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Absence of gut microbiota during early life affects anxiolytic Behaviors and monoamine neurotransmitters system in the hippocampal of mice

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ABSTRACT

The gut microbiome is composed of an enormous number of microorganisms, generally regarded as commensal bacteria. Resident gut bacteria are an important contributor to health and significant evidence suggests that the presence of healthy and diverse gut microbiota is important for normal cognitive and emotional processing. Here we measured the expression of monoamine neurotransmitter-related genes in the hippocampus of germ-free (GF) mice and specific-pathogen-free (SPF) mice to explore the effect of gut microbiota on hippocampal monoamine functioning. In total, 19 differential expressed genes (Htr7, Htr1f, Htr3b, Drd3, Ddc, Maob, Tdo2, Fos, Creb1, Akt1, Gsk3a, Pik3ca, Pla2g5, Cyp2d22, Grk6, Ephb1, Slc18a1, Nr4a1, Gdnf) that could discriminate between the two groups were identified. Interestingly, GF mice displayed anxiolytic-like behavior compared to SPF mice, which were not reversed by colonization with gut microbiota from SPF mice. Besides, colonization of adolescent GF mice by gut microbiota was not sufficient to reverse the altered gene expression associated with their GF status. Taking these findings together, the absence of commensal microbiota during early life markedly affects hippocampal monoamine gene-regulation, which was associated with anxiolytic behaviors and monoamine neurological signs.

1. Introduction

Gut microbiota is a huge microecosystem that influences a wide variety of physiological features and functions in the host [1,2]. Previous studies showed that commensal gut microbiota played a causal role in the onset of several enteric and metabolic diseases [3–5]. Recent animal studies have suggested that gut microbiota influences brain chemistry and behavior via the microbiota-gut-brain axis [6,7]. Previous studies also found that dysbiosis of the gut microbiome (characterized by significant changes in the relative abundance of Firmicutes, Actinobacteria and Bacteroidetes) might play a causal role in the development of depressive-like behaviors [8–10]. To evaluate a functional role for commensal gut bacteria in major depression disorder (MDD), the germ-free mice were colonized with the fecal microbiota from either healthy individuals or MDD patients, and were monitored the behaviors. Mice receiving the MDD fecal microbiota (characterized by significant changes in the relative abundance of Firmicutes, Actinobacteria and Bacteroidetes) showed depression-like behaviors and exhibited disturbances of microbial genes and host metabolites [8]. The microbiota-gut-brain axis has been confirmed to play an important role in brain function and behavior [11]. However, a deeper understanding of the connection between commensal microbiota and neuropsychiatric

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disorders is currently lacking.

Alterations of the monoamine neurotransmitter system were reported to be a key feature in the pathology of several neuropsychiatric disorders [12,13]. Previous study conducted a targeted metabolomics research on the mice model of depression, and revealed significant alterations in the levels of neurotransmitters [14]. Interestingly, recent studies have shown that the absence of gut microbiota could modify some brain neurotransmitters such as serotonin [6,11,15]. In addition, the abundant evidence suggested that the gut microbiota composition, the concentration of microbial products and gastrointestinal symptoms in humans are closely associated with disorders of the central nervous system (CNS), such as autism spectrum disorder and depression [16,17]. Based on these findings, we sought to systematically assess how the expression of genes involved in the monoamines neurotransmitters is shaped by commensal microbiota, and to further determine whether these specific changes are involved in the onset of neuropsychiatric disorders.

Gene expression profiling, a powerful molecular technique, has been widely used to analyze the expression levels of the entire genes in a specific tissue [18–20]. Germ-free (GF) mice represent an effective preclinical tool to investigate how commensal microbiota influences brain chemistry and behavior [6,21–23]. Here, we initially compared hippocampal gene expression patterns of GF mice and specific-pathogen-free (SPF) mice using Dopamine and Serotonin Pathway PCR array. Then, three key protein were selected and validated using Western blotting. In addition, some anxiety-like behavioral phenotypes were also chosen to confirm the bioinformatic results. Anxiety is a common symptom of neuropsychiatric disorders and altered anxiety-like behaviors have been consistently reported in GF mice in contrast to SPF mice [8,10]. As such, anxiety-like behaviors-open-field test (OFT) and novelty suppressed feeding (NSF)- were chosen here to validate the bioinformatic results. Finally, we sought to determine whether the aberrant genes expression and behavioral profile could be reversed following reconstitution of normal microbiota to adolescent GF mice.

2. Materials and methods

2.1. Animals

The GF procedure was performed as previously described [24], and the exact information for generating GF mice was provided in Supplementary File 1. Briefly, the Balb/c GF mice was generated via embryo transfer and group-housed in flexible film gnotobiotic isolators (Fengshi Laboratory Animal Equipment, Suzhou, China) housed in polycarbonate cages on sterile wood chips under a strict 12-h light/dark cycle (lights on at 7:30 AM) and a constant temperature of 21–22 °C and humidity of 55 ± 5%. The GF mice were given ad libitum access to a growth and reproduction standard rodent diet (GB-T 14924.3–2001, provided by Laboratory Animal Center of Third Military Medical University, Chongqing, China) sterilized by 60co gamma radiation (4 Mrad, Radiation Centre of Third Military Medical University, Chongqing, China) and sterilized water as previous study described [25,26]. The germ-free status of the mice was validated by cultures of aerobic and anaerobic microbes, and sequencing the 16 s rRNA gene from fecal and skin bacteria based on Chinese Laboratory Animal-Microbiological Standards and Monitoring (GB-T 14926A-2001) [27,28]. The SPF Balb/c mice were obtained from the Department of Laboratory Animal Science of the Third Military Medical University (Chongqing, China), and kept under the same feeding conditions with GF mice.

2.2. Ethical statement

All experiments in this study were approved by the Ethics Committee of Chongqing Medical University (approval no: 20130078), and all procedures were in accordance with the National Institutes of Health Guidelines for Animal Research (Guide for the Care and Use of Laboratory Animals) [29]. Special care was taken to minimize the number and suffering of animals.

2.3. Experimental protocol

The procedure for re-colonization was performed as previously described [11,30,31], which has previously demonstrated to be effective at restoring a normal microbiota. Briefly, 5–6-week male GF mice (n = 68) was randomly divided into two groups: One group (n = 33) of GF mice was removed from the GF facility to place them in cages with bedding material and fecal matter from SPF mice. The other group of GF mice was retained in the germ-free facility. A group of SPF BALB/c mice (n = 38) was obtained at the same age and sex.

Because behavioral tests may influence the gene expression [32], all of the three groups (GF, SPF, and colonized GF) were divided into three parts for separately used: one cohort (behavioral tests cohort) of GF (n = 22), CGF (n = 20), and SPF mice (n = 25) underwent behavioral tests; the second cohort (qPCR array cohort) of GF, CGF, and SPF mice (n = 9 per group) was subjected to PCR array analysis; and the third cohort (validation cohort) of GF, CGF, and SPF mice (n = 4 per group) were used for western blotting. All mice were tested and euthanized in adulthood at 8–9 weeks of age.

2.4. Sample collection and RNA extraction

The animals were anesthetized with chloral hydrate and immediately had the brain tissues removed. The hippocampal tissues were dissected out on an ice-cold plate, weighed, rapidly frozen with liquid nitrogen, and stored at −80 °C until used. Then, the obtained hippocampal was used for RNA extraction. The RNA extraction and real-time PCR microarrays procedures were carried out as described previously [33,34]. Briefly, hippocampal tissues from three mice were used to yield a pooled RNA sample, then three pooled RNA samples in each group were used to conduct real-time PCR microarrays. Total RNA was extracted using the Trizol extraction protocol (Invitrogen, Carlsbad, CA, USA) and purified using a RNeasy® MinElute™ Cleanup Kit (Qiagen, Valencia, CA, USA). RNA concentrations and purity were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

2.5. PCR microarrays

Total RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and complementary DNA (cDNA) was amplified by PCR using the 2× Super Array PCR Master Mix (SABiosciences, Frederick, MD, USA). A Qiagen ™ Mouse Dopamine and Serotonin Pathway PCR array (Cat. No. PAMM-158z, SABiosciences, Frederick, MD, USA) (n = 3/group) was used according to the manufacturer's recommended protocol as previously described [35,36]. Each array contains primers for 84 targets and 5 housekeeping genes and controls for RT and PCR reactions. These genes are mainly involved receptors, synthesis & degradation, neurotransmitter transporters, signal transduction pathways, and gene targets (Supplementary Table S1). Gene level data were normalized to average Ct of five housekeeping genes: Actb, B2m, Gapdh, Gusb, and Hsp90ab1. The acquired data were analyzed using the ΔΔCt method, through the web-based statistical analysis tool provided by the supplier (http://pcrdataanalysis.sabio-sciences.com/pcr/arrayanalysis.php).

2.6. Bioinformatics analysis

To predict the biological functions and relevant networks of significantly differentially expressed genes, the differential genes and corresponding fold changes were uploaded to the Ingenuity Pathway Analysis (IPA, https://www.qiagenbioinformatics.com). Canonical pathways and molecular interaction networks were generated based on

Table 1
The differential monoamine neurotransmitters genes between the experimental groups.

Gene	Function	GF/SPF		CGF/GF		CGF/SPF	
		Fold change	p-value	Fold change	p-value	Fold change	p-value
Genes involved in neurotransmitter receptors							
Htr7	serotonin receptors	1.22	0.002	0.78	**0.003**	0.95	0.214
Htr1f	serotonin receptors	1.53	0.002	0.82	**0.048**	1.26	**0.016**
Htr3b	serotonin receptors	3.27	0.000	0.77	0.728	2.52	0.149
Drd3	dopamine receptors	7.27	0.003	0.56	0.061	4.08	**0.016**
Genes involved in neurotransmitter transporters & metabolism							
Ddc	dopamine synthesis and degradation	1.44	0.032	1.09	0.35	1.57	**0.012**
Maob	serotonin synthesis and degradation	1.53	0.047	0.92	0.201	1.41	0.11
Tdo2	serotonin synthesis and degradation	2.96	0.001	0.72	0.053	2.13	**0.015**
Genes involved in signal transduction pathways							
Fos	cAMP/PKA pathway	0.49	0.002	1.42	0.091	0.69	**0.047**
Creb1	cAMP/PKA pathway	1.21	0.007	0.75	**0.001**	0.9	0.116
Akt1	PI3K/AKT pathway	1.13	0.034	0.85	0.036	0.96	0.455
Gsk3a	PI3K/AKT pathway	1.14	0.025	0.87	**0.028**	0.99	0.838
Pik3ca	PI3K/AKT pathway	1.08	0.044	0.96	0.49	1.04	0.555
Pla2g5	PLA2 pathway	2.86	0.002	0.34	**0.001**	0.96	0.876
Cyp2d22	PLA2 pathway	1.95	0.004	0.89	0.246	1.73	**0.003**
Grk6	G-protein coupled receptor regulation	0.92	0.046	0.9	0.22	0.83	**0.049**
Genes involved in downstream signaling							
Ephb1	dopamine and serotonin gene targets	1.39	0.005	0.78	0.053	1.08	0.391
Slc18a1	dopamine and serotonin gene targets	2.29	0.005	0.77	0.055	1.76	**0.006**
Nr4a1	dopamine and serotonin gene targets	0.58	0.000	1.44	**0.017**	0.83	**0.07**
Gdnf	dopamine and serotonin gene targets	3.47	0.003	0.68	**0.032**	2.37	**0.034**

the knowledge within the Ingenuity Pathway Knowledge Base.

KEGG Mapper analysis tool (http://www.kegg.jp/kegg/mapper.html) was used to construct the pathways of these genes relevant to the absence of gut microbiota. Search&Colour Pathway is an advanced version of the KEGG pathway mapping tool, where given objects (genes, proteins, compounds, glycans, reactions, drugs, etc.) are searched against KEGG pathway maps and found objects are marked in any background and foreground colors (bgcolor and fgcolor) [37].

2.7. Western blotting validation

An independent cohort of mice (n = 4/group) was used to validated the differential gen at the protein level. For Western blot, the procedures of electrophoresis, transfer, and immunodetection were conducted based on previously described procedures with minor modifications [38,39]. The hippocampal tissue was lysed in RIPA buffer with protease inhibitor cocktail (Roche, Mannheim, Germany). The protein was transferred from 10% SDS-PAGE to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) for blocking. The primary antibodies used were as follows: anti-dopa decarboxylase (1:1000, ab131282, Abcam, Cambridge, MA, USA); anti-Monoamine Oxidase B (1:1000, ab131045, Abcam, Cambridge, MA, USA); Anti-Dopamine Receptor D3 (1:1000, ab131045, Abcam, Cambridge, MA, USA); anti-GAPDH (1:5000, G9545, Sigma Aldrich, MO, USA). Anti-rabbit IgG (1:10000, 170–6515, Bio-Rad Laboratories, Redmond, WA, USA) were used as secondary antibodies. After immunodetection, the images were subjected to densitometric analysis performed using Quantity One software (Bio-Rad, Hercules, WA, USA).

2.8. Behavioral tests

The behavioral cohort of GF (n = 22), colonized GF (n = 20), and SPF mice (n = 25) was used to perform an OFT and NSF. The detailed protocols for assessing the two behaviors were in line with those in previous studies [8,10,26]. Briefly, GF and colonized GF mouse cages were removed from isolators and placed in a separate room with SPF mouse cages for at least 30 min before testing. All tests took place in a soundproof room to reduce stress between 8:00 and 12:00 a.m., unless otherwise noted. After each test, mice were returned to their home cages. Testing chambers were cleaned with disinfectant and 70% ethanol after each trial.

For OFT, each mouse was placed individually in the corner of an open-field box (45 × 45 × 30 cm) and allowed to explore freely for 6 min. Its spontaneous activities over the last 5 min were recorded using a video tracking system (SMART; Panlab SL, Barcelona, Spain). The total movement distance was used as an index of locomotor activity, while the time or distance spent in the center (inner 25% of the surface area) was used as an index of anxiety-like behavior [40,41].

For NSF, group-housed animals were deprived of food for 24 h and then placed in a temporary home cage for 30 min. For the test, individual mice were placed in a 45 × 45 × 30 cm open-field arena. A single food pellet (familiar laboratory mouse chow) was placed on a circular piece of white filter paper (12 cm in diameter) positioned in the center of the arena. Each mouse was placed in a corner of the arena and allowed to explore for up to 10 min. The trial ended when the mouse chewed some of the chow. Then, the mouse was immediately returned to its home cage and the food consumption during the subsequent 5 min was measured to eliminate the effect of appetite on the latency time.

2.9. Statistical analysis

The statistical analysis of gene expression in PCR array were performed as previously described [42,43]. Briefly, the statistical analysis of gene expression in PCR array were performed using the ΔΔCt method, through the web-based statistical analysis tool provided by the supplier (http://pcrdataanalysis.sabio-sciences.com/pcr/arrayanalysis.php). Multiple group comparisons were made using one-way ANOVA. All behavioral parameters, including OFT and NSF, displayed a non-normal distribution and were analyzed by non-parametric tests, and a p-value of < 0.05 was considered significant. All continuous variables were expressed as means ± standard errors of the mean. The statistical analyses were carried out using SPSS 17.0 for Windows (IBM Corp. Armonk, NY, USA).

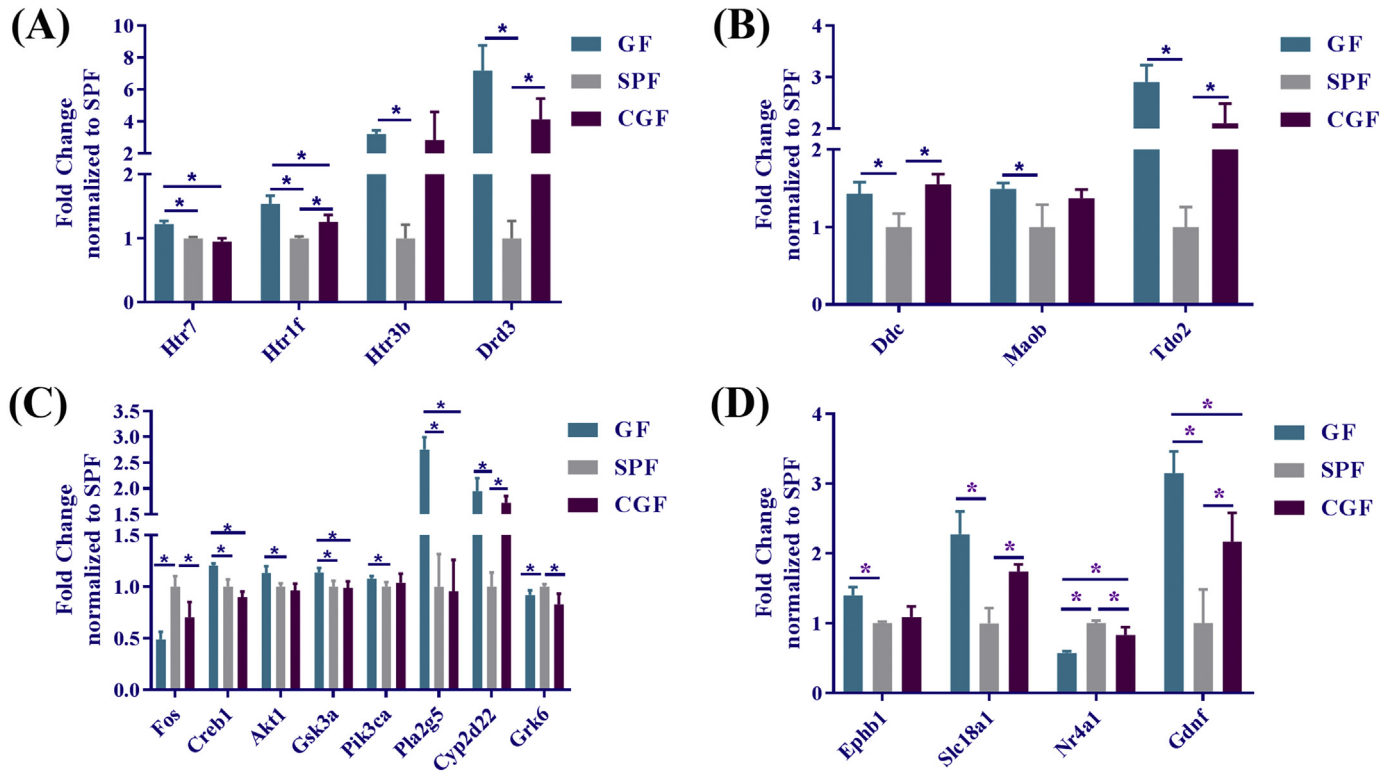

Fig. 1. Relative expression of genes involved in monoamine neurotransmitters in GF, CGF and SPF mice.
Abbreviation: Akt1, thymoma viral proto-oncogene 1; CGF, colonized GF; Creb1, CAMP-responsive element binding protein 1; Cyp2d22, cytochrome P450 family 2 subfamily d polypeptide 22; Ddc, dopa decarboxylase; Drd3, dopamine receptor D3; Ephb1, eph receptor B1; Fos, FBJ osteosarcoma oncogene; Gdnf, glial cell line-derived neurotrophic factor; GF, Germ-free; Grk6, G protein-coupled receptor kinase 6; Gsk3a, glycogen synthase kinase 3 alpha; Htr1f: serotonin receptor 1F; Htr3b, serotonin receptor 3B; Htr7: 5-hydroxytryptamine (serotonin) receptor 7; Maob, monoamine oxidase B; Nr4a1, nuclear receptor subfamily 4 group A member 1; Pik3ca, phosphatidylinositol 3-kinase catalytic alpha polypeptide; Pla2g5, phospholipase A2 group V; Slc18a1, solute carrier family 18 (vesicular monoamine) member 1; SPF, specific-pathogen-free; Tdo2, tryptophan 2,3-dioxygenase;
(A) Genes involved in neurotransmitter receptors; (B) Genes involved in neurotransmitter transporters & metabolism; (C) Genes involved in signal transduction pathways; (D) Genes involved in downstream signaling.
Data represent the mean ± SEM. *indicates $P < .05$ and FDR q-value < 0.1.

3. Results

3.1. Alteration of monoamine gene expression in GF mice hippocampus

To explore whether commensal microbiota modulates the expression of hippocampal monoamine neurotransmitter system genes, we systematically measured the expression of gene in the hippocampus of GF and SPF mice (Supplementary Table S2). In total, 19 genes that were differentially expressed between the two groups were identified (Table 1 Fig. 1). Compared with the SPF mice, the GF mice exhibited upregulation of the expression of the following genes: Htr7, Htr1f, Htr3b, Drd3, Ddc, Maob, Tdo2, Creb1, Akt1, Gsk3a, Pik3ca, Pla2g5, Cyp2d22, Ephb1, Slc18a1, and Gdnf. They were also associated with downregulation of Fos, Grk6, and Nr4a1. These differently expressed genes could be divided into four types: (i) serotonin and dopamine receptors (Htr7, Htr1f, Htr3b, Drd3); (ii) monoamine neurotransmitters transporters & metabolism (Ddc, Maob, Tdo2); (iii) monoamine neurotransmitters related signal transduction (Fos, Creb1, Akt1, Gsk3a, Pik3ca, Pla2g5, Cyp2d22, Grk6); (iv) downstream signaling of dopamine, serotonin synapse gene targets (Ephb1, Slc18a1, Nr4a1, Gdnf).

3.2. Bioinformatics analysis results of differentially expressed genes in GF mice hippocampus

To get a further understanding of these differentially expressed genes, the 19 differential hippocampal monoamine neurotransmitter related genes were uploaded to IPA for canonical pathways and biomolecular functions analyses. The differential genes in GF mice were involved in the following top 5 canonical pathway: serotonin receptor signaling, G-Protein coupled receptor signaling, Neuroinflammation signaling pathway, GDNF family ligand-receptor interaction, neurotrophin/TRK signaling (Fig. 2). To understand the relationships between the differential genes, correlation network was built through IPA database (Fig. 3A). The differently expressed genes involved in metabolism of dopamine and serotonin. In order to clearly show these identified differential genes, we built a pathway figure by KEGG Mapper analysis tool. Dopaminergic synapse and serotonergic synapse-related gene were the most significantly altered. A detailed construction of the pathways is shown in Fig. 4.

IPA platform also provided Diseases and BioFunctions enrichment analysis. These differential genes were significantly enriched in neurological disease and psychological disorder (Fig. 2). Molecular transport, cell-to-cell signaling and interaction, small molecule biochemisty were significantly enriched. The differently expressed genes were summarized in a network (Fig. 3B), showing that the absence of gut microbiota was associated with animal behavior and neurological signs.

3.3. Validation of bioinformatics analysis results of differentially expressed genes in GF mice hippocampus

Anxiolytic-like behaviors were chosen to validate the bioinformatics analysis results. In the OFT, the total movement distance of GF mice was significantly higher than that of SPF mice, suggesting higher locomotor activity in GF mice (Fig. 5A). Moreover, GF mice displayed

Top Canonical Pathways

Name	p-value	Overlap
Serotonin Receptor Signaling	3.44E-10	11.6 % 5/43
G-Protein Coupled Receptor Signaling	1.28E-07	2.2 % 6/277
Neuroinflammation Signaling Pathway	2.53E-07	1.9 % 6/311
GDNF Family Ligand-Receptor Interactions	6.17E-07	5.3 % 4/76
Neurotrophin/TRK Signaling	6.50E-07	5.2 % 4/77

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value range	# Molecules
Neurological Disease	1.48E-03 - 3.52E-12	17
Nutritional Disease	8.61E-04 - 1.97E-10	12
Psychological Disorders	1.40E-03 - 2.37E-09	14
Organismal Injury and Abnormalities	1.48E-03 - 4.97E-08	18
Skeletal and Muscular Disorders	1.09E-03 - 8.67E-08	14

Molecular and Cellular Functions

Name	p-value range	# Molecules
Molecular Transport	1.36E-03 - 3.28E-10	16
Cell-To-Cell Signaling and Interaction	9.11E-04 - 5.29E-10	19
Small Molecule Biochemistry	1.40E-03 - 6.11E-10	17
Cellular Movement	1.40E-03 - 7.49E-07	13
DNA Replication, Recombination, and Repair	9.11E-04 - 1.22E-06	6

Physiological System Development and Function

Name	p-value range	# Molecules
Behavior	9.35E-04 - 2.31E-07	12
Tissue Morphology	1.44E-03 - 8.52E-07	13
Embryonic Development	1.44E-03 - 1.22E-06	9
Nervous System Development and Function	1.27E-03 - 1.22E-06	12
Organismal Development	1.44E-03 - 1.22E-06	10

Fig. 2. Top canonical pathways, top diseases and biofunctions involved in the differential monoamine neurotransmitters genes.

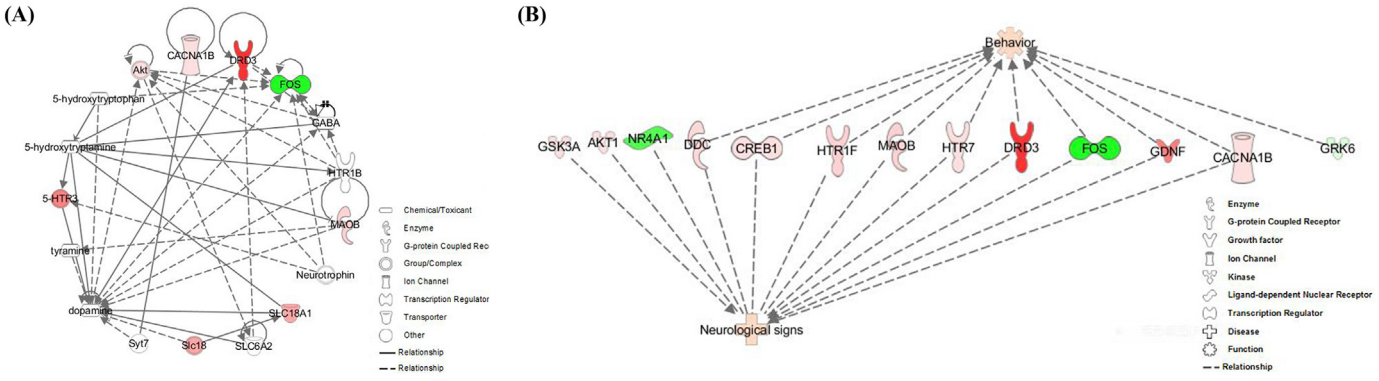

Fig. 3. (A) Correlation network of differential genes through the IPA database. (B) Molecular and cellular functions show that differential genes were associated with animal behavior and neurological signs.
Genes with red symbols were increased in Germ-free mice compared with control rats, while green symbols indicate decreases. Solid lines show direct interactions between the two components, while dotted lines show indirect interactions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

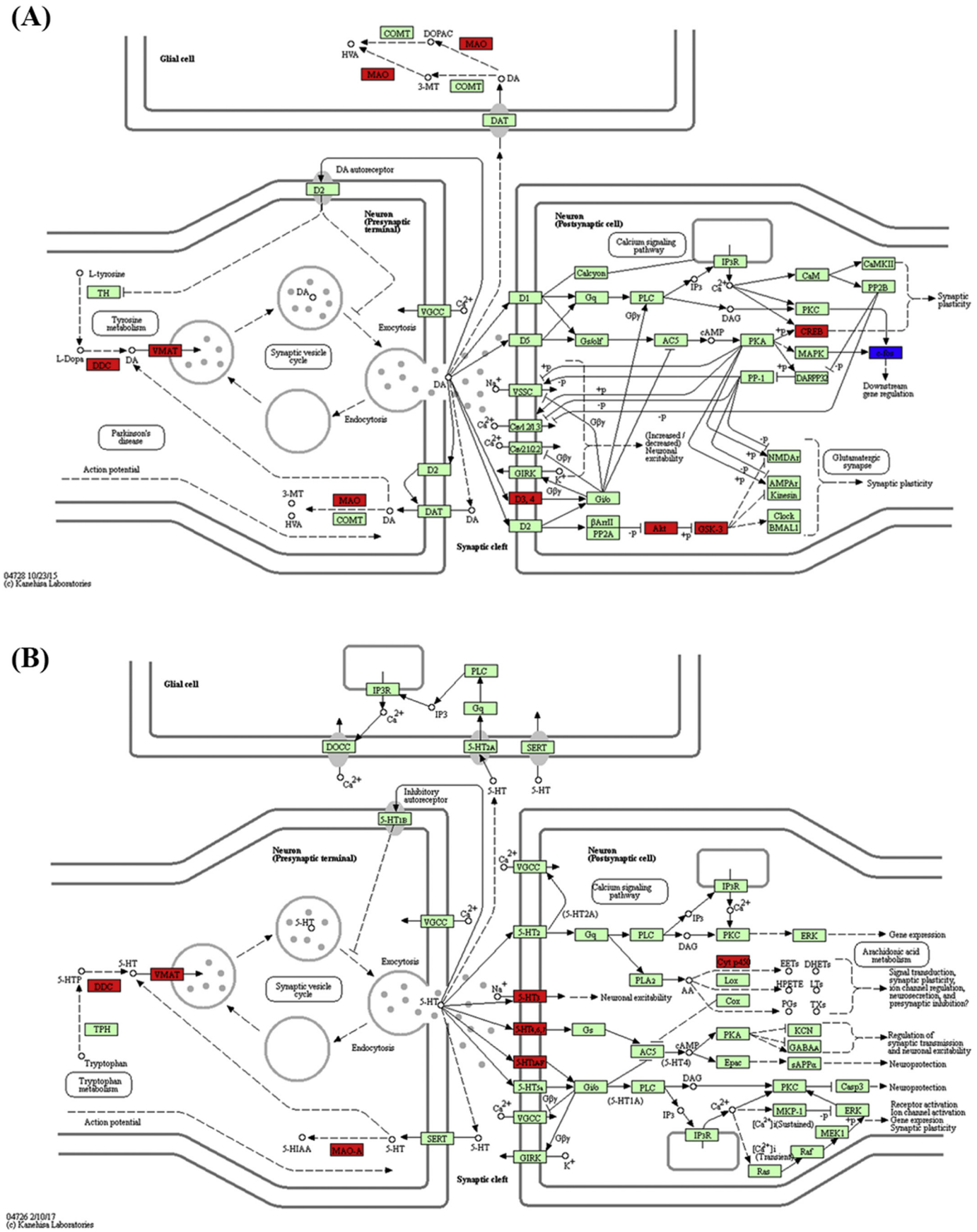

Fig. 4. KEGG map displaying commensal microbiota significantly influenced the expression of hippocampal dopaminergic synapse (A) and serotonergic synapse (B). Red colored boxes indicate upregulation in the germ-free (GF) group compared with control (CON), while blue-colored boxes indicate downregulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

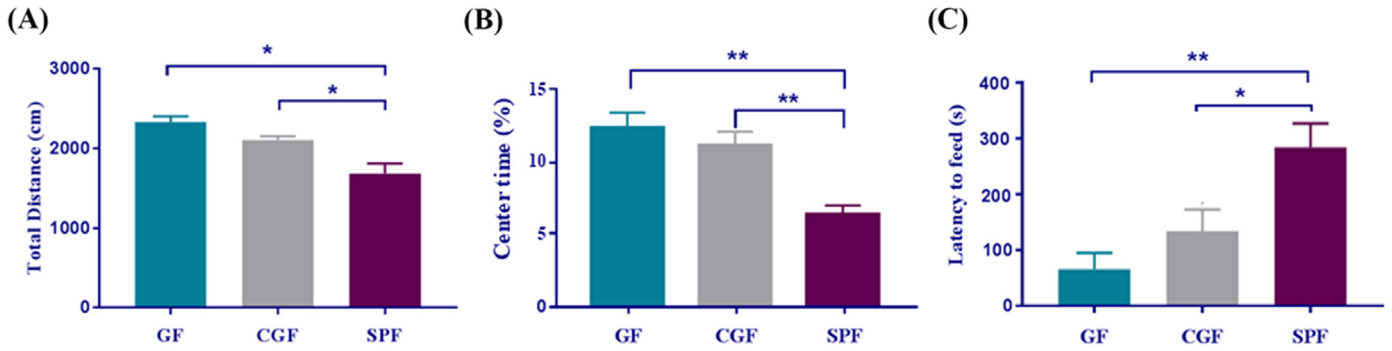

Fig. 5. Effect of commensal microbiota on anxiety behaviors.
Germ-free (GF) mice display increased motor activity and reduced anxiety, compared with specific-pathogen-free (SPF) mice. Reduced anxiety in GF animals, to a certain extent, is insufficient to reverse following restoration of the commensal microbiota. (A) The total distance in the OFT: The total movement distance was used as an index of locomotor activity. (B) The proportions of center time in the open-field test: The proportions of time in the center (inner 25% of the surface area) was construed as an index of anxiety-like behavior. (C) Latency to feed in novelty suppressed feeding, was construed as an index of anxiety-like behavior.
Data represent the mean ± SEM. *indicates $P < .05$, **indicates $P < .0$.

anxiolytic-like behavior, as evidenced by a significantly increased proportions of center time compared to SPF mice (Fig. 5B). In the NSF test, the latency to feeding was significantly decreased in GF mice relative to that in SPF mice, indicating less anxiety in the former group (Fig. 5C). There was no significant difference between the GF mice and SPF mice on the food consumption (Fig. S1).

The expression levels of Ddc, Maob, Drd3 were selected and validated using Western blotting in hippocampal tissue of mice. These results were in accordance with the changes found in PCR array and bioinformatics analysis results (Fig. 6).

3.4. Colonization of gut commensal microbiota to adolescent GF mice

To determine whether the differently expressed monoamine gene could be reversed by microbiota colonization, a set of GF mice recolonized with gut microbiota in adolescence (CGF mice, qPCR cohort) were used to profile the expression of hippocampal monoamine neurotransmitter system genes. A total of 10 of 19 (~53%) differently expressed genes were returned to their normal expression level in GF mice following recolonization, namely, Htr7, Htr3b, Maob, Creb1, Akt1, Gsk3a, Pik3ca, Pla2g5, Ephb1, Nr4a1. Htr1f, Drd3, Ddc, Tdo2, Fos, Cyp2d22, Grk6, and GDNF were not normalized by colonization (Table 1, Fig. 1). While, inconsistent with this finding, the colonization of adult GF mice could not reverse the abnormalities of behavioral phenotypes: compared with SPF mice, colonized GF mice showed increases in locomotor activity, anxiolytic-like behavior, and latency to feeding similar to those of GF mice (Fig. 5 A-C).

4. Discussion

The monoamine neurotransmitter system plays a critical role in regulating brain functions and behavior [44,45]. The relationship between tryptophan metabolism intermediates and affective disorder has recently been highlighted [46,47]. Here, we provide evidence that the absence of commensal microbiota at an early stage significantly influences the mice hippocampal monoamine neurotransmitter genes. Moreover, these genes whose expression was modulated by commensal microbiota were highly associated with anxiety-like behaviors; the introduction of microbiota into adolescent GF mice could normalize the expression of ~53% of aberrantly expressed genes. Coincidentally, chronic administration of probiotics to GF mouse model significantly increased the levels of both serotonin and dopamine in the striatum and induce changes in anxiety and depression-like behaviors [48,49]. These findings suggest that the expression of monoamine neurotransmitter-related genes may be greatly impacted by the absence of commensal microbiota during early life, which may be associated with the development of neuropsychiatric disorders.

GF status has been most consistently associated with anxiolytic phenotype, which were usually viewed as the hallmark of brain development and behavioral functions. Accompanied with the anxiolytic behaviors, there exist abnormal expression of neurobiochemical in GF stages [11,50–52]. Here, we found that the differently expressed genes which exist in dopaminergic synapse and serotonergic synapse (Fig. 4) could be divided into four types: monoamine neurotransmitter receptor, monoamine neurotransmitters transporters & metabolism, monoamine signal transduction pathway, and monoamine neurotransmitter gene targets. Our results demonstrated that commensal

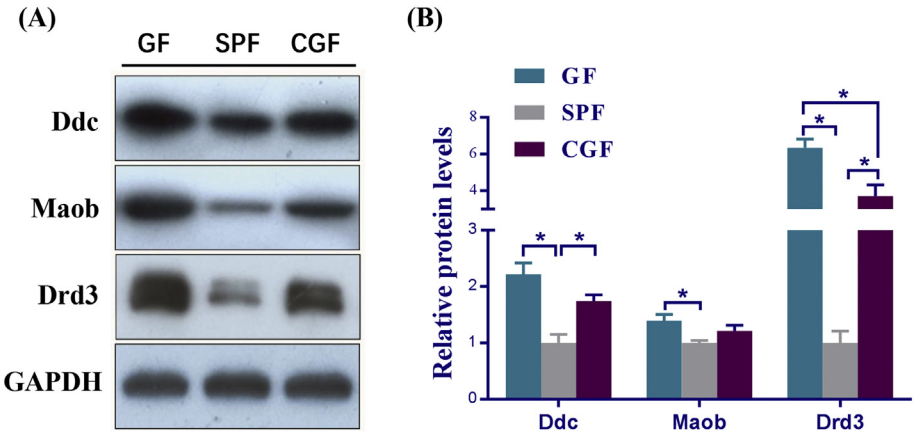

Fig. 6. Effect of the commensal microbiota on hippocampal Ddc, Maob, Drd3 expression.
Immunoblotting analysis of Ddc, Maob, Drd3 expression in the hippocampus of GF mice, CGF mice, SPF mice ($n = 4$/group). GAPDH was used as an internal control. (A) shows a representative Western blot result. (B) illustrated the fold difference of integrated absorbance of protein after normalization with GAPDH.
Data represent the mean ± SEM. *indicates $P < .05$.
Abbreviation: GF, Germ-free; CGF, colonized GF; SPF, specific-pathogen-free; Ddc, dopa decarboxylase; Maob, monoamine oxidase B; Drd3, dopamine receptor D3.

microbiota influences the whole monoamine neurotransmitter system. Disruption of monoamine neurotransmitter metabolism leads to diverse neurological manifestations in childhood, such as neurodevelopmental delay, pyramidal and extrapyramidal motor disorders, epilepsy, and neuropsychiatric features [53].

The bidirectional interaction between the gut microbiota and the brain occurs through various pathways including the serotonin neurotransmitter, neurotrophin, Hypothalamus-Pituitary-adrenal Axis, the immune system [54]. Consistent with our study, previous studies showed that central nervous system neurotransmission can be profoundly disturbed by the absence of gut microbiota, [11,55]. Gut microbiota plays an important role in determining the production and homeostasis of serotonin [15,56]. A few clinical and experimental clinical studies [57–59] have shown beneficial effects for probiotics or antibiotics administration on symptoms of patients with neuropsychiatric disorders, that could potentially be attributed to the change in gut microbiota. Our findings combined with previous GF animal studies consistently indicate that commensal microbiota may contribute to the onset of neuropsychiatric disorders by modulating the expression of hippocampal monoamine neurotransmitter system genes. Moreover, we found that the colonization of adolescent GF mice by microbiota cannot effectively normalize the aberrant expression of some genes and altered behavioral phenotypes. Consist with the protocol used in this study, previous studies demonstrated difference of the microbiota existed between the recipient and donor of gut microbiota [11,30,31]. These findings suggest that the absence of commensal microbiota may exert profound and sustained effects on the expression of hippocampal monoamine neurotransmitter genes, which could provide much valuable information for researchers to find out how the gut microbiota affects the neuropsychiatric disorders and vice versa.

There are some limitations that should be acknowledged. First, based on the differently expressed monoamine genes, targeted metabolomic studies should be performed to validate the assumed pathways. Second, the microbiota colonization was only performed using 5–6-week-old mice, while age can influence colonization patterns according to previous study [60]. A future study will have to conduct colonization in different periods, including neonatal, breast-fed, and weaning stage, to evaluate the effect of different time point intervention on the related genes. Behavior and gene expression of older mice should also be examined for long-term effects. Third, with locomotor activity difference existing in OFT, the NSF test results need to be validated in the future studies. Further studies involving the systematic performance of behavioral tests of GF mice are thus warranted.

5. Conclusion

In this study, we demonstrated the great impact of commensal microbiota on hippocampal monoamine neurotransmitter system genes, which may be involved in the development of neuropsychiatric disorders. The absence of gut microbiota could result in the reduced anxiety-like behaviors in GF mice and altered expression of hippocampal monoamine neurotransmitter system, which could not be reversed by colonizing with gut microbiota. Our results could provide original and valuable data for researchers to further study the microbiota-gut-brain axis.

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Author contributions statement

Conception and design of the study: PX, HW. Performed the experiments: JXP, FLD, WWL, BMY. Analyzed the data: HHW, PZ, FLD, JW. Collected samples: JXP, MXD, YYL. Drafting the article: JXP, PZ. Revision of the manuscript: JXP.

Conflict of interest

All authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jns.2019.03.027.

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