along with HSP70 and IDE proteins, Akt protein in the hippocampus of mice was detected. We found that Aβ-induced decline in p-Akt was restored by TCSNI application, as shown in Figure 3D. Taken together, these findings suggest that TCSNI induces mild thermal stress, which has the potential to upregulate

the expression of HSP70, IDE, and phosphorylated Akt in the hippocampus of Aβ-injected mice. This molecular response may contribute to the reduction of hippocampal Aβ levels and underlie the cognitive improvements observed in the treated mice, consistent with the mild stress-induced self-protective hypothesis.

This study proposes a novel periodic hyperthermic method, TCSNI, administered via nasal inhalation to induce mild stress as a potential treatment for AD. Stress response would trigger self-defense system [105], and depending on the intensity and duration of stress, cells would adapt or cause their death [106]. Studies have exhibited that preconditioning cells with non-lethal stress minimizes cell damage and improves cell survival [54,107]. The present study suggests an efficient way to induce mild stress in vivo with minimal impact on olfactory function using TCSNI, which has been shown to improve cognitive performance in mice in Y-maze and NOR tests. Moreover, TCSNI could reduce Aβ expression and significantly upregulate proteins related to Aβ degradation and cell survival, such as HSP70, IDE, and p-Akt, in the hippocampus of mice subjected to i.c.v. Aβ injection. Further studies are required to confirm the involvement of other stress-induced proteins, particularly those associated with neuroprotection and Aβ metabolism, and to validate these findings in other species and humans. Additionally, the potential combinatory effects of TCSNI with pharmacological treatments also warrant further investigation. Overall, this study provides new insights into the development of alternative therapeutic strategies for AD, highlighting the potential of TCSNI as a novel non-invasive intervention.

MATERIALS AND METHODS

Experimental animals and housing

C57BL/6 male mice (6–8 weeks old, 22–26 g) were purchased from National Laboratory Animal Center, and housed at National Taiwan University Animal Resources Center. The mice had ad libitum access to food and water and were maintained at a constant temperature of 23℃ ± 2℃, under a 12:12-hour light/dark cycle (lights switched on at 7:00 AM). All experimental techniques were executed based on established guidelines of the Animal Ethical Committee of National Taiwan University (approval protocol number: NTU-112-EL-00158). All mice were randomly assigned to two main groups. The first group was further divided into control, TCSNI, and HT sub groups, while the second group was divided into control, Aβ, and Aβ+TCSNI sub groups.

β-amyloid administration

$A\beta_{25\text{-}35}$ (Sigma-Aldrich; Merck KGaA) was prepared as stock solution at a concentration of 1 mg/mL in 0.9% sterile saline solution and fibril-aggregated at 37℃

for 7 days. On day 0, 10 μL of the aggregated form of Aβ was injected i.c.v. into the brains of the mice in the Aβ and Aβ-TCSNI groups, while the control group mice received an i.c.v. injection of 10 μL PBS.

HTSNI and TCSNI applications

This experiment utilized a novel nasal inhalation-based thermal stimulation system. As shown in Figure 4A, the system heats 3% isoflurane anesthetic gas by the water bath method and controls the duration of gas heating and cooling through a timer-controlled solenoid valve. In the HTSNI application, the timer-controlled solenoid valve was fixed to ensure a steady release of heated air, and the temperature was maintained at 47.5 ± 0.5℃ for 30 minutes. In the TCSNI application, the timer-controlled solenoid valve was programmed to execute a 10-cycle repetitive process, where each cycle consists of a 3-minute heating phase (47.5 ± 0.5℃), followed by a 1-minute cooling phase. The actual air temperature entering the mouse's nasal cavity is measured and monitored by a K-type thermocouple positioned at the end of the pipeline, with the measured temperature results shown in Figure 4B.

Figure 4. Experimental setup and design of TCSNI. (A) The TCSNI system was illustrated, which provides inhaling mildly heated air to deliver cyclic thermal stimulation. (B) The actual temperatures in the SH-SY5Y cells were measured using a K-type needle thermocouple at 10 sec intervals throughout the exposure durations of TC-HT and HT treatments.

Experimental design

For the 1st group, as shown in Figure 5, on day 7, 10, and 13, the respective thermal application was employed to the mice of the HTSNI and TCSNI groups. Buried food and odor sensitivity tests were conducted on day 15 and 16, respectively. For the 2nd group, 10 μL of Aβ solution was i.c.v.-injected into the brains of mice in the Aβ and Aβ+TCSNI groups on day 0, while control group mice received an i.c.v. injection of 10 μL PBS. On day 7, 10, and 13, the TCSNI thermal application was employed to the mice of different groups. The Y-maze test was performed at day 14, and the NOR tests were carried out at day 15 and 16. After the NOR test, mice were weighted and then euthanized to collect brain tissues.

Figure 5. TCSNI treatment and animal behavioral assessment. (A) For the 1st group, the thermal applications were employed to the mice of the HTSNI and TCSNI groups on days 7, 10 and 13. Buried food and odor sensitivity tests were assessed on day 15 and 16, respectively. (B) For the 2nd group, Aβ solution was injected

intracerebroventricular (i.c.v.) at day 0, and TCSNI application was applied at day 7, 10, and 13. The Y-maze test was performed at day 14, and the NOR tests were carried out at day 15 and 16, respectively. After the NOR test, mice were weighted and then euthanized for brain tissues collection.

Buried food test

On day 14 at 4 pm, food pellets were removed from the cages and the testing mice fasted overnight. The test was performed on day 15 at 10 am after 1-hour acclimatization in the testing room. Mice were then individually introduced into a clean cage containing 3 cm deep of clean bedding and allowed to acclimate to the cage for 5 minutes to reduce the interference from novel environment exploration during the test. A small piece of pellet with a diameter of about 5 mm was buried beneath 1 cm of bedding in a random corner of the cage, and the mouse was introduced into the cage. The site of mice placement and the location where the small pellet was buried remained constant. Time required for the mice to retrieve the small pellet with its front paws was measured in seconds (latency), with a maximum duration of 15 minutes.

Odor Sensitivity Test

The odor sensitivity test was performed on day 16 after 1-hour acclimatization in the testing room for the mice. Mice were then individually introduced into a clean cage, which was pre-placed with a dry cotton swab without bedding. The mice were allowed to acclimate to the cage for 30 minutes to reduce the interference from novel environment exploration during the test. After acclimatization, video recording began and lasted for 3 minutes, with the dry cotton swab was replaced by a cotton swab soaked in water. After 3 minutes, replace the cotton swab sequentially with diluted solutions at concentrations of 1/100,000, 1/10,000, 1/1,000, and 1/100. Sniffing behavior increases when the odor concentration reaches the olfactory threshold in mice. After acquiring all of the olfactory sensitivity and preference videos, a timer was used to time all investigations of cotton swabs within three minutes. The time of investigation is defined as any period when the mouse's nares are < 1 mm from the cotton swabs. The recorded latency would not include bouts when the mouse is not actively investigating, chewing, or licking the paper

Y-Maze test

The Y-maze test was employed to assess spatial working memory. The apparatus consists of three identical symmetric arms (40 cm × 13 cm × 6.5 cm) arranged at 120° angles from a central junction. Each mouse, with no prior familiarity with the maze,

was individually placed at the center and allowed to freely explore all three arms to test spontaneous alteration for 8 minutes. After each trial, the maze was cleaned with 70% alcohol to eliminate olfactory cues. The behavioral performance was recorded, and the maze arm entries were labeled ‘A’, ‘B’, and ‘C’. An arm entry was defined as the hind paws of mice completely entering the arm. A correct alternation was defined as sequential entry into the three different arms (i.e., ABC, ACB, BAC, BCA, CAB, and CBA) in overlapping triple sequences. The spontaneous alternation index was calculated using the following formula: (number of correct alternations) / (total number of arm entries – 2) × 100, and the total number of arm entries was also used as an indicator of general locomotor activity.

Novel object recognition test

The NOR test was conducted to evaluate recognition memory in mice across days 14 to 16 of the study. The test was performed in an open field arena (40 cm × 40 cm × 30.5 cm) over three consecutive days. On day 14 (habituation phase), each mouse was allowed to freely explore the empty arena for 8 minutes to acclimate to the testing environment. On day 15 (familiarization and short-term recognition memory test phase), two identical familiar objects (object A) were placed near the diagonal corners of the arena, and the assessed mouse was allowed to explore the arena for 8 minutes. After a 1-hour retention interval in the home cage, the mouse was returned to the arena for the short-term recognition memory test. During this phase, one of the original identical familiar objects (object A) was replaced with a novel object (object B). The mouse’s behavioral performance was recorded with an overhead video camera for 8 minutes. On day 16, to assess long-term recognition memory, the mice were again placed in the arena and allowed to explore the same familiar object from the training phase (object A) and a new novel object (object C) for 8 minutes. This design allowed the evaluation of both short-term and long-term object recognition memory. The familiar objects were possessing similar shapes, material composition, and coloration. The novel objects were objects of varying shapes, sizes, and colors in comparison to the familiar objects. After each assessment in all phases, all olfactory cues were removed by 70% ethanol. Object exploration was defined as the time spent by the mouse sniffing or touching the object with the nose and/or forepaws. The discrimination index was calculated as (Tn-To) / (Tn+To), where Tn and To represent the exploration time for the novel and familiar objects, respectively.

Brain tissue collection and western blot sample preparation

After NOR tests, mice were euthanized using carbon dioxide gas, and mice brains were extracted after confirmation of death and rinsed with PBS (HyClone; GE

Healthcare Life Sciences, Chicago, IL, USA) supplemented with 1% fresh protease inhibitor cocktail (EMD Millipore, Billerica, MA, USA). The hippocampus tissue was homogenized in 500 μL ice-cold RIPA lysis buffer (MilliporeSigma) containing phosphatase & protease inhibitors by the Q125 Sonicator (Qsonica, Newton, CT, USA), and the homogenates were centrifuged at 23,000×g at 4℃ for 30 minutes. The protein concentration levels of the samples were measured using the Bradford protein assay (cat. no. BRA222; BioShop Canada). Protein samples were stored at -80℃ for subsequent western blotting assessments.

Western blot analysis

20 μg of protein samples were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (MilliporeSigma) at 180 mA for 150 minutes. After blocking with 5% bovine serum albumin (BioShop Canada Inc.) in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 hour at room temperature, the membranes were incubated overnight with primary antibodies at 4℃ overnight. The specific primary antibodies against β-Amyloid (Aβ; cat. no. sc-28365; Santa Cruz Biotechnology), phospho-Akt (p-AKT; cat. no. 4060; Cell Signaling Technology, Inc.), Akt (AKT; cat. no. 9272; Cell Signaling Technology, Inc.), IDE (cat. no. ab133561; Abcam), HSP70 (cat. no. 4872; Cell Signaling Technology, Inc.), and β-actin (cat. no. GTX110564; GeneTex, Inc.) were used, with β-actin serving as the loading control for protein normalization. Following washing with TBST, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies, including goat anti-rabbit IgG (HRP) (cat. no. 111-035-003; Jackson ImmunoResearch Laboratories, Inc.) or goat anti-mouse IgG (HRP) (cat. no. GTX213111-01; GeneTex, Inc.). Dilutions of all primary and secondary antibodies were performed following the manufacturer's instructions. The membranes were visualized with an enhanced chemiluminescence substrate (Advansta, San Jose, CA, USA) and quantified using the Amersham Imager 600 imaging system (AI600; GE Healthcare Life Sciences).

Statistical analysis

All statistical results were presented as mean ± standard error of the mean (SEM) and analyzed using one-way analysis of variance (ANOVA) with Tukey's post hoc test in OriginPro 2022 software (version 2022; OriginLab Corporation, Northampton, MA, USA). A P-value of less than 0.05 was considered statistically significant.

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ABBREVIATIONS

AD	Alzheimer's disease
Aβ	β-amyloid
N-2-B	nose-to-brain
BBB	blood-brain barrier
HT	hyperthermia
TC-HT	thermal cycling hyperthermia
TCSNI	thermal cycling stimulation via nasal inhalation
HTSNI	hyperthermia stimulation via nasal inhalation
i.c.v.	intracerebroventricular
NOR	novel object recognition
HSP70	heat-shock protein 70
IDE	insulin-degrading enzyme

p-Akt	phosphorylated Akt
ROS	reactive oxygen species
ANOVA	analysis of variance

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Figure 1

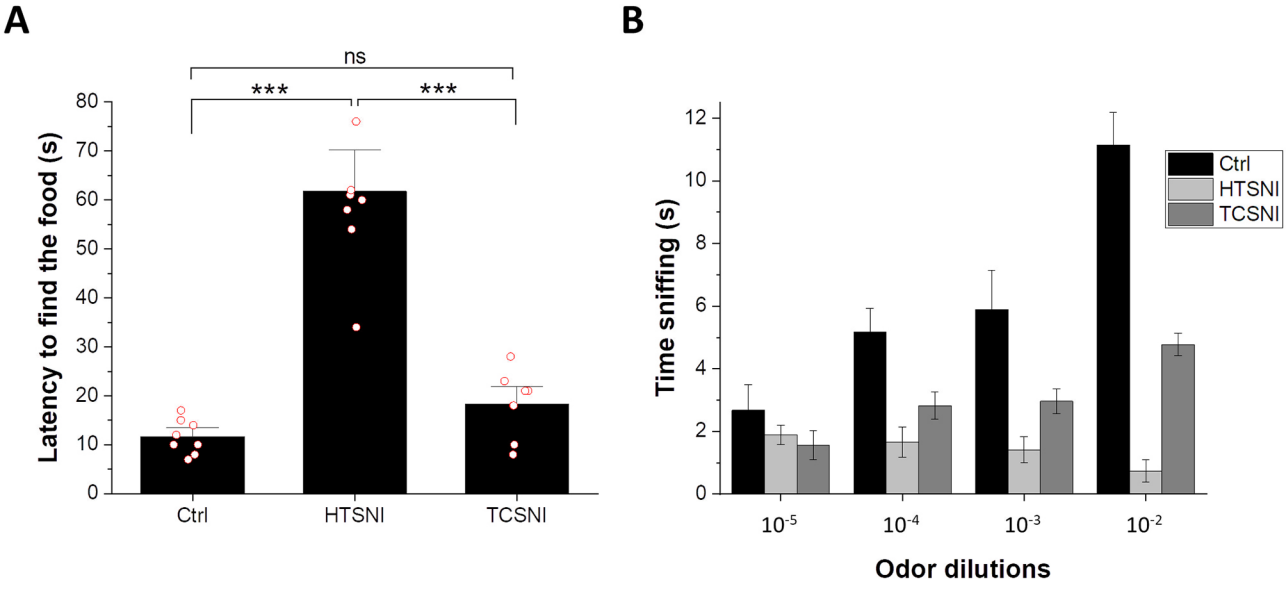

A
ns
***
***
Latency to find the food (s)
0
10
20
30
40
50
60
70
80
Ctrl
HTSNI
TCSNI
B
Time sniffing (s)
0
2
4
6
8
10
12
Ctrl
HTSNI
TCSNI
10-5
10-4
10-3
10-2
Odor dilutions

Figure 2

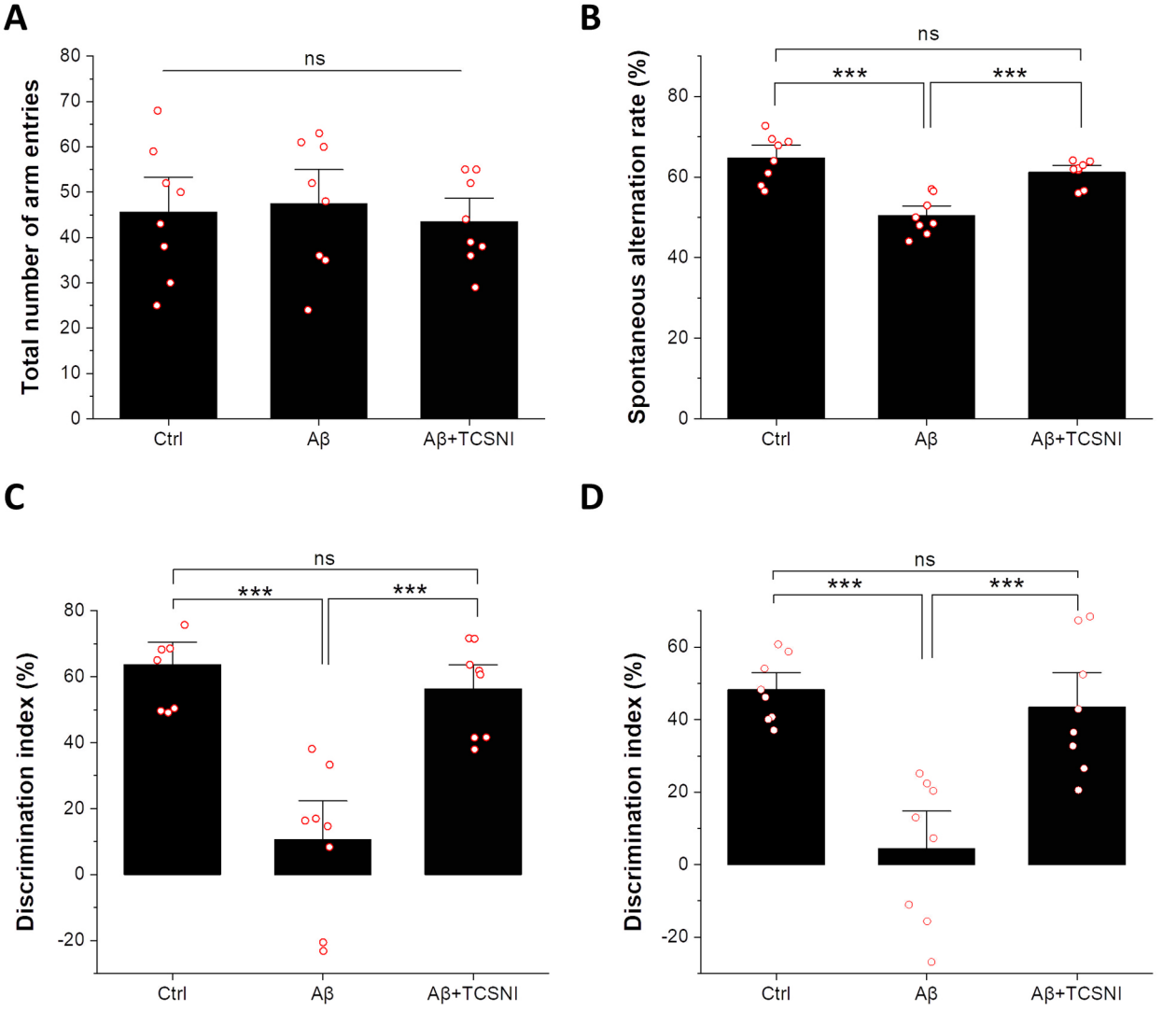

A
Total number of arm entries
80
70
60
50
40
30
20
10
0
ns
Ctrl
Aβ
Aβ+TCSNI
B
Spontaneous alternation rate (%)
80
60
40
20
0
ns
***
***
Ctrl
Aβ
Aβ+TCSNI
C
Discrimination index (%)
80
60
40
20
0
-20
ns
***
***
Ctrl
Aβ
Aβ+TCSNI
D
Discrimination index (%)
60
40
20
0
-20
ns
***
***
Ctrl
Aβ
Aβ+TCSNI

Figure 3

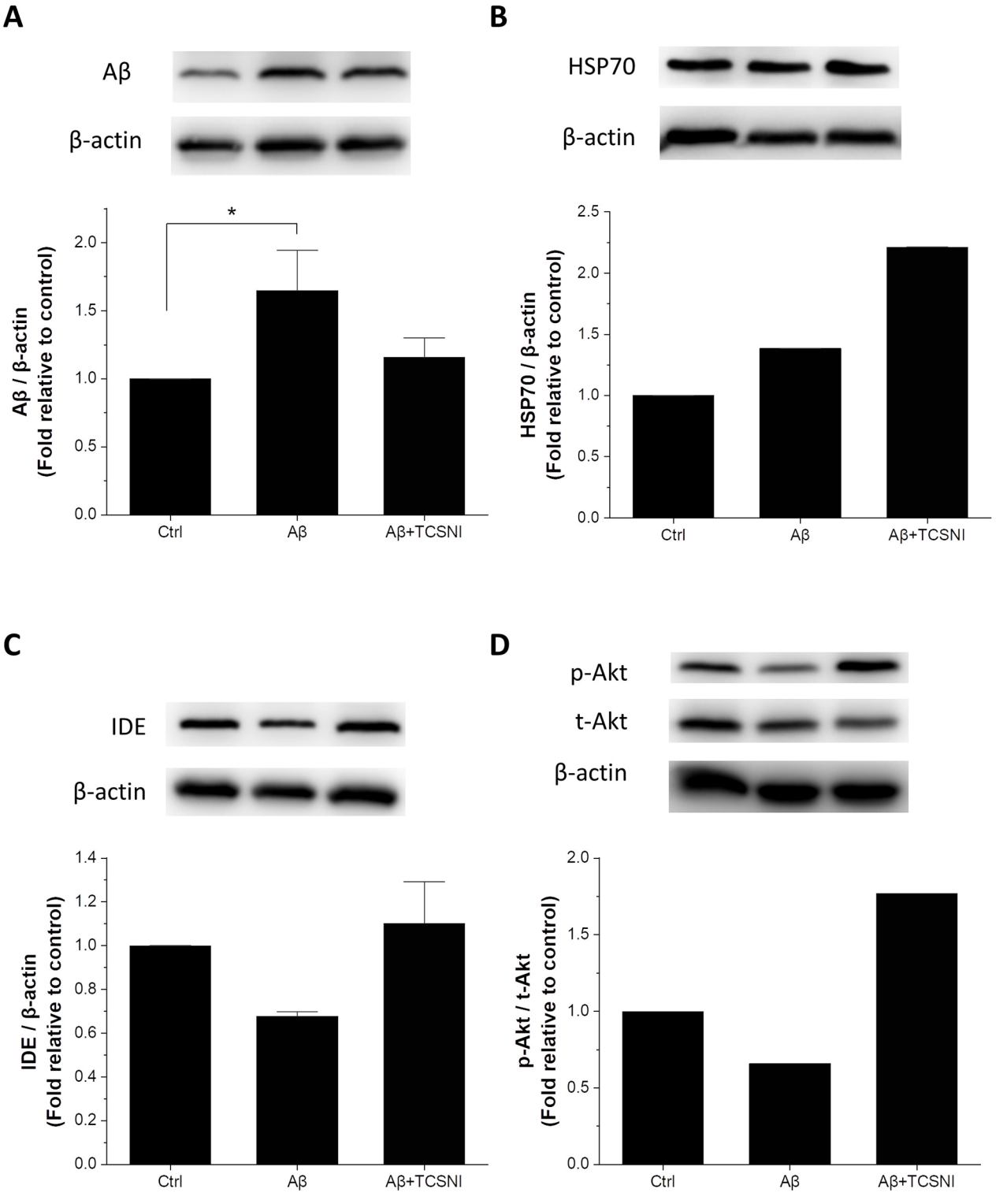
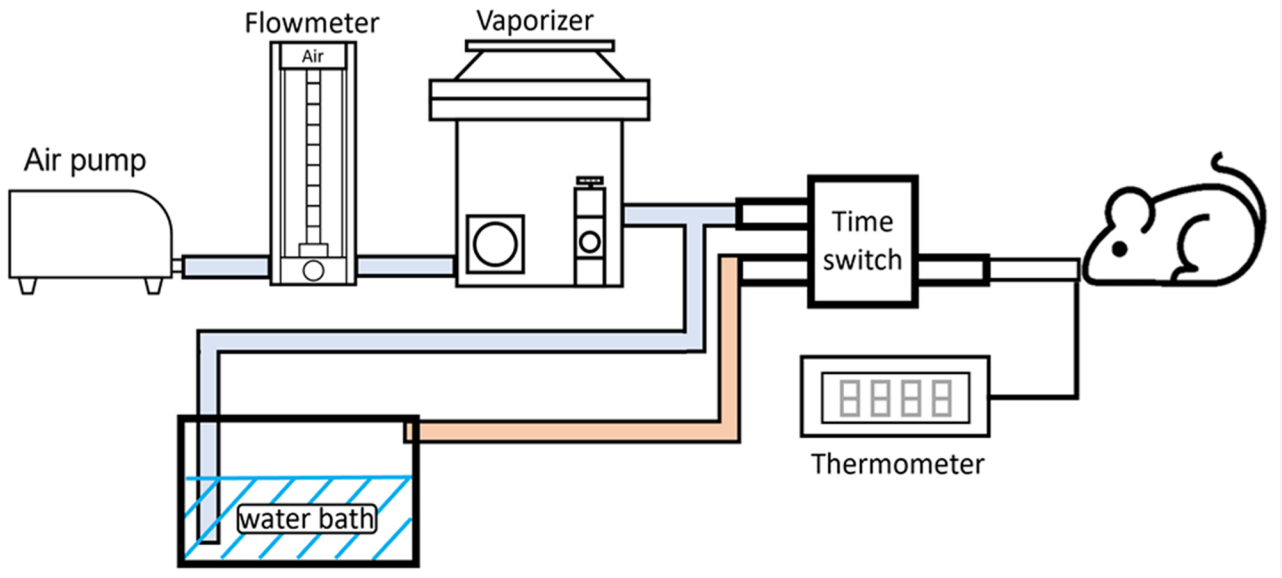

A
Aβ
β-actin
*
Aβ / β-actin
(Fold relative to control)
2.0
1.5
1.0
0.5
0.0
Ctrl
Aβ
Aβ+TCSNI
B
HSP70
β-actin
HSP70 / β-actin
(Fold relative to control)
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
Aβ
Aβ+TCSNI
C
IDE
β-actin
IDE / β-actin
(Fold relative to control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
Aβ
Aβ+TCSNI
D
p-Akt
t-Akt
β-actin
p-Akt / t-Akt
(Fold relative to control)
2.0
1.5
1.0
0.5
0.0
Ctrl
Aβ
Aβ+TCSNI

Figure 4

A


Flowmeter
Air
Vaporizer
Air pump
Time switch
Thermometer
water bath

B

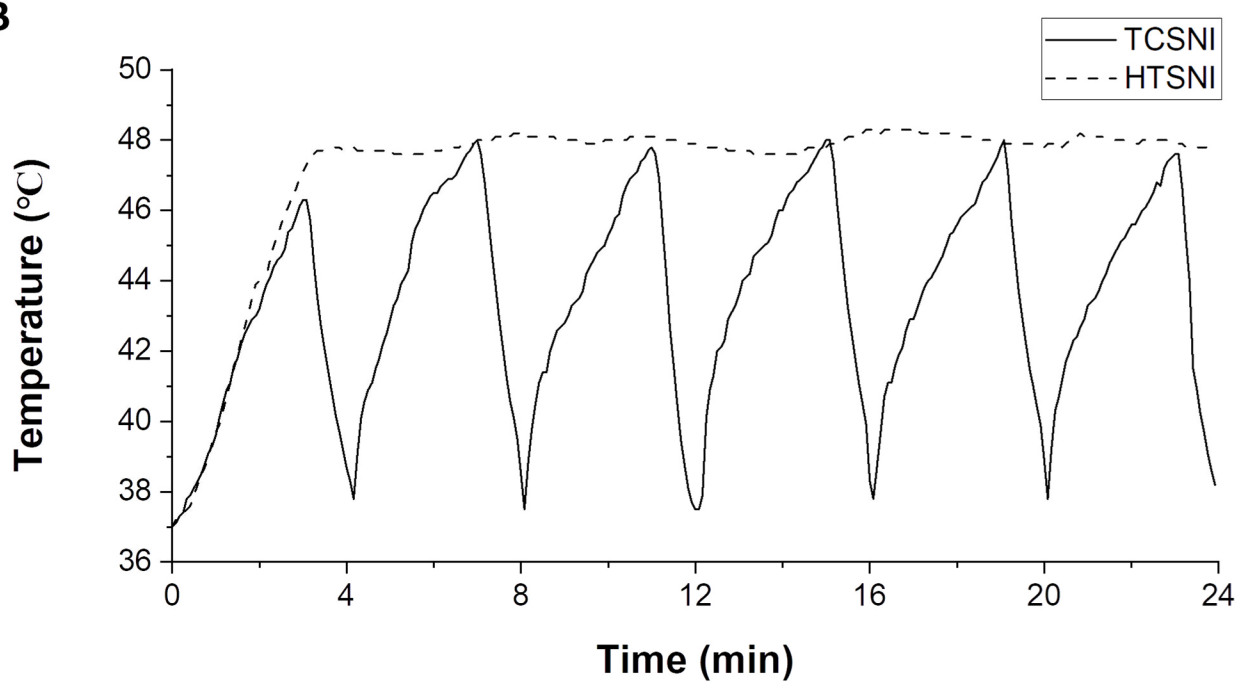
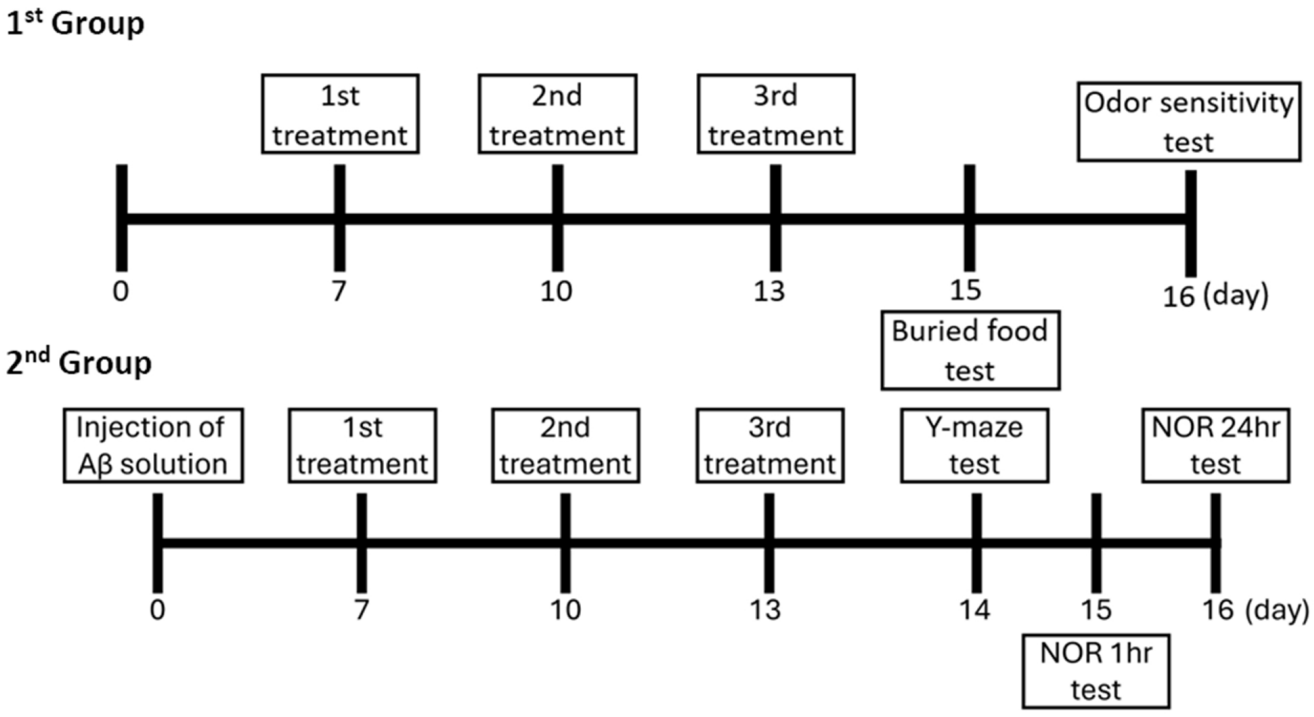

TCSNI
HTSNI
Temperature (°C)
50
48
46
44
42
40
38
36
0
4
8
12
16
20
24
Time (min)

Figure 5


1st Group
1st treatment
2nd treatment
3rd treatment
Odor sensitivity test
0
7
10
13
15
16 (day)
Buried food test
2nd Group
Injection of Aβ solution
1st treatment
2nd treatment
3rd treatment
Y-maze test
NOR 24hr test
0
7
10
13
14
15
16 (day)
NOR 1hr test