Caloric restriction preserves memory and reduces anxiety of aging mice with early enhancement of neurovascular functions

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ABSTRACT

Neurovascular integrity plays an important role in protecting cognitive and mental health in aging. Lifestyle interventions that sustain neurovascular integrity may thus be critical on preserving brain functions in aging and reducing the risk for age-related neurodegenerative disorders. Here we show that caloric restriction (CR) had an early effect on neurovascular enhancements, and played a critical role in preserving vascular, cognitive and mental health in aging. In particular, we found that CR significantly enhanced cerebral blood flow (CBF) and blood-brain barrier function in young mice at 5-6 months of age. The neurovascular enhancements were associated with reduced mammalian target of rapamycin expression, elevated endothelial nitric oxide synthase signaling, and increased ketone bodies utilization. With age, CR decelerated the rate of decline in CBF. The preserved CBF in hippocampus and frontal cortex were highly correlated with preserved memory and learning, and reduced anxiety, of the aging mice treated with CR (18-20 months of age). Our results suggest that dietary intervention started in the early stage (e.g., young adults) may benefit cognitive and mental reserve in aging. Understanding nutritional effects on neurovascular functions may have profound implications in human brain aging and age-related neurodegenerative disorders.

INTRODUCTION

Neurovascular functions, including cerebral blood flow (CBF) and blood-brain-barrier (BBB) function, play an important role on determining cognitive capability and mental health [1]. Studies have shown that neurovascular risk is highly associated with accelerated decline in language ability, verbal memory, attention and visuospatial abilities [2, 3]. Reduced CBF is linked to anxiety and depression [4-6], and impaired BBB is associated with neuroinflammation and synaptic dysfunction [7]. These neurovascular deficits are exacerbated with age [8] and in a more rapid and profound fashion in neurodegenerative disorders, including Alzheimer’s disease (AD) [9-12]. Interventions that are able to maintain neurovascular integrity are thus considered crucial for impeding age-related neurological disorders.

Caloric restriction (CR), without malnutrition, is the most studied intervention that has been shown to extend the longevity of a broad range of species [13-15]. In the central nervous system, CR has also been shown to induce anti-inflammatory mechanism, reduce oxidative stress and promote synaptic plasticity [16]. In aging, CR protects mitochondrial function, neuronal activity, brain...
volume size and white matter integrity [14, 17, 18]. Enhanced memory in elderly humans and aging animals has also been reported with CR [19-22]. In animal models of AD, CR reduces amyloid beta (Aβ) deposition and preserves memory [23, 24]. However, the impact of CR on CBF and BBB, and the interplay between in vivo neurovascular functions, cognitive aging, and mental health, remain unknown.

In this study, our primary goal was to identify age-related changes of neurovascular integrity in response to CR. We previously showed that CR is protective for CBF in old adult rodents [25]. Here we further determined whether CR shows early effects on neurovascular functions, and the potential changes in vascular signaling markers thereof, in young adult animals. Our secondary goal was to determine the correlation between neurovascular function, cognitive integrity, and mental health across the young and old mice. We hypothesized that CR has significantly protective effects on CBF and BBB, which may contribute to preserved neurovascular integrity, learning and memory, and reduced anxiety in aging. We used magnetic resonance imaging (MRI) to quantify in vivo CBF and confocal imaging to measure BBB function, and biochemical assays to determine neurovascular signaling markers. Behavioral tests were used to assess cognition, anxiety of the mice, and the correlation between behavioral and neurovascular outcomes. The findings from this study will enhance our understanding regarding the effectiveness of nutritional intervention on brain functions in aging.

RESULTS

Caloric restriction enhances neurovascular functions in young mice

We firstly determined the CBF and BBB changes in response to CR in young adult mice (5-6 months of age). We used MRI to measure CBF in mice fed with either ad libitum (AL) or 40% CR (N = 12 per group). Fig. 1A shows the group-averaged CBF images of AL and CR mice. The CBF level is colorized in a linear scale, indicating that CR mice have overall higher CBF compared to the AL mice. We did further CBF analyses in brain regions associated with cognitive functions (e.g., memory and learning) based on MRI structural imaging and mouse brain atlas. We found that young CR mice had significantly higher CBF in frontal cortex (p < 0.01; Fig. 1B) and hippocampus (p < 0.01, Fig. 1C), compared to young AL mice.

![Figure 1. Caloric restriction enhances neurovascular functions in young mice.](image-url)
BBB function was determined by measuring P-glycoprotein (P-gp) transport activity from cortical capillaries. P-gp is an ATP-driven transporter highly expressed at the BBB that facilitates clearance of \( \beta \)-amyloid (A\( \beta \)), a hallmark of AD. We previously established a confocal imaging-based assay to assess P-gp transport activity in freshly isolated brain capillaries from mice [26, 27]. This assay measures within capillary lumens accumulation of [N-\( \varepsilon \)(4-nitro-benzofurazan-7-yl)-D-Lys(8)]-cyclosporin A (NBD-CSA), a fluorescent P-glycoprotein substrate. Fig. 1D shows representative confocal images of capillaries incubated to steady state in medium containing 2 \( \mu \)M NBD-CSA; the intensity of fluorescence in the capillary lumen reflects the amount of NBD-CSA transported by P-gp. The corresponding quantitative results are shown in Fig. 1E. Young CR mice had enhanced P-gp transport activity (2.4 fold increase; \( p < 0.0001 \)) compared to AL mice.

**Caloric restriction enhances vascular signaling markers and shifts metabolism in young mice**

Caloric restriction has been shown to inhibit mammalian target of rapamycin (mTOR), a nutrient sensor, in response to cellular energy status and growth factors [28, 29]. We and others have previously showed that inhibiting mTOR signaling activates endothelial nitric oxide synthase (eNOS) and releases nitric oxide, a vasodilator, which in turn causes increased CBF [30, 31]. To determine whether the enhancement of neurovascular functions in the young adult mice is also associated with mTOR signaling, we measured the protein levels of mTOR and eNOS in capillaries isolated from young CR and AL mice (Fig. 2A). We found that, compared to AL mice, CR mice had significantly lower level of mTOR (decrease to 71.1 ± 1% over 100% controls; \( p < 0.01 \); Fig. 2B), but higher level of eNOS (increase by 146.5 ± 11.6% over 100% controls; \( p < 0.001 \); Fig. 2C), consistent with our previous findings [30]. We also measured P-gp protein expression levels (Fig. 2A). Similar to the results of P-gp activity, we found that CR mice had significant enhancement in P-gp protein levels compared to the AL mice (increase by 168.4 ± 23.1% over 100% controls; \( p < 0.001 \); Fig. 2D).

Reduced mTOR also implies metabolic status changes, we thus measured levels of the glucose transporter 1 (GLUT1) using Western blot (Fig. 2A). We observed a significant decrease of GLUT1 in young CR mice compared to the young AL mice (decrease to 70.9 ± 7.5% over 100% controls; \( p < 0.01 \); Fig. 2E). This is consistent with blood glucose results, showing that CR mice had significantly reduced blood glucose levels.
relative to the AL mice \((p < 0.01; \text{Fig. } 2F)\). In contrast, CR mice had significantly higher levels of blood ketone bodies compared to the AL mice \((p < 0.05, \text{Fig. } 2G)\). This is consistent with our previous findings that caloric restriction shifts metabolism from glucose to ketone bodies utilization \([17, 25]\).

**Caloric restriction decelerates the rate of decline of cerebral blood flow in aging mice**

We further determined the CBF and BBB function changes in response to CR in old adult mice \((18-20 \text{ months of age; 12 AL and 12 CR mice})\). Similar to the findings in the young mice, we found that old CR mice had significantly higher CBF compared to the old AL mice, both in the frontal cortex \((p < 0.01; \text{Fig. } 3A)\) and hippocampus \((p < 0.01, \text{Fig. } 3B)\). However, we did not observe a difference in P-gp activity between the two groups \((p > 0.05, \text{Fig. } 3C)\).

We calculated the age-related changes in CBF and BBB function. We found that in the frontal cortex, old AL mice had 43.13\% group averaged decline of CBF compared to the young AL mice (no variation was available due to cross-sectional comparison); In contrast, the old CR mice had only 22.28\% averaged reduction compared to the young CR mice (Fig. 3D).

Similar pattern was found in hippocampus – old AL mice had 45.10\% averaged decreases in CBF, whereas old CR mice had 28.13\% averaged reduction, compared to their youngittersmates (Fig. 3E). On the other hand, we found that there was a higher reduction rate of P-gp activity in the CR group (-75.33\%) compared with the AL group (-45.45\%; Fig. 3F). Collectively, we found that caloric restriction impeded the age-dependent decline of CBF but not P-gp activity (BBB function).

**Caloric restriction preserves learning and long-term memory of aging mice**

We used radial arm water maze (RAWM) to evaluate learning and spatial memory of the mice \([32, 33]\). Fig. 4A illustrates the RAWM assessment. The task for the mice was to identify the arm which contains a hidden platform. Wrong entries of the arms were recorded as errors. The protocol consisted of a two-day testing paradigm. Day 1 was the “learning” phase where mice went through three blocks (Blocks 1-3; 5 trials in each block) to test learning and short-term spatial memory. Day 2 was the “recall” phase where mice went through three additional blocks (Blocks 4-6) to test long-term memory after a 24-hour retention period to locate the platform. It is expected that after the two-day training, the mouse with intact memory could find the platform.
with minimal errors. In young mice, we saw no significant difference between AL and CR groups (Fig. 4B). In old mice, however, AL group made significantly more errors in the initial learning phase (Block 1; \( p < 0.01 \)) and the initial recall phase (Block 4; \( p < 0.01 \)), compared to the CR group (Fig. 4C). In addition, we found that old CR had similar performances in both Block 1 (Fig. 4D; \( p > 0.05 \)) and Block 4 (Fig. 4E; \( p > 0.05 \)) compared to the young mice (both AL and CR), suggesting that old CR mice had preserved learning and long-term memory abilities.

We used combined errors from Blocks 1 and 4 to further identify the correlation between cognitive function and CBF. We found that the errors made in RAWM had significantly inverse correlations with hippocampal CBF (\( r^2 = 0.29, p < 0.001 \); Fig. 4F) and frontal CBF (\( r^2 = 0.27, p < 0.001 \); Fig. 4G), indicating that level of CBF in cognition-associated brain regions is highly associated with learning and spatial memory performances.

**Caloric restriction reduces anxiety of aging mice**

We used elevated plus maze (EPM) to evaluate anxiety of the mice (Fig. 5A) [34]. The EPM consists of two open and two closed arms. Closed arms are perceived as safe zones, and thus mice with higher anxiety had tendency to stay in the closed arms. We determined the anxiety-related behavior by measuring the time spent in the closed arms over the 5 min. test session. For the young mice, we did not find significant differences between the CR and AL groups (\( p > 0.05 \); Fig. 5B), though CR mice had a trend of less time in the closed arms. In contrast, old AL mice spent significantly longer time in the closed arms compared to the old CR mice (\( p < 0.01 \); Fig. 5C), indicating higher anxiety of the old AL mice. Consequently, when comparing the age-related performances, the AL group showed higher increases in anxiety (31.43% group averaged) compared to the CR mice (12.54% group averaged) (Fig. 5D). We also found it had significant and inverse correlations between closed arm duration and CBF in hippocampus (\( r^2 = 0.40, p < 0.0001 \); Fig. 5E) and in frontal cortex (\( r^2 = 0.39, p < 0.0001 \); Fig. 5F).

**DISCUSSION**

To our knowledge, this is the first study to investigate the interplay between CR-induced changes of neurovascular integrity, cognitive function, and mental health.
health in aging using neuroimaging, behavioral assessments, and biochemical assays. In this study, we demonstrate (i) in young mice, CR enhanced CBF and BBB function, (ii) inhibited mTOR, which was associated with increased eNOS, reduced glucose metabolism, and increased blood ketone level; (iii) in the aging mice, CR preserved CBF, learning, long-term memory, and reduced anxiety; (iv) hippocampal and frontal CBF exhibited high association with cognitive performance and inversely correlated with anxiety level.

Our results indicate that young CR mice had significant enhancement of CBF and BBB function, which in turn may be associated with mTOR signaling changes induced by CR. In addition to modulating eNOS, we previously demonstrated that mTOR inhibition reduces proinflammatory cytokine that breaks down BBB, and thus restores BBB integrity and CBF in mice that carries human APOE4 gene [35]. Furthermore, mTOR is able to clean up misfolded proteins (such as Aβ and cerebral amyloid angiopathy) via regulation of autophagy [36], therefore, it can further reduce atherosclerosis and preserve neurovascular integrity, e.g., vascular density [30]. mTOR signaling also integrates the effects of brain-derived neurotrophic factor, which works with insulin/insulin-like growth factors and serotonin, to exert beneficial effects on vascular system by decreasing blood pressure, atherogenic lipids, inflammatory cytokines and oxidative stress, and increased cellular stress resistance [37, 38]. As shown in the present and previous studies, mTOR inhibition leads to metabolic shift from utilizing carbohydrate (e.g., glucose) to fatty acid (e.g., ketone bodies produced from fatty acids) [17, 25, 39, 40]. Increased levels of ketone bodies have shown to evoke CBF response, and reduce oxidative stress, neuroinflammation and Aβ [39, 41-44].

Increased CBF and ketone bodies metabolism suggest increased oxidative metabolism within the brain of young mice. Basal CBF is tightly coupled with cerebral metabolic rate of oxygen [45] and utilization of ketone bodies significantly elevate the oxygen utilization in mitochondria through beta-oxidation of fatty acid [40, 46]. This is supported by evidence from isolated mitochondria, showing CR enhances mitochondrial function and induces bioenergetic efficiency (7,19). This is also consistent with our previous imaging findings that old animals with chronic CR diet had preserved oxidative metabolism, mitochondrial functions

![Figure 5. Caloric restriction reduces anxiety of aging mice. (A) An illustration of the Elevated Plus Maze. The maze consists of four arms (two enclosed arms and two open arms) elevated 100 cm above the floor. Anxiety level was determined the time spent in the closed arms (conceived as a safe place) over a 5 minutes testing session. Closed arm duration (in seconds) of (B) Young AL and CR mice, and (C) Old AL and CR mice. (D) The age-dependent changes of anxiety level between AL and CR mice. Significant inverse correlation between closed arm duration and CBF in (E) hippocampus (r² = 0.40, p < 0.0001) and (F) frontal cortex (r² = 0.39, p < 0.0001). Color codes indicate the four groups of mice. Data are mean ± SEM. **p < 0.01; AL: ad libitum; CR: caloric restriction.](image-url)
(TCA cycle flux and ATP production), and neuronal activity (neurotransmission rate) compared to the old AL animals [18]. The increased oxidative metabolism, particularly in brain regions associated with cognition (e.g., frontal cortex and hippocampus), may also play a crucial role for neuronal and cognitive protections. Previous studies showed that cognition-associated brain regions have non-oxidative glycolysis exceeding the required needs of oxidative phosphorylation, a phenomenon known as aerobic glycolysis (AG) [47]. Excessive AG (or the "Warburg effect") is a key process that sustains T cell activation and differentiation and is involved in inflammatory-mediated conditions [48]. In line with this, the distribution of AG in normal young adults is spatially correlated with Aβ deposition in AD patients and cognitively normal individuals with elevated Aβ [49, 50]. Animal studies further demonstrated that Aβ plaque formation is an activity dependent process associated with AG [49, 51]. Therefore, increased oxidative metabolism in cognition-related regions may decrease AG and thus reduce the risk for AD, consistent with the findings in CR mice [23, 24].

The early-life neurovascular and neurometabolic changes may play a critical role in protecting physiological functionality and enhancing cognitive reserve with age [52]. Recent studies reported that lifestyle factors act as moderators for cognitive reserve to protect against AD in the elderly [52, 53]. The study suggested that neuroprotective mechanisms play a major role during early stages and compensatory mechanisms in later stages of the disease. In line with this, we observed that the CR mice had a slower reduction rate in CBF with age compared with the AL mice. As a result, the old CR mice had comparable levels of CBF compared to the young AL mice, similar to our previous findings in rats [25]. The preservation of hippocampal and frontal CBF were associated with improved cognition in the old CR mice. This is also consistent with our previous findings that restoration of CBF in young mice modeling human AD could potentially prevent their cognitive decline in aging [30, 35].

We further identified that CR reduced anxiety levels in aging mice. Similar findings were reported in a recent study, showing rhesus monkeys on a 30% CR diet demonstrated less anxious behavior than controls in different arousing contexts [54]. These results are consistent with other studies, indicating that mTOR inhibition is playing an important role on anxiety- and depressive-like behaviors [55]. Treating mice with rapamycin, an mTOR inhibitor, Halloran et al. observed reduced anxiety-like behavior, e.g., reduced thigmotaxis (swimming in close proximity to the pool wall), in mice with AD-like pathology. They also found a reduction in depressive-like behavior in the mice, e.g., floating during training phase of the Morris water maze and reduced time spent immobile on the tail suspension test. Similar effects have been reported for genetically modified mice with autistic-like behavior, showing that mTOR inhibition attenuated anxiety, hyperexcitability, abnormal social interaction, repetitive behavior and vocalizations of the mice [56, 57]. It has been shown that the changes in anxiety- and depressive-like behavior were correlated with increased levels of dopamine and dopamine metabolites in the midbrain [55]. Our study found that CBF was highly correlated (inversely) with anxiety level, which is consistent with previous findings [4-6].

We demonstrated that CR had beneficial effects on aging brain functions, potentially via the mTOR pathway. Genetic or pharmacological inhibition of mTOR signaling has shown to slow aging and extend lifespan in various species, and confer protection against many age-related pathologies [58]. Here we further show that activating the mTOR pathway could potentially protect brain function (both cognitive and non-cognitive types of behavior) in healthy aging by preserving neurovascular function. Specifically, mTOR inhibition was associated with increased eNOS, which may contribute to CBF enhancements in young CR mice and preservation in old CR mice. In AL mice (control), we found decreased P-gp protein expression and activity with age, which is consistent with previous literature findings in different mouse models [59-61]. In CR mice, however, calorie restriction did not preserve P-gp expression and activity with age. To this date it is unknown how blood-brain barrier P-gp activity is regulated in aging and how it can be preserved. Thus, more studies are needed to investigate potential signaling steps that lead to age-mediated reduction of P-gp at the BBB. Future studies will also need to further investigate the potential mechanism of CR effects on BBB function with age.

It has to be pointed out that we used a long-lived animal model (C57BL/6N) in the study. We recognize, however, that the CR effects may be strain- and genetics-dependent. Recent studies showed that CR had adversely impacted several inbred mice, including shortening lifespan and impairing fat storage with age [62, 63]. Therefore, it would be important for future studies to take into account genetic background to identify the effects of CR on brain aging. This is also applicable for humans, where each individual would have different responses to identical foods due to their genotype. Recent studies have shown that personalized diet could be prescribed based on the individual’s genetic response to post-meal blood sugar changes [64].
Precision nutrition would be very useful in the future to slow down brain aging and to prevent AD, and the progress can be monitored by in vivo neuroimaging and cognitive testing.

In conclusion, we used neuroimaging, behavioral assessments and biochemical assays to identify correlations between vascular integrity, cognitive functions, and mental health induced by CR in aging mice. We showed that neurovascular functions were enhanced in young CR mice, as well as preservation of CBF, cognition, and anxiety level in aging CR mice. Our study suggests that mTOR pathway may be critically involved in the process. These findings imply that dietary intervention started in the early stage (e.g., young adults) may benefit cognitive reserve in aging. Understanding nutritional effects on brain vascular, cognitive, and mental functions may have profound implications in human aging and other age-related neurodegenerative disorders. In the future, using in vivo MRI and cognitive assessments, we could be in a position to identify effective personalized nutritional interventions and treatment efficacy thereof to slow down brain aging and/or prevent dementia in humans.

**METHODS**

**Animals**

We used male C57BL/6N mice in the study as they demonstrated extended longevity with CR [65, 66]. Young (5–6 months) and old (18-20 months) adult mice were obtained from the National Institute on Aging Caloric Restriction Colony. At the National Institute on Aging, all mice were fed *ad libitum* [National Institutes of Health (NIH)-31 diet] until 14 weeks of age. The CR regimen was initiated by incremental caloric reduction of 10% at week 14, 25% at week 15, and reaching full 40% CR by week 16 with continuation of the diet over the lifetime. The vitamin-fortified NIH-31 [NIH-31 fortified] diet fed to CR mice provided 60% of the calories and additional vitamins supplement consumed by *ad libitum* mice. After arriving at our facilities, mice were housed individually (1 mouse per cage) in a specific pathogen-free facility. We determined the sample size with power analysis in order to perform the comparison at a 0.05 level of significance, with a 90% chance of detecting a true difference of all the measurements between the four groups. Twelve mice per group were used in the study. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky (UK) and in compliance with the ARRIVE guidelines [67].

**Cerebral blood flow measurement**

MRI experiments were performed on a 7T MR scanner (Clinscan, Bruker BioSpin, Germany) at the Magnetic Resonance Imaging & Spectroscopy Center of the University of Kentucky. Mice were anesthetized with 4.0% isoflurane for induction and then maintained in a 1.2% isoflurane and air mixture using a nose cone. Heart rate (90–110 bpm), respiration rate (50-80 breaths/min), and rectal temperature (37 ± 1 °C) was continuously monitored and maintained. T2-weighted structural images were acquired with field of view (FOV) =18 x18 mm², matrix = 256 x 256; slice thickness = 1 mm, 10 slices, repetition time (TR) =1500 ms, and echo time (TE) = 35 ms. Quantitative CBF (with units of mL/g per minute) was measured using MRI-based pseudo-continuous arterial spin labeling (pCASL) techniques. A whole body volume coil was used for transmission and a mouse brain surface coil was placed on top of the head for receiving. Paired control and label images were acquired in an interleaved fashion with a train of Hanning window-shaped radiofrequency pulses of duration/spacing = 200/200 μs, flip angle = 25° and slice-selective gradient = 9 mT/m, and a labeling duration = 2100 ms [68]. The images were acquired by 2D multi-slice spin-echo echo planner imaging with FOV =18 x18 mm², matrix =128 x 128, slice thickness = 1 mm, 10 slices, TR = 4,000 ms, TE = 35 ms, and 120 repetitions. pCASL image analysis was employed with in-house written codes in MATLAB (MathWorks, Natick, MA) to obtain quantitative CBF [69].

**Radial arm water maze**

As shown in Fig. 3A, the RAWM task can be used to measure both spatial working memory and spatial reference memory [32, 33]. The RAWM task was conducted in the Rodent Behavioral Core (RBC) of UK as described previously [17], following a 2-day testing paradigm. A staggered training schedule was used, running the mice in cohorts of ten mice, while alternating the different cohorts through the trials over day 1 and day 2 of the test. This alternating protocol was used to avoid the learning limitations imposed by massed sequential trials and to avoid fatigue that may result from consecutive trials. Geometric extra-maze visual cues were fixed throughout the study on three sides of the curtains. Visual platform trials were included in the training, and were used to determine if visual impairment could be a cofounding variable. The mouse performance was recorded by EthoVision XT 8.0 video tracking software (Noldus Information Technology). Data were analyzed by the EthoVision software for the number of incorrect arm entries, which are defined as errors. The video was reviewed for each
mouse to ensure that the mice did not employ nonspatial strategies, such as chaining, to solve the task.

**Elevated plus maze**

The EPM was also performed at RBC of UK. The maze consists of four arms (two enclosed arms and two open arms) elevated 100 cm above the floor (Fig. 5A). The time that mouse spent in the closed arms and open arms of the maze were recorded automatically over the 5 min test session by EthoVision XT 8.0 video tracking software (Noldus Information Technology).

**Blood-brain barrier function determination and Western blotting**

**Capillary isolation**

Brain capillaries were isolated from mice according to a previously described protocol [26, 70]. Briefly, mice were euthanized by CO₂ inhalation and decapitated; brains were immediately harvested and collected in ice-cold DPBS buffer supplemented with 5 mM D-glucose and 1 mM Na-pyruvate, pH 7.4. Brains were dissected by removing meninges, choroid plexus and white matter, and homogenized in DPBS. The brain homogenate was mixed with Ficoll® and centrifuged at 5,800g for 20 min at 4°C. The capillary pellet was resuspended in 1% BSA buffer and first passed through a 300 µm nylon mesh and then through a 27 µm nylon mesh. Capillaries retained by the 27 µm nylon mesh were collected and washed with DPBS buffer, and used for experiments.

**P-glycoprotein transport activity**

Isolated capillaries were incubated for 1 h at room temperature with 2 µM NBD-CSA (custom-synthesized by R. Wenger (Basel, Switzerland)) in DPBS buffer. Ten capillary images were acquired by confocal microscopy (Leica TSP SP5 Confocal Microscope with Environmental Chamber, 63 × D-Water UV objective, numerical aperture 1.2, 488-nm line of an argon laser, Leica Microsystems). Confocal images were analyzed by quantitating luminal NBD-CSA fluorescence with Image J software (v.1.45s; Wayne Rasband, NIH). Specific, luminal NBD-CSA fluorescence was taken as the difference between total luminal fluorescence and fluorescence in the presence of the P-glycoprotein-specific inhibitor PSC833 (5 µM, Novartis, Basel, Switzerland) [71].

**Western blotting and quantification**

To determine protein expression, isolated brain capillaries were homogenized in tissue lysis buffer containing protease inhibitor cocktail. Homogenized brain capillary samples were centrifuged at 10,000 g for 15 min at 4°C, followed by a centrifugation of the denucleated supernatants at 100,000 g for 90 min at 4°C. Pellets (crude brain capillary plasma membranes) were resuspended and protein concentrations were determined using the Bradford protein assay. Western blots were performed using the NuPage™ electrophoresis and blotting system from Invitrogen. Blotting membranes were incubated overnight with antibody to P-gp (C219; MA1-26528, ThermoFisher, 1µg/ml), mTOR (ab134903, Abcam, 1µg/ml), GLUT1 (ab652, Abcam, 1µg/ml), and β-actin (ab8226 from Abcam, 1:1000, 1 µg/ml). Proteins were detected using SuperSignal™ West Pico Chemoluminescent substrate (Pierce, Rockford, IL, USA) and protein bands were visualized with a BioRad Gel Doc™ XRS imaging system.

**Blood glucose and ketone bodies measurements**

When the mice were sacrificed, blood samples were collected in 500 µl lithium heparin 12.5 IU Terumo Capiject Capillary blood collection tubes (Vacutainer K2 EDTA) to avoid blood coagulation. A total of 1–2 µl of blood sample were used to measure blood glucose level using a blood glucose meter and a test strip (Clarity Plus, Boca Raton, FL, USA). Another 10 µl of blood sample was used for ketone bodies level measurement using a STAT-Site M (β-Hydroxybutyrate) meter and a test strip (Standbio Ketosite STAT-Site M-β HB, Boerne, TX, USA).

**Statistics**

Statistical analyses were performed using GraphPad Prism 6 (GraphPad, San Diego, CA, USA). All data are expressed as mean ± SEM. Results were assessed using two-way analysis of variance (ANOVA). Tukey’s test was further used as a post hoc test to detect between-group differences. Values of p < 0.05 were considered statistically significant.

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**CONFLICTS OF INTEREST**

The authors of this manuscript have no conflict of interests to declare.
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