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Research Article

# Changes of Dopamine and Tyrosine Hydroxylase Levels in the Brain of Germ-free Mice

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**Background:** Dopamine (DA) is one of the most important catecholamine neurotransmitters in the central nervous system. The degeneration and deletion of dopaminergic neurons are closely linked to Parkinson's disease (PD) and other psychiatric or neurological diseases. Several studies have been suggesting that intestinal microorganisms are associated with the occurrence of central nervous diseases, including diseases that are closely related to dopaminergic neurons. However, the intestinal microorganism's regulation of dopaminergic neurons in the brain is largely unknown.

**Objectives:** This study aimed to investigate the hypothetical differences of DA and its synthase tyrosine hydroxylase (TH) expression in different parts of the brain of germ free (GF) mice.

**Materials and Methods:** Several studies in recent years have shown that commensal intestinal microbiota promotes changes in DA receptor expression, DA levels, and affects this monoamine turnover. Germ free (GF) and specific pathogen free (SPF) C57b/L male mice were used to analyze TH mRNA and expression levels, and DA levels in the frontal cortex, hippocampus, striatum, and cerebellum, using real time PCR, western blotting, and ELISA tools.

**Results:** Compared with SPF mice, the TH mRNA levels were decreased in the cerebellum of GF mice, while the TH protein expression was tended to increase in the hippocampus, and conversely showed significant decrease in the striatum. The average optical density (AOD) of TH immunoreactive nerve fibers and the number of axons in striatum of mice in GF group were significantly lower than that in SPF group. Compared with SPF mice, the DA concentration in the hippocampus, striatum and frontal cortex of GF mice was decreased in GF mice.

**Conclusion:** The changes of DA and its synthase TH in the brain of GF mice showed that the absence of conventional intestinal microbiota had certain regulatory effects on central dopaminergic nervous system, which is considered helpful for studying the effect of commensal intestinal flora on diseases related to impaired dopaminergic nerve system.

*Key words.* Catecholamine neurotransmitters, Dopaminergic nervous system, Intestinal microorganisms

## 1. Background

Dopamine (DA) is one of the most important catecholamine neurotransmitters in the central nervous system, playing an important role in the regulation of brain's activity, cognition, emotion, positive reinforcement, ingestion, endocrine and many other functions (1). Tyrosine hydroxylase (TH) is an initial enzyme in DA biosynthesis and is also a rate-limiting enzyme. In the animal brain, TH is mainly distributed in the dopaminergic neurons, and its degeneration and deletion can lead to Parkinson's disease (PD) (2). Parkinson's disease (PD) is a common neurodegenerative disorder

characterized by progressive physical limitations such as tonicity, bradykinesia, tremor, and functional impairment. So far, PD has been diagnosed mainly by clinical motor symptoms (3). Also, Dopaminergic dysfunction is closely linked to drug addiction, learning and memory impairment, attention deficit hyperactivity syndrome and other psychiatric and neurological diseases (2, 4).

In recent years, several studies have suggested that intestinal flora is associated with the occurrence of a variety of central nervous system diseases (5), including the diseases that are closely related to dopaminergic neurons. There are many studies that have been reporting a correlation between intestinal flora and PD. For instance, the abundance of prevotella in the feces of PD patients was significantly decreased (6). The decreased prevotella abundance and the increased enterobacter abundance were associated with the severity of postural instability and gait difficulty. Forsyth (7) reported that the intestinal permeability of patients with newly diagnosed PD in the PD group was significantly higher than that in the control group, and this change was related to the effect of Escherichia coli on intestinal mucosa. These examples suggest that the intestinal flora disorders are closely related to the pathogenesis of PD. Through clinical observation, it has been found that there is also a certain relationship between the intestinal flora and drug addiction. In this context, studies revealed that a long-term use of morphine could change the composition of intestinal flora, impair the intestinal barrier function, and lead to bacterial translocation, causing tolerance of the body to opioids (8, 9). In addition, antibiotic use has cleared the intestinal flora in mice resulting in an increase in their sensitivity to cocaine (10). Moreover, the intestinal microflora could affect the nervous system by altering hippocampal neurogenesis in adult mice, thus affecting learning as well as memory (11, 12). We also found that the microbial diversity (α diversity) of patients with attention deficit hyperactivity disorder (ADHD) is significantly reduced, and these patients had elevated levels of bacteroides (13). All these evidences strongly support that the gut microbiota is key to the pathophysiology of these neuropsychiatric disorders with impaired dopaminergic neurotransmission.

## 2. Objective

However, it is still unclear whether the intestinal flora affects the dopaminergic nervous system. So far, only a few studies have investigated how the intestinal microorganisms regulate DA and TH in the brain. Several studies in recent years have shown that commensal intestinal microbiota promotes changes in D.

A receptor expression, DA levels, and affects this monoamine turnover. Diaz Heijtz *et al*. observed a higher striatal dihydroxyphenylacetic acid/dopamine (DOPAC/DA) ratio in adult germ-free mice compared to specific pathogen-free (SPF) controls, in addition to higher D1 mRNA expression in the hippocampus of germ-free mice, while lower transcript levels of this receptor were found in the striatum and NAcc compared to SPF controls (14). Furthermore, Crumeyrolle-Arias *et al*. observed a significant decrease in DOPAC and DA in the frontal cortex (15). The above studies suggest that the presence or absence of conventional intestinal microbiota may have a significant impact on the dopaminergic nervous system. Therefore, this study aimed to explore whether there are any abnormalities in DA and TH in different parts of the brain of sterile mice by comparing germ free (GF) mice with specific pathogen-free (SPF) mice.

## 3. Materials and Methods

### *3.1. Materials*

GF and SPF C57BL/6 mice (male, 6-week-old) were obtained from the Slac Laboratory (Shanghai, China). The GF mice were placed in a sterile isolator and tested for aseptic conditions weekly by a microscope and by aerobic and anaerobic culture of fresh fecal samples. All GF mice were supplied with autoclaved water and fed a regular G-irradiation (50 kGy) diet (Shanghai Institute of Biochemistry and Cell Biology). The mice were maintained at 20-24 °C and a 12-hour light/dark cycle (07:30 turn on the light) with a humidity of 55-65%. All experiments were conducted in accordance with relevant guidelines and regulations, and this study was approved by the Animal Welfare and Ethics Group of the Ministry of Experimental Animal Science, Fudan University (201901005Z).

Eight C57BL/6 mice were involved in the experiment, 4 in GF group and 4 in SPF group. At the time of sampling, each mouse was euthanized by cervical dislocation, and the brain was quickly removed and freeze-dried for TH mRNA, western blotting and DA ELISA assay with three independent experiments.

The frontal cortex, hippocampus, striatum, and cerebellum were dissected on ice, weighed and then frozen. All brain samples were stored in a sterile cryopreservation tube, and then were stored at -80 °C until analysis.

*3.2. RT-PCR Procedures*

Total RNA was extracted from the brain tissues by Trizol reagent (Invitrogen). According to the manufacturer's instructions of RT-PCR kit (KAPA Biosystems), the upstream and downstream primers of each index were 1 μL, and the total reaction system was 20 μL. Actin was used as an internal reference, the upstream primer sequence of TH was GCCAGTCCGTTCCTTCAAGA, and the downstream primer sequence was GCCAGT CCGTTCCTTCAAGA. The upstream primer sequence of actin was GAGATTCACCAGC; and the downstream primer sequence was ATGTCACCACCGATC. The reaction conditions of RT-PCR were as follows: pre-degeneration at 95 °C for 10 min, 1 cycle; degeneration at 95 °C for 10 s, annealing at 59 °C for 30 s, extension at 72 °C for 15 s, with a total of 45 cycles; extension at 70 °C for 10 min after the last cycle. After that, the amplification was carried out on 1% agarose gel electrophoresis. The integrated optical density (IOD) of target RNA amplification products and internal reference actin amplification products were analyzed by gel imaging system, and the ratio of the two (IOD target gene/IOD actin) was calculated as the relative expression of target RNA.

*3.3. Western Blotting*

The total protein of all parts of the brain was extracted by treating with RIPA lysis buffer. The concentration was determined by bicinchoninic acid (BCA). Equal amount of protein was obtained for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The protein was then transferred onto the PVDF membranes. The membrane was sealed at room temperature for 1 hour in 5% skimmed milk, and then was hybridized overnight at 4 °C with anti-TH antibody (abcam 1:1000). The membrane was then hybridized with sheep anti-mouse antibody (Jackson 1:2000) labeled with horseradish peroxidase (HRP), and then was hybridized at room temperature for 1 hour. The membrane was exposed, developed and fixed in chemiluminescent solution. β-Actin antibody was used as an internal reference.

*3.4. Immunohistochemistry*

After dehydration, permeabilization, wax-dip, embedding, sectioning, bake sheet, dewaxing with xylene, and dehydration with gradient alcohol followed by fixation with 4% paraformaldehyde for 12 h, the brain specimens were sliced into 5 μM sections. Three separate slices were collected from four parts of each mouse (frontal cortex, hippocampus, striatum, and cerebellum). The sections were processed with immunostaining for TH (rabbit anti-tyrosine hydroxylase, Abcam, 1:1000 dilution) according to SP (streptavidin-peroxidase) method, diaminobenzidine (DAB) staining, hematoxylin re-staining and neutral gum sealing. TH positive cells were stained with cytoplasm, while DAB staining showed brown.

*3.5. Image Analysis*

Three random microscopic fields in each section from each group were viewed photographed with the ×20 under the microscope (Nikon, CI-S). As many tissues as possible filled the whole field of vision to ensure that the background light of each picture was consistent. The TH positive cells or nerve fibers were selected from each field under a 200-fold field of view, the percentage of positive cells was counted, and the average value for both sides was taken. Image Pro Plus 6.0 (media cybernetics, Inc., Rockville, MD, USA) image analysis software was used to analyze and calculate the integrated optical density (IOD) and average optical density (AOD) of TH positive staining area by selecting the typical brown color as the uniform criterion for positive judgment. AOD = IOD / measurement area. Image pixels were set to 2448*2048. Experiment and counting were performed without knowing the condition identities (blind).

*3.6. Enzyme-Linked Immunosorbent Assay (ELISA)*

DA ELISA kit (LSBio, No. LS-F39204) was used for analysis. According to the instructions, the protein levels of DA in brain tissue were measured. The brain tissues in the striatum, hippocampus, frontal cortex and cerebellum were distinguished on ice, and were cut into tiny fragments. Each tissue was then weighed, and the lysis buffer (200 μL) was added per 20 mg tissue. The mixture was then centrifuged at 12000 rpm for 5 minutes. After that, 10 μL of supernatant was removed, and was diluted 10 times with 10% PBS. The supernatant (10 μL) was then taken for determining the OD value and content. The

DA concentration was expressed by ng.g$^{-1}$.

*3.7. Statistical Analyses*

The data of the current study were analyzed using SPSS software version 13.0 (IBM Corporation, Armonk, NY, USA). Mean differences of parametric values between groups were compared by independent t-test. Mann-Whitney test was used to analyze non-normal distributed data. Based on the two-tailed analysis, results with a P value less than 0.05 were statistically significant (2).

## 4. Results

*4.1. Lower TH mRNA Expression in the Cerebellum of GF Mice*

The mRNA levels of TH in the cerebellum showed a significant difference between the two groups (P<0.05), but no significant differences were observed in the frontal cortex, hippocampus and striatum between the two groups. Compared with SPF control group (1.37±0.27), the mRNA expression of TH in cerebellum of GF mice was decreased (0.63±0.08, P < 0.05), (**Fig. 1**).

*4.2. Higher TH Protein Expression in the Hippocampus and Lower in the Striatum of GF Mice*

The TH expression in the hippocampus and striatum showed significant differences between the two groups (P<0.05), but no significant differences were observed in the TH expression in the frontal cortex and cerebellum. Compared with SPF group (hippocampus: 0.0612, striatum: 1.511±0.08), the TH expression in the striatum of GF mice was decreased (0.632±0.181, P < 0.05), and was significantly increased in the hippocampus (0.6986), (P<0.05, **Fig. 2 and Fig. 3**)

*4.3. Lower Immunostaining Cells in GF Mice Brain*

In general, brain species in SPF group showed more TH immuno-positive cells and more conspicuous coloration than GF group (**Fig. 4, Supplementary 1 and 2**). The average optical density (AOD) of TH immunoreactive nerve fibers in striatum of mice in GF group was significantly lower than that in SPF group (P=0.004, **Fig. 5**, **Supplementary 1 and 2**). The number of axons under high magnification of 400X was also significantly lower than that in SPF group (P=0.002, **Fig. 6**, **Supplementary 1 and 2**).

*4.4. Lower DA Concentration in the GF Mice Brain*

DA concentrations in the frontal cortex, striatum and hippocampus of mice in GF group were (247.8 ±78.77) ng.g$^{-1}$, (724.1 ±68.6) ng.g$^{-1}$ and (557.6 ±160.9) ng.g$^{-1}$g, respectively, while those in SPF group were (803.4 ±199.1) ng.g$^{-1}$, (1301.1 ±188.8) ng.g$^{-1}$ and (1430.2±279.6) ng.g$^{-1}$, respectively. These results suggested that the DA concentration in GF group was significantly lower than that in control group, showing significant difference in the between the two groups (P<0.05). (**Fig. 7**).

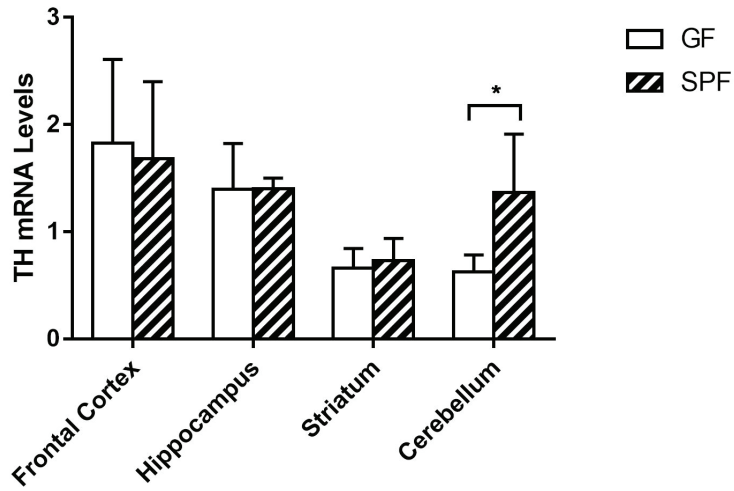


**Figure 1. TH mRNA expression in frontal cortex, hippocampus, striatum and cerebellum of GF and SPF mice.** Compared with SPF mice, GF mice had a higher TH mRNA expression in the cerebellum area (n = 4). **P<0.05 compared with SPF mice.

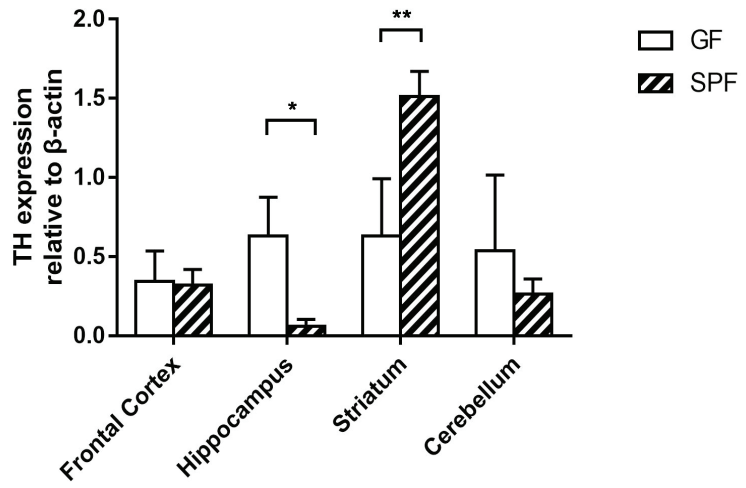


**Figure 2. GF mice showed higher expression of TH proteins in the hippocampus and lower expression in the striatum** (n = 4). *P<0.05 compared with SPF mice. **. P<0.01 compared with SPF mice

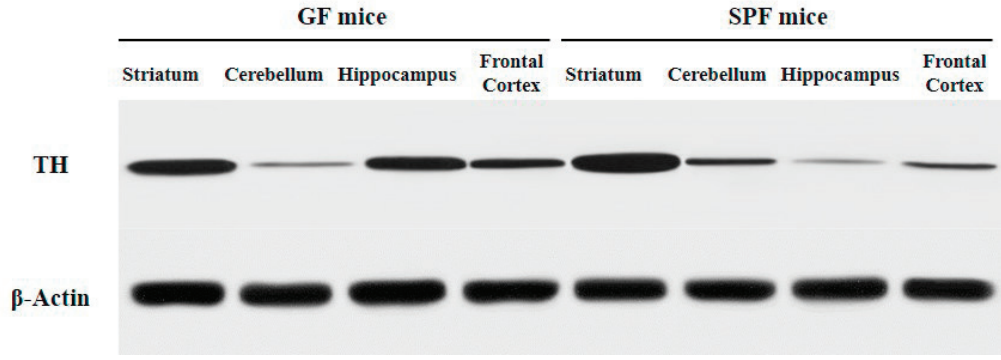


**Figure 3. Representative Western blot films for TH and β-Actin protein expression in the striatum, Cerebellum, hippocampus and frontal cortex of GF and SPF mice** (for further details, see Fig.2).

## 5. Discussion

This study supported the fact that the normal intestinal microorganisms influence the changes in the brain's dopaminergic nervous system. The results of this study suggested that compared with SPF mice, the TH mRNA expression in the cerebellum of GF mice was decreased, while the protein expression level in the striatum of GF mice showed significant decrease in the striatum. Compared with SPF mice, the DA concentration in the hippocampus, striatum and frontal cortex of GF mice was decreased. The above results suggested that the dopaminergic nervous system in different parts of the brain might be affected by intestinal flora.

In animal brain, TH is mainly distributed in neurons containing catecholamine, which is considered as a key enzyme in DA synthesis pathway. Also, it is associated with the occurrence of many neurological diseases, such as PD, schizophrenia, bipolar disorder, hyperactivity disorder and so on (16). Current studies have shown that normal intestinal flora mainly affects the central nervous system through three pathways of gut-brain axis (immunity, endocrine and vagus nerve) (17). For example, the intestinal flora imbalance releases intestinal pro-inflammatory cytokines, leading to inflammation

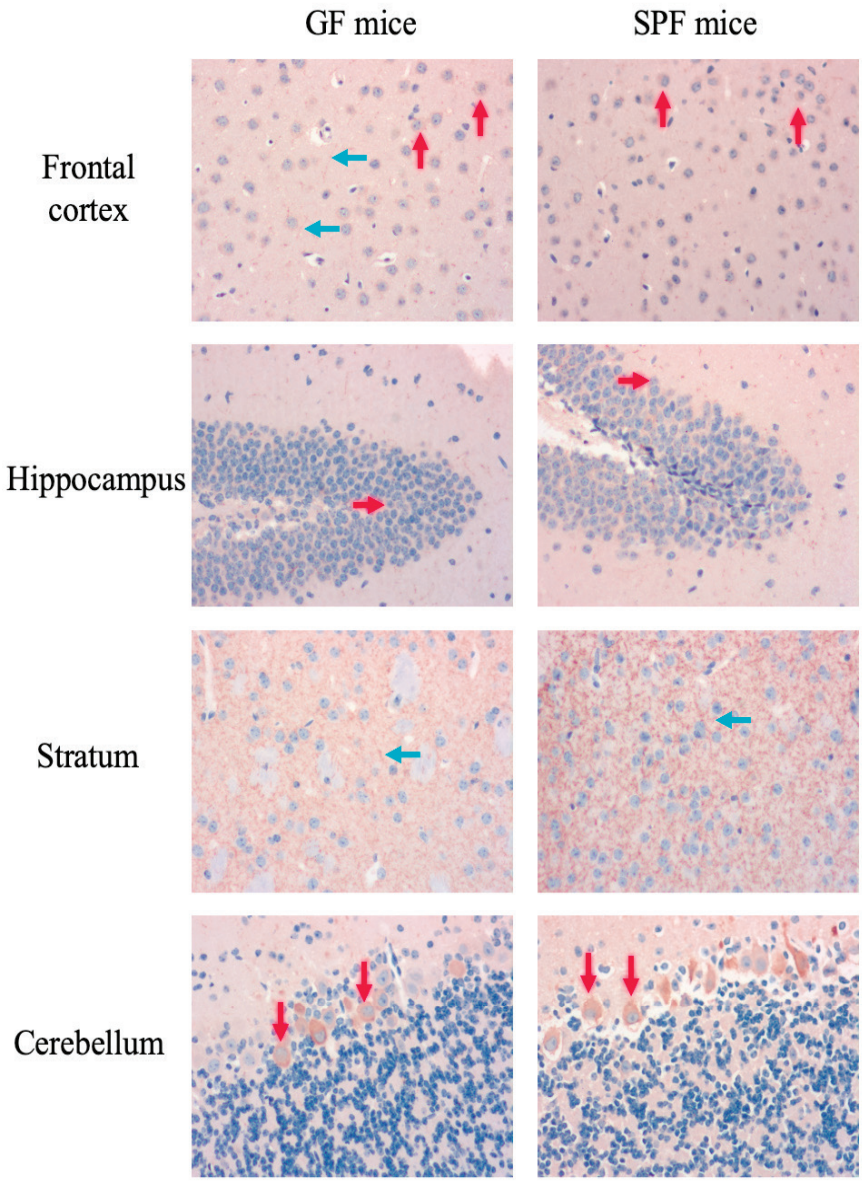


**Figure 4. Representative images of TH immunohistochemistry** (magnification, x400; Pixels: 2488 × 2048). Red arrows indicate the stained neurons and blue arrows indicate the stained neurites.

(18). On one hand, the cytokines produced by intestinal flora enters the circulatory system through intestinal mucosa, directly affecting the brain function through the blood-brain barrier transport system (19, 20). On the other hand, there are microglia in the brain parenchyma, white blood cells in cerebrospinal fluid, macrophages and dendritic cells in the periventricular, choroid plexus and meninges, expressing TLRs on the surface of these cells. These in turn generate microorganism-associated molecular patterns (MAMPs), releasing cytokines to produce neurotoxic effects (21, 22). In addition, the intestinal tract is considered to be the largest endocrine organ in the human body. Intestinal microbial flora can regulate the secretion of multiple hormones by intestinal endocrine cells and then the information exchange occurs between the intestine and the brain (23). Furthermore, intestinal microorganisms and their metabolites can enter the central nervous system through the vagus nerve by affecting the intestinal nervous system (24).

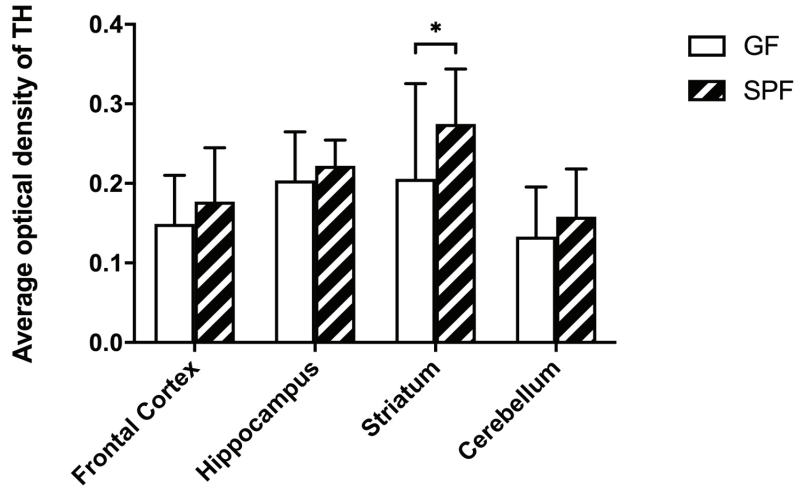


**Figure 5.** Quantitative analysis of TH immunoreactive neurons and fiber showed that the average optical density (AOD) of TH immunoreactive nerve fibers in striatum of mice in GF group was significantly lower than that in SPF group (P=0.004, n = 4).

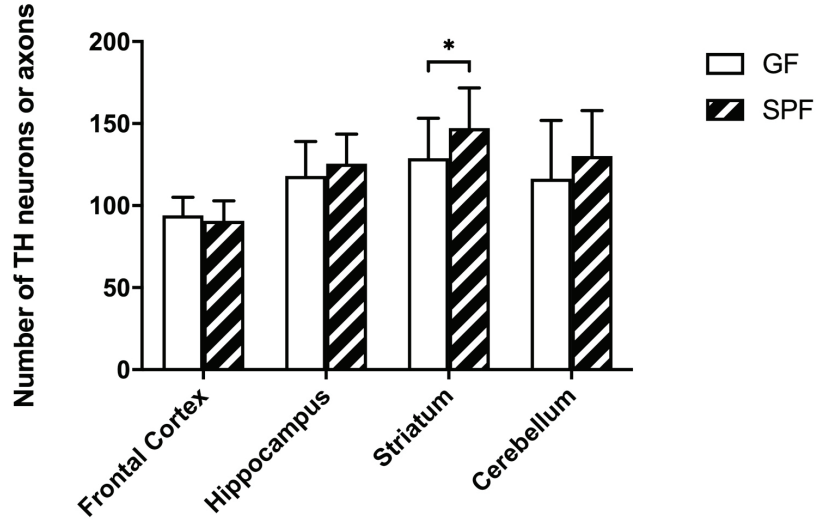


**Figure 6.** The number of TH-positive neurons or axons in the striatum areas was higher in SPF mice than that in of GF group (P=0.002, n=4).

The intestinal flora may affect the TH in the nervous system by the above three pathways, where each pathway might be parallel and interact with each other. Although the above mechanisms might help explain the changes of TH in the brain caused by intestinal flora, they cannot explain the different trends in specific areas. This study found that the TH mRNA expressions in the cerebellum and the protein expression level in the striatum of GF mice were down-regulated, while the protein expression level in the hippocampus was up-regulated. Previous studies have suggested that the inflammatory response induced by microglia can lead to the damage of dopaminergic neurons (25). The distribution of microglia in the central nervous system is specific to regions, and there are different microglial phenotypes in different regions of the central nervous system of healthy adult mice (26, 27). Moreover, previous studies have found that the microglia interact with various components of the microflora-intestine-brain axis (28, 29). Therefore, microglia might be involved in the changes of TH in the intestinal microbial-induced central nervous system in specific regions.

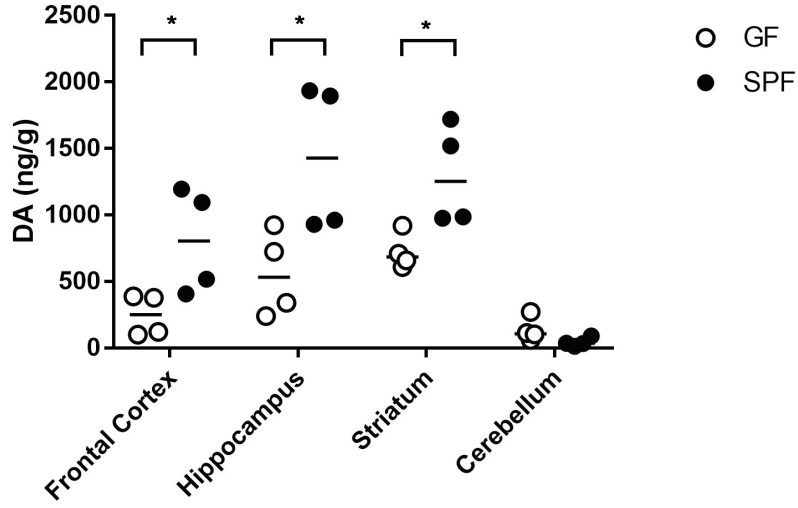


**Figure 7. DA concentrations in the SPF mice and GF mice were measured with described methods.** DA concentration of the frontal cortex, striatum and hippocampus in GF mice was significantly lower than that in SPF mice (n = 4). *P<0.05 compared with SPF mice.

This study also suggested that the intestinal flora might also affect the changes of DA concentrations in different parts of the brain. According to a study, the changes of DA concentration in the brain of GF mice originated from the changes of DA synthase (15). However, the changes of TH and DA in different brain regions were not synchronized in this study, considering that these changes might be related to different conversion rates of DA in the brain. Nishino *et al*. observed that the DA conversion rates in the prefrontal cortex, plasma, striatum and brainstem were decrease in male BALB/c GF mice (30). Interestingly, Diaz Heijtz *et al.* reported high conversion rates of NE, DA and 5-HT in the striatum of male NMRI mice (14). Therefore, the effects of animal genetic background and intestinal microflora on DA concentration and conversion rate in the brain require further studies. Interestingly, it has been demonstrated that chronic impairment of vagus nerve function led to a decrease in the DA activity in the striatum (31). Lactobacillus PS128 may increase DA concentration in the striatum of GF mice through vagus nerve pathway (32). Therefore, we speculated that the changes of DA concentration observed in various brain regions might be mediated by vagus nerve activity, and this hypothesis needed further confirmation.

## 6. Conclusion

The changes of DA and its synthase TH in the brain of GF mice showed that the absence of intestinal microbiota had certain regulatory effect on central dopaminergic nervous system, which is considered helpful for studying the effect of commensal intestinal flora on diseases related to impaired dopaminergic nerve system. In future, our team will continue to study the mechanism of this regulation and further explore the relationship between dopaminergic nervous system and intestinal flora.

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**Conflicts of Interest**

All of the authors report no conflicts of interest in this work.

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