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Blood–brain barrier disruption and edema formation due to prolonged starvation in wild-type mice

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Abstract:

INTRODUCTION: Different types of diseases have been treated by restricted caloric intake or fasting. Although during this long time, fasting protective measures, for example, supplements, are given to the patients to protect vital organs such as the liver and kidney, little attention is given to the brain. The current research aims to investigate hypoglycemia due to prolonged fasting disrupts blood–brain barrier (BBB) in mice.

MATERIALS AND METHODS: Immunohistochemistry (IHC) and *in situ* hybridization (ISH) techniques were used to examine the expression of different genes. Evans blue extravasation and wet–dry technique were performed to evaluate the integrity of BBB and the formation of brain edema, respectively.

RESULTS: We confirmed that hypoglycemia affected mice fasting brain by examining the increased expression of glucose transporter protein 1 and hyperphosphorylation of tau protein. We subsequently found downregulated expression of some genes, which are involved in maintaining BBB such as vascular endothelial growth factor (*VEGF*) in astrocytes and *claudin-5* (a vital component of BBB) and *VEGF* receptor (*VEGFR1*) in endothelial cells by ISH. We also found that prolonged fasting caused the brain endothelial cells to express lipocalin-2, an inflammatory marker of brain endothelial cells. We performed Evans blue extravasation to show more dye was retained in the brain of fasted mice than in control mice as a result of BBB disruption. Finally, wet–dry method showed that the brain of prolonged fasted mice contained significantly higher amount of water confirming the formation of brain edema. Therefore, special attention should be given to the brain during treatment with prolonged fasting for various diseases.

CONCLUSIONS: Our results demonstrated that hypoglycemia due to prolonged fasting disrupts BBB and produces brain edema in wild-type mice, highlighting the importance of brain health during treatment with prolonged fasting.

Keywords:

Blood–brain barrier, *Claudin-5*, Evans blue, Hypoglycemia, Lipocalin-2

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Introduction

Different types of diseases have been treated by restricted caloric intake or fasting, for example, rheumatoid arthritis,[1] obesity,[2,3] type-2 diabetes,[3] schizophrenia,[4] various neurological diseases,[5] prevention and treatment of cancer,[6] and irritable bowel syndrome (IBS).[7] Moreover, continuous fasting was considered beneficial for some diseases such as IBS. In a study, a group of people suffering from IBS were treated with pharmacotherapy and psychotherapy with a period of 10 consecutive days of fasting therapy (FT). Although, nowadays, prolonged fasting is not a suggestive therapy for IBS, the results showed that FT has beneficial effects on intractable patients with IBS.[7]

Blood–brain barrier (BBB) is the protective barrier around the brain which separates the

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circulating blood from the extracellular fluid of the brain. This barrier is often compromised in different conditions such as neurodegenerative diseases, stroke, epilepsy, diabetes, traumatic brain injury, and many others.[8] Since glucose is the main energy-providing molecule for normal brain activity,[9] it is a life-threatening condition, in which blood glucose can drop to levels that jeopardize brain functions.[10] Several decades ago, it had been reported that hypoglycemia causes disruption of BBB in insulin-induced hypoglycemic animals[11] as well as electrically induced seizure in hypoglycemic rats.[12]

Brain astrocytes play the most vital roles in brain energy delivery, production, utilization, and storage.[13] When neuronal activity is increased, and the energy demand exceeds supply during acute hypoglycemia, astrocyte glycogen is converted to lactate through glucose, which is transported to the neurons ensuring proper functions of the brain.[14] Therefore, sustained low serum glucose levels will affect astrocytic metabolism and interfere with its function to maintain the BBB integrity.[15-17] Maintenance of BBB is accomplished by different secreted molecules from astrocytes such as vascular endothelial growth factor (*VEGF*), sonic hedgehog (Shh), angiopoietin 1 and 2, and many others whose receptors are expressed in the vascular endothelial cells.[17] Proper balance of these astrocytic secreted proteins is essential to maintain BBB. For example, lack of the Shh signaling compromises BBB integrity[18] and excessive secretion of *VEGF* from reactive astrocytes disrupts BBB.[19] Sustained *VEGF* expression at the proper level is crucial for the vascular homeostasis in the adult brain and other tissues.[20,21]

In the present study, we showed that prolonged fasting (3 or 4 days) induces abnormal genes' expression in astrocytes (such as *VEGF*) and endothelial cells (such as *claudin-5, VEGFR1,* and *lipocalin-2* [*Lcn2*]) of blood vessels in wild-type mice. Since *Lcn2* is an inflammatory stress marker expressed by endothelial cells, we checked and found BBB disruption and edema formation in prolonged fasting wild-type mice. Our study suggests that necessary precautions should be taken to maintain proper brain function before starting long-term fasting as a treatment for other diseases.

Materials and Methods

Mice

Wild-type mice were produced in our laboratory from inbred breeding pairs of wild-type male and female mice. The day of birth was considered to be postnatal day 0 (P0). Mice aged P30 and weighted 22–38 g were taken for experiments. All the procedures were approved by the Animal Research Committee at Jahangirnagar University (No. BBEC, JU/M 2024/06, 116, dated on 18.06.2024), and the Guide for the Care and Use of Laboratory Animals of the Institute for Laboratory Animal Research was followed.

Mice fasting

To induce hypoglycemia, wild-type mice were fasted for a prolonged period of 3 or 4 consecutive days (3 day fasting [3DF] or 4DF) as previously described.[22] Fasting was started at P26 or P27 or P29, and then mice were sacrificed at P30 after 96 h or 72 h or 24 h of fasting for 4DF, 3DF, and 1DF, respectively.

Histology

Paraffin sections were used for immunohistochemistry (IHC) and *in situ* hybridization (ISH). To make paraffin sections, mice at P30 were anesthetized with an intraperitoneal injection of a lethal dose of sodium pentobarbital (125 mg/kg body weight) and were then perfused transcardially with 2 ml of 0.01M PBS, followed by 20–25 ml of 0.1M phosphate buffer containing 4% paraformaldehyde (PFA). Brains were removed and immersed in 4% PFA overnight, and the embedding procedure was performed on the next day. Consecutive 10 µm thick coronal sections were then cut using a rotary microtome (HM 325; Microm).

ISH was performed on paraffin sections as previously described.[23] The following mouse ISH probes were used: *Glut1* (*Slc2a1*, Genbank accession number [GAN]: NM_011400), *Claudin-5* (*Cldn5*, GAN: NM_013805; a kind gift from Dr. Mikio Furuse),[24] *VEGF* (*Vegfa*, GAN: NM_001287056.1 and NM_001287057.1), *VEGFR1* (*Flt-1*; GAN: D88689.1), and *Lcn2* (*Lcn2*, *24p3*, or *NGAL*; GAN: NM_008491).

IHC was performed as previously described,[23] using the following antibodies and dilutions: mouse monoclonal anti-AT-8 (Innogenetics, Belgium, 1:200), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) (Nichirei, Tokyo, Japan, 1:1000), rabbit monoclonal anti-von Willebrand Factor (vWF) (Abcam, 1:50), rabbit polyclonal anti-Iba1 (WAKO, 1:500), and peroxidase-labeled anti-mouse or anti-rabbit IgG (Medical and Biological Laboratories, 1:200). Transmitted light images and fluorescent images were taken with an Olympus BX53 microscope connected to a CCD camera (DP74; Olympus).

Evans blue extravasation

To assess the integrity of the BBB, Evans blue extravasation strategy was used[25] with some modifications. Evans blue dye (1% diluted in PBS; Sigma-Aldrich) was transcardially injected, which was subsequently transferred into the brain by the circulatory system. Then, PBS was transcardially injected until the solution flowing out of the right atrium became clear. After removing

the brain, each brain was washed in PBS, weighed, and homogenized in 1 ml of 50% trichloroacetic acid solution. The supernatant was collected after centrifugation at 10,000 g for 10 min.[25] Finally, the optical density (OD) of the collected supernatant Evans blue solution was measured at 620 nm spectrophotomically.

Brain edema measurement

For the assessment of brain edema, we measured the percentage of brain water content. The percentage of brain water content was determined using the wet–dry method[26-28] with minor modifications. Mice were sacrificed by decapitation, and the brains were quickly removed. The wet weight of each brain was measured and recorded. The brain was then completely dried by heating at 80°C for 48 h, after which the dry weight of the brain was measured and recorded. The percentage of water content (H_2O%) was calculated as follows:

$$H_2O\ \% = 100\times [(\text{wet weight} - \text{dry weight})/\text{wet weight}]$$

Statistics

All data were analyzed using Microsoft Excel-based *t*-tests (Microsoft, Seattle, WA, USA). The results were presented as mean ± standard error of the mean. $P < 0.05$ was considered statistically significant.

Results

Prolonged fasting of 4 days decreases both body weight and blood glucose level of wild-type mice. To examine the condition of BBB in prolonged fasting, we choose 4 days (96 h) of continuous fasting[22] as a maximum level because of ethical issues. We also examined fasting mice for 3 days (72 h) to compare as well as to verify results. In control mice supplied with regular food, there was a significant increase in body weight (at 0 h 34.78 ± 1.12 g/mouse and at 72 h 36.78 ± 1.44 g/mouse; $P < 0.05$) after 72 h from the time of starting fasting [Figure 1a], whereas fasting for 72 h significantly decreases the body weight of wild-type mice (at 0 h 36.08 ± 1.36 g/mouse and at 72 h 30.85 ± 0.88 g/mouse; $P < 0.001$) [Figure 1b]. We also found that 96 h of fasting significantly decreases the body weight of wild-type mice (at 0 h 30.00 ± 3.02 g/mouse and at 96 h 22.25 ± 1.70 g/mouse; $P < 0.01$) [Figure 1c]. When we compared the body weight of two different fasting mice (72 h and 96 h), we found even 1 day more fasting can significantly decrease (72 h 30.85 ± 0.88 and 96 h 22.25 ± 1.70 g/mouse; $P < 0.001$) the body weight (data not shown).

Fasting reduced the blood glucose level. We measured the fasting blood glucose level in our experimental animals, which showed that 3 days fasting (3DF, 72 h; control 14.55 ± 0.87, 3DF 9.35 ± 0.46 mmol/L per mouse; $P < 0.01$) as well as 4 days fasting (4DF, 96 h; control 16.43 ± 0.85, 4DF 5.90 ± 0.88 mmol/L per mouse; $P < 0.01$) significantly reduced the blood glucose level [Figure 1d and e]. Like body weight, blood glucose level was also significantly decreased from 72 to 96 h of fasting (3DF 9.35 ± 0.46, 4DF 5.90 ± 0.88 mmol/L per mouse; $P < 0.05$) [Figure 1f]. These data suggested that hypoglycemia reduced the body weight as well as circulating blood glucose in experimental animals.

Hypoglycemia induced upregulation of glucose transporter protein (*GluT1*) and hyperphosphorylation of *tau protein* in the brain of prolonged fasted mice. The expression of GluT1 was upregulated in the brain when there was systemic hypoglycemia.[29,30] We took the advantage of this finding to examine that systemic low glucose levels induced hypoglycemia in the brain of prolonged fasted mice or not. We used ISH to observe the expression of *GluT1* mRNA in the brain section of fasted mice [Figure 2A]. The results showed, as expected, upregulated expression of *GluT1* mRNA in the endothelial cells of BBB and in astrocytes of prolonged fasted mice. Again, since hypoglycemia[31] or starvation[32] induces tau hyperphosphorylation (p-tau) in the brain, we checked p-tau expression in the fasted mice brain by AT-8 immunohistochemistry (IHC) to validate the results obtained from *GluT1* ISH. As expected, p-tau was not induced in the wild-type mice serving as control, whereas p-tau was highly induced in the brain of fasted mice [Figure 2B]. All these data suggested that hypoglycemia due to prolonged fasting produced a hypoglycemic brain in the wild-type mice.

Decreased expression of *claudin-5* and increased expression of *Lcn2* in the blood endothelial cells of prolonged fasted mice. It had been reported that hypoglycemia can induce the cerebrovascular permeability,[11,12,33] and recently, it was reported that hypoglycemia decreases the expression of *claudin-5*, one of the major tight junction proteins involved in the formation and maintenance of BBB, in *in vitro* culture of brain endothelial cell line.[34,35] Therefore, we first examined the expression of *claudin-5* by ISH in the fasted mice brain. As reported, *claudin-5* expression was decreased in the prolonged fasted mice brain when compared with the brain of wild-type control mice [Figure 3A]. We also confirmed that *claudin-5* ISH signals were positive for only von Willebrand Factor (vWF, a marker of endothelial cells) but not for GFAP-positive astrocytes and Iba1-positive microglia [Supplementary Figure 1A]. There were reports that acute exposure to low glucose rapidly induced endothelial dysfunction in the human endothelium *in vitro*,[36] and hypoglycemia induces oxidative stress and inflammation, leading to endothelial dysfunction in some human type I diabetes patients.[37] The acute phase protein *Lcn2* was upregulated in inflammation, infection, and many other injuries,[38] and *Lcn2* had been reported to be expressed by brain-inflamed

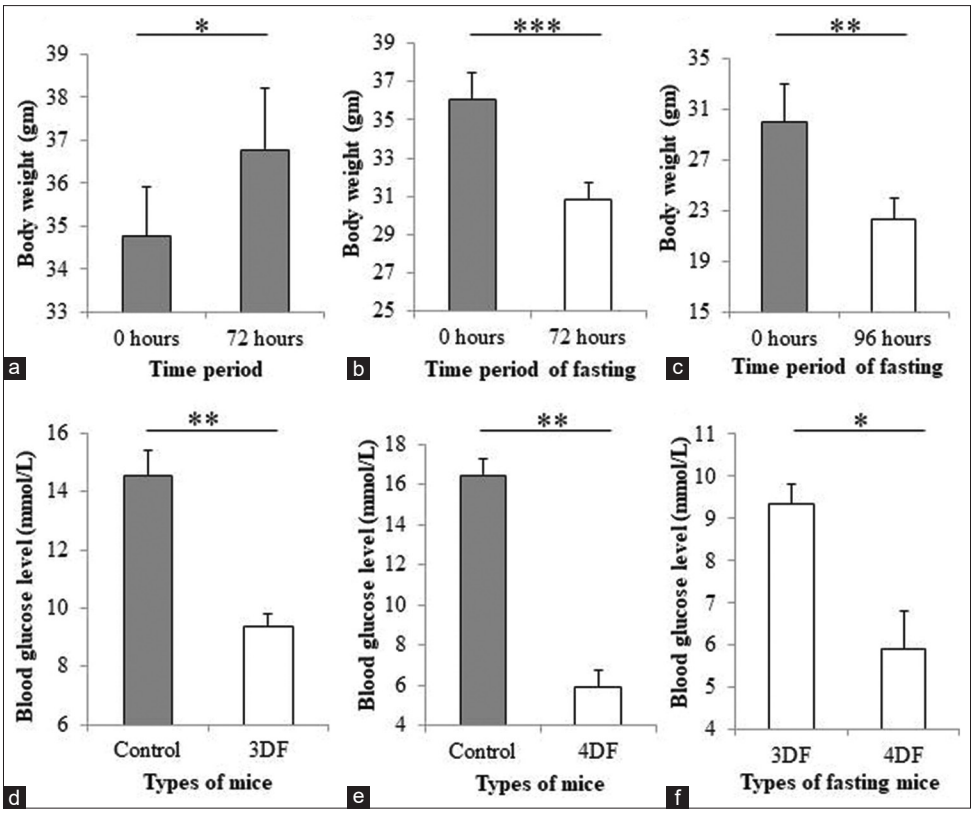

Figure 1: Body weight and blood glucose level of wild-type mice decrease due to prolonged fasting. (a) Body weight was found to be significantly increased for control mice supplied with regular food for 72 h, but (b) there was a significant ($P < 0.001$) decrease in body weight due to prolonged fasting of 72 h. (c) For another group of mice fasted for 96 h, body weight was found to be significantly ($P < 0.01$) decrease. (d and e) Fasting causes a significant decrease in the blood glucose level of wild-type mice fasted for both 3 consecutive days, 3 day fasting (3DF) ($P < 0.01$), and 4DF ($P < 0.01$). (f) Blood glucose level was found to be also significantly ($P < 0.05$) decreased when mice were fasted for 4DF compared with 3DF. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Microsoft Excel-based *t*-test. 3DF: 3-day fasting, 4DF: 4-day fasting

endothelial cells after intraperitoneal administration of lipopolysaccharides,[39] intracerebral hemorrhage,[40] and transient middle cerebral artery occlusion.[41] We chose *Lcn2* protein to evaluate inflammation in the endothelial cells of the blood vessels in the fasted mice and examined *Lcn2* expression in fasted mice by ISH. We found upregulated expression of *Lcn2* mRNA in the endothelial cells of micro- and macroblood vessels [Figure 3B, red arrowheads] of fasted mice when comparing with wild-type control mice. Both claudin-5 (tight junction protein involved to maintain BBB) decreased expression and *Lcn2* (inflammatory protein reported to involve in BBB leakage) increased expression indicated the abnormalities of the endothelial cells of prolonged fasted mice.

Another important player to maintain BBB is the *VEGF* synthesized by astrocytes and sustained *VEGF* expression at the proper level is crucial for the vascular homeostasis in the adult brain and other tissues.[20,21] Therefore, we examined the expression of *VEGF* by ISH in fasted mice and compared with the wild type. We found downregulation of *VEGF* mRNA expression in the prolonged fasted mice brain when compared with the wild type [Figure 3C]. Astrocytes were identified as cell types expressing *VEGF* in both wild-type and fasted mice by double staining with *VEGF* ISH and GFAP IHC [Figure 3C,D, insets]. Since astrocytes maintained BBB[15,16] by secreting a variety of molecules including *VEGF* and blood endothelial cells expressed *VEGF* receptors (*VEGFR1*),[17] we examined the expression of *VEGFR1* by ISH. Like *VEGF* expression, its receptor (*VEGFR1*) expression was also decreased in the prolonged fasted mice [Figure 3D]. Endothelial cells were found as cell types expressing *VEGFR1* receptor [Supplementary Figure 1B]. These data suggested that hypoglycemia may cause abnormalities in vascular homeostasis by downregulated expression of *VEGF* and its receptors, which were required for normal vascular structure and function.[42]

BBB disruption and brain edema formation in prolonged fasting mice. It was well known that astrocytes worked as pivotal players in brain energy delivery, production, utilization, and storage[13] and that glucose tightly controls astrocytic morphology and functions.[43] Again, the acute phase protein *Lcn2* was upregulated in inflammation, infection, and many other injuries,[38] and *Lcn2* had been reported to be expressed by brain endothelial cells in different abnormal and experimental conditions,[39-41] which resulted in BBB disruption; therefore, all of our findings motivated us to investigate BBB disruption and

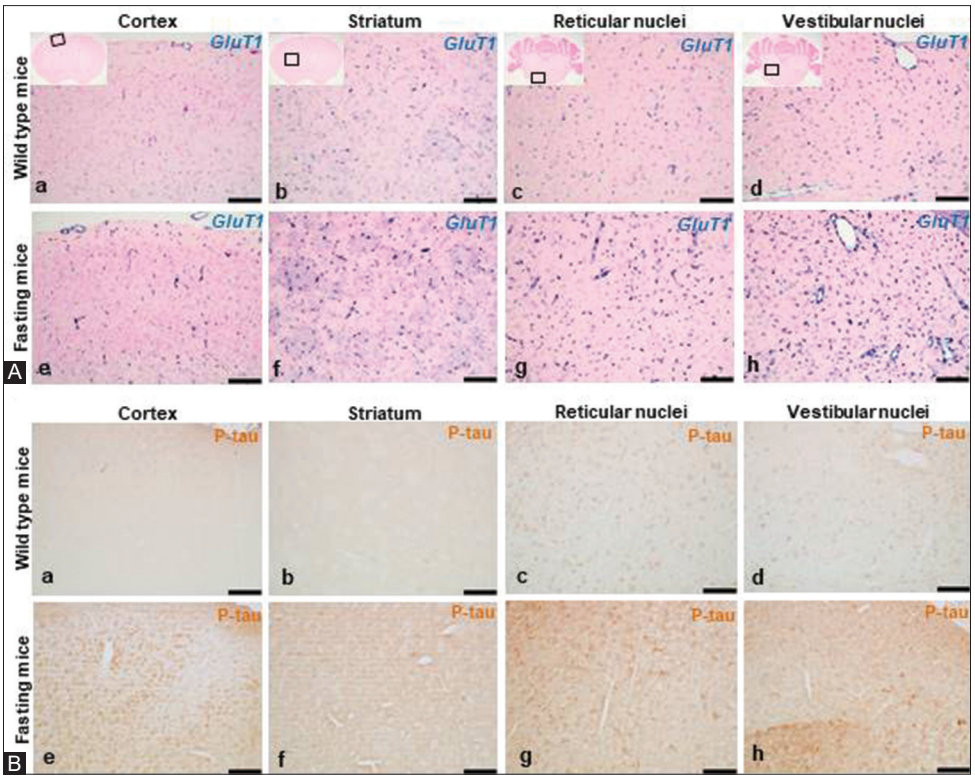

Figure 2: Hypoglycemia observed in wild-type mice due to prolonged fasting affecting the brain glycemic status. (A) Glucose transporter protein 1 *in situ* hybridization showed upregulation of this mRNA in the wild-type fasted mice brain (e-h) compared with wild-type mice brain (a-d) with normal feeding indicating hypoglycemic brain in prolonged fasted mice. Sections were counterstained with nuclear fast red. (B) P-tau (AT-8) immunohistochemistry also revealed hyperphosphorylation of tau protein in the wild-type fasted mice brain (e-h) compared with wild-type mice brain (a-d) with normal feeding. Scale bars: 100 μm

brain edema formation in prolonged fasted mice. To investigate BBB integrity, the Evans blue extravasation strategy was used.[25] The results showed high retention of this dye in the brain tissue of prolonged fasted mice than in control [Figure 4A], which was subsequently quantified by measuring the fluorescence of Evans blue dye spectrophotometrically, which is significantly higher in prolonged fasted mice [Figure 4B; control, 0.17 ± 0.03 OD/mice; 4DF mice 0.38 ± 0.07 OD/mice; $P < 0.05$], indicating BBB disruption in fasted mice. Compromised BBB led to brain edema, which formation was examined by wet–dry method.[26-28] Although 3DF mice did not show significant water leakage from blood vessels to brain parenchyma [Figure 4C; control, 79.15% ±0.49% brain water content; 3DF mice 78.21% ±0.35% brain water content; $P > 0.05$], a significant difference of water content was found in 4DF mice when comparing with control [Figure 4D; control, 79.72% ±0.24% brain water content; 4DF mice 77.70% ±0.16% brain water content; $P < 0.05$]. Together, all these data indicated that prolonged fasting mice were suffering from brain edema as a result of BBB disruption.

Discussion

Nowadays, restricted caloric intake or fasting is considered a treatment for several diseases, including 10 consecutive days of fasting for the treatment of IBS.[7] This prolonged fasting of treatment motivated us to examine the status of BBB during prolonged fasting since this BBB barrier is often compromised in different conditions.[8] There are two major findings of the current study. First, prolonged fasting (96 h) of wild-type mice causes abnormal blood homeostasis with downregulation of some genes (*claudin-5*, *VEGF*, and its receptors) which are involved in maintaining BBB integrity. Second, disturbed blood homeostasis and *Lcn2*-positive stressed blood endothelial cells lead to the disruption of BBB and formation of brain edema. Therefore, the present study uncovers the impact of prolonged fasting on the brain.

Our investigation begins to determine if systematic hypoglycemia induced by prolonged fasting has an impact on the brain. Upregulated expression of *GluT1* mRNA and p-tau protein [Figure 2A and B] in the brain of prolonged fasted mice indicates systemic low glucose levels are producing hypoglycemic brain since both of these two genes are highly expressed in the brain during hypoglycemia and starvation.[29-32] Recently, it was reported that hypoglycemia downregulates the expression of *claudin-5 in vitro*,[34,35] and we also found downregulated expression of *claudin-5* mRNA in the prolonged fasted hypoglycemic mice brain in comparison with the wild-type control mice brain [Figure 3A] indicating vulnerability of the BBB in the fasted mice.

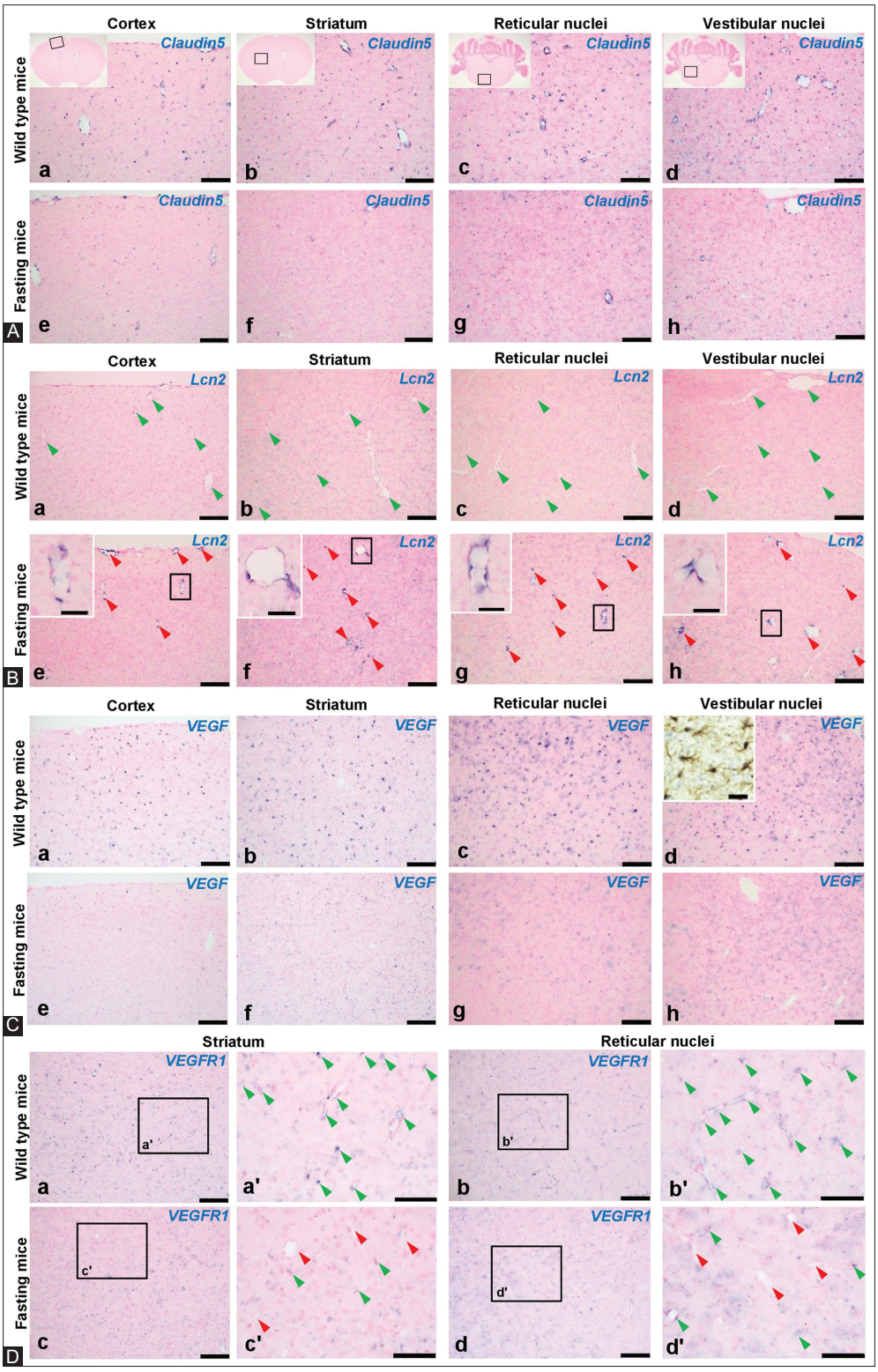

Figure 3: Effects of hypoglycemia on prolonged fasting wild-type mice brain. (A) *Claudin-5* mRNA expressions were decreased in prolonged fasting wild-type brain (e-h) as revealed by *in situ* hybridization (ISH). (B) Abnormalities in the endothelial cells of blood vessels were detected by *Lcn2* ISH in the hypoglycemic brain of prolonged fasting mice (e-h). Green and red arrowheads indicate *Lcn2* negative endothelial cells in normal feeding wild-type mice and *Lcn2*-positive endothelial cells in prolonged fasting wild-type mice, respectively. Small black rectangles were shown in high magnification as the top left insets. (C) vascular endothelial growth factor (*VEGF)* ISH showed that *VEGF* mRNA expression was reduced in the brain of prolonged fasting mice (e-h). Inset (d) shows double staining of *VEGF* ISH and GFAP IHC indicating GFAP-positive astrocytes are *VEGF*-producing cells. (D) Expression of *VEGFR1* was also found to be reduced in the brain of prolonged fasting mice as detected by *VEGFR1* ISH. Black rectangles of a, b, c, and d were shown in high magnification as indicated. Green arrowheads indicate positive signals for ISH and red arrowheads indicate negative signals for ISH. All sections were counterstained with nuclear fast red after ISH. Scale bars: 100 μm, 50 μm (inset of C and a'-d' of D). *Lcn2: Lipocalin-2*, *GluT1*: Glucose transporter protein 1, *VEGF:* Vascular endothelial growth factor, *VEGFR1: VEGF* receptor 1

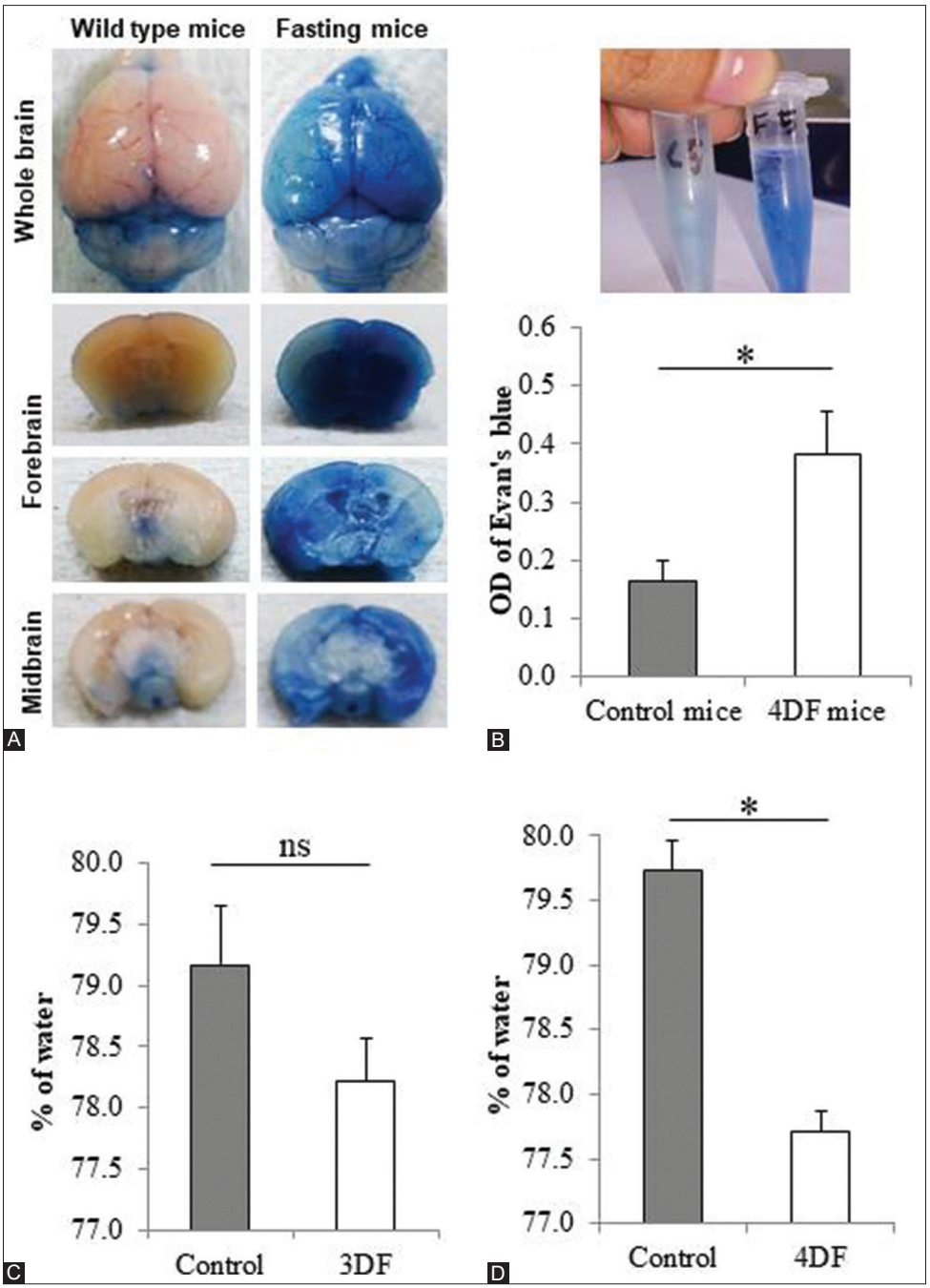

Figure 4: Blood–brain barrier disruption and brain edema formation in prolonged fasting mice. (A) Evans blue extravasation was done for both normal-feeding wild-type mice and prolonged fasting mice. After extravasation, brains were removed and sliced to show the brain parenchyma retained the dye. (B) Upper panel shows the brain supernatant after Evans blue extravasation study. Figure clearly shows the prolonged fasting mice brain retained more Evans blue dye (right) than the normal feeding control mice (left). The lower panel shows a significantly ($P < 0.05$) higher amount of Evans blue dye was retained in the prolonged fasted mice brain than in normal-feeding control mice. (C and D) Although the dry–wet method did not show significant differences in the % of water in the prolonged fasting mice brain when compared with normal feeding control mice for 3-day fasting but showed significant ($P < 0.05$) differences for 4DF. ns: Not significant, 3DF: 3 day fasting, 4DF: 4 day fasting, *P<0.05, Microsoft Excel-based *t* test.

Astrocyte-derived *VEGF* can disrupt *claudin-5*,[19,44] and hypoglycemia upregulates the expression of *VEGF in vitro*;[45,46] therefore, we checked the expression of *VEGF* mRNA by ISH. Surprisingly, we found downregulation of *VEGF* mRNA in the astrocytes of the whole brain of prolonged fasted mice by ISH [Figure 2C]. Recently, it was reported that 1DF increases the tanycytic *VEGF* expression in the third ventricle which boosts blood–hypothalamus barrier plasticity.[47] However, *VEGF* expression in other brain regions except tanycytes after 1DF was not reported in the article. Therefore, we checked the expression of *VEGF* in other brain regions of 1DF wild-type mice using our ISH probe. As reported, 1DF increases tanycytic expression of *VEGF* in the third ventricle [Supplementary Figure 2d], but most importantly, in other brain regions such

as hippocampus and cortex, the *VEGF* expression was decreased compared with normal feeding control [Supplementary Figure 2e and f]. However, the expression of *VEGF* decreases in the tanycytes as well as in the hippocampus and cortex of 4DF mice brain [Supplementary Figure 2g-i]. It suggests differential expression of brain *VEGF* in hypoglycemic condition. Next, we examined the expression of one of the *VEGF* receptors, *VEGFR1*, binding with which *VEGF* exerts its functions.[48] As indicated by ISH experiment, the expression of *VEGFR1* receptor was downregulated [Figure 3D] in the prolonged fasted mice when compared with wild-type control mice, and their expression was mainly found in the endothelial cells [Supplementary Figure 1B]. Since astrocytes secrete a variety of molecules including *VEGF,* and receptors for *VEGF* are expressed by endothelial cells;[17] lack of either *VEGF* or its receptors will block the *VEGF* signaling which can lead to vascular abnormalities.[42]

All data indicate there might be some abnormalities in the blood homeostasis due to prolonged starvation. We looked for the expression of *Lcn2* since it is expressed by brain endothelial cells after intraperitoneal administration of lipopolysaccharides,[39] intracerebral hemorrhage,[40] and transient middle cerebral artery occlusion,[41] resulting in BBB disruption. ISH showed that *Lcn2* was upregulated in the prolonged fasted mice brain; specifically in the endothelial cells of blood vessels [Figure 3B] suggesting inflammation in these cells when compared with normal feeding mice.

Finally, we examined the integrity of the BBB by Evans blue extravasation and found increased Evans blue dye retention in the prolonged fasted mice brain, indicating a compromised BBB [Figure 4A and B]. In addition, the wet–dry method showed an edematous amount of water content in the prolonged fasted mice brain [Figure 4D] validating the presence of compromised BBB in prolonged fasted mice.

Limitations

This study explains the necessity of the management of blood-brain barrier (BBB) during hypoglycemia. We showed prolonged starvation disrupts BBB. Though we used different approaches to examine the disruption of BBB, however, it is necessary to examine the disrupted BBB by electron microscopy (EM).

Conclusions

Our data showed that the BBB is disrupted, and brain edema occurs in the prolonged fasted mice. Further analysis should be done to investigate what are the probable causes of low expression of *VEGF* by the astrocytes and *VEGFR1* by brain endothelial cells at the genomic level due to hypoglycemia.

Author contributions

MIH performed most of experiments, MIH contributed to designing experimental plan, writing manuscript, MH to histological analyses, MH, MA, SS and AA worked on Evan's blue and wet-dry experiments, respectively.

Declaration of Helsinki

All the ethical issues are solved by following the ethical guidelines or principles mentioned in "Declaration of Helsinki" for medical research involving human and other animal subjects originally developed in 1975 and revised in 2000.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of interest

There are no conflicts of interest.

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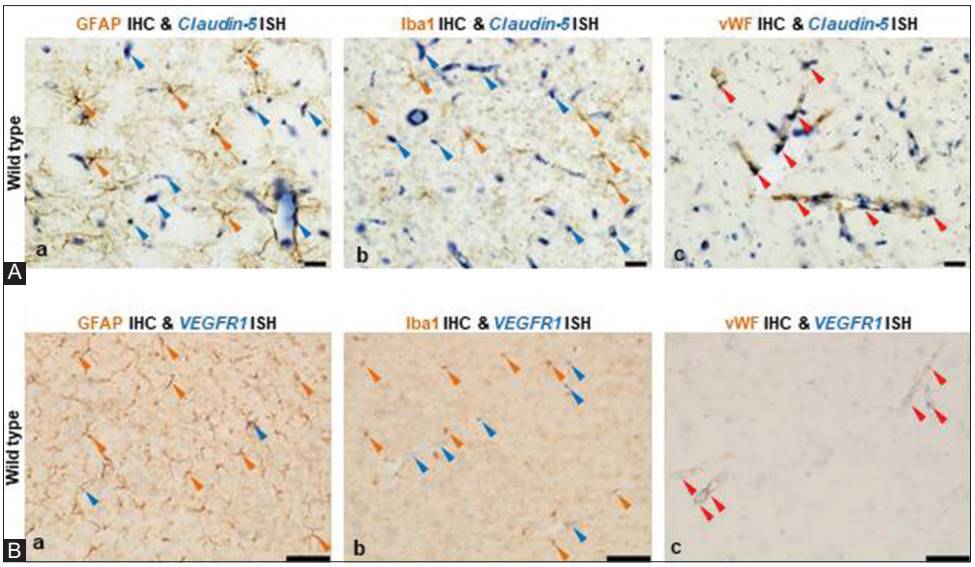
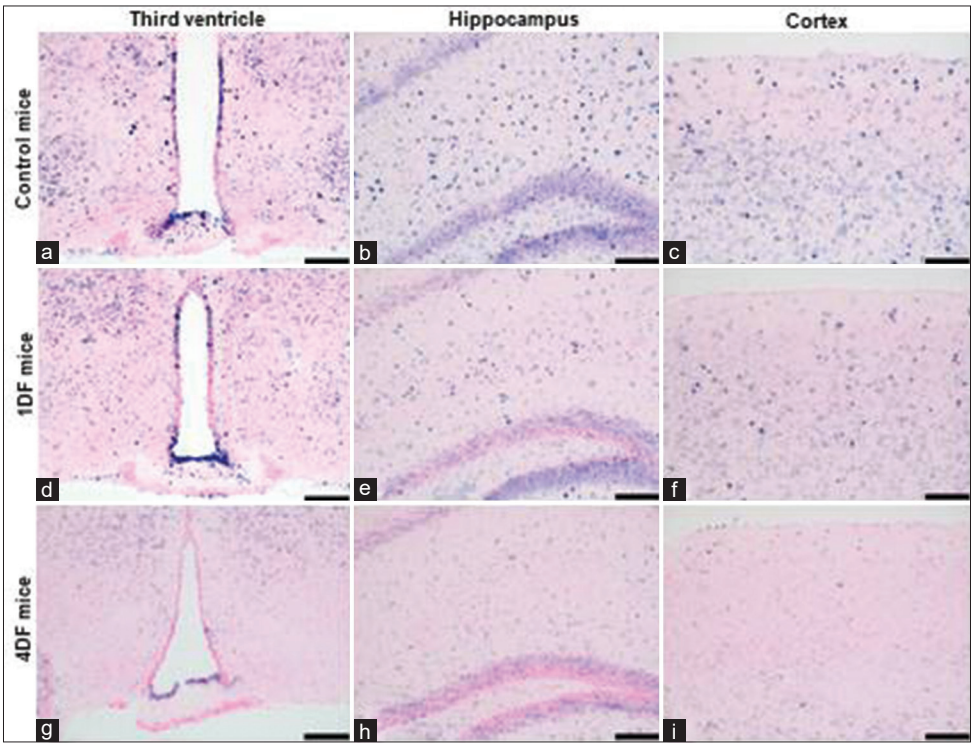
References

1. Sköldstam L, Magnusson KE. Fasting, intestinal permeability, and rheumatoid arthritis. Rheum Dis Clin North Am 1991;17:363-71.
2. Kollar EJ, Atkinson RM. Responses of extremely obese patients to starvation. Psychosom Med 1966;28:227-46.
3. Zubrzycki A, Cierpka-Kmiec K, Kmiec Z, Wronska A. The role of low-calorie diets and intermittent fasting in the treatment of obesity and type-2 diabetes. J Physiol Pharmacol 2018;69:663-83. [doi: 10.26402/jpp. 2018.5.02].
4. Faulkner G, Soundy AA, Lloyd K. Schizophrenia and weight management: A systemic review of interventions to control weight. Acta Psychiatr Scand 2003;108:324-32.
5. Phillips MC. Fasting as a therapy in neurological disease. Nutrients 2019;11:2501.
6. Brandhorst S, Longo VD. Fasting and caloric restriction in cancer prevention and treatment. Recent Results Cancer Res 2016;207:241-66.
7. Kanazawa M, Fukudo S. Effects of fasting therapy on irritable bowel syndrome. Int J Behav Med 2006;13:214-20.
8. Obermeier B, Daneman R, Ransohoff RM. Development, maintenance and disruption of the blood-brain barrier. Nat Med 2013;19:1584-96.
9. Mergenthaler P, Lindauer U, Dienel GA, Meisel A. Sugar for the brain: The role of glucose in physiological and pathological brain function. Trends Neurosci 2013;36:587-97.
10. Klip A, Hawkins M. Desperately seeking sugar: Glial cells as hypoglycemia sensors. J Clin Invest 2005;115:3403-5.

11. Oztaş B, Küçük M, Sandalci U. Effect of insulin-induced hypoglycemia on blood-brain barrier permeability. Exp Neurol 1985;87:129-36.
12. Oztaş B, Camurcu S. Blood-brain barrier permeability after electrically induced seizure in normoglycemic, hypoglycemic, and hyperglycemic rats. Psychiatry Res 1989;29:151-9.
13. Bélanger M, Allaman I, Magistretti PJ. Brain energy metabolism: Focus on astrocyte-neuron metabolic cooperation. Cell Metab 2011;14:724-38.
14. Falkowska A, Gutowska I, Goschorska M, Nowacki P, Chlubek D, Baranowska-Bosiacka I. Energy metabolism of the brain, including the cooperation between astrocytes and neurons, especially in the context of glycogen metabolism. Int J Mol Sci 2015;16:25959-81.
15. Janzer RC, Raff MC. Astrocytes induce blood-brain barrier properties in endothelial cells. Nature 1987;325:253-7.
16. Kuchler-Bopp S, Delaunoy JP, Artault JC, Zaepfel M, Dietrich JB. Astrocytes induce several blood-brain barrier properties in non-neural endothelial cells. Neuroreport 1999;10:1347-53.
17. Alvarez JI, Katayama T, Prat A. Glial influence on the blood brain barrier. Glia 2013;61:1939-58.
18. Alvarez JI, Dodelet-Devillers A, Kebir H, Ifergan I, Fabre PJ, Terouz S, *et al*. The hedgehog pathway promotes blood-brain barrier integrity and CNS immune quiescence. Science 2011;334:1727-31.
19. Argaw AT, Asp L, Zhang J, Navrazhina K, Pham T, Mariani JN, *et al*. Astrocyte-derived VEGF-A drives blood-brain barrier disruption in CNS inflammatory disease. J Clin Invest 2012;122:2454-68.
20. Lee S, Chen TT, Barber CL, Jordan MC, Murdock J, Desai S, *et al*. Autocrine VEGF signaling is required for vascular homeostasis. Cell 2007;130:691-703.
21. Domigan CK, Warren CM, Antanesian V, Happel K, Ziyad S, Lee S, *et al*. Autocrine VEGF maintains endothelial survival through regulation of metabolism and autophagy. J Cell Sci 2015;128:2236-48.
22. Hashimoto T, Cook WS, Qi C, Yeldandi AV, Reddy JK, Rao MS. Defect in peroxisome proliferator-activated receptor alpha-inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting. J Biol Chem 2000;275:28918-28.
23. Horie M, Watanabe K, Bepari AK, Nashimoto J, Araki K, Sano H, *et al*. Disruption of actin-binding domain-containing dystonin protein causes dystonia musculorum in mice. Eur J Neurosci 2014;40:3458-71.
24. Morita K, Sasaki H, Furuse M, Tsukita S. Endothelial claudin: Claudin-5/TMVCF constitutes tight junction strands in endothelial cells. J Cell Biol 1999;147:185-94.
25. Han F, Shirasaki Y, Fukunaga K. Microsphere embolism-induced endothelial nitric oxide synthase expression mediates disruption of the blood-brain barrier in rat brain. J Neurochem 2006;99:97-106.
26. Zhao J, Moore AN, Clifton GL, Dash PK. Sulforaphane enhances aquaporin-4 expression and decreases cerebral edema following traumatic brain injury. J Neurosci Res 2005;82:499-506.
27. Shohami E, Novikov M, Mechoulam R. A nonpsychotropic cannabinoid, HU-211, has cerebroprotective effects after closed head injury in the rat. J Neurotrauma 1993;10:109-19.
28. Higashida T, Kreipke CW, Rafols JA, Peng C, Schafer S, Schafer P, *et al*. The role of hypoxia-inducible factor-1α, aquaporin-4, and matrix metalloproteinase-9 in blood-brain barrier disruption and brain edema after traumatic brain injury. J Neurosurg 2011;114:92-101.
29. Koranyi L, Bourey RE, James D, Mueckler M, Fiedorek FT Jr., Permutt MA. Glucose transporter gene expression in rat brain: Pretranslational changes associated with chronic insulin-induced hypoglycemia, fasting, and diabetes. Mol Cell Neurosci 1991;2:244-52.
30. Kumagai AK, Kang YS, Boado RJ, Pardridge WM. Upregulation of blood-brain barrier GLUT1 glucose transporter protein and mRNA in experimental chronic hypoglycemia. Diabetes 1995;44:1399-404.
31. Lee CW, Shih YH, Wu SY, Yang T, Lin C, Kuo YM. Hypoglycemia induces tau hyperphosphorylation. Curr Alzheimer Res 2013;10:298-308.
32. Yanagisawa M, Planel E, Ishiguro K, Fujita SC. Starvation induces tau hyperphosphorylation in mouse brain: Implications for Alzheimer's disease. FEBS Lett 1999;461:329-33.
33. Hsu DW, Hedley-Whyte ET. Effects of insulin-induced hypoglycemia on cerebrovascular permeability to horseradish peroxidase. J Neuropathol Exp Neurol 1980;39:265-84.
34. Sajja RK, Prasad S, Cucullo L. Impact of altered glycaemia on blood-brain barrier endothelium: An *in vitro* study using the hCMEC/D3 cell line. Fluids Barriers CNS 2014;11:8.
35. Zhao F, Deng J, Yu X, Li D, Shi H, Zhao Y. Protective effects of vascular endothelial growth factor in cultured brain endothelial cells against hypoglycemia. Metab Brain Dis 2015;30:999-1007.
36. Wang J, Alexanian A, Ying R, Kizhakekuttu TJ, Dharmashankar K, Vasquez-Vivar J, *et al*. Acute exposure to low glucose rapidly induces endothelial dysfunction and mitochondrial oxidative stress: Role for AMP kinase. Arterioscler Thromb Vasc Biol 2012;32:712-20.
37. Ceriello A, Novials A, Ortega E, Canivell S, La Sala L, Pujadas G, *et al*. Glucagon-like peptide 1 reduces endothelial dysfunction, inflammation, and oxidative stress induced by both hyperglycemia and hypoglycemia in type 1 diabetes. Diabetes Care 2013;36:2346-50.
38. Jha MK, Lee S, Park DH, Kook H, Park KG, Lee IK, *et al*. Diverse functional roles of lipocalin-2 in the central nervous system. Neurosci Biobehav Rev 2015;49:135-56.
39. Ip JP, Noçon AL, Hofer MJ, Lim SL, Müller M, Campbell IL. Lipocalin 2 in the central nervous system host response to systemic lipopolysaccharide administration. J Neuroinflammation 2011;8:124.
40. Ni W, Zheng M, Xi G, Keep RF, Hua Y. Role of lipocalin-2 in brain injury after intracerebral hemorrhage. J Cereb Blood Flow Metab 2015;35:1454-61.
41. Jin M, Kim JH, Jang E, Lee YM, Soo Han H, Woo DK, *et al*. Lipocalin-2 deficiency attenuates neuroinflammation and brain injury after transient middle cerebral artery occlusion in mice. J Cereb Blood Flow Metab 2014;34:1306-14.
42. Yang Y, Zhang Y, Cao Z, Ji H, Yang X, Iwamoto H, *et al*. Anti-VEGF- and anti-VEGF receptor-induced vascular alteration in mouse healthy tissues. Proc Natl Acad Sci U S A 2013;110:12018-23.
43. Lee CY, Dallérac G, Ezan P, Anderova M, Rouach N. Glucose tightly controls morphological and functional properties of astrocytes. Front Aging Neurosci 2016;8:82.
44. Argaw AT, Gurfein BT, Zhang Y, Zameer A, John GR. VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown. Proc Natl Acad Sci U S A 2009;106:1977-82.
45. Sone H, Kawakami Y, Okuda Y, Kondo S, Hanatani M, Suzuki H, *et al*. Vascular endothelial growth factor is induced by long-term high glucose concentration and up-regulated by acute glucose deprivation in cultured bovine retinal pigmented epithelial cells. Biochem Biophys Res Commun 1996;221:193-8.
46. Satake S, Kuzuya M, Miura H, Asai T, Ramos MA, Muraguchi M, *et al*. Up-regulation of vascular endothelial growth factor in response to glucose deprivation. Biol Cell 1998;90:161-8.
47. Langlet F, Levin BE, Luquet S, Mazzone M, Messina A, Dunn-Meynell AA, *et al*. Tanycytic VEGF-A boosts blood-hypothalamus barrier plasticity and access of metabolic signals to the arcuate nucleus in response to fasting. Cell Metab 2013;17:607-17.
48. Wittko-Schneider IM, Schneider FT, Plate KH. Brain homeostasis: VEGF receptor 1 and 2-two unequal brothers in mind. Cell Mol Life Sci 2013;70:1705-25.



Supplementary Figure 1: Endothelial cells of blood vessels give positive signals for *claudin-5*, vascular endothelial growth factor receptor (*VEGFR1*). (A) Double staining of *claudin-5 in situ* hybridization (ISH) with IHC of different cell-specific markers showed that *claudin-5* ISH signals were not co-localized with Glial Fibrillary Acidic Protein positive astrocytes (a) and Iba1 positive microglia (b) but co-localized with vWF-positive endothelial cells (c). (B) Double staining of *VEGFR1* ISH with immunohistochemistry (IHC) of different cell-specific markers revealed that *VEGFR1* receptor of *VEGF* is expressed by vWF-positive endothelial cells (c). Brown arrowheads indicate respective single positive cells for each IHC, blue arrowheads indicate respective single positive cells for each ISH, and red arrowheads indicate respective double positive cells for ISH and IHC. Scale bars: 50 μm (B), 20 μm (A). GFAP: Glial Fibrillary Acidic Protein, IHC: Immunohistochemistry, ISH: *In situ* hybridization, *VEGFR1:* Vascular endothelial growth factor receptor 1, vWF: Von Willebrand Factor, 4DF: 4 day fasting



Supplementary Figure 2: Expression of vascular endothelial growth factor (*VEGF*) increased in the tanycytes of the third ventricle but decreased in other brain regions after 1 day fasting (1DF). *VEGF in situ* hybridization was performed in the forebrain of normal feeding control mice (a-c), 1DF mice (d-f) and 4DF mice (g-i). Tanycytic expression of *VEGF* was increased in 1DF mice (a and d) but decreased in 4DF mice (a, d, and g), whereas hippocampal and cortical expression of *VEGF* was gradually decreased after 1DF and 4DF when compared with normal feeding control mice (b-c, e-f, and h-i). 4DF: 4-day fasting, 1DF: 1-day fasting