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# Metabolic Profiling in Plasma and Brain Induced by 17$\beta$-Estradiol Supplementation in Ovariectomized Mice

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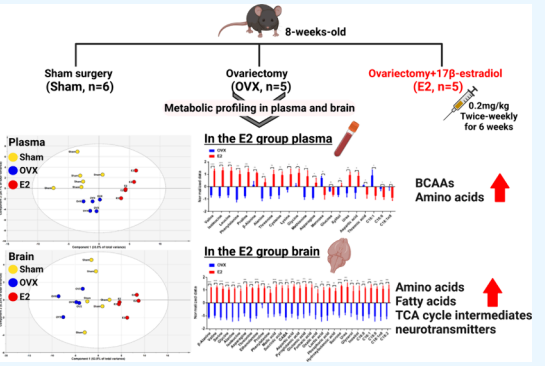
**ABSTRACT:** 17$\beta$-Estradiol is an ovarian hormone that regulates energy circulation and storage by acting on the central nervous system. However, the metabolic differences between the blood and brain when stimulated by 17$\beta$-estradiol are poorly understood. Moreover, research using menopause-induced models to investigate primary metabolites in the blood and brain is limited. Thus, this study aimed to identify metabolic changes in the plasma and brain resulting from 17$\beta$-estradiol supplementation in an estrogen-deficient mouse model. Three groups of mice were utilized: sham-operated mice (Sham), ovariectomized mice (OVX), and ovariectomized mice that received a weekly supplementation of 17$\beta$-estradiol (E2). Plasma and brain samples from these mice were subjected to metabolic analysis using gas chromatography−time-of-flight−mass spectrometry. Compared with the plasma samples from the Sham and OVX groups, the plasma samples from the E2 group contained higher contents of branched-chain amino acids (BCAAs), such as valine, isoleucine, and leucine. Meanwhile, the brain samples from the E2 group contained higher contents of most metabolites, including BCAAs, neurotransmitters, tricarboxylic acid cycle intermediates, and fatty acids, than those from the two other groups. This study is the first to reveal differences in energy metabolism induced by 17$\beta$-estradiol supplementation through brain metabolic profiling of ovariectomized mice, emphasizing the importance of brain metabolic profiling in menopausal hormone research.




## 1. INTRODUCTION

Estrogen is a vital mediator of various physiological processes in the nervous, immune, and cardiovascular systems of the body.[1] Estrogen deficiency is common in women during menopause and is associated with conditions such as osteoporosis, metabolic dysfunction, and neurological diseases.[2,3] 17$\beta$-Estradiol, one of the primary estrogens produced in the ovaries, regulates energy homeostasis and is closely linked to health issues in postmenopausal women.[4,5] Historically, 17$\beta$-estradiol has been widely used in menopausal hormone therapy (MHT) to prevent and manage these conditions. However, in recent years, the need for comprehensive studies on MHT has become clear owing to a better understanding of the timing, method, and age-dependent side effects of its administration.[6,7] Human menopause typically spans 4−5 years and is challenging to study because of the diversity of environmental and genetic factors. Therefore, rodents, which share certain characteristics with humans and have shorter lifespans, are suitable models for studying menopause. Specifically, the ovariectomized (OVX) mouse model is valuable for researching and comprehending the hormonal changes resulting from menopause because it surgically induces estrogen deficiency.[8]

Metabolomics can explain biological changes through analysis of the concentration and pathways of metabolites that act as reactants and products in metabolic reactions.[9] Hormones transported to the brain via the bloodstream play essential roles in various bodily functions, including the regulation of energy metabolism and immune responses.[10] The blood−brain barrier (BBB) is the most tightly regulated interface among the brain and circulatory system. The BBB, which is composed of the lipophilic membrane, selectively allows hydrophilic substances transported in the blood to maintain homeostasis in the central nervous system (CNS) environment.[11,12] In particular, 17$\beta$-estradiol is transported through the blood−brain barrier (BBB) to the hypothalamus of the brain, where it interacts with various neurons to regulate metabolism.[13] Therefore, it is noteworthy that 17$\beta$-estradiol passes through the BBB and affects the central nervous system. This is why it is important to understand the metabolic differences between blood and the brain affected by 17$\beta$-estradiol. However, little is known about the effects of 17$\beta$-estradiol supplementation on brain metabolism.

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Table 1. Relative Retention Times (RRT) and Mass Spectrometry Data of Low-Molecular-Weight Metabolites in the Plasma and Brain

| compound | RT[a] | RRT[b] | quantification ion[c] | mass fragment[d] |
|---|---|---|---|---|
| pyruvic acid | 04:56.3 | 0.427 | 174 | 89, 115, 174 |
| lactic acid | 05:02.1 | 0.436 | 117 | 117, 147, 191 |
| alanine | 05:31.9 | 0.479 | 116 | 116, 147, 190 |
| oxalic acid | 05:57.8 | 0.516 | 147 | 117, 133, 147 |
| hydroxybutanoic acid | 06:09.3 | 0.533 | 233 | 147, 177, 205 |
| valine | 06:44.7 | 0.584 | 144 | 144, 156, 218 |
| urea | 07:03.4 | 0.611 | 147 | 147, 189 |
| serine1 | 07:11.2 | 0.622 | 116 | 116, 132, 147 |
| ethanolamine | 07:16.0 | 0.629 | 174 | 100, 147, 174 |
| glycerol | 07:16.6 | 0.630 | 147 | 147, 177, 205 |
| phosphoric acid | 07:17.8 | 0.632 | 299 | 211, 299, 314 |
| leucine | 07:18.5 | 0.633 | 158 | 102, 147, 158 |
| isoleucine | 07:31.3 | 0.651 | 158 | 147, 158, 218 |
| proline | 07:36.7 | 0.659 | 142 | 142, 143, 147 |
| glycine | 07:40.0 | 0.664 | 174 | 147, 174, 248 |
| succinic acid | 07:44.3 | 0.670 | 147 | 147, 149, 247 |
| fumaric acid | 08:04.5 | 0.699 | 245 | 143, 147, 245 |
| serine2 | 08:07.1 | 0.703 | 116 | 100, 147, 204 |
| threonine | 08:21.1 | 0.723 | 291 | 117, 218, 291 |
| $\beta$-alanine | 08:47.0 | 0.760 | 174 | 147, 174, 248 |
| malic acid | 09:14.9 | 0.800 | 147 | 100, 147, 218 |
| aspartic acid | 09:32.0 | 0.825 | 100 | 100, 147, 232 |
| methionine | 09:35.4 | 0.830 | 176 | 128, 147, 176 |
| pyroglutamic acid | 09:38.5 | 0.835 | 156 | 147, 156, 230 |
| GABA[e] | 09:39.9 | 0.837 | 304 | 147, 174, 304 |
| cysteine | 09:51.2 | 0.853 | 220 | 100, 147, 220 |
| glutamic acid | 10:20.7 | 0.895 | 246 | 128, 156, 246 |
| phenylalanine | 10:28.8 | 0.907 | 218 | 100, 192, 218 |
| asparagine | 10:44.7 | 0.930 | 116 | 116, 132, 231 |
| xylitol | 11:01.4 | 0.954 | 217 | 103, 147, 217 |
| ribitol | 11:03.9 | 0.958 | 217 | 103, 147, 217 |
| glutamine | 11:31.3 | 0.997 | 156 | 147, 156, 245 |
| L-2-chlorophenylalanine[f] | 11:33.2 | 1.000 | 218 | 147, 218, 226 |
| citric acid | 11:47.0 | 1.020 | 273 | 147, 273, 348 |
| fructose1 | 12:06.5 | 1.048 | 103 | 103, 147, 217 |
| fructose2 | 12:10.3 | 1.054 | 103 | 103, 147, 217 |
| mannose | 12:12.7 | 1.057 | 319 | 147, 205, 319 |
| glucose1 | 12:17.4 | 1.064 | 319 | 147, 205, 319 |
| glucose2 | 12:26.3 | 1.077 | 319 | 147, 205, 319 |
| lysine | 12:31.8 | 1.085 | 317 | 156, 174, 317 |
| C16:1 | 13:16.8 | 1.149 | 311 | 117, 132, 311 |
| C16:0 | 13:24.2 | 1.160 | 313 | 117, 132, 313 |
| inositol | 13:36.0 | 1.177 | 305 | 147, 217, 305 |
| C18:2 | 14:25.7 | 1.249 | 337 | 129, 262, 337 |
| C18:1n9 | 14:27.5 | 1.251 | 339 | 117, 129, 339 |
| tryptophan | 14:27.7 | 1.252 | 202 | 147, 202, 218 |
| C18:0 | 14:35.8 | 1.263 | 341 | 117, 132, 341 |
| sucrose | 16:31.7 | 1.431 | 217 | 147, 217, 361 |
| cholesterol | 19:28.0 | 1.685 | 329 | 129, 329, 368 |

[a]Retention time (m:s). [b]Relative retention time (retention time of analyte/retention time of L-2-cholorophenylalanine). [c]Specific mass ion used for quantification. [d]Three highest intensity ions. [e]GABA, 4-aminobutyric acid; C16:1, palmitoleic acid; C16:0, palmitic acid; C18:2, linoleic acid; C18:1n9, oleic acid; C18:0, stearic acid. [f]Internal standard.

Hence, our study aimed to identify metabolic changes in the plasma and brain resulting from 17$\beta$-estradiol supplementation in an estrogen-deficient mouse model. This study is the first to identify these metabolic changes in the OVX mouse brain, highlighting the importance of metabolomics in MHT. Additionally, it serves as a foundation for further research on the OVX mouse model using metabolite analysis.

## 2. MATERIALS AND METHODS

**2.1. Experimental Design and Sample Collection.** Eighteen eight-week-old female C57BL/6J mice (DBL, Eumseong, Korea) weighing 19−21 g were accommodated in a controlled chamber with constant temperature and humidity (25 °C and 55 ± 5%) under a 12-h light/dark cycle (light exposure from 7:00 to 19:00). The mice had ad libitum access to tap water and standard chow pellets (DBL). All animal care and experimental procedures were performed in compliance with the protocols approved by the Institutional Animal Care and Use Committee at Incheon National University (permission number: INU-ANIM-2020−04). After a week acclimatization period, the mice were randomly assigned to different groups: sham-operated mice (sham, $n$ = 6), ovariectomized mice (OVX, $n$ = 6), and ovariectomized mice that received a supplementation of 17$\beta$-estradiol (E2, n =


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Table 2. Effect of 17$\beta$-Estradiol Supplementation for 6 Weeks on General Characteristics of Mice after Surgery (Means ± SD)[a]

| | sham | OVX | E2 |
|---|---|---|---|
| initial body weight (g) | 20.67 ± 0.58 | 20.02 ± 0.26 | 20.60 ± 0.32 |
| after surgery body weight (g) | 22.15 ± 0.81*** | 25.16 ± 0.77 | 24.56 ± 0.87 |
| final body weight (g) | 25.28 ± 0.16*** | 28.70 ± 1.48 | 27.44 ± 1.92 |
| uterus weight (g) | 0.18 ± 0.03**** | 0.03 ± 0.02 | 0.08 ± 0.01*** |
| liver weight (g) | 1.25 ± 0.08 | 1.24 ± 0.05 | 1.24 ± 0.08 |
| SC-WAT (g) | 0.27 ± 0.09**** | 0.76 ± 0.05 | 0.50 ± 0.14** |
| PM-WAT (g) | 0.48 ± 0.07**** | 1.52 ± 0.24 | 0.84 ± 0.29** |

[a]**$p$ < 0.01,***$p$ < 0.001, ****$p$ < 0.0001 OVX versus sham or E2. SC-WAT, subcutaneous white adipose tissue; PM-WAT, parametrial white adipose tissue; sham, sham-operated mice; OVX, ovariectomized mice; and E2, ovariectomized mice that received twice-weekly supplementation of 17$\beta$-estradiol.

6). The mice were anesthetized through intraperitoneal (ip) injection of 0.3% tribromoethanol (0.3 mg/kg, Sigma-Aldrich, Saint Louis, MO, USA). The ovaries of the mice in the OVX and E2 groups were removed by cutting the anterior uterine horns. In the sham group, dorsoventral incisions were made through the skin, muscles, and periosteum, similar to those in the OVX animals but without removing the ovaries. However, several ovariectomized mice died during the surgery. Therefore, excluding the sham group (sham, $n$ = 6), the remaining 10 mice were divided into two groups according to hormone supplementation (OVX, $n$ = 5; E2, $n$ = 5). After a 3 week recovery period, the mice were received i.p. injections of either vehicle (0.9% saline) or 17$\beta$-estradiol (0.2 mg/kg twice weekly, Sigma-Aldrich) for 6 weeks. The dose and duration of 17$\beta$-estradiol supplementation were determined based on previous studies related to estrogen treatment.[14,15] Body weight changes were monitored every week for 9 weeks to assess the recovery after ovariectomized surgery and evaluate the effectiveness of 17$\beta$-estradiol supplementation. At the end of the body weight monitoring period, blood samples were collected from the anesthetized mice using a 24G syringe. The blood samples were immediately placed into 2 mL tubes containing 20 $\mu$L of heparin (100 $\mu$g/$\mu$L, Sigma-Aldrich). Plasma samples were obtained by centrifuging total blood at 3,500 rpm for 15 min at 4 °C. Following blood collection, the brain, liver, subcutaneous white adipose tissue (SC-WAT), parametrial white adipose tissue (PM-WAT), and uterus were quickly extracted from the mice and then weighed to compare general characteristics among the groups. All extracted tissues were flash-frozen in liquid nitrogen for subsequent analyses, and all samples were stored at −80 °C until further processing.

**2.2. Sample Extraction and Analysis of Metabolites.** Low-molecular metabolites, such as amino acids, organic acids, sugars, sterols, and fatty acids, were obtained from the plasma and brain in this study and then analyzed.[16] To extract low-molecular metabolites, 500 $\mu$L of methanol−chloroform (3:1, $v/v$) solution was added to blood (50 $\mu$L) and half brain (approximately 200 mg) samples in 2 mL tubes. The half brain samples were added with approximately 200 mg of beads (425−600 $\mu$m) and then agitated (Mini Bead beater 96, BioSpec products, USA) twice for 20 s. Then, the tubes containing the brain samples were added with 500 $\mu$L of methanol−chloroform, producing 1 mL of extraction solvent. Afterward, 30 $\mu$L of L-2-chlorophenylalanine (0.3 mg/mL), which serves as an internal standard (IS), was added to the plasma and brain samples, respectively. The tubes were vortex mixed for 10 s, stored in a deep freezer at −20 °C for 10 min, sonicated for 10 min at 20 °C, and then centrifuged at 14,000 × $g$ for 15 min at 4 °C. The supernatant was transferred to a fresh tube and then concentrated for 2 h in a vacuum concentrator (CVE−3110, EYELA, Tokyo, Japan). The concentrated sample underwent derivatization, first for 90 min at 37 °C in 100 $\mu$L of methoxyamine hydrochloride in pyridine (20 mg/mL), and then treated with 100 $\mu$L of $N,O$−bis(trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane at 60 °C for 60 min. The brain samples were freeze-dried for 16 h before derivatization. The tubes containing the derivatized samples were centrifuged at 14,000 × $g$ for 15 min at room temperature. The supernatant was passed through a 0.5 $\mu$m hydrophobic membrane filter and then transferred to a glass insert in an autosampler vial. Gas chromatography−time-of-flight−mass spectrometry (GC−TOF−MS) was used to analyze the low-molecular metabolites. An Agilent 7890A GC system (Agilent Technologies, Santa Clara, CA, USA) was connected to a Pegasus GC−TOFMS Benchtop (LECO, St. Joseph, MI, USA). Helium gas was passed through the GC at a rate of 1 mL/min using the CP-Sil 8 CB Low Bleed/MS (30 m × 0.25 mm, 0.25−$\mu$m i.d. film thickness; CP 5860, Agilent) column. Split mode was used to inject 1 $\mu$L of sample at a ratio of 1:15, with an inlet temperature of 230 °C. The oven's temperature was set to begin at 80 °C for 2 min, ramped up to 320 °C at a rate of 15 °C/min, and then held for 10 min. The temperatures of the transfer line and ion source were set at 250 °C, respectively. The spectral data were scanned on an 85−600 $m/z$ mass scale. Based on the specified ions, the ratio of the relative peak area to that of the IS was calculated for quantification (Table 1).

**2.3. Statistical Analysis.** The GC−TOF−MS data were normalized using unit variance scaling. Principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS−DA) were performed using SIMCA package 14.1 software (Umetrics, Ume, Sweden). The predictability and goodness of fit of the OPLS−DA model were assessed using $Q^2$ and $R^2Y$, respectively, where values exceeding 0.5 indicated significant metabolic differences between the two groups. The $R^2Y$ and $Q^2$ values ranged from 0 to 1. Statistical analysis was performed with Student's $t$-test using Prism GraphPad 9.0 (GraphPad, San Diego, CA, USA). A $p$-value less than 0.05 in the Student's $t$-test denoted a significant difference between the means of the compared groups. Heat maps were generated using Multi-Experiment Viewer version 4.9.0 (MeV) and pathway enrichment analysis was performed using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/).


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## 3. RESULTS AND DISCUSSION

**3.1. Effects of 17β-Estradiol Supplementation on Physical Conditions.** In this study, we measured the body weight, liver, uterus, and WAT of the mice to assess their physical changes in response to 17β-estradiol supplementation. Body weight was monitored weekly from baseline until sample collection, whereas the liver, uterus, and WAT were weighed during sample collection (Table 2). Sixteen mice with an initial average weight of 20.4 ± 0.5 g were randomly allocated into three groups. Three weeks after surgery, the average body weight of mice in the OVX group was 25.2 ± 0.8 g, which was similar to that of mice in the E2 group (24.6 ± 0.9 g) that underwent the same surgery (Table 2). On the other hand, the average body weight of mice in the OVX group was significantly greater than that of mice in the sham group (22.2 ± 0.8 g, $p < 0.001$). These results are consistent with

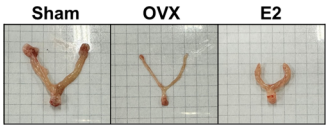


**Figure 1.** Representative uterine phenotype photos of each group (sham, sham-operated mice; OVX, ovariectomized mice; and E2, ovariectomized mice that received twice-weekly supplementation of 17β-estradiol).

**Table 3. Composition and Content (Ratio/g) of Low-Molecular-Weight Metabolites in the Plasma of Mice**

| | contents (average ± SD, ratio/g) | | |
|---|---|---|---|
| compound | sham | OVX | E2 |
| pyruvic acid | 1.56 ± 0.56 | 2.11 ± 0.22 | 2.42 ± 0.45 |
| lactic acid | 81.77 ± 15.37 | 63.54 ± 7.47 | 67.6 ± 3.78 |
| alanine | 33.03 ± 6.57 | 23.6 ± 2.60 | 32.08 ± 4.00 |
| oxalic acid | 80.55 ± 22.78 | 60.6 ± 2.47 | 59.32 ± 4.47 |
| hydroxybutanoic acid | 0.93 ± 0.40 | 1.31 ± 0.42 | 1.56 ± 0.27 |
| valine | 9.42 ± 2.43 | 8.47 ± 1.05 | 17.18 ± 1.33 |
| urea | 76.7 ± 14.02 | 74.14 ± 8.94 | 95.86 ± 6.85 |
| serine | 1.13 ± 0.38 | 0.83 ± 0.26 | 0.83 ± 0.25 |
| ethanolamine | 0.58 ± 0.13 | 1.18 ± 0.19 | 1.31 ± 0.11 |
| glycerol | 11.70 ± 1.67 | 14.68 ± 1.12 | 13.34 ± 1.96 |
| phosphoric acid | 12.4 ± 4.82 | 10.54 ± 2.34 | 12.81 ± 2.34 |
| leucine | 6.42 ± 1.28 | 5.67 ± 0.97 | 12.53 ± 1.85 |
| isoleucine | 3.84 ± 0.73 | 3.20 ± 0.41 | 7.26 ± 0.95 |
| proline | 3.84 ± 1.75 | 2.79 ± 0.72 | 8.18 ± 1.41 |
| glycine | 4.62 ± 0.78 | 9.33 ± 1.61 | 13.87 ± 2.67 |
| succinic acid | 2.31 ± 2.20 | 1.15 ± 0.37 | 1.42 ± 0.27 |
| fumaric acid | 0.23 ± 0.12 | 0.22 ± 0.04 | 0.25 ± 0.06 |
| threonine | 0.59 ± 0.20 | 0.44 ± 0.08 | 0.89 ± 0.17 |
| β-alanine | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.02 ± 0.00 |
| malic acid | 1.44 ± 0.69 | 1.30 ± 0.28 | 1.63 ± 0.33 |
| aspartic acid | 0.12 ± 0.03 | 0.11 ± 0.04 | 0.17 ± 0.02 |
| methionine | 1.67 ± 0.44 | 1.17 ± 0.24 | 2.19 ± 0.58 |
| pyroglutamic acid | 9.10 ± 0.98 | 10.76 ± 1.24 | 11.16 ± 0.53 |
| cysteine | 0.16 ± 0.04 | 0.14 ± 0.02 | 0.21 ± 0.03 |
| glutamic acid | 0.62 ± 0.14 | 0.59 ± 0.12 | 0.63 ± 0.11 |
| phenylalanine | 1.95 ± 0.24 | 1.52 ± 0.15 | 2.35 ± 0.24 |
| asparagine | 0.56 ± 0.16 | 0.35 ± 0.03 | 0.53 ± 0.11 |
| xylitol | 0.09 ± 0.04 | 0.05 ± 0.01 | 0.07 ± 0.01 |
| ribitol | 0.05 ± 0.01 | 0.05 ± 0.00 | 0.05 ± 0.00 |
| glutamine | 7.76 ± 1.19 | 8.23 ± 0.77 | 7.24 ± 0.26 |
| citric acid | 1.91 ± 0.30 | 1.86 ± 0.14 | 1.71 ± 0.09 |
| fructose | 0.38 ± 0.06 | 0.40 ± 0.04 | 0.47 ± 0.12 |
| mannose | 1.48 ± 0.41 | 1.76 ± 0.11 | 1.26 ± 0.26 |
| glucose | 75.27 ± 18.19 | 55.57 ± 1.27 | 50.24 ± 2.68 |
| lysine | 1.29 ± 0.29 | 1.45 ± 0.13 | 1.96 ± 0.32 |
| C16:1 (palmitoleic acid) | 0.06 ± 0.02 | 0.09 ± 0.03 | 0.04 ± 0.02 |
| C16:0 (palmitic acid) | 1.54 ± 0.33 | 1.19 ± 0.09 | 0.97 ± 0.11 |
| inositol | 2.31 ± 0.14 | 2.77 ± 0.33 | 3.30 ± 0.48 |
| C18:2 (linoleic acid) | 0.20 ± 0.05 | 0.12 ± 0.01 | 0.10 ± 0.02 |
| C18:1n9 (oleic acid) | 0.36 ± 0.10 | 0.27 ± 0.05 | 0.18 ± 0.04 |
| tryptophan | 5.70 ± 0.62 | 6.04 ± 0.54 | 5.66 ± 0.82 |
| C18:0 (stearic acid) | 0.94 ± 0.20 | 0.61 ± 0.13 | 0.53 ± 0.12 |
| sucrose | 0.03 ± 0.01 | 0.04 ± 0.02 | 0.04 ± 0.01 |
| cholesterol | 5.43 ± 1.70 | 3.54 ± 0.30 | 3.80 ± 0.32 |



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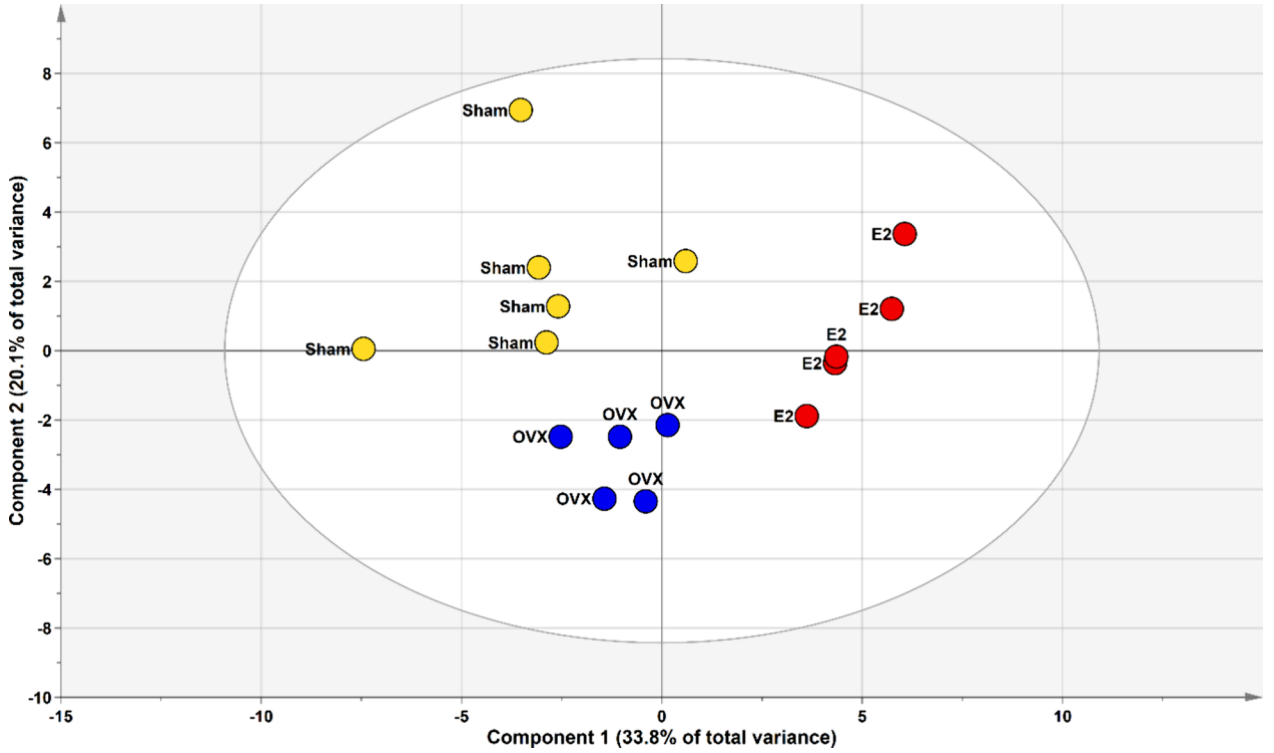


**Figure 2.** Score plot of the PCA model obtained from 44 metabolites in the plasma samples of the mice from the sham, OVX, and E2 groups (yellow, sham; blue, OVX; red, E2). Sham, sham-operated mice; OVX, ovariectomized mice; and E2, ovariectomized mice that received twice-weekly supplementation of 17$\beta$-estradiol.

previous studies showing that ovariectomized mice gained more body weight compared to sham-operated mice due to decreased systemic metabolism and energy metabolism.[17,18]

After 6 weeks of 17$\beta$-estradiol supplementation in the mice in the E2 group, the body weight, liver, uterus, WAT, and uterine phenotypes of the mice in all groups were confirmed (Figure 1 and Table 2). The evaluation of uterine phenotype to assess the effectiveness of ovariectomy and 17$\beta$-estradiol supplementation was successful. The uterine size in the OVX group was dramatically smaller than that in the sham group. The E2 group showed an intermediate size between those of the OVX and sham groups (Figure 1). Uterine weight followed a similar pattern as the observed phenotype: sham group, 0.18 $\pm$ 0.03 g ($p < 0.0001$); OVX group, 0.03 $\pm$ 0.02 g; and E2 group, 0.08 $\pm$ 0.01 g ($p < 0.001$; Table 2). These results are consistent with previous findings that estradiol regulates uterine development and ovariectomy causes an atrophic uterine phenotype.[19−21] The final body weights were as follows: sham group, 25.3 $\pm$ 0.2 g; OVX group, 28.7 $\pm$ 1.5 g; and E2 group, 27.4 $\pm$ 1.9 g. The mice in the OVX group had the highest body weight, whereas those in the sham group had the lowest. SC-WAT and PM-WAT were also highest in the OVX group, followed by the E2 and sham groups. Notably, a significant difference in WAT weight was found between the OVX and E2 groups.

The SC-WAT in the E2 group was 0.5 $\pm$ 0.1 g ($p < 0.01$), which was significantly lower than that in the OVX group (0.8 $\pm$ 0.0 g). The PM-WAT in the E2 group ((0.8 $\pm$ 0.3 g) was also significantly lower ($p < 0.01$) than that in the OVX group (1.5 $\pm$ 0.2 g). By contrast, no significant difference in liver weight was found among the three groups (Table 2). These results are consistent with previous findings that 17$\beta$-estradiol supplementation in the OVX model reduces WAT by inducing browning of WAT in mice.[22−24] Comprehensively, we have shown that the effects of ovariectomy on body weight and uterine development align with those reported in prior research.[17−21] Furthermore, our study suggests that 17$\beta$-estradiol supplementation effectively reduces WAT accumulation in the OVX mouse model.[22−24]

**3.2. Metabolite Comparison of Plasma.** Metabolomics analysis of the plasma samples collected from the three groups of mice was performed to determine the changes in plasma metabolites under hormone deficiency and supplementation. Using GC−TOF−MS, we identified 44 metabolites in the plasma of the three mouse groups, including 19 amino acids, 8 organic acids, 4 sugars, 3 sugar alcohols, 1 steroid, 5 fatty acids, and 4 other metabolites (Table 3). Applying plasma data to the PCA model resulted in a score plot that separated the data into groups (Figure 2). PCA, an unsupervised statistical technique, was employed to identify differences between metabolic phenotypes.[25] The sham group with ovaries and the OVX group without ovaries were separated. The E2 group was differentiated from the other groups due to 17$\beta$-estradiol supplementation. These results confirmed the metabolic distinctness of the Sham, OVX, and E2 groups. They also highlighted that the metabolic mechanisms changed with ovary removal and subsequent 17$\beta$-estradiol supplementation.

After verifying the metabolic separation of the three groups using PCA, we employed OPLS−DA to precisely assess the metabolic differences between the two groups. OPLS−DA is a supervised statistical method that categorizes variables based on their associations with class identifiers.[26] Additionally, a Student's $t$-test was performed to verify the significance of the two groups and visualized in a bar graph with metabolites having a $p$-value below 0.05 (Figure 3). The OPLS−DA model score plot between the OVX and sham groups had an $R^2Y$


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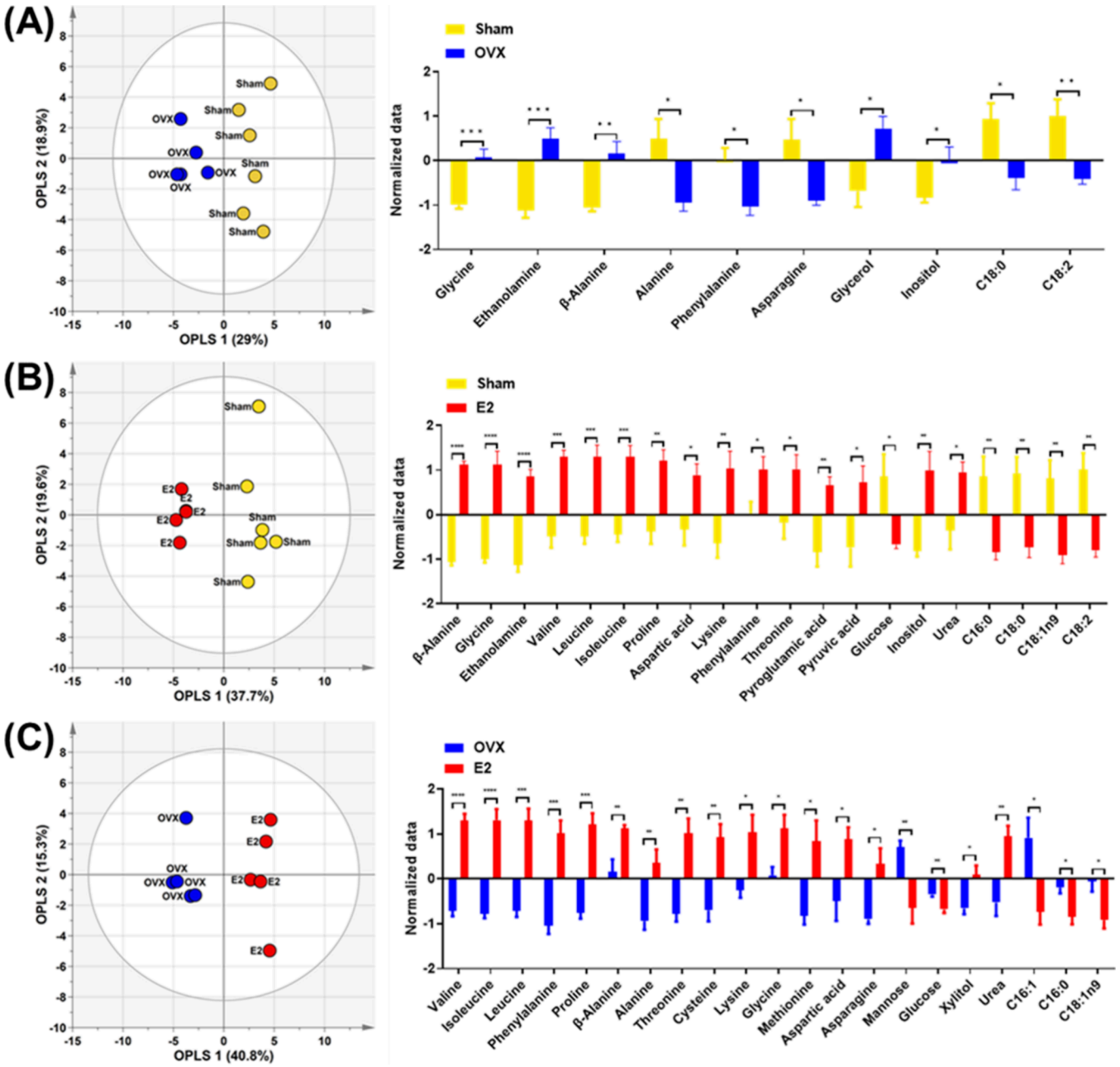


**Figure 3.** OPLS-DA model score plots and Student's *t*-test bar graphs based on the 44 metabolites obtained from the plasma samples of the mice from the (A) sham and OVX groups, (B) sham and E2 groups, and (C) OVX and E2 groups. Sham, sham-operated mice; OVX, ovariectomized mice; and E2, ovariectomized mice that received twice-weekly supplementation of 17$\beta$-estradiol. *$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001, ****$p$ < 0.0001.

value of 0.893 and a $Q^2$ value of 0.463. Additionally, Student's *t*-test results showed that 10 metabolites were significantly different between the OVX and sham groups (Figure 3A). Compared with the sham group, the OVX group had lower alanine and phenylalanine levels. This result is consistent with the characteristics of amino acid metabolism in the blood of postmenopausal women.[27] In addition, the OVX group had lower contents of C18:0 and C18:2 than did the sham group. Previous study has reported that reduced fatty acid metabolism (especially unsaturated fatty acids) is associated with impaired bone metabolism in OVX mouse.[28] Consequently, the levels of plasma metabolites changed, depending on the presence or absence of ovariectomy.

OPLS−DA analysis of the E2 and Sham groups showed more distinct differences, with an $R^2Y$ value of 0.962 and a $Q^2$ value of 0.853. Student's *t*-test results revealed significant differences in the levels of 19 metabolites between the E2 and Sham groups. Compared with the sham group, the E2 group had higher levels of branched-chain amino acids (BCAAs), such as valine, leucine, and isoleucine (Figure 3B).

In addition, the E2 and OVX groups showed notable differences, as evidenced by their high $R^2Y$ and $Q^2$ values of 0.963 and 0.809, respectively. Student's *t*-test results revealed significant differences in the levels of 21 metabolites between the E2 and OVX groups. The levels of amino acids, including BCAAs, were significantly higher in the E2 group than in the OVX group (Figure 3C). The plasma metabolite results in the


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Table 4. Composition and Content (Ratio/g) of Low-Molecular-Weight Metabolites in the Brain of Mice

| compound | content (Average ± SD, Ratio/g) | | |
|---|---|---|---|
| | sham | OVX | E2 |
| pyruvic acid | 0.28 ± 0.06 | 0.26 ± 0.03 | 0.30 ± 0.03 |
| lactic acid | 53.33 ± 1.88 | 46.53 ± 3.83 | 58.54 ± 2.87 |
| alanine | 27.69 ± 1.19 | 23.38 ± 2.40 | 31.92 ± 0.79 |
| oxalic acid | 31.88 ± 3.81 | 26.46 ± 2.79 | 34.67 ± 3.11 |
| hydroxybutanoic acid | 0.17 ± 0.05 | 0.15 ± 0.03 | 0.23 ± 0.02 |
| valine | 4.19 ± 0.48 | 3.49 ± 0.34 | 5.48 ± 0.24 |
| urea | 40.83 ± 3.29 | 37.18 ± 2.22 | 55.17 ± 5.91 |
| serine | 1.87 ± 0.12 | 1.57 ± 0.09 | 2.16 ± 0.11 |
| ethanolamine | 2.98 ± 0.35 | 2.14 ± 0.28 | 4.15 ± 0.47 |
| glycerol | 9.48 ± 1.02 | 8.43 ± 0.66 | 10.99 ± 1.13 |
| phosphoric acid | 80.47 ± 3.12 | 72.74 ± 6.25 | 91.33 ± 1.57 |
| leucine | 2.45 ± 0.18 | 1.72 ± 0.28 | 2.26 ± 0.23 |
| isoleucine | 2.54 ± 0.28 | 1.96 ± 0.23 | 2.87 ± 0.18 |
| proline | 5.77 ± 0.45 | 6.17 ± 0.27 | 6.96 ± 0.21 |
| glycine | 25.76 ± 3.23 | 17.81 ± 1.34 | 31.09 ± 2.20 |
| succinic acid | 18.36 ± 2.60 | 16.55 ± 1.39 | 23.76 ± 1.43 |
| fumaric acid | 2.09 ± 0.29 | 1.89 ± 0.19 | 2.86 ± 0.21 |
| threonine | 1.19 ± 0.03 | 0.97 ± 0.08 | 1.37 ± 0.03 |
| $\beta$-alanine | 1.13 ± 0.22 | 0.65 ± 0.07 | 1.71 ± 0.05 |
| malic acid | 7.80 ± 0.71 | 7.03 ± 0.51 | 9.99 ± 0.51 |
| aspartic acid | 11.39 ± 0.36 | 10.71 ± 0.69 | 13.57 ± 0.45 |
| methionine | 1.86 ± 0.23 | 1.39 ± 0.24 | 1.95 ± 0.09 |
| pyroglutamic acid | 43.76 ± 2.71 | 38.96 ± 2.67 | 50.83 ± 2.58 |
| 4-Aminobutyric acid | 9.49 ± 0.34 | 7.90 ± 0.61 | 11.11 ± 0.70 |
| cysteine | 1.78 ± 0.31 | 1.64 ± 0.22 | 2.50 ± 0.29 |
| glutamic acid | 48.85 ± 1.65 | 45.08 ± 3.38 | 58.57 ± 3.01 |
| phenylalanine | 2.11 ± 0.23 | 1.89 ± 0.12 | 2.29 ± 0.13 |
| asparagine | 1.21 ± 0.07 | 1.14 ± 0.05 | 1.43 ± 0.06 |
| xylitol | 0.30 ± 0.03 | 0.32 ± 0.02 | 0.39 ± 0.03 |
| ribitol | 0.25 ± 0.02 | 0.25 ± 0.01 | 0.28 ± 0.02 |
| glutamine | 18.38 ± 3.26 | 18.71 ± 2.70 | 18.11 ± 2.82 |
| citric acid | 0.94 ± 0.13 | 0.99 ± 0.18 | 1.13 ± 0.13 |
| fructose | 1.16 ± 0.33 | 1.10 ± 0.14 | 1.20 ± 0.15 |
| mannose | 0.02 ± 0.00 | 0.02 ± 0.00 | 0.02 ± 0.00 |
| glucose | 0.49 ± 0.16 | 0.49 ± 0.07 | 0.50 ± 0.09 |
| lysine | 1.41 ± 0.04 | 1.16 ± 0.11 | 1.27 ± 0.04 |
| C16:1 (palmitoleic acid) | 0.11 ± 0.01 | 0.09 ± 0.02 | 0.15 ± 0.02 |
| C16:0 (palmitic acid) | 9.96 ± 0.62 | 8.14 ± 1.44 | 12.38 ± 0.54 |
| inositol | 27.75 ± 0.82 | 25.71 ± 1.69 | 30.1 ± 1.69 |
| C18:2 (linoleic acid) | 0.08 ± 0.01 | 0.05 ± 0.01 | 0.10 ± 0.01 |
| C18:1n9 (oleic acid) | 2.60 ± 0.23 | 1.62 ± 0.46 | 3.55 ± 0.44 |
| tryptophan | 0.92 ± 0.08 | 0.95 ± 0.14 | 0.90 ± 0.09 |
| C18:0 (stearic acid) | 9.10 ± 0.45 | 7.00 ± 1.35 | 11.28 ± 0.74 |
| sucrose | 0.66 ± 0.07 | 0.49 ± 0.06 | 1.04 ± 0.05 |
| cholesterol | 34.36 ± 1.33 | 31.16 ± 2.17 | 35.72 ± 1.75 |

E2 group support the previous finding that low levels of BCAAs in the blood are associated with early menopause in patients with symptoms of estrogen deficiency.[29] Comprehensively, the metabolic changes in the plasma due to 17$\beta$-estradiol supplementation were greater than those due to the presence or absence of ovarian removal. These results suggest that 17$\beta$-estradiol supplementation can alleviate the decrease in plasma BCAA contents caused by estrogen deficiency.

**3.3. Metabolite Comparison of Brain.** Using GC−TOF−MS, we identified 45 metabolites in the brains of the three groups of mice, including 20 amino acids, 8 organic acids, 4 sugars, 3 sugar alcohols, 1 steroid, 5 fatty acids, and 4 others (Table 4). By applying the brain metabolite data to the PCA model, a score plot was derived that effectively separated the data into groups (Figure 4). Specifically, distinct changes in the metabolite content were observed in each group in the following order: OVX, sham, and E2. This result suggests that the three groups exhibited distinct metabolic profiles, with the most significant variations observed depending on the presence or absence of 17$\beta$-estradiol supplementation.

The OPLS−DA score plot of the OVX and Sham groups was clearly separated by the presence or absence of ovariectomy, unlike in the plasma, with an $R^2Y$ value of 0.927 and a $Q^2$ value of 0.689. Student's $t$-test results revealed significant differences in the levels of 22 metabolites between the OVX and sham groups (Figure 5A). In the OVX group,



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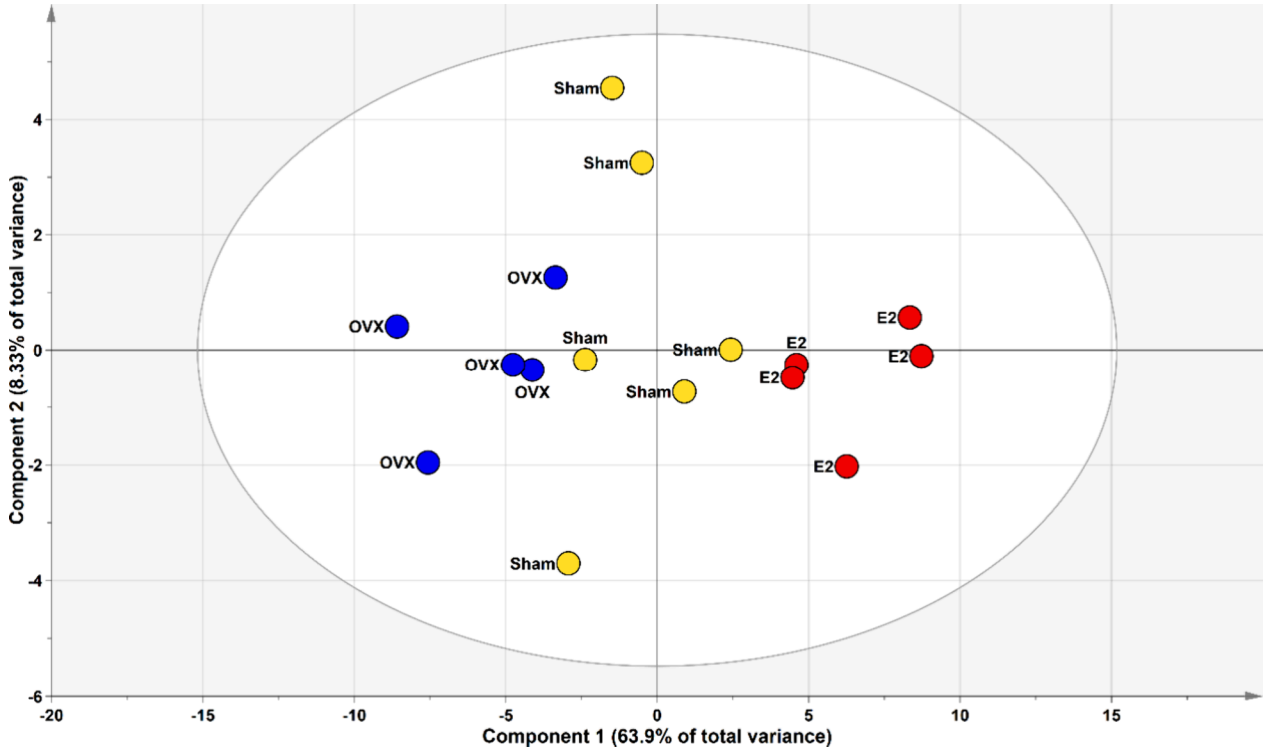


**Figure 4.** Score plot of the PCA model obtained from 45 metabolites in the brain samples of the mice from the sham, OVX, and E2 groups (yellow, Sham; blue, OVX; red, E2;). Sham, sham-operated mice; OVX, ovariectomized mice; and E2, ovariectomized mice that received twice-weekly supplementation of 17$\beta$-estradiol.

low levels of amino acids, such as BCAAs, glucogenic amino acids (methionine and threonine), and ketogenic amino acids (lysine and leucine), were detected. The balance between amino acids and nitrogen is important for the CNS. BCAAs are nitrogen donors in the brain; they are rapidly transported across the BBB and play an important role in metabolic homeostasis.[30] Additionally, glucose is used as the main energy source in the brain, whereas ketones serve as an alternative energy source.[31] Glucogenic and ketogenic amino acids in the brain are important from the perspective of energy metabolism. Therefore, the decrease in the levels of BCAAs, glucogenic amino acids, and ketogenic amino acids in the OVX group suggested potential metabolic problems related to estrogen deficiency.

The sham and E2 groups had $R^2Y$ and $Q^2$ values of 0.977 and 0.904, respectively. Student's *t*-test results revealed significant differences in the levels of 27 metabolites between the sham and E2 groups (Figure 5B). The levels of most amino acids, organic acids, and fatty acids were higher in the E2 group than in the sham group. Notably, the levels of neuronal and signaling substances, such as aspartic acid, glutamic acid, $\gamma$-aminobutyric acid (GABA), and glycine, were significantly higher in the E2 group than in the Sham group. Aspartic acid, which is primarily concentrated in the mouse pituitary gland, facilitates the release of gonadotropin-releasing hormone from the hypothalamus.[32] Glutamic acid serves as an excitatory neurotransmitter in the CNS and plays vital roles in cognitive, memory, and learning functions.[33] Furthermore, GABA is a primary inhibitory neurotransmitter in the brain. Together with glutamic acid, an excitatory neurotransmitter, it regulates the balance between inhibition and excitation essential for brain function.[34,35] Glycine is a major neurotransmitter that acts on the CNS.[35] These results suggest that 17$\beta$-estradiol supplementation can increase neurotransmitter levels, potentially affecting brain function and hormonal regulation.

The difference was even greater between the OVX and E2 groups. The OPLS−DA score plot of the E2 and OVX groups was clearly separated by the presence or absence of 17$\beta$-estradiol supplementation, with an $R^2Y$ value of 0.99 and a $Q^2$ value of 0.921. Student's *t*-test results revealed significant differences in the levels of 31 metabolites (Figure 5C). Compared with the OVX group, the E2 group had significantly higher levels of BCAAs, glucogenic amino acids, ketogenic amino acids, ketone bodies (hydroxybutanoic acid), neurotransmitters, and tricarboxylic acid (TCA) cycle intermediates. In particular, unlike the plasma, the brains of the E2 group had higher contents of fatty acids and TCA cycle intermediates. This phenomenon appears to be closely related to the thermogenesis of brown adipose tissue (BAT). BAT is regulated by the central and peripheral nervous systems and plays an important role in stimulating thermogenesis.[13] In BAT thermogenesis, fatty acids act as the main energy source and help reduce body fat by browning white fat.[22,23] In the present study, the E2 group had a WAT weight significantly lower than that of the OVX group (Table 2). These results suggest that 17$\beta$-estradiol supplementation can stimulate the CNS and promote BAT thermogenesis, thereby reducing WAT weight. Furthermore, TCA cycle activity is highly correlated with fatty acid absorption and oxidation.[36,37] Therefore, an increase in the levels of TCA-cyclic metabolites and fatty acids in the brain suggests active fatty acid absorption and oxidation in the brain through the TCA cycle. Comprehensively, 17$\beta$-estradiol supplementation impacts neurotransmitter levels and stimulates the CNS. Furthermore, it influences BAT thermogenesis and potentially affects TCA cycle activity, fatty acid uptake, and oxidation in the brain. This comprehensive analysis


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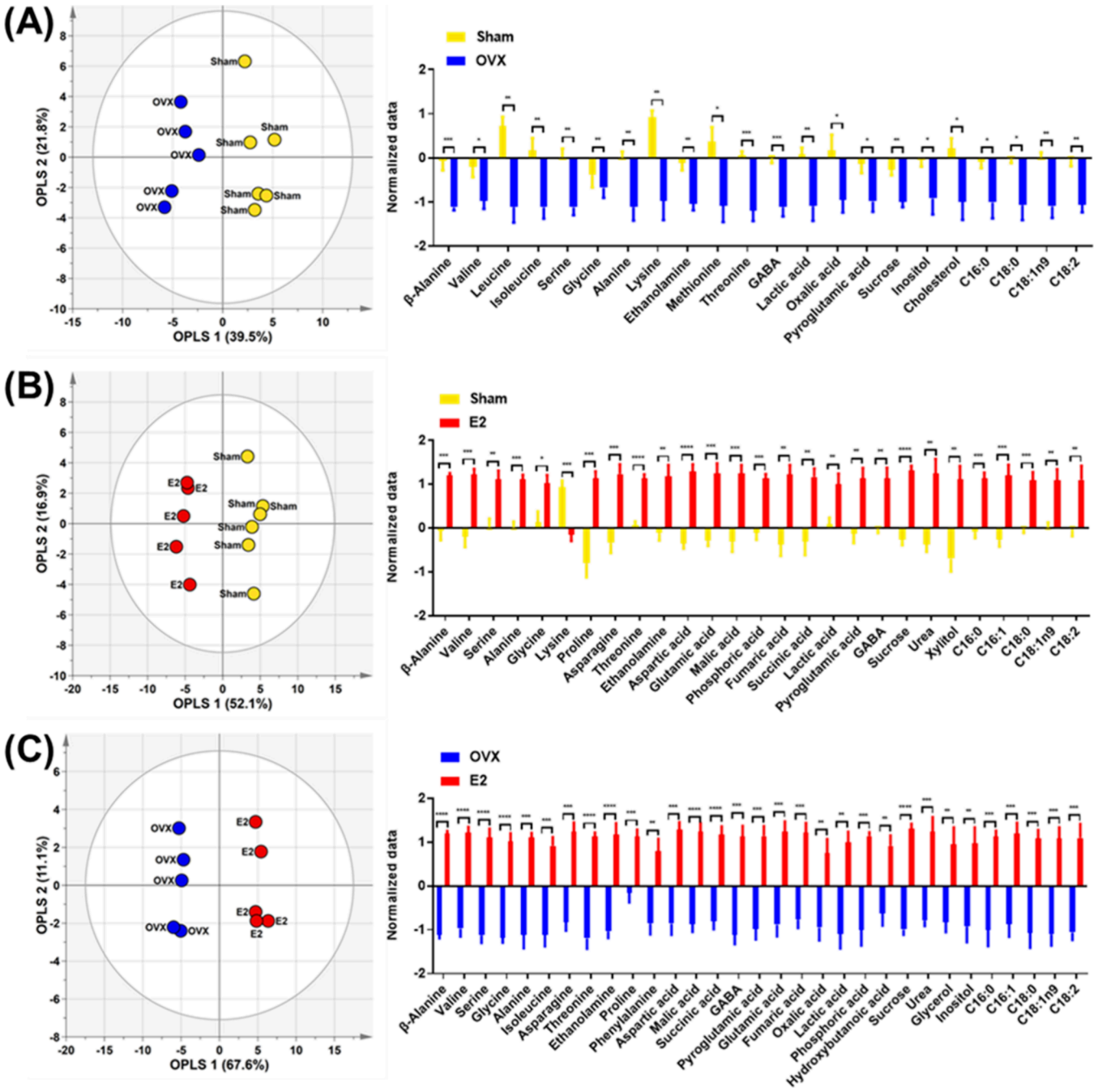


**Figure 5.** OPLS-DA model score plot and Student's *t*-test bar graphs based on the 45 metabolites obtained from the brain samples of the mice from the (A) sham and OVX groups, (B) sham and E2 groups, and (C) OVX and E2 groups. Sham, sham-operated mice; OVX, ovariectomized mice; and E2, ovariectomized mice that received twice-weekly supplementation of 17$\beta$-estradiol. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

provides valuable insights into the complex metabolic changes in the brain associated with estrogen deficiency and supplementation in mice.

**3.4. Energy Metabolism due to 17$\beta$-Estradiol Supplementation in an OVX Mouse Model.** Supplementation of 17$\beta$-estradiol to the OVX mouse model significantly increased metabolic levels in the plasma and brain. Pathway and enrichment analyses were performed to investigate the significance of metabolic changes in serum and brain affected by 17$\beta$-estradiol supplementation in the OVX mouse model (Figure S1). Thirty-two metabolic pathways were significantly enriched in the plasma of mice in the OVX and E2 groups ($p < 0.05$). Among them, the pathways of valine, leucine, and isoleucine biosynthesis and phenylalanine metabolism were the most significantly enriched ($p < 0.001$) (Figure S1A). Furthermore, in the brain of mice in the OVX and E2 groups, there was an enrichment of significant metabolites in thirty-seven metabolic pathways (Figure S1B). Five metabolic pathways are quite noteworthy: $\beta$-alanine metabolism, D-amino acid metabolism, valine, leucine and isoleucine biosynthesis, alanine, aspartate, and glutamine metabolism, and TCA cycle ($p < 0.01$).

Figure 6 shows an overview of metabolites of significant pathways based on previous analysis results. The metabolic differences related to energy metabolism are shown (Figure 6). $\beta$-Alanine significantly increased in the plasma and brain of the



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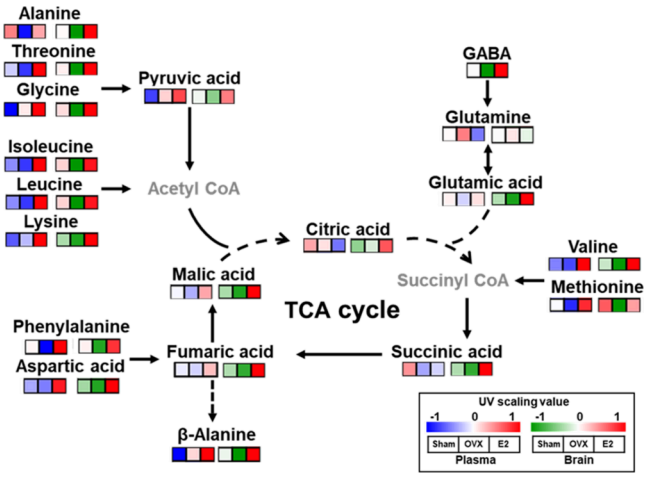


**Figure 6.** Overview of metabolic alteration in serum and brain affected by 17$\beta$-estradiol supplementation in the OVX mouse model. Metabolites with $p < 0.05$ are shown in normalized data using UV scaling. UV scaling values range from −1.0 to 1.0. Each square is represented as the mean of the normalized data. Red indicates normalized values greater than 0, blue or green indicates normalized values less than 0, and gray indicates undetected metabolites. Sham, sham-operated mice; OVX, ovariectomized mice; and E2, ovariectomized mice that received twice-weekly supplementation of 17$\beta$-estradiol.

E2 group. $\beta$-Alanine is a component of carnosine that inhibits skeletal muscle-related aging, and an increase in $\beta$-alanine increases muscle carnosine content.[38] Carnosine neutralizes age-related neurodegenerative diseases and helps stabilize the nervous system by regulating the neurotransmitter system.[39] Therefore, 17$\beta$-estradiol supplementation appears to enhance $\beta$-alanine activity, suggesting potential benefits for muscle and nervous system health. $\beta$-Alanine is produced from aspartic and fumaric acid. Aspartic acid was significantly increased in both the plasma and brain of the E2 group. However, TCA cycle intermediates, including fumaric acid, did not show significant changes in the plasma of the E2 group (Figure 6). Thus, it is insufficient to explain the relationship between 17$\beta$-estradiol supplementation and energy metabolism. In contrast to the plasma, the brain of the E2 group showed significant increases in the levels of TCA cycle intermediates, such as malic, fumaric, and succinic acid. Serving as the primary metabolic pathway that fuels the body, the TCA cycle generates approximately two-thirds of the total energy in the body.[40] In addition, the levels of amino acids, such as isoleucine, threonine, and methionine, which produce acetyl-CoA and succinyl-CoA, were increased in the brain samples from mice in the E2 group (Figure 6). Amino acid deamination produces intermediates of the TCA cycle. Ultimately, each cycle intermediate helps create the next intermediate, and amino acids help create intermediates, thereby activating the entire cycle. Decreased metabolic activity is closely associated with the development of various diseases, such as metabolic syndrome and diabetes, which occur during menopause.[41,42] These results suggest that supplying 17$\beta$-estradiol in a state of estrogen deficiency activates metabolism. This is consistent with previous studies showing that 17$\beta$-estradiol supplementation in postmenopausal women activates metabolic regions of the brain.[43] In the present study, 17$\beta$-estradiol supplementation in estrogen-deficient models notably increased the energy metabolism in the plasma and brain. The meticulous examination of brain metabolites revealed noteworthy elevations in the levels of TCA cycle intermediates and associated amino acids. This comprehensive brain metabolite analysis indicates the robust activation of pivotal metabolic pathways responsible for generating a substantial portion of the body's energy.

In conclusion, the effects of 17$\beta$-estradiol supplementation on the plasma and brain metabolites of OVX mice were successfully evaluated using metabolomics techniques. After 6 weeks of 17$\beta$-estradiol supplementation, reduction of WAT and recovery of atrophic uterus were confirmed through body weight and uterine phenotype. Metabolic differences were confirmed by using multivariate analysis. In particular, the OPLS−DA model revealed statistically significant differences between the OVX and E2 groups. In the plasma, the E2 group had higher amino acid levels than did the OVX group. In the brain, the E2 group had significantly higher contents of metabolites including TCA cycle intermediates, neurotransmitters, amino acids, and fatty acids. This study is the first to reveal differences in energy metabolism induced by 17$\beta$-estradiol supplementation through brain metabolic profiling of OVX mice, underscoring the importance of brain tissue metabolic profiling in menopausal hormone research. It may also serve as a basis for further studies of brain metabolic profiles under varied hormonal supplementation conditions.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c10399.

Pathway and enrichment analyses in serum and brain affected by 17$\beta$-estradiol supplementation in the OVX mouse model (PDF)

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### Author Contributions

#S.H.Y. and Y.J.K. have contributed equally to this work and share first authorship. S.H.Y. performed formal analysis, data curation, and writing−original draft. Y.J.K. performed methodology, formal analysis, and writing−review. H.R.Y. and S.U.P. performed methodology and resource gathering. J.G.K performed conceptualization, resource gathering, supervision, and writing−review and editing. J.K.K. performed conceptualization, supervision, and writing−review and editing.

### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

Sham, sham-operated mice; OVX, ovariectomized mice; E2, ovariectomized mice that received twice-weekly supplementation of 17$\beta$-estradiol; BCAA, branched-chain amino acid; MHT, menopausal hormone therapy; BBB, blood-brain barrier; SC-WAT, subcutaneous white adipose tissue; PM-WAT, parametrial white adipose tissue; BAT, brown adipose tissue; PCA, principal component analysis; OPLS-DA, orthogonal partial least-squares discriminant analysis; CNS, central nervous system; TCA, tricarboxylic acid; GC-TOF-MS, gas chromatography−time-of-flight−mass spectrometry; GABA, 4-aminobutyric acid; C16:1, palmitoleic acid; C16:0, palmitic acid; C18:2, linoleic acid; C18:1n9, oleic acid; C18:0, stearic acid

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