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Comprehensive analysis of the gut microbiome and post-translational modifications elucidates the route involved in microbiota-host interactions

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ABSTRACT

The gut microbiome interacts with the host to maintain body homeostasis, with gut microbial dysbiosis implicated in many diseases. However, the underlying mechanisms of gut microbe regulation of host behavior and brain functions remain unclear. This study aimed to elucidate the influence of gut microbiota on brain functions via post-translational modification mechanisms in the presence or absence of bacteria without any stimulation. We conducted succinylome analysis of hippocampal proteins in germ-free (GF) and specific pathogen-free (SPF) mice and metagenomic analysis of feces from SPF mice. These results were integrated with previously reported hippocampal acetylome and phosphorylome data from the same batch of mice. Subsequent bioinformatics analyses revealed 584 succinylation sites on 455 proteins, including 54 up-regulated succinylation sites on 91 proteins and 99 down-regulated sites on 51 proteins in the GF mice compared to the SPF mice. We constructed a panoramic map of gut microbiota-regulated succinylation, acetylation, and phosphorylation, and identified cross-talk and relative independence between the different types of post-translational modifications in modulating complicated intracellular pathways. Pearson correlation analysis indicated that 13 taxa, predominantly belonging to the Bacteroidetes phylum, were correlated with the biological functions of post-translational modifications. Positive correlations between these taxa and succinylation and negative correlations between these taxa and acetylation were identified in the modulation of intracellular pathways. This study highlights the hippocampal physiological changes induced by the absence of gut microbiota, and proteomic quantification of succinylation, phosphorylation, and acetylation, contributing to our understanding of the role of the gut microbiome in brain function and behavioral phenotypes.

Keywords: Gut microbiota; Hippocampal protein; Post-translational modifications; Succinylation; Acetylation; Phosphorylation

INTRODUCTION

Microbial communities in natural and host-associated environments are large and delicate ecosystems comprised of trillions of bacteria, archaea, fungi, microbial eukaryotes, and viruses (Marcelino et al., 2020). These communities play significant roles in human health and disease (Fan and & Pedersen, 2021). As the largest microbial community in the human body, the gut microbiota not only regulates host metabolism, neurological signaling, immune responses, gut hormone secretion, and gastrointestinal function, but also

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protects against infection (Fan & Pedersen, 2021; Wei et al., 2019).

The conventional brain-centric perspective proposed that the peripheral system was unidirectionally driven by the central nervous system (CNS), with the influence of peripheral metabolic homeostasis and immune balance on the CNS largely neglected or simply overlooked (Morais et al., 2021). However, in more recent years, research has begun to recognize that microorganisms can influence the brain through their ability to produce and modify many metabolic, immunological, and neurochemical factors in the gut, which ultimately impact the nervous system (Lukić et al., 2019). Although the exact mechanisms involved remain unclear, accumulating evidence has demonstrated that gut microbiota dysbiosis can adversely affect behavior and neural biochemistry through the microbiota-gut-brain axis (Cryan & Dinan, 2012; Fung et al., 2017; Long-Smith et al., 2020). In previous studies, we showed that the gut microbiota modulates gene transcription and protein expression (Chen et al., 2019; Liu et al., 2020; Rao et al., 2021; Zeng et al., 2016; Zhou et al., 2020) and drives metabolic alterations in the brains of rodents and nonhuman primates (Li et al., 2018a; Wang et al., 2020a; Zheng et al., 2021). The gut microbiota varies with lifecycle and exerts profound influences on the host (Yang et al., 2022), with bacterial infection found to precipitate stress-induced memory dysfunction (Gareau et al., 2011). Research has also shown that the maternal gut microbiome can modulate fetal neurodevelopment and thalamocortical axon genesis in mice (Vuong et al., 2020) and maternal sleep deprivation can induce gut microbial dysbiosis (Yao et al., 2022). However, two crucial questions remain to be addressed. First, how does the gut microbiota influence host behavior and brain function via the gut-brain axis? Second, which key taxa are responsible for mediating the interactions between the microbiota and host.

Germ-free (GF) animals, which lack microbiota and are free from bacterial contamination, serve as suitable models for exploring host responses to disordered, normal, or absent flora. GF mice have been an invaluable tool for investigating microbiota-host interactions (Cryan & Dinan, 2012; Cryan et al., 2019; Foster & McVey Neufeld, 2013; Long-Smith et al., 2020; Zheng et al., 2019) and exhibit cognitive deficits, altered sociability, and modifications in neurogenesis, neurotransmitter, synapse, and neuronal activity-related genes (Cryan et al., 2019; Luczynski et al., 2016; Schretter, 2020; Wang et al., 2020a). We have also previously identified decreased depressive and anxiety-like behavior in GF mice accompanied by molecular and neurochemical changes (Chen et al., 2017b, 2019; Zeng et al., 2016; Zheng et al., 2016). However, although earlier studies have confirmed the role of gut microbiota in regulating host behavior and brain function, the precise mechanisms remain largely unknown.

Proteins are pivotal biomolecules necessary for mediating diverse biological functions. Most cellular proteins require further modification to perform their specific functions (Fang et al., 2018). Post-translational modifications (PTMs), with the addition of covalent bonds or enzymatic modification on amino acid side chains or at the C- or N-termini of proteins (Gao et al., 2019), play crucial roles in modulating protein function by regulating their activity, localization, and interaction. Protein PTMs are critical regulators of cellular function and activity, including responses to environmental stimuli (Zhu et al., 2022). In our previous work using GF mice, we found that an absence of microbiota induces global dysregulation in protein phosphorylation and acetylation (Wang et al., 2020b; Yu et al., 2021), while gut microbiota dysbiosis induces changes in phosphorylation, acetylation, and succinylation in hippocampal proteins (Liu et al., 2021; Wang et al., 2020b). These findings suggest that PTMs may help reveal the mechanisms underlying gut microbiota regulation of brain function and behavior. However, little is known regarding the extent to which PTMs are influenced by gut microbiota and which type of PTM is more susceptible to gut microbiota.

In the current study, proteomic quantification of lysine succinylation in the hippocampus of GF and specific pathogen-free (SPF) mice was performed using tandem-mass-tag (TMT) labeling followed by high-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The resulting succinylome was integrated with our previously published acetylome and phosphorylome data from the same batch of GF and SPF mice (Wang et al., 2020b; Yu et al., 2021) and a panoramic map of succinylation, phosphorylation, and acetylation of hippocampal proteins in GF mice was constructed. To further explore how the gut microbiota regulates this panoramic map of PTMs, metagenomics sequencing was used to analyze gut microbiota composition in SPF mice, with results then integrated with the PTM data to reveal microbial/PTM interactions.

MATERIALS AND METHODS

Animals

Eight-week-old GF and SPF male Kunming mice (*n*=8 per group, 30–40 g) were provided by the Laboratory Animal Center at the Third Military Medical University (Chongqing, China), as previously described (Wang et al., 2020b). The GF mice were housed in sterile conditions using flexifilm gnotobiotic isolators and fed with irradiated food and sterile water. Stool samples from the GF mice were collected weekly and examined by polymerase chain reaction (PCR) and culture-based methods to verify GF status. The SPF mice were housed in a standard animal facility. All mice were maintained under consistent environmental conditions: noise less than 60 dB, 12 h light/dark cycle (lights on at 0800–2000h), temperature of 22–23 °C, and relative humidity of 50%±5%. All regulations regarding the use of research animals were observed and all protocols were approved by the Ethics Committee of Chongqing Medical University (Chongqing, China; Approval No. 2017013).

Sample preparation, TMT labeling, and high-performance liquid chromatography (HPLC) fractionation

Sample preparation, TMT labeling, and HPLC fractionation procedure were performed as described previously (Liu et al., 2021; Wang et al., 2020b). Briefly, GF and SPF mice were anesthetized and perfused before sacrifice by decapitation. The hippocampus was immediately dissected on ice, frozen in liquid nitrogen, and stored at −80 °C. Proteins were extracted and their concentrations were determined using a BCA kit, followed by trypsin digestion. Each peptide sample was labeled with the respective TMT reagent (based on the manufacturer's protocols, Thermo Scientific, USA) and pooled samples were desalted with Strata X C18 SPE column (Phenomenex, USA), dried by vacuum centrifugation, and fractionated by high pH reverse-phase HPLC (Agilent

300Extend C18 column, 5 μm particles, 4.6 mm ID, 250 mm length, see Supplemental Materials for further details).

Affinity enrichment of Ksucc-modified peptides and LC-MS/MS analysis

Ksucc-modified peptides were enriched by dissolving tryptic peptides in NETN buffer (100 mmol/L NaCl, 1 mmol/L EDTA, 50 mmol/L Tris-HCl, 0.5% NP-40, pH 8.0), followed by incubation with pre-washed succinylated antibody beads at 4 °C overnight with gentle shaking. The beads were washed four times with NETN buffer and twice with ddH_2O, with the bound peptides then eluted with 0.1% trifluoroacetic acid (TFA). Eluted fractions were combined and vacuum-dried. The peptides were desalted with C18 ZipTips (Millipore, USA), according to the manufacturer's instructions, and LC-MS/MS was subsequently performed (see Supplemental Materials for further details).

Conserved sequence analysis

Conserved sequences comprising amino acids at specific locations within modify-21-mers (encompassing 10 amino acids both upstream and downstream of the modification site) in all protein sequences were analyzed using the motif-x algorithm. The minimal number of peptides occurring in one motif "occurrence" was 20, and the significance threshold value was 0.0000001. Motif-based clustering was visualized as a heatmap. Icelogo (https://iomics.ugent.be/icelogoserver/) was used to examine the properties of amino acids surrounding the modification sites using *t*-test (P<0.05), and the "choosing scoring system" was set to "percentage difference".

Secondary structure prediction and cellular localization of Ksucc proteomes

The sequence profiles of target proteins were used to predict secondary structures of succinylation, acetylation, and phosphorylation using NetSurfP-2.0 (Klausen et al., 2019) (http://www.cbs.dtu.dk/services/NetSurfP-2.0/). Predictions with a minimum probability of 0.5 were then selected. The Wilcoxon's test was used to calculate *P*-values and Wolfpsort software (https://www.wolfpsort.hgc.jp/) was used to predict subcellular localization of succinylated, acetylated, and phosphorylated proteins in the mouse hippocampus.

Functional annotation and pathway analysis

Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using Omicsbean (http://www.omicsbean.cn/). *P*-values were calculated using Fisher's exact test (hypergeometric test), with P<0.05 indicating significance.

Prediction of phosphorylation kinase-substrate and kinase activities

Site-specific kinase-substrate relationships (ssKSRs) were predicted using iGPS1.0 software and the GPS2.0 algorithm5. Protein-protein interaction (PPI) information served as the contextual factor to filter out potential false-positives. The parameter "Interaction" was set to "Exp./String" and a "medium" threshold was selected. Kinase activity was predicted by gene set enrichment analysis (GSEA) and normalized enrichment scores (NES) were considered to represent kinase activity scores. Kinases predicted to have positive or negative activity and significantly differentially expressed phosphorylation sites were used to construct a kinase-substrate regulatory network.

PPI network analysis and construction of multiple PTM regulatory networks

PPI networks were constructed to identify succinylated, acetylated, and phosphorylated proteins using the STRING database (http://string-db.org/), with visualization performed using Cytoscape (v.3.9.1).

Metagenomic analysis of fecal samples

Total genomic DNA was extracted from fecal samples of SPF mice (8 weeks old, n=6), according to the manufacturer's instructions. The DNA was fragmented to an average size of approximately 400 bp for paired-end library construction using NEXTFLEX® Rapid DNA-Seq (Bioo Scientific, USA). Paired-end sequencing was performed on the Illumina NovaSeq 6000 platform (Illumina, USA) at Majorbio Bio-Pharm Technology (China). Data were analyzed using the Majorbio Cloud Platform (www.majorbio.com). Open reading frames (ORFs) from each assembled contig were predicted by Prodigal/MetaGene (http://metagene.cb.k.u-tokyo.ac.jp/, see Supplementary Materials for further details).

Correlation between gut microbiome and PTMs

Gene taxonomy was identified from the NR (RefSeq non-redundant proteins) database in the bacterial domain. The operational taxonomic unit (OTU) table was collapsed at the species level and filtered for rare taxa by only including taxa with an average relative abundance of at least 0.1% in all samples (Dayama et al., 2020). In total, 134 taxa (species) were identified. Overlapping KEGG pathways shared between modified proteins and genes corresponding to microbiota were screened. Pearson correlation analysis of overlapping pathways and the 134 taxa was performed.

Statistical analysis

All statistical analyses (*t*-test, Fisher's exact test, Pearson correlation analysis) were conducted using IBM SPSS software (v.21.0; SPSS, USA), with P<0.05 indicating statistical significance.

RESULTS

Global landscape of microbial regulation of succinylation, acetylation, and phosphorylation proteomes in mouse hippocampus

Succinylome and metagenomic analyses were integrated with previously reported lysine acetylome and protein phosphorylome data from the hippocampus of GF mice (Wang et al., 2020b; Yu et al., 2021). We aimed to reveal the role of the gut microbiota in PTM regulation from both physiological and biological perspectives. The study design and workflow are shown in Figure 1. In total, 584 succinylation sites were found on 455 proteins with a distribution of mass error near zero (<0.02 Da), indicating mass accuracy appropriate for MS data (Figure 2A). Most peptides ranged between seven and 20 amino acids in length (Figure 2B), suggesting satisfactory sample preparation. Between 1–9 Ksuc sites were found per peptide, with most containing only one (Figure 2C). A fold-change cutoff of >1.2 or <0.83 and a *P*-value threshold of <0.05 were used to select differentially modified sites based on volcano plots, including 54 up-regulated succinylation sites on 91 proteins and 99 down-regulated succinylation sites on 51 proteins in GF mice compared to SPF mice (Figure 2D; Supplementary Table S1). Most succinylated protein sites enriched in oxidative phosphorylation were down-regulated,

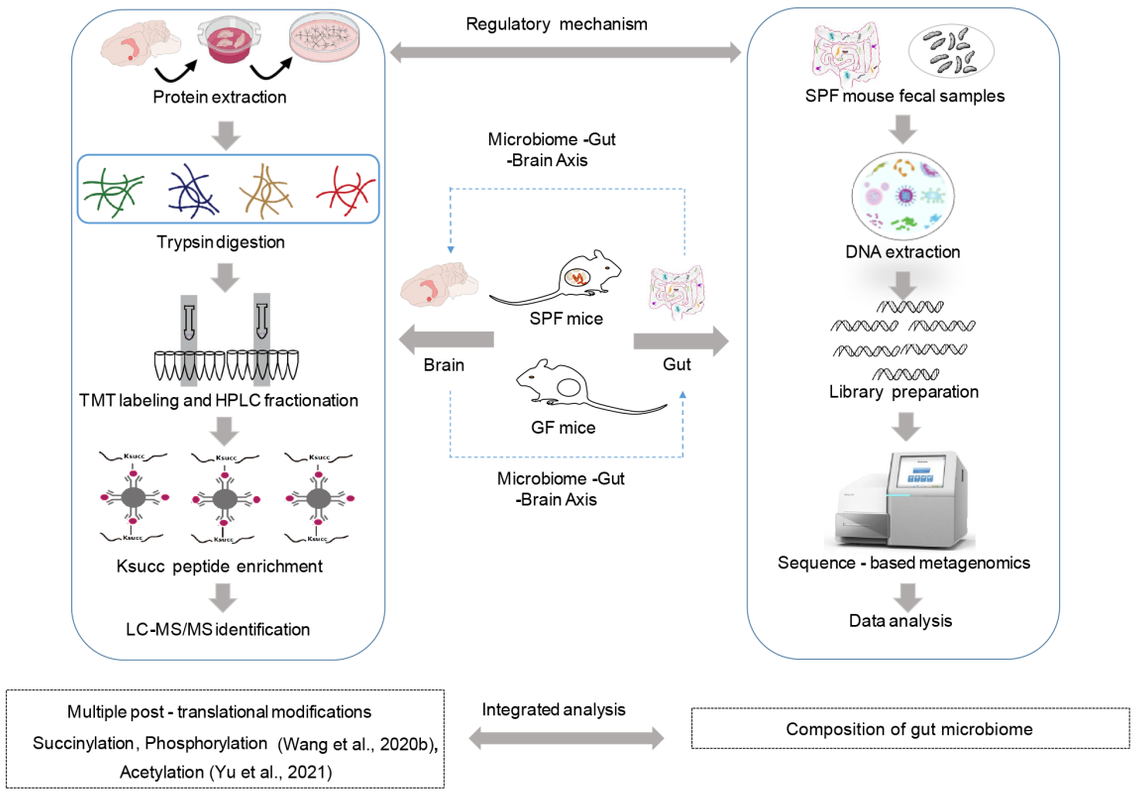

Figure 1 Experimental workflow

GF, germ-free; SPF, specific pathogen-free; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

including Atp5a1, Atp6v1e1, Atp5h, and Ndufv3.

Acetylation and phosphorylation were more susceptible to regulation by the gut microbiome than succinylation in GF mice. In total, 986 acetylation sites on 543 acetylated proteins and 6 945 sites on 2 370 phosphorylated proteins were identified in GF mice (Wang et al., 2020b) (Figure 2F), including 106 up-regulated phosphorylation sites on 88 proteins, 503 down-regulated phosphorylation sites on 349 proteins (Supplementary Table S2), 138 up-regulated acetylation sites on 104 proteins, and 43 down-regulated acetylation sites on 32 proteins (Supplementary Table S3). Overlap analysis identified 84 proteins co-modified by phosphorylation, succinylation, and acetylation (Figure 2E), with four proteins differentially expressed between GF and SPF mice (Figure 2F), including two up-regulated (Figure 2G) and one down-regulated (Figure 2H). Interactions between succinylation and acetylation were analyzed, revealing competitive binding at lysine residues. Of these, 63 sites were modified by both succinylation and acetylation (Figure 2I), eight of which were differentially modified between GF and SPF mice (Figure 2J). These findings provide a comprehensive overview of multiple PTMs in hippocampal proteins regulated by gut microbiota.

Motif analysis of microbial modified sites

Using the motif-x algorithm, conserved sequences surrounding the modified sites were analyzed. A preference for Pro and Lys residues at the +1 position, Glu residues between the −3 and −1 position and at the +2 position, and Asp residues at the +2 position relative to the Ksucc sites was observed (Figure 3A). Thus, *EE*Ksucc* motifs were the most conserved sequences for Ksucc sites (Figure 3B; Supplementary Table S4). The Ksucc*E motif has been identified as the most conserved sequence for Ksucc sites regulated by gut microbiota dysbiosis in a mouse model of depression (Liu et al., 2021). This suggests different structural preferences for different subtypes of gut microbiota dysbiosis.

Prediction of secondary structures for different PTMs

To further ascertain whether the function and structure of proteins were influenced by gut microbiota under physiological conditions, analyses were conducted on proteins showing differential PTMs. In total, 40.36% of Ksucc sites were found in ordered regions (34.12% in α-helices and 6.24% in β-strands), while 59.63% of sites were located in disordered regions (Figure 3C). Previous studies identified Kac sites primarily in disordered regions (62.1% in coils), with a smaller proportion in ordered regions (31.63% in α-helices and 6.26% in β-strands) (Wang et al., 2020b; Yu et al., 2021) (Supplementary Figure S1). Our results also showed 83.32% of serine phosphorylation sites were located in disordered regions, while only 16.66% of sites were located in ordered regions (11.68% in α-helices and 4.98% in β-strands); 81.96% of threonine phosphorylation sites were located in disordered regions, while 18.02% of sites were located in ordered regions (11.91% in α-helices and 6.11% in β-strands) (Supplementary Figure S2). Gut microbiota-modified succinylation sites were more prevalent in α-helices, while acetylation or phosphorylation sites were more prevalent in disordered coils. Surface accessibility analysis identified fewer phosphorylation, acetylation, or succinylation sites compared with corresponding non-modified residues. Thus, phosphorylation, acetylation, and succinylation produced by gut microbiota under physiological conditions may influence the functions and surface properties of proteins.

Subcellular localization of different PTMs

Subcellular localization predictions for differentially expressed

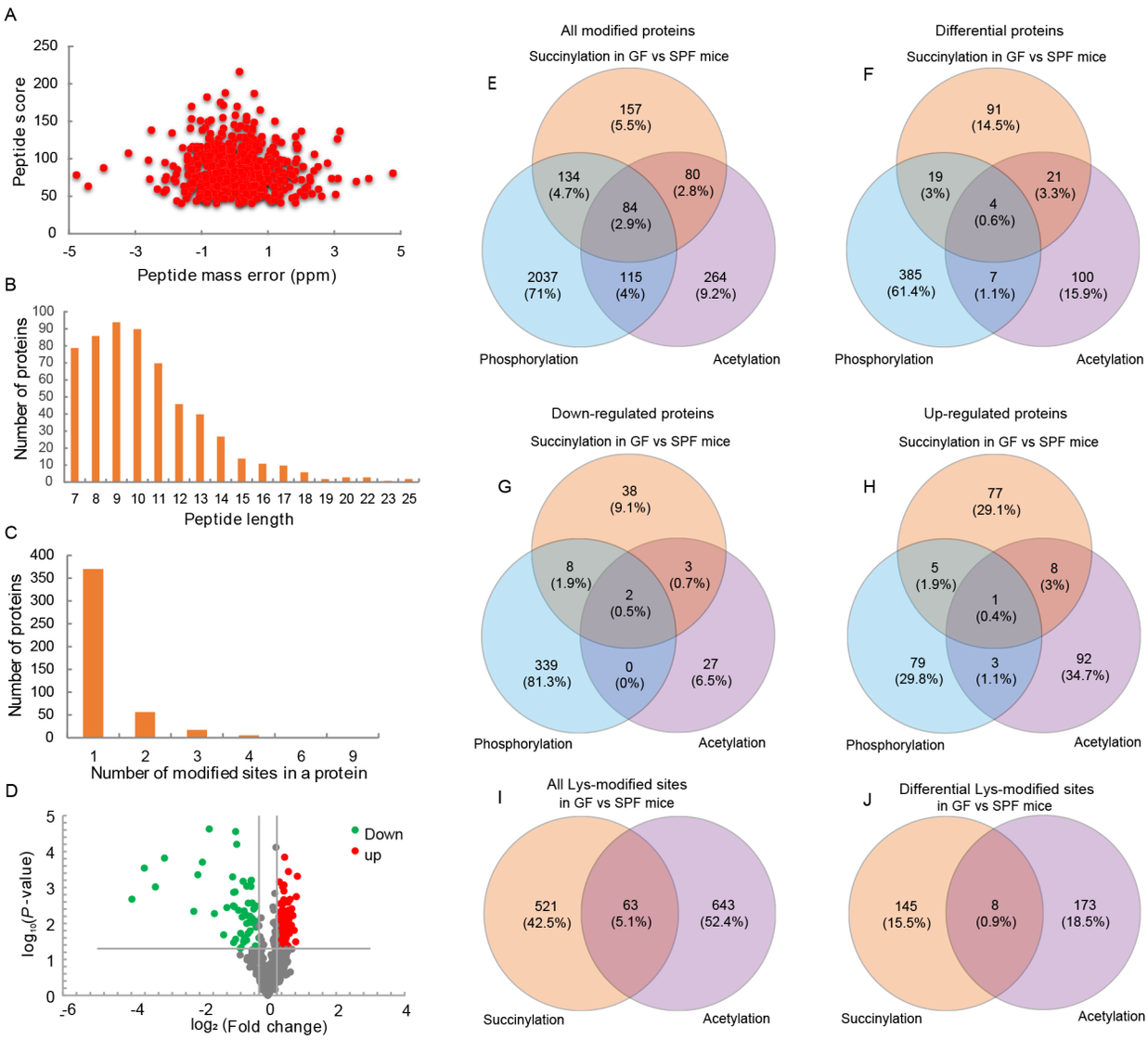

Figure 2 Phosphorylation, acetylation, and succinylation proteome profiles in hippocampal tissue from GF mice

A: Distribution of mass error of all succinylated peptides. B: Distribution of peptide lengths of all succinylated peptides. C: Number of succinylation sites within each modified protein. D: Volcano plot of differentially expressed succinylated proteins in GF vs. SPF mice. E: Venn diagram showing total number of common and distinct hippocampal proteins modified by phosphorylation, acetylation, and succinylation in GF vs. SPF mice. F: Venn diagram showing number of common and distinct differentially expressed hippocampal proteins modified by phosphorylation, acetylation, and succinylation in GF vs. SPF mice. G: Venn diagram showing number of common and distinct down-regulated hippocampal proteins modified by phosphorylation, acetylation, and succinylation in GF vs. SPF mice. H: Venn diagram showing number of common and distinct up-regulated hippocampal proteins modified by phosphorylation, acetylation, and succinylation in GF vs. SPF mice. I: Venn diagram showing overlap between Lys acetylation and succinylation sites in GF vs. SPF mice. J: Venn diagram showing overlap between differentially expressed Lys acetylation and succinylation sites in GF vs. SPF mice.

succinylated, acetylated, and phosphorylated proteins showed that Ksucc modifications were abundant in the cytoplasm (84 proteins, 63%), plasma membrane (47 proteins, 35%), nucleus (31 proteins, 23%) and mitochondria (24 proteins, 18%) (Figure 3D). Earlier studies identified gut microbiota-regulated acetylated proteins in the mitochondria (35%), cytoplasm (32%), and nucleus (Wang et al., 2020b; Yu et al., 2021). Most microbiota-regulated phosphorylated proteins were localized in the nucleus (181 proteins, 55%), plasma membrane (56 proteins, 17%), cytoplasm (49 proteins, 14%), and mitochondria (17 proteins, 5%) (Wang et al., 2020b). The patterns suggest a prominence of Ksucc modifications in cytoplasmic proteins, Kac sites in mitochondrial proteins, and phosphorylation in nuclear proteins. These findings suggest that the gut microbiota may exert regulatory effects on various PTMs across distinct cellular compartments.

Functional annotation and enrichment analysis of different PTMs

GO functional annotations were conducted for all modified proteins. The analysis produced classifications in the cell component (CC) category, biological process (BP) category (including single-organism cellular processes, cellular component organization, and biogenesis), and molecular function (MF) category (Supplementary Figure S4).

Previous studies have reported GO annotations for acetylated and phosphorylated proteins (Wang et al., 2020b; Yu et al., 2021). In our study, succinylated proteins were enriched in cytoplasmic part (80%), single-organism cellular

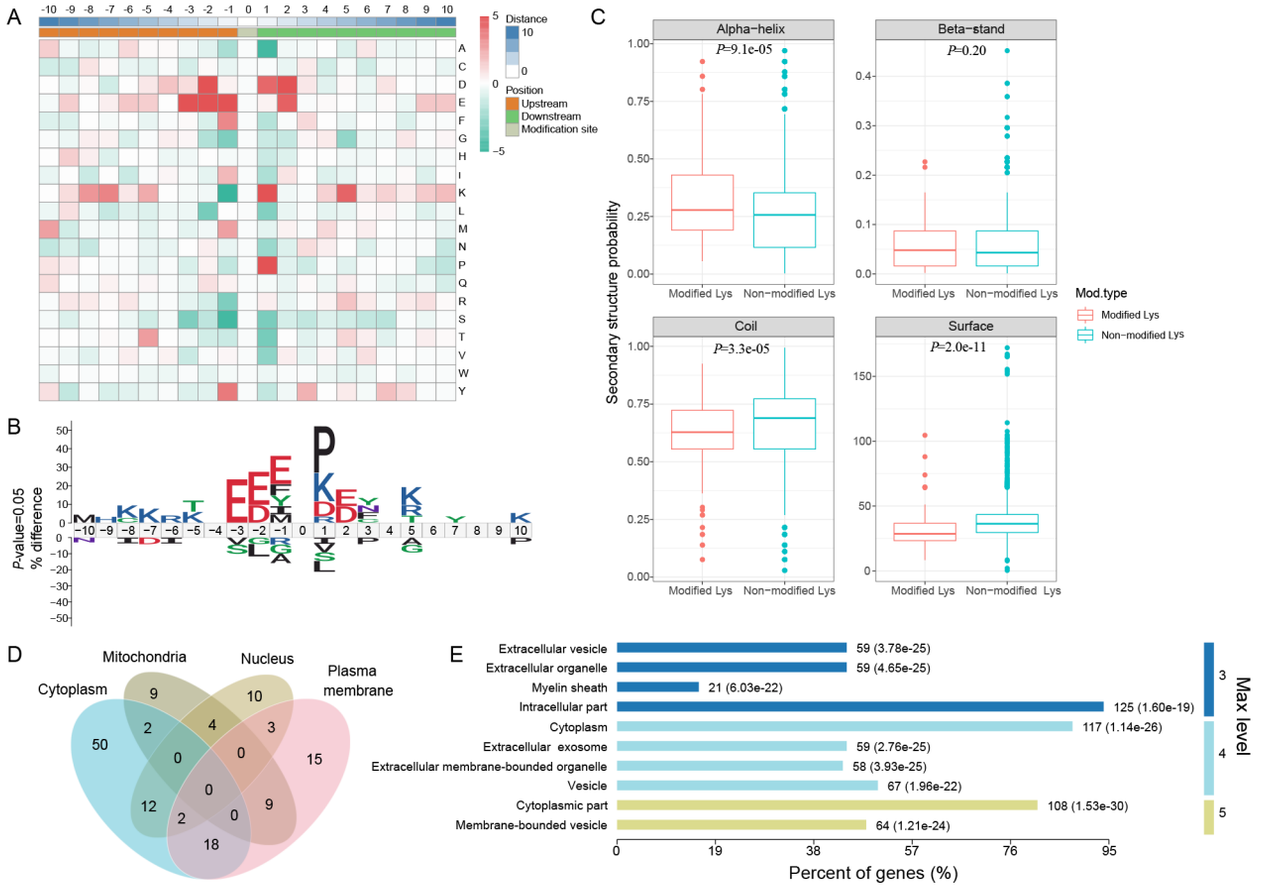

Figure 3 Properties of gut microbiota-regulated succinylation proteome

A: Heatmaps showing features of flanking sequences for all Lys succinylation sites. B: IceLogo diagram showing over-represented amino acids around succinylation site. C: Secondary structure distribution and surface accessibility prediction of significant succinylation sites. D: Venn diagram showing cellular localization of differentially succinylated proteins. E: Bar graph showing cellular component enrichment based on GO annotation analysis of differential succinylated proteins. Max level means maximal annotated level of this term in GO graph (tree) and number indicates depth of GO term level.

process (29%), and protein binding within the CC, BP, and MF categories, respectively (Supplementary Figure S5).

KEGG pathway enrichment analysis showed that succinylated proteins were primarily enriched in metabolic pathways, carbon metabolism, and oxidative phosphorylation (Supplementary Table S5), while acetylated proteins were mainly enriched in carbon metabolism, tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (Supplementary Table S6), and phosphorylated proteins were mainly enriched in glutamatergic synapse, synaptic vesicle cycle, cAMP signaling pathway, and oxytocin signaling pathways (Supplementary Table S7). These observations suggest that different PTMs play distinct roles in different biological processes, with acetylation predominantly affecting metabolic pathways and phosphorylation primarily impacting intracellular signaling pathways.

Overlap analysis revealed the shared pathways among the three PTMs, including oxytocin signaling, glucagon signaling, long-term potentiation, endocrine, and other factor-regulated calcium reabsorption, synaptic vesicle cycle, and dopaminergic synapse pathways (Figure 4). Notably, both phosphorylation and succinylation influenced the GnRH signaling, cholinergic synapse, and axon guidance pathways. Both phosphorylation and acetylation regulated the GABAergic synapse and glutamatergic synapse pathways, while succinylation and acetylation regulated the carbon metabolism and oxidative phosphorylation pathways (Supplementary Table S8).

Construction of PPI network for modified proteins

To investigate the complicated interactions between differentially modified proteins and their synergistic effects on functional pathways, a PPI network of differentially expressed succinylated, acetylated, and phosphorylated proteins was generated using high-confidence interactions (≥0.7) with STRING. Highly connected node clusters, indicative of interactions with other modified proteins, were defined using the MCODE plug-in. Four such clusters, which spanned various pathways, including oxidative phosphorylation, ribosome, PI3K-Akt signaling pathway, and TCA cycle, were identified (Figure 5). Among these pathways, oxidative phosphorylation and TCA cycle were modified by succinylation and acetylation, while ribosome and PI3K-Akt signaling pathway were modified by phosphorylation.

The energy production pathways, oxidative phosphorylation, and TCA cycle were further explored. Most oxidative phosphorylation proteins were modified by acetylation and/or succinylation, with only NADH dehydrogenase (ubiquinone) Fe-S protein 8 (Ndufs8), a core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I), modified by phosphorylation (Figure 6). TCA cycle proteins were mainly modified by acetylation, except for the pyruvate dehydrogenase complex component dihydrolipoamide

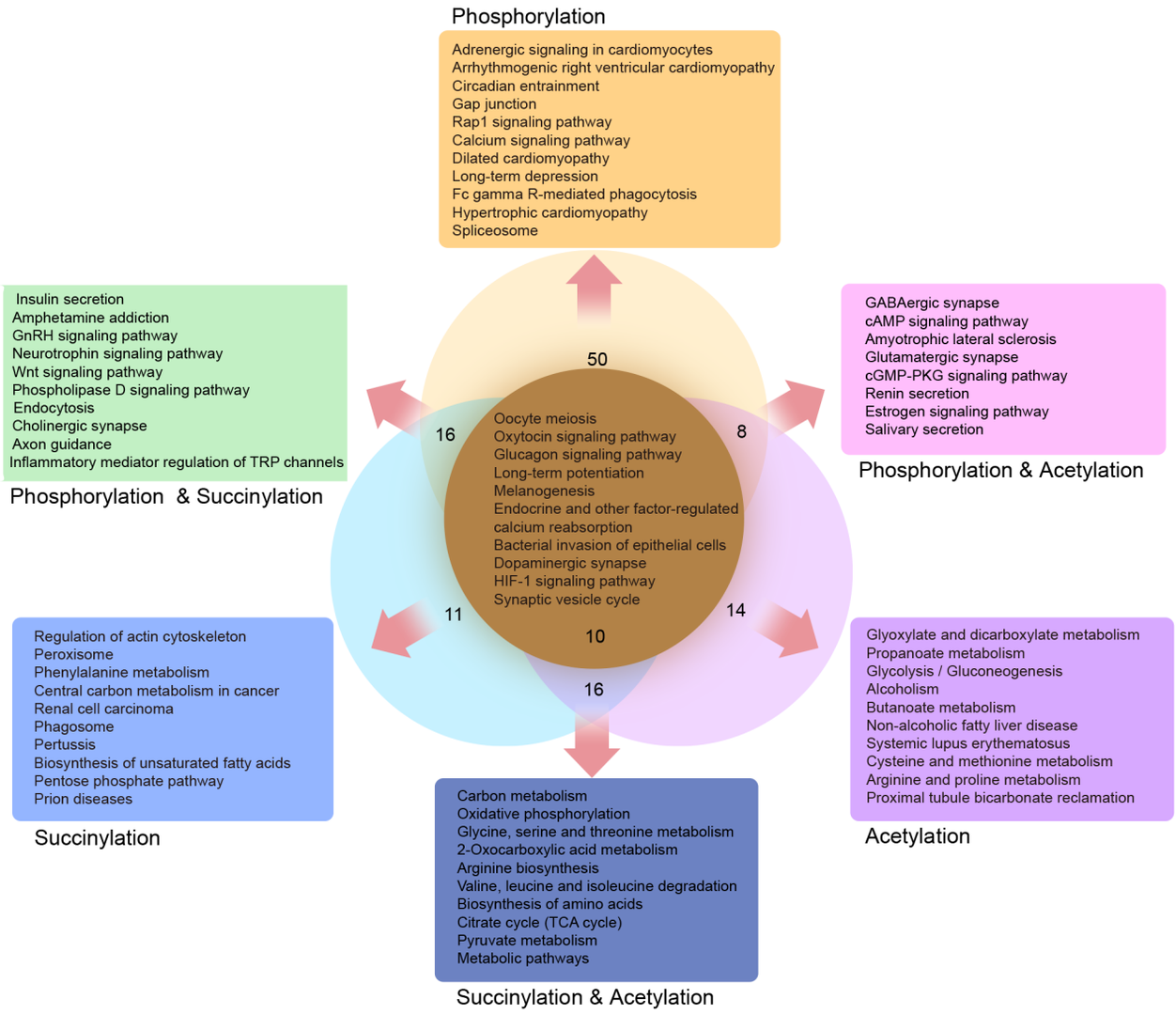

Figure 4 KEGG pathway overlap among differentially expressed phosphorylated, acetylated, and succinylated proteins

Venn diagram showing number of common and distinct proteins modified by phosphorylation, acetylation, and succinylation. Brown area indicates common KEGG pathways enriched in phosphorylated, succinylated, and acetylated proteins; blue area indicates unique KEGG pathways enriched in succinylated proteins; purple area indicates unique KEGG pathways enriched in acetylated proteins; and orange area indicates unique KEGG pathways enriched in phosphorylated proteins.

dehydrogenase (Dld), which was modified by both succinylation and acetylation. These findings highlight both cross-talk as well as specific roles for different PTMs in modulating intracellular pathways.

Comprehensive analysis of gut microbiome and PTMs

KEGG pathway analysis was conducted on gut microbial genes to reveal specifically enriched pathways (Supplementary Table S9). Further overlap analysis identified eight shared pathways between microbial genes and modified proteins, including biosynthesis of amino acids, glycolysis/gluconeogenesis, cysteine and methionine metabolism, alanine, aspartate and glutamate metabolism, pyruvate metabolism, ribosome, oxidative phosphorylation, and 2-oxocarboxylic acid metabolism. Pearson correlation analysis indicated that 13 of the 134 taxa were significantly correlated with these common pathways, which were enriched in 37 differentially expressed succinylated and acetylated proteins. These common pathways were used as a “bridge” to illustrate correlations between gut microbiota and modified proteins (Figure 7). Most taxa belonged to Bacteroidetes, Muribaculaceae_bacterium, *Bacteroides_acidifaciens*, Porphyromonadaceae_bacterium, and *Alistipes*_sp._Z76. A positive correlation between succinylation and taxa was found in these pathways, while a negative association was detected between acetylation and taxa. In the absence of gut microbiota, the GF mice showed reduced pathway activity, decreased succinylation, and increased acetylation. These findings suggest that specific taxa may play roles in modulating brain functions via PTM regulation.

DISCUSSION

The gut microbiota is associated with various biological processes essential for maintaining CNS and peripheral nervous system homeostasis. Various molecular and signaling pathways enable bidirectional communication between the gut microbiota and brain, thus establishing the microbiota-gut-brain axis (Osadchiy et al., 2019; Schoch et al., 2022). Advancements in sequencing technology have provided a powerful approach for exploring the associations between the gut microbiome and brain diseases and facilitating the

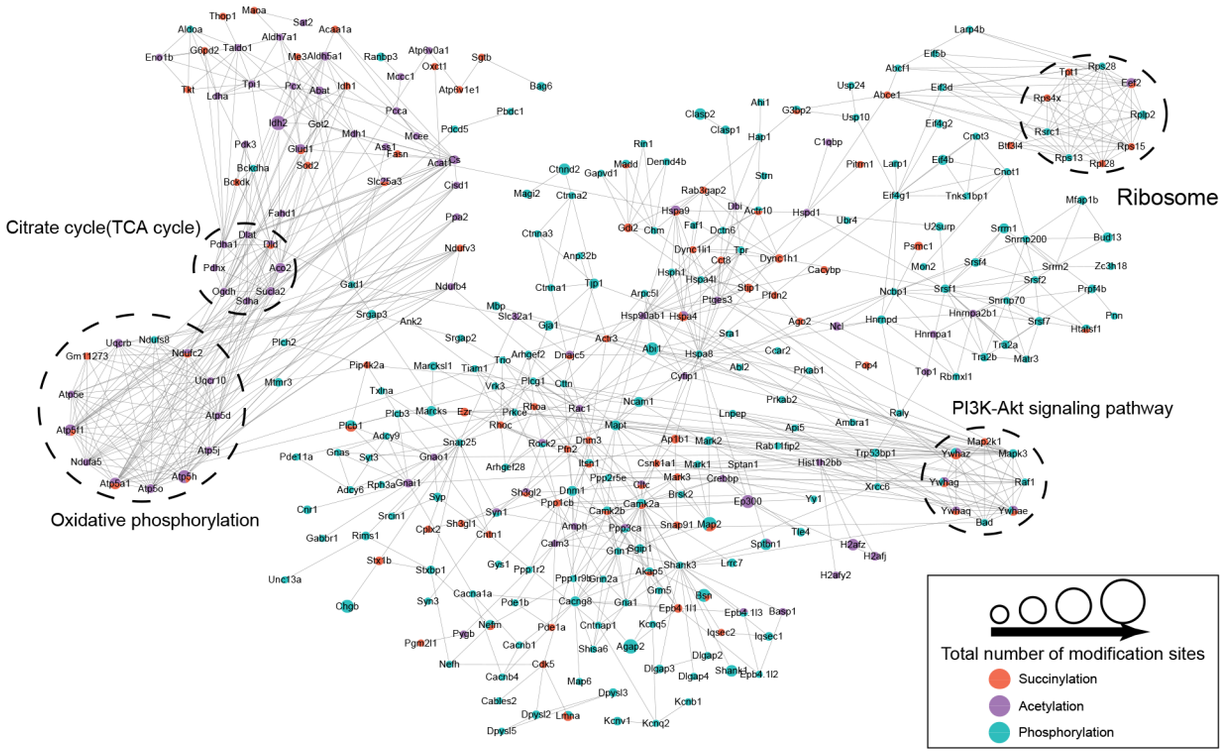

Figure 5 PPI network analysis of differentially expressed phosphorylated, acetylated, and succinylated proteins

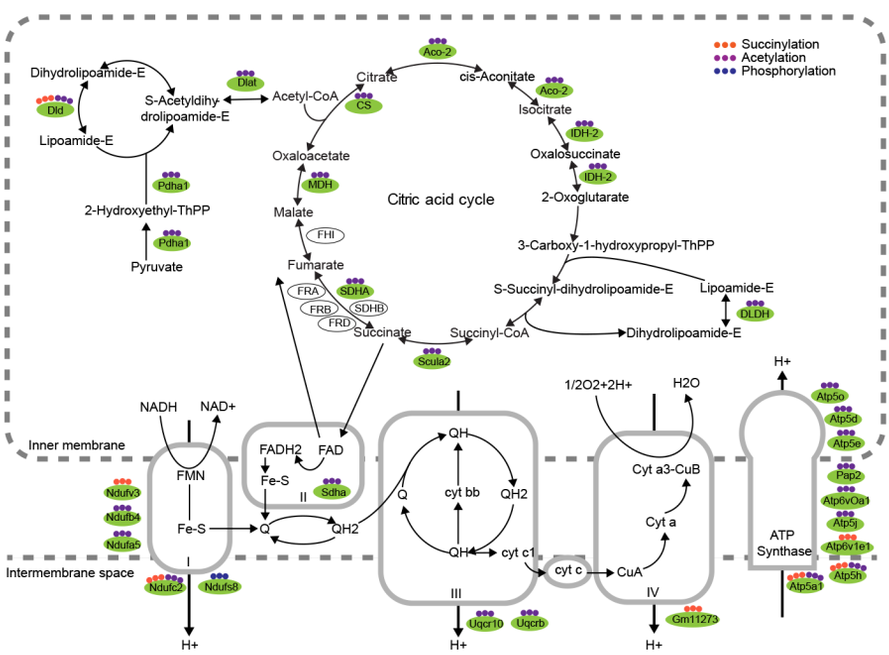

Figure 6 Details of phosphorylation, acetylation, and succinylation alterations in relative proteins involved in oxidative phosphorylation pathway

development of novel interventions targeting the gut to regulate brain function and create "gut treatments for brain diseases".

In practice, there is a pressing need to elucidate both the identity and the mechanisms through which the gut microbiota governs brain function. While previous studies have focused on changes in gut microbiota and regulatory roles during specific diseases in patients or animal models (Parker et al., 2022), structural variants in the gut microbiome during a state of disease cannot be ruled out (Zeevi et al., 2019). Microbial strains may exhibit different genomic structures under different pathological and physiological states (Durrant & Bhatt, 2019; Wang et al., 2021); this complexity adds to the challenge of developing gut microbiota-based interventions for brain diseases using gut microbiota obtained from healthy donors. Thus, exploring the composition of gut microbiota and their interactions with the brain under physiological conditions is vital, but remains relatively understudied.

PTMs are sensitive to environmental stimuli (Zhu et al., 2022). By analyzing global PTM profiles, one can potentially

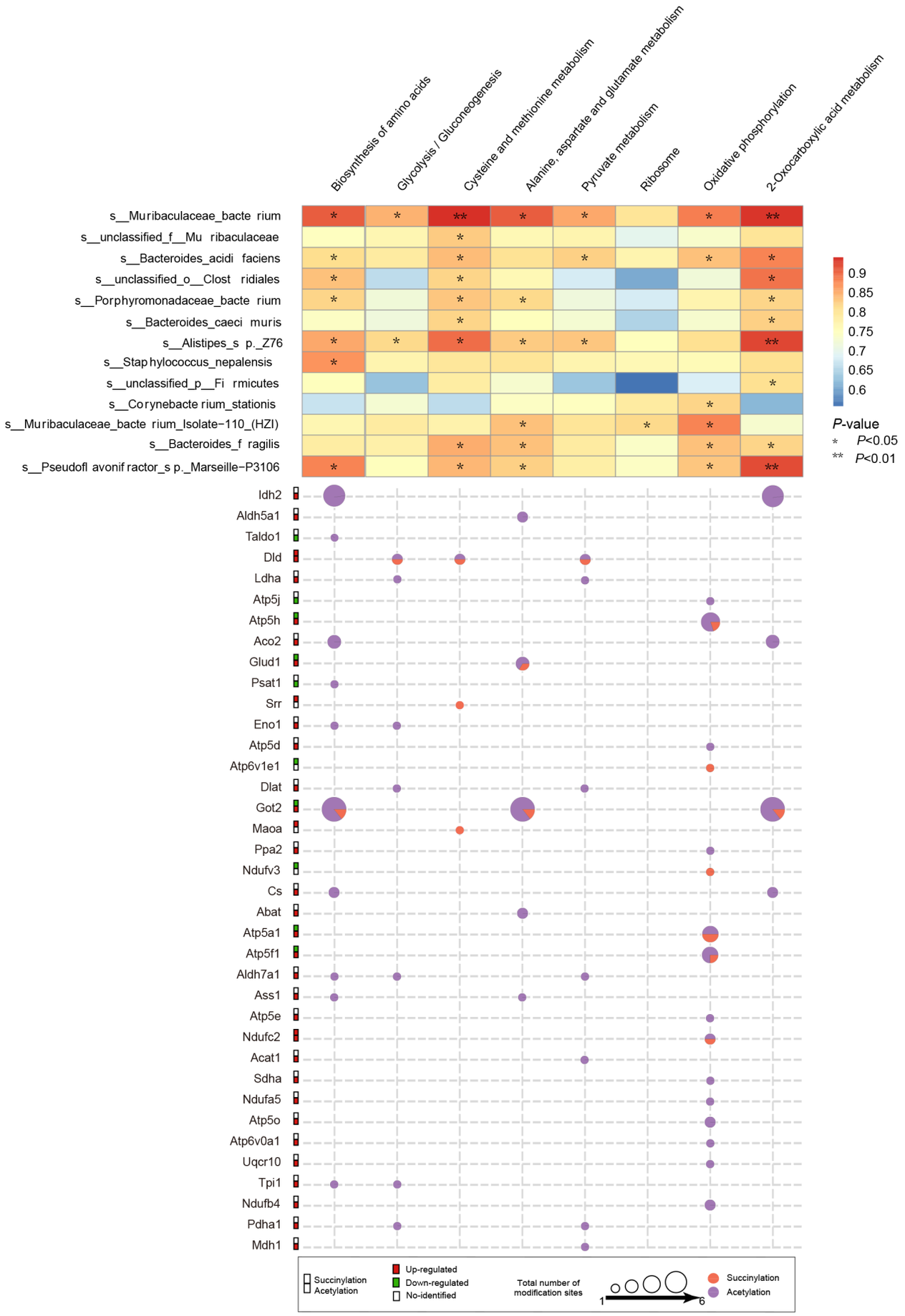

Figure 7 Integration analysis of gut microbiota and protein post-translational modifications

elucidate the mechanisms by which the gut microbiota regulates brain function and behavioral phenotypes. Here, we generated a panoramic map of the microbiota-regulated succinylome, acetylome, and phosphorylome in the hippocampus of GF and SPF mice to explore the regulatory role of the gut microbiota in modulating PTMs and better understand the gut-brain axis from a PTM perspective.

Our results showed that acetylation and phosphorylation were more susceptible to gut microbiome effects than succinylation and together constituted the vast majority of PTMs, as shown in previous research (Ebert et al., 2022). Phosphorylation, catalyzed by more than 800 protein kinases, is one of the most pervasive PTM in eukaryotic cells, affecting up to one third of the proteome (Humphrey et al., 2015; Pieroni et al., 2020) and showing involvement in almost every cellular process (Humphrey et al., 2015). Acetylation is involved in physiology and disease, including DNA damage repair, gene transcription, autophagy, and metabolism (Narita et al., 2019). In earlier work, we noted that the deacetylases, NAD-dependent deacetylase sirtuin 1 (SIRT1) and SIRT2, were down-regulated in the hippocampus of GF mice compared to SPF mice (Liu et al., 2021), while SIRT5, which regulates de-succinylation, remained unaffected. This may account for the observed dominance of acetylation over succinylation in GF mice.

In addition, previous research has shown that succinylation regulated by the gut microbiota tended to occur near acidic amino acids in cytoplasmic proteins, while acetylation was prominent in mitochondrial proteins and phosphorylation was dominant in nuclear proteins, underlining the specific biological processes tied to each PTM type (Liu et al., 2021). We also identified distinct pathways enriched by different PTMs. Notably, peroxisome and biosynthesis of unsaturated fatty acids were associated with succinylated proteins. In contrast, glycolysis/gluconeogenesis and cysteine and methionine metabolism with acetylated proteins and calcium signaling pathway, Fc gamma R-mediated phagocytosis, and spliceosome were associated with phosphorylated proteins. Cross-talk between different PTM types was also evident in the nervous system. For example, neurotransmitter release, including cholinergic, GABAergic, glutamatergic, and dopaminergic systems, were co-modified by at least two types of PTMs. Neurotransmitters play many roles in the CNS (Wu et al., 2022), and disruptions in their function are hallmark features of neuropsychiatric diseases (Snyder & Ferris, 2000) such as depression (Duman et al., 2019). Neurotransmitters also mediate bidirectional gut-brain communication (Lai et al., 2021; Mittal et al., 2017). Thus, the gut microbiota appears to regulate brain function via cross-talk of PTMs affecting proteins in various biological processes.

In contrast to phosphorylation, both acetylation and succinylation target lysine, suggesting an increased potential for cross-talk between them. Our results showed that many proteins involved in metabolic pathways, such as oxidative phosphorylation and the TCA cycle, are co-modified by acetylation and succinylation. Oxidative phosphorylation and the TCA cycle are pivotal processes in living organisms, representing the main mechanisms initially providing energy for neuronal activity (Hall et al., 2012; Wang et al., 2019). Oxidative phosphorylation comprises five multi-subunit complexes, designated CI–CV. Previous studies have identified the occurrence of phosphorylation on CI–CV, acetylation on CI, CII, and CV, and glycosylation on CI, CII, and CV (Chen et al., 2008; Guo et al., 2017; Koopman et al., 2013). In the present study, the absence of gut microbiota precipitated increased acetylation levels of complexes, particularly adenosine triphosphate (ATP) synthase subunits (CV), such as Atp5d, Atp5h, Atp5o, Atp5a1, ATP5f1, Atp5e, as well as decreased activity and mitochondrial respiration (Rahman et al., 2014; Vassilopoulos et al., 2014). Impairment of ATP synthase increases the NAD+/NADH ratio, subsequently reducing protein succinylation (Chen et al., 2017a) and regulating Ndufv3, Ndufb4, Ndufa5, Ndufc2, and Ndufs8. This may explain the divergent patterns of acetylation and succinylation observed for certain related proteins, like Atp5h, Atp5a1, and Atp5f1, in our results. Key rate-limiting enzymes of the TCA cycle, including citrate synthase (Krug et al., 2019), isocitrate dehydrogenase (IDH), and α-ketoglutarate dehydrogenase (α-KGDH), were enriched with acetylation and succinylation sites, underscoring the regulatory role of gut microbiota in energy metabolism.

These energy-producing pathways are also co-modified by acetylation and succinylation in mice introduced with gut microbiota from patients diagnosed with major depressive disorder (Liu et al., 2021), accompanied by a down-regulation in the expression of pathway-relevant genes (Qi et al., 2020). However, even for the same pathway, the source of gut microbiota, whether from pathological and physiological conditions, can differentially regulate host health. Our previous work revealed differences in the modification of complex subunits in the oxidative respiratory chain and enzymes in the TCA cycle when modified by gut microbiota from patients versus healthy individuals (Liu et al., 2021; Wang et al., 2020b; Yu et al., 2021). Furthermore, gut microbiota-produced butyrate can induce the phosphorylation of Ser37 and Tyr105, acetylation of Lys305, and succinylation of Lys311 on pyruvate kinase isoform 2 (PKM2), a key enzyme in pyruvate metabolism, to suppress the proliferation of colorectal cancer cells (Bettaieb et al., 2013; Li et al., 2018b; Lv et al., 2011; Wang et al., 2017; Yang et al., 2012). Depression-associated taxa can induce hyper-succinylation of Lys224 and Lys247 of PKM, without affecting acetylation and phosphorylation (Liu et al., 2021). Notably, these changes are absent under normal physiological states. Even when focusing on the same modification site of a specific protein, discrepancies emerge between pathological and physiological states. For example, depression-related gut microbiota dysbiosis can reduce acetylation of Lys321 on the Cs protein (Liu et al., 2021), whereas an absence of gut microbiota can enhance its acetylation. These findings suggest that different taxa may target different aspects for brain function regulation, and the mechanisms underlying microbiota-host interactions should be explored in further studies.

Bacteroidetes and Firmicutes are the dominant phyla in the gut (The Human Microbiome Project Consortium, 2012), with Bacteroidetes comprising over half of the gut microbiome (Qin et al., 2010; The Human Microbiome Project Consortium, 2012) and playing a key role in providing their host with energy (Johnson et al., 2017). In this study, we found a significant correlation between Bacteroidetes bacteria and metabolic pathways related to energy metabolism, including Muribaculaceae_bacterium, *Bacteroides_acidifaciens*, and *Alistipes*_sp._Z76, which participate in glycolysis/gluconeogenesis and pyruvate metabolism processes. Many energy-related metabolic pathways are influenced by taxa from various phyla. For example, Bacteroidetes and

Firmicutes exhibit synergistic effects on oxidative phosphorylation, while *Bifidobacterium* modulates metabolic processes and enhances mitochondrial activity in gut regulatory T cells (Tregs) (Sun et al., 2020), suggesting that a group of gut microorganisms, rather than just a single microorganism, modulates physiological processes essential for health (Miyauchi et al., 2020). Negative correlations between acetylation and taxa suggest that such taxa may reduce the activity of enzymes involved in energy metabolism by promoting the acetylation of relevant proteins (Rahman et al., 2014; Vassilopoulos et al., 2014). Our study highlights several potential targets associated with these taxa of interest, indicating possible gut-brain axis mechanisms through which the gut microbiota affects host health by modifying protein function.

This study has several limitations to mention. First, to avoid potential confounders arising from the influence of sex hormones, only male mice were used. Second, while the SPF mice used for PTM analysis and for metagenomes were the same age and housed in the same environmental conditions, they were not from the same batch. Third, although our results are promising and were obtained using highly reliable methods, experimental validation has not yet been performed. Further studies involving different brain regions and including female mice are necessary to understand how the gut microbiome influences host health via PTMs.

CONCLUSIONS

In conclusion, we used GF and SPF mice to determine proteomic changes in the hippocampus, particularly quantification of succinylation, phosphorylation, and acetylation, arising from the absence of gut microbiota. Our objective was to clarify the role of gut microbiota on brain functionality via PTM dynamics under physiological conditions. We further constructed a panoramic map of gut microbiota-regulated succinylation, acetylation, and phosphorylation and identified cross-talk/relative independence between different types of PTMs in modulating intracellular pathways. Notably, we identified positive correlations between succinylation and taxa and negative correlations between acetylation and taxa, with implications for the modulation of intracellular pathways. These findings should help advance our understanding of how the gut microbiome regulates brain function and behavioral phenotypes by modulating PTMs under physiological conditions.

DATA AVAILABILITY

The raw metagenomics sequencing reads can be downloaded from the NCBI (PRJNA1003305), China National Center for Bioinformation (PRJCA018916), and Science Data Bank databases (DOI: 10.57760/sciencedb.j00139.00058).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

H.W., P.J., and P.X. conceived and designed the study, H.Y.W. and L.X.L. wrote the manuscript, X.Y.C. and Y.D.Z. analyzed the data, W.W.L., W.X.L., L.W., and X.L.M. revised the manuscript. All authors read and approved the final version of the manuscript.

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