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# Intrahippocampal administration of $A\beta_{1-40}$ impairs spatial learning and memory in hyperglycemic mice

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**Abstract**

Age-related neurodegenerative dementia, particularly Alzheimer's disease (AD), may be exacerbated by several interacting risk factors including genetic predisposition, beta amyloid (Aβ) protein accumulation, environmental toxins, head trauma, and abnormal glycolytic metabolism. We examined the spatial learning and memory effects of $A\beta_{1-40}$ administration on hyperglycemic mice by their performance in the Morris water maze. Chronic hyperglycemia was induced in male C57BL/6J mice to mimic diabetes mellitus by intraperitoneal injection of streptozotocin (STZ), which specifically destroys pancreatic β-islet cells. Ten days after STZ treatment, intrahippocampal infusion of vehicle, monomer, or oligomer $A\beta_{1-40}$ was given to these hyperglycemic mice. Our results demonstrate that in comparison with vehicle or monomer $A\beta_{1-40}$, oligomer $A\beta_{1-40}$ induced significant deficits of spatial learning and memory in hyperglycemic mice. Apoptotic signals were identified in the CA1 and dentate gyrus of hippocampus in hyperglycemic mice. Aβ accumulation, oxidative stress, and apoptosis in the CA1 region were more intensive in hyperglycemic mice than that in normoglycemic mice after acute treatment with oligomer $A\beta_{1-40}$ peptide treatment. These results indicate that CA1 apoptosis was enhanced by oxidative stress resulting from accumulation of Aβ. Considered together, these findings suggest that hyperglycemic mice are more vulnerable to the Aβ-induced-oxidative stress than normal subjects. We therefore propose that Aβ accumulation would be enhanced by hyperglycemia, and the oxidative stress caused by Aβ accumulation would in turn enhance the AD symptoms.


*Keywords:* Alzheimer's disease; Streptozotocin; Diabetes mellitus; Spatial reference memory; Hippocampus; β-Amyloid

## 1. Introduction

Alzheimer's disease (AD) is a slowly progressive neurodegenerative disease accompanied with dementia. The beta amyloid (Aβ), a peptide of 39–43 amino acids resulting from proteolytic cleavage of amyloid precursor protein (APP), plays an important role in the AD brain. Deposition of Aβ is an early and critical event in the pathogenesis of AD (Selkoe, 1997), first forming in temporal cortical regions including the hippocampus (Ball et al., 1985; Duyckaerts, Uchihara, Seilhean, He, & Hauw, 1997; Hyman, Van Hoesen, Damasio, & Barnes, 1984), a region implicated in memory formation (Hyman, Van Hoesen, Kromer, & Damasio, 1986; Squire, 1986; Wallenstein, Eichenbaum, & Hasselmo, 1998). It was proposed that Aβ aggregates to form neurotoxic plaques, which leads to neurodegeneration accompanied by dementia. Studies in APP transgenic mice have supported the hypothesis that memory loss is related to Aβ (Chen et al., 2000; Gordon et al., 2001; Hsiao et al., 1955; Janus et al., 2000; Morgan et al., 2000). However, there is no consensus about which form of Aβ is responsi-

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ble for AD. Various forms of Aβ, characterized by their aggregation states, are found in the brains of APP transgenic mice of different ages (Kawarabayashi et al., 2001). Recent data show that the more harmful physical forms of Aβ are small, still soluble and diffusible aggregates of low molecular weight (Dahlgren et al., 2002; Kayed et al., 2003; Stefani & Dobson, 2003; Walsh et al., 1999; Walsh et al., 2002). Therefore, the mechanism underlying Aβ neuronal toxicity is still poorly understood despite several recent proposals (Butterfield, Drake, Pocernich, & Castegna, 2001; Eckert et al., 2003; Lashuel, Hartley, Petre, Walz, & Lansbury, 2002; Lue et al., 1999; Mattson et al., 1992).

Mild to moderate impairments of cognitive functioning have been reported in patients with diabetes mellitus (DM) (Awad, Gagnon, & Messier, 2004). Previous evidence suggests that the hyperglycemia is the major "toxic" effect in the development of diabetic end-organ damage to brain in Type I and Type II diabetes (Gispen & Biesswls, 2000). Diabetes can be induced experimentally in mice by streptozotocin (STZ) administration, which develops a delayed and/or progressive hyperglycemia, insulitis, and severe destruction of β cells (Dominique et al., 2004; Edward, 1982; John et al., 2001; Matthew, Smyth, Patrick, Brian, & Carl, 1994). However, the mechanism of any correlation between hyperglycemia and Aβ as a cause of Alzheimer's disease in DM patient is still unknown.

The risk for developing a neurodegenerative disorder increases with age and may be associated with an excessive generation of reactive oxygen species (ROS) and oxidative stress (Toren & Nikki, 2000). As a main pathway of neuronal death, apoptosis is an active form of cell degeneration and is executed by caspase proteins. There is a growing consensus that ROS is a potent inducer of apoptosis, and apoptosis contributes to the loss of neurons during normal aging (Zhang & Herman, 2002). To evaluate the interactions between abnormal glucose metabolism and Aβ in vivo, we have created an animal model to evaluate the vulnerability of chronically hyperglycemic subjects to the damage of intrahippocampal $A\beta_{1-40}$ administration.

From observation of neuropathology, we found that Aβ accumulation, oxidative stress, and apoptosis in the CA1 region were more intense in hyperglycemic mice than in normoglycemic mice after acute oligomer $A\beta_{1-40}$ treatment. The STZ-induced hyperglycemia group showed induction of apoptosis in the dentate gyrus of the hippocampus. We also found the impairment of spatial reference learning and memory was only seen in hyperglycemic mice treated with oligomer $A\beta_{1-40}$. These results suggest that cell loss in the dentate gyrus and CA1 regions of the hippocampus by the hyperglycemia with oligomer $A\beta_{1-40}$-treated mice induced an impairment of spatial reference learning and memory. Furthermore, we also suggest that Aβ accumulation, oxidative stress, and apoptosis in the CA1 region of the hippocampus is enhanced by hyperglycemia which then leads to increased impairment of spatial reference learning and memory.

## 2. Materials and methods

### 2.1. Animals

Male C57BL/6J mice (6–8 weeks) were purchased from the National Breeding Center for Laboratory Animals (Nankang, Taiwan) and group-housed in a vivarium maintained at 20–25 °C with 60% relative humidity. Food and water were provided ad libitum. A light/dark cycle of 12/12 h was installed with lights on at 7:00 AM. The procedure adhered to Guidelines for Care and Use of Experimental Animals and was approved by the Institutional Animal Care and Use Committee of National Taiwan University, Taipei, Taiwan. Subjects were divided into six groups: (1) normoglycemic mice treated with double-distilled water, (2) normoglycemic mice treated with monomer $A\beta_{1-40}$, (3) normoglycemic mice treated with oligomer $A\beta_{1-40}$, (4) hyperglycemic mice treated with double-distilled water, (5) hyperglycemic mice treated with monomer $A\beta_{1-40}$, and (6) hyperglycemic mice treated with oligomer $A\beta_{1-40}$. Each group had 9–12 mice.

### 2.2. Experiment timeline

After one week of adaptation to the home cage, mouse body weight and blood glucose were measured on days 1, 10, 15, and 23. Streptozotocin (STZ) or vehicle control was injected into mice on day 1. Oligomer $A\beta_{1-40}$, monomer $A\beta_{1-40}$, and vehicle were injected into the hippocampal CA1 region of mice on day 11. The water maze pretraining, training, testing, and probe trial were performed on days 16–22. Mice were killed for immunohistochemical analyses on day 23.

### 2.3. Hyperglycemia procedure

After animals were weighed, blood samples were obtained by tail prick and glucose levels were measured by a commercial glucometer (Accu-Check Active; Roche) before any treatment (Dominique et al., 2004). STZ (Sigma; 200 mg/kg in 0.1 ml of sodium citrate buffer, pH 4.5) was intraperitoneally injected into non-fasting mice within 15 min to induce chronic hyperglycemia. The normoglycemic groups were injected with an equivalent volume of the citrate buffer. Ten days later, blood glucose levels and body weight were measured. The STZ-treated subjects who failed to develop hyperglycemia (defined as blood glucose concentrations $>200$ mg/dl) were not used. Untreated normoglycemic and STZ-treated hyperglycemic subjects were infused with oligomer $A\beta_{1-40}$, monomer $A\beta_{1-40}$, or vehicle (double-distilled water) in the CA1 region of the hippocampus.

### 2.4. Preparation of oligomer and monomer $A\beta_{1-40}$

The $A\beta_{1-40}$ was dissolved in 1 ml of double-distilled water to a concentration of 0.23 mM and incubated at 37 °C for 7 days to allow oligomer formation as previously described (Minako et al., 2003). Monomer $A\beta_{1-40}$ was freshly prepared in 1 ml of double-distilled water to a concentration of 0.23 mM.

### 2.5. Animal surgery

Mice were weighed and blood glucose measured before surgery. They were anesthetized with pentobarbital (50 mg/kg; MTC Pharmaceuticals, Cambridge) and placed in a stereotaxic instrument (DKI-900, David Kopf Instruments, CA). An incision was made in scalp and hole was drilled in the skull over the injection site. The 30-gauge-needle was lowered into the dorsal hippocampus. Coordinates for the anterior–posterior (from bregma), medial–lateral (from midline), and dorsal–ventral (from surface of the skull) axes were $-2.3$, $\pm 2.5$, and $-1.5$ mm, respectively. Bilateral intrahippocampal infusion was administrated via a 10.0 μl Hamilton microsyringe with a 30-gauge needle fitted to the arm of the stereotaxic instrument. Double-distilled water as vehicle for peptides was used in the study as a control infusion. A 0.6 μl volume of oligomer $A\beta_{1-40}$, freshly made $A\beta_{1-40}$ peptide solution, or double-distilled water alone (as a vehicle

control) was slowly infused at a rate of 0.2 μl/min. After an additional 5 min, to assure adequate diffusion, the needle was slowly retracted. Four days post-surgery, all mice were weighed and blood glucose measured. On the fifth day, mice were trained and tested on the Morris water maze.

### 2.6. Morris water maze (MWM)

Spatial memory was evaluated with a conventional MWM, commonly adopted for studying cognitive deficits in APP transgenic mice (Janus & Westaway, 2001). The water maze apparatus consisted of a circular pool (1.2 m in diameter and 0.47 m high) made of white plastic. The pool was filled to a depth of 20 cm with water (24–25 °C) made opaque by the addition of non-toxic white paint. During conventional MWM training, an escape platform (10 cm in diameter) made of white plastic, with a grooved surface for better grip, was submerged 0.5 cm underneath the water surface. Cues of various types provided distant landmarks in the testing area of the room. The swim path of a mouse during each trial was recorded by a video camera suspended 2.5 m above the center of the pool and connected to a video tracking system. One day before the spatial training commenced, all mice underwent pretraining (day 16) to familiarize them with the requirements of the test. Each mouse was first placed on a visible platform located in the center of the pool and allowed to remain there for 20 s. In the following three 60-s trials, mice were released into the water facing the wall of the pool from semirandomly chosen cardinal compass points. If a mouse failed to swim to the platform or stay on it for 20 sec, it was placed on the platform by an experimenter. The mice were given a 4-day training (days 17–20) with four 60-s training trials (inter-trial interval: 20–30 min) per day. The hidden platform was always placed in the same location of the pool (Northeast quadrant) throughout the training period. During each trial, from quasi-randomly chosen cardinal compass points, the mouse was released into the water facing the pool wall. After climbing onto the platform, the mouse was allowed to rest on it for 20 s. On day 21, 24 h after the last training trial, all mice were given three testing trials to measure the time to climb onto the hidden platform. On day 22, all mice were given one probe trial to evaluate their spatial memory for the platform.

### 2.7. Histology and immunohistochemistry

Immediately after the behavioral test, mice were overdosed with pentobarbital and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline. Brains were removed and post-fixed with 4% paraformaldehyde overnight and then placed in 30% sucrose in phosphate-buffered saline for 2 days. Brains were serially sectioned at 30 μm on a cryostat. Histology and immunohistochemistry were performed to assess the location of the infusion needle tip and to identify any anatomical and neurochemical abnormalities induced by the STZ and oligomer $A\beta_{1-40}$ treatments. A rabbit anti-β-amyloid$_{1-40}$ polyclonal antibody (Chemicon, CA, 1:200 dilution) staining was used to detect the presence of Aβ, using standard procedures. Specific antibodies were used to assess oxidative stress (MnSOD, Upstate, CA, 1:200 dilution) and caspase 3 expression (Chemicon, 1:40 dilution).

For immunohistochemistry, free-floating sections were immunostained. In brief, sections were rinsed in 0.1 M phosphate-buffered saline (PBS) three times (10 min/wash). Endogenous peroxidase activity was blocked by incubation with 3% $H_2O_2$ for 30 min, and sections were then washed in PBS three times (10 min/wash). Non-specific epitopes were then blocked by incubation in 5% normal goat serum and 0.1% Triton X-100 in PBS for 2 h. Sections were incubated in primary antibodies overnight at room temperature and then washed three times in phosphate-buffered saline for 10 min/wash. Secondary antibodies were applied to the sections by a linking reagent (DAKO, CA) for 1 h. Immunostaining was highlighted using substrate-chromogen solution and DAB oxidation. All sections were mounted on coated slides and coverslipped for light microscopy. The extent of $A\beta_{1-40}$ accumulation, oxidative stress, and apoptosis in the CA1 region of the hippocampus were quantitated by two experimenters who were unaware of the experimental condition for these slices. Images were digitized with a CCD camera. A stage micrometer was used to calibrate the pixel-to-μm conversion and a double-blind paradigm was used to calculate all areas. The percentage of staining area of the $A\beta_{1-40}$ accumulation, oxidative stress, and apoptosis in the CA1 region of the hippocampus was analyzed by research-based digital image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD, USA).

### 2.8. Statistical analysis

To determine the effects of STZ, body weight and blood glucose were analyzed by one-way ANOVA test followed by post hoc LSD multiple range tests for comparison among different time point in each treatment. The same analysis was used to evaluate statistical differences for the timing of the escape latencies among groups of different treatments. Furthermore, acquisition of spatial navigation response and searching the target quadrant in water maze pool were analyzed by mixed two-way ANOVA tests followed by post hoc LSD multiple range tests for comparison among treatments in different training days. For the immunostainings, we used two-tailed Student's *t*-tests to assess the staining area of different groups. The Statistical results are expressed as means ± SEM.

## 3. Results and discussion

### 3.1. Hyperglycemia was induced by the STZ treatment

The results of body weight and blood glucose measurements are shown in Fig. 1. As expected, the body weights were decreased in the 3 STZ-treated groups ($p < .05$) but not in the sodium citrate-treated control groups (Fig. 1A). Blood glucose levels were significantly increased at day 10, 15, and 23 compared to the level of day 1 when mice were not yet treated with STZ ($p < .05$; Fig. 1B). These results were consistent with previous studies (John et al., 2001; Dominique et al., 2004). Therefore, we confirmed that hyperglycemia was successfully induced in these mice by a single high dose injection of STZ. Mouse blood glucose levels and body weights were monitored throughout the study.

### 3.2. Impaired spatial reference learning and memory of oligomer $A\beta_{1-40}$-treated hyperglycemic mice

Hyperglycemic and normoglycemic mice that were naïve to the water maze showed no deficits in swimming abilities or climbing onto a visible platform during pretraining (data not shown). In subsequent tests on whether treatment of $A\beta_{1-40}$ to hyperglycemic mice resulted in a functional deficit in spatial learning and memory, $A\beta_{1-40}$ (freshly prepared monomer or 7 day-incubated oligomer) or vehicle was infused into the bilateral intrahippocampal CA1 of mice with hyperglycemia or normoglycemia. In addition, the preparation of monomer and oligomer $A\beta_{1-40}$ was confirmed with the Western blot in this study (data not shown). We found that only hyperglycemic mice given oligomer $A\beta_{1-40}$ had significantly impaired performance in spatial reference learning and memory compared to the other groups ($F(5, 65) = 2.399$, $p = .047$) (Fig. 2A).

We also evaluated the effects of oligomer $A\beta_{1-40}$ on acquisition of the spatial navigation response. The statistical analysis showed significant difference between groups of hyperglycemia and normoglycemia ($F(1,19) = 6.2192$, $p = .022$, Fig. 2B) and training days ($F(3,19) = 4.9450$,

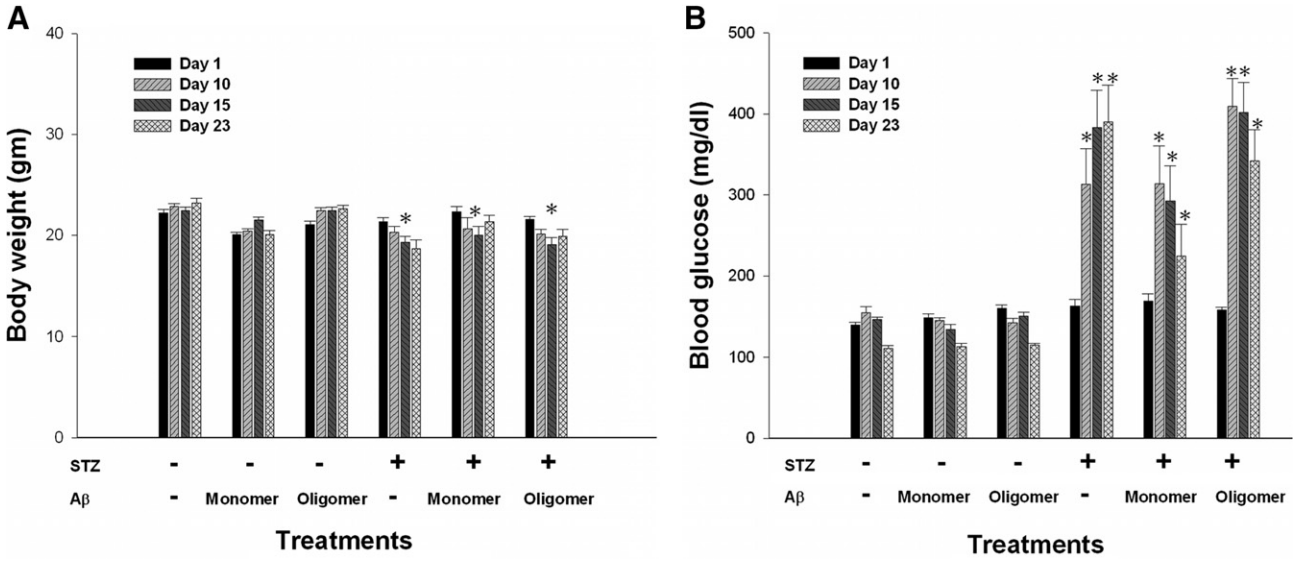


Fig. 1. Hyperglycemia in C57BL/6J male mice was induced by STZ treatment. (A) Body weights examined before (day 1) and after STZ injection (days 10, 15, and 23) and (B) Blood glucose levels examined before (day 1) and after STZ injection (days 10, 15, and 23). Reduced body weight and significantly increased blood glucose revealed the effectiveness of hyperglycemic induction by STZ. Error bars indicate standard error of the means. $*p < .05$.

$p = .0042$), and insignificant interaction effect in group × training days ($F(3, 19) = 0.2922$, $p = .8308$). Therefore, we suggested that hyperglycemic mice with oligomer $A\beta_{1-40}$ injection were slower in learning ability than normoglycemic mice injected with oligomer $A\beta_{1-40}$. We further identified that the speed and extent of learning were significantly different between hyperglycemic mice treated with different Aβ. Statistical analysis by mixed two-way ANOVA reveals significant effects among different Aβ treatments ($F(2, 6) = 5.0137$, $p < .05$) and training days ($F(3, 97) = 3.5490$, $p < .05$), and insignificant interaction effect of group × training days ($F(6, 97) = 0.8319$, $p > .05$). In fact, the escape latency of the oligomer $A\beta_{1-40}$-treated hyperglycemic mice did not reach the same asymptotic level as the monomer- or vehicle-treated mice (Fig. 2C). The average swim velocity during the 4 training days was similar among all hyperglycemic mice (Fig. 2D; $p > .05$), indicating that infusion of Aβ peptides did not grossly affect their sensory or locomotor activities. To confirm the lack of spatial memory in the hyperglycemia group with oligomer $A\beta_{1-40}$, we conducted probe test and measured the time spent in the different quadrants. An animal that has learned the location of the platform should spend more time in the target quadrant than any other quadrants. Compared to the other groups, mice in the hyperglycemic group treated with oligomer $A\beta_{1-40}$-treated mice spent less time in the target quadrant but more time in the opposite quadrant ($F(3, 36) = 4.162$, $p = .012$; Fig. 2E).

The results of behavioral analysis showed that acute intrahippocampal CA1 administration of oligomer $A\beta_{1-40}$ induced a pronounced impairment of spatial memory in the hyperglycemic mice compared to monomer $A\beta_{1-40}$ or vehicle treatment. These results were consistent with previous studies that glucose metabolism is decreased in AD patients (Buchsbaum et al., 1991; Duara et al., 1986). Several studies also suggest that metabolic dysfunction may increase susceptibility to neurodegeneration or contribute to amyloid neurotoxicity in AD pathogenesis (Broe et al., 1990; Henderson, 1988; Matthew et al., 1994). In normoglycemic mice, however, injection of oligomer $A\beta_{1-40}$ resulted in a non-statistically significant loss of spatial memory compared to the mice injected with vehicle or monomer $A\beta_{1-40}$. The result shows that the acute oligomer $A\beta_{1-40}$ treatment only partially affected spatial memory but not spatial learning in the normoglycemic mice. The soluble Aβ oligomers, but not fibrils or monomers, have recently been considered responsible for cognitive dysfunction prior to the formation of senile plaques in transgenic mouse overexpressing human APP (Chapman et al., 1999; Hsiao et al., 1955; Kayed et al., 2003). However, the dose and the structure of the Aβ peptides still are unclear in these transgenic studies. A recent study suggests that specific assemblies, particular trimers, are selective inhibitors of certain forms of hippocampal long-term potentiation (Townsend, Shankar, Mehta, Walsh, & Selkoe, 2006). In addition, Ishibashi, Tomiyama, Nishitsuji, Hara, and Mori (2006) also suggest that accumulated oligomer Aβ, but not fibrillar Aβ, is closely associated with synaptic failure, which is the major cause of cognitive dysfunction. Townsend et al. (2006) also suggests that long-term potentiation in juvenile mice is resistant to the effects of Aβ oligomers. Therefore, we suggest that acute single oligomer Aβ treatment might not result in significant damage to spatial learning and memory in younger normoglycemic mice. Furthermore, hyperglycemia could enhance the impairment of the acute oligomer $A\beta_{1-40}$ in spatial learning and memory.

In addition, we also found that STZ alone did not affect spatial learning and memory. Previous study has suggested that hyperglycemia is unlikely to be the only factor in the development of cognitive impairments in Type II DM (Biessels & Kappelle, 2005). Furthermore, another experimental evidence indicates that the behavioral and neurophysiological consequences of DM are accentuated by aging

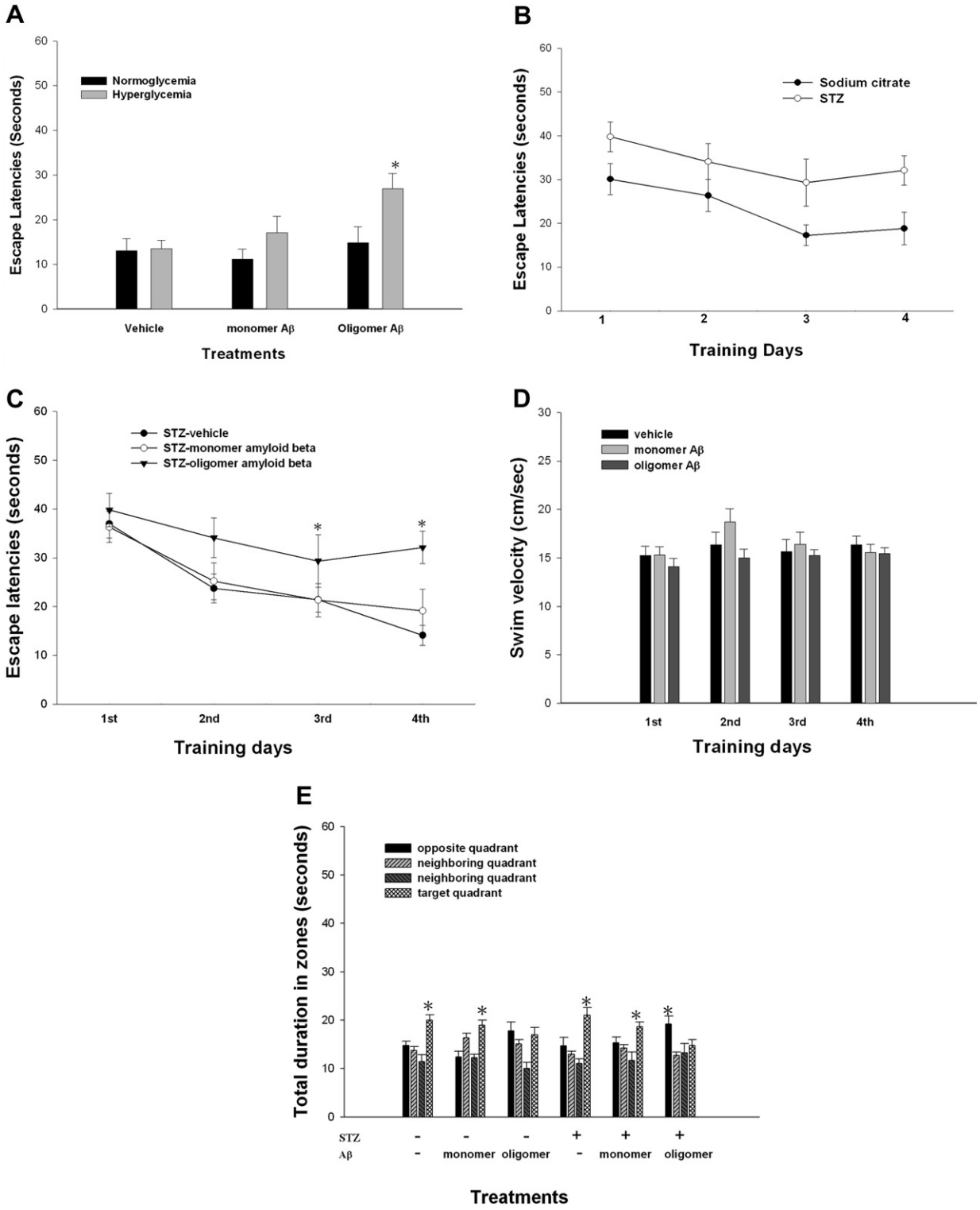


Fig. 2. Effect of $A\beta_{1-40}$ peptide on performance of water maze task. The 4 training days were scheduled on days 17–20. The 3 testing trials were carried out on day 21. (A) Hyperglycemic mice injected with oligomer $A\beta_{1-40}$ showed significant impairment in the spatial reference memory compared to mice treated with other treatments. (B) The escape latency of oligomer-treated mice in the water maze during the 4 training days. The oligomer $A\beta_{1-40}$ injected hyperglycemic mice showed a slower learning ability than the normoglycemic mice ($p < .05$). (C) The escape latency of hyperglycemic mice in the water maze during the 4 training days. The oligomer $A\beta_{1-40}$ injected hyperglycemic mice showed a slower learning ability than the monomer or vehicle-treated hyperglycemic mice. (D) Swim velocity of hyperglycemic mice in the water maze during the 4 training days. No significant difference was identified among the 3 groups of mice with different Aβ treatments. (E) The performance of mice during probe trial conducted on day 23. The hyperglycemic mice with oligomer $A\beta_{1-40}$ injection spent less time in the target quadrant than in the other 3 quadrants. These results show that hyperglycemia accelerated the impairment of the spatial reference learning and memory with oligomer $A\beta_{1-40}$ treatment. Double-distilled water was used as vehicle treatment in the study. Error bars indicate standard error of the means. *$p < .05$.

(Kamal, Biessels, Duis, & Gispen, 2000). Previous studies also suggest that mice with diabetes induced for one week by STZ treatment had no nerve alterations, as total fiber number and myelinated fiber size were compatible between hyperglycemic and control mice (Dominique et al., 2004; Soroku et al., 2001). It has also been reported that at least one-month duration of diabetes is strictly required for development of behavioral disturbances in passive avoidance and Y-maze tasks in STZ-treated rats (Mehrdad, Mohammad, Mohammad, & Tourandokht, 2006). Rats treated with STZ for 8 weeks showed increased cognitive deficits (Chandrashekhar, Vijay, & Shrinivas, 2006). Yan et al. (2004) showed that significant organ damage in hyperglycemic mice 100 days after STZ treatment. In the present study, the duration of STZ treatment should not induce the impairment of the spatial learning and memory in mature mice. Therefore, our results suggest that acute oligomer $A\beta_{1-40}$ enhances the impairment of spatial learning and memory in the hyperglycemic mice, but not in normoglycemic mice.

### 3.3. Increasing Aβ accumulation, apoptotic signal, and oxidative stress in the CA1 regions of hyperglycemic mice injected with oligomer $A\beta_{1-40}$

At first, we confirmed that the infusion site in each mouse was exactly in the CA1 region of the hippocampus

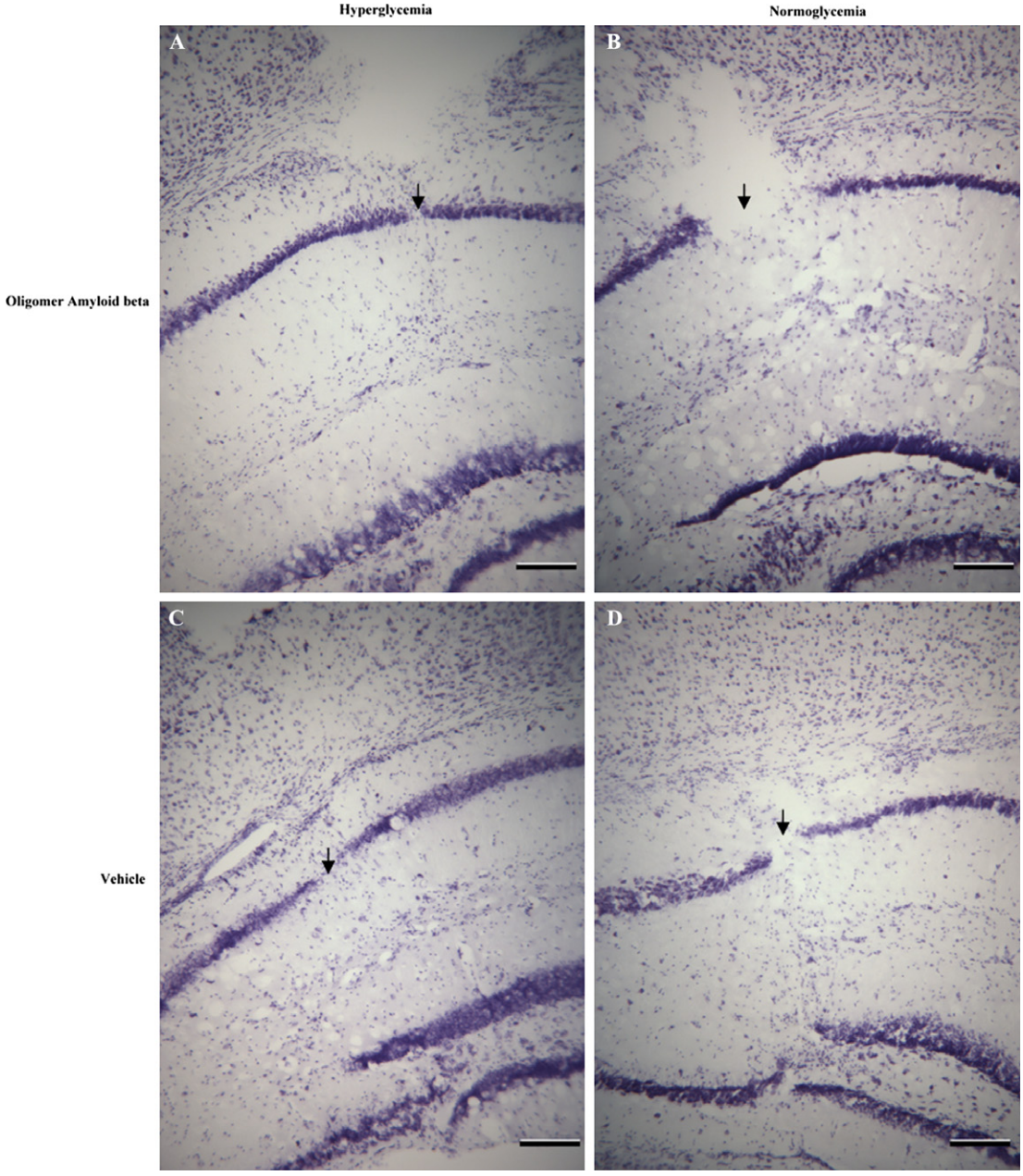


Fig. 3. The $A\beta_{1-40}$ injection sites in the CA1 region is confirmed by cresyl violet staining. (A) Oligomer $A\beta_{1-40}$ injection into hyperglycemic mice. (B) Oligomer $A\beta_{1-40}$ injection into normoglycemic mice. (C) Vehicle injection into hyperglycemic mice. (D) Vehicle injection into normoglycemic mice. Arrows indicate the injection site. Scale bar = 100 μm. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

by cresyl violet staining of mouse brains (Fig. 3). Immunohistochemical analyses showed that Aβ depositions were identified in both of the hyperglycemic and normoglycemic mice at the CA1 region after infusion with oligomer $A\beta_{1-40}$ (Figs. 4A-1 and A-2). However, the extent of the Aβ accumulation was more evident in the hyperglycemic mice than in the normoglycemic mice ($p < .001$; Fig. 4B). No positive staining of Aβ accumulation was observed in vehicle-treated mice (Figs. 4A-3 and A-4). Therefore, we suggested that acute oligomer $A\beta_{1-40}$ might result in increased accumulation of $A\beta_{1-40}$ around CA1 region in hyperglycemic mice compared to normoglycemic mice.

Because a common feature of AD pathology is neuron loss, we used caspase-3 immunostaining to detect the presence of apoptosis in mice with impaired spatial reference memory. Although caspase-3 immunopositive reactions were identified in the CA1 regions of both hyperglycemic and normoglycemic mice treated with oligomer $A\beta_{1-40}$, the apoptotic CA1 region in hyperglycemic mice was greater than that in normoglycemic mice ($p < .001$; Figs. 5A-1, A-2, and B). Apoptotic signals in the dentate gyrus subregion of hippocampus were also observed in the hyperglycemic mice treated with oligomer $A\beta_{1-40}$ or vehicle (Figs. 5A-1 and A-3). No signal, however, was observed in the normalglycemic

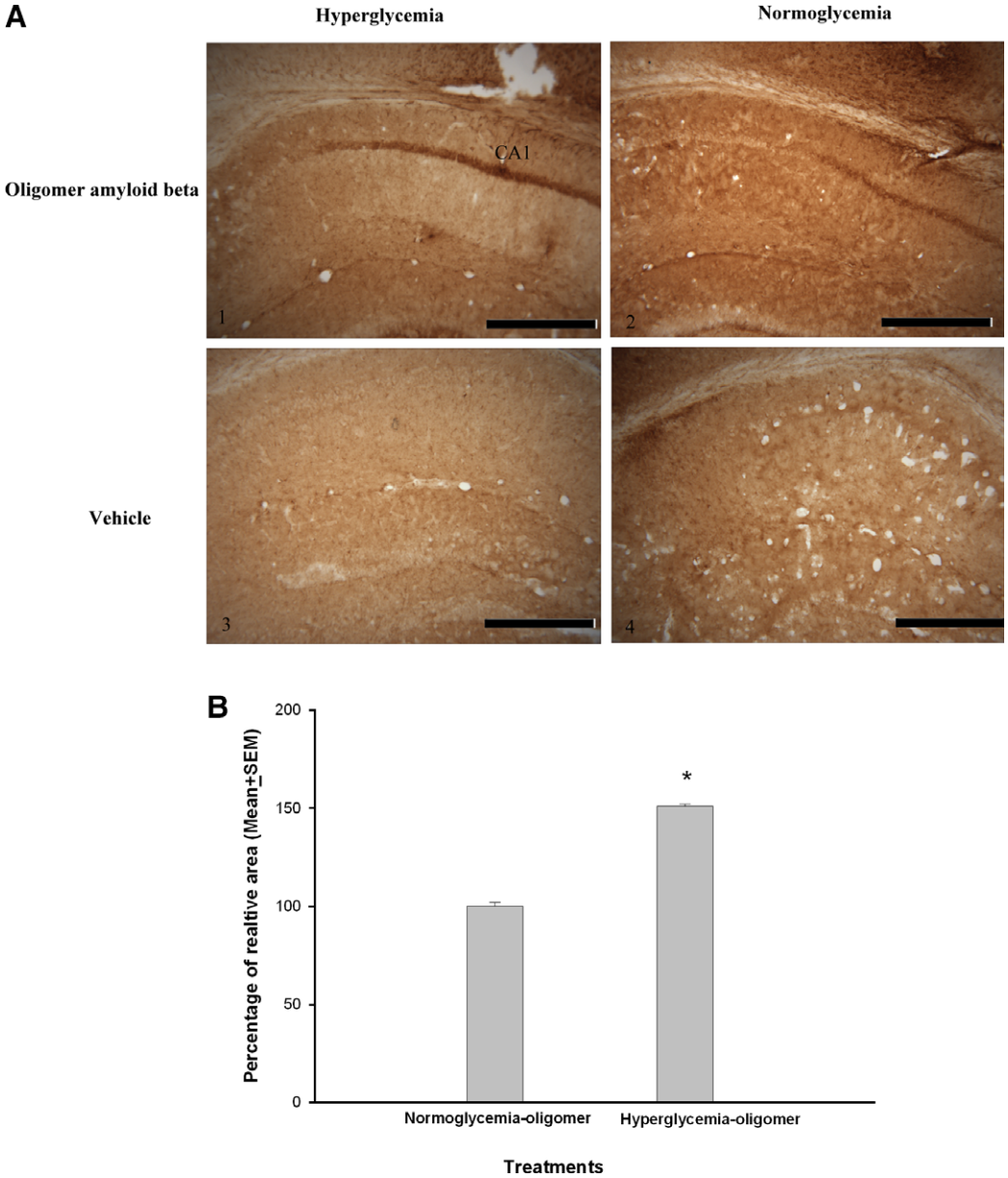


Fig. 4. Immunohistochemistry of $A\beta_{1-40}$ deposition in the CA1 region of hippocampus. (A) The $A\beta_{1-40}$ immunoreactive granules by DAB staining in the CA1 region of the hippocampus of the mice. (1) Oligomer $A\beta_{1-40}$ injection into hyperglycemic mice, (2) oligomer $A\beta_{1-40}$ injection into normoglycemic mice, (3) vehicle injection into hyperglycemic mice, (4) vehicle injection into normoglycemic mice. Scale bar = 100 μm. (B) The percentage of staining area of the $A\beta_{1-40}$ deposition in the CA1 region. Compared to the normoglycemic mice treated with oligomer $A\beta_{1-40}$ ($n = 3$), there was a significantly increased area of $A\beta_{1-40}$ deposition in the CA1 of hyperglycemic mice treated with oligomer $A\beta_{1-40}$. *$p < .001$.

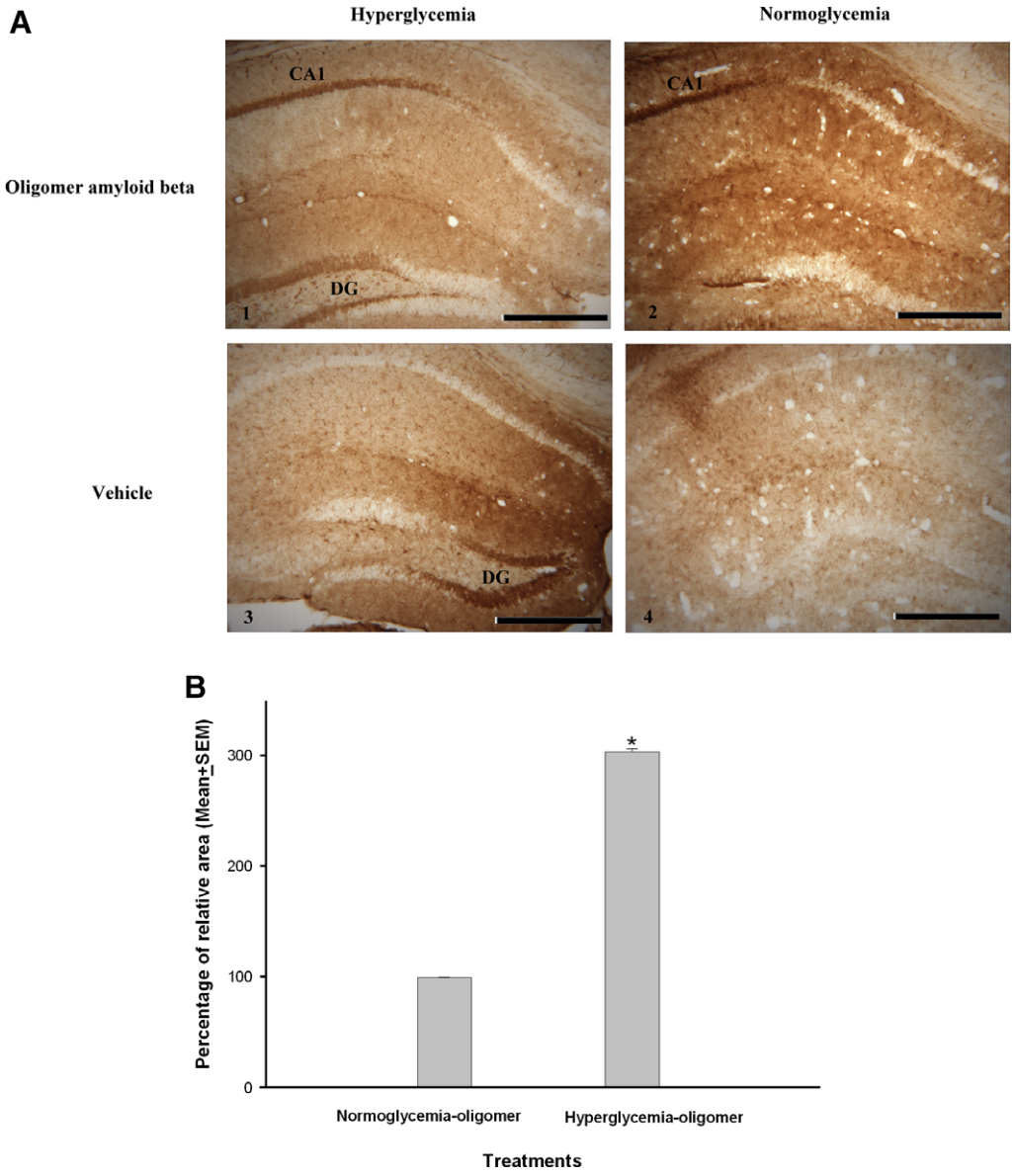


Fig. 5. Results of caspase-3 signal showing apoptosis in the subregions of the mouse hippocampus. (A) Caspase-3 immunoreactivity in the hippocampus of the mice. (1) Oligomer $A\beta_{1-40}$ injection into hyperglycemic mice, (2) oligomer $A\beta_{1-40}$ injection into normoglycemic mice, (3) vehicle injection into hyperglycemic mice, (4) vehicle injection into normoglycemic mice. Caspase-3 positive signals are observed in the CA1 and the dentate gyrus (DG) subregions of the hippocampus in the hyperglycemic mice. Normoglycemia show the caspase-3 positive signals only in the CA1 subregion of the hippocampus with oligomer $A\beta_{1-40}$-treated mice. Scale bar = 100 μm. (B) The percentage of relative area in the CA1 of the mice. Compared to the normoglycemia group treated with oligomer $A\beta_{1-40}$ ($n = 3$), hyperglycemia group treated with oligomer $A\beta_{1-40}$ ($n = 3$) showed a significant cell loss in CA1 region. *$p < .001$.

mice infused with vehicle even though there was some cell loss at the infusion site (Fig. 5A-4). Furthermore, in examining the correlation between apoptosis and oxidative stress, we found MnSOD immunopositive reaction in the CA1 region of hyperglycemic and normoglycemic mice injected with oligomer $A\beta_{1-40}$ (Figs. 6A and B). The oxidative stress signal in the CA1 region is correlated to the result of the $A\beta_{1-40}$ accumulation and caspase-3 staining. No signal was identified in the dentate gyrus subregion of the hyperglycemic mice (Figs. 6A-1 and A-3), although apoptotic signal was previously observed in this region. There was no staining observed in CA1 region of hyperglycemic or normoglycemic mice injected with vehicle (Figs. 6A-3 and A-4).

Our findings from the serial immunostaining for $A\beta_{1-40}$, caspase-3, and MnSOD suggest that the increasing $A\beta_{1-40}$ accumulation induced more apoptotic CA1 neuron in hyperglycemic mice than that in the normoglycemic mice. Previous evidence also indicates that around 70% of neurons in the CA1 region of the hippocampus die during the progression of AD (West, Coleman, Flood, & Troncoso, 1994). Therefore, we propose that the interaction between the hyperglycemia and oligomer $A\beta_{1-40}$ enhanced the damage of the CA1 subregion over the "safe threshold". In

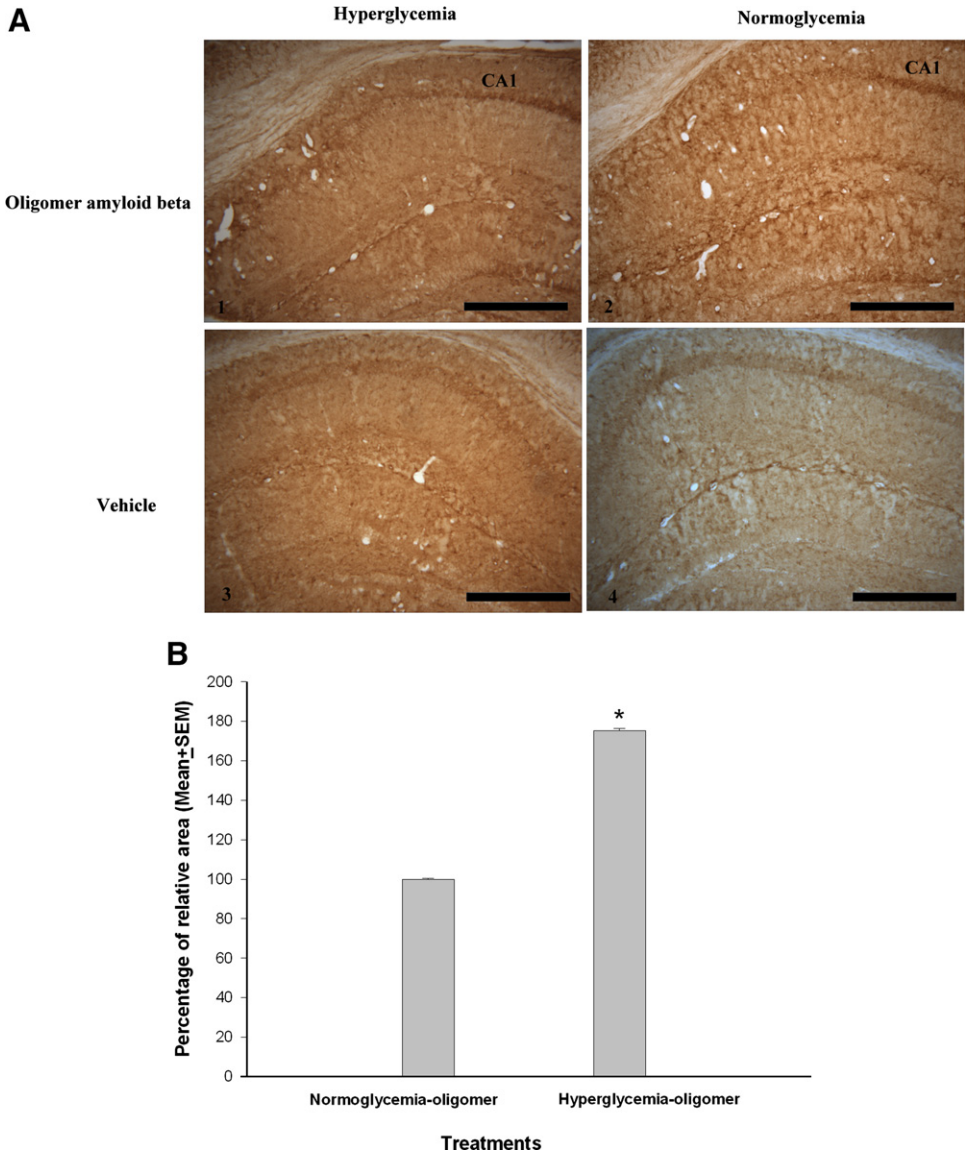


Fig. 6. The results of MnSOD staining show oxidative stress occurred in mouse brains. (A) MnSOD immuno reactivity in the CA1 region of the hippocampus. (1) oligomer $A\beta_{1-40}$ injection into hyperglycemic mice, (2) oligomer $A\beta_{1-40}$ injection into normoglycemic mice, (3) vehicle injection into hyperglycemic mice, (4) vehicle injection into normoglycemic mice. Positive oxidative stress signal is observed in the CA1 subregion of the hippocampus only in oligomer $A\beta_{1-40}$ treated hyperglycemic or normoglycemic mice. Scale bar = 100 μm. (B) The percentage of relative area of the normoglycemia and hyperglycemia-treated oligomer $A\beta_{1-40}$ mice in the CA1 region. Compared to the normoglycemia group-treated oligomer $A\beta_{1-40}$ ($n = 3$), hyperglycemia-group treated with oligomer $A\beta_{1-40}$ ($n = 3$) show a significant oxidative stress in CA1 region. *$p < .001$.

addition, we found the oxidative stress of the CA1 region was also more evident in hyperglycemic mice than in normoglycemic mice. Therefore, these data suggested that acute intrahippocampal CA1 administration of oligomer $A\beta_{1-40}$ induced more severe damage in hyperglycemic mice than in normoglycemic mice through increasing Aβ accumulation, oxidative stress, and apoptotic neuron. One previous study also suggests a positive feedback loop between the oxidative damage and glucose metabolic dysfunction (Munch et al., 1998). A previous dose response experiment doses ranging from 0.01 to 5 μM confirmed the selective vulnerability of CA1 to soluble oligomeric Aβ (Kim et al., 2003). Evidence showed that brain tissues from AD patients have more nerve cells with activated caspase-3 than do those from people who died of other causes (Stadelmann et al., 1999). Su, Zhao, Anderson, Srinivassan, and Cotman (2001) reported that neurons with caspase-3 are found in brains of AD mice or cultured nerve cells. Aβ, in ways that are inhibited by free radical antioxidants like vitamin E, causes brain cell protein oxidation, lipid peroxidation, reactive oxygen species formation, and other oxidative stress responses, suggesting that this peptide is a source of oxidative stress in brain (Butterfield, Hensley, Harris, Mattson, & Carney, 1994).

Furthermore, we also found cell apoptosis in the dentate gyrus of hyperglycemic mice injected with oligomer $A\beta_{1-40}$ or vehicle. These results are consistent with previous pathological studies in humans and animals showing hyperglyce-

mia preferentially induces neuronal death in the CA1, subiculum, and dentate gyrus of the hippocampus, as well as in the superficial layers of the cortex, or in the striatum (Auer & Siesjo, 1993). We did not, however, observe any MnSOD immunoreactivity in the dentate gyrus region of these mice. A study of 3- and 12-month old rats showing nerve conduction deficits after STZ treatment revealed no changes in antioxidant enzymes except for increased catalase in 12-month-old rats (Kishi, Nickander, Schmelzer, & Low, 2000). Therefore, we suspect that the apoptosis in the dentate gyrus was induced by STZ treatment through another signaling pathway instead of from oxidative stress in CA1 region arising from accumulation of Aβ.

The anatomical and behavioral data from our study suggest that hyperglycemia itself induced the apoptosis in the dentate gyrus of the hippocampus, but had no effect on spatial learning and memory. However, hyperglycemia group with oligomer $A\beta_{1-40}$ treated mice induced more accumulation of $A\beta_{1-40}$, oxidative stress, and apoptosis in the hippocampal CA1 neurons. Many studies have suggested that lesions in the CA1 and dentate gyrus of the hippocampus induce the impairment of spatial learning and memory. The hippocampus has been well known to play a critical role in certain types of learning, including spatial learning (Jarrard, 1973; Morris, Garrud, Rawlins, & O'Keefe, 1982; Shengming et al., 2005). Specific cells within the hippocampus become selectively activated when an animal is replaced in particular locations within its environment (Wilson & McNaughton, 1993). Rats displayed dysfunctions in spatial memory when various sites within the hippocampus were lesioned (Stubley, Mungall, & Wright, 1994). Within the hippocampus, cells of the dentate gyrus serve to restrict or amplify signals that originate in extra-hippocampal sites and propagate into the hippocampus proper (Lothman, Bertram, & Stringer, 1991). The CA1 region of hippocampus plays significant roles in associational memories (Volpe, Davis, Towle, & Dunlap, 1992; Wood, Mumby, Pinel, & Philips, 1993). Therefore, hyperglycemia in oligomer $A\beta_{1-40}$-treated mice induced the impairment of spatial learning and memory.

In summary, our results not only provide the animal model to evaluate in detail the behavioral and neuroanatomical effects of the interaction between abnormal glucose metabolism and $A\beta_{1-40}$ in vivo but also provide experimental support for the epidemiological literature indicating that the amyloid accumulation and metabolic dysfunction may interact to exacerbate the pathogenesis of AD.

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