Metabolomic Analysis Identifies Lactate as an Important Pathogenic Factor in Diabetes-associated Cognitive Decline Rats

Authors
Liangcai Zhao, Minjian Dong, Mengqian Ren, Chen Li, Hong Zheng, and Hongchang Gao

Correspondence
gaohc27@wmu.edu.cn

In Brief
The study reveals in chronic diabetes conditions, excess lactate secretion in hippocampus play an inhibitory function on cognition ability, through lactate receptor (GPR81)-depended mechanism. Besides, we proposed hypothesis of “lactate shuttle in chronic diabetic state.” And lactate dehydrogenase-A may be one of drug’s targets for cognitive dysfunction.

Graphical Abstract

Highlights
- Quantitative metabolomics identified elevated lactate levels in diabetic brains.
- Levels and enzymatic activity of LDH-A were also found significantly up-regulated.
- GPR81 dependent PKA-CREB regulated cognition decline in the diabetic rats.
- Mechanistic insights into role of lactate in diabetes-associated cognitive decline.
Metabolomic Analysis Identifies Lactate as an Important Pathogenic Factor in Diabetes-associated Cognitive Decline Rats*

Liangcai Zhao‡§, Minjian Dong‡§, Mengqian Ren‡, Chen Li‡, Hong Zheng‡, and Hongchang Gao‡¶

Diabetes mellitus causes brain structure changes and cognitive decline, and it has been estimated that diabetes doubles the risk for dementia. Until now, the pathogenic mechanism of diabetes-associated cognitive decline (DACD) has remained unclear. Using metabolomics, we show that lactate levels increased over time in the hippocampus of rats with streptozotocin-induced diabetes, as compared with age-matched control rats. Additionally, mRNA levels, protein levels, and enzymatic activity of lactate dehydrogenase-A (LDH-A) were significantly upregulated, suggesting increased glycolysis activity. Importantly, by specifically blocking the glycolysis pathway through an LDH-A inhibitor, chronic diabetes-induced memory impairment was prevented. Analyzing the underlying mechanism, we show that the expression levels of cAMP-dependent protein kinase and of phosphorylated transcription factor cAMP response element-binding proteins were decreased in 12-week diabetic rats. We suggest that G protein-coupled receptor 81 mediates cognitive decline in the diabetic rat. In this study, we report that progressively increasing lactate levels is an important pathogenic factor in DACD, directly linking diabetes to cognitive dysfunction. LDH-A may be considered as a potential target for alleviating or treating DACD in the future.

Molecular & Cellular Proteomics 17: 2335–2346, 2018. DOI: 10.1074/mcp.RA118.000690.

Diabetes mellitus (DM) is the most common metabolic disease in the world and can adversely affect multiple organs. Both type 1 and type 2 diabetes patients are more likely to develop cognitive decline than healthy people (1–4). The reported cognitive dysfunctions include impairments in memory, executive function, language, and processing speed (4–7). Diabetes-associated cognitive decline (DACD) was first reported in 2006 (8) and received increasing acceptance and attention since then. Efforts to understand the pathophysiological changes that underlie the development and progression of DACD are of vital importance for the development of novel treatments or prevention measures.

Many studies have associated glucotoxicity with learning and memory dysfunction in diabetes, as it is known that hyperglycemia can induce neuronal dysfunction through augmented oxidative stress, excessive release of cytokines, activation of protein kinase C, and increased flux of the polyol pathway (9). Hyperglycemia is an important factor in the development of cognitive decline in diabetic patients (10). Moreover, altered levels of metabolites, including free fatty acids, lipids, and advanced glycation end products generated by nonenzymatic glycation, are also known to initiate and aggravate pathological damages (11, 12). However, the key pathogenic factors of the disease are still unclear, and the metabolic changes in specific brain regions need to be further investigated.

Metabolomic analysis is an important platform used to measure and identify key metabolites related to pathological conditions. It has been extensively applied for the exploration of potential biomarkers and has provided crucial insights into the pathogenesis of diseases (13–16). Using this technology, Prentice K. J. et al. originally identified the furan fatty acid metabolite 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) to be significantly elevated in gestational diabetic patients (17, 18). By nuclear magnetic resonance (NMR)-based metabolomics, we have previously shown that the development of DACD is related to alterations in glucose metabolism and to the impaired glutamate-glutamine cycle in the hippocampus of the diabetic dyslipidemia mouse model (db/db mice) (19). Furthermore, by employing 13C NMR with labeled glucose and acetate as substrates, we observed an altered rate of particular metabolic pathways (i.e., increased glycolysis) in the diabetic animals (20, 21). By direct analysis of the metabolic features of purified astrocytes cultured in a high-glucose environment, time-dependent lactate production and the utilization of specific amino acids were identified (22, 23). Nevertheless, determining the causal link...
Role of Lactate in Diabetic Brains

between these metabolic alterations and the disease requires further exploration.

In the present study, we found that the anaerobic glycolysis metabolite lactate is significantly elevated in a time-dependent manner in hippocampal extracts of rats with streptozotocin (STZ)-induced diabetes. Furthermore, we confirmed elevated glycolysis in the hippocampus of diabetic rats by measuring the activity and the expression levels of lactate dehydrogenase-A (LDH-A). In addition, the possibility that astrocytes release excess lactate in diabetic conditions, which could affect learning and memory, was also explored.

Experimental Procedures

Animal Treatment—Male Sprague-Dawley rats weighing 220 ± 15 g (eight weeks old) were purchased from SLAC Laboratory Animal Co. Ltd. Shanghai, China. All animals were kept in a specific pathogen-free facility of the Laboratory Animal Center of Wenzhou Medical University (Wenzhou, China) with regulated temperature and humidity. During the whole experimental procedure, the rats were fed with certified standard rat chow and tap water ad libitum. All animal treatments were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

After a 12-h fast, rats were randomly selected and injected intraperitoneally (i.p.) with STZ (Sigma-Aldrich), which was freshly prepared in citrate buffer (0.1 M, pH 4.5) at a single dose of 70 mg/kg body weight. Control rats were injected with the same volume of vehicle (citrate buffer). Two days after STZ administration, blood glucose concentrations were measured and the rats that had glucose concentrations higher than 16.70 mmol/l were defined as diabetic (21). Diabetic rats were analyzed two weeks, eight weeks, 12 weeks, and 15 weeks after STZ administration, while age-matched rats without STZ administration were used as control rats.

Male 15-week-old db/db (BKS.Cg-m+/+ Leprdb/J, n = 11) and WT (C57BLKS/J-m+/+ db, n = 15) mice were purchased from the Model Animal Research Center of Nanjing University. All animals were kept in a specific pathogen-free colony of the Laboratory Animal Center of Wenzhou Medical University (Wenzhou, China) with regulated temperature and humidity. During the whole experimental procedure, rats were fed with certified standard rat chow and tap water ad libitum. All animal treatments were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. db/db mice were sacrificed by decapitation, and tissue samples were immediately dissected, snap-frozen in liquid nitrogen, and stored at −80 °C until use. The induction of hypoglycemia in STZ-induced diabetic rats was carried out by insulin (10 ml: 400 IU, i.p. Becton Dickinson & Company, China) at a dose of 40 IU/kg body weight (24).

The abbreviations used are: DACD, diabetes-associated cognitive decline; CREB, CAMP-response element-binding protein; NMR, nuclear magnetic resonance; STZ, streptozotocin; PLS-DA, partial least squares-discriminant analysis; PC, principal components; MRI, magnetic resonance imaging; LDH, lactate dehydrogenase; MWM, Morris water maze; BDNF, brain-derived neurotrophic factor; MCTs, monocarboxylate transporters; Arc; activity-regulated cytoskeleton-associated protein; EGR, early growth response; GAP, growth-associated protein; SYP, synaptophysin; PSD, postsynaptic density protein; TCA, tricarboxylic acid; HBSS, Hank’s balanced salt solution.

Morris Water Maze Test—Rats were subjected to the Morris water maze (MWM) test following previously published methods (25, 26). Briefly, the test was conducted in a circular pool (pool diameter, 110 cm; pool height, 30 cm), filled with water that was made opaque with nontoxic paint and had a temperature of 26 ± 1 °C. The circular escape platform (platform diameter, 7 cm) was submerged 1 cm below the water surface. Cues were hung at four locations (north, west, south, and east corners) of the swimming pool wall. The rats were trained for four consecutive days, and on each training day, they swam four trials (starting from a different initial placement each time) for 60 s or until they located and climbed onto the hidden escape platform (within 60 s). The rats that failed to find the platform within 60 s were guided to the platform by the operator. In addition, the rats were tested in a single 90-s probe trial without the platform on the last training day. The swimming path length, the escape latency, and the swimming velocity were recorded by a computer system.

Magnetic Resonance Imaging—Rats were anesthetized by mechanically administered gas anesthesia (1–3% isoflurane mixed with 0.5 l/min oxygen) for magnetic resonance imaging (MRI). MRI was performed in a 3.0 Tesla MR scanner (Philips Achieva, Netherlands) by placing the rats into a micro-imaging coil. T2-weighted images were acquired by rapid acquisition with the following OAX T2 TSE spin echo sequence: TR/TE = 3,198/80 ms, field of view = 4 cm, matrix = 200 × 216, section thickness = 0.5 mm, intersection gap = 0.14 mm, NEX = 8. MRI was performed at 12 and 15 weeks after the STZ treatment.

Sample Collection and 1H NMR Spectra Acquisition—The hippocampi and other tissues were dissected immediately after euthanasia of the rats, snap-frozen in liquid nitrogen, and stored at −80 °C, until use. The preparation of brain samples and extracts and the acquisition of 1H-NMR spectra were performed as described previously (21, 27, 28). High-resolution 1H-NMR spectra were obtained on a Bruker AVANCE III 600 spectrometer operating at 600.13 MHz equipped with a triple resonance probe. The lyophilized samples were dissolved in 450 μl D2O (which included 0.1 mM TSP as a reference for chemical shift) and then transferred into 5-mm NMR tubes. Both spectra were acquired with an 8.0-s relaxation delay and a 32-K data point. The acquisition time was 5.44 s, and in total 2,048 scans for intracellular samples and 256 scans for extracellular samples were performed. A one-dimensional ZGPR pulse sequence was applied to achieve satisfactory water suppression in aqueous extracts.

Multivariate Pattern Recognition Analysis—The 1H NMR spectra were phase- and baseline-corrected and integrated to binning data with a size of 0.01 ppm, from 0.4 to 10.0 ppm, using the Bruker Topspin 2.1 software package. For the NMR spectra recorded in hippocampal extracts, the region 4.69–5.04 of δ was removed to eliminate artifacts related to the residual water resonance. The remaining spectral segments were normalized to the total sum of the spectral intensity to compensate for variations in the total sample volume. The normalized integral values were then subjected to multivariate pattern recognition or quantitative analysis using the SIMCA-P+ V12.0 software package (Umetrics, Umea, Sweden). Data were visualized by the score plots of the first two principal components (t1 and t2) to provide the 2D information, where the position of each point represents one sample.

Primary Cultures of Rat Cortical Neurons and Astrocytes—Primary neurons were cultured as previously described with some modifications (29). Briefly, neuron cultures were prepared from less than 24-h-old Sprague-Dawley rat pups. The cerebral cortical tissues were dissected under sterile conditions and the meninges were removed under the dissecting microscope. Tissues were minced and then digested with 0.25% trypsin for 10 min at 37 °C. Digestion was...
terminated by DMEM, which contained 10% fetal bovine serum. The cell suspension was passed through a 200-mesh cell strainer to obtain a single-cell suspension. The cells were plated in poly-L-lysine coated Falcon flasks (75 cm²) at a density of 2 × 10⁵ cells/ml after counting the cells by using a hemocytometer before culturing them in a humidified incubator with 5% CO₂ at 37 °C (Thermo Fisher Scientific, USA). After allowing the cells to settle down for 12 h, they were washed once with HBSS and the culture medium was replaced by fresh Neurobasal medium that was supplemented with B27 (1×), 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. To prevent excessive proliferation of astrocytes, cytosine arabinoside was added to a final concentration of 10 μM for 12 h after 48 h in culture. After that, the neurons were cultured for another four days. Half of the medium was exchanged twice a week during the culturing period. The neurons were assessed by immunostaining for the neuronal marker microtubule-associated protein-2 and the astrocyte marker glial fibrillary acidic protein. Glucose concentration in the culture media was 5.5 mM.

Primary astrocytes were cultured as previously described with some modifications (30). Briefly, cultures were prepared from 2–3-day-old Sprague-Dawley rat pups. Cerebral cortical tissues were dissected under sterile conditions. Astrocytes were seeded in 24 culture flasks (10⁶ cells/flask) and five 24-well plates (10⁶ cells/well) for examination of intracellular and extracellular lactate levels, respectively. Cells were washed once with HBSS 24 h after seeding, and the culture medium was replaced by fresh DMEM containing 5.5 mM glucose for the control group or 25 mM for the high-glucose group (31–33).

Cell Metabolite Extraction—Cells were harvested after rapid quenching at the end of high-glucose stimulation. Quenched cells were pelleted by centrifugation and rinsed with HBSS before they were transferred to a centrifuge tube. Subsequently, cell pellets were resuspended in 450 μl of ice-cold CH₃OH:CHCl₃ (v/v 2:1) and sonicated on ice for 30 min. Afterward, another 450 μl ice-cold CHCl₃: H₂O (v/v 1:1) were added to form an emulsion, which was left on ice for 15 min before being centrifuged at 12,000 rpm for 20 min (4 °C). The upper phase was collected, lyophilized, and stored at −80 °C until NMR analysis (two flasks/sample, n = 6/group).

The five plates corresponded to the five time intervals (0, 2, 24, 48, and 72 h). Culture medium (1 ml) was transferred from each well into individual 15 ml centrifuge tubes at each time point after exposure to high glucose. Then, 3 ml ice-cold CH₃OH:CHCl₃ (v/v 2:1) were added, followed by 1 ml ice-cold CHCl₃. This solution was vigorously mixed for 60 s on ice before being centrifuged at 8,000 rpm for 20 min at 4 °C. Finally, the collected supernatants were lyophilized and stored at −80 °C for further NMR analysis.

LDH Activity Determination—Isolated rat hippocampi were homogenized in protein lysis buffer and debris was eliminated by centrifugation at 12,000 rpm for 10 min at 4 °C. The protein concentrations in all samples were determined by the Bradford protein assay kit (Bio-Rad). Spectrophotometric assays were then used to determine LDH activity converting pyruvate (P) to lactate (L) and L→P in a tunable microplate reader (Spectramax M5; Molecular Devices) with the corresponding software (Soft Max Pro) (34). For determination of LDH activity, an LDH kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used according to the manufacturer’s protocol. The reverse transcription step was carried out using the M-MLV RT kit (Invitrogen). CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) was used for q-PCR analysis. The complete set of primers is listed in Table S1.

Western Blotting—Following addition of sample loading buffer, protein samples were electrophoresed and then electrotransferred onto PVDF membranes. The bands were blocked for one hour at room temperature with fresh 5% nonfat milk in Tris-buffered saline containing Tween 20 (TBST) and then incubated with specific primary antibodies diluted in TBST, for 16–18 h, at 4 °C. Following three washes with TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h and the immunoreactive bands were visualized using an enhanced chemiluminescence kit. The bands were quantified using Image-Pro Plus 6.0 software.

Tissue Preparation for Immunohistochemistry—For preparation of fresh frozen sections for immunohistochemistry, the rats were killed by cervical dislocation and their brains were dissected, rapidly frozen on dry ice, and stored at −80 °C. The frozen tissues were embedded in mounting medium (Tissue-Tek; Sakura Finetek). Sections of 14 μm were cut on a Microm Model HM 500 m Cryostat (Microm), collected onto Super Frost slides (Menzel Gläser), and stored until use.

Intracerebroventricular Injection—Ten-week-old diabetic or control rats received intracerebroventricular injections of the LDH inhibitor oxamate (10 μg dissolved in ultrapure water at a final concentration of 0.4 mg/ml, Sigma Aldrich) once daily for 14 days. The oxamate-injected rats were subjected to the MWM test at 12 weeks after the initial STZ treatment.

Statistics—Data are presented as means ± standard deviation, unless stated otherwise. The statistical significance was analyzed using a two-tailed Student’s t test or one-way ANOVA followed by Dunn’s test for multiple group comparison where appropriate (SPSS, Inc.). Statistical significance was defined as p < 0.05.

RESULTS

Determination of Cognitive Decline in Diabetic Rats—To determine the occurrence of cognitive decline in rats with STZ-induced diabetes, MWM, immunohistochemistry, MRI, and RT-PCR were carried out at different time points. Fig. 1A–1C show the swimming behavior data of the diabetic and age-matched control rats. We found that at the time points of eight and 12 weeks after STZ treatment, the diabetic rats had longer escape latency during the original MWM test and fewer platform crossings during the probe trial. Cross-sectional MRI studies showed that 12-week diabetic rats displayed larger hyperintensities in the lateral ventricle regions, compared with age-matched control rats, suggesting the appearance of brain atrophy (Fig. 1D). Moreover, hippocampus expression levels of glial fibrillary acidic protein, an indicator of astrocyte reac-
Activity, were up-regulated at all time points checked (Figs. S1 and S2). In addition, the mRNA levels of biomarkers like brain-derived neurotrophic factor (BDNF), c-Fos, activity-regulated cytoskeleton-associated protein (Arc), presynaptic and postsynaptic plasticity proteins, i.e. early growth response-1 (EGR-1), growth-associated protein 43 (GAP43), synaptophysin (SYP), postsynaptic density protein-93 (PSD-93), and PSD-95, that underlie the molecular basis of learning and memory, were markedly down-regulated in 12-week diabetic rat samples compared with samples from age-matched controls, but not in two-week diabetic rats (except c-Fos and BDNF, which were also altered at two weeks) indicating the occurrence of DACD (Fig. 1E, Fig. S3).

**Metabolomic Analysis**—We used $^1$H NMR spectrometry to examine 16 metabolites in hippocampal extracts from STZ and vehicle-treated rats at two, eight, and 12 weeks post STZ administration (representative spectra are shown in Fig. 2A). To characterize the metabolic changes that occur at different stages of diabetes progression, the mean trajectory was calculated based on partial least squares discriminant analysis (PLS-DA) of the spectra. The trajectory exhibited a distinct separation between the diabetic and control groups along the principal component 1 (PC1) and PC2 direction (Figs. 2B and 2C). Additionally, the diabetic rats showed a clear trajectory plot during the period from two weeks to 12 weeks, in contrast to the age-matched controls. The corresponding loading plot showed that the contents of myo-inositol, fumarate, and

**Fig. 1.** **Streptozotocin administration induces diabetes-associated cognitive decline in rats.** (A–C) Behavior changes were studied using Morris water maze test and representative records from spatial memory test of diabetes mellitus (DM) and control rats at two, eight, and 12 weeks ($n=5–8$ for each group) are shown here. (A) The graph illustrates the escape-latency behavior of diabetic and control rats during the four days of the MWM test. (B) Representative swim track of differently treated rats. (C) The number of platform crossing by the diabetic rats is decreased at eight weeks and 12 weeks. (D) MRI T2 images are presented to visualize the volume of the brain and the position of the volume of interest (VOI) in the lateral ventricles of eight-week and 12-week diabetic and control rats. The VOI is magnified eight times in the bottom right corner of each image and the lateral ventricle zone is delineated by the red line. (E) RT-PCR determination of mRNA levels of brain-derived neurotrophic factor (BDNF), c-fos, activity-regulated cytoskeleton-associated protein (Arc), and early growth response-1 (EGR-1) in hippocampal extracts of diabetic and control rats at two and 12 weeks after treatment. Data represent mean ± S.D. from five rats in each group. Significance was determined by two-tailed unpaired Student’s t test of diabetic rats versus controls and denoted with *$p<0.05$, **$p<0.01$. 

**Role of Lactate in Diabetic Brains**

2338

Molecular & Cellular Proteomics 17.12
Lactate were significantly higher, while succinate, glutamine, glutamate, and citrate were decreased in 12-week diabetic rats (Fig. 2D). In the hippocampus samples, lactate levels were increased by 30% in eight-week diabetic rats, and a nearly twofold increase of lactate was apparent in 12-week diabetic rats, as compared with age-matched controls (Fig. 3). In addition, levels of most of the cerebral intermediates or products related to the Krebs cycle were decreased with the exception of fumarate. These results suggest that high anaerobic glycolysis and low tricarboxylic acid (TCA) cycle activity are pathological features of the DACD rat hippocampus.

Next, we measured hippocampal lactate concentrations in two-week and 12-week diabetic rats using ELISA to confirm the NMR findings. Our results showed that lactate levels are higher in 12-week but not in two-week diabetic rats as

**Fig. 2. Multivariate pattern recognition analysis of 1H nuclear magnetic resonance (NMR) analysis of hippocampus extracts from diabetic and control rats.** (A) Representative 1H NMR spectrum obtained from diabetic specimen and controls at two, eight, and 12 weeks post STZ administration. Keys: Lac, lactate; Ala, alanine; GABA, gamma-aminobutyric acid; NAA, N-acetyl aspartic acid; Glu, glutamate; Gin, glutamine; Pyr, pyruvate; Suc, succinate; Citr, citrate; Cre, Creatine; Cho, choline; GPC, Glycerophosphocholine; PC, Phosphocholine; Tau, Taurine; ml, Myo-inositol. (B) PLS trajectory of metabolic profiles of the control group (black pentagrams) and of the diabetic group (red triangles) at different time intervals \([R^2X = 0.757, R^2Y = 0.956, Q^2 = 0.837]\). (C) PLS-DA scores plotted based on 1H NMR spectra from diabetes rats at two, eight, and 12 weeks post STZ administration. \([R^2X = 0.579, R^2Y = 0.818, Q^2 = 0.432]\). (D) Univariate analysis of metabolome data from all NMR spectra. Student’s t test \(p\) values and fold changes are shown as a Volcano plot. Each dot indicates one metabolite. Metabolites involved in glycolysis and Krebs cycle are indicated in red or blue, respectively. See also the legend of (A), \(n = 5–8\) per group, \(p < 0.05\): -Log_{10}(p value) > 1.3.
compared with age-matched controls (Fig. S4). Due to the strong similarities between DACD and diabetes, we examined whether lactate levels were also elevated in other tissues or in other diabetic animal models (Fig. S5). In different brain regions of the 12-week STZ diabetic rats, such as the cortex and the striatum, lactate levels were also significantly elevated. A similar increasing trend of lactate levels was found in hippocampal extracts of 17-week old db/db mice (type 2 DACD model). However, lactate levels in urine, serum, and heart tissue of rats with STZ-induced diabetes were decreased or not altered. Interestingly, in hypoglycemic rats treated with insulin, hippocampal levels of lactate displayed a trend for reduction ($p = 0.062, n = 6$, Fig. S5). The abovementioned results show that the elevation of lactate levels in diabetic animals is independent of the tissue, organ, animal age, and STZ toxicity.

Assessment of LDH Activity and Expression—The functioning of neurons in the brain depends on glycolysis and as a consequence, neurons must replenish NAD$,^+$, which is accomplished by increasing pyruvate (P) to lactate (L) conversion by lactate dehydrogenase (LDH; EC 1.1.1.27) (36). There are five isoenzymes, LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5. Each isoform is composed of M and H subunits, which are the gene products of LDH-A and LDH-B, respectively (Fig. 4A). LDH-A is mainly responsible for the P→L conversion, while LDH-B is responsible for the L→P conversion. Using a microplate reader to measure enzymatic activity, we found in 12-week diabetic rats a 31% increase of P→L conversion and a 13% increase of L→P conversion, as compared with age-matched controls. This corresponds to a total of 20% increase of P→L conversion in anaerobic glycolysis. Interestingly, in two-week diabetic rats, L→P
conversion is the prevailing direction of conversion (Figs. 4B and 4C).

Our RT-PCR data showed that LDH-A levels are up-regulated by 90% in 12-week diabetic rats, while they are not altered in two-week diabetic rats, as compared with the corresponding controls (Fig. 4D). Quantitative Western blot analysis confirmed that 12-week diabetic rats had a significant increase in hippocampal LDH-A expression compared with age-matched control rats (Figs. 4E and 4F). These results, coupled with the LDH gene expression and enzymatic activity changes, indicate that the observed higher lactate levels in the hippocampi of diabetic rats were the result of a metabolic shift to increased lactate production in diabetic rats as determined by quantification of LDH-A activity and expression.

**FIG. 4.** Functional metabolic shift to increased lactate production in diabetic rats as determined by quantification of LDH-A activity and expression. (A) Schematic visualization of the difference in the amino acid sequence between subunits M (LDH-A) and H (LDH-B) and of their combination in the five different isoforms of LDH (LDH 1 to 5). (B) Quantification of LDH enzymatic activity in the hippocampus from two- and 12-week diabetic and control rats. Quantification of LDH activity determined from both pyruvate and lactate sides of the reaction indicated a metabolic shift to increased lactate production in diabetic rats with time. (C) The P:L ratio is a simplified reflection of the LDH activity (IU/mg protein) of all isoenzymes during interconversion of P→L. (D) Real-time PCR analysis of LDH-A and LDH-B mRNA expression levels in the hippocampus of two-week and 12-week diabetic rats versus control rats. Data show that mRNA levels shift to up-regulation of LDH-A production in 12-week diabetic rats. (E) Protein expression levels of LDH-A in the hippocampus of two-week and 12-week diabetic rats and their controls, as determined by Western blotting. (F) Graph of the Western blotting quantification performed by software analysis, illustrating that LDH-A is significantly increased in 12-week diabetic rats. (G) Immunohistochemical determination of LDH-A expression in the different hippocampal regions (CA1, CA2, CA3, and dentate gyrus) of diabetic and control rats. Data represent mean ± S.D. from n = 6 rats in each group. *p < 0.05, **p < 0.01 versus controls.
shift to anaerobic glycolysis, where large amounts of lactate are being produced from pyruvate in an environment of consistent hyperglycemia. Furthermore, we determined by immunohistochemistry staining that LDH-A was highly expressed mainly in CA1 and CA2 regions of the 12-week diabetic rats, while it was neither detected in the CA3 and dentate gyrus regions of 12-week rats, nor in the hippocampi of the two-week diabetic rats (Fig. 4).

**Determination of PKA-CREB Expression**—The activation of G protein-coupled receptors 81 (GPR81) by lactate has been shown to modulate CREB through the inhibition of cAMP and PKA-CREB activity (37). To determine the mechanism through which lactate decreases c-Fos and Arc biosynthesis, we examined the protein expression levels of c-Fos and Arc in hippocampal extracts from different time points of diabetic rats and control rats. In 12-week diabetic rats, PKA and CREB phosphorylation activity was significantly inhibited compared with age-matched controls (Fig. 5), which was not the case in two-week diabetic rats, indicating inhibited PKA-CREB transduction under chronic diabetic conditions.

**Lactate Transport Examination**—It is known that lactate is transported through the cell membrane by proton-linked monocarboxylate transporters (MCTs) (38). In the brain, MCT4 is expressed on astrocytes that are glycolytic and can supply other cells with lactate, while MCT1 is mainly detected in endothelial cells of the brain’s blood vessels and in oligodendrocytes (39). MCT2 is found in the cell bodies, dendrites, and axons of neurons (39, 40). In our study, we performed RT-PCR to determine the mRNA levels of MCT1, MCT2, and MCT4 in the hippocampi of the different diabetic groups. The results showed significantly decreased MCT4 levels in 12-week diabetic rats, compared with age-matched controls, which may reflect the compensatory response of astrocytes in an environment of excess lactate. Conversely, no changes in MCT2 were detected either in two-week or in 12-week diabetic rats, also indicating no alterations of lactate transport into neurons (Figs. 6A–6C).

To confirm the transport process, lactate contents were determined in the intra- and extracellular compartments of astrocytes and neurons, respectively, under high-glucose challenge. We found significantly elevated lactate levels in the extracellular compartments of astrocytes, as well as a faster rate of lactate efflux (Fig. 6D). Interestingly, we determined reverse directions of lactate transport into neurons (influx), under high-glucose conditions. These results suggest that in a high-glucose environment, or under diabetic conditions, excess lactate originates from astrocytes and not from neurons.

**Pharmacological Intervention**—To determine the causal role of lactate signaling in cognition impairment, LDH-A inhibitor oxamate was intracerebroventricularly injected into ten-week diabetic rats once daily for 14 days. This treatment recovered the levels of lactate in the cortex or midbrain regions to control values (Figs. 7A–7C). Furthermore, we found that the increased latency of the diabetic rats in the MWM test was significantly recovered by oxamate treatment, compared with vehicle-injected diabetic rats (Figs. 7D–7H). Additionally, mRNA and protein levels of molecular markers related to cognition, such as BDNF, Arc, PSD-95, and Syp were accordingly recovered (Figs. 7I–7M).

**DISCUSSION**

In the central nervous system, lactate is considered as equivalent to glucose regarding its access to the TCA cycle in neurons and it also serves as a precursor for the synthesis of neurotransmitters (41). Furthermore, lactate also plays a signaling role in the brain (42). Recently, it was revealed that Montreal cognitive assessment scores of clinical subjects...
were negatively correlated to lactate levels in the cerebrospinal fluid but not in the blood, strongly suggesting that excess cerebral lactate is related to cognitive decline (43). In this study, we proposed one model that during early stages of diabetes in rodents, there is increased gliosis, accelerated gliosis, and elevated extracellular lactate, which is available to the surrounding neurons. However, at later stages of diabetes, due to mitochondrial dysfunction, lactate utilization is reduced and it accumulates in the extracellular space. This excess of lactate causes neuronal dysfunction, by modulating the GPR81-PKA-CREB signaling pathway, which finally leads to decrease of synaptic plasticity, down-regulation of proteins related to memory function (i.e. c-Fos, Arc, BDNF), and cognitive decline (Fig. 8).

Employing NMR-based metabolomics, we detected that lactate levels increase over time in hippocampal extracts from diabetic rats. By determining the activity of the key proteins of glycolysis, LDH-A and LDH-B, we identified L→P conversion to be dominant at the two-week stage of diabetes, which switched to the opposite direction at the 12-week stage. These results are consistent with results from our previous 13C NMR studies on the same diabetic model, in which lactate C2 was enriched and the pyruvate recycling pathway was switched to the opposite direction at the 12-week stage. In this study, we employed an in vitro primary cell culture system in high-glucose conditions to recapitulate the in vivo diabetic environment. Indeed we detected higher extracellular lactate levels. Lactate was also increased in astrocytes but not in neurons. According to the ‘lactate shuttle’ hypothesis, lactate is a metabolic substrate released under physiological conditions by glial cells (mainly astrocytes) and is taken up by the adjacent neurons (53). In our previous study using [2-13C]acetate as a tracer substrate, we demonstrated in the same diabetic rat model reduced lactate supply to neurons, which was accompanied by lactate accumulation in astrocytes (21). In this study, we employed an in vitro primary cell culture system in high-glucose conditions to recapitulate the in vivo diabetic environment. Indeed we detected higher extracellular lactate levels. Lactate was also increased in astrocytes but not in neurons. Accordingly, in hippocampal extracts of 12-week diabetic rats, we found decreased levels of MCT1 and MCT4, but increased glycolysis and inhibited pyruvate recycling occur, resulting in the accumulation of lactate in the brain.

Lactate secreted from astrocytes is available for transport by MCT2 into neurons for energy supply or it may accumulate in the extracellular space and exert pathological function. In peripheral adipocytes, it was reported that the effects of lactate in lipolysis involve GPR81 (44) and PKA-CREB signal transduction (37). CREB is one of the most important transcription factors, activated by its phosphorylation on serine 133. This activation regulates the expression of regulatory immediate early genes that are responsible for long-term synaptic plasticity and memory formation (45). In this study, we confirmed that the expression levels of PKA-pCREB and of molecules implicated in memory function are down-regulated in 12-week diabetic rats, while being normal in two-week diabetic rats. These results strongly suggest GPR81-mediated memory impairment in chronic diabetic conditions, which is indicative of DACD. The results are consistent with previous in vitro experiments of neuronal activity suppression (46, 47). However, here we provide evidence that GPR81 mediates lactate signaling by inhibiting the PKA-CREB pathway in the diabetic rat brain, which has not been documented in previous studies.

In fact, lactate is not just a metabolic substrate for neurons, but it also functions as a gliotransmitter (46), an autocrine hormone (48), and a receptor agonist (47). For example, it was reported that glycolysis leads to the production of ATP, which modulates selective neuronal membrane conductance through ATP-sensitive potassium channels (KATP) (49, 50). Lactate is also known to stimulate synaptic plasticity-related gene expression in neurons by the modulation of N-methyl-D-aspartate receptor activity and its downstream signaling cascade comprising extracellular signal-regulated kinases 1 and 2 (ERK1/2) (51). Moreover, it was reported that lactate shuttling between astrocytes and neurons is probably required for long-term memory formation (52). The activation of different signal transduction pathways, the different time course, and dose-related effects may contribute to the contradictory results on the role of lactate in brain function, which needs to be further explored in future studies.

According to the ‘lactate shuttle’ hypothesis, lactate is a metabolic substrate released under physiological conditions by glial cells (mainly astrocytes) and is taken up by the adjacent neurons (53). In our previous study using [2-13C]acetate as a tracer substrate, we demonstrated in the same diabetic rat model reduced lactate supply to neurons, which was accompanied by lactate accumulation in astrocytes (21). In this study, we employed an in vitro primary cell culture system in high-glucose conditions to recapitulate the in vivo diabetic environment. Indeed we detected higher extracellular lactate levels. Lactate was also increased in astrocytes but not in neurons. Accordingly, in hippocampal extracts of 12-week diabetic rats, we found decreased levels of MCT1 and MCT4, but increased glycolysis and inhibited pyruvate recycling occur, resulting in the accumulation of lactate in the brain.

Lactate secreted from astrocytes is available for transport by MCT2 into neurons for energy supply or it may accumulate in the extracellular space and exert pathological function. In peripheral adipocytes, it was reported that the effects of lactate in lipolysis involve GPR81 (44) and PKA-CREB signal transduction (37). CREB is one of the most important transcription factors, activated by its phosphorylation on serine 133. This activation regulates the expression of regulatory immediate early genes that are responsible for long-term synaptic plasticity and memory formation (45). In this study, we confirmed that the expression levels of PKA-pCREB and of molecules implicated in memory function are down-regulated in 12-week diabetic rats, while being normal in two-week diabetic rats. These results strongly suggest GPR81-mediated memory impairment in chronic diabetic conditions, which is indicative of DACD. The results are consistent with previous in vitro experiments of neuronal activity suppression (46, 47). However, here we provide evidence that GPR81 mediates lactate signaling by inhibiting the PKA-CREB pathway in the diabetic rat brain, which has not been documented in previous studies.

In fact, lactate is not just a metabolic substrate for neurons, but it also functions as a gliotransmitter (46), an autocrine hormone (48), and a receptor agonist (47). For example, it was reported that glycolysis leads to the production of ATP, which modulates selective neuronal membrane conductance through ATP-sensitive potassium channels (KATP) (49, 50). Lactate is also known to stimulate synaptic plasticity-related gene expression in neurons by the modulation of N-methyl-D-aspartate receptor activity and its downstream signaling cascade comprising extracellular signal-regulated kinases 1 and 2 (ERK1/2) (51). Moreover, it was reported that lactate shuttling between astrocytes and neurons is probably required for long-term memory formation (52). The activation of different signal transduction pathways, the different time course, and dose-related effects may contribute to the contradictory results on the role of lactate in brain function, which needs to be further explored in future studies.
not MCT2, also suggesting that changes in lactate transport was only related to astrocytes, and not to neurons.

Due to its critical role in sustaining a high rate of glycolysis, LDH-A was suggested as a potential therapeutic target for DACD. By pharmacological treatment, we showed that cognition decline in diabetic rats was reversed by the LDH-A inhibitor oxamate, suggesting that lactate is implicated in DACD. Interestingly, previous studies have shown that lactate is involved in many physiological and pathological processes in the brain, such as appetite (54), mental disorders (55), neurotransmitter transporter function (56), and protection from brain damage (57), as well as cognition (38, 47, 51, 52).

In conclusion, our study reveals that glycolysis activity is elevated in the hippocampal region during the late stages of diabetes progression, but not during early stages. Consistently, the expression levels and activity of LDH-A are increasing during disease progression in the diabetic rat brain, suggesting an important role of glycolysis and LDH-A in DACD and the potential for LDH-A to be examined as an early diagnostic marker or as a therapeutic target. Nonetheless, the exact molecular mechanisms that can induce the lactate-mediated cognitive dysfunction need further elucidation.

DATA AVAILABILITY

The NMR spectrometry data have been deposited to the KiMoSys repository (http://kimosys.org) with the dataset identifier Data EntryID 91.
Role of Lactate in Diabetic Brains

**Fig. 8.** Working model of lactate production and transport in neuron-astrocyte compartments in the chronic diabetic state. In the later stages of diabetes, due to gliosis and glycolysis enhancement, excess lactate levels are transported out of the astrocytic cytoplasm by MCT1 and MCT4. This is accompanied by reduction of lactate uptake by adjacent neurons. Thus, lactate accumulates in the extracellular space. This leads to neuronal dysfunction by affecting the GPR81-PKA-CREB signal transduction pathway, which finally results in decreased levels of immediate early genes (IEGs, i.e. c-Fos, Arc), BDNF, and other proteins related to learning and memory and finally resulting in cognitive decline in diabetic rats.

* This work was supported by National Natural Science Foundation of China Grants 81770830, 81771386, and 21575105 and Zhejiang Provincial Natural Science Foundation Grants LY17H160049 and LQ18H160027. The authors declare no competing financial interests.

† To whom correspondence should be addressed: Institute of Metabonomics & Medical NMR, School of Pharmaceutical Sciences, Wenzhou Medical University, Wenzhou 325035, China, Tel: 86 577 86699715. E-mail: gaohc27@wmu.edu.cn.

§ Co-first authors.

Author contributions: L.Z. and H.G. designed research; L.Z. and M.D. performed research; L.Z. and H.G. wrote the paper; and M.R., C.L., and H.Z. analyzed data.

REFERENCES

1. Yaffe, K., Falvey, C., Hamilton, N., Schwartz, A. V., Simonick, E. M., Satterfield, S., Cauley, J. A., Rosano, C., Launer, L. J., Strotmeyer, E. S., and Harris, T. B. (2012) Diabetes, glucose control, and 9-year cognitive decline among older adults without dementia. *Arch. Neurol.* **69**, 1170–1175

2. Okereke, O. I., Kang, J. H., Cook, N. R., Gaziano, J. M., Manson, J. E., Buring, J. E., and Grodstein, F. (2008) Type 2 diabetes mellitus and cognitive decline in two large cohorts of community-dwelling older persons. *J. Am. Geriatr. Soc.* **56**, 1028–1036

3. Yaffe, K., Blackwell, T., Kanaya, A. M., Davidowicz, N., Barrett-Connor, E., and Krueger, K. (2004) Diabetes, impaired fasting glucose, and development of cognitive impairment in older women. *Neurology* **63**, 658–663

4. Manschot, S. M., Biessels, G. J., de Valk, H., Agra, A., Rutten, G. E., van der Grond, J., Kappelle, L. J., and Utrecht Diabetic Encephalopathy Study, G. (2007) Metabolic and vascular determinants of impaired cognitive performance and abnormalities on brain magnetic resonance imaging in patients with type 2 diabetes. *Diabetologia* **50**, 2388–2397

5. Fischer, A. L., de Frias, C. M., Yeung, S. E., and Dixon, R. A. (2009) Short-term longitudinal trends in cognitive performance in older adults with type 2 diabetes. *J. Clin. Exp. Neuropsychol.* **31**, 809–822

6. Samaras, K., Lutgers, H. L., Kochan, N. A., Crawford, J. D., Campbell, L. V., Wen, W., Slavin, M. J., Baune, B. T., Lipnicki, D. M., Brodaty, H., Trollor, J. N., and Sachdev, P. S. (2014) The impact of glucose disorders on cognition and brain volumes in the elderly: The Sydney Memory and Ageing Study. *Age* **36**, 977–993

7. Gregg, E. W., Yaffe, K., Cauley, J. A., Rolka, D. B., Blackwell, T. L., Narayan, K. M., and Cummings, S. R. (2000) Is diabetes associated with cognitive impairment and cognitive decline among older women? Study of Osteoporotic Fractures Research Group. *Arch. Intern. Med.* **160**, 174–180

8. Mijnhout, G. S., Scheitens, P., Diamant, M., Biessels, G. J., Wessels, A. M., Simsek, S., Snoek, F. J., and Heine, R. J. (2006) Diabetic encephalopathy: A concept in need of a definition. *Diabetologia* **49**, 1447–1448

9. Yagihashi, S., Mizukami, H., and Sugimoto, K. (2011) Mechanism of diabetic encephalopathy: Where are we now and where to go? *J. Diabetes Invest.* **2**, 18–32

10. Wang, S. B., and Jia, J. P. (2014) Oxytmarine attenuates diabetes-associated cognitive deficits in rats. *Acta Pharmacol. Sin.* **35**, 331–338

11. Yaffe, K., Lindquist, K., Schwartz, A. V., Vitartas, C., Vittinghoff, E., Satterfield, S., Simonick, E. M., Launer, L., Rosano, C., Cauley, J. A., and Harris, T. (2011) Advanced glycation end product level, diabetes, and accelerated cognitive aging. *Neurology* **77**, 1351–1356

12. Sima, A. A. (2010) Encephalopathies: the emerging diabetic complications. *Acta Diabetol.* **47**, 279–293

13. Nicholson, J. K., Lindon, J. C., and Holmes, E. (1999) “Metabonomics”: Understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. * Xenobiota** **29**, 1181–1189

14. Lu, J., Xie, G., Jia, W., and Jia, W. (2013) Metabolomics in human type 2 diabetes research. *Front. Med.* **7**, 4–13

15. Chen, W. L., Wang, J. H., Zhao, A. H., Xu, X., Wang, Y. H., Chen, T. L., Li, J. M., Mi, J. Q., Zhu, Y. M., Liu, Y. F., Wang, Y. Y., Jin, J., Huang, H., Wu, D. P., Li, Y., Yan, X. J., Yan, S. I., Li, J. Y., Wang, S., Huang, X. J., Wang, B. S., Chen, Z., Chen, S. J., and Jia, W. (2014) A distinct glucose metabolism signature of acute myeloid leukemia with prognostic value. *Blood* **124**, 1645–1654

16. Luo, P., Yin, P., Hua, R., Tan, Y., Li, Z., Qiu, G., Yin, Z., Xie, X., Wang, X., Chen, W., Zhou, L., Wang, X., Li, Y., Chen, H., Gao, L., Lu, X., Wu, T., Wang, H., Niu, J., and Xu, G. (2017) A large-scale, multi-center serum metabolite biomarkers identification study for the early detection of hepatocellular carcinoma. *Hepatology* **67**, 662–675

17. Prentice, K. J., Luu, L., Allister, E. M., Liu, Y., Jun, S. L., Sloop, K. W., Hardy, A. B., Wei, L., Jia, W., Fantus, I. G., Sweet, D. H., Sweeney, G., Retnakaran, R., Dai, F. F., and Wheeler, M. B. (2014) The furan fatty acid metabolite CMPF is elevated in diabetes and induces beta cell dysfunction. *Cell Metabolism* **19**, 653–666

18. Liu, Y., Prentice, K. J., Eversley, J. A., Hu, C., Batchuluun, B., Leavey, K., Hansen, J. B., Wei, D. W., Cox, B., Dai, F. F., Jia, W., and Wheeler, M. B.
Role of Lactate in Diabetic Brains

(2016) Rapid elevation in CMFP may act as a tipping point in diabetes development. Cell Rep. 14, 2889–2900

27. Hu, W., Yang, Y., Dong, B., Zheng, H., Lin, X., Du, Y., Li, X., Zhao, L., and Gao, H. (2016) Metabonomics profiles delineate potential role of gluta- mate-glutamine cycle in db/db mice with diabetes-associated cognitive decline. Mol. Brain 9, 40

28. Zheng, H., Zheng, Y., Wang, D., Cai, A., Lin, Q., Zhao, L., Chen, M., Deng, M., Ye, X., and Gao, H. (2017) Analysis of neuron-astrocyte metabolic cooperation in the brain of db/db mice with cognitive decline using 13C NMR spectroscopy. J. Cereb. Blood Flow Metab. 37, 332–343

29. Wang, N., Zhao, L. C., Zheng, Y. J., Lin, L., Xiang, Y., Yan, Z. H., Bai, G. H., Ye, J. Y., and Zhao, L. (2015) Induction of triglyceride accumulation and mitochondrial maintenance in muscle cells by lactate. Sci. Rep. 6, 33732

30. Bergersen, L. H. (2015) Lactate transport and signaling in the brain: potential therapeutic targets and roles in body-brain interaction. J. Cereb. Blood Flow Metab. 35, 176–185

31. Rafik, A., Boulland, J. L., Halestrap, A. P., Ottersen, O. P., and Bergersen, L. (2003) Highly differential expression of the monocarboxylate transporters MCT2 and MCT4 in the developing rat brain. Neuroscience 122, 677–688

32. Portela, L., Brochier, A. W., Hämäläinen, M. J., Casarini, D. E., Takahashi, T., countertops, K., and Magistretti, P. J. (2011) Development of a water-maze procedure for studying spatial learning in the rat. J. Neurosci. Methods 11, 47–60

33. Butcher, S. P., Sandberg, M., Hagberg, H., and Hamberger, A. (1987) Cellular origins of endogenous amino acids released into the extracellular fluid of the rat striatum during severe insulin-induced hypoglycemia. J. Neurochem. 48, 722–728

34. Zhao, Q., Matsumoto, K., Tsuneyama, K., Tanaka, K., Li, F., Shibahara, N., Miyata, T., and Yokozawa, T. (2011) Diabetes-induced central cholinergic neuron loss and cognitive deficit are attenuated by tacrine and a Chinese herbal prescription, kangen-karyu: Elucidation in type 2 cholesterosis. Mol. Nutr. Food Res. 55, 1112–1122

35. Morris, R. (1984) Developments of a water-maze procedure for studying through increased permeability of connexin 43 hemichannels. Cell Metab. 677–688

36. Ross, J. M., O¨ berg, J., Brene´ , S., Coppotelli, G., Terzioglu, M., Pernold, K., Goiny, M., Sitnikov, R., Kehr, J., Trifunovic, A., Larsson, N. G., Hoffer, P. H., and Olson, L. (2010) High brain lactate is a hallmark of aging and altered by shift in the lactate dehydrogenase A/B ratio. Proc. Natl. Acad. Sci. U.S.A. 107, 20087–20092

37. Sun, J., Ye, X., Xie, M., and Ye, J. (2016) Induction of triglyceride accumulation and mitochondrial maintenance in muscle cells by lactate. Sci. Rep. 6, 33732

38. Bergersen, L. H. (2015) Lactate transport and signaling in the brain: potential therapeutic targets and roles in body-brain interaction. J. Cereb. Blood Flow Metab. 35, 176–185

39. Rafik, A., Boulland, J. L., Halestrap, A. P., Ottersen, O. P., and Bergersen, L. (2003) Highly differential expression of the monocarboxylate transporters MCT2 and MCT4 in the developing rat brain. Neuroscience 122, 677–688

40. Portela, L., Brochier, A. W., Hämäläinen, M. J., Casarini, D. E., Takahashi, T., countertops, K., and Magistretti, P. J. (2011) Development of a water-maze procedure for studying spatial learning in the rat. J. Neurosci. Methods 11, 47–60

41. Morris, R. (1984) Developments of a water-maze procedure for studying through increased permeability of connexin 43 hemichannels. Cell Metab. 677–688

42. Ross, J. M., O¨ berg, J., Brene´ , S., Coppotelli, G., Terzioglu, M., Pernold, K., Goiny, M., Sitnikov, R., Kehr, J., Trifunovic, A., Larsson, N. G., Hoffer, P. H., and Olson, L. (2010) High brain lactate is a hallmark of aging and altered by shift in the lactate dehydrogenase A/B ratio. Proc. Natl. Acad. Sci. U.S.A. 107, 20087–20092

43. Sun, J., Ye, X., Xie, M., and Ye, J. (2016) Induction of triglyceride accumulation and mitochondrial maintenance in muscle cells by lactate. Sci. Rep. 6, 33732

44. Lauritzen, K. H., Morland, C., Puchades, M., Holm-Hansen, S., Hagelin, E. M., Lauritzen, F., Attammal, R., Storm-Mathisen, J., Gjedde, A., and Bergersen, L. H. (2014) Lactate receptor sites link neurotransmission, neurovascular coupling, and brain energy metabolism. Cereb. Cortex 24, 2784–2796

45. Ross, J. M., O¨ berg, J., Brene´ , S., Coppotelli, G., Terzioglu, M., Pernold, K., Goiny, M., Sitnikov, R., Kehr, J., Trifunovic, A., Larsson, N. G., Hoffer, P. H., and Olson, L. (2010) High brain lactate is a hallmark of aging and altered by shift in the lactate dehydrogenase A/B ratio. Proc. Natl. Acad. Sci. U.S.A. 107, 20087–20092

46. Sun, J., Ye, X., Xie, M., and Ye, J. (2016) Induction of triglyceride accumulation and mitochondrial maintenance in muscle cells by lactate. Sci. Rep. 6, 33732

47. Bergersen, L. H. (2015) Lactate transport and signaling in the brain: potential therapeutic targets and roles in body-brain interaction. J. Cereb. Blood Flow Metab. 35, 176–185

48. Rafik, A., Boulland, J. L., Halestrap, A. P., Ottersen, O. P., and Bergersen, L. (2003) Highly differential expression of the monocarboxylate transporters MCT2 and MCT4 in the developing rat brain. Neuroscience 122, 677–688

49. Portela, L., Brochier, A. W., Hämäläinen, M. J., Casarini, D. E., Takahashi, T., countertops, K., and Magistretti, P. J. (2011) Development of a water-maze procedure for studying spatial learning in the rat. J. Neurosci. Methods 11, 47–60

50. Morris, R. (1984) Developments of a water-maze procedure for studying through increased permeability of connexin 43 hemichannels. Cell Metab. 677–688

51. Morris, R. (1984) Developments of a water-maze procedure for studying through increased permeability of connexin 43 hemichannels. Cell Metab. 677–688

52. Ross, J. M., O¨ berg, J., Brene´ , S., Coppotelli, G., Terzioglu, M., Pernold, K., Goiny, M., Sitnikov, R., Kehr, J., Trifunovic, A., Larsson, N. G., Hoffer, P. H., and Olson, L. (2010) High brain lactate is a hallmark of aging and altered by shift in the lactate dehydrogenase A/B ratio. Proc. Natl. Acad. Sci. U.S.A. 107, 20087–20092

53. Sun, J., Ye, X., Xie, M., and Ye, J. (2016) Induction of triglyceride accumulation and mitochondrial maintenance in muscle cells by lactate. Sci. Rep. 6, 33732