Diminished lactate utilization in LDHB-deficient neurons leads to impaired long-term memory retention

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Abstract
The brain's substantial energy consumption primarily arises from the continuous activity of its neurons, which are involved in information processing, transmission, and storage. The astrocyte-neuron lactate shuttle (ANLS) suggests that neurons preferentially utilize lactate over glucose due to its higher efficiency. Astrocytes are proposed to supply neurons with lactate through LDHA expression, while neurons predominantly express LDHB to facilitate lactate oxidation. In this study, LDHB-deficient neurons demonstrated reduced capacity to utilize lactate, which correlated with impaired long-term memory retention in LDHB-deficient mice. However, these mice retained normal short-term memory and showed no macroscopic brain anomalies or neuronal cell death. In a high-glucose environment, the energy metabolism of LDHB-deficient neurons remained typical, suggesting that lactate is not the sole or primary substrate for neuronal energy metabolism, with glucose and other substrates sufficiently supporting the requirements. The observed expression of LDHA in neurons further corroborates the glucose-driven energy metabolism in these cells.

Procedure
Animal study
LDHB knockout (KO) mice and their wild-type (WT) counterparts, aged between 12-14 weeks and 72-89 weeks, were used for this study. The LDHB KO mice, bred on a C57BL/6 genetic background, were created by disrupting the LDHB gene via the cre/loxP approach. All experimental procedures involving these animals received approval from Ajou University Medical Center's Laboratory Animal Research Center (Approval number: AJIRB-BMR-SMP-13-181). Mice were maintained in an environment with a stable temperature of 22±2°C, humidity at 55±10%, and a 12-hour light cycle (7:00 to 19:00 h). They were provided with ad libitum access to food and water. Mice displaying abnormal behaviors or with a discernible rough coat were excluded to mitigate individual variability. Throughout the study, investigators remained blinded to the group assignments. All animal procedures were approved by the institutional review board of Ajou University School of Medicine (2021-0057).

Neuron cell culture
Cortical and hippocampal tissues were isolated from E14 embryos of wild-type (WT) and LDHB-deficient (KO) mice. Following dissection, the tissues were centrifuged at 1,300 rpm for 3 minutes. The supernatant was subsequently discarded, and the resulting pellet was gently resuspended multiple times in 1 mL of plating medium (PM) using a flame-polished Pasteur pipette. Cells were then stained with trypan blue for counting and adjusted to a seeding density of 1 X 10⁶ cells/mL. These cells were cultured on plates pre-coated with a poly-D-lysine solution. For tissue isolation, a minimum essential medium (MEM) supplemented with 200 mM glucose was employed. The PM, composed of MEM with 200 mM glutamine, 5% fetal bovine serum, and 5% horse serum, was used to plate the cells. After 2 days in culture, neurons
were treated with 5 μM cytosine arabinoside (ARA-C) in growth medium (MEM supplemented with 200 mM glutamine and 5% horse serum) to inhibit non-neuronal cell proliferation. Subsequent experiments, including the lactate consumption assay and biochemical analyses (ATP measurement, evaluation of mitochondrial membrane potential, and assessment of reactive oxygen species production), were conducted after an additional 5 days of culturing. For the lactate consumption rate assay, the culture medium was replaced with lactate-rich medium (MEM containing 5 mM glucose and 20 mM lactate). Lactate concentrations in the medium were determined every 6 hours to calculate the lactate consumption rate. For biochemical analyses, neurons were cultured for an additional 24 hours in a lactate-rich medium.

**Measurement of intracellular ATP**

ATP levels were quantified using the ATPlite™ Luminescence Assay System 1000 Assay Kit (PerkinElmer, Waltham, MA, USA), based on the luminescence generated by the reaction of ATP with luciferase and D-luciferin. Cells were lysed using the ATPLite lysis buffer (comprising NaOH and 4 ml/l of 100 Mriton X-100) through a combination of shaking and sonication, as per the manufacturer's guidelines. Post-lysis, samples were centrifuged at 13,000 rpm for 5 min at 4 °C. From the resulting supernatant, 10 μl was combined with 65 μl of dilution buffer (a 2:0 ratio of PBS:lysis buffer) and 15 μl of substrate solution in a 96-well microplate. After incubating under dark conditions for 10 min, luminescence intensity was recorded. ATP concentrations were then normalized to protein levels in each sample.

**Measurement of MMP**

Mitochondrial membrane potential (MMP) of intact neurons was assessed using tetramethylrhodamine methyl ester (TMRM; Molecular Probes, Life Technologies), a cell-permeable fluorescent dye that selectively accumulates in mitochondria due to their negative charge. Adherent cells had their culture medium aspirated, were washed with PBS, and then resuspended in DMEM containing a nal TMRM concentration of 10 μM (prepared by adding 5 μl of 1 mM TMRM to 500 μl DMEM). Cells were incubated at 37 °C for 30 minutes. Notably, cells were pre-treated with CCCP for 10 minutes before TMRM staining. Fluorescence was subsequently analyzed via flow cytometry using a FACS Vantage system (Becton Dickinson).

**Measurement of ROS**

Intracellular levels of reactive oxygen species (ROS) were assessed using the fluorescent probe 2',7'-dichlorodihydrofluorescein (CM-H2DCFDA; Molecular Probes). Cells were washed with PBS, pelleted, and subsequently resuspended in PBS containing 5 μM CM-H2DCFDA. Following incubation at 37 °C for 30
minutes, fluorescence was analyzed via flow cytometry using a FACS Vantage system (Becton Dickinson).

**LDH isozyme pattern assay**

The lactate dehydrogenase (LDH) isozyme profile was characterized using nondenaturing Tris-glycine polyacrylamide gel electrophoresis (PAGE). Cell proteins were lysed in a buffer containing 100 mM K2HPO4, 30 mM KF, 1 mM EDTA, and a protease inhibitor cocktail. Following homogenization, the lysate underwent centrifugation at 13,000 rpm at 4 °C for 15 minutes. The resultant supernatant served as the sample and was consistently maintained on ice. For gel loading, each sample was mixed with an equal volume of 40% sucrose and bromophenol blue for visualization. Each well of the gel received 10 μg of the prepared sample, and the electrophoresis was conducted using an SDS-free Tris-glycine buffer. Subsequent to electrophoresis, the gel underwent staining with a developer solution composed of lactate (3.24 mg/ml), β-nicotinamide adenine dinucleotide (NAD⁺; 0.3 mg/ml), nitroblue tetrazolium (NBT; 0.8 mg/ml), and phenazine methosulfate (PMS; 0.167 mg/ml), all dissolved in a 10 mM Tris-HCl buffer (pH 8.5). The staining process continued at 37 °C until desired band visibility was achieved, typically around 30 minutes or longer. The staining reaction was halted using a 5% acetic acid solution.

**Western blot assay**

Proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer and their concentrations were determined using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, USA). For electrophoresis, proteins were resolved on sodium dodecyl sulfate-polyacrylamide gels with equal amounts of protein (μg) loaded per lane. Following electrophoresis, proteins were transferred onto either polyvinylidene difluoride (PVDF) or nitrocellulose membranes. The membranes were then probed using specific primary antibodies. The loading control was validated using an actin antibody at a 1:5000 dilution. Bound primary antibodies were detected with a horseradish peroxidase-conjugated secondary antibody and visualized using enhanced chemiluminescence (ECL) reagent. The utilized primary antibodies, including anti-actin (ab8226), anti-LDHB (ab75167), anti-LDHA (EP1566Y), were sourced from Abcam (Cambridge, UK).

**Magnetic resonance spectroscopy**

Magnetic resonance spectroscopy (MRS) was conducted on additional mice (WT n=6, KO n=7). Mice were anesthetized using 2% isoflurane in a 70% nitrous oxide and 30% oxygen mix, delivered via a vaporizer. During anesthesia, rectal temperature was maintained at 37.0±0.5°C using an automated heating pad linked to a rectal probe. MRS was performed using a 15.2 T preclinical MRI machine (Bruker,
Germany) at the Center for Neuroscience Imaging Research, Sungkyunkwan University (Suwon, South Korea). A 60 mm volume coil facilitated excitation, while an 18 mm quadrature surface coil was employed for signal detection (Bruker). A single voxel (2 X 2 X 2 mm^3) was positioned over the right hippocampus. MRS scans utilized the Point-Resolved Spin Echo Sequence (PRESS) method, with an echo time of 35 ms and a total acquisition time of 10 min (TR/TE = 2500/3.5 ms, 256 averages) to capture the metabolite spectrum with water suppression. Metabolite spectra were assessed by the lactate to N-acetyl aspartate ratio using the Image J software.

**Morris water maze test**

Spatial learning and memory were assessed using the Morris water maze. The setup included a 90 cm diameter white pool with a 10 cm x 25 cm transparent platform positioned 1.0 cm beneath the water surface. The water was maintained at a consistent 22 ± 0.5° C. During the learning phase (days 1-5), mice underwent three daily trials of 60 seconds each, with the platform's location unchanged and a uniform starting position for all subjects. Mice unable to locate the platform within 60 seconds were manually guided to it for a 10-second period. Five days post learning phase, the time spent by mice in the former platform zone, now sans platform, was recorded. Swim trajectories and related metrics were captured using the Smart 3.0 software in tandem with the Panlab Harvard apparatus (Barcelona, Spain).

**Open field test**

Movement was assessed using the open field test, employing a white acrylic box measuring 40 cm x 40 cm x 30 cm. Mice were individually positioned in the box's center and permitted 1 minute of free exploration. Locomotor activity was automatically recorded using video-tracking, commencing immediately upon mouse placement in the chamber. Line crossings were quantified by counting the instances the mouse's right hind leg contacted a field line. Additionally, the frequency of mouse movements and rearings were recorded to validate movement variations.

**Y maze test**

Short-term memory was assessed using the Y maze test. The test apparatus, constructed from black acrylic, featured three arms converging at a central point, forming a 'Y' shape. Each arm's end had a unique mark for differentiation. Mice were positioned at the convergence of the arms, and their arm entries were recorded for a duration of 8 minutes. Spontaneous alternation was determined using the formula: (actual alternation x 100%) / (total alternation-1)
**Novel object recognition test**

Non-spatial working memory was assessed using an object recognition test. The test employed a white acrylic box measuring 40 cm x 40 cm x 30 cm. The objects designated for discrimination were plastic-made and varied in shape and color, specifically a blue cube and a black cylinder. On the day preceding the test, mice were habituated to the environment with a 5-minute free exploration of the box. An hour before the main test, mice explored two identical objects (blue cubes) for 10 minutes, followed by a 3-minute exploration of a new object (black cylinder). The exploration time of both familiar (F) and new (N) objects was captured automatically using the Smart 3.0 software in conjunction with the Panlab Harvard apparatus (Barcelona, Spain). The discrimination index (%) was derived using the formula: \( \frac{N - F}{N + F} \times 100 \). The object preference (%) was computed as \( \frac{N}{N + F} \times 100 \).

**Tissue processing**

Mice were intraperitoneally anesthetized with 10% chloral hydrate and subsequently perfused transcardially with 1X phosphate buffered saline followed by 4% paraformaldehyde. Their brains were extracted and post-fixed in 4% paraformaldehyde at 4°C for a minimum of 24 hours, then dehydrated in 30% sucrose. Brain tissues were sectioned coronally at 20 \( \mu \)m using a Leica cryostat (CM3050S; Wetzlar, Germany) and preserved in a storage solution at 4°C.

**Cresyl violet staining**

Sections were mounted on coated slides for cresyl violet staining and air-dried overnight. After rehydration in distilled water, they were stained with 0.1% cresyl violet for 5 minutes. Subsequently, sections were rinsed in 70% ethanol, dehydrated through an ethanol and xylene series, and then coverslipped using vectamount. Neuronal cells in the hippocampal CA1 region were quantified using a Nikon microscope (SMZ745T, Japan) at 200X magnification. Cell counts were obtained from selected fields measuring 0.64 x 0.48 mm\(^2\). The cumulative count from the CA1 pyramidal layer on a single slide was averaged over three sections per animal. Results were expressed as group averages. As per Ooigawa, Nawashiro et al. (2006), while non-dark neurons denote healthy cells, dark neurons indicate aberrant cells with compact, dense morphology. The combined total of non-dark and dark neurons represented the total number of hippocampal CA1 cells.

**DAPI staining**

Nuclei were labeled with 4′,6-diamino-2-phenylindole (DAPI; Vector, CA, USA). Observations were made using a Zeiss fluorescence microscope (Axiovert 200M; Oberkochen, Germany) at 400X magnification.
and images captured with an AxiocamMR3 (Zeiss). CA1 pyramidal nuclei counts were obtained from fields measuring 0.347 x 0.260 mm², with averages derived from three distinct CA1 pyramidal regions per animal. The thickness of the CA1 pyramidal nuclei was quantitatively analyzed by measuring width length using Image J software.

**Immunohistochemistry**

Brain tissue sections were mounted on slides and rinsed with phosphate-buffered saline. Endogenous peroxidase activity was inhibited using 0.3% hydrogen peroxide for 5 minutes and 0.25% triton X-100 for 10 minutes. Sections were blocked with 10% normal serum for 1 hour, then incubated with anti-8-hydroxyguanosine (8-OHdG; 1:500, ab62623, Abcam, USA) and anti-rabbit glial fibrillary acidic protein (GFAP; 1:500, Z0334, Dako, USA) antibodies overnight at 4°C. Post incubation, sections were rinsed and exposed to Alexa Fluor 555 goat anti-rabbit IgG (A21428, Invitrogen, USA) or delight 549 anti-mouse IgG (Vector) for 2 hours at room temperature. Sections were then mounted using vectashield hard set containing DAPI (Vector). Imaging was performed with a Zeiss fluorescence microscope (Axiovert 200M; Oberkochen, Germany) at 100X and 400X magnification, and images were captured with an AxiocamMR3 (Zeiss). Quantitative analysis of 8-OHdG involved measuring mean density values in pyramidal CA1 neurons, normalized to LDHB WT mice values. For GFAP quantification, the area of immunopositive cells in CA1 Str. Radiatum was evaluated within fields measuring 1.38 x 1.04 mm². All measurements were conducted using Image J software.

**Statistical analysis**

Data are expressed as mean ± SEM. Statistical significance was determined using an unpaired Student’s t-test. A p-value less than 0.05 was deemed statistically significant. All statistical evaluations were conducted with GraphPad Prism version 5.01 (La Jolla, USA).