Formaldehyde induces diabetes-associated cognitive impairments

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ABSTRACT: Patients with type 2 diabetes mellitus (T2DM) often develop cognitive impairments and have an increased risk of developing Alzheimer's disease. Hyperglycemia is a major characteristic of T2DM, but how elevated glucose levels lead to cognitive decline remains elusive. Here, we report that patients with T2DM and mutations in the formaldehyde (FA)-degrading enzyme aldehyde dehydrogenase 2 (*ALDH2*) gene had higher levels of FA and more severe dementia. Injection of FA induced hyperglycemia and cognitive deficits in rats. Ablation of gene expression of *ALDH2*, the main enzyme to oxidize FA, resulted in abnormally high levels of hippocampal FA, leading to hyperglycemia and cognitive impairments as well as potentiating streptozotocin-induced diabetes development in *ALDH2* knockout mice. We found that FA interacts with insulin to form FA-insulin adducts, and these FA-insulin adducts caused insulin deficiency, contributing to memory decline in diabetic rodent models. Reduction of FA by transgenic overexpression of human *ALDH2* attenuates hyperglycemia and alleviates cognitive deficits in diabetic mouse models. These findings suggest that excess FA plays a critical role in mediating diabetes-related dementia. Targeting FA and its metabolizing enzyme ALDH2 may be a valid approach for preventing and treating dementia in diabetes mellitus.—Tan, T., Zhang, Y., Luo, W., Lv, J., Han, C., Hamlin, J. N. R., Luo, H., Li, H., Wan, Y., Yang, X., Song, W., Tong, Z. Formaldehyde induces diabetes-associated cognitive impairments. FASEB J. 32, 3669–3679 (2018). www.fasebj.org

KEY WORDS: ALDH2 · hyperglycemia · dementia · Alzheimer

Diabetes mellitus is a devastating metabolic disorder, and patients with diabetes often experience significant cognitive impairments. Approximately 70% of patients with diabetes eventually develop Alzheimer's disease (AD) and related dementia, and patients with AD have a higher than normal tendency to develop type 2 diabetes mellitus (T2DM) (1). Cognitive decline is a common pathologic characteristic in T2DM and AD (2). However, the molecular mechanisms underlying diabetes-induced cognitive impairments and the link between diabetes and AD remain unclear. As the major pathologic hallmark of diabetes, hyperglycemia has been considered the key contributor to cognitive decline in patients with diabetes (3).

Formaldehyde (FA) is a common environmental pollutant that is also produced by the human body during normal metabolism. The endogenous reactions that generate FA include methanol oxidation, histone demethylation, and methylamine deamination (4, 5). Growing evidence suggests that excess FA is a critical factor contributing to age-related or pathologic memory loss (6, 7). Compared with healthy control subjects, patients with AD have significantly increased FA concentrations in both hippocampi and urine, which are inversely correlated to patient's Mini–Mental State Examination (MMSE) scores (8). Elevated FA levels have also been observed to be associated with cognitive decline in AD animal models (8, 9). High levels of FA induce tau hyperphosphorylation (10) and suppress hippocampal long-term potentiation (LTP) (11).

ABBREVIATIONS: Aβ, amyloid β; AD, Alzheimer's disease; ALDH2, aldehyde dehydrogenase 2; FA, formaldehyde; fluo-HPLC, HPLC with fluorescence detection; GLUT3, glucose transporter type 3; *hALDH2*, human aldehyde dehydrogenase 2; HG, high glucose; KO, knockout; L-STZ, lower dose of streptozotocin; LTP, long-term potentiation; MMSE, Mini–Mental State Examination; STZ, streptozotocin; T1/2DM, type 1/2 diabetes mellitus; Tg, transgenic; WT, wild type

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Mitochondrial aldehyde dehydrogenase 2 (ALDH2) is the main enzyme responsible for oxidizing FA (12) and is ubiquitously expressed to promptly metabolize excess FA (13). Individuals carrying *ALDH2* variants are vulnerable to neural damage and to developing AD (14, 15). Moreover, aged *ALDH2*$^{-/-}$ mice exhibit AD-like pathologies, including increases in oligomeric amyloid β protein (Aβ), synaptic loss, vascular pathologies, and cognitive deficits (16). In AD mouse models, both the transcription and translation of ALDH2 are markedly reduced (17, 18). Interestingly, recent studies have shown that *ALDH2* mutations are closely associated with an increased risk of diabetes (19). In addition, a decline in ALDH2 activity has been detected in streptozotocin (STZ)-induced diabetes model rats (20). Furthermore, FA has been found to affect glucose metabolism (21), elevate blood glucose levels (22, 23), and cause memory decline (6). These data strongly suggest that alterations in ALDH2-mediated FA metabolism and accumulated FA may play a pathologic role in contributing to cognitive decline and AD pathogenesis during diabetes.

In the present study, patients with diabetes and *ALDH2* polymorphisms were analyzed. We found that higher levels of FA which result from loss of ALDH2 activity in T2DM are associated with the more severe hyperglycemia and cognitive impairments. We also found that FA induces hyperglycemia and cognitive impairment, whereas reduction of FA by transgenic (Tg) overexpression of human (*h*)*ALDH2* attenuates hyperglycemia and alleviates cognitive deficits in both T1DM and T2DM model mice. Our research provides evidence that targeting FA and its metabolizing enzyme ALDH2 may be an effective approach for preventing and treating dementia in diabetes.

MATERIALS AND METHODS

Participants

Participants were recruited from Beijing Geriatric Hospital and 2 homes for the elderly in Beijing, China, between 2013 and 2015 in accordance with approved procedures and guidelines of the Clinical Ethics Committee at Capital Medical University. Written informed consent was obtained from each participant, either directly or from his or her guardian, before participation. Participants who refused to provide blood or urine samples, had a life-threatening illness, or were unable to participate in the assessments were excluded from the entire survey. The mean age of the 153 individuals (80 elderly patients with diabetes and 73 age-matched control subjects) was 72.66 ± 3.88 yr. Cognitive function was assessed by performing the MMSE. Morning urine and venous blood samples were collected for biochemical analysis.

DNA extraction and human genotyping analysis

DNA was extracted from fresh blood by using a genomic DNA extraction mini kit and stored at −80°C. Human genotyping was performed by using the DNA primers: forward, 5′-CAAATTACAGGGTCAACTGCT-3′; reverse, 5′-CCACACTCACAGTTTTCTCTT-3′. Agarose gel electrophoresis was performed.

Generation of *ALDH2*$^{-/-}$ mice and human *ALDH2* Tg mice

All animal protocols were approved by the Biologic Research Ethics Committee at Capital Medical University. Three-month-old male C57BL/6 mice were housed in a temperature-controlled room under a 12-h light-dark cycle with access to water and food *ad libitum*. To generate *ALDH2*$^{-/-}$ mice, a DNA fragment of the targeting vector (pGT-5, 3 mg/ml) was microinjected into the fertilized eggs taken from superovulated C57BL/6 females. The injected eggs were then transferred to the oviducts of C57BL/6 pseudopregnant females to produce male chimeric mice. All offspring were tested for the presence of the knockout (KO) and/or wild-type (WT) *ALDH2* alleles by PCR. *Human ALDH2*$^{+/+}$ Tg mice were generated by using the CAG promoter expression system. The human *ALDH2* (*hALDH2*) gene was amplified by PCR using the DNA primers F1: 5′-GGAAGATGTGGACAAGGCAG-3′; R1: 5′-ATGCCACTTTGTCCACATCC-3′ to generate a 560 bp product. ALDH2 enzymatic activity was measured as described in a previous study (20).

Detection of FA, glucose, and hippocampal insulin levels

Blood and morning urine samples from the human subjects were taken before breakfast. After centrifugation (8000 *g*, 4°C, 10 min), blood and urine FA levels were measured by using HPLC with fluorescence detection (fluo-HPLC). Blood was obtained from the tail vein of rodents. Blood glucose levels were then measured by using OneTouch II (LifeScan, Inc., Milpitas, CA, USA). Hippocampal insulin levels of the rodents were assessed by using a Radioimmunoassay Kit (China Institute of Atomic Energy, Beijing, China).

FA and STZ treatments

The FA lethal dose 50% values were measured in male mice. Mice (n = 10, each group) were given an injection of 10% formalin in physiologic saline in doses ranging from 0.099 to 0.249 g/kg. A lethal dose of FA was defined as the amount causing death within 90 min. *ALDH2*$^{-/-}$, WT, and *hALDH2*$^{+/+}$ mice were intraperitoneally injected with STZ in a low dose (50 mg/kg) or a high dose (200 mg/kg). Mice with blood glucose levels >13 mM were considered diabetic. After 6 wk of treatment, the Morris water maze test was performed as previously described (24).

Electrophysiologic recording *in vivo*

Hippocampal LTP recordings and intracerebroventricular injection of FA (0.45 mM) in Sprague-Dawley rats (n = 10/group) were performed according to our previous reports (11).

High glucose-, STZ-, and FA-induced diabetes rat models

For acute treatment, healthy adult male Sprague-Dawley rats (180 ± 2.5 g) were housed under a 12 h light-dark cycle and randomly divided into 2 groups (n = 8/group) then intraperitoneally injected with saline or FA (0.45 mM) for 7 consecutive days. For chronic treatment, adult rats were randomly divided into 4 groups with 60 in each group. Each group was then divided into 6 groups (n = 10/group). Rats were intraperitoneally injected with a single dose of STZ (60 mg/kg) or daily injected with saline (10 mM), glucose (1 g/kg), or FA (60 mg/kg) for

8 consecutive weeks. Insulin (2 U/kg/d) was subcutaneously injected into STZ-diabetes rats or FA-injected rats. After 8 wk of treatment, animals were subjected to the Morris water maze test or detected with global DNA 5-methylcytosine or ^{3}H-glucose uptake.

Chronic high glucose or STZ treatment combined with a high-fat diet induced T2DM characteristics in *hALDH2*$^{+/+}$ mice

A group of *hALDH2*$^{+/+}$ mice were injected with glucose (1 g/kg) for 6 wk. To generate a T2DM mouse model, C57BL/6 or *hALDH2*$^{+/+}$ mice (n = 10/group) were intraperitoneally injected with a low-dose STZ (40 mg/kg) for 3 consecutive days and fed a high-fat diet (D12492-high fat diet; Research Diets Inc., New Brunswick, NJ, USA, and Thermo Fisher Scientific, Waltham, MA, USA) for 6 wk. Blood glucose levels and body weight were checked once a week.

Structural and functional measurement of insulin and FA-insulin adducts

Electrophoresis

Human insulin (Sigma-Aldrich, St. Louis, MO, USA) was diluted to 0.4 mM in PBS and then incubated with FA (0.5 mM; Sigma-Aldrich) at 37°C for 0, 1, 12, and 24 h. FA-insulin adducts were mixed with a loading buffer containing glycerol and SDS and then analyzed by Tricine-SDS-PAGE.

Immunoblotting analysis

Immunoblotting assays were performed by using an mAb toward insulin (1:1000 dilution). Washes, secondary antibody, and detection procedures were performed by using the BM Chemiluminescence Western Blotting Kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions.

Thioflavin T–binding assays

Insulin was incubated at 37°C in the presence of FA for 1 h. The signal was obtained by measuring thioflavin T emission at 482 nm.

Fluorescence spectrometer

Insulin samples were continuously excited at 276 nm (λex) using the Hitachi F-4600 fluorescence spectrophotometer setup (Hitachi High-Technologies, Tokyo, Japan). The excitation and emission slit widths were set at 5.0 nm. The detected emission fluorescence of insulin is at 297 nm (λ_{em}), mainly derived from tyrosine-19 in the A-chain of insulin. The D value ($\Delta\lambda$) between the excitation and emission wavelengths was set at 20 or 65 nm. The voltage was 600 V.

Confocal microscopy

Primary hippocampal neurons were dissociated from 18-d-old embryos. Cultured neurons were then incubated with normal insulin or FA-insulin adducts. Because Ca^{2+} is involved in insulin-mediated glucose uptake, intracellular Ca^{2+} influx was detected by using Fluo-3 AM (Thermo Fisher Scientific) and imaged by using a laser scanning confocal microscope.

Glucose uptake

Assessment of ^{3}H-2-deoxy-D-glucose in cultured hippocampal neurons and slices (n = 6/group) was performed as previously described (25).

Quantification of global methylation levels in the hippocampi

Nuclear DNA was extracted. The global DNA methylation (5-methylcytosine) of hippocampal homogenates (n = 6/group) was quantified by using an Ultra Kit (Epigenetek, Farmingdale, NY, USA).

Experimental design and statistical analysis

All data were analyzed by using IBM SPSS v.19.0 (IBM SPSS Statistics, IBM Corp., Armonk, NY, USA) software. The χ^2 test was used to determine whether the *ALDH2* genotypes were in Hardy-Weinberg equilibrium. The clinical characteristics and biomarkers were compared by using the χ^2 test for categorical variables and ANOVA for continuous variables. The correlations among urine/blood FA samples, blood glucose levels, and MMSE scores were analyzed by descriptive statistics-correlation coefficient. The changes in the response amplitudes of LTP were analyzed by using mixed design ANOVAs. The statistical significance of the Morris water maze test results was analyzed with repeated measures ANOVA followed by Tukey's *post hoc* analysis. For other experiments, statistical significance was determined by using the Student's t test (for independent or dependent samples, as appropriate), with $P < 0.05$ (2-tailed) considered significant. Data are reported as mean ± SEM.

RESULTS

Higher levels of FA associated with cognitive impairments in T2DM with *ALDH2*^{2*2}

ALDH2 mutations have been suggested to be associated with increased diabetes risk, and ALDH2 is the main enzyme that oxidizes excess FA. To examine the effect of *ALDH2* variants on diabetes-associated dementia, 80 elderly patients with T2DM and 73 age-matched control subjects were recruited in this study. The information on age, sex, education level, blood glucose values, and FA levels in blood and urine samples are shown in **Table 1**. G487A mutations in *ALDH2* were found and classified into 3 genotypes in the patients with T2DM: normal or typical homozygote *ALDH2*^{1*1} (GG), heterozygote *ALDH2*^{1*2} (GA), and homozygous mutant *ALDH2*^{2*2} (AA). The distribution of *ALDH2* genotypes was consistent with Hardy-Weinberg equilibrium. However, *ALDH2* variants in the patients with diabetes were strongly associated with elevated FA concentrations in blood and urine samples, increased blood glucose levels, and reduced scores on the MMSE (**Fig. 1*A*–*H***). Blood ALDH2 activity was negatively correlated with urine FA levels (R = −0.638) (Fig. 1*I*), suggesting that an *ALDH2* mutation leads to FA accumulation. Endogenous FA levels were negatively correlated with MMSE scores (R = −0.575) and positively correlated with blood glucose levels (R = 0.673) (Fig. 1*J, K*), whereas blood glucose levels were negatively correlated with MMSE scores

TABLE 1. *T2DM with ALDH2 deficiency exhibit excess FA, hyperglycemia, and cognitive decline*

Variable	Diabetes (n = 80)	Control (n = 73)	P
Age (yr)	72.14 ± 4.15	73.18 ± 3.62	>0.05
Sex (male/female)	33/47	31/42	>0.05
Education (yr)	8.73 ± 1.67	6.92 ± 2.46	>0.05
Blood glucose (mM)	9.78 ± 1.14	5.37 ± 0.04	<0.01
Blood FA (mM)	0.087 ± 0.002	0.078 ± 0.003	<0.01
Urine FA (mM)	0.044 ± 0.02	0.031 ± 0.001	<0.01
MMSE scores	15.17 ± 1.14	28.19 ± 0.09	<0.01
Genotype: $ALDH2^{1.1}$	23 (28.75%)	52 (71.23%)	<0.01
Genotype: $ALDH2^{1.2}$	36 (45.00%)	16 (21.91%)	<0.05
Genotype: $ALDH2^{2.2}$	21 (26.25%)	5 (6.84%)	<0.01
Allele: ALDH2*1	82 (51.25%)	120 (82.19%)	<0.01
Allele: ALDH2*2	78 (48.75%)	26 (17.80%)	<0.01

($R = -0.291$) (Fig. 1*L*). These findings suggest that the higher FA levels which result from loss of ALDH2 activity in T2DM are associated with more severe hyperglycemia and cognitive impairments.

FA treatment induces hyperglycemia and cognitive deficits in rats

To examine the effect of FA on glucose metabolism and cognitive function, we injected healthy adult male Sprague-Dawley rats with 0.45 mM FA, a pathologic concentration previously reported in STZ-induced diabetes rat models (11). An intraperitoneal injection of FA for 7 d had no effect on body weight but significantly increased blood glucose levels as well as blood and hippocampal FA levels (**Fig. 2*A–D***). To examine the effect of FA on synaptic plasticity, we assessed the LTP *in vivo*. Intracerebroventricular injection of 0.45 mM FA markedly reduced average field excitatory postsynaptic potential amplitudes of LTP in the injected rats compared with control rats ($P < 0.01$) (Fig. 2*E*).

Furthermore, the rats were subjected to the Morris water maze test, and we found that there was no difference in vision and mobility between the FA-injected rats and control rats in the visible platform tests. However, the FA-injected rats exhibited a marked increase in escape latency on acquisition d 3–6 compared with control rats ($P < 0.01$) (Fig. 2*F*). In the probe trial on the last day of testing, the FA-injected rats spent less time and had a shorter distance in the target quadrant than control rats ($P < 0.01$) (Fig. 2*G, H*). These results indicate that injection of FA induces hyperglycemia and cognitive deficits in the healthy rats.

STZ induces elevated FA levels and cognitive deficits in *ALDH2* KO mice

To further investigate the effect of FA on blood glucose level and cognitive function, we generated *ALDH2* KO ($ALDH2^{-/-}$) mice. ALDH2 protein expression was abolished in $ALDH2^{-/-}$ mice (**Fig. 3*A, B***). Disrupting *ALDH2* expression resulted in increased blood and hippocampi FA levels in the $ALDH2^{-/-}$ mice (Fig. 3*C, D*). Previous studies have identified that diabetes can be induced in WT mice using a single high dose of STZ (≥200 mg/kg) (26). Here, we examined whether a lower dose of STZ (L-STZ, 50 mg/kg) could induce diabetes in $ALDH2^{-/-}$ mice. The results showed that L-STZ treatment led to weight loss (Fig. 3*E*) and hyperglycemia (Fig. 3*F*) in $ALDH2^{-/-}$ mice but not in WT mice 6 wk posttreatment. These data indicate that FA accumulation due to loss of ALDH2 potentiates STZ-induced diabetes development in $ALDH2^{-/-}$ mice.

We then examined the effect of L-STZ on cognitive function in $ALDH2^{-/-}$ mice using the Morris water maze test. In the visible platform tests, there were no differences in vision or mobility between L-STZ–injected $ALDH2^{-/-}$ mice (L-STZ KO) and L-STZ–injected WT mice (L-STZ WT). However, there was a significant difference in escape latency on acquisition d 4–6 between L-STZ KO and L-STZ WT mice during the Morris water maze test ($P < 0.01$) (Fig. 3*G*). In the probe trial on the last day of testing, L-STZ KO mice spent less time in the target quadrant than L-STZ WT mice ($P < 0.01$) (Fig. 3*H*). These data suggest that abolishing *ALDH2* expression resulted in accumulation of endogenous FA, contributing to cognitive impairment in mice with hyperglycemia induced by STZ.

Excess FA facilitates hyperglycemia and cognitive impairment by interacting with insulin

Previous studies have shown that reactive aldehydes play an important role in the deterioration of DM, and hydroxynonenal can interact with insulin to form hydroxynonenal–insulin adducts, leading to decreased levels of reactive insulin (27). Therefore, we investigated whether FA affects insulin structure and activity and examined the effects of 8-wk 0.45 mM FA injection on cognitive function in Sprague-Dawley rats.

First, we found that FA induced a time-dependent aggregation with several higher MW bands detected above the insulin monomer both *in vitro* and *in vivo* (**Fig. 4*A, B***). In addition, the result of thioflavin T assay indicates that these aggregates are mainly formed by the interactions between FA and insulin instead of insulin self-aggregation (Fig. 4*C*). Using fluorescence spectrometry to detect alterations in insulin structure, we found that FA-insulin adducts exhibited a time-dependent decrease in fluorescence intensities, and the length of treatment times was negatively correlated with fluorescence intensities ($R = -0.924$) (Fig. 4*D, E*). Hence, FA can react with insulin and form FA-insulin adducts both *in vitro* and *in vivo*.

We next compared the activity between FA-insulin adducts and normal insulin. The results showed that FA-insulin adducts induced a lower level of intracellular Ca^{2+} influx compared with normal insulin (Fig. 4*F*). These adducts also inhibited glucose uptake in cultured hippocampal neurons (Fig. 4*G*). FA-insulin adducts had lower activity in reducing blood glucose levels compared with normal insulin when injected into STZ-induced diabetes rats (Fig. 4*H*).

To further examine whether excess FA affects blood glucose level and cognitive function by interacting with

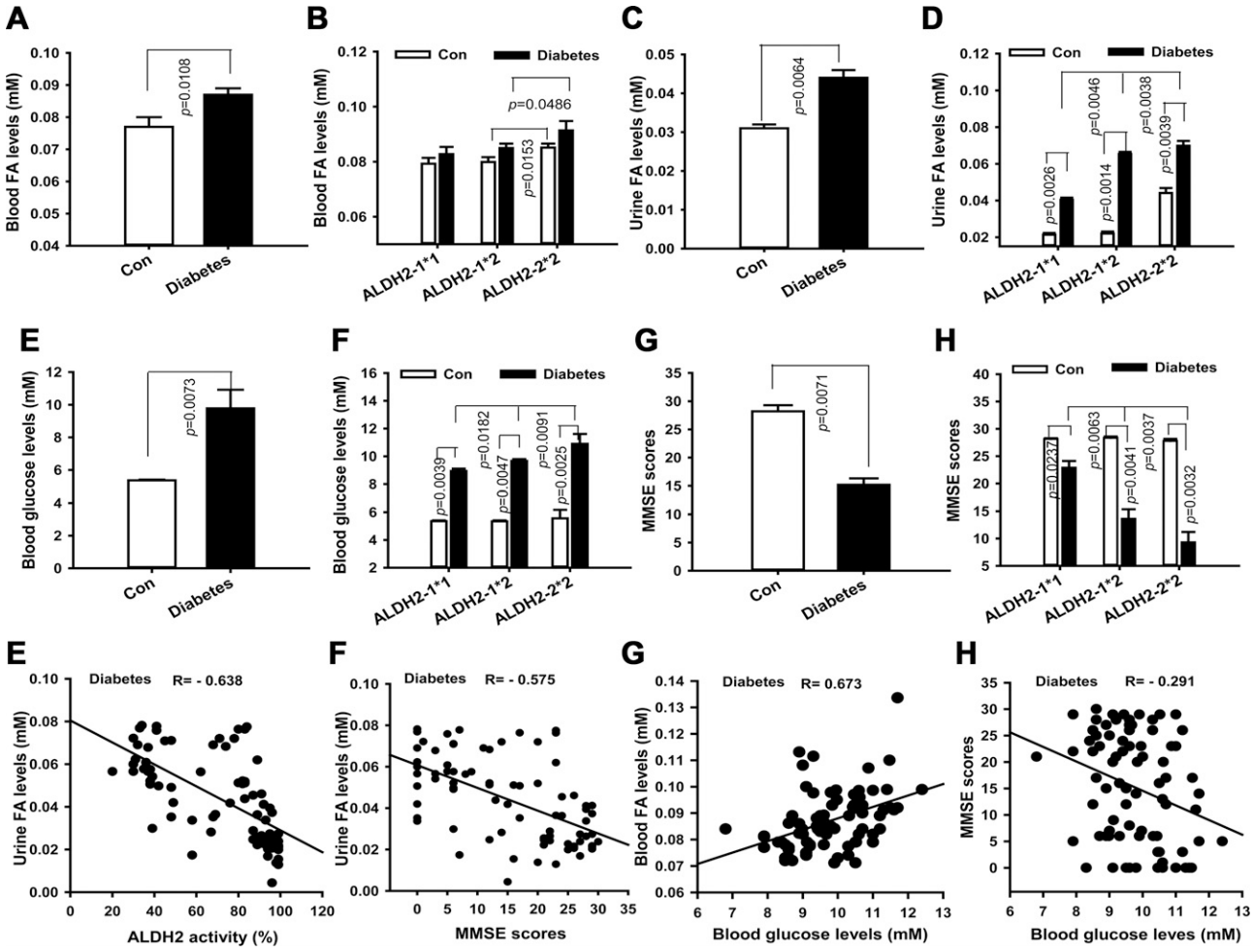

Figure 1. Effects of *ALDH2* mutations on glucose status and cognitive function in control (Con) individuals and patients with diabetes. *A–D*) FA levels in blood (*A*, *B*) and urine (*C*, *D*) were quantified by performing fluo-HPLC. *E–H*) Blood glucose levels (*E*, *F*) and MMSE scores (*G*, *H*) in the control and diabetes groups. *I*, *J*) The correlation between the urine FA level and ALDH2 activity and MMSE score. *K*) The correlation between blood FA level and blood glucose concentration. *L*) The correlation between the MMSE score and blood glucose level. Statistical analysis: 1-way ANOVA with *post hoc* Newman-Keuls tests, and descriptive statistics-correlation coefficient.

endogenous insulin, FA with or without insulin was injected into healthy adult Sprague-Dawley rats for 8 wk. Results showed that insulin injection markedly reduced the high blood glucose levels and increased neuronal glucose uptake in the hippocampi of FA-injected rats (Fig. 4*I, J*). Moreover, insulin treatment significantly improved the spatial memory in the FA-injected rats, which showed shorter escape latency on d 3–6 during the Morris water maze test and spent more time in the target quadrant in the probe trial on the last day than the rats injected only with FA ($P < 0.01$) (Fig. 4*K, L*). These results indicate that FA-induced insulin deficiency contributes to memory decline in diabetic rodent models.

Hyperglycemia induces memory loss by gradually increasing FA generation

To investigate the molecular mechanism underlying cognitive impairments in the patients with T2DM, we injected high glucose (HG) into healthy adult Sprague-Dawley rats and used STZ-diabetes rats as positive controls. Notably, chronic 8-wk HG injection, as well as STZ treatment, induced a significant elevation in hippocampal FA levels (>0.4 mM) and blood glucose levels in rats (**Fig. 5*A, B***). Moreover, after 8 wk, the Morris water maze test was performed. Results showed that there was no difference in mobility, but a significant difference in escape latency occurred on d 4–6 between the diabetes model rats and control rats ($P < 0.01$) (Fig. 5*C*). In the probe trial, both STZ- and HG-injected rats spent less time in the target quadrant than control rats on d 7 ($P < 0.01$) (Fig. 5*D*). Furthermore, the time spent in the target quadrant was negatively correlated with the FA levels in the hippocampi of the rats (Fig. 5*E*).

We next explored the relationship between hyperglycemia and endogenous FA accumulation. Previous studies have shown that chronic hyperglycemia can induce global DNA demethylation, and DNA demethylation leads to FA generation (28–30). We found that in the hippocampi of STZ- and HG-injected rats, global 5-methylcytosine levels were markedly decreased and negatively correlated with FA levels ($R = -0.518$; $R = -0.887$)

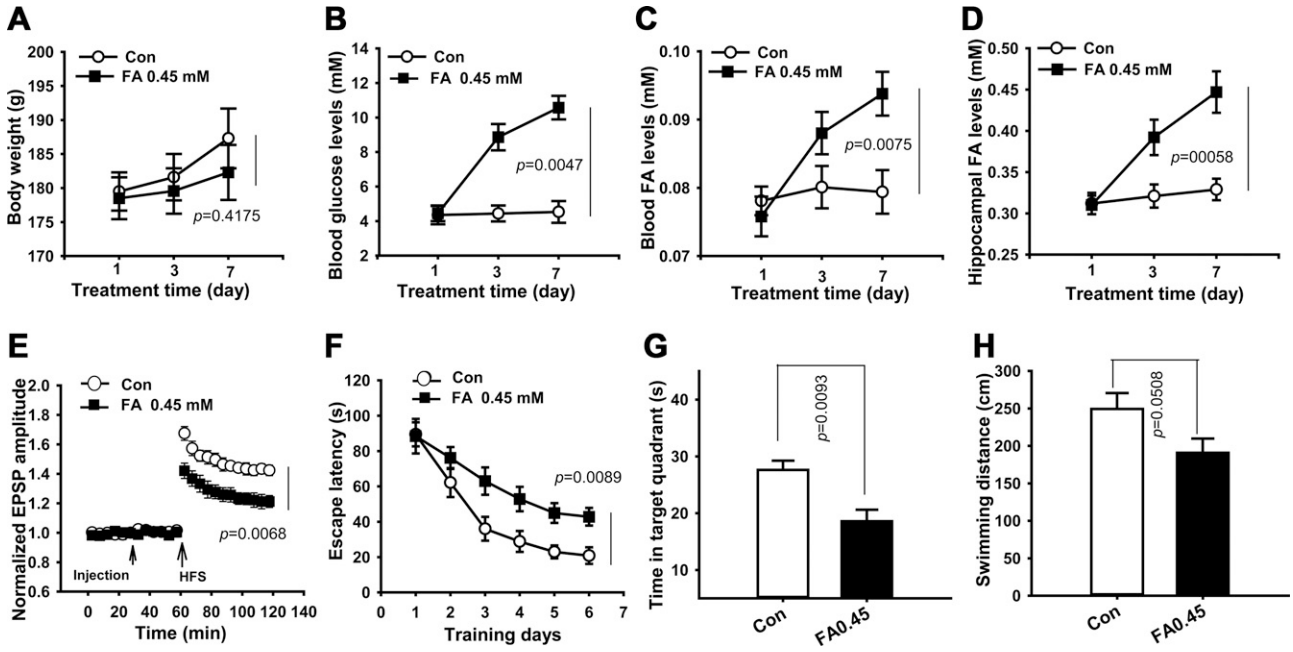

Figure 2. Exogenous application of FA impaired LTP and spatial memory in Sprague-Dawley rats. *A*, *B*) Body weight (*A*) and blood glucose levels (*B*) were measured. *C*, *D*) Blood and hippocampal FA concentrations were quantified by performing fluo-HPLC. *E*) Hippocampal LTP was analyzed by *in vivo* LTP recording. *F*–*H*) Spatial memory was evaluated by performing the Morris water maze test; $n = 8$/group. Data are the means ± SEM. Statistical analysis: 2-way ANOVA with Tukey's *post hoc* test, and 1-way ANOVA with *post hoc* Newman-Keuls tests. Con, control; EPSP, excitatory postsynaptic potential.

(Fig. 5*F, G*), indicating that chronic hyperglycemia induces FA generation by eliciting global DNA demethylation. The elevated FA in HG-injected rats was not derived from loss of ALDH2 activity compared with that of STZ-injected rats after 8 wk of injections (Fig. 5*H*).

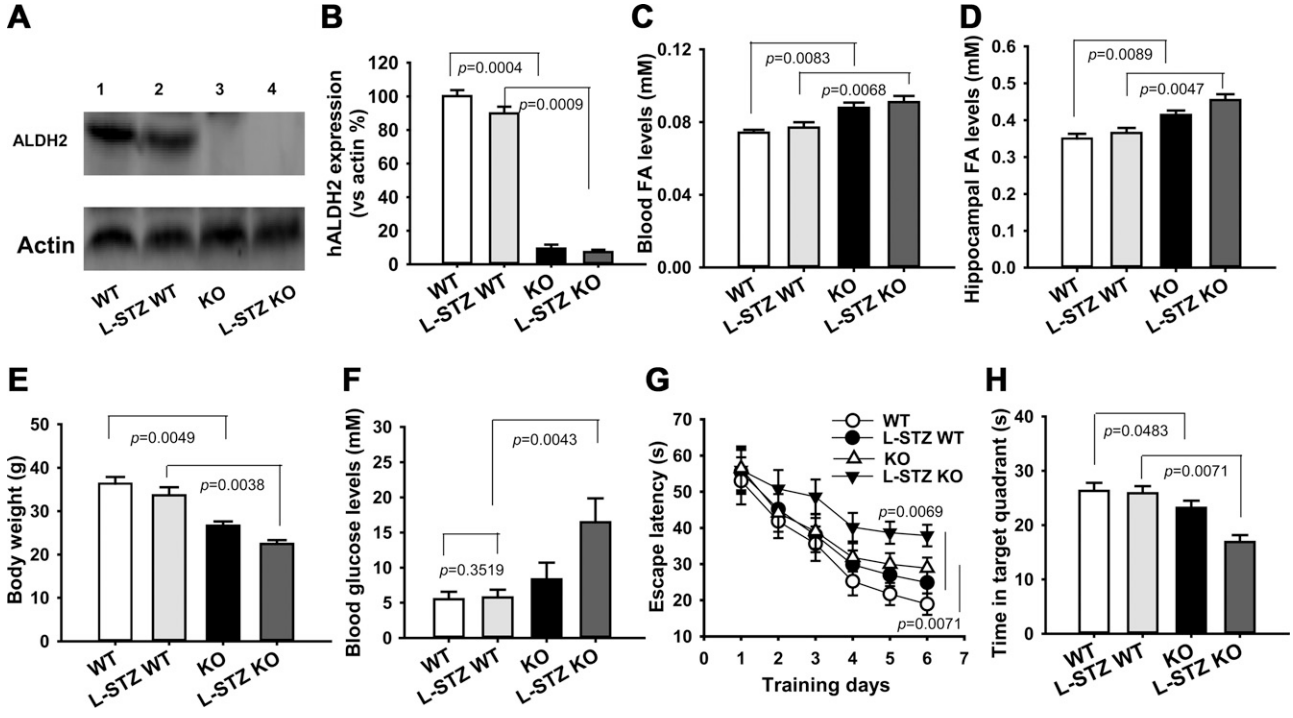

Figure 3. *ALDH2*$^{-/-}$ mice with excess FA were more sensitive to STZ toxicity. *A*) Brain lysates from WT mice, *ALDH2*$^{-/-}$ (KO) mice, WT mice injected with L-STZ (L-STZ WT), and *ALDH2*$^{-/-}$ mice injected with L-STZ (L-STZ KO) were blotted for ALDH2. *B*) Brain ALDH2 expressions were quantified. *C*, *D*) Blood and hippocampal FA concentrations were detected by performing fluo-HPLC. Body weight (*E*) and blood glucose levels (*F*) were measured. *G*, *H*) The Morris water maze was performed for 7 consecutive days to evaluate spatial memory of the mice; $n = 10$/group. Data are means ± SEM. Statistical analysis: 2-way ANOVA with Tukey's *post hoc* test, and 1-way ANOVA with *post hoc* Newman-Keuls tests.

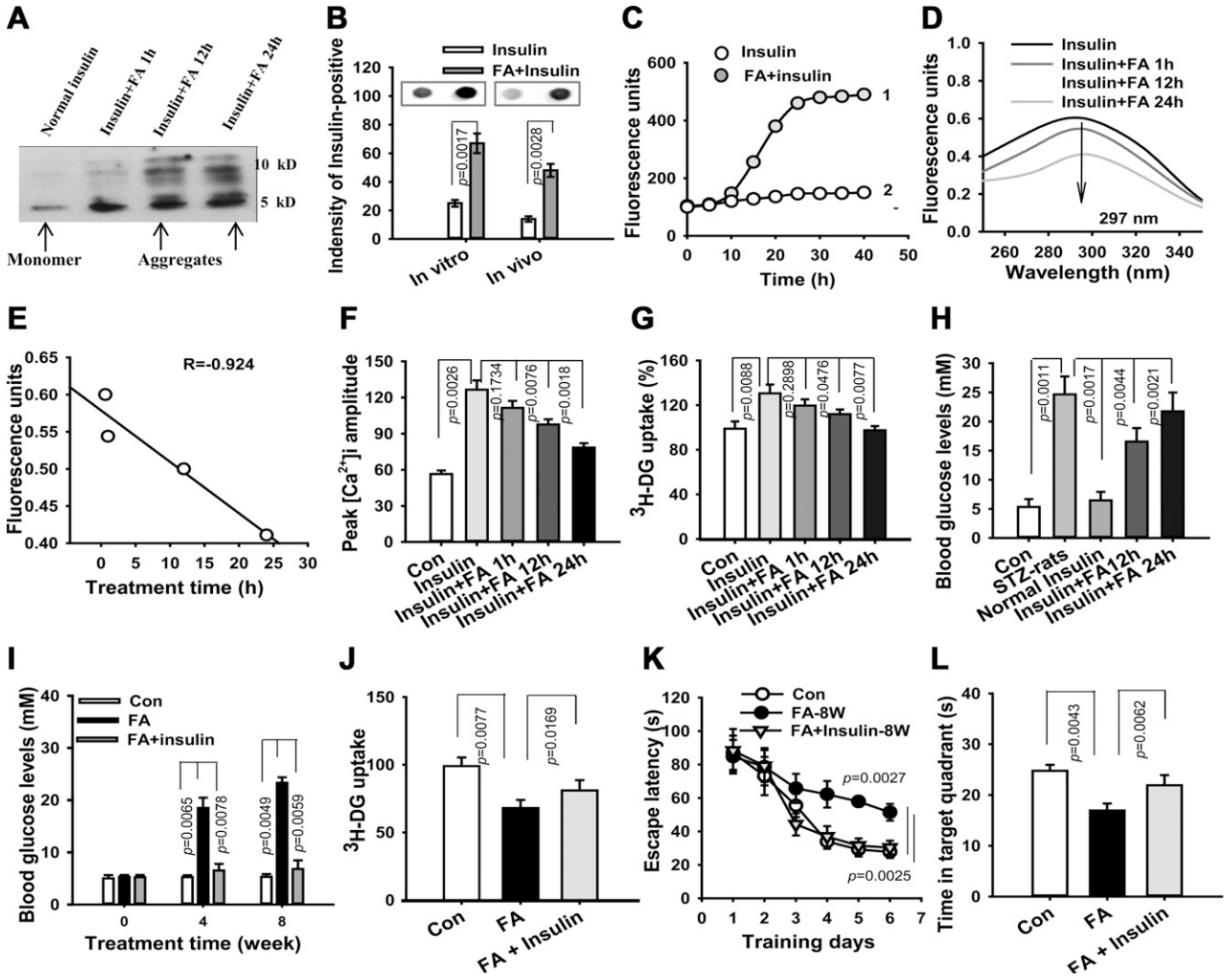

Figure 4. FA aggregates with insulin, leading to insulin dysfunction and memory deficits. *A*) FA-insulin aggregates were detected by electrophoresis. *B*) FA-insulin aggregates were analyzed by immunoblotting with insulin antibody. *C–E*) Fluorescence intensity of insulin was detected by conducting staining with thioflavin T. *F*) Intracellular Ca^{2+} influx was scanned by a confocal laser. *G*) The ability of glucose uptake in cultured hippocampal neurons was quantified by a radioimmunoassay kit with ^{3}H-DG. Results are expressed as the mean ± SEM of 3 independent studies. *H, I*) Blood glucose levels in the rats were detected. *J*) Glucose uptake in the hippocampi of the rats was measured by using a radioimmunoassay kit with ^{3}H-DG. *K, L*) Spatial memory was detected by performing the Morris water maze test for 7 consecutive days; $n = 10$/group. Data are the means ± SEM. Statistical analysis: 2-way ANOVA with Tukey's *post hoc* test, and 1-way ANOVA with *post hoc* Newman-Keuls tests. Con, control.

Reducing FA by overexpressing human *ALDH2* improves glucose state and cognitive function in diabetic mice

Our data here provide strong evidence that FA exacerbates hyperglycemia and induces memory deficits in the diabetic animal models, suggesting that reduction of FA could have protective effect to alleviate glucose levels and improve cognitive function. To examine this issue, we generated *hALDH2*$^{+/+}$ Tg mice, which overexpress *hALDH2*. The mRNA levels of the exogenous *hALDH2* gene were markedly increased in the blood of Tg mice (**Fig. 6*A***). Overexpression of *hALDH2* significantly reduced hippocampal FA levels in *hALDH2*$^{+/+}$Tg mice (Fig. 6*B*). These data show that *hALDH2* overexpression can decrease FA accumulation.

Both WT and *hALDH2*$^{+/+}$ Tg mice were treated with multiple low doses of STZ and fed with a high-fat diet to induce T2DM. Interestingly, the T2DM model mice with overexpression of *hALDH2* (Tg-T2DM) exhibited markedly lower blood glucose levels, as well as blood and hippocampal FA levels, than T2DM model mice without overexpression of *hALDH2* (Fig. 6*C–E*). Compared with WT STZ-injected (WT-STZ) mice, STZ-injected *hALDH2*$^{+/+}$Tg (Tg-STZ) mice exhibited a significant increase in hippocampal insulin level (Fig. 6*F*). Moreover, hippocampal insulin levels were negatively correlated with blood and hippocampal FA levels in all groups of the mice (Fig. 6*G*). In the visible platform tests, there were no differences in vision or swimming speed between Tg-STZ and WT-STZ mice, but the *hALDH2*$^{+/+}$Tg-STZ mice had a significantly shorter escape latency from d 4 to 6 compared with WT-STZ mice during the Morris water maze test ($P < 0.01$) (Fig. 6*H*). In the probe trial, the Tg-STZ mice also spent more time in the target quadrant compared with WT-STZ mice ($P < 0.01$) (Fig. 6*I*).

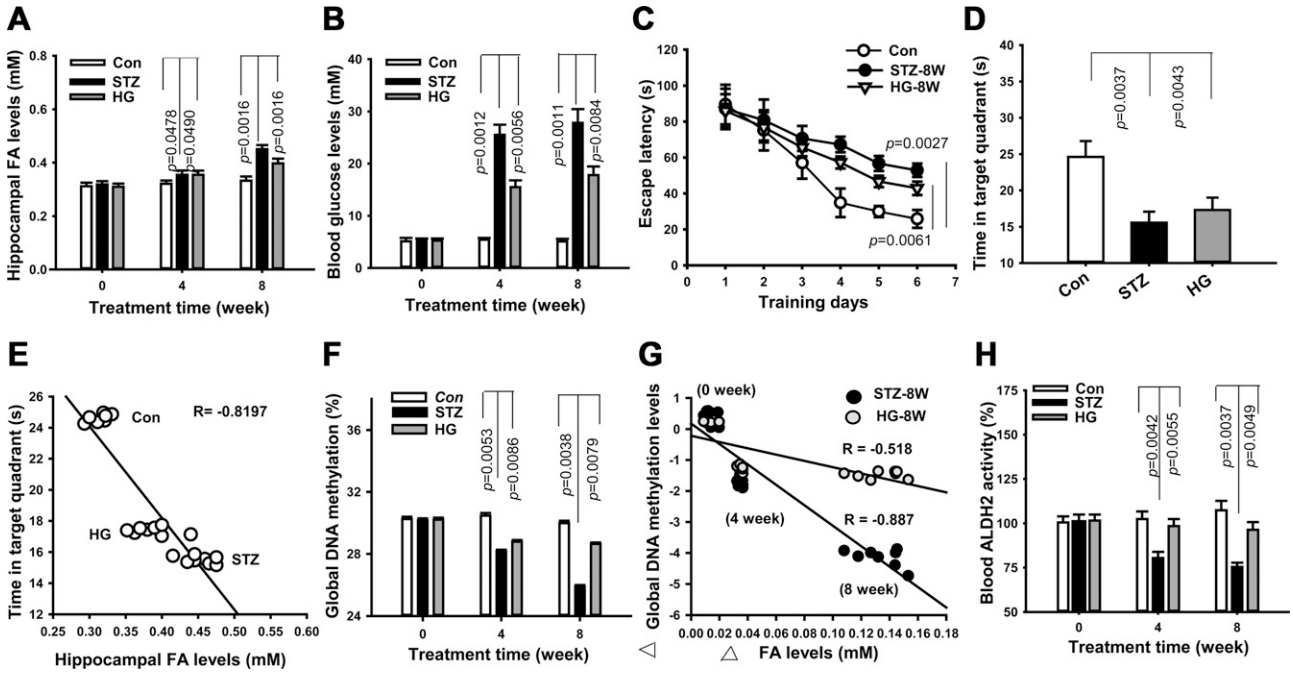

Figure 5. Hyperglycemia-induced FA accumulation and memory deficits in SD rats. Hippocampal FA levels (*A*) and blood glucose levels (*B*) in the rats were detected. *C*, *D*) Spatial memory was evaluated by performing the Morris water maze test. *E*) The correlation between the hippocampal FA level and the time spent in target quadrant during the Morris water maze test. *F*, *G*) The correlation between DNA demethylating levels and hippocampal FA levels. *H*) Blood ALDH2 activity was analyzed by ELISA; $n = 10$/group. Data are means ± SEM. Statistical analysis: 2-way ANOVA with Tukey's *post hoc* test, and 1-way ANOVA with *post hoc* Newman-Keuls tests. Con, control.

Similarly, overexpression of *hALDH2* reversed hyperglycemia and cognitive deficits in T2DM mice or the mice injected with HG (Fig. 6*J–L*). Taken together, our data show that removing excess FA by overexpressing *hALDH2* attenuates hyperglycemia and improves cognitive function in both T1DM and T2DM mice.

DISCUSSION

AD is the most common neurodegenerative disorder leading to dementia, and its pathologic features include amyloid plaques and neurofibrillary tangles (31–33). Cognitive decline is one of the major consequences of diabetes, and growing evidence supports a strong link between AD-related dementia and diabetes (24, 34–37). The prevalence of mild cognitive impairments is dramatically increased in individuals with diabetes (38, 39), and diabetes also significantly accelerates the progression from mild cognitive impairments to dementia (40). Consistent with these studies, our clinical survey revealed that individuals experiencing T2DM had markedly lower MMSE scores compared with nondiabetic healthy control subjects. Patients with diabetes display impaired global cognition, episodic memory, semantic memory, and working memory (41). However, the mechanism underlying the cognitive deficits in patients with diabetes remains unclear. Hyperglycemia has been considered the main candidate contributing to cognitive impairments. In the present study, we found that chronic hyperglycemia increased hippocampal FA generation by inducing global DNA demethylation. More importantly, we showed that the accumulation of FA induced by chronic hyperglycemia facilitated cognitive decline during diabetes.

Previous studies have shown that FA administration by inhalation or injection significantly impairs learning and memory in experimental animals (42–44). In this study, we showed that injection of exogenous FA into healthy Sprague-Dawley rats or accumulation of endogenous FA by ablating *ALDH2* expression in *ALDH2*$^{-/-}$ mice resulted in significant cognitive impairments. Oxidization (degradation) of FA is mainly dependent on 2 enzymes (ALDH2 and cytosolic alcohol dehydrogenase). Under a glutathione-depleted condition in diabetes (45), ALDH2 (K_m values in the range of 0.5 mM for FA), but not alcohol dehydrogenase (a glutathione-dependent FA degrading enzyme; K_m values: 2.6 μM for FA) (12), plays a more important role in degrading pathologic concentrations of FA (13). Thus, we knocked out the *ALDH2* gene in the mice to induce endogenous accumulation of FA. We found that *ALDH2* KO mice resulted in excess FA, and these mice were more vulnerable to developing hyperglycemia and its associated cognitive decline compared with WT mice. Taken together, these studies suggest that FA accumulation is one of the critical pathologic factors contributing to diabetes-related cognitive impairments. To further determine whether reduction of excess FA can successfully rescue cognitive deficits in the diabetic mice, we scavenged excess FA by overexpression of *hALDH2* in the *hALDH2*$^{+/+}$ mice. Our results showed that restoration

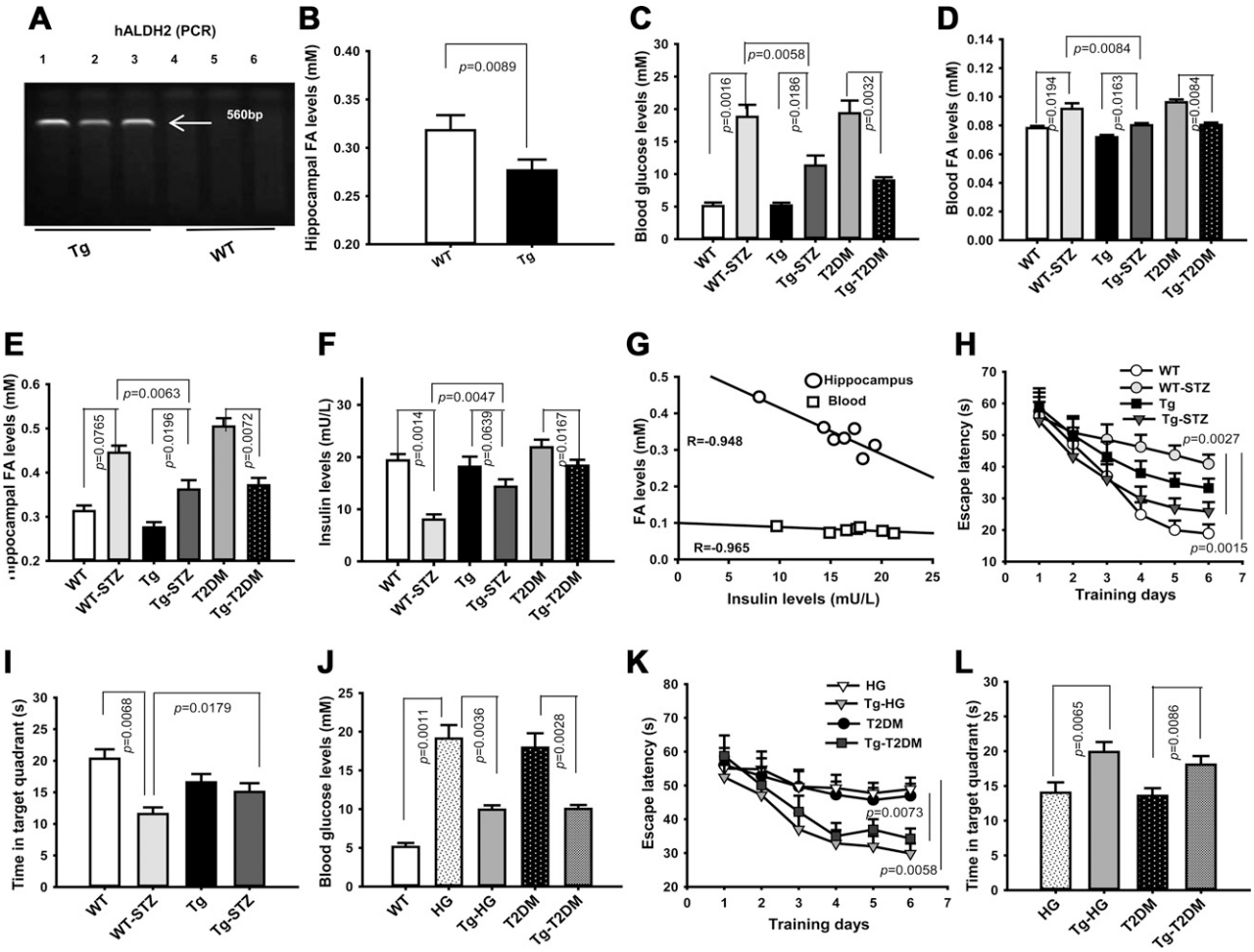

Figure 6. Overexpression of *hALDH2* ameliorated hyperglycemia and cognitive deficits in diabetic mice. *A*) The mRNA levels of *hALDH2* were detected by performing PCR. *B*) Fluo-HPLC was performed to detect FA levels in hippocampi from WT and *hALDH2*$^{+/+}$ Tg mice. *C*) Detection of blood glucose levels in WT mice, WT mice with STZ injection (WT-STZ), *hALDH2*$^{+/+}$ Tg mice, Tg mice with STZ injection (Tg-STZ), WT mice fed with high-fat diet as a T2DM model, and Tg mice fed with high-fat diet (Tg-T2DM). *D*, *E*) Blood and hippocampal FA concentrations were detected. *F*) Insulin levels were measured by ELISA. *G*) The correlation between FA and insulin levels. *H*, *I*) Spatial memory of WT, WT-STZ, Tg, and Tg-STZ mice was evaluated by performing the Morris water maze test. Blood glucose levels (*J*) and spatial memory (*K*, *L*) of WT mice injected with high concentration of glucose (HG), Tg mice with HG injection (Tg-HG), T2DM, and Tg-T2DM mice were detected; $n = 10$/group. Data are means ± SEM. Statistical analysis: 2-way ANOVA with Tukey's *post hoc* test; 1-way ANOVA with *post hoc* Newman-Keuls tests.

of hippocampal FA levels to normal could significantly reverse diabetes-induced cognitive impairments.

Insulin signaling plays an important role in facilitating glucose transporter type 4–mediated glucose uptake in adipose tissue and skeletal muscle, which is the major cellular mechanism disposing of excess glucose load to maintain glucose homeostasis (46, 47). Therefore, the FA-induced insulin deficiency impairs glucose transport in the peripheral system, leading to severe hyperglycemia. Unlike muscle and fat cells, neurons uptake glucose mainly *via* glucose transporter type 3 (GLUT3) (48). Although GLUT3 is considered insulin-independent, recent studies have identified that insulin treatment dramatically stimulates GLUT3 translocation to the plasma membrane, which potentiates depolarization with high extracellular K^+ to increase neuronal glucose uptake (49). Consistent with this finding, our study also identified that reduction of insulin by FA markedly decreased glucose uptake in the cultured neurons as well as hippocampal neurons in the FA-injected mice, resulting in cognitive deficits in these mice.

Insulin has been found to enhance synaptic plasticity, reduce brain inflammation and oxidative stress, and inhibit Aβ generation and aggregation as well as tau hyperphosphorylation (50–52). Therefore, both insulin deficiency and dysfunction have been found to promote cognitive deficits in patients with diabetes and AD (53, 54). Consistent with these studies, we also found that FA treatment led to the cognitive deficits in rats by interacting with the endogenous insulin to impair the insulin signaling. Accordingly, addition of exogenous insulin improved cognitive performance in the FA-injected rats.

In summary, we found that FA accumulation facilitates cognitive deficits during diabetes, suggesting that reduction of FA expression may be a promising drug target for preventing and/or reversing diabetes-related dementia. FJ

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AUTHOR CONTRIBUTIONS

W. Song and Z. Tong designed the studies; T. Tan, Y. Zhang, C. Han, J. N. R. Hamlin, H. Luo, and H. Li performed the experiments; Y. Zhang, W. Luo, J. Lv, Y. Wan, X. Yang, W. Song, and Z. Tong analyzed data; Z. Tong provided reagents; and T. Tan, Y. Zhang, W. Song, and Z. Tong wrote the manuscript.

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