

# Restoration of Spatial Learning Through Oral Administration of Lipopolysaccharides in Diabetes-related Cognitive Dysfunction

HIROYUKI INAGAWA<sup>1,2,3</sup>, MASATAKA ODA<sup>1</sup>, VINDY TJENDANA TJHIN<sup>1</sup>,  
CHIE KOHCHI<sup>1,2</sup> and GEN-ICHIRO SOMA<sup>1,2,3</sup>

<sup>1</sup>Control of Innate Immunity, Collaborative Innovation Partnership, Kagawa, Japan;

<sup>2</sup>MacrophI Inc., Kagawa, Japan;

<sup>3</sup>Research Institute for Healthy Living, Niigata University  
of Pharmacy and Applied Life Sciences, Niigata, Japan

**Abstract.** *Background/Aim:* In a previous report, our group showed that oral administration of lipopolysaccharides (LPS) from *Pantoea agglomerans* can prevent the progression of streptozotocin (STZ)-induced diabetes-related cognitive dysfunction (DRCD) in mice without causing significant side-effects. However, the treatment effects of oral administration of LPS to DRCD remain unknown. *Materials and Methods:* We modified our previous animal experimental model to investigate whether oral administration of LPS can recover cognitive function after DRCD onset. *Results:* The Morris water maze (MWM) revealed a significant decrease in learning and memory abilities at 13 days after intracerebroventricular administration of STZ, thereby providing evidence of the occurrence of DRCD in the animal model. Oral administration of LPS (1 mg/kg per day) started after cognitive impairment was observed. After 28 days of treatment, mice receiving LPS via the oral route showed significant recovery of spatial learning ability, a symptom of early dementia, while only a trend toward recovery was seen for spatial memory compared to the untreated group. *Conclusion:* These results, limited to MWM, suggest that oral administration of LPS is a promising therapeutic strategy for restoring decreased spatial learning ability.

*Correspondence to:* Gen-Ichiro Soma, Control of Innate Immunity, Collaborative Innovation Partnership, 2217-16 Hayashi-cho, Takamatsu Kagawa, 761-0301, Japan. Tel: +81 878139201, Fax: +81 878139203, e-mail: gensoma@shizenmeneki.org

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Diabetes is a global health problem, affecting 537 million people around the world. Moreover, diabetes-related cognitive dysfunction (DRCD) is one of the most serious complications of diabetes (1), and its lack of prevention or treatment poses a major risk to global health. In this study, we investigated the effects of oral administration of lipopolysaccharides (LPS) from *Pantoea agglomerans* on the treatment of DRCD.

Our previous study revealed that oral administration of LPS from *P. agglomerans* (OAL) prevents high-fat diet-induced cognitive dysfunction in senescence-accelerated mouse prone 8 (SAMP8) mice (2). In another study, we also observed that OAL prevents streptozotocin (STZ)-induced DRCD in mice without causing any significant side effects, as evidenced by the Morris water maze (MWM) (3). Although the treatment efficacy of OAL in DRCD has not yet been established, expectations for its treatment efficacy are high because it has been shown to be safe and have few side effects (4). Building on our previous findings, the present study aimed to investigate whether OAL improves cognitive performance in spatial learning in mice with STZ-induced DRCD using MWM.

## Materials and Methods

**Animals.** Six-week-old male C57BL/6 mice (20–24 g) were purchased from SLC, Inc. (Shizuoka, Japan) and acclimated for five days. All mice (four–five mice per cage) were maintained under specific pathogen-free conditions in a temperature- and humidity-controlled room under a 12-h light/dark cycle with unrestricted access to food and water. Mouse Diet D12450B, containing negligible LPS levels, was acquired from Research Diets, Inc. (New Brunswick, NJ, USA).

The experiments in this investigation were reviewed and approved by the Animal Care and Use Committee of the Control of Innate Immunity CIP (Approval No. 21-19). All animals were



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handled according to the “Law for the Humane Treatment and Management of Animals Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain” (Ministry of the Environment, Japan), the Guidelines for Proper Conduct of Animal Experiments (the Science Council of Japan), and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions (Ministry of Education, Culture, Sports, Science and Technology, Japan). The mice were anesthetized with 4% isoflurane using the NARCOBIT-E type 2 inhalation anesthesia system (Natsume Seisakusho Co., Ltd., Tokyo, Japan) and euthanized by cervical dislocation at the end of the experiment.

**STZ administration.** STZ was purchased from Sigma-Aldrich (St. Louis, MO, USA). After isoflurane anesthesia, the mice were fixed in a micromanipulator (SMM-100; Narishige, Tokyo, Japan) using the SR-5M-HT brain stereotaxic apparatus (Narishige). Next, all animals received a single injection of STZ (0.2 mg/mouse, dissolved in 5 ml saline) into the right lateral ventricle with the IMS-20 microinjector (Narishige) (STZ-administered mice,  $n=18$ ). The stereotaxic coordinates were +0.3 mm anterior, +1.0 mm lateral (right), and +2.5 mm ventral from the bregma. After skin suture, an antibiotic ointment containing 20 mg/g chloramphenicol, 5 mg/g fradiomycin, and 100,000 U/g nystatin (Daiichi Sankyo Healthcare Co., Ltd., Tokyo, Japan) was applied to the wounds of the experimental mice. Control sham mice (saline-administered mice,  $n=9$ ) were administered 5 ml of saline into the right lateral ventricle. After surgery, all mice were monitored daily for pain/discomfort and infections using the previously mentioned guidelines.

**MWM.** The MWM was performed 13 days post-STZ injection to assess spatial learning and memory in the STZ-treated and sham mice, as previously described (3). The experimental apparatus consisted of a circular tank with an inner diameter of 88 cm, a height of 40 cm, and a removable transparent circular platform with a diameter of 10 cm. The pool was filled with water to a height of 30 cm, maintained at  $23\pm 1^\circ\text{C}$ , and made opaque with white ink. The pool area was divided into four equal quadrants, and cards with different shapes (circle, square, triangle, or cross) were placed on the wall of each quadrant. The platform was submerged 1 cm below the water surface and placed approximately in the center of one quadrant (target quadrant). Each mouse consecutively participated in a pretraining session (one day), training sessions (four days), and a probe session (one day). The pretraining session was conducted three days before the first training session to familiarize the mice with the escape platform. The mice were placed on the platform for 20 s, allowed to swim freely for 30 s, and then assisted in swimming back to the platform.

The training sessions and probe test were conducted daily for five days. Four trial runs were conducted each day during the training sessions. In each trial, the mouse was randomly released into the water at a starting position facing the pool wall. The mouse was given 60 s to find the escape platform. Once the animal found the platform, it was allowed to stay on it for 20 s. The spatial learning ability of each mouse was assessed using escape latency, defined as the time spent to find the platform. If the mouse did not find the platform within 60 s, it was assisted and allowed to rest on the platform for 20 s, and the escape latency time was recorded as 60 s. The average escape latency time of the four trials for each mouse was calculated. The probe

test was conducted one day after the last training day to assess the spatial reference memory ability of the mice. The platform was removed from the pool, and each mouse was released into the water from the quadrant opposite the target quadrant. Then, the mice were allowed to swim freely for 60 s. The swimming paths of the mice were recorded using a video camera suspended above the center of the pool. Animal Tracker software (5) was used to visualize the swimming trajectory of each mouse and measure the time spent swimming in the target quadrant.

The second MWM was conducted 24 days after the first oral LPS administration to determine whether LPS could restore the spatial learning and memory abilities of the STZ-induced DRCD mice. No pretraining session was conducted in the second MWM.

**LPS characteristics.** LPS derived from *P. agglomerans* (Macrophil Inc. Kagawa, Japan) was purified to over 99%, according to methods previously described (6). LPS from *P. agglomerans* has considerably low protein and nucleic acid contamination, with values of 0.5% and less than 0.35% (w/w), respectively. LPS also activates macrophages in small amounts, with an effective concentration ( $EC_{50}$ ) of 1.6 ng/ml (6).

**LPS treatment.** LPS was dissolved in sterile distilled water and administered orally at a concentration of 1 mg/kg BW per day. The concentration of LPS in the drinking water was adjusted weekly to ensure that each mouse received the corresponding dose based on its average BW and amount of water consumption (2). LPS was orally administered starting 17 days after STZ injection until the end of the experiment.

**Statistical analysis.** The statistical analysis was performed using BellCurve for Excel ver. 4.01 (Social Survey Research Information, Tokyo, Japan). The escape latency and total time spent in the target quadrant data were recorded as the mean  $\pm$  standard error of the mean. Student's *t*-test was used to compare the differences between the saline and STZ groups. The differences between the three groups of mice (saline, STZ, and STZ+LPS) were analyzed using one-way analysis of variance followed by the Tukey-Kramer multiple comparison test. *p*-Values  $< 0.05$  were considered significantly significant.

## Results

**Experimental system for verifying treatment effect.** In our previous study, we showed that OAL can prevent STZ-induced DRCD in mice (4). STZ was administered intracerebroventricularly (*icv*) one week after continuous OAL, and three weeks later, learning and memory abilities in mice with STZ-induced DRCD were evaluated using the MWM. Figure 1A shows the protocol we used to evaluate the preventive effect of OAL on STZ-induced DRCD (the preventive experiment). Although this experimental system can verify the preventive effect of OAL on DRCD onset, it does not allow for the verification of the treatment effect of OAL for established DRCD.

To investigate the therapeutic effect of OAL on DRCD, we first attempted to establish a protocol that would allow us to analyze the therapeutic effect by changing the timing of OAL administration against the background of a

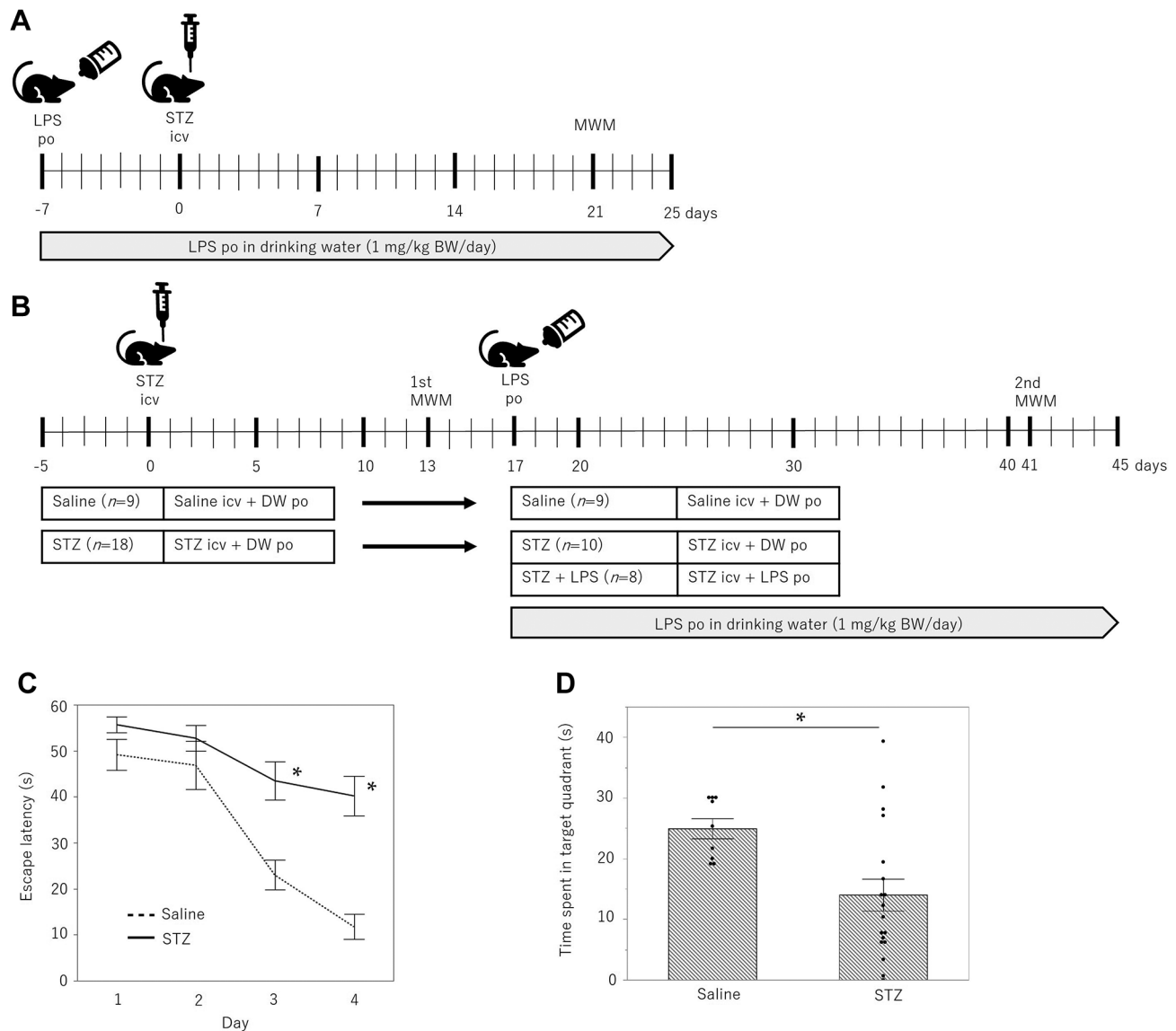


Figure 1. (A) Protocol of the preventive experiment. STZ was administered *icv* 1 week after continuous OAL, and three weeks later, learning and memory abilities in mice with STZ-induced DRCD were evaluated using the MWM. OAL: Oral administration of LPS from *P. agglomerans*; BW: body weight; DRCD: diabetes-related cognitive dysfunction; LPS: lipopolysaccharide; MWM: Morris water maze; *icv*: intracerebroventricularly; *po*: per os. (B) Protocol of the treatment experiment. STZ was administered intracerebroventricularly on Day 0 to the STZ groups. An equivalent amount of saline was administered *icv* to the saline group. The first MWM was started on Day 13 to confirm that DRCD had been induced in the STZ. The STZ group was divided into STZ and STZ +LPS groups after the first MWM. LPS was added to the drinking water of the STZ +LPS group starting from Day 17 until the end of the experiment. The second MWM was started on Day 41. The spatial recognition ability of the STZ-induced DRCD mice compared to the saline control group was assessed using the MWM performed 13 days after STZ administration (the first MWM). (C) Escape latency of the STZ and saline groups during the 4 training days. (D) Average time spent in the target quadrant by the STZ and saline groups in the probe test. (STZ group, n=18, saline group, n=9). Data are presented as average  $\pm$  standard error of the mean. \* $p < 0.01$  for Student's *t*-test.

previously reported protocol for evaluating the preventive effect of (3). Therefore, we administered *icv* STZ to mice, and starting 13 days later, we assessed whether DRCD was established in the mice using the MWM (the first MWM). Subsequently, OAL was administered to mice exhibiting

impaired learning and memory abilities, as evidenced by the MWM. Figure 1B shows the protocol used to evaluate the treatment effect of OAL on STZ-induced DRCD (the treatment experiment). The two protocols differed in the timing of the OAL. In the preventive experiment, OAL was

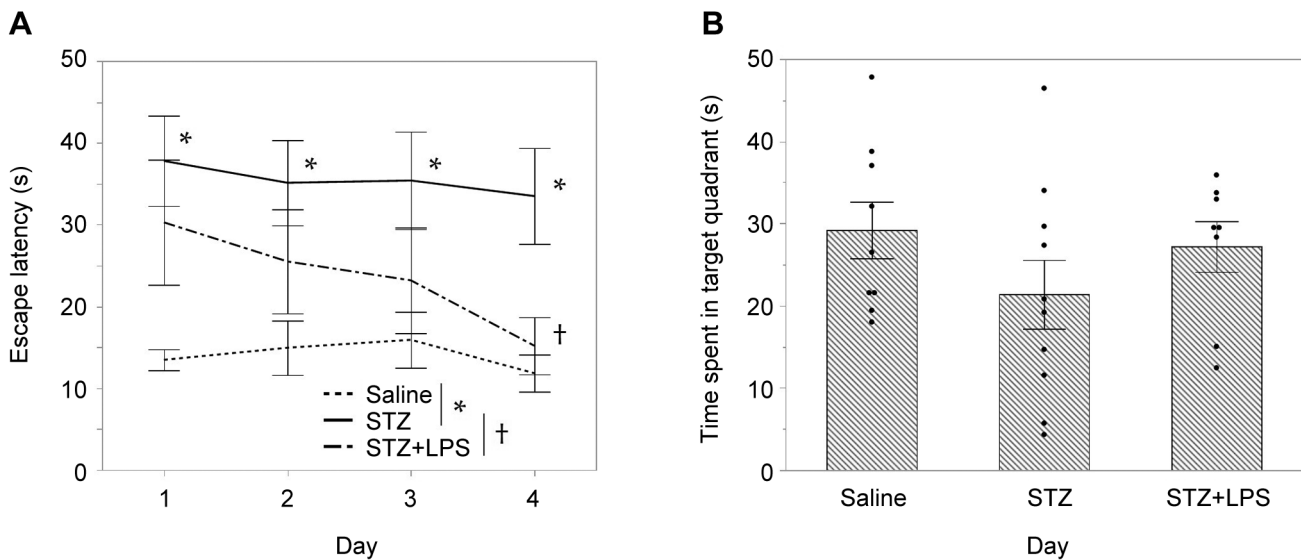


Figure 2. Effect of oral administration of LPS on the STZ-induced DRCD mice. Mice with *icv* STZ-induced impaired spatial learning and memory were given water or LPS for 28 days, and their spatial learning and memory were assessed the second time using the MWM. (A) Escape latency of the STZ, STZ+LPS, and saline groups during the four training days. (B) Average time spent in the target quadrant by the STZ, STZ+LPS, and saline groups in the probe test. (STZ group,  $n=10$ , STZ+LPS group,  $n=8$ , and saline group,  $n=9$ ). Data are presented as average  $\pm$  standard error of the mean. \* $\dagger p < 0.05$  for one-way analysis of variance followed by the Tukey-Kramer multiple comparison test. LPS: Lipopolysaccharide; *icv*: intracerebroventricularly.

to be started seven days prior to STZ administration, whereas in the treatment experiment, OAL was to be administered at 17 days after STZ administration.

The results of the first MWM from Day 13 after *icv* STZ administration are shown in Figure 1C and 1D. During the training days, the escape latency of the STZ-administered mice was significantly longer than that of the saline-administered mice on both Day three ( $p=0.003$ ) and Day four ( $p < 0.001$ ). In addition, the STZ-administered mice spent significantly less time in the target quadrant than did the saline-administered mice ( $p=0.002$ ).

Consequently, the results of the first MWM showed that spatial learning memory was impaired at around 13-17 days after *icv* STZ administration, providing evidence of the onset of DRCD. These mice served as a DRCD model to verify the efficacy of the treatment effect of OAL on established DRCD.

**Treatment effect of oral LPS administration.** Of the 18 of *icv* STZ-administered mice, eight received OAL at a dose of 1 mg/kg body weight (BW) daily from after 17 days following the *icv* administration of STZ, and the remaining 10 received sterile water orally as a control for the same period according to the treatment experiment protocol (Figure 1B). The effect of OAL on DRCD was evaluated by the MWM from 24 days after starting OAL (the second MWM).

As shown in Figure 2A, the STZ-administered mice had significantly longer escape latency and lower learning ability on all four days during the training session compared with the saline-treated mice in the second MWM (Days one-four;  $p=0.008$ ,  $p=0.021$ ,  $p=0.04$ , and  $p=0.004$ , respectively). Moreover, the escape latency gradually decreased in the STZ+LPS group with each training session in the second MWM (Figure 2A). The results of the second MWM showed no significant differences in escape latency between the STZ+LPS and saline groups during the training (Figure 2A). The STZ+LPS group showed significantly shorter escape latency on the fourth day of training than did the STZ group in the second MWM ( $p=0.019$ , Figure 2A). In the probe test of the second MWM (Figure 2B), the STZ+LPS group showed a trend toward recovery of spatial reference memory and a slight increase in time spent in the target quadrant compared with the STZ group; however, the differences among groups were not statistically significant.

Thus, the shorter escape latency time and longer time spent in the target quadrant of the OAL group during the second MWM compared to the sterile distilled water group suggest that OAL has a treatment effect against STZ-induced DRCD. Furthermore, the treatment effect of OAL against STZ-induced DRCD was observed at around 24-28 days after starting OAL. No significant increase or decrease in the BW of the mice due to OAL was observed (data not shown).

## Discussion

To investigate whether OAL is useful in the treatment of DRCD, we modified the protocol to examine the efficacy of OAL in preventing DRCD (Figure 1A) and started OAL after confirming the onset of DRCD after the *icv* administration of STZ (Figure 1B). With this protocol, we succeeded in creating a model in which restoration from DRCD could be examined (Figure 1C and 1D, Figure 2A and 2B). The major difference between the two protocols is that in the prevention experiment, OAL was started before STZ *icv* administration, whereas in the treatment experiment, OAL was started after the onset of DRCD had been confirmed after STZ *icv* administration. The results of the treatment experiment suggest that OAL is useful in restoring learning ability from DRCD and may also restore memory ability (Figure 2A and B).

The loss of hippocampal function is a hallmark of early-stage dementia, which is associated with the loss of learning and memory abilities (7-9). Our previous work showed that prophylactic OAL prevents the decline of both spatial learning and spatial memory in SAMP8 mice with accelerated aging as well as *icv* STZ-administered mice (diabetic dementia), as evidenced by the MWM (2, 3, 10, 11). Despite the controversy surrounding the contrast between the decline in spatial learning and spatial memory in animals and cognitive decline in humans, it is well established that cognitive decline in both animals and humans is associated with hippocampal dysfunction (9). Therefore, animal models are useful for assessing the early loss of learning and memory functions in patients with dementia.

In this study, we used the MWM to investigate the treatment effects of OAL on STZ-induced DRCD in mice, which complements our previous report (3) and expands the usefulness of OAL. In our previous study, we demonstrated that OAL prevents STZ-induced DRCD by promoting the expression of membrane-bound colony-stimulating factor (CSF1) in the leukocytes of peripheral blood, which, in turn, activates CSF1 receptors in the brain microglia (3). However, further studies are needed to determine whether OAL promotes CSF1 expression in leukocytes after STZ-induced DRCD.

Microglia activation plays a pivotal neuroprotective role by removing metabolic waste products from the brain. Our recent study showed that OAL (0.01-1mg/kg for 7 days) activates resident peritoneal macrophages and enhances phagocytic activity *via* the TLR4 signaling pathway (12). In addition, microglia in OAL-treated mice were shown to promote phagocytosis and significantly reduce metabolic waste products, such as amyloid- $\beta$  and denatured tau protein, in the brain (3, 11). Furthermore, interleukin 10 enhanced by OAL induces microglia to an anti-inflammatory form (13), and heat shock proteins exhibit neuroprotective and anti-

inflammatory effects in the brain (14), making them a potential target for DRCD. Therefore, OAL may not only prevent DRCD, but may also exert therapeutic effects by removing amyloid- $\beta$  and tau proteins, which are causative agents of dementia, and by inducing anti-inflammatory responses and tissue repair.

The multifaceted activation of tissue macrophages by OAL may be a factor in the preventive and treatment effects of DRCD. The results of the present study suggest that OAL has a treatment effect on diabetic dementia that acts through a novel mechanism involving brain waste removal. Therefore, OAL may be a potential therapeutic agent for treating mild dementia due to diabetes.

As shown in Figure 2A, the OAL-administered mice showed significantly shorter escape latency in the MWM, suggesting that OAL restores spatial learning in an animal model. The hippocampus is a critical brain region for memory, spatial cognition, and learning. The hippocampal cornu ammonis 1 (CA1) region is particularly important for long-term memory formation, spatial cognition, and decision-making (15-18). Moreover, place cells, a type of pyramidal cell found in the CA1 region, are essential for understanding animal movement mechanisms and cognitive behavior in space (17, 19). The activity pattern of place cells reveals that the role of these neurons is associated with spatial cognition and corresponds to a specific location in space (20). The present results suggest that LPS restores spatial learning ability by improving the function of place cells in the CA1 region in mice. However, this study only evaluated the LPS in the MWM, and further studies incorporating additional dementia assessment systems are needed to elucidate the mechanism by which LPS enhances spatial learning.

DRCD is one of the most serious complications of diabetes (1), and approximately 55 million people worldwide currently have Alzheimer's disease (AD) (21). Although treating AD is a clinical challenge, current approaches to treatment can slow the onset and progression of the disease (22, 23). While acetylcholinesterase inhibitors (24) and glutamate receptor antagonists (25) slow the progression of dementia, these drugs cannot improve cognitive function in the affected patients. Tau protein modulators (26) and prion protein inhibitors (27) have also shown some promise for the treatment of dementia, but they are still experimental and in the early stages of drug development. Moreover, although dexmedetomidine (28), pioglitazone (29), curcumin (30), rapamycin (31), and melatonin (32) have shown potential for treating dementia in animal models, they have not yet been tested in clinical trials. Aducanumab (33, 34) has attracted increasing attention as an antibody-drug conjugate; however, it is worth noting that such conjugates can cause harm if not properly eliminated from the body.

Recently, lecanemab was approved in the United States and Japan for the treatment of mild cognitive impairment

and mild dementia due to AD (35, 36). Lecanemab is a humanized IgG1 monoclonal antibody that binds with high affinity to amyloid- $\beta$  soluble protofibrils. In a multicenter, double-blind, phase three trial, lecanemab reduced markers of amyloid in early AD (mild cognitive impairment or mild dementia due to AD) and resulted in moderately less cognitive decline (37). Therefore, lecanemab is expected to be the first pharmaceutical drug for early AD, but its effect is to inhibit the progression of cognitive decline, not recovery. In addition, some concerns remain about the adverse events of amyloid-related imaging abnormalities (37).

Unlike pharmaceutical drugs, which cannot be administered as a preventive measure against dementia, LPS is safe (4) and can be preventatively administered as a dietary supplement, suggesting that OAL is safer and more effective than pharmaceutical agents against dementia. In addition, because our results suggest that OAL restores the function of impaired place cells, OAL may be a potential treatment for mild dementia. Restoring cognitive ability in people with dementia remains a worldwide challenge. We present this brief report to support the findings of our previous study and provide a foundation for future research into developing the first therapeutic strategy for restoring spatial learning.

## Conclusion

This study elucidates the therapeutic potential of oral lipopolysaccharides (LPS) from *P. agglomerans* for reversing spatial learning impairments in a mouse model of diabetes-related cognitive dysfunction (DRCd). This restoration of cognitive function, underscores the relevance of LPS as a promising non-pharmaceutical intervention for cognitive deficits associated with diabetes. Future research should focus on understanding the underlying mechanisms and exploring the consistency of these effects across different models and stages of cognitive decline. This could potentially lead to novel dietary-based therapies for managing cognitive impairments in diabetic populations, thereby improving quality of life and reducing the burden of diabetes complications.

## Conflicts of Interest

The Authors declare no conflicts of interest.

## Authors' Contributions

H.I., M.O., C.K. and G-I.S. conceptualized the study and coordinated the experiments. H.I., V.T., C.K., M.O. and G-I.S. reviewed and edited the manuscript. All Authors have read and agreed to the published version of the manuscript.

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