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Acutely increased β-hydroxybutyrate plays a role in the prefrontal cortex to escape stressful conditions during the acute stress response

Hyeonwi Son [a, 1], Ji Hyeong Baek [a, 1], Jae Soon Kang [a], Soonwoong Jung [a], Hye Jin Chung [b], Hyun Joon Kim [a, *]

[a] Department of Anatomy and Convergence Medical Sciences, Institute of Health Sciences, Bio Anti-aging Medical Research Center, Gyeongsang National University Medical School, 15 Jinju-daero 816 Beongil, Jinju, Gyeongnam, 52727, Republic of Korea
[b] College of Pharmacy and Research Institute of Pharmaceutical Sciences, Gyeongsang National University, 501 Jinju-daero, Jinju, Gyeongnam, 52828, Republic of Korea

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ABSTRACT

Ketone bodies can be increased in the blood under certain physiological conditions, but their role under such conditions remains to be clarified. In the present study, we found the increment and usage of β-hydroxybutyrate (BHB) in the prefrontal cortex (PFC) during acute stress. BHB levels increased in the blood and PFC after 30-min acute immobilization stress, and BHB dehydrogenase 1 increased in the PFC simultaneously, but not in the hippocampus. Moreover, increased levels of acetyl-CoA, pyruvate carboxylase, and glutamate dehydrogenase 1 were found in the PFC, implicating the metabolism of increased BHB in the brain. Thus, we checked the levels of glutamate, glutamine, and GABA and found increased levels of glutamate and glutamine in the stressed group compared with that in the control group in the PFC. Exogenous administration of BHB enhanced struggling behaviors under stressful conditions. Our results suggest that the metabolism of BHB from peripheral blood in the PFC may contribute to acute stress responses to escape stressful conditions.

1. Introduction

Appropriate behavioral responses are important to protect life when facing risky and/or inescapable situations. To execute the appropriate behaviors, the brain requires a lot of energy. Although the brain represents only 2% of the body's mass, it consumes 20% of the whole body's energy [1,2]. Nevertheless, the brain cannot store energy by itself and must be supplied energy from the peripheral blood stream. Metabolites formed in peripheral organs, such as glucose, lactate, and ketone bodies that contain energy, are transported into the brain via the blood stream [3].

Ketone bodies comprise β-hydroxybutyrate (BHB) and acetoacetate synthesized by the liver from the fatty acid [4]. Indeed, the brain activates the sympathetic nervous system when it needs energy, resulting in the release of fatty acids from adipose tissue. The fatty acids are converted into ketone bodies in the liver, and then ketone bodies are transported to the brain and other organs via the blood stream [5]. BHB is the most abundant ketone body in mammals.

Ketone bodies are oxidized in the brain not only to produce ATP but also to increase the total pool of glutamatergic and GABAergic neurotransmitters (glutamate, glutamine, and GABA) [6–11]. Their supplementation and utilization are activated under specific conditions such as during the developmental period and starvation [3,4,12]. However, no evidence has supported the use of ketone bodies in the brain during acute stress responses.

During acute stress responses, the brain requires a lot of energy. When organisms encounter threatening circumstances, they perform suitable behaviors to escape from such situations for survival. These stress responses are mediated by hypothalamus-pituitary-adrenal (HPA) axis activation [13]. When animals face a threat, an activated HPA axis elevates glucocorticoids in the blood, eventually affecting the prefrontal cortex (PFC) and hippocampus (HI) [14–17], accompanied by a higher energy expenditure and enhanced glutamatergic signaling [18,19].

Ketone bodies support energy production but also provide a backbone of neurotransmitters [6–11]. Recently, psychosocial stress conditions were reported to increase the levels of blood

* Corresponding author.
E-mail address: kimhj@gnu.ac.kr (H.J. Kim).
[1] These authors contributed equally to this work.

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ketone bodies [20]. However, it remains unclear how the brain consumes peripheral ketone bodies under inescapable stressful situations. Therefore, we investigated whether the brain uses ketone bodies and the role of excess ketone bodies in the brain under inescapable stressful circumstances, such as during acute stress responses.

2. Materials and methods

2.1. Animals

Male 9-week-old C57BL/6J mice (specific-pathogen-free grade; KOATEC, Pyeongtaek, Korea) were singly housed in a temperature-controlled (25 ± 2 °C) vivarium on a 12-h light-dark cycle (lights on at 6:00 a.m.) with access to food and water ad libitum. Acute immobilization stress (AIS) was conducted using a restrainer as previously described [21,22]. All the mice were habituated to the room for 1 week before the experiments. Animal use procedures were performed in accordance with the National Institutes of Health (NIH, Bethesda, MD, USA) guidelines and with an approved protocol (GNU-180619-M0029) from the Gyeongsang National University Institution Animal Care & Use Committee (GNU IACUC).

2.2. Determination of the BHB and Acetyl-CoA levels

To measure the BHB and acetyl-CoA levels, the mice were decapitated under CO_2 anesthesia to collect the blood and brain between 8:00 to 10:00 a.m. Blood was collected in vacutainers containing K_3EDTA and plasma was isolated by centrifugation at 1000×*g* for 15 min at 4 °C. The PFC and HI were dissected from the brain on ice, frozen in liquid nitrogen, and stored at −80 °C until analysis. Quantification of BHB was performed using the BHB (Ketone Body) colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA). Acetyl-CoA levels in the prefrontal cortex were measured using a PicoProbe Acetyl-CoA fluorometric assay kit (K317-100, Biovision, Milpitas, CA, USA) according to the manufacturer's protocol.

2.3. Western blot analysis

Western blot analysis was conducted as previously described [23,24] with some modifications. Briefly, the collected PFC and HI were homogenized and centrifuged for 30 min at 12,000 rpm. The protein samples were separated by SDS-PAGE and then transferred to a PVDF membrane (Bio-rad, Hercules, CA, USA). The membranes were blocked with 5% skim milk and incubated with primary antibodies (anti-BDH1, 1:2000; anti-PC, 1:2000; anti-GDH1, 1:1,000, Santa Cruz Biotechnology, Dallas, TX, USA). Each band density was read using FUJI-FILM Multi Gauge software (Fujifilm, Tokyo, Japan). The densities were normalized using the α-tubulin density as an internal control.

2.4. Measurements of amino acids

The amino acid levels in the PFC and plasma were measured without derivatization by liquid chromatography-mass spectrometry (LC-MS)/MS as previously described [25] with slight modifications. The tissue homogenates and blood samples were centrifuged (12,000 rpm, 30 min), and the tissue supernatants or plasma samples were mixed with an internal standard (L-Glu-d5) and diluted with the mobile phase. Five-microliter aliquots of the diluted samples were injected into an LC-MS/MS system (Agilent 6460; Agilent, Singapore). A SeQuant ZIC®-HILIC column (2.1 × 100 mm, 3.5 μm, 100 Å) was used for separation by gradient elution with the mobile phase comprising 0.1% aqueous formic acid and acetonitrile. The multiple reaction monitoring detection method was employed to detect amino acids (*m*/*z* 148 → 84 for Glu, *m*/*z* 147 → 84 for Gln, *m*/*z* 104 → 87 for GABA, and *m*/*z* 153 → 88 for the internal standard).

2.5. Tail suspension test (TST)

The TST was conducted as previously described [23]. The mobile time was measured 30 min after D,L-β-hydroxybutyrate i.p. injection. The mobile time (threshold: 6%) and highly mobile time (threshold: 20%) were analyzed using EthoVision software (Noldus Information Technology, Wageningen, The Netherlands). Folding numbers were counted manually by video when a mouse bent its body more than 45°.

2.6. Statistical analysis

Statistical significance was determined by two-tailed, unpaired Student's *t*-tests at $p < 0.05$. Data summaries and statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Acute immobilization stress increases β-hydroxybutyrate and β-hydroxybutyrate dehydrogenase 1 levels in the PFC

We immobilized mice using a restrainer [22] and then exposed the mice to a bright (200 lux) and open area for 30 min. This acute immobilization stress (AIS) significantly increased β-hydroxybutyrate (BHB) in the blood and PFC compared with those in the control group, but not in the HI (Fig. 1A: $t_{(9)} = 2.742$, $p = 0.0228$, B: $t_{(9)} = 2.690$, $p = 0.0248$). The first step of the catabolism of β-hydroxybutyrate in the brain is oxidation by β-hydroxybutyrate dehydrogenase 1 (BDH1) to acetoacetate [4]. Thus, we examined BDH1 expression and found increased BDH1 levels in the PFC of the stressed group (STR) compared with that in the control group (CTL) (Fig. 2A; $t_{(9)} = 3.302$, $p = 0.0092$), but not in the HI. Using immunohistochemistry, the increased BDH1 expression was confirmed in the prelimbic cortex in the PFC (Fig. 2B; $t_{(10)} = 2.828$, $p = 0.0179$).

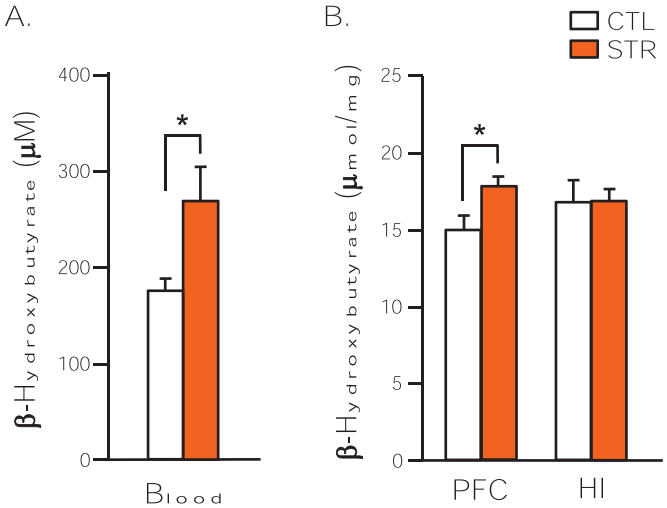

Fig. 1. Acute stress increases β-hydroxybutyrate levels in the blood and prefrontal cortex (PFC). The β-hydroxybutyrate level increased in the blood (A) and PFC (B), but not in the hippocampus (HI) (B), of 30-min restrained mice (STR) compared with control mice (CTL). n = 5–6/group. All the values are expressed as means ± SEM. *p < 0.05, Student's *t*-test.

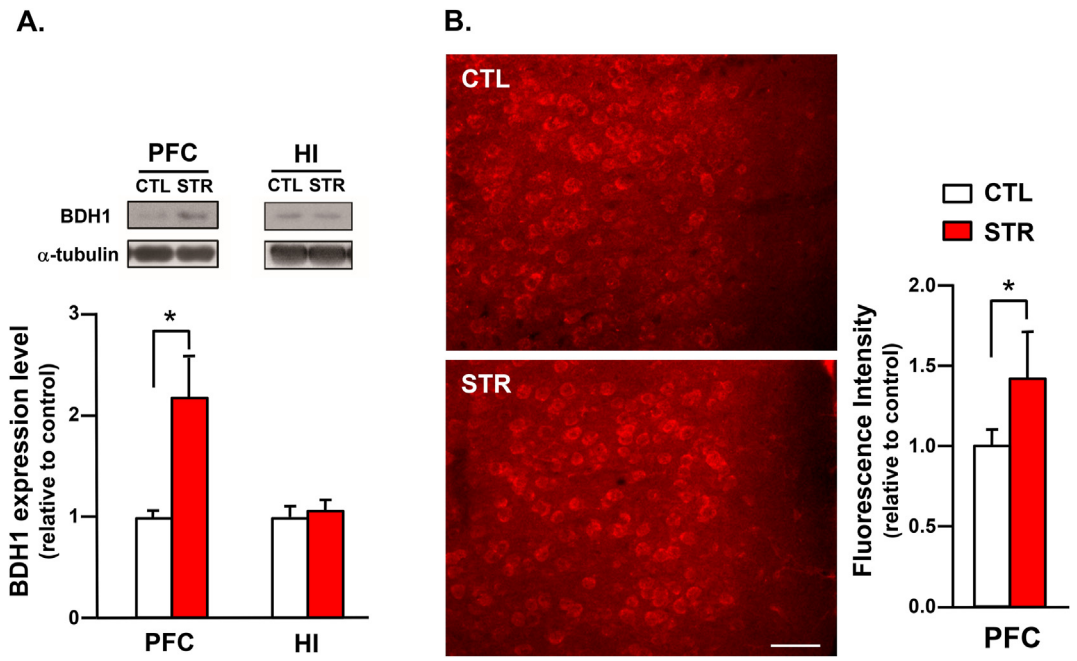

Fig. 2. Acute stress increases β-hydroxybutyrate dehydrogenase 1 (BDH1) levels in the prefrontal cortex (PFC). (A) BDH1 expression increased in the PFC of 30-min restrained mice compared with that in control mice, but not in the hippocampus (HI). n = 5–9/group. Upper panel: representative images of western blotting. (B) The BDH1 increase in the PFC by acute stress was confirmed by immunohistochemistry. n = 6/group. All the values are expressed as means ± SEM. *p < 0.05, Student's *t*-test.

These results suggest that the catabolism of BHB from blood occurred in the PFC during acute stress responses.

3.2. 30-min AIS increases anaplerotic metabolism in the PFC

Increased ketone bodies are catabolized to acetyl-CoA in the brain that is used in the TCA cycle for terminal oxidation. The acetyl-CoA in the brain results in enhanced anaplerotic metabolism to increase glutamate (Glu), glutamine (Gln) and GABA levels [4,8]. To confirm whether BHB increased by AIS affects anaplerotic metabolism, we assessed the acetyl-CoA, pyruvate carboxylase (PC) and glutamate dehydrogenase 1 (GDH1) levels in the PFC (Fig. 3). Increased levels of acetyl-CoA (Fig. 3A, $t_{(10)} = 2.936$, $p = 0.0149$), PC (Fig. 3B, $t_{(8)} = 2.893$, $p = 0.0201$) and GDH1 (Fig. 3C, $t_{(9)} = 3.452$, $p = 0.0072$) were observed in the PFC of the STR group compared with that of the CTL. Moreover, we evaluated the levels of Glu, Gln, and GABA in the PFC and HI and found that the levels of Glu and Gln were significantly increased in the PFC of STR group (Fig. 3D; Glu: $t_{(6)} = 2.476$, $p = 0.0481$; 3E Gln: $t_{(6)} = 3.032$, $p = 0.0230$), but we found no difference in the HI (Fig. 3E).

3.3. Exogenous BHB is transported into the PFC and affects acute stress response behaviors

To assess whether increased BHB levels affect behaviors during acute stress responses, we examined effortful behaviors during inescapable stressful conditions, such as the tail suspension test. For this purpose, we administered BHB via i.p. at different doses (0, 5, 10, 20, and 40 mmol/kg) and measured the changes in the blood and PFC (Fig. 4A and B). Expectedly, exogenous BHB transported into the brain and the BHB concentration in the PFC were significantly increased with more than 10 mmol/kg of BHB injection ($F_{(4, 24)} = 9.633$, $p < 0.0001$; 0 vs. 10 mmol/kg, $p = 0.0357$; 0 vs. 20 mmol/kg, $p = 0.0011$; 0 vs. 40 mmol/kg, $p = 0.0002$). In the blood, the concentration of BHB also significantly changed with the administration of more than 10 mmol/kg ($F_{(4, 22)} = 146.7$, $p < 0.0001$; 0 vs. 10 mmol/kg, $p = 0.0004$; 0 vs. 20 mmol/kg, $p < 0.0001$; 0 vs. 40 mmol/kg, $p < 0.0001$) (Fig. 4A and B). Thus, we determined the dose of administration as 10 mmol/kg to increase BHB significantly in the blood and PFC. The administered BHB (10 mmol/kg) affected the effortful behaviors (Fig. 4C, D, and E). The TST is considered an inescapable acute stress, and mice normally make efforts to escape the stressful condition, which has been used to measure helplessness, a condition of depressiveness. The BHB-administered mice showed a longer mobile duration (Fig. 4C, $t_{(12)} = 2.643$, $p = 0.0215$) even with a highly mobile time (threshold over 20%) (Fig. 4D, $t_{(12)} = 2.193$, $p = 0.0487$) and folding number (Fig. 4E, $t_{(12)} = 3.520$, $p = 0.0042$) than the saline-administered group.

4. Discussion

The brain, which lacks energy storage, receives energy sources such as glucose, lactate, and fat-derived ketone bodies from peripheral organs [3]. The use of ketone bodies in the brain is highly associated with the evolution of brain function [1], indicating that the fat use of the brain is important for the animal's survival. However, there is a lack of understanding of the use of ketone bodies in the brain, particularly under stressful conditions. Our results provide evidence that the brain uses fat-derived ketone bodies during the acute stress response, a function that may closely related to struggles to overcome the stressful condition.

Ketone bodies comprise BHB and acetoacetate. They are synthesized in the liver mitochondria through the oxidation of fatty acids from adipose tissue under starvation, developmental stage, and psychosocial stress [20,26]. The interconversion between BHB and acetoacetate is rapid [27]. BHB constitutes up to 70% of the ketone bodies and is a non-volatile and stable compound [26]. Thus, BHB is a representative indicator of ketone bodies and BHB level is expected to linearly reflect the total ketone concentration. Thus, many studies of the effects of ketone bodies on brain functions have been performed based on the BHB levels in the brain [28–32]. In the present study, the blood BHB level increased by

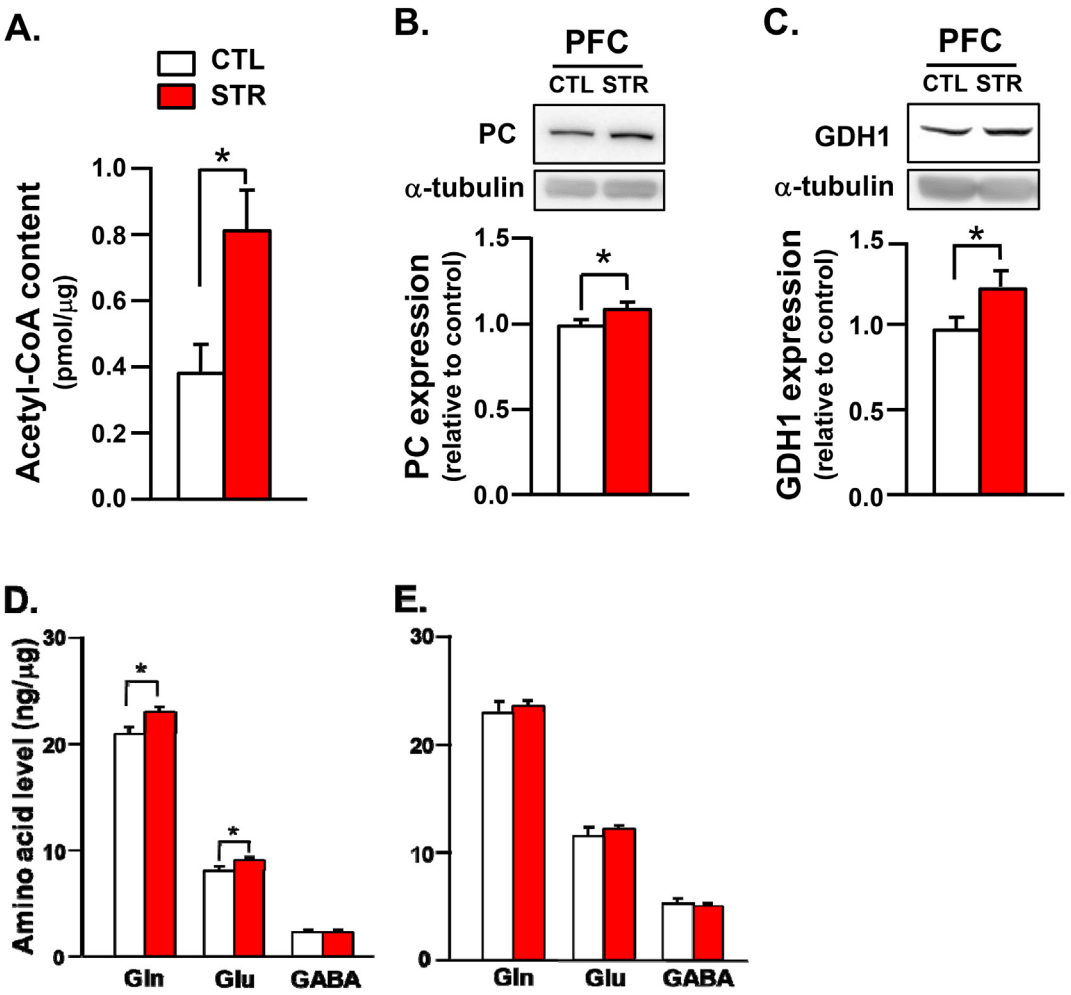

Fig. 3. Acute stress increases β-hydroxybutyrate (BHB) metabolism in the prefrontal cortex (PFC). BHB metabolite levels (A: acetyl-CoA) and expression of BHB metabolism-related catalases (B: pyruvate carboxylase, PC; C: glutamate dehydrogenase 1, GDH1) were increased in the PFC by 30-min acute stress. n = 6/group in A-C. Glutamate (Glu) and glutamine (Gln) levels also increased in the PFC (D) of the restrained mice compared with that in control mice, but not in the hippocampus (HI) (E). The GABA level did not change in the PFC and HI. n = 4/group in D-E. All the values are expressed as means ± SEM. *p < 0.05, Student's *t*-test.

approximately 70% after 30-min AIS compared with that of control mice and the increased BHB level was only found in the PFC, but not in the HI at the same time (Fig. 1). This finding suggests a role for BHB in the PFC during acute stress responses.

Ketone bodies have various functions in the brain. Blood ketones can be taken up by tissues requiring energy and converted to acetyl-CoA, which is used to produce ATP. However, it is becoming apparent that BHB and its metabolites are involved in gene expression, lipid metabolism, neuronal function, and the metabolic rate as well as in energy production [4,12,33].

Acetyl-CoA converted from ketone bodies enters the TCA cycle, leading to energy production and anaplerosis [4]. A previous study reporting that exogenous BHB has anticonvulsant activity showed that BHB acutely modulates brain neurotransmissions. For example, ketone bodies stimulate glutamate decarboxylase activity, elevating the GABA content in synaptosomes [34]. Additionally, ketone body-induced alterations in TCA cycle metabolites (oxaloacetate, citrate, succinyl-CoA, and α-ketoglutarate) favor forming glutamate over aspartate. Thus, BHB in the PFC can be used for neurotransmitter production [34,35].

In the adult brain, anaplerosis increases the glutamate and GABA levels [36]. Intravenously infused BHB was also reported to rapidly produce glutamate and glutamine in the human brain [37]. In a previous study, we found that glutamate and glutamine increase glutamatergic signaling [23]. In the present study, the levels of BHB and glutamate-glutamine increased in the PFC within 30 min of AIS (Figs. 1 and 4). Therefore, we hypothesized that the increased level of BHB in the PFC stimulated glutamatergic signaling affecting behaviors during acute stress response related to PFC function.

Acute stress enhances PFC activity accompanied by increased glutamatergic signaling and energy use [38], consistent with our study findings of increased BDH1 and acetyl-CoA levels in the PFC, indicating the increased use of ketone bodies in the PFC of mice during acute stress responses. Because ketone bodies contribute to the production of both energy and neurotransmitters in the brain at the same time, our study suggests that using ketone bodies in the PFC contributes to effortful behaviors to cope with an inescapable stressful condition. We found that an artificial increase in ketones increases effortful behaviors under tail suspension conditions. These results indicate that ketone body metabolism in the PFC contributes to efforts to escape from unpleasant situations.

Our previous studies showed that insufficient glutamatergic signaling in the PFC caused depressive behaviors that were tested using the forced swim test and TST [23,39]. A longer immobile time in these tests could be considered helplessness, a representative symptom of major depressive disorder [40]. However, glutamine supplements and optogenetic stimulation for glutamatergic signaling reversed depressive behaviors and increased the mobile

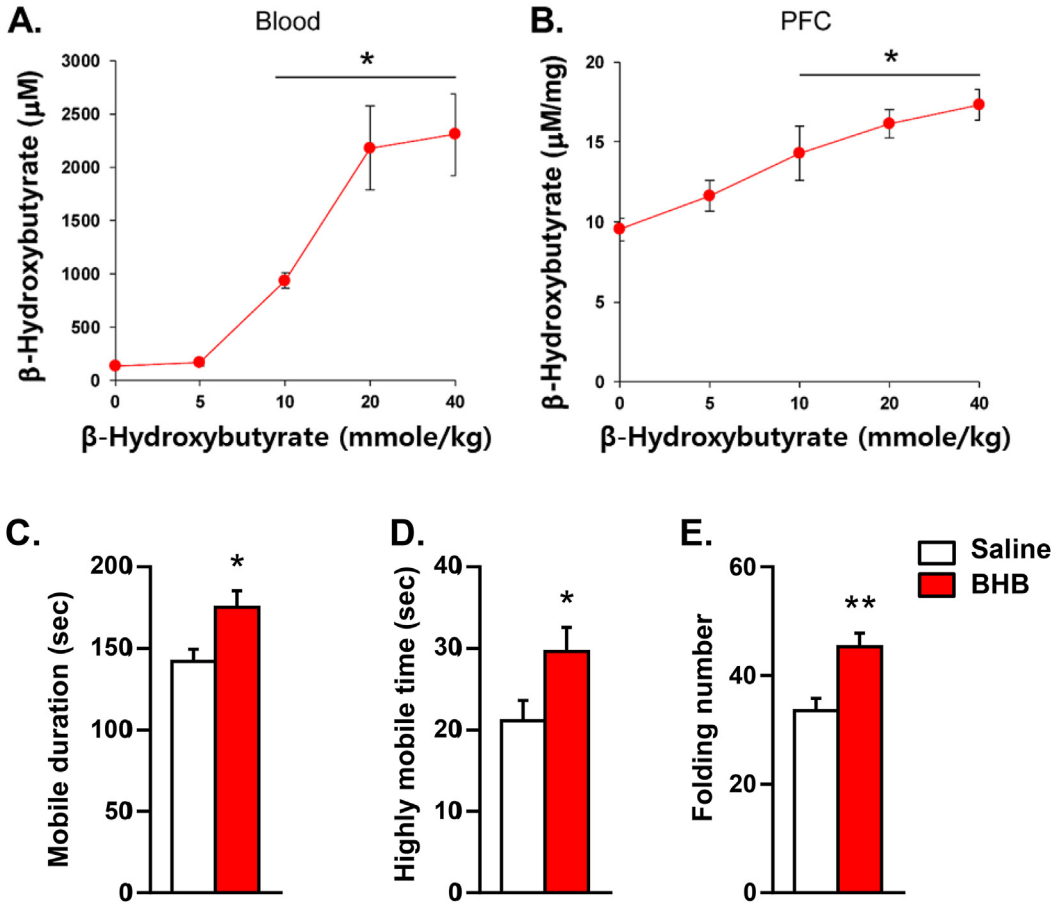

Fig. 4. Artificially increased β-hydroxybutyrate (BHB) in the prefrontal cortex (PFC) increases effortful behavior. BHB levels increased in the blood (A) and PFC (B) of BHB-injected mice in a dose-dependent manner (n = 5–7/group). The BHB injection increased mobile duration (C), highly mobile duration (D), and folding number (E) in the tail suspension test (n = 7/group). All the values are expressed as means ± SEM. *p < 0.05, Student's t-test.

time [23,39], indicating that enhancement of glutamatergic signaling in the PFC induced will and effortful behaviors for escape. Based on these previous results, we hypothesized that excessive BHB in the PFC might increase glutamate and glutamine levels and then stimulate glutamatergic signaling, resulting in increased mobile time during TST. As expected, we found that artificially increased BHB in the PFC, relevant to 30-min AIS (Figs. 1 and 4), led to a longer mobile duration and folding number during the TST—i.e., effortful behaviors to escape stressful condition. Therefore, AIS-induced BHB in the PFC might be used as a metabolic source for glutamatergic signaling required for acute stress responses.

Modern humans face more challenges every day than during the ancient days of hunting. Thereafter, the importance of brain function is increasing for the well-being of modern humans. In challenging situations, the function of the brain can be maintained by extreme energy supplies from peripheral metabolites. The use of fat in the brain is considered an important factor in the evolution of brain function. The present study provides a piece of evidence that the PFC uses ketone bodies in relation to survival behaviors under acute stress conditions.

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