**Title page**

**Full title:** Diabetes impairs hippocampal function via advanced-glycation-end product–mediated new neuron generation in animals with diabetes-related depression

**Running title:** Diabetes impairs hippocampus via AGEs.

**Authors:**

**Shao-hua Wang,** Ph.D, The Department of Endocrinology, affiliated ZhongDa Hospital of Southeast University, No.87 DingJiaQiao Road, Nanjing, PR China, 210009 E-mail: wshgyj@yahoo.com.cn tel:0086-25-83285148; fax: 0086-25-83285132

**Zi-lin Sun (Corresponding author),** Ph.D, M.D. The Department of Endocrinology, affiliated ZhongDa Hospital of Southeast University, No.87 DingJiaQiao Road, Nanjing, PR China, 210009 E-mail: sunzilin1963@yahoo.com.cn tel:0086-25-83285148; fax: 0086-25-83285132

**Yi-jing Guo,** Master, Ph.D student .The Department of Neurology, affiliated ZhongDa Hospital of Southeast University, No.87 DingJiaQiao Road, Nanjing, PR China, 210009 E-mail: janegyj@yahoo.com.cn tel:0086-25-83272027; fax: 0086-25-83285132

**Yang Yuan,** Ph.D student, The Department of Endocrinology, affiliated ZhongDa Hospital of Southeast University, No.87 DingJiaQiao Road, Nanjing, PR China, 210009 E-mail: yang_yuan000@sina.com.cn tel:0086-25-83285148; fax: 0086-25-83285132

**Bing-quan Yang,** Master, The Department of Endocrinology, affiliated ZhongDa Hospital of Southeast University, No.87 DingJiaQiao Road, Nanjing, PR China, 210009 E-mail: bqyang@163.com tel:0086-25-83285148; fax: 0086-25-83285132

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Name and address of person to whom reprint requests should be addressed:

Zi-lin Sun, Ph.D, M.D. The Department of Endocrinology, affiliated ZhongDa Hospital of Southeast University, No.87 DingJiaQiao Road, Nanjing, PR China, 210009 E-mail: sunzilin1963@yahoo.com.cn tel:0086-25-83285148; fax: 0086-25-83285132

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Conflict of interest

The authors of this manuscript declare that there are no actual or potential financial or other conflicts of interest related to the submitted manuscript.
Abstract

The diabetes-induced reduction of neurogenesis in hippocampal dentate and its reversal with antidepressant medications implies a potential mechanism for diabetes-related depression and cognitive decline. In the following article, the role of advanced glycation end products (AGEs) in hippocampal neurogenesis deficits in diabetic animals with depression has been further explained in the light of an in vitro study. Diabetes was induced in animals with the use of streptozotocin (55 mg/kg, i.p.), and the animals then divided into those with and those without depression-like behaviors as analyzed by behavioral tests. The AGE formation inhibitor aminoguanidine (10 mg/kg) was administrated for an additional 4 weeks. Proliferating cells, their survival, and their phenotype fate were monitored with bromodeoxyuridine labeling and confocal laser microscopy. The presence of AGE peptides was determined with the use of a flow injection assay. Animals with diabetes and depressive symptoms displayed a reduction in hippocampal neurogenesis and an elevated serum level of AGE peptides, both of which were reversed by a 4-week regimen of aminoguanidine (10 mg/kg, i.p.), which inhibits AGE formation; in addition, the depressive behaviors were improved. These findings provided in vivo evidence that diabetes impairs hippocampal function via the AGE-mediated generation of new neurons. This likely represents a putative mechanism that is responsible for diabetes-related depression and cognitive decline, and it suggests a potential approach for future research.

Keywords: Diabetes mellitus; Hippocampus; Neurogenesis; Advanced glycation end products, Neural stem cells; Depression
INTRODUCTION

As a result of high-calorie diets and sedentary lifestyles, diabetes mellitus (DM) is rapidly becoming more prevalent in the world (Reaven, 2005). Many organs and organ systems are adversely affected by diabetes, including the brain, which undergoes changes that may increase the risk of depression and cognitive decline (Greenwood and Winocur, 2005; Messier, 2005).

The hippocampal subgranular zone in the adult brain is identified as an endogenous resource of neural stem cells. These cells are self-renewing and multipotent progenitor cells that are able to differentiate into new neurons and glial cells, thus allowing the addition of new neurons to persist into adulthood (Gage, 2000; Gould and Gross, 2002). Adult neurogenesis, which is the process of generating functionally integrated neurons from progenitor cells in the dentate gyrus of the hippocampus, is associated with affective disorders and depression (Jacobs et al., 2000; Kempermann and Kronenberg, 2003). The production of these new neurons in the brain during adulthood is a plastic event that is modulated by several factors, including strain, gender, aging, hormones, neurotransmitters, neuropeptides, growth factors, the environment, physical activity, learning, dietary restrictions, stress, drugs (e.g., antidepressants) and several pathological situations (e.g., ischemia, seizures, inflammation, degenerative diseases) (Gross, 2000; Abrous et al., 2005; Ming and Song, 2005; Dranovsky and Hen, 2006;). Data from studies of animal models further suggest that defective adult neurogenesis is present in individuals with diabetes; this condition is thus hypothesized to be an etiological factor of depression and cognitive deficits (Stranahan et al., 2008; Zhang et al., 2008). However, the mechanisms responsible for depression and cognitive dysfunction in patients with diabetes have not been elucidated.

Humans with poorly controlled diabetes show hyperglycemia accompanied by an accelerated
rate of advanced glycation end product (AGEs) formation and accumulation. AGEs in various
tissues are known to progress during normal aging and at an accelerated rate in individuals with
diabetes (Yamagishi et al., 2003). A recent understanding of this process has confirmed that AGEs
play important roles in the pathogenesis of diabetic complications and neurodegenerative
disorders, including Alzheimer disease (Yamagishi and Imaizumi, 2005). Our *in vitro* study
previously demonstrated that AGEs downregulated the proliferating cells and their neurogenic
differentiation (accepted by Molecular and Cellular Endocrinology). So the question remains:
Does diabetes impair hippocampal function via the AGE-mediated generation of new neurons,
which probably represents a putative mechanism that is responsible for diabetes-related depression
and cognitive decline?

In this article, we provide direct evidence that elevated AGE levels contribute to the
impairment of hippocampal neurogenesis and to behavioral deficits in rodent models of
insulin-deficient diabetes with depressive behaviors. These findings will deepen the current
understanding of the pathogenetic roles of AGE-mediated effects on neuron generation in
diabetes-related depression and cognitive decline.

**MATERIALS AND METHODS**

**Animals**

Adult male Sprague–Dawley rats (Medical College of Southeast University, China),
weighing 210–250 g, were used in the experiments. Animals were housed two per cage (40 cm
long × 25 cm wide × 15 cm high) with food and water available ad libitum unless otherwise
indicated. All rats were kept on a 12-h light–dark cycle (lights on at 7 a.m.) in the same colony.
room, with temperature (21 ± 2 °C) and humidity (55%) remaining constant. All experiments were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985).

**Experimental Design and Drug Administration**

During the 2-week adaptive period, the animals were trained to consume a 1% (w/v) sucrose solution. The training consisted of three 1-h baseline sucrose tests sessions following 20 h food and water deprivation. On the basis of their sucrose intake in final baseline test, animals were randomly assigned into two matched groups: nondiabetic control (CON; n = 10) and diabetes (DM, n = 30). Diabetes was induced by a single intraperitoneal injection of streptozotocin (55 mg/kg body weight, Sigma, St. Louis, MO). CON rats were injected with vehicle alone. Diabetes was verified at 72 h after streptozotocin injection by evaluating blood glucose levels with the use of glucose-oxidase reagent strips (Lifescan, Milpitas, CA). Rats having a blood glucose level of ≥300 mg/dl (16.7 mmol/l) were considered to be diabetic. All studies were conducted after diabetic verification.

Four weeks after the verification of diabetes (Week 4 showing the same as following), DM group animals were subsequently randomly assigned to two experimental groups according to relative sucrose intake. Reduced preference for sucrose solution are considered a putative indicator of anhedonia (Moreau, 1997; Willner et al., 1992), which represents behavioural change that might be regarded as behavioural correlates of depression-like symptoms in humans. Specifically, anhedonia was defined as a reduction in sucrose intake relative to control group intake and baseline intake. Details were described as follows in the Results section: diabetes without depression-like behavior (DM+N, n = 10) and diabetes with depression-like behavior
The DM+D group was divided into two subgroups: DM+D only (n = 10) and DM+D+AG (n = 10). The DM+D+AG rats received daily treatment with the AGE cross-link inhibitor aminoguanidine (10 mg/kg) for an additional 4 weeks.

Weeks 4 and 8 after diabetic verification, blood glucose and AGE-peptides (AGE-P) levels were measured. To avoid the effects of sucrose intake on blood glucose levels, preference sucrose test was performed three days before Weeks 4 and 8. Since sucrose preference is very likely to be impacted upon by the diabetic condition independently of any relationship with depression, forced swim test was arranged to avoid sucrose intake as one single measure of depression-like behavior one day before Weeks 4 and 8. Hippocampal neurogenesis including cells proliferation, survival and differentiation was detected on Week 8. To label proliferating cells, bromodeoxyuridine (BrdU, Sigma), 50 mg/kg in saline i.p., was given twice daily, at 8-h intervals, on consecutive days before Week 8. Rats were killed the day after the last dose. To label proliferating cells for long-term studies on survival and differentiation, animals were injected with BrdU (50 mg/kg, i.p.) three times a day at 4-h intervals on 4 consecutive days after Week 6 and sacrificed at Week 8.

**Behavioral Tests**

Behavioral tests contained sucrose preference test and forced swim test, and were operated and scored by experienced observers who were blind to the design of the study.

The sucrose preference test was used to operationally determine anhedonia, which is considered as a core symptom of depression. The test was performed according to the method described previously with minor modifications (Wang et al., 2008). In brief, before testing, animals were food and water deprived for 20 h. Intake of water or a sucrose solution (1%) was
measured by weighing two pre-weighted bottles before testing and after 1 h. The relative sucrose intake (g/g) was the absolute sucrose intake per gram of rat body weight, whereas sucrose preference was calculated according to the following ratio: sucrose intake (g)/sucrose intake (g) + water intake (g).

The modified rat forced swim test was conducted essentially as described by Detke et al. (Detke et al., 1995). Briefly, rats were placed individually for 15 min in polypropylene vessel (60 cm long x 40 cm wide x 40 cm high) with tap water level of 30 cm (25±1°C). This ensured that the rat’s feet did not touch the floor of the vessel and that it could not climb out of it. Twenty-four hours after their first exposure, the animals were replaced in the swim apparatus for 5 min, and the session was recorded by two trained testers. Climbing behavior consisted of upward-directed movements of the forepaws along the side of the swim chamber. Swimming behavior was defined as movement (usually horizontal) throughout the swim chamber, which also included crossing into another quadrant. Immobility was assigned when no additional activity was observed other than that required to keep the rat’s head above the water. Prior to statistical analyses, data from each of the two testers were averaged for each session.

The Determination of AGE-peptides by Flow Injection Assay

The determination of AGE-P by flow injection assay (FIA) according to the method described previously (Sun et al., 2001). Briefly, The 20-ml serum samples were mixed with 480 µl of trichloroacetic acid (0.15 mol/L) and 100 µl of chloroform in microcentrifuge tubes. The tubes were shaken vigorously and then were centrifuged (10 min, 13,000×g). The 20 ml of the aqueous layer was injected to sample injector loop of High performance liquid chromatography (Shimadzu,
Japan). The flow rate was 0.5 ml/min and spectrofluorometric detector was set with emission wavelength at 440 nm and excitation wavelength at 370 nm for detection of AGE-P. The samples were analyzed in duplicate, and peak height mode was used for signal measurement. Standard AGE-P were diluted as 0.1, 0.5, 1, 5, 10, 50, 100 mg/L and performed for preparing calibration curve and calculating sample AGE-P as described above. The value was defined as U/mL, 1.0 U/mL is equal to standard AGE-P obtained from hydrolysis of 1.0 mg/L AGE-BSA.

**Immunohistochemistry**

Immunohistochemistry staining for detection of BrdU-labeled nuclei, coronal brain sections (40µm) were pretreated with 0.1M citric acid, pH6.0 at 75 °C for 45 min, followed by 2M HCl for 20 min at 37 °C, and rinsed in 0.1M boric acid for 10 min. Sections were incubated in 3% H₂O₂ for 15 min, and in blocking solution (3% normal goat serum/1% BSA/0.3% Triton X-100 in PBS) for 2 h, and with mouse mono-clonal anti-BrdU antibody (Chemicon, 1:100) at 4 °C overnight. After washing step, sections were incubated with biotinylated goat anti-mouse secondary antibody (Sigma, 1:100) and placed in avidin–peroxidase complex solution for 1 h, respectively. The horseradish peroxidase reaction was detected with 0.05% diaminobenzidine and 0.03% H₂O₂. To determine the phenotype of proliferating cells, double immunofluorescence was conducted to detect the colocalization of BrdU with either neuron specific nuclear protein (NeuN) or Glial fibrillary acidic protein (GFAP). Serial sections were incubated with 2M HCl for 30 min at 37 °C, After washing, sections were incubated in blocking solution, then with mouse mono-clonal anti-BrdU antibody (Chemicon, 1:150 dilution) either with rabbit mono-clonal anti-NeuN (Chemicon, 1:50) or with rabbit mono-clonal anti-GFAP (Neomarker, 1:100) overnight at 4°C.
After washing, sections were incubated for 1 h with rhodamine-conjugated goat anti mouse IgG (Vector, 1:100) and FITC-conjugated goat anti-rabbit IgG (Vector, 1:100).

**Image Acquisition and Data Analysis**

The number of BrdU-labeled cells revealed by immunohistochemistry were quantified with a bright-field microscope at 50× magnification, by an investigator blind to treatment history, on 6 coronal sections of 40µm thickness spaced 240µm apart within the hippocampus (bregma −2.12 mm to bregma −6.30 mm) (Paxinos and Watson, 1997). BrdU-labeled cells were counted in the granule cell layer and subgranular zone, defined as a two-cell body width along the base of the granule cell layer and considered part of the granule cell layer. More than two cells away from the granule cell layer were classified as being located in the hilar region. The numbers of labeled cells were expressed as the mean number of BrdU-positive cells per section. The numbers of BrdU/NeuN and BrdU/GFAP double-labeled cells in the granule cell layer and hilar, was quantified by using Zeiss LSM510 laser scanning microscope (Carl Zeiss) equipped with an 40× objective at excitation/emission wavelengths of 535 nm/565 nm(rhodamine, red), 470 nm/505 nm(FITC, green). The BrdU-positive cells were first randomly selected in dentate gyrus and analyzed in their entire z-axis with a 0.5µm step to exclude false double-labeling. A minimum of 50 BrdU-labeled cells were analyzed from each rat, and numbers of double labeled cells were expressed as percentages of the total number of BrdU-labeled cells.

**Statistical Analysis**

The data was analyzed using Graph Pad Prism version 4.0. Values are presented as means ±
S.D. for the indicated experiments. Blood glucose, quantity of immunofluorescent signal data and behavioral tests data were performed by using one-way ANOVA. AGE-P levels were analyzed by using a repeated measurement ANOVA with treatment (control, streptozotocin induction) and week (baseline, week 4) as two factors. To detect significant differences among the experimental groups and weeks, (one-way or two way) ANOVA was supported by the Bonferroni post hoc tests for multiple, comparisons, with $P < 0.05$ considered significant.

**RESULTS**

**Blood Glucose Level and Behavioral Tests**

Four weeks after diabetic verification, depressive diabetic rats (DM+D) rats demonstrated a reduction in sucrose intake as compared with the DM group ($52.3\% \pm 2.8\%$ vs. $69.6\% \pm 2.3\%$; $P < .001$; $n = 20$ and 10, respectively) and their baseline intake values ($26.3\% \pm 2.2\%$ decreased; $n = 20$). A forced swim test confirmed depressive behaviors, including increased immobility time and decreased swimming and climbing behaviors in the DM+D rats (Tables 1 and 2). DM+D rats had a blood glucose level of $20.7 \pm 2.1$ mmol/L, which was increased by 27.8% as compared with DM animals.

At week 8, the reduction in sucrose intake persisted in the DM+D animals, and the forced swim test also confirmed depression-like behaviors among them; however, all of these conditions were reversed after 4 weeks of aminoguanidine administration (see Tables 1 and 2). The DM+D rats had a blood glucose level of $20.2 \pm 1.6$ mmol/l, which was increased by 29.5% as compared with the DM+N animals. Aminoguanidine reduced the blood glucose levels of the DM+D rats ($18.4 \pm 0.5$ mmol/l vs. $20.2 \pm 1.6$ mmol/l), but the results did not reach the
statistical significance ($P > .05$).

**The Level of Serum AGE Peptides**

Two-way ANOVA showed significant effects of streptozotocin induction and time (all $P < .0001$) on the elevated levels of serum AGE-P in DM rats during the 4 weeks after the onset of diabetes. At week 8, the high levels of serum AGE-P persisted in both DM+N and DM+D animals as compared with the control animals (all $P < .0001$). Aminoguanidine reduced the increased levels of AGE-P in the DM+D animals ($2.56 \pm 0.12$ vs. $3.48 \pm 0.07$ U/mL, $P < .001$). All of these results are shown in Table 3.

**Proliferation**

Proliferating cells measured with BrdU were located exclusively near the subgranular zone, and they frequently formed clusters after injection. Eight weeks after diabetic verification, the number of BrdU-labeled cells was reduced in DM animals with or without depressive behaviors as compared with the controls ($13.0 \pm 1.9$, $21.8 \pm 1.3$ vs. $29.8 \pm 3.2$; all $P < .001$). Cell proliferation was reduced by 56% and 27%, respectively. The DM+D animals displayed an apparent reduction in their numbers of proliferating cells as compared with the DM+N animals ($P < .001$), and this reduction could be reversed with the use of aminoguanidine ($19.2 \pm 1.9$ vs. $13.0 \pm 1.9$, $P < .001$). All of these results are shown in Figure 1.

**Survival and Differentiation**

According to the BrdU-labeling protocol for the study of survival and differentiation, the
BrdU-positive cells in each group were found to be mainly located in the granule cell layer, with a few cells being present in the hilus. The nuclei of these cells had a uniform oval shape that is typical of granular neurons. The phenotype of BrdU-positive cells was determined with the use of the markers for NeuN and GFAP. BrdU and NeuN signals were detected in the granule cell layer. The majority of BrdU-positive cells showed a neuronal phenotype that was indicated by the coexpression of NeuN and BrdU (Figure 2). As compared with the control sample, DM+N and DM+D animals exhibited not only a decrease in the number of newborn cells (by 15% and 44%, respectively) but also a significant decrease in the proportion of BrdU-positive cells costained with NeuN (by 21% and 38.1%, respectively). The numbers of both BrdU-positive cells and those cells that were costained with NeuN were lower in DM+D animals than they were in the DM+N animals (all \(P < .001\)), and all of these conditions were reversed by aminoguanidine (all \(P < .001\), Figure 3). However, there were no differences among the four experimental groups with regard to the numbers of BrdU-positive cells that were costained with GFAP.

**DISCUSSION**

AGEs play pathogenetic roles in diabetic complications and neurodegenerative disorders, including Alzheimer disease. Our *in vitro* study previously demonstrated the downregulated effects of AGEs on the proliferating cells and their neurogenic differentiation, thus raising the possibility that diabetes impairs hippocampal function via AGE-mediated negative effects on the generation of new neurons from neural stem cells. In the present study, we further provide *in vivo* evidence to test this hypothesis. The most striking finding was that rats with streptozotocin-induced diabetes and depression-like behaviors displayed a reduction in
hippocampal neurogenesis and an elevated serum level of AGE-P. These conditions were reversed
after 4 weeks of treatment with aminoguanidine, which is the crosslink inhibitor of AGE
formation; the depression-like behaviors among these animals were improved simultaneously.
These findings further the understanding of the potential mechanisms of AGEs responsible for
diabetes-related depression and cognitive decline. The sucrose preference test was used to
operationally determine anhedonia, the “core” symptom of depression. Forced swim test was also
adopted to measure depression-like behaviour, and importantly, to avoid sucrose preference as a
single index to assess depression, for sucrose intake is very likely to be impacted upon by the
diabetic condition independently of any relationship with depression.

Hippocampal neurogenesis is strongly diminished in the present streptozotocin-induced
model. These findings are in conformance with the previous independent studies of cell
proliferation (Jackson-Guilford et al., 2000; Saravia et al., 2004; Beauquis et al., 2006; Beauquis
et al., 2008), survival (Beauquis et al., 2006; Beauquis et al., 2008), and the generation of new
neurons (Zhang et al., 2008). Hypoglycemia can induce a transient increase in proliferation, but it
can also cause a subsequent progenitor cell loss in the dentate gyrus of the rat (Suh et al., 2005) or
impair hippocampal long-term potentiation (Yamada et al., 2004). All of these findings indicate
that anomalies of glucose homeostasis in diabetes may play a critical role in modulating
hippocampal neurogenesis. Alternatively, overt hyperglycemia may exert its own effects during
adulthood or even during pregnancy, when it has been shown to influence brain development
(McCall, 1992; McEwen et al., 2002).

The evidence for the combined impairment of proliferation, survival, and neuronal
differentiation in diabetes is consistent with the serum level of AGE-P as a result of hyperglycemia.
AGEs are highly reactive, and they continue to react with nearby amino groups, thus producing both intra- and intermolecular crosslinks (Makita et al., 1994). A flow injection assay system was developed to detect low-molecular-mass AGEs (AGE-P). With good precision and recovery, it was suggested by our previous study that AGE-P assayed by flow injection assay were superior to AGEs determined by ELISA and fluorescence spectroscopy (Liu et al., 2000). As an inhibitor of AGE formation, aminoguanidine reduced the increased levels of AGE-P in depressive diabetic animals, and this simultaneously inhibited hippocampal neurogenesis in these animals. The findings further indicated that one possible mechanism of hippocampal neurogenesis impairment in diabetes seems to be via an increased serum level of AGE-P. The deposition of these covalent adducts on various macromolecules has been reported to contribute to the development of the complications of aging and diabetes through both direct chemical-mediated (covalent crosslink formation) and cell-surface-receptor–mediated pathways (Vlassara and Palace, 2002). In our previous in vitro study, we found that AGEs downregulated the proliferation and neurogenic differentiation of neural stem cells and that the peroxisome proliferator-activated receptor (PPAR) γ-mediated the AGE regulation of neural stem cell proliferation (with the exemption of differentiation). The exact signaling pathway need to be further investigated.

A streptozotocin model of type 1 diabetes, which is characterized by low insulin and high plasma glucose levels, affects not only the body’s overall metabolism but also brain activity. Neuronal proliferation and the subsequent integration of new neurons into the hippocampal circuit are regulated processes that are affected by high serum levels of AGE-P, and they can have profound effects on an animal’s behavior. A reduced preference for sucrose solution, which is a putative indicator of anhedonia (Willner et al., 1992; Moreau, 1997), may be regarded as a
behavioral correlation of depressive symptoms in animals with diabetes.

Two facts led to the hypothesis that declining hippocampal neurogenesis and depressive symptoms may be causally connected in diabetes. First, diabetic animals with depressive behavior displayed reduced numbers of proliferating cells and new neurons. Alternatively, the increase in diabetes-related neurogenesis may have the potential to become a drug target for aminoguanidine for the alleviation of symptoms; previous studies have indicated that improved neurogenesis mediates antidepressant action and alleviates symptoms among animals with streptozotocin-induced diabetes. The present findings again indicate a role for AGE-P in the mediation of hyperglycemia in diabetes-related hippocampal neurogenesis impairment. In addition, various studies have suggested that the loss of hippocampal neurons may contribute to impairments in mood and cognition and in the continual formation of new networks encoding new memories. It is helpful to further understand that hippocampal neurogenesis would have a plausible role in the depressive behaviors associated with diabetes. Although the possible long-term benefit of diabetes-induced neurogenesis bears further investigation, the correlating lower levels of neuron formation could lead to less adaptive behavior and have a depressive affect.

The current study demonstrates the downregulated effects of AGEs on the proliferating cells and their neurogenic differentiation, although the interpretation of the data presented here has certain limitations. For example, although BrdU is the preferred technique for labeling newborn cells, the putative factors involved in BrdU-labeled cells cannot be excluded, because BrdU can incorporate itself into nicked or damaged DNA that is undergoing repair. These findings test, in vivo, the possibility that diabetes impairs hippocampal function via the negative effects of AGEs on the generation of new neurons, which probably represents a putative association with
diabetes-related depression-like behaviours. Further studies is indispensable to provide the precise mechanism of the roles of AGEs in diabetes-related neurophysical and neurodegenerative disorders. In addition, the serum level of AGE-P may serve as a useful marker for monitoring pathological processes and the progression of depressive disorders in diabetic patients. The inhibition of AGE formation and the reduction of increased levels of AGE-P among animals with diabetes demonstrate potential as therapeutic targets.

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Table 1. Relative Sucrose Intake in All Groups at Baseline, 4 and 8 Weeks after Diabetic Verification

<table>
<thead>
<tr>
<th>Group(n)</th>
<th>Baseline</th>
<th>Week 4</th>
<th>Week 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON(10)</td>
<td>69.6±2.3</td>
<td>71.2±2.2</td>
<td>71.0±2.2</td>
</tr>
<tr>
<td>DM+N(10)</td>
<td>70.2±2.4</td>
<td>69.6±3.1</td>
<td>67.4±1.9</td>
</tr>
<tr>
<td>DM+D(10)</td>
<td>71.2±3.1</td>
<td>51.8±2.8 ab</td>
<td>49.2±1.9 ab</td>
</tr>
<tr>
<td>DM+D+AG(10)</td>
<td>70.6±2.5</td>
<td>52.8±2.9 ab</td>
<td>58.4±3.4 c</td>
</tr>
</tbody>
</table>

Values are means±S.D. See text for results of relevant statistical tests. CON, control; DM+N, streptozotocin-induced diabetic rats without depression-like behaviour; DM+D, streptozotocin-induced diabetic rats with depression-like behaviour; AG, aminoguanidine.

a p < 0.001 DM+N, DM+D vs. respective CON value.
b p < 0.001 DM+D vs. DM+N value.
c p < 0.001 DM+D+AG vs. DM+D value.

Table 2. Forced swim test in All Groups at 4 and 8 Weeks after Diabetic Verification

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Immobility time (s)</th>
<th>Swimming time (s)</th>
<th>Climbing time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 4</td>
<td>Week 8</td>
<td>Week 4</td>
</tr>
<tr>
<td>CON(10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM+N(10)</td>
<td>44.9±8.3</td>
<td>43.9±8.4</td>
<td>128.1±7.2</td>
</tr>
<tr>
<td>DM+D(10)</td>
<td>41.0±6.8</td>
<td>41.7±7.4</td>
<td>128.3±6.4</td>
</tr>
<tr>
<td>DM+D+AG(10)</td>
<td>98.2±8.7 ab</td>
<td>42.5±6.2 c</td>
<td>96.8±7.2 ab</td>
</tr>
</tbody>
</table>

Values are means±S.D. See text for results of relevant statistical tests. CON, control; DM+N, streptozotocin-induced diabetic rats without depression-like behaviour; DM+D, streptozotocin-induced diabetic rats with depression-like behaviour; AG, aminoguanidine.

a p < 0.001 DM+D, DM+D+AG vs. respective CON value.
b p < 0.001 DM+D, DM+D+AG vs. DM+N value.
c p < 0.001 DM+D+AG vs. DM+D value.

Table 3. Serum Level of AGE-peptides in All Groups at Baseline, 4 and 8 Weeks after Diabetic Verification

<table>
<thead>
<tr>
<th>Group(n)</th>
<th>Baseline (U/mL)</th>
<th>Week 4 (U/mL)</th>
<th>Week 8 (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON(10)</td>
<td>0.99±0.04</td>
<td>1.00±0.04</td>
<td>1.00±0.05</td>
</tr>
<tr>
<td>DM+N(10)</td>
<td>1.02±0.05</td>
<td>3.46±0.19 ab</td>
<td>3.48±0.08 a</td>
</tr>
<tr>
<td>DM+D(10)</td>
<td>0.99±0.04</td>
<td>3.49±0.13 a</td>
<td>3.48±0.07 a</td>
</tr>
<tr>
<td>DM+D+AG(10)</td>
<td>1.00±0.05</td>
<td>3.45±0.09</td>
<td>2.56±0.12 b</td>
</tr>
</tbody>
</table>

Values are means±S.D. See text for results of relevant statistical tests. CON, control; DM+N, streptozotocin-induced diabetic rats without depression-like behaviour; DM+D, streptozotocin-induced diabetic rats with depression-like behaviour; AG, aminoguanidine.

a p < 0.001 DM+N, DM+D vs. respective CON value.
b p < 0.001 DM+D+AG vs. DM+D value.
**Figure legend**

FIG. 1. Representative images illustrating bromodeoxyuridine (BrdU) immunostaining for proliferating cells in dentate gyrus (DG) of the brains (upper, original magnification, 20×), and of brains of all experimental rats (low, original magnification, 20×). BrdU labelling cells disperse mainly in the subgranular zone (SGZ), rarely in either the granule cell layer (GCL) or the hilus (Hilar). CON: Control rats injected with vehicle alone; DM+N: streptozotocin-induced diabetic rats without depressive-like behaviour; DM+D: streptozotocin-induced diabetic rats with depressive-like behaviour; DM+D+AG: aminoguanidine (AG, 10mg/kg) administrated in DM+D rats for 4 weeks. BrdU (Sigma), 50 mg/kg in saline i.p., was given twice daily, at 8-h intervals, on consecutive days before Week 8. Rats were killed the day after the last dose.

FIG. 2. Confocal microscopy of a representative double-stained hippocampal section showing cells labelled with bromodeoxyuridine (BrdU) (Left, red), neuron specific nuclear protein (NeuN) (Centre, green); BrdU and NeuN labelling are shown in both isolated and overlapping cases (Right). The experiment was repeated thrice, and similar results were obtained each time (original magnification, 20×). CON: Control rats injected with vehicle alone; DM+N: streptozotocin-induced diabetic rats without depressive-like behaviour; DM+D: streptozotocin-induced diabetic rats with depressive-like behaviour; DM+D+AG: aminoguanidine (AG, 10mg/kg) administrated in DM+D rats for 4 weeks. Animals were injected with BrdU (50 mg/kg, i.p.) three times a day at 4-h intervals on 4 consecutive days after Week 6 and sacrificed at Week 8.

FIG. 3. Survival (A) and differentiation (B) of proliferating cells in dentate gyrus (DG) of the brains of all experimental rats. CON: Control rats injected with vehicle alone; DM+N: streptozotocin-induced diabetic rats without depressive-like behaviour; DM+D: streptozotocin-induced diabetic rats with depressive-like behaviour; DM+D+AG: aminoguanidine (AG, 10mg/kg) administrated in DM+D rats for 4 weeks. Animals were injected with BrdU (50 mg/kg, i.p.) three times a day at 4-h intervals on 4 consecutive days after Week 6 and sacrificed at Week 8. \( ^a p < 0.001 \) DM+N, DM+D vs. CON value; \( ^b p < 0.001 \) DM+N vs. DM+D; \( ^c p < 0.001 \) DM+D+AG vs. DM+D value. Values are means ± SD.
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