

Regulation of Gut Microbiota Disrupts the Glucocorticoid Receptor Pathway and Inflammation-related Pathways in the Mouse Hippocampus

Xuechen Rao^{1,2†}, Lanxiang Liu^{2,3†}, Haiyang Wang^{2,4†}, Ying Yu^{2†}, Wenxia Li², Tingjia Chai², Wei Zhou², Ping Ji⁴, Jinlin Song⁴, Hong Wei^{5*} and Peng Xie^{2,6*}

¹College of Biomedical Engineering, Chongqing Medical University, Chongqing 400016, ²NHC Key Laboratory of Diagnosis and Treatment on Brain Functional Diseases, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, ³Department of Neurology, Yongchuan Hospital of Chongqing Medical University, Chongqing 402160, ⁴College of Stomatology and Affiliated Stomatological Hospital of Chongqing Medical University, Chongqing 401147, ⁵Department of Laboratory Animal Science, College of Basic Medical Sciences, Third Military Medical University, Chongqing 400038, ⁶Department of Neurology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China

An increasing number of studies have recently indicated the important effects of gut microbes on various functions of the central nervous system. However, the underlying mechanisms by which gut microbiota regulate brain functions and behavioral phenotypes remain largely unknown. We therefore used isobaric tags for relative and absolute quantitation (iTRAQ)-based quantitative proteomic analysis to obtain proteomic profiles of the hippocampus in germ-free (GF), colonized GF and specific pathogen-free (SPF) mice. We then integrated the resulting proteomic data with previously reported mRNA microarray data, to further explore the effects of gut microbes on host brain functions. We identified that 61 proteins were upregulated and 242 proteins were downregulated in GF mice compared with SPF mice. Of these, 124 proteins were significantly restored following gut microbiota colonization. Bioinformatic analysis of these significant proteins indicated that the glucocorticoid receptor signaling pathway and inflammation-related pathways were the most enriched disrupted pathways. This study provides new insights into the pathological mechanisms of gut microbiota-regulated diseases.

Key words: Germ-free, Gut microbiota, Proteomics, Hippocampus, Glucocorticoid receptor pathway, Inflammation-related pathways

INTRODUCTION

The human gastrointestinal tract contains approximately one trillion microorganisms, most of which are bacteria, belonging to more than 1,000 bacterial species [1]. Recent studies have sug-

gested that the gut microbiota can have dramatic effects on the development and function of the host brain [2]. Moreover, accumulating studies have revealed bidirectional communication between the gut microbiota and the brain, and have proposed a novel conceptual model of a “microbiota–gut–brain axis” [3-5]. Through this axis, the gut and the brain may communicate via multiple mechanisms, including immune responses, the vagus nerve, short-chain fatty acids, endocrine signaling, and tryptophan metabolism [2, 6]. Many studies have found a high prevalence of mood disorders, such as depression and/or anxiety, in patients with gastrointestinal disorders including irritable bowel syndrome and inflammatory bowel disease [7, 8]. In our previous studies, we demonstrated the effects of the gut microbiota on brain function. For example, gut

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*To whom correspondence should be addressed.

Peng Xie, TEL: 86-023-68485490, FAX: 86-023-68485111
e-mail: xiepeng@cqmu.edu.cn

Hong Wei, TEL: 86-023-68752051, FAX: 86-023-68752051
e-mail: weihong63528@163.com

†These authors contributed equally to this work.

microbiota may modulate depression- and anxiety-like behaviors in mice through the hypothalamic–pituitary–adrenal (HPA) axis [9], and alter hippocampal RNA regulation and lipid metabolism [10, 11]. Furthermore, colonization of gut microbiota early in life might facilitate neurodevelopment via protein kinase C–cAMP response element-binding protein (CREB) signaling [12]. Sex-specific differences in gut microbiota composition in patients with major depressive disorder (MDD) were identified [13]. In general, disturbances in gut–brain axis may be highly correlated with pathophysiology of mood disorders.

Germ-free (GF) mice, who are born and raised without any exposure to microbes, allow us to better understand the effects of gut microbes on the host brain. Our previous studies have found that the GF mice exhibited antidepressant-like and anti-anxiety-like behaviors when comparing with SPF mice [10, 12, 14, 15]. Although colonized GF (CGF) mice—a complementary approach to explore the potential effects of the gut microbiota on brain function—showed no normalization of behavioral changes, the expression levels of numerous lncRNAs, miRNAs and mRNAs were significantly restored [11, 16, 17]. In addition, when transplanting fecal samples from patients with major depressive disorder and healthy controls to GF mice, respectively, the “depression microbiota” received mice showed significant depressive- and anxiety-like behaviors compared with “healthy microbiota” received mice [15], suggesting a causative role of gut microbiota dysbiosis in the onset of depression. However, the mechanism of gut microbiota drives anxiety- and depression-like behaviors is complex and still unclear. In the present study, we therefore focused our attention on the hippocampus, which is a crucial brain region that has for decades been recognized as critical in the pathogenesis of depression [18].

Isobaric tags for relative and absolute quantitation (iTRAQ) is a relatively practical protein detection method with high accuracy, sensitivity, and reproducibility. This method can be used to identify differentially expressed proteins in biological samples under different pathological conditions that are potential to uncover the underlying mechanisms of diseases. Using this approach, we have previously identified numerous differentially expressed proteins in the olfactory bulb of the gut microbiota-remodeled mice model of depression [19], and in the hippocampus of the chronic social defeat stress mice model, and identified the potential targets for depression treatment [20]. To investigate the underlying mechanisms by which gut microbes influence the depression-related brain function and behaviors, we used iTRAQ-based quantitative proteomic analysis to obtain proteomic profiles of the hippocampus of GF, CGF, and SPF mice, followed by the bioinformatics analysis. In addition, we integrated our current proteomic data with previ-

ously reported mRNA microarray data from GF vs. SPF mice [14] to further explore the underlying mechanisms.

MATERIALS AND METHODS

Animals

Nine-week-old male GF and SPF ($n=10$ per group) BALB/c mice were provided by the animal experimental center of the Third Military Medical University (Chongqing, China). GF mice were fed in flexible film plastic isolators. All conditions were kept sterile and verified to meet the Chinese Laboratory Animal Microbiological Standards and Monitoring (GB 14922.2-2011) by testing the feces and skin of the GF mice. To successfully colonize some of the GF mice, we moved 5-week-old male GF mice ($n=10$) into cages with bedding materials and fecal pellets from SPF mice for 3 weeks, thus generating CGF mice. In previous studies, this method has been demonstrated to effectively restore the normal microbiota [21]. The CGF, GF, and SPF mice were housed in a 12-hour light/dark environment to fully simulate a circadian rhythm (lights on from 07:30~19:30), with a constant temperature of 21~22°C and a relative humidity of 55%±5%. Both water and autoclaved standard mice chow of the same formulation were available *ad libitum* for all animals. This study was approved by the Ethics Committee of Chongqing Medical University and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978).

Sample collection and preparation

The mice were anesthetized with 10% chloral hydrate (400 mg/kg) followed by perfusion, and killed in random order by cervical dislocation [9]. The brain was quickly removed from the cranium, and the hippocampus was immediately stripped out and frozen in liquid nitrogen, and then stored at -80°C until used in the assay. To avoid any circadian impact on the results, all mouse brains were collected at the same time of day (between 09:00 and 11:00) [22]. For processing, samples were first subjected to liquid nitrogen grinding. Subsequently, each sample was dissolved and adequately homogenized in 1 ml of SDT buffer. All of the sample lysates were then sonicated on an ice-water mixture at 80 W for 7 s, followed by a pause for 8 s, which was repeated six times. After ultrasonic extraction, the lysates were centrifuged at 14,000×g for 30 minutes at 4°C. Next, the supernatant was decanted and passed through a 0.22 µm filter. A bicinchoninic acid assay was used to determine protein concentrations. For the iTRAQ labeling and strong cation exchange (SCX) fraction, samples were pooled within each treatment group to minimize inaccuracies caused by individual differences.

iTRAQ labeling and SCX fraction

This part of the experiment was performed in a similar manner to that of previous studies [23, 24]. In line with the manufacturer's instructions, pooled samples were extracted and digested, and the tryptic peptides were labeled using the iTRAQ Reagent-8PLEX Multiplex Kit. The samples were labeled with different iTRAQ tags. Subsequently, the labeled peptides from each group were mixed at equal ratios, and SCX chromatography was then used for further fractionation.

Liquid chromatography (LC)-mass spectrometry (MS)/MS and data analysis

The SCX fractions were subjected to LC-MS analysis and separated using a Thermo Easy-nLC binary buffer system. The composition of the buffer was buffer A (0.1% formic acid) and buffer B (consisting of 84% acetonitrile and 0.1% formic acid). The samples were first loaded into reversed-phase columns (20 mm×100 μm, 5 μm-C18). Next, analytical columns (75 μm×100 mm, 3 μm-C18) were used to separate the mixed peptides at a flow rate of 300 nL/minute for 60 minutes. The specific settings of the liquid-phase linear gradient was set as follows: minutes 0~55 with buffer B from 0% to 55%; minutes 55~57 with buffer B from 55% to 100%; and then buffer B maintained at 100% during minutes 57~60. Subsequently, each specimen was isolated by capillary high-performance LC and applied to a Q-Exactive mass spectrometer for protein identification. The specific detection method was as follows. The positive ion and parent ion were scanned in the range of 300 to 1,800 m/z. The first resolution of MS was 70,000 at m/z 200, and the secondary resolution of MS was 17,500 at m/z 200. The maximum ion injection times were fixed at 10 and 60 ms, respectively. After a full MS scan, the MS data required for further analysis were extracted from the top 10 most abundant precursor ions. The underfill ratio was 0.1% and the normalized collision energy was 30 eV. The protein identification and quantitative analysis methods were performed using Mascot 2.2 and Proteome Discoverer 1.4 software based on the UniProt (uniprot_Mouse_77271_20150609) database. The Mascot search parameters were set as reported in a previous study [24].

Bioinformatics analysis

For further analysis, the significantly differentially expressed proteins were selected using the following thresholds: $p < 0.05$, fold change > 1.2 or < 0.83 . To explore the functions of the differentially expressed proteins, we first used Gene Ontology (GO) enrichment analysis, which was performed using OmicsBean [25]. This describes the properties of genes and gene products in an organism over three aspects: biological process (BP), cellular component

(CC), and molecular function (MF). We used Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA; www.ingenuity.com) to identify significant dysfunctional canonical pathways and networks to obtain a better understanding of the pathophysiological effects of differentially expressed proteins on host [26]. We then mapped the differentially expressed proteins onto the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (v11.0; https://string-db.org/) to construct the protein-protein interactions (PPI) network. The PPI network was visualized by Cytoscape (v3.7.2), and the hub proteins were analyzed using the CytoHubba plug-in [27-29]. In addition, we integrated our present proteomic data with previously reported mRNA microarray data [14] to reveal the mechanisms by which the gut microbiota regulates brain functions and behavioral phenotypes.

RESULTS

Comparative proteome analysis

We used the well-established iTRAQ-based strategy to investigate proteomic changes in the hippocampus of GF, SPF, and CGF mice. The quantified protein sequence information was extracted in batches from the UniProtKB database. A total of 5,514 proteins were acquired. Based on the thresholds described above, numerous proteins were identified to be differentially expressed in GF vs. SPF, CGF vs. SPF, and CGF vs. GF comparisons, respectively. We performed an overlapping analysis of the differentially expressed proteins among these three comparison groups, and found that 42 common proteins were differentially expressed across the three comparisons (Fig. 1A). However, only 4 upregulated proteins (Fig. 1B) and 17 downregulated proteins (Fig. 1C) were identified with concordant direction of fold change across the three comparisons.

Functional analysis of differentially expressed proteins in the absence of gut microbiota

In present study, we found that the absence of gut microbiota results in significant changes in hippocampal protein expression in GF mice. Of the acquired 5,514 proteins, a total of 61 proteins were identified upregulation and 242 were downregulation in the GF mice compared with the SPF mice (Supplementary Table 1). To explore the biological functions of these significant proteins, we firstly performed GO annotations for these differentially expressed proteins. As a result, the response to organic cyclic compound was the most significantly annotated term in BP category at level 5 (p -value=5.31E-10, Fig. 2A), most of these proteins were annotated as cytosolic proteins in CC category (p -value=2.77E-17, Fig. 2B), and protein kinase binding was the most significant related function in

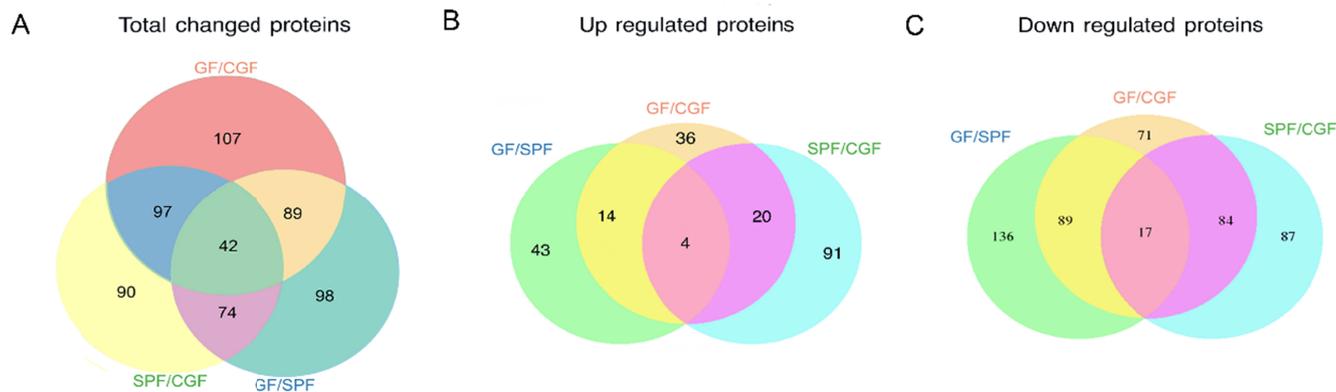


Fig. 1. Overall overlapping analysis for the differentially expressed proteins in the three comparison groups. (A) Venn diagram of the differentially expressed proteins in the three comparison groups. (B) Venn diagram of the upregulated proteins in the three comparison groups. (C) Venn diagram of the downregulated proteins in the three comparison groups.

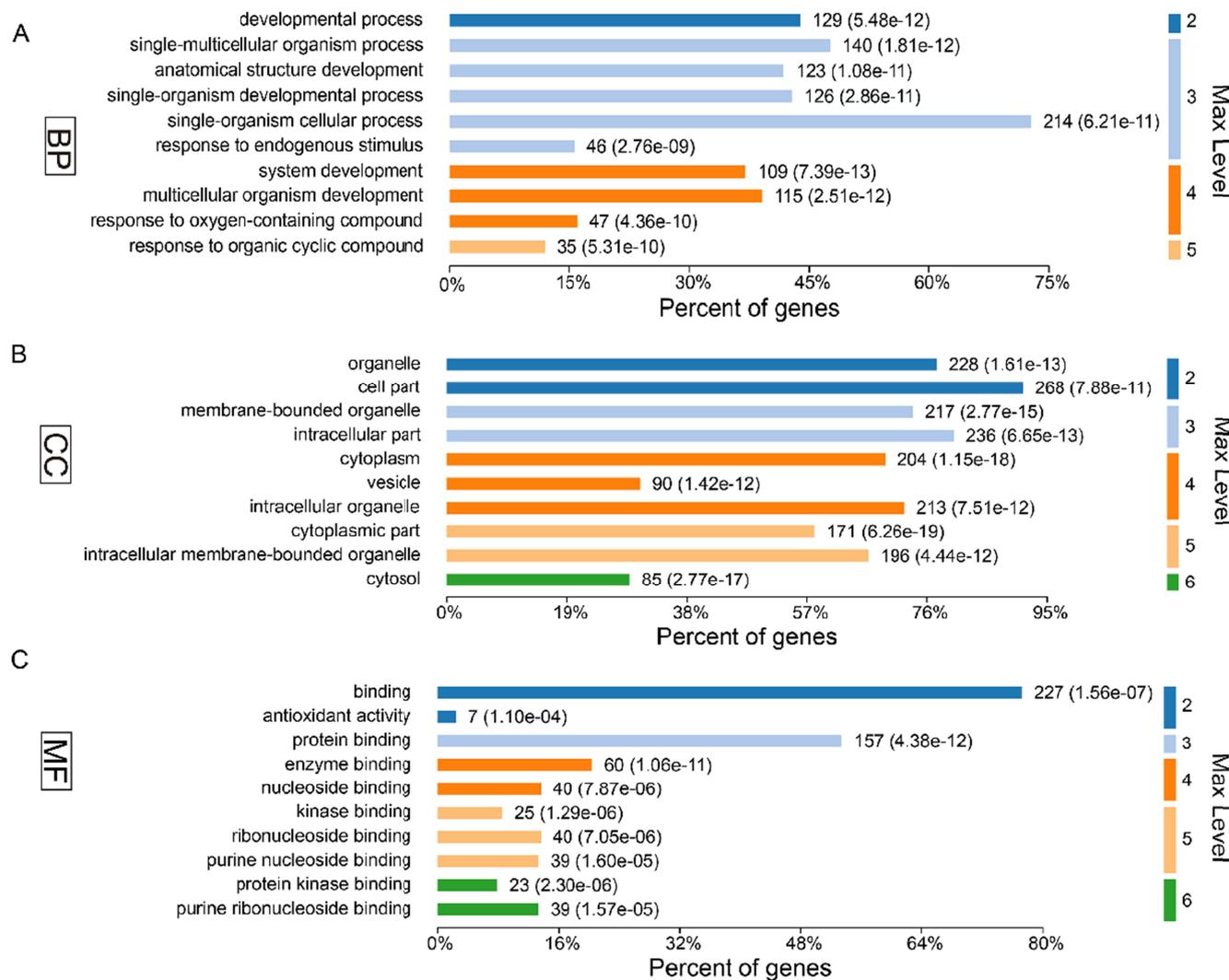


Fig. 2. Gene ontology annotations of the total 303 differentially expressed proteins for (A) biological process, (B) cellular component and (C) molecular function.

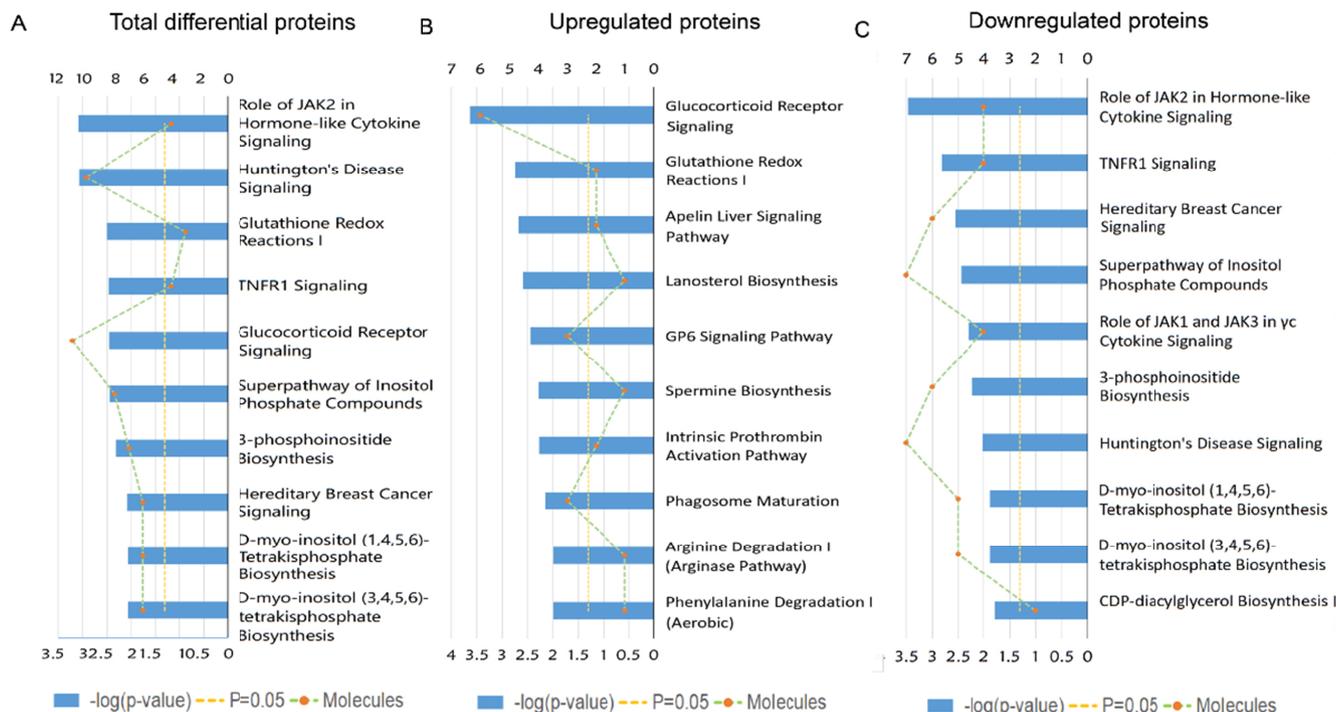


Fig. 3. Ingenuity Pathway Analysis (IPA) analysis of the differentially expressed proteins in the GF vs. SPF comparison group. (A) IPA analysis of the 303 differentially expressed proteins in the GF vs. SPF comparison group. (B) IPA analysis of the 61 upregulated proteins in the GF vs. SPF comparison group. (C) IPA analysis of the 242 downregulated proteins in the GF vs. SPF comparison group.

MF category (p-value=2.30E-06, Fig. 2C).

To get a better understanding of underlying mechanisms by which the absence of gut microbiota regulates the brain functions and behaviors of host. We performed functional enrichment pathway analysis for all these significant proteins, as well as the upregulated and downregulated proteins, respectively, using IPA software. The results revealed that the role of Janus kinase 2 (JAK2) in hormone-like cytokine signaling (p-value=8.13E-04), Huntington's disease signaling (p-value=8.51E-04), glutathione redox reaction I (p-value=3.16E-03), Tumor necrosis factor receptor 1 (TNFR1) signaling (p-value=3.47E-03), and glucocorticoid receptor signaling (p-value=3.55E-03) were the top five disturbed functional pathways (Fig. 3A). However, the upregulated proteins were primarily enriched in the glucocorticoid receptor signaling pathway (p-value=2.29E-04, Fig. 3B), and the downregulated proteins were mainly involved in the role of JAK2 in hormone-like cytokine signaling and in TNFR1 signaling pathways (p-value=3.39E-04 and p-value=1.51E-03, respectively, Fig. 3C).

Protein interactions play an important role in regulating cells and their signaling pathways. We thus performed the PPI network analysis to further understand the interactions among these differentially expressed proteins in GF vs. SPF mice. As a result, a total of 282 significant proteins were annotated in STRING database.

Using the CytoHubba plug-in, ten top-rank hub proteins were identified, and the PPI network of these hub proteins with their directly interacted proteins were shown in Fig. 4A, and that of the ten hub proteins per se in Fig. 4B. Of these hub proteins, POLR2B and POLR2H are the subunits of DNA-dependent RNA polymerase II, and play critical roles in the transcription. Interestingly, AQR, LSM3, BUD31, SART1 and SF1 are important components of spliceosome that catalyzes the pre-mRNA splicing. The down-regulations of these spliceosome related proteins in GF mice compared with the SPF mice, suggesting the inhibition of pre-mRNA alternative splicing, this findings was consistent with that of our previous study focused on the phosphorylation modification changes in the hippocampus of GF mice [30].

Functional analysis of proteins restored following gut microbiota colonization

To further explore the effects of gut microbiota on the protein expression of host. We analyzed the restored proteins following gut microbiota colonization that were characterized by their expression in opposite directions when examined in GF vs. SPF and CGF vs. GF comparisons, and were recognized as microbiota-specific proteins. As a result, a total of 124 proteins were significantly restored after gut microbiota colonization. Of all these 124

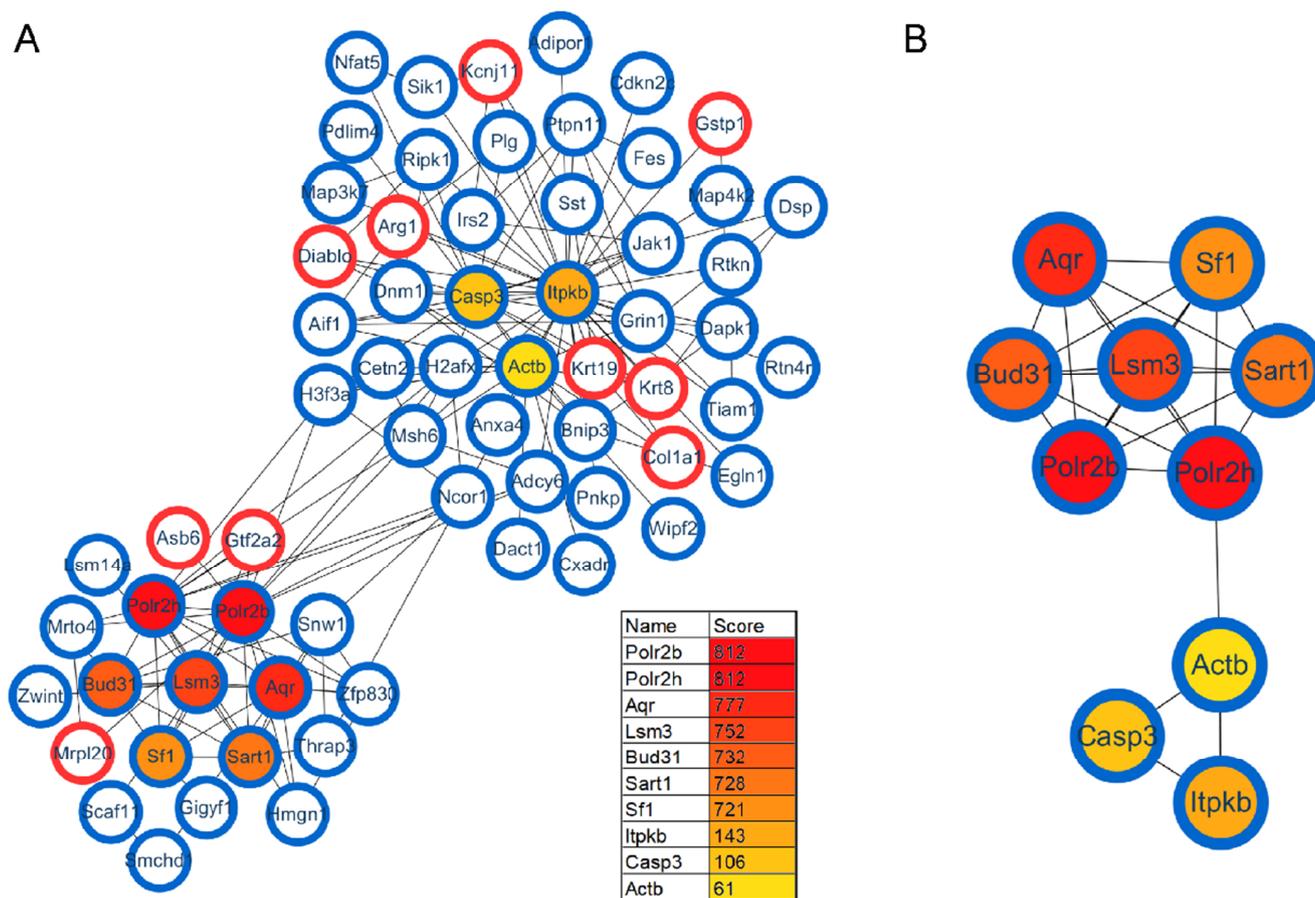


Fig. 4. Protein–protein interaction (PPI) analysis of differentially expressed proteins in the GF vs. SPF comparison group. (A) The PPI network of the ten hub proteins with their directly interacted proteins. (B) The PPI network of the ten hub proteins per se.

restored proteins, 18 were upregulated in GF mice compared with SPF mice, and 106 were downregulated. The expression levels of these restored proteins in the GF, SPF, and CGF mice are displayed in Supplementary Table 2. In the GO annotation analysis, these restored proteins were primarily related to the response to organic cyclic compounds in BP category at level 5 (p -value=4.81E-06, Fig. 5A), annotated in intracellular membrane-bound organelles in CC category at level 5 (p -value=3.05E-07, Fig. 5B), and had protein kinase binding functions in MF category at level 6 (p -value=3.23E-04, Fig. 5C). Moreover, we performed functional pathway analysis using IPA software to further explore the biological functions of these 124 microbiota-specific proteins (Fig. 6A and 6B), and found that glucocorticoid receptor signaling (p -value=1.51E-03) and TNFR1 signaling (p -value=2.04E-03) pathways were the primarily enriched disrupted pathways (Fig. 6C). This was consistent with the results found in mice with an absence of gut microbiota, as described in the previous section.

As a complementary approach to evaluate the restored protein-related pathways after gut microbiota colonization, we performed

functional analysis of differentially expressed proteins in GF vs. CGF comparison. A total of 74 proteins were identified upregulation and 261 were downregulation in the GF mice compared with the CGF mice (Supplementary Table 3). The functional enrichment analysis showed that the AMP-activated protein kinase (AMPK) signaling pathway was the most significantly associated disrupt pathway (p -value=7.59E-04, Fig. 6D). Several preclinical evidences do support the anti-inflammatory actions of AMPK [31]. In the PPI network analysis, the hub proteins with their directly interacted proteins were shown in Fig. 7A, and ten hub proteins were identified (Fig. 7B), e.g., AQR, LSM3, POLR2B, and ZFP830. Several preclinical evidences do support the anti-inflammatory actions of AMPK [31]. In the PPI network. Interestingly, the AQR and LSM3 are also the important components of spliceosome.

Integrated analysis of the proteomic and microarray data from GF mice

As described in the previous sections, these differentially ex-

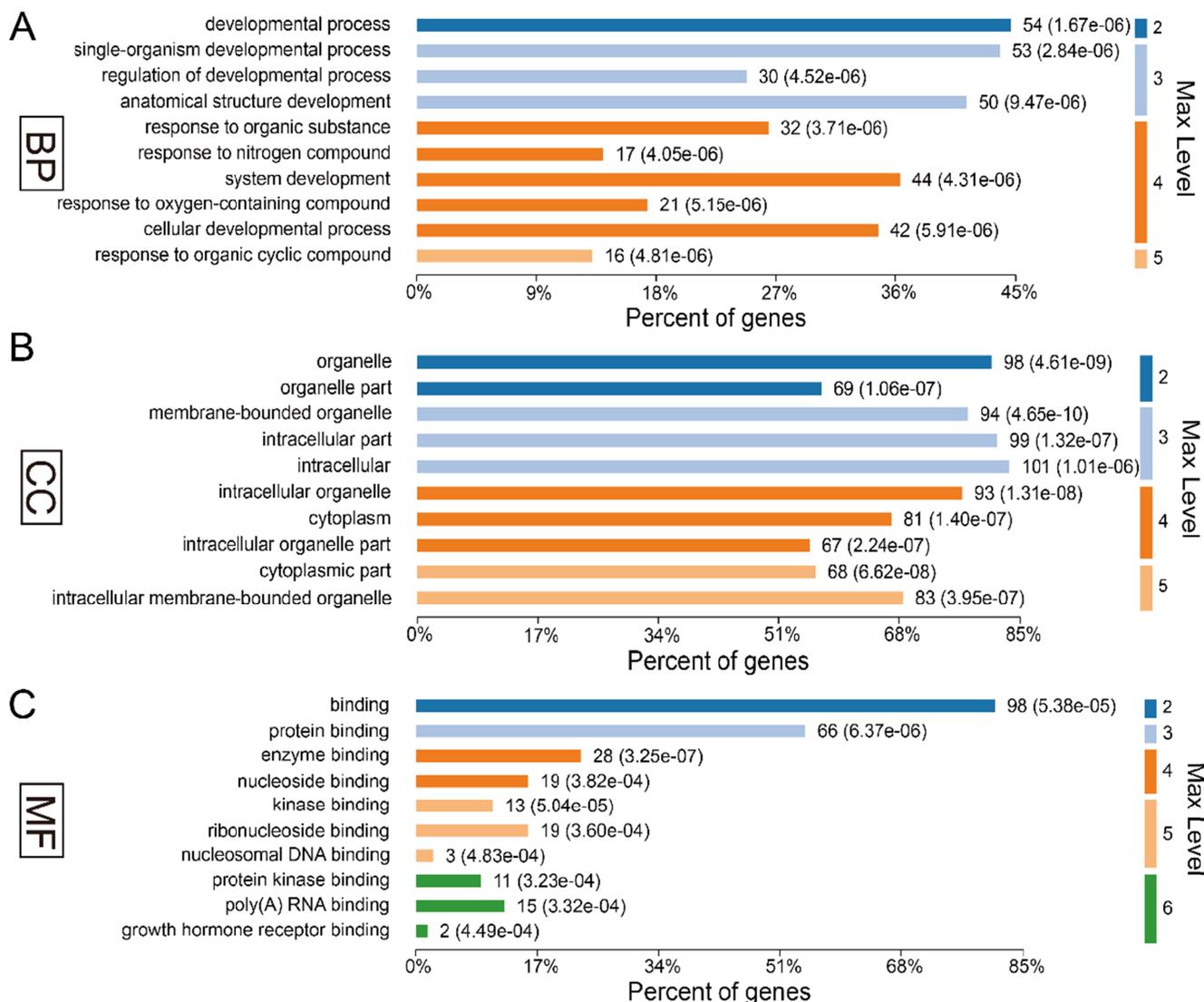


Fig. 5. Gene ontology annotations of the 124 restored differentially expressed proteins for (A) biological process, (B) cellular component and (C) molecular function.

pressed proteins, especially the upregulated proteins, were primarily enriched in the glucocorticoid receptor signaling pathway, indicating that the absence of gut microbiota may enhance the activity of the glucocorticoid receptor signaling pathway through upregulating the expression of related proteins. To further understand the underlying mechanisms by which gut microbiota regulate functions of glucocorticoid receptor signaling pathway, we performed an integrated analysis of the present proteomic results combined with previously reported microarray data that analyzed changes in the expression of glucocorticoid receptor signaling pathway genes [14]. The dysfunctions of glucocorticoid receptor signaling pathway are associated with disturbed inflammatory response [32]. Stirringly, the present results also indicated the disturbances

in inflammation-related pathways in GF mice, that were the role of JAK2 in the hormone-like cytokine signaling pathway and the TNFR1 signaling pathway, and all these inflammation-related proteins were all downregulated in GF mice compared with SPF mice. Finally, the glucocorticoid receptor signaling and inflammation-related pathways were displayed visually in Fig. 8.

DISCUSSION

Gut microbiota plays an important role in regulating the human health and diseases. Increasing evidence proved the potential effects of gut microbiota on mental disorders, e.g., depression and anxiety [33, 34]. However, the underlying mechanisms by which

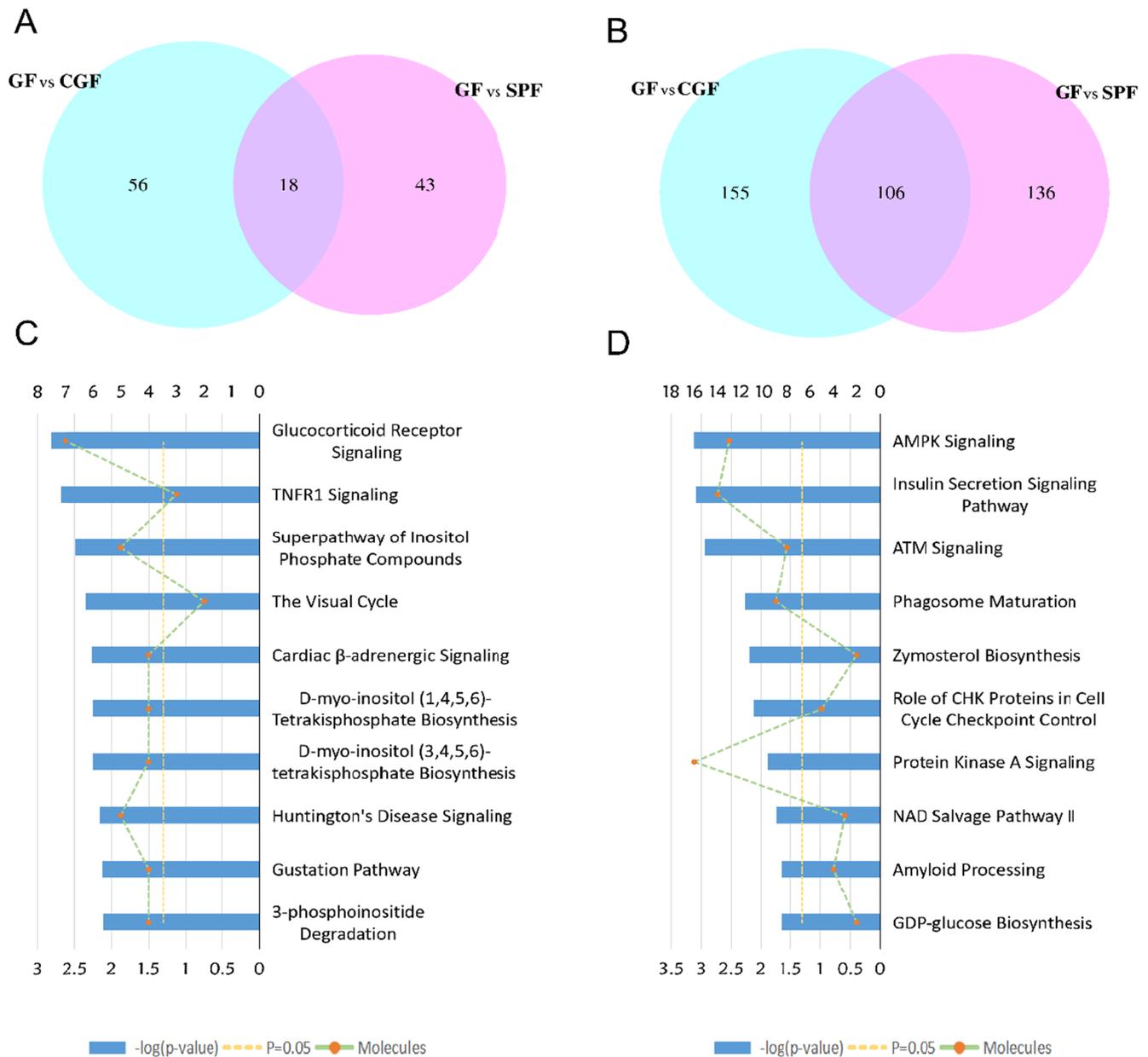


Fig. 6. Overlapping analysis for differentially expressed proteins in GF mice compared with SPF mice and Bioinformatic analysis of the 124 restored proteins. (A) Overlapping Venn diagram of the upregulated proteins in the GF vs. SPF and GF vs. CGF comparison groups. (B) Overlapping Venn diagram of the downregulated proteins in the GF vs. SPF and GF vs. CGF comparison groups. (C) Ingenuity Pathway Analysis (IPA) analysis of the 124 restored proteins. (D) Ingenuity Pathway Analysis (IPA) analysis of the 335 proteins for GF vs. CGF comparison.

the gut microbiota influence the host's brain functions and behaviors are complex and remain little understood. Thus, we used iTRAQ-based quantitative proteomic approach to analyze the protein expression changes in the hippocampus of GF, SPF and CGF mice, to further explore the underlying mechanisms at protein level. In present study, inbred line mice were used to mitigate the confounding effects of undefined variation in genetic backgrounds, we found that a total of 303 proteins were dysregulated in

GF mice compared with SPF mice, and 124 proteins were significantly restored after gut microbiota colonization. These significant proteins were primarily associated with glucocorticoid receptor signaling and inflammation-related pathways.

As previously reported [35], a complementary approach using CGF mice revealed the restoration of transcript expression, with no normalization of depression- and anxiety-like behavioral changes. Being different from transplanting fecal samples from

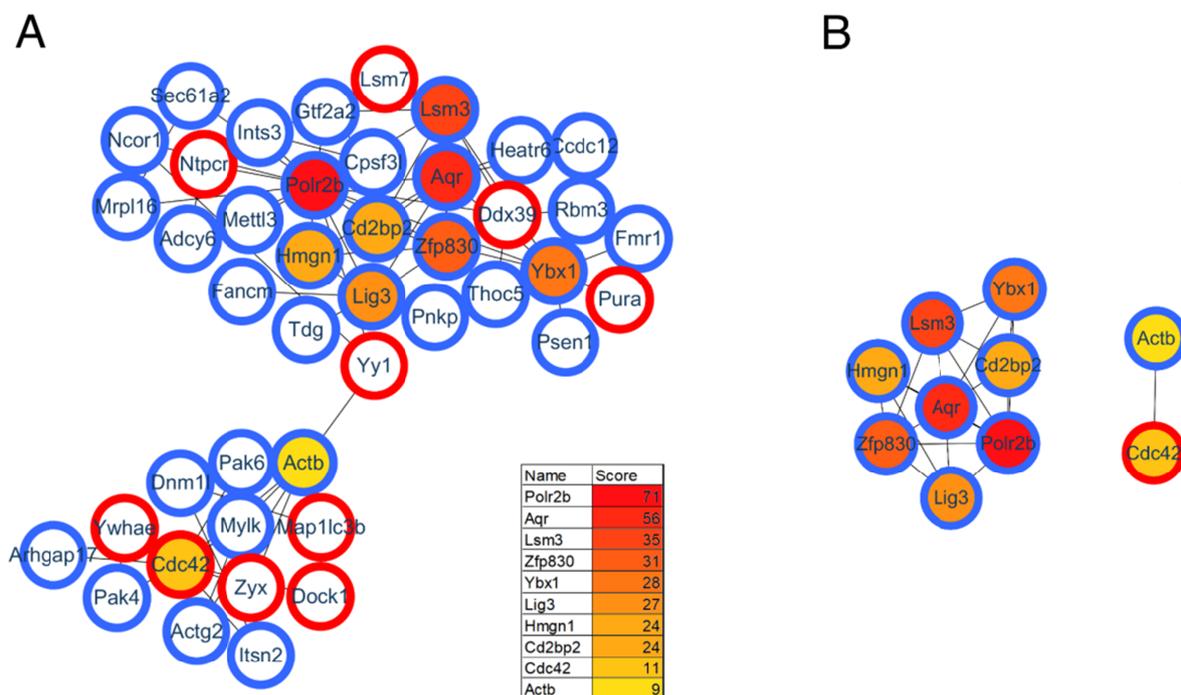


Fig. 7. Protein-protein interaction analysis for GF vs. CGF comparison group. (A) The PPI network of the ten hub proteins with their directly interacted proteins. (B) The PPI network of the ten hub proteins per se.

MDD patients to GF mice that caused significant depressive- and anxiety-like behaviors [15], the CGF mice were naturally colonized with gut microbiota from SPF mice, and this procedure has previously demonstrated to be effective at restoring a normal microbiota [21, 36]. These results suggest that the disturbances in the glucocorticoid receptor signaling and inflammation-related pathways are regulated by gut microbiota, rather than by the depression- or anxiety-like behaviors per se. And previous studies have reported that depression is strongly related to disrupted inflammatory processes [37]. There is growing evidence showed that depression is highly prevalent in infectious and autoimmune diseases, and this co-morbidity cannot be attributed only to the psychological distress of the disease [38]. Moreover, depressed patients consistently have higher levels of pro-inflammatory cytokines, acute phase proteins, chemokines, and cellular adhesion molecules [39, 40]. It has also been reported that an absence of gut microbiota may lead to behavioral abnormalities in mice via the HPA axis, which suggests that gut microbiota are an environmental factor that can regulate the pressure response of the HPA axis. For example, after the same restraint stress, serum adrenocorticotropic hormone levels are significantly higher in GF mice than in SPF mice [22, 41]. Taken together, these results indicate that disturbances in glucocorticoid receptor signaling and inflammation-related pathways in the hippocampus are involved in mechanisms

underlying gut microbiota–host interactions in mice.

In the glucocorticoid receptor signaling pathway, Fbpb5, a co-chaperone that participates in the regulation of glucocorticoid receptor sensitivity and the efficiency of the negative feedback process of the HPA axis [42], was upregulated in GF mice compared with SPF mice. FKBP5 and other components form an inactive glucocorticoid receptor (GR) multiprotein complex in the cytoplasm that is released after glucocorticoid combines with GR (GLGR). A previous study consistently found that GF mice had significantly higher levels of Fkbp5 transcripts in the pituitary compared with SPF unstressed mice [43]. It has also been reported that stress upregulates Fkbp5 in the pituitary of male mice compared with unstressed animals [44], although this result may have been caused by the particular stress model that was used. In the present study, RNA polymerase II (RNA pol II), Nuclear receptor corepressor (NCOR), and components of the SWI/SNF complex were downregulated in GF mice compared with SPF mice, and expression levels were restored after gut microbiota colonization. In contrast, Transcription initiation factor IIA (TF IIA) was upregulated in GF mice compared with SPF mice. The transcriptional regulatory complex formed by RNA pol II, TF IIA, and other proteins can interact with GLGR dimers and further influence downstream gene expression. In addition, nuclear factor of activated T cells (NFAT), a transcription factor involved in the transcriptional

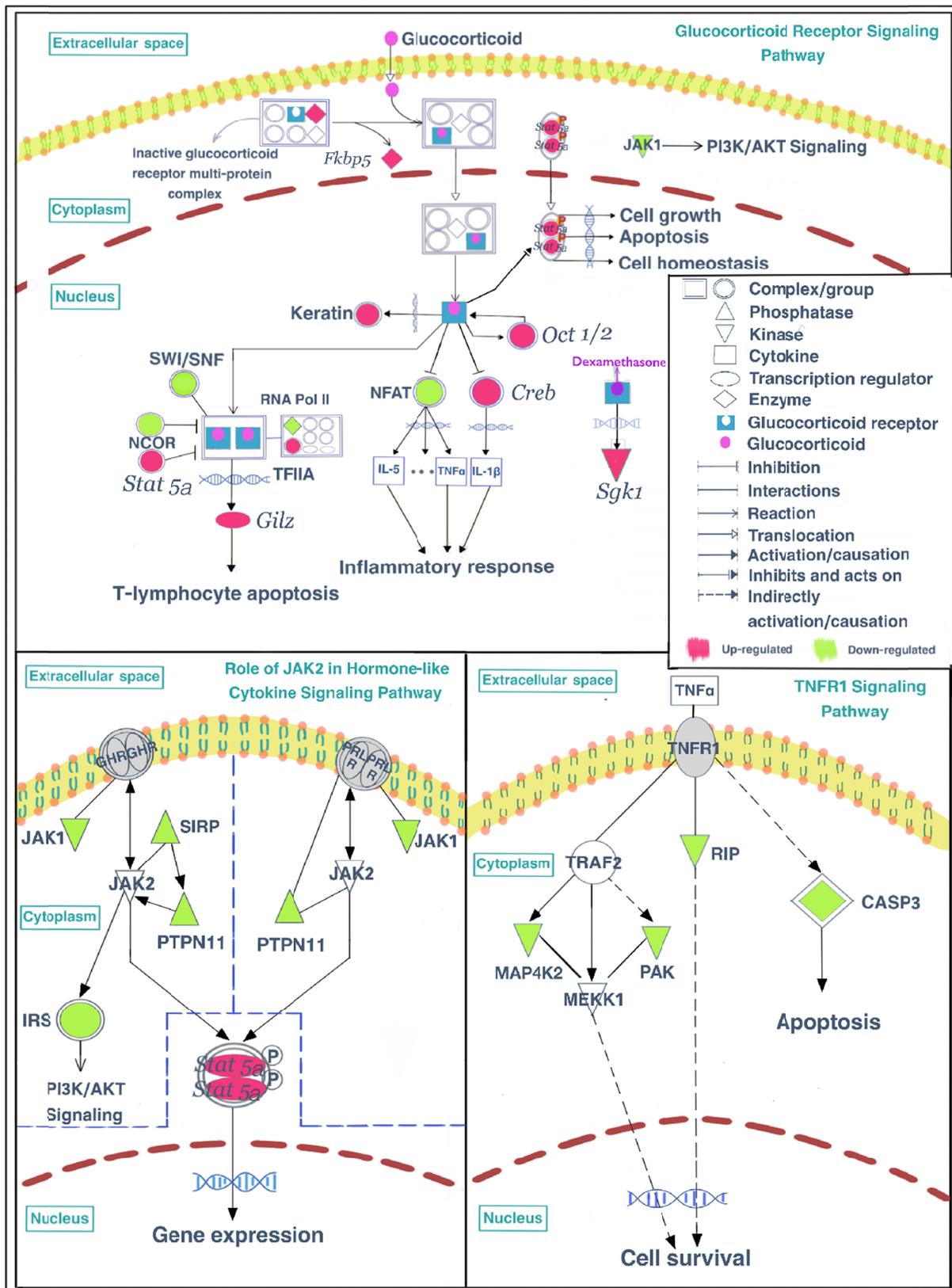


Fig. 8. Mapped proteins and genes in the glucocorticoid receptor signaling pathway, the role of JAK2 in the hormone-like cytokine signaling pathway, and the TNFR1 signaling pathway.

regulation of inflammatory genes, was downregulated in GF mice compared with SPF mice. Moreover, Stat5a and Creb can regulate the activation of transcription, and were upregulated in GF mice in the previous study [14]. Phosphorylated STAT5a protein in the nucleus promotes cell growth, apoptosis, and cell homeostasis functions. cAMP responsive element binding protein 1 (CREB) can promote the synthesis of IL1- β and is repressed by the GLGR complex, followed by the disruption of the inflammatory response. Studies have found that treatment with *Bifidobacterium breve* CCFM1025 can reduce depression- and anxiety-like behavior in mice, as well as decrease the active HPA axis and inflammatory response. However, increased IL1- β in the hippocampus of stressed mice was not reduced with this treatment [45]. This result is consistent with our findings of upregulated CREB in the hippocampus of GF mice. Additionally, a previous study has reported that commensal microbiota cause an upregulation in the expression of cytokines, including IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, TNF, and IFN, in the colon [43]. Considering the antidepressant- and antianxiety-like behaviors in GF mice compared with SPF mice [14], together with the finding that GLGR dimers can regulate the inflammatory response through gene regulation [46-49], it appears that the inflammatory response may play an important role in the mechanisms by which gut microbiota regulate depression- and anxiety-related behaviors.

In inflammation-related pathways, four proteins were enriched in the role of JAK2 in the hormone-like cytokine signaling pathway, they were Janus kinase 1 (JAK1), signal regulatory protein alpha (SIRP), protein tyrosine phosphatase non-receptor type 11 (PTPN11) and insulin receptor substrate 2 (IRS). Four proteins were enriched in the TNFR1 signaling pathway, they were receptor interacting serine/threonine-protein kinase 1 (RIP), Mitogen-activated protein kinase kinase kinase 2 (MAP4K2), p21 (CDKN1A)-activated kinase 6 (PAK), and Caspase-3 (CASP3). These inflammation-related proteins were all downregulated in GF mice compared with SPF mice. Interestingly, a previous study reported that upregulated cytokine expression *in vivo* can lead to the increased secretion of steroid hormones by activating the HPA axis [50], thus increasing glucocorticoids in the entire body. Conversely, changes in the activity of the glucocorticoid receptor signaling pathway can lead to corresponding changes in the inflammatory pathway [51]. The present proteomic results revealed that the upregulated proteins in the GF vs. SPF comparison were primarily enriched in the glucocorticoid receptor signaling pathway, while the downregulated proteins were highly involved in inflammation-related pathways. Moreover, previous reports have suggested that anti-inflammatory treatments, such as glucocorticoids, which have been used clinically for decades as potent anti-

inflammatory and immunosuppressive agents [52], may produce antidepressant effects [53, 54]. Together, these findings suggest that an enhancement of anti-inflammatory functions and a reduction in inflammatory activity may play a synergistic role in the generation of antidepressant and antianxiety behaviors in GF mice.

Some limitations in the present study need to be acknowledged. First, only hippocampal tissue was analyzed in the present study. Future research thus needs to include multiple brain regions to gain a comprehensive understanding of the underlying mechanisms of microbial–host interactions. In addition, cellular-specificity analysis is warranted to get better insight into the role of each hippocampal cell subtype in the microbiota-directed effects within the brain. Second, only male mice were analyzed for proteomic changes in this study, and further studies including both male and female mice are needed to identify any sex-specific effects of gut microbiota on host brain functions. Third, further researches analyzing the genetic background of mouse are required to evaluate its effects on experimental results.

In conclusion, numerous differentially expressed proteins were identified through a proteomic profiling analysis of hippocampal tissue from GF, SPF, and CGF mice, and these significant proteins were mainly related to the glucocorticoid receptor signaling pathway and inflammation-related pathways revealed by bioinformatics analysis. The present results indicate that these disrupted pathways may be involved in the underlying mechanisms by which gut microbiota regulate brain functions and behavioral phenotypes. This study provides new insights into the pathological mechanisms of gut microbiota-regulated diseases.

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CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

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