

# **11-plex DiLeu Isobaric Labeling Enables Quantitative Assessment of Brain Region Protein-Association Networks Impacted by the Gut Microbiome**

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## **Abstract**

Gut microbiota can regulate host brain functions and influence various physiological and pathological processes through the brain-gut axis. To systematically elucidate the intervention of different gut environments on different brain regions, we implemented an integrated approach that combines 11-plex DiLeu isobaric tags with a “BRIDGE” normalization strategy to comparatively analyze the proteome of six brain regions in germ-free (GF)- and conventionally raised (ConvR)-mice. A total of 5,945 proteins were identified and 5,656 were quantifiable, while 1,906 of them were significantly changed between GF- and ConvR-mice. 281 proteins were filtered with FC greater than 1.2 in at least one brain region, of which heatmap analysis showed clear protein profile disparities both between brain regions and gut microbiome conditions. Gut microbiome impact is most overt in the hypothalamus and the least in thalamus region. Collectively, this approach allows an in-depth investigation of the induced protein changes by multiple gut microbiome environments in a brain region- specific manner. This comprehensive proteomics work improves the understanding of brain region protein-association networks impacted by the gut microbiome and highlights the critical roles of brain-gut axis.

**Keywords:** Gut microbiota; DiLeu isobaric tag; Proteomics; Quantitation; Brain-gut regulation

## INTRODUCTION

The gut microbiota-brain axis is a bidirectional communication system between the gut microbiota and central nervous system (CNS)<sup>1-2</sup>. Neurotransmitters such as serotonin and acetylcholine produced by the gut microbiota can influence the development of the nervous system through gut microbiota-brain axis, while it can also alter the level of pro- and anti-inflammatory cytokines to regulate host physiology, metabolism, immunity, and even behavior. At the same time, its own compositions can also be altered due to the two-way regulation of gut microbiota-brain axis. Gut microbiota dysbiosis may contribute to many behavioral and neurological disorders such as Alzheimer's disease<sup>3-4</sup>, Parkinson's disease<sup>5</sup>, depression<sup>6-7</sup> and anxiety<sup>8-9</sup>. Therefore, normal function of the gut microbiota-brain axis is essential to the homeostasis of the nervous system, especially the CNS<sup>10</sup>.

Currently, the roles of microbiota in anxiety-related behaviors, impaired social cognition and stress responses, etc. have been widely investigated<sup>11-14</sup>. Studies have shown that a symbiotic gut-brain coregulatory axis can link the gut microbiota to neurogenic control in the hippocampus region<sup>14</sup>. In another study, several key brain regions, including amygdala, striatum, cerebellum, hippocampus and cortex were dissected and the gene expression were evaluated and compared, demonstrating that the microbiota was necessary for appropriate and dynamic regulation of myelin-related genes with clear implications on cortical myelination at an ultrastructural level<sup>15</sup>.

Proteomic analysis to study the intervention of gut microbiota on the proteome of different brain regions enables a global and systemic understanding of such regulation by gut microbiota-brain axis. Mass spectrometry (MS)-based approaches are powerful tools to identify thousands of proteins in complex samples and to precisely quantify protein expression

between animals with different gut microbiota<sup>16</sup>. In our previous work, germ-free (GF)-mice were employed to investigate the relative changes in neuropeptides and proteins in the hypothalamus region compared to conventionally raised (ConvR)-mice. As expected, the functions of these altered neuropeptides and the pathways of the proteins involved were related to various essential behaviors, including brain development, memory, and learning<sup>17</sup>. In this work, *N*, *N*-dimethyl leucine (DiLeu) tags for quantitative proteomics analysis were utilized, which featured more compact structures and higher intensity of generated reporter ions compared to commercial tags such as tandem mass tags (TMT) and isobaric tags for relative and absolute quantification (iTRAQ). This novel design contributes to improved confidence in peptide identification and quantification, and the tags are more readily synthesized in-house with higher yields and lower cost<sup>18-20</sup>. The multiplexed isobaric tags allow multiple samples to be analyzed in a single assay, facilitating high-throughput quantification of thousands of proteins. When the number of samples exceeds the multiplexing capability, “BRIDGE” samples are often beneficial to more reliable quantification between batches. It has been reported that a total of 82 samples were quantitatively analyzed in 11 experiments using 10-plex TMT isobaric tags, of which two tags were used to label “BRIDGE” samples for normalization<sup>21</sup>. Likewise, using iTRAQ tags, a “BRIDGE” sample was also introduced for sample analysis at two locations<sup>22</sup>. Inspired by this concept, we reason that introducing a “BRIDGE” sample as an internal reference could alleviate the variation when quantifying samples from batch to batch using DiLeu tags.

In this work, we developed a method based on 11-plex DiLeu tagging combined with the “BRIDGE” strategy to perform a comprehensively quantitative proteomic analysis of six brain

regions in mice with distinct gut microbiota environments. Of these, DiLeu 118c was used to label the “BRIDGE” sample, and the other 10-plex DiLeu tags were used to label 5 GF- and 5 ConvR-mouse samples per brain region. The method developed in this work allows simultaneous profiling of protein changes in GF- and ConvR- mice from both the same and different brain regions. This work describes an effective strategy for high-throughput in-depth proteome profiling of the brain different gut microbiota environments and provides a comprehensive data resource for future neuroscience research.

## **EXPERIMENTAL SECTION**

### **Chemicals and Reagents**

All isotopic reagents used for DiLeu synthesis were purchased from Isotec (Miamisburg, OH, USA). Mass spectrometry-grade trypsin and dithiothreitol (DTT) were obtained from Promega (Madison, WI, USA). Ammonium bicarbonate, urea, dichloromethane (DCM), ACS grade methanol (MeOH), Optima UPLC grade ACN, Optima UPLC grade water and Optima LC/MS grade formic acid (FA) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Sodium cyanoborohydride ( $\text{NaBH}_3\text{CN}$ ), formaldehyde ( $\text{CH}_2\text{O}$ ), L-leucine, hydrogen chloride gas (HCl), tris hydrochloride (tris HCl), trifluoroacetic acid (TFA), iodoacetamide (IAA), triethylammonium bicarbonate (TEAB), N, N-dimethylformamide (DMF), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMM), N-methylmorpholine (NMM), heptafluorobutyric acid (HFBA), dimethyl sulfoxide (DMSO) and bovine serum albumin (BSA) were bought from Sigma-Aldrich (St. Louis, MO, USA). Hydroxylamine solution was purchased from Alfa Aesar (Ward Hill, MA, USA). DiLeu reagents were

synthesized in the laboratory based on our previous work.

### **Animal Experiment, Tissue Extraction and DiLeu Labeling**

Animal care and research protocols were approved by the UW–Madison Animal Care and Use Committee. Mice were housed in groups in temperature-controlled chambers with a 12-hour light: 12-hour dark cycle and received *ad-libitum* access to water and food. Mice were fed autoclaved LabDiet #5021 (Purina Mills, Inc. Richmond, IN). Twenty-one-week-old male C57BL/6 GF-mice and ConvR-mice (n=5/group) were euthanized by CO<sub>2</sub> inhalation and bloodletting after 4-h of fasting. Their brains were dissected and subsequently heated rapidly by Denator™ to reduce postmortem degradation, after which the cortex, hippocampus, hypothalamus, spinal cord, striatum and thalamus regions were isolated, immediately snap frozen in liquid nitrogen, and stored at -80°C until analysis.

Brain tissues were lysed by sonication in the extraction buffer (4% SDS, 100 mM Tris/Base pH 8.0), and protein concentrations were determined by the BCA assay. After adding DTT to 10 mM, each sample containing 200 µg protein was incubated for 0.5 h at room temperature. The samples were then alkylated with 55 mM iodoacetamide (IAA) for 30 min in the dark, and the reaction was quenched by 5 mM DTT. Proteins were precipitated overnight by addition of 5.5 times the volumes of 80% acetone, then rinsed with 80% acetone and dried in air. Dried pellet was resuspended in 8 M urea, 30 mM NaCl, 50 mM Tris (pH = 8), 5 mM CaCl<sub>2</sub> and a protease inhibitor cocktail tablet (Roche Diagnostics). The sample was sonicated in an ice-water bath for 20 min, centrifuged at 14,000 × g for 5 min, and the supernatant was collected. Afterwards, the supernatant was diluted with 50 mM Tris solution (pH = 8) to a urea concentration of less than 1 M. The protein was digested with trypsin at a protein/enzyme ratio

of 50:1 for 16 h at 37°C. The digestion was quenched by adding 10% trifluoroacetic acid (TFA) to a pH lower than 3, followed by a desalting step using a SepPak C18 solid-phase extraction cartridge (Waters).

A detailed method for the syntheses of 11-Plex DiLeu tags was reported by Frost et al <sup>20</sup>. The DiLeu labeling was performed as follows. Tags and peptide digests were added to the labeling solution in a 20:1 ratio by weight and vortexed for 2 h at room temperature. Equivalent amounts of digested peptides from 10 mice were pooled as internal reference. DiLeu reagents (115a, 115b, 116a, 116b, 116c, 117a, 117b, 117c, 118a, 118b, and 118c) were used to randomly label the digests from the different brain regions of GF-mice (labeled with 116a, 116b, 116c, 117a, and 117b) and ConvR-mice (labeled with 115a, 115b, 117c, 118a, and 118b), respectively. The internal reference was labeled with DiLeu reagent 118c, which was reserved for “BRIDGE” samples. Next, hydroxylamine at a concentration of 0.25% was added to quench the labeling reaction, and the peptide mixtures were pooled at the same ratio and dried down. The combined samples were acidified with HFBA to a concentration of 0.5% and washed with SCX SpinTips (Protea Biosciences, Morgantown, WV) to remove unreacted DiLeu reagent and reaction byproducts of the labeled peptides, and then pooled samples were divided into four fractions by high pH fractionation using C18 column (Phenomenex).

### **LC-MS Analysis**

Samples analysis was performed using a Dionex Ultimate 3000 nanoLC system (Thermo Scientific) with a Q Exactive HF hybrid quadrupole-Orbitrap mass spectrometer (QE HF, Thermo Fisher Scientific, San Jose, CA). Labeled tryptic peptides were dried in vacuum and dissolved in water containing 3% ACN and 0.1% FA. Peptides were loaded onto a

microcapillary column with an inner diameter of 75  $\mu\text{m}$  made with an integrated emitter tip and packed with 15 cm of Bridged Ethylene Hybrid C18 particles (1.7  $\mu\text{m}$ , 130  $\text{\AA}$ , Waters). Mobile phase A and B consisted of water containing 0.1% FA and ACN containing 0.1% FA, respectively. Gradient elution separation was performed over 120 min using 3% to 30% mobile phase B at a flow rate of 300 nL/min. A survey scan of peptide precursors from 300 to 1500  $m/z$  was performed at RP 60,000 with an AGC target of  $1 \times 10^6$  and maximum IT of 100 ms. The top 10 precursors were then selected for HCD MS<sup>2</sup> analysis at RP 60,000 with an isolation width of 2.0 Da, an NCE of 30, an AGC target of  $1 \times 10^5$  and a maximum IT of 100 ms. Precursors were dynamically excluded for 15 s with a tolerance of 10 ppm.

## Data Analysis

The qualitative and quantitative analysis of DiLeu-labeled peptides was processed using MaxQuant (1.6.0.1). Raw files were searched in MaxQuant with the UniProt mouse complete database (February, 2023), selecting trypsin as the enzyme and allowing for two missed cleavages. The search was performed with a precursor mass tolerance of 25 ppm and a fragment mass tolerance of 0.03 Da. Static modifications included DiLeu tags on peptide N-termini (+145.12801 Da) and carbamidomethylation of cysteine residues (+57.02146 Da), while dynamic modifications included DiLeu labeling on lysine residues, oxidation of methionine residues (+15.99492 Da), deamidation of asparagine and glutamine residues (+0.98402 Da), and methylation of C termini and aspartic acid, glutamic acid, histidine, lysine, arginine, serine, and threonine residues (+14.01565 Da). Peptide-spectrum matches (PSMs) were validated using percolator against a 1% FDR (false discovery rate) based on q-values.

Peptide quantitation was performed using MaxQuant with a reporter ion integration

tolerance of 20 ppm for the most confident centroid. Only PSMs containing all 11 reporter ions were considered and a minimum of one quantified peptide was used to determine the quantitative ratios of the protein. Proteomic reporter ion ratio values were exported to Excel workbook format (Redmond, CA). The UniProt Gene Ontology Annotation database (<http://www.ebi.ac.uk/GOA/>) was used.

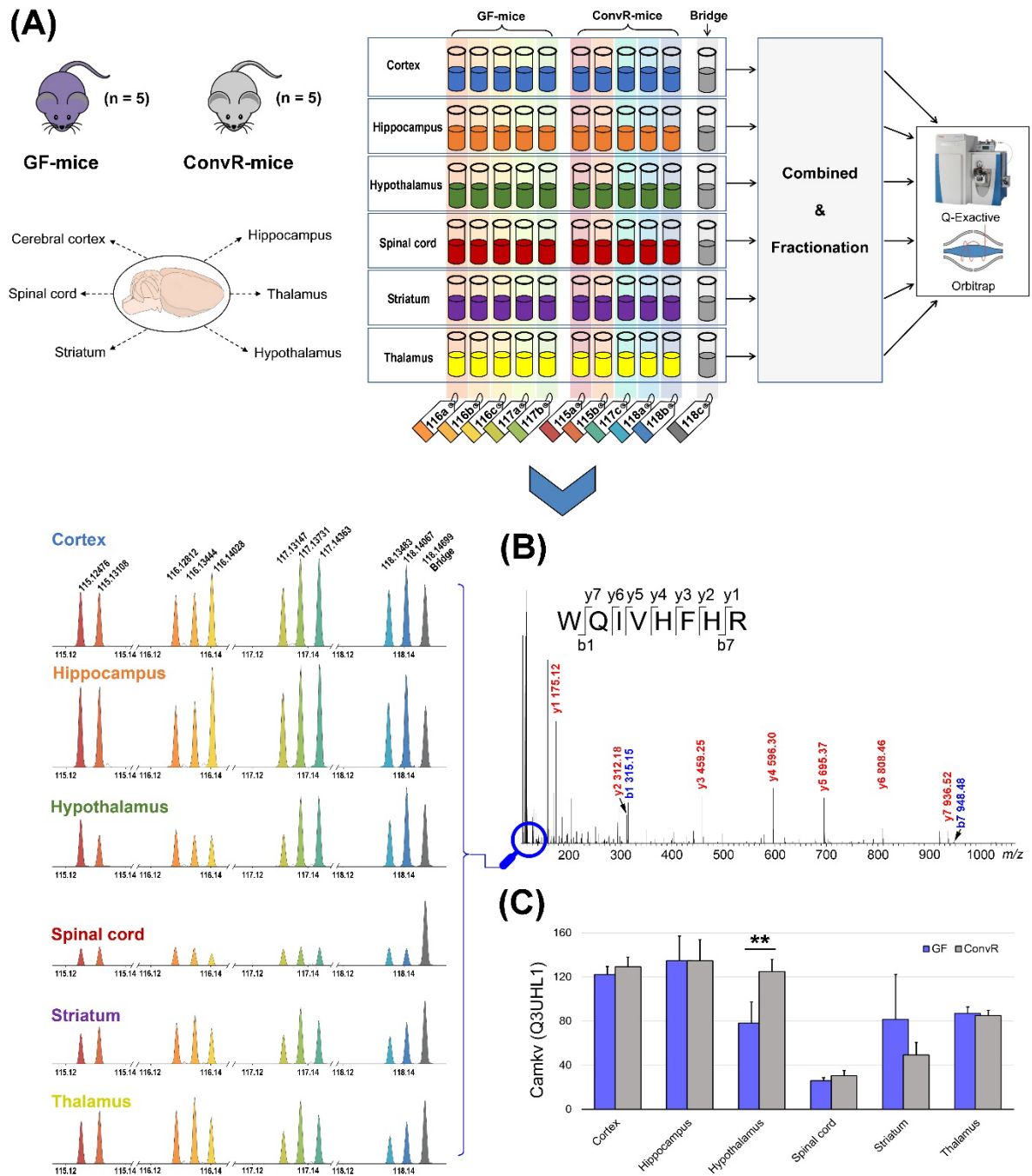
The intensity of proteins in “BRIDGE” samples is specified as 100. In the same brain region, the intensity of proteins is calculated as  $(Intensity_{\text{Samples}}/Intensity_{\text{BRIDGE}}) \times 100$ , i.e. the relative intensity of proteins in each sample can be normalized by the intensity of the “BRIDGE” sample labeled with the DiLeu 118c reagent. Thus, the average value of the quantified proteins can be calculated from the normalization of the independent BRIDGE-samples. Quantification of normalized proteins from different samples was analyzed by Excel and SPSS 16.0 software. The student’s *t*-test (two-tailed) was performed for comparisons between two independent groups of samples. A *p*-value of < 0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

### **Schematic illustration of BRIDGE-based multiplexed quantification of proteins in six brain regions**

Bottom-up proteomics sample preparation was used in the workflow for BRIDGE-based 11-plex quantification of proteins from six brain regions as shown in **Figure 1**. Proteins were extracted from brain tissues and digested into shorter peptides, which were then labeled with DiLeu isobaric tags. GF-mice were labeled with 5-plex DiLeu tags and all six brain regions of each mouse were labeled with the same DiLeu tag. ConvR-mice were labeled with the other 5-

plex DiLeu tags in the same way. Thus, a total of 60 brain samples from 5 GF-mice and 5 ConvR-mice were labeled with 10-plex DiLeu tags. To prepare “BRIDGE” reference sample, 60 trypsin-digested brain samples were first pooled in equal amounts and labeled with the 11<sup>th</sup> DiLeu tag, and then this pooled sample was equally divided into 6 portions, each of which was added to the samples from each brain region as “BRIDGE”. Afterwards, samples from the same brain region were combined and fractionated for analysis by QE-HF. The abundance of 10-plex tags normalized based on the 11<sup>th</sup> DiLeu tag (118c, BRIDGE) could be used for protein quantification in different brain regions. Using the proposed scheme, we achieved simultaneous analysis of the proteome of all brain regions in GF- and ConvR-mice, which helps to reveal the proteome changes in different brain regions under different gut microbiota environments. This study further elucidates the effects of gut microbiome changes on the regulation of brain functions, including memory, learning, circadian rhythm, etc.



**Figure 1.** Experimental workflow of quantitative proteomic analysis for proteins from six mouse brain regions under different gut microbiome environments using “BRIDGE” based DiLeu isobaric labeling strategy. (A) Germ-free (GF)-mice and conventionally raised (ConvR)-mice were euthanized, and the Cerebral cortex, Hippocampus, Hypothalamus, Spinal cord, Striatum and Thalamus regions were harvested. Samples were labeled with DiLeu isobaric for quantitative analysis. The “BRIDGE” samples were labeled with DiLeu 118c tag and used as

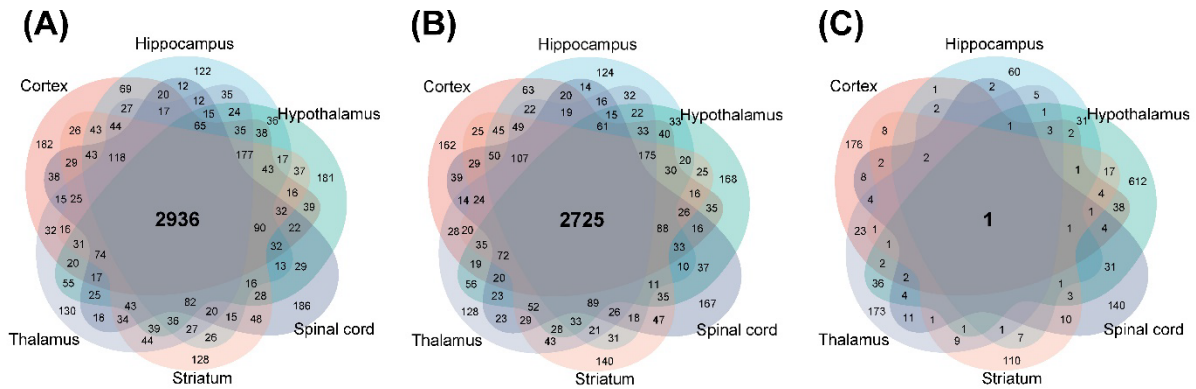
a reference to normalize all proteins from different regions. (B) MS<sup>2</sup> spectrum of 11-plex DiLeu-labeled peptide WQIVHFHR acquired from different regions with the reporter ion peaks shown in the left panel. (C) The relative abundance of CaM kinase-like vesicle-associated protein (Camkv, Q3UHL1) in different brain regions from GF- and ConvR-mice.

### **Quantitative proteomic analysis of six brain regions in GF- and ConvR-mice**

We employed a “BRIDGE” based isobaric labeling strategy to quantify and compare proteins from six brain regions simultaneously using 11-plex DiLeu tags. A total of 60 samples from five GF- and five ConvR-mice were analyzed. As shown in **Figure 2(A)**, a total of 5,945 proteins were identified in six brain regions (**Supplemental data 1**), of which 2,936 were shared by the six brain regions examined. A total of 36,939 peptides were identified in six brain regions (**Figure S1**), of which 8,162 peptides were shared, and 2,527, 1,208, 1,925, 2,312, 1,024 and 1,198 peptides were detected exclusively in the Cortex, Hippocampus, Hypothalamus, Spinal cord, Striatum and Thalamus, respectively. In addition, 182, 122, 181, 186, 127 and 130 proteins were uniquely detected in the Cortex, Hippocampus, Hypothalamus, Spinal cord, Striatum and Thalamus, respectively, suggesting a region-specific proteome landscape in mouse brain.

As shown in **Supplemental data 1**, a total of 5,656 proteins could be quantified in each brain region. To compare the relative amounts of the same proteins present simultaneously in different brain regions, we used the BRIDGE samples for normalization: by setting the amount of each protein in the BRIDGE samples for each brain region to 100, the amount of the proteins in the GF- and ConvR-groups from each brain region would have a value relative to the BRIDGE sample. This is a normalization process using BRIDGE samples in which the same

proteins are present at the same level in different brain regions, ensuring comparable protein levels in different brain regions. Venn diagram shows that 2,725 of these quantifiable 5,656 proteins are shared among different brain regions (**Figure 2B**). In addition, 162, 124, 168, 167, 140 and 128 proteins were detected exclusively in the Cortex, Hippocampus, Hypothalamus, Spinal cord, Striatum and Thalamus, respectively.



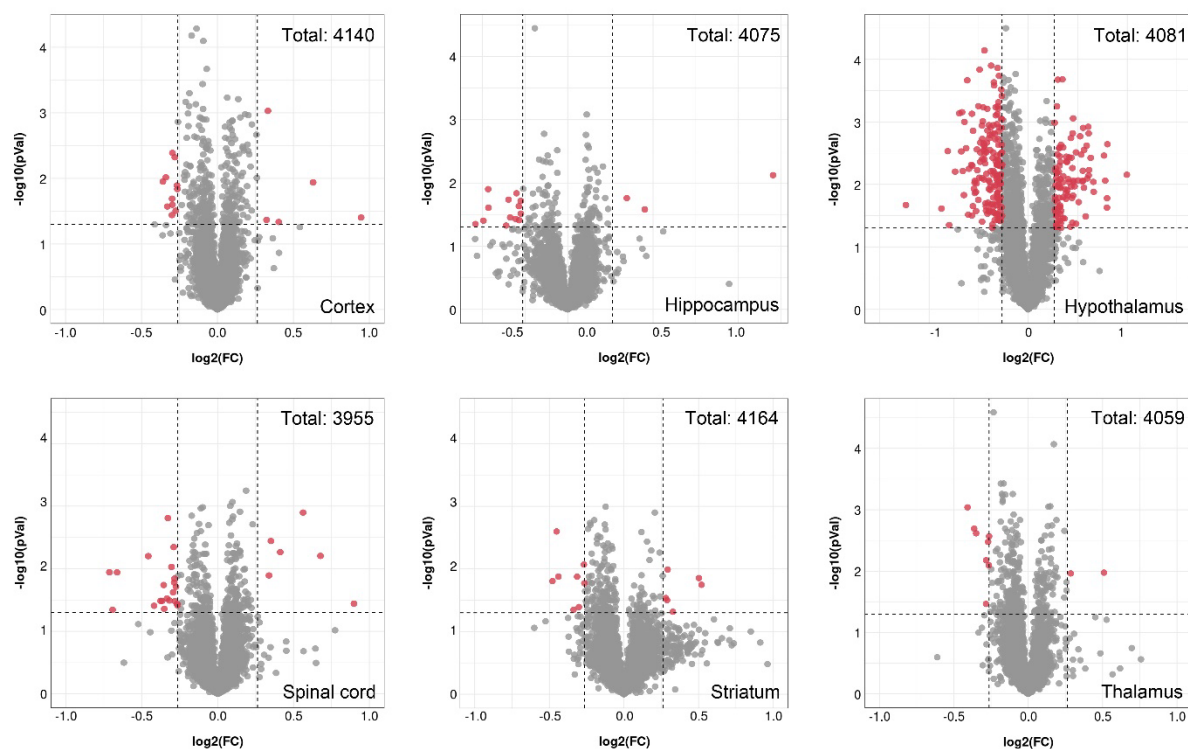
**Figure 2.** Venn diagrams of proteins identified from different brain regions. (A) Distribution of all 5,945 proteins identified in each brain region; (B) Distribution of the quantified 5,656 proteins in each brain region; (C) Distribution of the significantly changed 1,906 proteins between GF- and ConvR-mice in each brain region.

Furthermore, by comparing the results of proteomic experiments in GF- and ConvR-mice in different gut environments, 1,906 proteins were found to be significantly altered ( $p < 0.05$ ), of which 288, 122, 798, 230, 182 and 286 proteins were detected in the Cortex, Hippocampus, Hypothalamus, Spinal cord, Striatum and Thalamus, respectively. Among them, 176, 60, 612, 140, 110 and 173 proteins were exclusively detected in each of the above brain regions, which

sums up to 1,266 proteins accounting for 66.4% of all significantly altered proteins. In addition, only one protein, NAD(P) transhydrogenase (Q61941), was shared by all brain regions (**Figure 2C**). These results suggest that gut microbiome may affect different brain regions through distinct pathways. Therefore, certain proteins or functions in each brain region might be regulated by different gut environments.

Although some proteins changed significantly in different gut environments of GF- and ConvR- mice, their fold changes were relatively modest. To find the key differentially expressed proteins, we set an average fold change (FC) greater than 1.2 as the threshold according to the literature, and filtered a total of 355 proteins from 1,906 significantly changed proteins, of which 16, 16, 272, 27, 14 and 10 proteins were found in the Cortex, Hippocampus, Hypothalamus, Spinal cord, Striatum and Thalamus, respectively, as shown in **Figure 3**.

In addition, threshold of  $FC > 1.5$  and  $p < 0.05$  was set to screen proteins specific to certain brain regions. As shown in **Figure S2**, 2 proteins in the Cortex, 1 protein in the Hippocampus, 30 proteins in the Hypothalamus, 5 proteins in the Spinal cord were selected, while there was no protein selected in the Striatum and Thalamus.



**Figure 3.** Volcano plots showing  $-\log_{10} p$ -values plotted against  $\log_2$  ratio (GF-/ConvR-) in different brain regions. The horizontal line represents the  $t$ -test threshold of significance assigned ( $p < 0.05$ ). The vertical lines mark the threshold of 1.2-fold up and down-regulated proteins. Five biological replicates ( $n = 5$ ) were used.

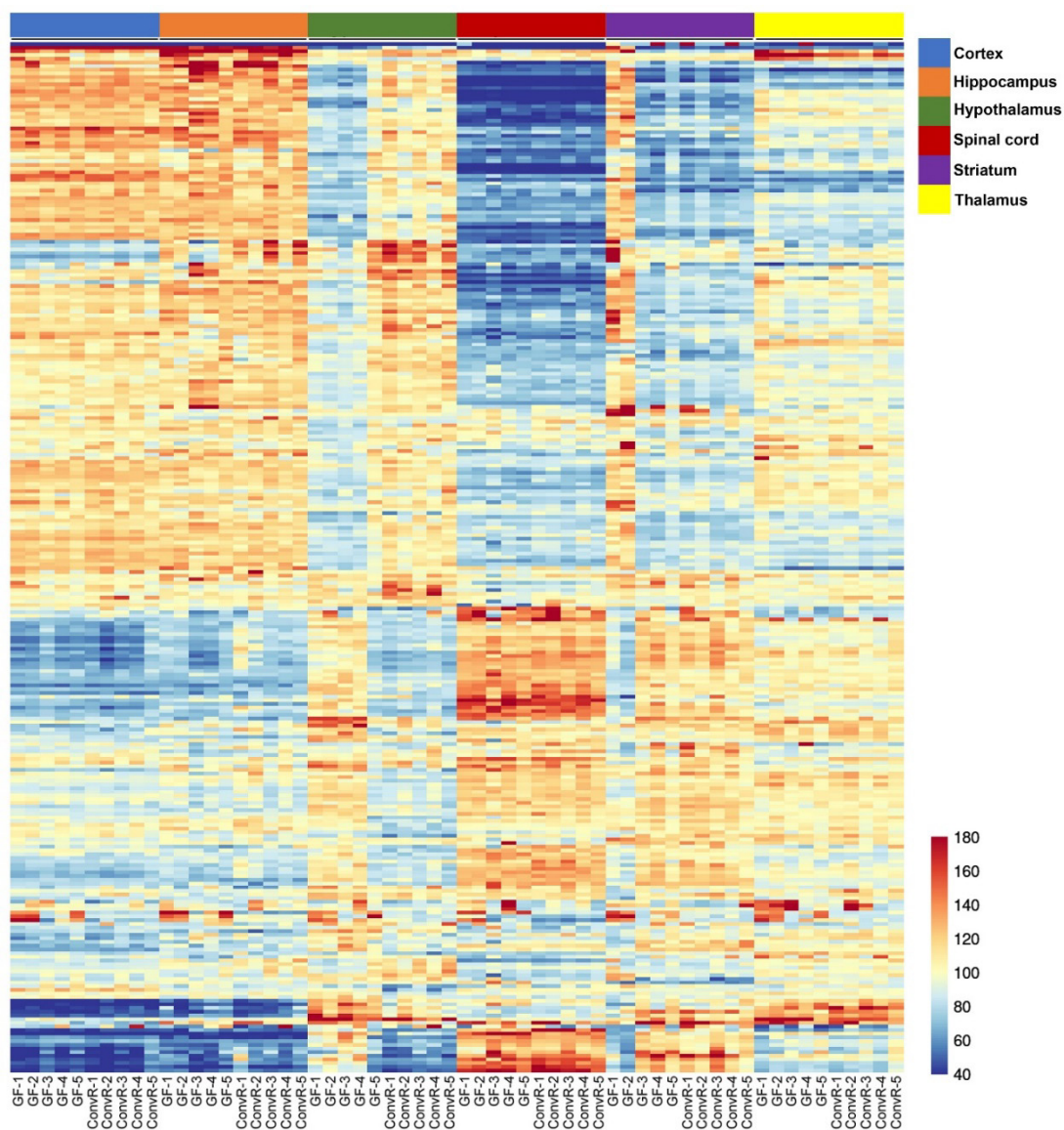
### Comparison of proteomes between six brain regions in GF- or/and ConvR-mice

Of the 2,725 proteins that could be quantified simultaneously in all six brain regions in GF-/ConvR-mice, 281 proteins were filtered for FC values greater than 1.2 in at least one brain region, and significant change ( $p < 0.05$ ) was not a necessary selection parameter. Heatmap of these 281 proteins shown in **Figure 4** and **Supplemental data 2** depicts differences in protein profiles between GF- and ConvR-mice, and between different brain regions of the same type of mice. The results show that protein differences between brain regions are greater than that between GF and ConvR mice, which demonstrates again the present strategy is capable of comparatively analyzing protein levels from biological samples with multiple variables. The

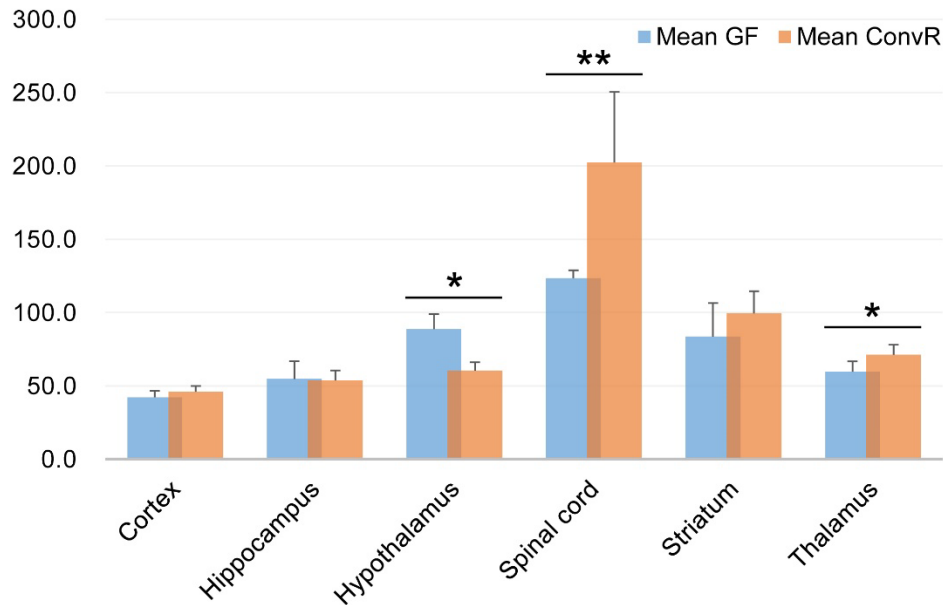
Cortex and Hippocampus regions showed similar profiles, while the Spinal cord and Striatum regions showed similar profiles. However, the protein profiles of the Hypothalamic differed from all other brain regions, and there were significant differences between the GF- and ConvR- groups within the Hypothalamic region. This suggests that protein abundance and functions in the Hypothalamus are strongly influenced by gut microbiome interventions, but not in other brain regions.

Furthermore, we found 23 proteins exhibit significantly ( $p < 0.05$ ) different expression level between GF- and ConvR-mice in at least one brain region (**Supplemental data 3**), with FC > 1.2 was not a necessary selection parameter. These proteins were detected in all 60 samples, reflecting differences not only between brain regions of the same type of mice, but also between the same brain regions of GF- and ConvR-groups. Taking Glial fibrillary acidic protein (Gfap, P03995) as an example (**Figure 5**), in the Hypothalamus Gfap level was significantly higher in GF- than that in ConvR-mice ( $p < 0.01$ ), while in the Spinal cord and Thalamus it was significantly higher in ConvR- than that in GF-mice ( $p < 0.05$ ,  $p < 0.05$ ). In both GF- and ConvR-mice, the Cortex Gfap level was significantly lower than that in the Hypothalamus ( $p < 0.01$ ), Spinal cord ( $p < 0.01$ ), Striatum ( $p < 0.01$ ) and Thalamus ( $p < 0.01$ ).

Next, we looked deeper into the protein levels in different groups or regions, as well as the functions of these representative proteins, which were of particular interest in this work. We found that Gfap level was highest in the Spinal cord in both GF- and ConvR-mice. Consistent to our results, Yang et al. also reported previously that as an intermediate filament (IF) III protein, Gfap is uniquely found in astrocytes of the central nervous system and is highly expressed in the brain, especially in the Spinal cord<sup>23</sup>.



**Figure 4.** Heatmap of 281 selected proteins from six brain regions.



**Figure 5.** The relative abundance of representative protein Glial fibrillary acidic protein (Gfap, P03995) in different brain regions from GF- and ConvR-mice. The vertical coordinate represents the relative amount of normalized protein, with BRIDGE's value of 100.

In addition, sodium- and chloride-dependent GABA transporter 3 (GAT3, P31650) showed relatively higher levels in the Hypothalamus, Spinal cord, Striatum and Thalamus than the Cortex and Hippocampus. GAT3 plays an important role in regulating extracellular GABA levels in the brain, and it is preferentially distributed in astrocytes<sup>24-25</sup>. GABA metabolism is particularly mediated by GAT3 uptake and is utilized to support astrocyte glutamine synthesis<sup>26</sup>. At the molecular level, it was found that attenuating astrocyte  $\text{Ca}^{2+}$  signaling increased GAT3 functional expression and protein levels<sup>27</sup>. Neurogranin (Nrgn, P60761) is a small protein usually expressed in pyramidal cells of the Hippocampus and Cortex, which has been shown to be involved in synaptic plasticity, synaptic regeneration and long-term potentiation mediated by calcium- and calmodulin-signaling pathways<sup>28</sup>. In this work, Nrgn showed significantly higher level in the Cortex and Hippocampus than the other regions.

Synaptotagmin-2 (Syt2, P46097) was identified as the major  $\text{Ca}^{2+}$  sensor at GABAergic basket cell (BC) to Purkinje cell (PC) synapses, and selective use of Syt2 as release sensor at BC-PC synapses ensured rapid and efficient feedforward inhibition in cerebellar microcircuits<sup>29</sup>. In this work, the relative level of Syt2 is higher in the Spinal cord and Striatum than in other brain regions, and the level of Syt2 is higher in the GF-Hypothalamus than in the ConvR-Hypothalamus. We also found in our experiments that a  $\text{Zn}^{2+}$  uptake transporter, ZIP12, was identified in all six regions. ZIP12 has been reported to play a critical role in neuronal development and neurulation and is highly expressed in both the human brain and the mouse nervous system<sup>30</sup>. Calcium/calmodulin-dependent protein kinase type II (Camk2, P11798) showed significantly higher abundance in the Cortex and Hippocampus, whereas in the Hypothalamus and Striatum, the Camk2a level was much lower. It has been reported that the activity and subcellular localization of Camk2 are critical for synaptic plasticity and learning-memory<sup>31</sup>.

### **KEGG pathway enrichment analysis**

To further investigate proteins that were exclusively present in different brain regions, KEGG pathway enrichment analysis was used to unveil pathways or functions they are closely associated with (**Supplemental data 4**). As shown in **Figure 2(A)**, 182, 122, 181, 186, 127 and 130 proteins were detected exclusively in the Cortex, Hippocampus, Hypothalamus, Spinal cord, Striatum and Thalamus, respectively. KEGG pathway analysis suggests these proteins (182) in the Cortex are involved in glycan degradation, cell cycle, sphingolipid metabolism and adhesion junctions. Proteins (122) exclusively in Hippocampus are involved in pathways including circadian entrainment, glycerophospholipid metabolism, cAMP signaling pathway,

cholinergic synapse and calcium signaling pathway. Proteins (181) unique to Hypothalamus are involved in circadian rhythm, thyroid hormone signaling pathway, GnRH secretion, ubiquitin-mediated proteolysis and sphingolipid signaling pathways. Proteins (186) in the Spinal cord region are involved in pathways including peroxisome, vascular smooth muscle contraction, circadian rhythm, tight junction, cholinergic synapse, long-term depression, fatty acid degradation, sphingolipid metabolism, neuroactive ligand-receptor interaction and calcium signaling pathways. Proteins exclusively present in the Striatum (127) and Thalamus (130) are involved in metabolic, GnRH secretion and ribosome, peroxisome pathways, respectively. Collectively, the results revealed that proteins exclusively present in different brain regions are responsible for corresponding different physiological functions. In addition, biological process, cellular component and molecular function enrichment analysis are also shown in **Supplemental data 4**.

To explore the differences in protein levels of each brain region in different gut microbiome environments and the possible pathways involved, KEGG enrichment analysis was performed for proteins with  $FC > 1.2$  between GF- and ConvR-groups. The results showed that proteins with  $FC > 1.2$  in the Cortex (28) and Spinal cord (50) were not involved in any significantly enriched pathway. Proteins with  $FC > 1.2$  in the Hippocampus (52) were involved in Dopaminergic synapse, cAMP signaling pathway and GABAergic synapse. Proteins (335) in the Hypothalamus were involved in synapse pathways (Glutamatergic, Dopaminergic, Cholinergic and GABAergic synapse), signaling pathways (Calcium, cAMP, cGMP-PKG, GnRH, Wnt, Phospholipase D, etc.) and long-term dementia, circadian rhythm or long-term depression related to memory and learning. Proteins (152) in the Striatum were also involved

in related synapse and signaling pathways. In the Thalamus, 37 proteins were involved in neuroactive ligand-receptor interaction pathways.

The KEGG pathway enrichment analysis of 281 proteins in heatmap also suggested that these proteins were involved in synapse pathways such as glutamatergic, dopaminergic, cholinergic, GABAergic and serotonergic synapses, and in signaling pathways including cAMP, oxytocin, cGMP-PKG, Calcium, GnRH, MAPK, Wnt, etc. In addition, long-term depression, long-term potentiation, circadian rhythms and synaptic vesicle cycles, which are closely related to learning, memory, and neurotransmitter release, were also enriched, similar to our previous reports<sup>32-34</sup>. Several neuropeptide precursors, such as ProSAAS, secretogranin-1, secretogranin-2, secretogranin-3, neuroendocrine protein 7B2, chromogranin-A and phosphatidylethanolamine-binding protein 1, were detected in each region, but only ProSAAS was significantly altered between GF- and ConvR-mice in the Hypothalamus region ( $P < 0.01$ ). Neuropeptides from ProSAAS, such as big SAAS, little SAAS, PEN and big LEN have been reported to be associated with food intake and circadian rhythms<sup>17, 34</sup>.

In summary, we found that the effects on central nervous system due to the gut microbiome environment changes were most obvious in the Hypothalamus and Striatum, followed by the Hippocampus and Thalamus, while the effects on the Cortex and Spinal cord were not significant. The effects on the Hypothalamus and Striatum were mainly related to various signal synapse and transmission, learning and memory, circadian rhythm, etc.

## CONCLUSIONS

In this work, a method for comprehensive analysis of proteomes in the six brain regions from

mice raised under different gut microbiota environments was developed. 10-plex DiLeu tags were used to label five GF- and five ConvR-mice, and the 11<sup>th</sup> DiLeu channel was used as the “BRIDGE” sample, which was applied for normalizing samples from different brain regions. With the present strategy, 5,656 proteins were identified from simultaneous quantitative analysis of 60 brain samples in six different regions. This comprehensive proteomics analysis describes the molecular influences on brain tissues under different gut microbiome environments, and therefore can provide new insights into the physiological functions and pathological relevance of the interactions between gut microbiome and central nervous system.

#### **Associated contents**

#### **Supporting information**

The Supporting Information is available free of charge at the ACS Publications website:

<https://pubs.acs.org>

**Supplemental data 1.** All 5,945 identified proteins in six brain regions.

**Supplemental data 2.** Relative abundance of 281 selected proteins from six brain regions.

**Supplemental data 3.** 23 representative proteins from six brain regions, which exhibit significantly ( $p < 0.05$ ) different expression level between GF- and ConvR-mice in at least one brain region.

**Supplemental data 4.** KEGG, biological process, cellular component and molecular function enrichment analysis.

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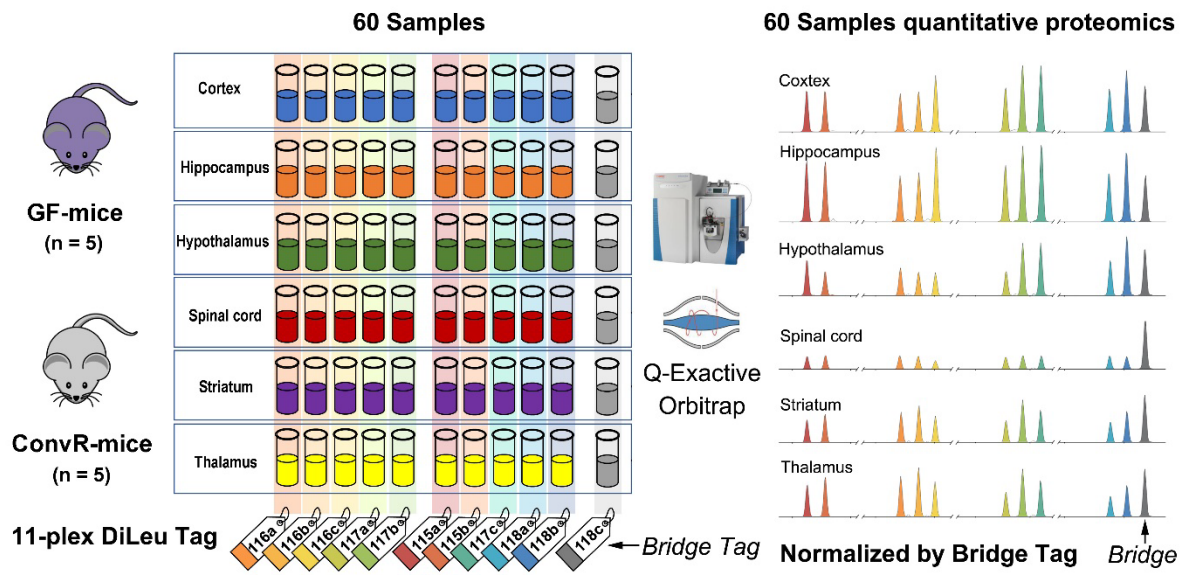
## **Notes:**

The authors declare no competing financial interest.

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## Table of Content Graphic



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