Gut microbiota regulates autism-like behavior by mediating vitamin B6 metabolism in EphB6-deficient mice

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Abstract
Background Autism spectrum disorder (ASD) is a developmental disorder with limited effective pharmacological treatments for the core autistic symptoms so far. Increasing evidences, especially the clinical studies in ASD patients, suggest a functional link between gut microbiota and development of ASD. However, the mechanisms linking gut microbiota and brain dysfunctions (gut-brain axis) in ASD are still not well-established. With genetic mutations and down-regulated expression in patients with ASD, EPHB6, which is also important in homeostasis of gut, has been generally considered to be a candidate gene for ASD. Nonetheless, the role and mechanism of EPHB6 involved in regulating gut microbiota and development of ASD have been unclear.

Results Here, we found deletion of EphB6 induced autism-like behavior and disturbed gut microbiota in mice. More importantly, transplanting fecal microbiota from EphB6-deficient mice resulted in autism-like behavior in antibiotics-treated C57BL/6J mice. Meanwhile, transplanting fecal microbiota from wild-type mice ameliorated autism-like behavior in EphB6-deficient mice. At the metabolic levels, disturbed gut microbiota led to vitamin B6 and dopamine defects in EphB6-deficient mice. At the cellular levels, excitation/inhibition (E/I) imbalance in medial prefrontal cortex was induced by gut microbiota-mediated defects of vitamin B6 metabolism in EphB6-deficient mice.

Conclusions Our study uncovers a key role for gut microbiota in regulation of autism-like social behavior by mediating vitamin B6 metabolism, dopamine synthesis and E/I balance in EphB6-deficient mice, suggesting new strategies for understanding and treatment of ASD.

Background
Autism spectrum disorder (ASD), affecting approximately 1% of the population around the world, is mainly characterized with impaired social interaction and communication, and restricted and repetitive behavior [1]. So far, although early behavioral and educational interferences show effective ameliorative roles on autistic symptoms of ASD patients, there are limited effective pharmacological therapies for the treatment of core autistic symptoms [1, 2].

Accumulating evidences show that gut-brain-microbiota axis plays a key role in regulating homeostasis of human body. Gut microorganisms have been reported to participate in a lot of
neuropsychiatric disorders, such as anxiety disorders, depression [3] and epilepsy [4]. In most ASD patients, changed gut microorganisms and serious gastrointestinal problems are observed [5–7]. Interestingly, several studies have found the important role of gut microbiota in modulating ASD-like phenotypes of mice [8–10]. A clinical study shows that microbiota transfer therapy can improve gastrointestinal problems and autistic symptoms of ASD patients, who are 7 to 16 years old, and this benefit can last for two years [11, 12]. These studies suggest that gut-brain-microbiota axis may have a significant impact on the development of ASD. However, how gut microbiota contributes to dysregulation of brain function is still not well-characterized.

EPHB6, belonging to the Eph family of receptor tyrosine kinases, locates on chromosome 7q. In 1998, a two-stage genome research on susceptibility loci in autism found transcripts mapping to the chromosome 7q region predisposing to autism, including EPHB6 [13]. After that, more studies suggested EPHB6 as a candidate of ASD-associated-gene [14–16] and recent genomic studies have found the mutation of EPHB6 in some ASD patients [17, 18]. Most importantly, EPHB6 has been found to be with a down-regulated expression in ASD patients in transcriptome analysis [19, 20]. Although EPHB6 plays an important role in regulating Eph receptor signaling networks, T cell functions, development of intestinal epithelium and epithelial homeostasis [21–23], the role and mechanisms of EPHB6 involved in regulating gut microbiota and ASD are still unclear.

In our study, we find EphB6 is functionally associated with ASD and regulates autism-like social behavior by gut microbiota-mediated vitamin B6 and dopamine metabolism. More importantly, we establish the functional link between dysregulated gut microbiota and excitation/inhibition (E/I) imbalance in medial prefrontal cortex (mPFC), a key gut-brain functional axis, in EphB6-deficient mice.

**Results**

Deletion of EphB6 led to autism-like behavior and gut microbial disturbance in mice

As a candidate gene associated with ASD, whether and how EPHB6 works in ASD is still unclear. To answer these questions, we established EphB6 knockout mice. We found a deletion of EphB6 in different tissues, including colon, brain, lung and spleen, in EphB6 knockout mice (KO mice) compared
with EphB6\textsuperscript{+/+} (wild-type, WT) mice (Additional file 1: Figure S1c-d). However, the weight of brain and body, the length of body and daily dietary consumption were similar between the two groups of mice in spite of the deletion of EphB6 (Additional file 1: Figure S1e-h).

Patients with ASD often display repetitive stereotyped behavior and social deficits. Interestingly, we found KO mice spent more time on self-grooming compared with WT mice (Fig. 1a). While in marble burying test, KO mice buried similar marbles with WT mice (Additional file 1: Figure S1j). In social partition test, KO mice spent less time on sniffing at partition, no matter the familiar or novel mouse was put in, than WT mice (Fig. 1b). In three-chambered social approach task, KO mice spent similar time in chambers with an unfamiliar mouse or inanimate object (Fig. 1d), also KO mice showed less preference for the social mouse (stranger 1) over the object than the WT mice (Fig. 1f-g). While the novel social partner (stranger 2) was put into the empty wire cage, KO mice still spent similar time in the two chambers (Fig. 1e) and showed less preference for the novel mouse over the familiar mouse than WT mice (Fig. 1h). These results confirmed the abnormalities of social interaction in KO mice sufficiently. Olfactory cues have been generally considered to be of the most importance in communication among mice [24, 25]. In olfactory habituation/dishabituation test, repeated presentation of cotton swabs saturated by same odor caused less and less time spent sniffing at cotton swabs and presentation of cotton swab saturated by a new odor caused increased time spent sniffing both in WT and KO mice. However, when introducing a cotton swab saturated with social odor, KO mice showed less interest in social odors than WT mice (Fig. 1i). These results implied the communication deficits in KO mice although the ability to discriminate and habituate different odors was normal.

Patients with ASD are often accompanied with other mental diseases, such as hyperactivity, anxiety and intellectual disability. In open field test, KO mice showed same locomotor activities and spent almost same time in center area compared with WT mice (Additional file 1: Figure S1k-l). While in elevated-plus-maze test, KO mice spent less time in open arm and more time in closed arm compared with WT mice (Fig. 1j), which implied that KO mice displayed anxiety-like behavior. In morris water maze, KO mice had the normal spatial learning and memory as the WT mice (Additional file 1: Figure
Collectively, mice with deletion of EphB6 showed autism-like behavior, including stereotyped behavior and social deficits, accompanied with anxiety-like behavior, but no evidence of intellectual disability.

Eph/ephrin signaling has been reported to modulate gut epithelial development and homeostasis. Also, it has been generally accepted that many ASD patients have gastrointestinal (GI) symptoms [5, 6, 26] and a changed composition of gut microbiota [7]. Then we want to know whether mice with deletion of EphB6 will suffer from GI problems. To measure intestinal permeability by FITC-dextran, we found the significantly increased intestinal permeability in EphB6-deficient mice compared with WT mice (Fig. 1k). Accordingly, the mRNA expression of Cldn4, one member of tight junction molecules, was decreased in colon of KO mice compared with WT mice (Fig. 1l). In addition, we detected that mRNA expression of IL-1β, as a proinflammatory factor, was dramatically increased and IL-6, which has anti-inflammatory effect, was decreased in colon of KO mice compared with WT mice (Fig. 1m). GI problems in KO mice was not accompanied with morphological changes in small intestine, colon, kidney, liver, thymus and lung (Additional file 1: Figure S1p).

The integrity of intestinal mucosa was important to maintain the balance of ecological environment in animals’ gut. Then we detected the fecal microbial populations of mice using 16S rRNA gene sequencing. There were no differences in microbial species richness and diversity between the two groups (Fig. 1n-o). Notably, principle coordinates analysis of Bray-Curtis distance showed fecal microbiota of KO mice clustered differently from WT mice (Fig. 1p), which predicted a different gut microbial composition between the two groups. In phylum level, the differences between the two groups were caused by the decreased Deferribacteres and Proteobacteria in fecal microbiota of KO mice (Fig. 1q). In general, our results indicated deletion of EphB6 resulted in increased intestinal permeability and changes of gut microbial composition in mice.

Many studies have referred that GI problems and abnormal behavior of ASD always appear parallelly in patients [5]. Then we wondered who came first in KO mice. We found the microbial species richness and diversity were also similar between WT and KO mice which were 3-week-old or 4-week-old (Additional file 1: Figure S2a-b). In principle coordinates analysis, gut microbiota of 4-week-old KO
mice clustered differently from 4-week-old WT mice (Additional file 1: Figure S2d), while gut microbiota of 3-week-old WT and KO mice clustered similarly (Additional file 1: Figure S2c). At the same time, 4-week-old KO mice, but not 3-week-old KO mice, showed increased self-grooming and decreased interest in social odor compared with even-aged WT mice (Additional file 1: Figure S2e-g). These results further implied the possible relation between the abnormal behavior and gut microbial dysbiosis in mice with deletion of EphB6. Transplantation of fecal microbiota from EphB6-deficient mice caused autism-like behavior in SPF C57BL/6J mice

ASD is generally considered to be a neuro-developmental disorder, postnatal developmental disorder can also cause autism in patients [27], and postnatal mutation of Nrxn1 in neurons led to autism-like behavior in mice [28]. Also gut microbiota of ASD patients could induce autism-like behavior in mice [7]. So, to study the relation between gut microbial dysbiosis and autism-like behavior in mice with deletion of EphB6, we gavaged the fecal microbiota from 8-week-old male WT or KO mice to 3-week-old SPF male C57BL/6J mice for a week (Fig. 2a). Three weeks after the gavage of fecal microbiota, gut microbial composition in SPF C57BL/6J mice treated with fecal microbiota from WT and KO mice was different (Fig. 2b-d). More interestingly, C57BL/6J mice with the gastric perfusion of fecal microbiota from KO mice displayed increased self-grooming (Fig. 2e) and decreased social behavior (Fig. 2f-h) compared with control mice. While in open field test and elevated-plus-maze test, the two groups of mice behaved similarly (Additional file 1: Figure S2h-j). Furthermore, we gavaged orally the suspending solution of fecal microbiota from WT or KO mice to antibiotic-pretreated SPF male C57BL/6J mice. After pretreatment with antibiotics for 5 days, fecal microbiota of 8-week-old male WT or KO mice was gavaged orally to 3-week-old SPF male C57BL/6J mice for 5 days (Fig. 2j). About 2 weeks after fecal microbial colonization, similarly, we found gut microbiota of SPF C57BL/6J mice treated with fecal microbiota from KO mice clustered differently from control mice (Fig. 2k-m). Then we found C57BL/6J mice with gastric perfusion of fecal bacteria from KO mice showed increased self-grooming (Fig. 2n) and decreased social behavior (Fig. 2o-r). Also, the two groups of mice behaved similarly in open field test and elevated-plus-maze test (Additional file 1: Figure S2k-m). What’s more,
fecal microbiota from 4-week-old KO mice, but not 3-week-old, induced increased self-grooming and social deficits in 3-week-old SPF C57BL/6J mice compared with C57BL/6J mice gavaged with fecal microbiota from even-aged WT mice (Additional file 1: Figure S2n-s). Collectively, fecal microbiota from EphB6-deficient mice caused more self-grooming and impaired social behavior in C57BL/6J mice. Then we wondered whether gut microbiota still played the role in autism-like behavior in adult mice. To begin, we gavaged orally a mixture of antibiotics to 6-week-old male SPF C57BL/6J mice for a week. And we found antibiotic treatment disrupted the gut microbiota greatly and induced decreased self-grooming and social deficits in young adult C57BL/6J mice (Fig. 3a-i). These results indicated us that gut microbiota was related with autism-like behavior even in adult mice and different gut microbiota probably contributed to different behaviors, such as self-grooming and social behavior.

Then, we gavaged the fecal microbiota from 8-week-old male WT or KO mice directly to 6-week-old SPF male C57BL/6J mice for a week. And we found fecal microbiota from KO mice also induced disturbed gut microbiota, more self-grooming and social deficits in adult C57BL/6J mice (Fig. 3j-r). Basically, our results indicated the important role of gut microbiota in autism-like behavior, even in adult mice.

Transplantation of fecal microbiota from wild-type mice ameliorated autism-like behavior in adult EphB6-deficient mice

Until now, there has no studies focusing on the effectiveness of microbiota transplantation in adult ASD patients. Then we gavaged orally the fecal microbiota from 8-week-old male WT mice to 8-week-old KO mice for a week. A week later, we found the gut microbiota of KO mice gavaged with fecal microbiota of WT mice clustered differently from KO mice gavaged with sterile PBS (Fig. 4b). In phylum level, we found the relative abundance of Deferribacteres was increased in KO mice gavaged with fecal microbiota of WT mice (Fig. 4c-d). And in species level, we found Mucispirillum, which is a genus in the phylum Deferribacteres, was increased in KO mice treated with fecal microbiota of WT mice (Fig. 4e). Also, fecal microbiota transplantation ameliorated the decreased relative abundance of Prevotellaceae_UCG-001 and the increased relative abundance of Lactobacillales in KO mice (Fig. 4f-g).
Then functionally, we found, after being gavaged with fecal microbiota from WT mice, KO mice showed decreased self-grooming (Fig. 4h) and increased social behavior (Fig. 4i-l). These results indicated that gut microbial dysbiosis was responsible for autism-like behavior in mice with deletion of EphB6.

Gut microbiota-mediated vitamin B6 metabolism regulated social behavior in EphB6-deficient mice

Considering the abnormal behaviors were probably because of the problem of brain, so we tried to figure out how gut microbiota affected brain and subsequently caused autism-like behavior in EphB6-deficient mice.

First, we tried to find the key region of brain affected by dysregulated gut microbiota in mice with deletion of EphB6, which was responsible for autism-like behavior. Studies on ASD patients or mouse models show that hippocampus, cerebellum and mPFC have been implicated in ASD [29, 30]. After being processed with three-chambered social approach task, we found the protein expression of c-Fos was significantly increased in mPFC of KO mice compared with WT mice (Additional file 1: Figure S3a-c). ASD has been generally considered to be caused by an increased ratio of synaptic excitation and inhibition and ASD children exhibit elevations in resting state neuronal activity [31]. So, whether mPFC was modulated by gut microbiota in KO mice needed to be further investigated. Because mPFC tissue was too small for some experiments and we used PFC tissue of mice in our next study.

The first question we asked was whether the bacteria could modulate mPFC directly. Unfortunately, we did not detect bacterial DNA or any bacterial colonies in PFC tissues of WT or KO mice (Additional file 1: Figure S3d-e). Unexpectedly, we found metabolites of gut microbiota from KO mice also induced social deficits in C57BL/6J mice (Additional file 1: Figure S3f-k). Were there some substances had been affected by gut microbial dysbiosis that caused social deficits in KO mice?

To found the metabolites that had been significantly changed, we detected metabolites in target tissue, that is PFC of KO mice, using non-targeted metabolomics strategies. Surprisingly, the metabolites in PFC were significantly different between the two groups of mice using orthogonal partial least squares discriminant analysis (Fig. 5a). KEGG pathway analysis showed 4 pathways that were significantly enriched in the differentially changed metabolites, including vitamin B6 metabolism
pathway because of decreased relative abundance of pyridoxamine (PM) and pyridoxal 5′-phosphate (PLP) in PFC of KO mice (Fig. 5b-d).

Vitamin B6 in body is mainly from diet and gut bacteria's synthesis and then is absorbed in intestine. Then we detected the level of vitamin B6 in feces, blood and PFC of mice. The increased level of pyridoxine (PN) in feces, decreased level of PM and PLP in plasma, and decreased level of PLP in PFC were found in EphB6-deficient mice (Fig. 5e-j). A week after being gavaged with fecal microbiota from WT mice, KO mice had decreased level of PN in feces, increased level of PM and PLP in plasma and increased level of PLP in PFC compared with KO mice gavaged with sterile PBS (Fig. 5e-j). These results indicated that gut microbiota regulated the level of vitamin B6 in feces, blood and PFC of mice. Then we wondered if vitamin B6 supplementation could ameliorate the autism-like behavior of KO mice. However, the intragastric supplementation of vitamin B6 did not ameliorate social deficits of KO mice (Additional file 1: Figure S4a-b). While one hour after being injected with 1 mg PLP intraperitoneally, KO mice had increased level of PLP in plasma (Fig. 5l), and increased social behavior (Fig. 5n-p) compared with control mice. While self-grooming (Fig. 5m) and social novelty (Fig. 5q) were not changed in KO mice after the injection of PLP. Also, the injection of 1 mg or 2 mg PLP intraperitoneally had no effect on social behavior of C57BL/6J mice (Additional file 1: Figure S4c-e). Moreover, after being fed without vitamin B6 for two weeks, C57BL/6J mice had decreased level of PLP in plasma and decreased social behavior (Fig. 5r-u). Conclusively, our results hinted that there was a relation between gut microbiota-mediated defects of vitamin B6 metabolism and social deficits in EphB6-deficient mice.

Gut microbiota-mediated vitamin B6 metabolism regulated dopamine in PFC in EphB6-deficient mice

Then we tried to clarify how the decreased vitamin B6 induced social deficits in mice. Vitamin B6, as a co-factor, has been implicated in more than 140 biochemical reactions in cells, including biosynthesis and catabolism of amino acid and neurotransmitters [32]. As the most important active substances in brain, we first detected the neurotransmitters in PFC of mice by high performance liquid chromatography (HPLC) and found similar levels of glutamate, GABA, glycine, aspartic acid, serine and glutamine, among WT and KO mice gavaged with sterile PBS or fecal microbiota from WT mice
Interestingly, we found a decreased dopamine and an increased 5-HT in PFC of KO mice compared with WT mice (Fig. 6b). When treated with fecal microbiota from WT mice, KO mice had an increase in level of dopamine, but similar level of 5-HT, in PFC compared with KO mice gavaged with sterile PBS. While the level of noradrenaline, epinephrine and DOPAC were similar among the three groups of mice. More excitingly, after being gavaged with fecal microbiota from KO mice, the level of dopamine in PFC of SPF C57BL/6J mice had a decrease compared with C57BL/6J mice gavaged with fecal microbiota from WT mice (Additional file 1: Figure S5a-c). Also, we found the injection of PLP intraperitoneally increased the level of dopamine in PFC of KO mice (Fig. 6c) and deficiency of vitamin B6 decreased the level of dopamine in PFC of SPF C57BL/6J mice (Fig. 6d). Briefly, these results indicated that gut microbiota-mediated vitamin B6 metabolism could affect the level of dopamine in PFC of mice.

To answer whether decreased dopamine contributed to autism-like behavior in EphB6-deficient mice and considering the fast metabolism of dopamine in brain, we injected the agonists of dopamine receptors into mPFC of mice. While deletion of EphB6 had no effect on mRNA expressions of dopamine receptors or tyrosine hydroxylase (Th) in mPFC or ventral tegmental area (VTA) (Fig. 6e).

As dopamine D1 receptor (D1R) had the highest expression in mPFC and then followed by dopamine D2 receptor (D2R) (Fig. 6f), we injected the D1R agonist (SKF38393) or D2R agonist (quinpirole) into mPFC of mice. We found KO mice had increased social behavior (Fig. 6g-j) after being injected with SKF38393 compared with KO mice injected with artificial cerebrospinal fluid (ACSF). While there were not any differences in C57BL/6J mice injected with ACSF or SKF38393 (Additional file 1: Figure S5d-f). Differently, quinpirole did not increase social behavior in KO mice (Additional file 1: Figure S5g-i).

What’s more, D1R antagonist induced a decreased social behavior in C57BL/6J mice (Fig. 6k-n). In short, these results proposed dysregulated gut microbiota and vitamin B6 metabolism led to autism-like behavior by D1Rs-mediated pathway in EphB6-deficient mice.

Gut microbiota regulated E/I balance in mPFC of EphB6-deficient mice

D1Rs are generally considered to modulate GABAergic inhibition in PFC [33]. Also, imbalance between excitation and inhibition (E/I) in synaptic transmission and neural circuits has been implicated in ASD
Moreover, the correction of E/I imbalance can normalize key autistic phenotypes in animal models of ASD [37].

Then to further investigate the cellular mechanism underlying gut microbiota-mediated autism-like behavior in EphB6-deficient mice, we recorded spontaneous excitatory postsynaptic currents (sEPSCs) and spontaneous inhibitory postsynaptic currents (sIPSCs) of mPFC pyramidal neurons, in WT and KO mice treated with sterile PBS or fecal microbiota from WT mice. The amplitude and frequency of sEPSCs were similar among the groups (Fig. 7a-e). The amplitude of sIPSCs was also similar among the groups, while the frequency of sIPSCs was decreased in KO mice and was rescued by transplantation of fecal microbiota from WT mice (Fig. 7f-j). Additionally, we found a decreased frequency of sIPSCs in pyramidal neurons of mPFC in C57BL/6J mice gavaged with fecal microbiota of KO mice (Additional file 1: Figure S6a-h). D1R agonist, at a concentration of 10 µM, increased the frequency of sIPSCs in pyramidal neurons of mPFC in KO mice (Fig. 7k-n), while the same concentration of D1R agonist had no effect on sEPSCs or sIPSCs recorded in pyramidal neurons of mPFC in WT mice (Additional file 1: Figure S6i-l). Collectively, these results indicated that gut microbiota modulated E/I balance, which was possibly regulated by dopamine, in pyramidal neurons of mPFC in EphB6-deficient mice.

Discussion

Increasing evidences, especially the clinical studies in ASD patients, suggest a functional link between gut microbiota and development of ASD. Besides, genomic and transcriptome studies for ASD patients discover many candidate genes for ASD. However, the functions of ASD-associated genes and the mechanisms linking gut microbiota and brain dysfunctions (gut-brain axis) in ASD are still not well-established.

First in our study, we uncover EphB6 is an ASD-associated gene functionally. EPHB6 has been suggested as a candidate gene for ASD for a long time [15–17] and is found to be down-regulated in ASD patients [19, 20]. Here, using our transgenic mouse models, we found deletion of EphB6 induced autism-like behavior in mice that mimicked the core symptoms of ASD patients fairly well. Using whole-genome sequencing and transcriptome analysis, researchers have found more than 1000
genes that are associated with ASD, including EPHB6 [15–18], EPHA1 [15], EPHB2 [38]. Our study uncovers the functional role of EphB6 in ASD and suggests EphB6-deficient mice can be used as a new mouse model of ASD.

Secondly, we find gut microbial dysbiosis is required for autism-like behavior in EphB6-deficient mice. Most ASD patients have serious GI problems [5, 6, 26] and changed composition of gut microbiota [39, 40]. Moreover, microbiota transfer therapy can improve GI and autistic symptoms in ASD children [11, 12]. Our study also suggests the probable role of gut microbiota on treating core symptoms of adult ASD patients. Eph families have the important role in regulating epithelial homeostasis by the interaction with epithelial cell adhesion and junction proteins [22]. Cldn4 can interact with EphA2 and ephrin-B1 to affect the tight junction integration [41, 42]. AF-6 can be recruited to cell–cell contacts in MDCK and 293T cells by interacting with Eph receptors, including EphB6 [43, 44]. The ablation of EphB6 may induce the dysregulated interaction between Eph families and junction proteins that leads to increased intestinal mucosal permeability and then gut microbial dysbiosis in mice.

Thirdly, we find dysfunction of vitamin B6 metabolism is key for gut microbiota-mediated autism-like behavior in EphB6-deficient mice. We found the decreased level of vitamin B6 in plasma and PFC of EphB6-deficient mice was rescued by transplantation of fecal microbiota from wild-type mice. Moreover, injection of vitamin B6 intraperitoneally rescued social deficits of EphB6-deficient mice. More interestingly, PLP has been found to have an unbelievable low level in ASD children compared with controls [45]. Date to 1960s, a lot of clinical studies have used vitamin B6 to treat ASD children, and most studies have reported the improved autistic symptoms in ASD children [46–48]. While there are also reports of the non-effect of vitamin B6 on ASD patients [49]. Considering the complicated causes of ASD, we think vitamin B6 is effective for a part of ASD patients, such as ASD patients with down-regulated expression of EPHB6. Vitamin B6 cannot be synthesized by the body itself and the source of vitamin B6 in body is mainly from diet and bacteria’s synthesis via intestinal absorption. So, normal intestinal functions are important for the homeostasis of vitamin B6 in body. The intestinal absorption of vitamin B6 is pH dependent with higher uptake at acidic compared with alkaline pHs [50]. The more alkaline environment in gut of EphB6-deficient mice (Additional file 1: Figure S1i) may
cause decreased absorption of vitamin B6. How the changed bacteria affect the gut pH and vitamin B6 level in feces and blood need more exploration. Overall, our study finds a new modulated role of gut microbiota on vitamin B6 and proves the ameliorative role of gut microbiota-mediated vitamin B6 on social deficits in EphB6-deficient mice.

Finally, we functionally establish the mechanisms linking gut microbiota and brain dysfunctions (gut-brain axis) in EphB6-deficient mice. Gut-brain axis has been generally considered to be involved in psychiatric diseases. However, there are few studies on how brain is specially regulated by gut microbiota. In our study, we found dopamine in PFC of EphB6-deficient mice was regulated by gut microbiota-mediated vitamin B6. In ASD patients, there are lower medial prefrontal dopaminergic activity [51]. After being given vitamin B6, autistic children have a reduced urinary homovanillic acid, which suggests an improved dopamine metabolism [52]. These studies suggest the regulated role of vitamin B6 in dopaminergic metabolites in ASD patients. Previously, Sgritta reported the modulated VTA plasticity by Lactobacillus reuteri in ASD mouse models [10]. Here, our study showed a new regulatory role of gut microbiota on dopamine in PFC by modulating vitamin B6 in EphB6-deficient mice. What’s more, we found the ameliorative role of D1R agonists in social behavior and the modulated E/I balance in mPFC by gut microbiota in EphB6-deficient mice. Activating D1Rs in PFC can increase frequency of sIPSCs of pyramidal neurons, while D2R agonist does not have the same effect [33]. The modulation of social behavior by D1Rs was probably because of its modulation of GABAergic inhibition in EphB6-deficient mice. Collectively, our study indicates decreased dopamine is induced by dysregulated gut microbiota-mediated defect of vitamin B6 and then contributes to E/I imbalance and social deficits in EphB6-deficient mice.

Conclusions
In summary, our study uncovers a key role for gut microbiota in autism-like behavior of EphB6-deficient mice. Mechanistically, gut microbiota-mediated defect of vitamin B6 metabolism regulates autism-like social behavior by decreasing dopamine levels and inducing E/I imbalance in mPFC in EphB6-deficient mice. Our study suggests a new ASD mouse model, proves the important role of gut microbiota in genetic factor-induced autism and provides a new insight into gut-brain-microbiota axis.
Methods

Mice

All the mice used in our experiments were male. SPF C57BL/6J mice (3-8 weeks old) were obtained from Animal Experiment Center of Southern Medical University in Guangzhou of China. EphB6-deficient mice were obtained using the embryonic stem (ES) cells that were inserted with EphB6\textsuperscript{tm1e(KOMP)Wtsi} vector (IKMC project number: 49365). The injection of ES cells and obtaining of chimeric mice were operated by subsidiary of Cyagen Biosciences Inc. in Guangzhou of China. The chimeric mice were crossed with SPF C57BL/6J mice and its offspring (EphB6\textsuperscript{+/} mice) were kept being crossed with SPF C57BL/6J mice until the fifth generation was born. Then EphB6\textsuperscript{+/} mice were crossed with each other to obtain EphB6\textsuperscript{+/-} mice and EphB6\textsuperscript{-/-} mice. All the mice were raised at a controlled appropriate SPF condition with the temperature at 24 ± 1°C and humidity at 50% to 70% separately and with 12 hr light-dark cycles by turning lights on from 8:00 a.m. to 8:00 p.m.. The animals were housed in groups of 4-5 in plastic cages (Exhaust Ventilated Closed-System Cage Rack). Standard sterile diet and drinking water for raising mice were used to feed the mice. The EphB6 ablation mice were genotyped at 2-week-old using the primers 5'-CTCTGCAAGTGAGACACTTTTCC-3' and 5'-AGCCAGTCTCTCCTACCTGTTTTGG-3' for the wild-type band, 5'-CTCTGCAAGTGAGACACTTTTCC-3' and 5'-CGTGGTATCGTTATGCGCCT-3' for the mutant band and weaned at 3-week-old. All procedures treated with mice were in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals in China.

Fecal microbiota transplantation

Fresh feces of healthy male EphB6\textsuperscript{+/-} and EphB6\textsuperscript{-/-} mice (8 mice for each group from at least 3 cages) were collected from the disinfected anus into new sterile tubes every day before the experiment to promise microbial vitality [4]. Then the fresh feces were weighted, mixed with sterile PBS at a dilution ratio of 1 mg/10 μL or 1 mg/20 μL and centrifuged at 900 x g for 3 min. The supernatant was collected and gavaged orally to each mouse (10 mL/kg) for 5 or 7 consecutive days. All the mice were handled aseptically.
The dilution ratio of 1 mg/20 μL was used to treat 3-week-old SPF C57BL/6J mice and the dilution ratio of 1 mg/10 μL was used to treat 6-week-old SPF C57BL/6J mice and 8-week-old EphB6+/+ and EphB6−/− mice.

For fecal microbiota or metabolite transplantation, after the fresh feces were weighted, mixed with sterile PBS at a dilution ratio of 1 mg/10 μL and centrifuged at 4000 x g for 10 min, the supernatant and precipitate were both collected. After being filtered by the filter with a pore size of 0.22 μm (Cat# SLGP033RS, Millipore, Darmstadt, Germany), the supernatant was orally gavaged to each mouse (10 mL/kg) for 7 consecutive days. After being resuspended in sterile PBS, centrifuged at 900 x g for 3 min, washed by sterile PBS twice, the precipitate was orally gavaged to each mouse (10 mL/kg) for 7 consecutive days.

**Antibiotics treatment**

Vancomycin (50 mg/kg, CAS: 123409-00-7, MP bio, California, USA), neomycin (100 mg/kg, CAS: 1405-10-3, MP bio) and metronidazole (100 mg/kg, CAS: 443-48-1, MCE, New Jersey, USA) were mixed using sterile drinking water [4]. Then the mixture was orally gavaged to 3-week-old or 6-week-old SPF C57BL/6J mice twice a day for 5 or 7 consecutive days and the amount of infusion was based on the weight of mice. The mixture was prepared every day and used freshly. During the treatment, ampicillin (1 mg/mL, CAS: 69-52-3, MP bio) was added into the drinking water of mice and changed with fresh solution every 3 days. For 3-week-old SPF C57BL/6J mice, the antibiotics treatment lasted for 5 days [53]. All the mice were handled aseptically.

**Western blot analysis**

After abdominally anesthetized with phenobarbital sodium (60 mg/kg), the brain of mouse was quickly removed, put into an ice-cold mouse brain mold (Cat# 68713, RWD, Shenzhen, China) and sliced. Then posterior mPFC, hippocampus and cerebellum of mice were cut out. The total proteins of tissues were extracted using the lysis buffer (Cat# P0013B, Beyotime, Shanghai, China) and boiled in protein loading buffer. Equal amounts of the denatured protein samples were electrophoresed in 6-10% polyacrylamide gel containing 0.1% SDS and transferred to polyvinylidene fluoride (PVDF) membranes with pore size of 0.45 μm (Cat# IPVH00010, Millipore). Then the PVDF membranes were incubated
with primary antibodies at 4°C for at least 12 hr. After that, the samples were incubated with secondary antibodies for about 2 hr at room temperature (Cat# BA1050 and Cat# BA1054, Boster, California, USA). The desired signals were visualized by Quantitative FluorChem SP Imaging System (Alpha Innotech, California, USA). Intensities of bands were quantified by ImageJ software and signal values of corresponding band of Gapdh were considered as internal controls. The following primary antibodies were used: EphB6 (1:500, Cat# ab54656, Abcam, Cambridge, UK), c-Fos (1:500, Cat# MABE329, Millipore) and Gapdh (1:5000, Cat# 60004-1-ig, Proteintech, Chicago, USA).

**Quantitative reverse transcription PCR (qRT-PCR)**

After anesthetized with phenobarbital sodium (60 mg/kg), different tissues of mice were quickly removed and put into liquid nitrogen, including colon, colonic epithelium, spleen and lung. Then posterior mPFC and VTA were sectioned out using ice-cold mouse brain mold (Cat# 68713, RWD). qRT-PCR was performed accordingly [54] by using a 7500 real-time PCR system (ABI, California, USA) and SYBR Premix Ex Taq (Cat# RR420A, Takara, Osaka, Japan). Normalized to the mRNA expression level of Gapdh or Actb, the mRNA expressions of other genes were evaluated using the method of \( \Delta\Delta C_t \). All primers used in qRT-PCR were listed in Additional file 2: Table S1.

**Hematoxylin-eosin staining**

The staining of different tissues of mice with haematoxylin and eosin was performed accordingly [54]. Briefly, the tissues were immersed into 4% formaldehyde immediately for 24 hr. Then tissues were embedded in paraffin, sectioned, and stained with haematoxylin and eosin. The stained sections were observed using an optical microscope (Olympus, Tokyo, Japan).

**Intestinal permeability assay**

Mice were fasted for 4 hr before experiment, then FITC-dextran (50 mg/mL, Cat# 46944, Sigma Aldrich, Missouri, USA) was gavaged to mice (600 mg/kg) [8]. 4 hr after the oral gavage, the blood of mouse was collected by cardiac puncture. Then the blood was placed at room temperature for 1 hr before being centrifuged at a speed of 3000 rpm for 10 min. Then the supernatant was transferred to a new tube and centrifuged at a speed of 12000 rpm for 10 min at 4°C. The supernatant, which was the serum, was diluted with equal volume of PBS and 100 μL diluted serum was added to a 96-cell
microplate. The concentration of FITC in serum was determined by Varioskan LUX microplate reader (Thermo Fisher Scientific, Massachusetts, USA) with an excitation of 485 nm and an emission wavelength of 528 nm. The serial diluted FITC-dextran (0, 0.5, 1, 2, 4, 6, 8, 10 μg/μL) was used as standards. Serum of mice administered with PBS was used as negative controls.

**Vitamin B6-deficient mouse model**

The formula of diet with normal vitamin B6 or without vitamin B6 was based on previous study [55]. Then 6-week-old SPF male C57BL/6J mice were fed with the diet with or without vitamin B6 for two weeks.

**Behavioral studies**

Mice used for experiments were male and naive. Mice were handled for 3 days before the experiments and habituated in the experiment room for at least 30 min before each test [56]. Mice were performed with different behavioral tests with a sequence or different mice were used for different behavioral tests which were mentioned in the figure legends. The sequence of different behavioral tests was self-grooming test, olfactory habituation/dishabituation test, three-chambered social approach task, marble burying test, open field test, social partition test, elevated plus maze and morris-water-maze test. Different behavioral tests were done with an interval of at least 2 days. Self-grooming test was performed as previously described [57]. Generally, mouse was first placed in an empty crystal cage to habituate the cage for 10 min, then the time each mouse spent on self-grooming was recorded during next 10 min by a double-blind experienced experimenter. Self-grooming included face-wiping, scratching/rubbing of head and ears, and full-body grooming. Between each trial, the apparatus was cleaned by 30% ethyl alcohol in water. Marble burying test was performed as previously described [58]. Mouse was placed into an animal cage filled with fresh wood chip bedding with the depth of 5 cm. Regular pattern of glass marbles (5 rows of 4 marbles), which were placed 4 cm apart from each other, were regularly placed under the bedding and mouse was allowed to explore for 30 min. The number of buried (more than 50 percent of their depth in bedding) marbles was counted. Social partition test was performed as previously described [59]. Mouse was individually housed in
one side of the cage which was divided by a clear perforated partition with 0.6 cm-diameter holes, and the other side of cage was housed with a sex- and age- matched C57BL/6J mouse for 24 hr before experiment. At the first trial, the total time that the experimental mouse spent on sniffing partition with the familiar mouse on the other side during 5 min was recorded. Then the familiar mouse was replaced with a sex- and age- matched unfamiliar C57BL/6J mouse, the total time that the experimental mouse spent on sniffing partition with the unfamiliar mouse during 5 min was recorded. In the last trial, unfamiliar mouse was instead replaced by familiar mouse and the time that the experimental mouse spent on sniffing partition as first trial was recorded. The time spent on sniffing partition in the three trials was recorded by a double-blind experienced experimenter.

Olfactory habituation/dishabituation test was performed as previously described [60]. Mouse was placed into a clean usual animal cage with thin bedding in a fresh room for 30 min before test. One swab saturated with water was given to mouse for 2 min, and then quickly replaced by another swab saturated with water for the following 2 min, then third swab saturated with water was given to mice for another 2 min quickly. Then other odors were given to mouse similarly. The sequence of given odors was water, almond extract, imitation banana flavor, odor of soiled bedding from mice and odor of soiled bedding from another cage of mice. Water, almond extract (dilution of 1:100) and imitation banana flavor (dilution of 1:100) were regarded as unsocial odors, while soiled bedding with the excrement of sex- and age-matched unfamiliar C57BL/6J mice were regarded as social odors. The time each mouse spent on sniffing the odorant swabs in every 2 min trial was recorded by a double-blind experienced experimenter.

Three-chambered social approach task was performed as previously described [61]. The apparatus was divided into three rectangular clear chambers (60 cm x 40 cm x 22 cm) by two walls on which had two removable doorways (8 cm x 5 cm) that allowed mouse to access each chamber freely. After habituated to the middle chamber for 5 min, the mice were allowed to explore the three chambers freely for 10 min. For the sociability test, an age- and sex-matched C57BL/6J mouse was placed in the wire cage in one chamber while the wire cage in the other chamber was empty. Then the dividers were raised and the experimental mouse was allowed to freely explore all three chambers for 10 min.
For the social novelty test, another age- and sex-matched C57BL/6J mouse was placed in the empty wire cage described above. And the experimental mouse was originally placed in the center of the chamber and allowed to explore freely for 10 min after doorways were removed. Between each trial, the apparatus was cleaned by 70% ethyl alcohol in water. Time spent in each chamber and heat maps were calculated using EthoVison XT software (Noldus, Wageningen, Netherlands). And the time spent on sniffing the wire cages which represented the social approach behavior of mice was calculated by a double-blind experienced experimenter.

In open field test, mouse was placed in the center of an open field chamber (40 cm × 40 cm × 30 cm) [62]. Exploratory behavior of mice was assessed by a session of 30 min and total distance was automatically recorded and analyzed by a VersaMax animal behavioral monitor system (Omnitech Electronics, Nova Scotia, Canada).

Elevated-plus-maze test was performed accordingly [62]. Briefly, mouse was put into the center of elevated-plus-maze which was consisted of two opposing open arms (30 cm × 5 cm × 0.5 cm), two opposing enclosed arms (30 cm × 5 cm × 15 cm) and a central platform (5 cm × 5 cm) for 5 min. The time spent in different arms and entries into different arms were recorded by EthoVison XT software (Noldus).

Morris water maze test was performed as before [63]. Generally, 4 trials were given to each mouse every day for 5 days. In each trial, the searching time for the mouse was no more than 1 min. A stay on the platform was 15 s. Intervals between each trial were no less than 1 min. On the sixth day, the probe test was performed by removing the platform and recording the swimming paths of mice in 1 min. The swimming paths of mice during the learning and test period were analyzed by EthoVison XT software (Noldus).

**16S rRNA gene sequencing**

Fecal samples of the experimental mice were collected and stored at −80°C before being performed. Using QIAamp Fast DNA Stool Mini kit (Cat# 51604, QIAGEN, Venlo, Netherlands), genomic DNA of samples were extracted. The purity and concentration of the extracted DNA were detected using agarose gel electrophoresis. Bacterial DNA was amplified with the primers targeting V3-V4 regions (5'
TACGGRAGGCAGCAG-3', 5'-GGGTATCTAATCCT-3'). Then DNA was sequenced using MiSeq PE300 platform (Illumina, California, USA) by oe biotechnology company in shanghai. The raw data were treated and processed using QIIME software package (version 1.8.0). Then represent sequences of OTU were blasted in Silva database (version 123). The alpha diversity and beta diversity were analyzed using QIIME software package (version 1.8.0).

**Metabolomic analysis**

For untargeted metabolite analysis, plasma and PFC of mice were prepared and deproteinized with methanol. Then the samples were analyzed using liquid chromatography-mass spectrometry by oe biotechnology company in shanghai. UPLC-Q-TOF/MS (ACQUITY UPLC I-Class, Waters, Massachusetts, USA) and ESI-QTOF/MS (Xevo G2-S Q-TOF, Waters) were used. The chromatographic column was the ACQUITY UPLC BEH C18 Column (1.7 µm, 2.1 mm X 100 mm, Waters). Mobile phase A was water contained with 0.1% formic acid and mobile phase B was acetonitrile contained with 0.1% formic acid. The gradient elution was 1%-5% mobile phase B in 0-1 min, 5%-30% mobile phase B in 1-2 min, 30%-60% mobile phase B in 2-3.5 min, 60%-90% mobile phase B in 3.5-7.5 min, 90%-100% mobile phase B in 7.5-9.5 min, 100% mobile phase B in 9.5-12.5 min, 100%-1% mobile phase B in 12.5-12.7 min, 1% mobile phase B in 12.7-16 min. The spectrum signal of samples was acquired by electrospray ionization using positive and negative ionization modes. The data were pretreated using progenesis QI (Waters) and then multivariate statistical analysis was performed using SIMCA software (version 14.0, Umetrics, Umeå, Sweden). The enriched pathway analysis of changed metabolites was performed using KEGG database (http://www.genome.jp/KEGG/pathway.html) and R (version 3.4.1).

For targeted metabolic analysis, PFC was pretreated with 0.4 M perchloric acid which contained 0.04% EDTA and 100 µL plasma was pretreated with 50 µL 5% trichloroacetic acid. 1 M NaOH was added to samples to quench acid.

For the analysis of amino acid neurotransmitters, high performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) was used, with fluorescence detection system (Prominence RF-20A/20Axs, Shimadzu) and the C18 chromatographic column (Eclipse AAA, 4.6 x 150 mm, 5 µm, Agilent, California, USA). All the used reagents and liquid were chromatographically pure. Mobile phase A
contained 20 mM sodium acetate solution (pH7.2), methyl alcohol and tetrahydrofuran which were at a volume ratio of 400:95:5. Mobile phase B contained 20 mM sodium acetate solution (pH7.2) and methyl alcohol which were at a volume ratio of 120:380. The gradient elution was 0%-63% mobile phase B in 0-10 min, 63% mobile phase B in 10-12 min, 63%-100% mobile phase B in 12-12.01 min, 100% mobile phase B in 12.01-17 min, 100%-0% mobile phase B in 17-18 min, 0% mobile phase B in 18-21 min. The temperature of column was set as 35°C. The flowing rate of mobile phase was 0.8 mL/min. The excitation wavelength was set as 340 nm, and the emission wavelength was set as 455 nm. The derivatization reagent contained o-phthalaldehyde (OPA, 5 mg, CAS: 643-79-8, Sigma-Aldrich), methyl alcohol (120 μL), β-mercaptoethanol (10 μL) and borate buffer (0.2 M, pH 9.2, 1mL) and was kept out of light. Data were recorded by INT7. The standards, including glutamic acid (CAS: 56-86-0, Sigma-Aldrich), gamma-aminobutyric acid (CAS: 56-12-2, Sigma-Aldrich), glycine (CAS: 56-40-6, Sigma-Aldrich), aspartic acid (CAS: 56-84-8, Sigma-Aldrich), serine (CAS: 56-45-1, Sigma-Aldrich) taurine (CAS: 107-35-7, Sigma-Aldrich) and glutamine (CAS: 56-85-9, Sigma-Aldrich), were prepared at the concentrations of 7.8125, 15.625, 31.25, 62.5, 125 and 250 ng/mL. The concentrations of amino acid neurotransmitters in different samples were acquired according to the concentrations of standards.

For the analysis of monoamine neurotransmitters, HPLC contained with electrochemical detection system (DECADE life, Antec Scientific, Zoeterwoude, Netherlands) was used, including the chromatographic column (Accucore C18, 150 x 2.1 mm, 2.6 μm, Thermo Scientific). All the used reagents and liquid were chromatographically pure. Mobile phase was prepared with deionized water and MeOH with a volume ratio of 9:1, containing NaH₂PO₄ (100 mM), sodium octane sulfonate (0.74 mM), Na₂EDTA (0.027 mM) and KCl (2 mM). The temperature of column was set at 35°C. And the flowing rate of mobile phase was set at 0.2 mL/min. The standards, including epinephrine (CAS: 51-43-4, Sigma-Aldrich), noradrenaline (CAS: 108341-18-0, Sigma-Aldrich), dopamine (CAS: 62-31-7, Sigma-Aldrich), 3,4-dihydroxyphenylacetic acid (CAS: 102-32-9, Sigma-Aldrich), homovanillic acid (CAS: 306-08-1, Sigma-Aldrich), 5-Hydroxyindole-3-acetic acid (CAS: 54-16-0, Sigma-Aldrich) and 5-
hydroxytryptamine (CAS: 153-98-0, Sigma-Aldrich), were prepared at the concentrations of 0.5, 1, 25, 125 and 250 ng/mL. The concentrations of monoamine neurotransmitters in different samples were acquired according to the concentrations of standards.

For the analysis of pyridoxal 5′-phosphate and pyridoxamine, TSQ Quantiva combined with Prelude SPLC System (Thermo Fisher Scientific) were used. All the used reagents and liquid were chromatographically pure. First, the separation of substances was performed using Prelude SPLC System with the C18 chromatographic column (Water Acquity UPLC HSS T3, 2.1 x 100 mm, 1.7 μm). Mobile phase A contained 0.2% formic acid. Mobile phase B was methyl alcohol. The gradient elution was 0%-50% mobile phase B in 0-2 min, 50%-95% mobile phase B in 2-3.5 min, 95% mobile phase B in 3.5-5.5 min, 95%-0% mobile phase B in 5.5-6.5 min. The temperature of column was set as 40°C. The flowing rate of mobile phase was 0.25 mL/min. Data were recorded using positive-ion electrospray ionization and the selected reaction monitoring mode. For pyridoxal 5′-phosphate, precursor ion was m/z 248.03, product ion was m/z 150.071 and collision energy was 16.067 V. For pyridoxamine, precursor ion was m/z 169.152, product ion was m/z 152.111 and collision energy was 12.124 V. Data were acquired and processed with TraceFinder software (version 3.3 sp1, Thermo Fisher Scientific). The standards, including pyridoxal 5′-phosphate (CAS: 41468-25-1, Sigma-Aldrich) and pyridoxamine (CAS: 524-36-7, Sigma-Aldrich), were prepared at the concentrations of 0.1, 0.5, 1, 6.25, 12.5, 25, 50 and 100 ng/mL. The concentrations of pyridoxal 5′-phosphate and pyridoxamine in different samples were acquired according to the concentrations of standards.

**Bacterial culturing**

PFC of mice were brought out aseptically and homogenized in PBS using sterile magnetic beads. Then the homogenates were painted on the Luria-Bertani solid medium and cultured for 24 hr at 37°C.

**DNA extraction of bacteria**

The PFC of mice were brought out aseptically and the genomic DNA of the tissue was extracted using PureLink genomic DNA kit (Cat# K1820-01, Invitrogen, California, USA). Then the DNA was amplified using bacterial universal primers (5′-AGAGTTTGATCATGGCTCAG-3’, 5′-CCGGGAACGTATTCACC-3’) [64]. The DNA of *Escherichia coli* was used as positive control.
**Stereotaxic surgery and drug microinjection**

The stereotaxic surgery was performed to implant brain infusion cannula into mPFC of adult male mice based on the published protocols [62]. After being anesthetized by phenobarbital sodium (60 mg/kg), the mouse was placed in a stereotaxic frame (RWD) and a hole with the diameter of 1 mm was drilled with a dental drill on the skull of mouse according to the adjusted coordinates of bilateral mPFC (AP: +1.84 mm, ML: ± 0.4 mm, DV: -2.2 mm). Then the brain infusion cannula (Cat# 62004, RWD) was carefully put into the drilled hole and fixed by glass ionomer cement. After the operation, the mice were resuscitated on an electric blanket and then put back into the original cage. After a recovery of 7 days, the catheter cap (Cat# 62104, RWD) was removed and the injection needle (Cat# 6220, RWD4) was inserted into the catheter after a disinfection with 75% alcohol. The injection needle was connected with a microsyringe through a polyethylene tube (Cat# 62320, RWD), and the drug was injected into the mPFC at a speed of 0.1 μL/min controlled by a microsyringe pump (Cat# R404, RWD). The total volume of the injected drug was 0.3 μL. After the injection of drug, the injection needle was kept being inserted into the catheter for 5 min to facilitate the complete diffusion and absorption of the drug. Behavioral test was performed 30 min after administration. The drugs used in this experiment were SKF38393 (CAS: 62717-42-4, MCE) and quinpirole (CAS: 524-36-7, Sigma-Aldrich).

**Slice preparation**

Male mice were decapitated after anesthetized by phenobarbital sodium (60 mg/kg). Brains were removed quickly and then placed into the ice-cold modified ACSF containing (in mM): 26 NaHCO₃, 10 glucose, 10 MgSO₄, 2 KCl, 1.3 NaH₂PO₄, 0.2 CaCl₂ and 250 sucrose. Slices containing mPFC (300 μm) were prepared using a VT-1200S vibratome (Leica, Wetzlar, Germany) in ice-cold modified ACSF. And then slices were transferred into the storage chamber containing the regular ACSF (in mM) (126 NaCl, 26 NaHCO₃, 10 glucose, 2 CaCl₂, 3 M KCl, 1 MgSO₄, and 1.25 NaH₂PO₄) at 31°C for 1 hr and then were removed to room temperature (25 ± 1°C) for 1 hr before being recorded. All solutions were saturated with 95% O₂/5% CO₂ (vol/vol) during the slice preparation [65].
**Electrophysiology**

The neurons in mPFC were obtained using an infrared (IR)-differential interference contrast (DIC) microscope (ECLIPSE FN1, Nikon, Tokyo, Japan). To record sEPSCs, pipettes (input resistance: 3-7 MΩ) were filled with an intracellular solution containing (in mM) 105 K-gluconate, 30 KCl, 10 phosphocreatine, 10 HEPES, 4 ATP-Mg, 0.3 EGTA, and 0.3 GTP-Na (pH 7.3, 285 mOsm). When recording sEPSCs, the GABA<sub>A</sub> receptors were blocked with 20 µM bicuculline methiodide (CAS:40709-69-1, TOCRIS, Minneapolis, USA). When recording sIPSCs, the holding potentials were 0 mV, pipettes (input resistance: 3-7 MΩ) were filled with an intracellular solution containing (in mM) 110 Cs<sub>2</sub>S, 2 CaCl<sub>2</sub>, 5 EGTA, 5 HEPES, 5 TEA, 5 ATP-Mg (pH 7.35, 285 mOsm). Data were recorded by a multiClamp 700B (Molecular Devices, San Jose, USA), digitized at 10 kHz and filtered at 3 kHz. Data were collected when the series resistance fluctuated within 20% of the initial values and analyzed by pClamp 10.2 software (Molecular Devices) [65]. For the treatment of D1R agonist, SKF-38393 (10-50 µM) was applied for 5 min.

**Statistical analyses**

All statistical analyses were performed with SPSS statistical software (version 20.0). Sample size was determined according to previously published studies [4, 8-10]. No animals were excluded. The normality of all data was analyzed using Kolmogorov-Smirnov test. Levene’s test was used for the test of equal variances. For the data with normal distributions, two-tailed and unpaired Student’s t-test was performed to analyze two independent groups with equal variance. Two-tailed and unpaired Student’s t-test with Welch’s correction was used to analyze two independent groups with unequal variance. One-way ANOVA was performed to analyze multiple groups with only one variable and the differences between groups were performed with LSD pairwise comparison. When the variances were unequal, Dunnett’s T3 pairwise comparison was used to analyze the difference between groups. For the data without normal distributions, Mann-Whitney U test was used to analyze two independent groups, Kruskal-Wallis test was used to analyze multiple groups. Two-way repeated measures ANOVA was used to analyze different groups with two variables, including the sniffing on different odors,
latency to the platform and total distance in open field test. All results showed were mean ± SEM, n represented the number of independent biological replicates and p value < 0.05 was considered significant. The statistical methods and statistical values of each result were presented in Additional file 3: Table S2.

Abbreviations

Abx: pre-treated with antibiotics (ampicillin, vancomycin, neomycin, metronidazole)
ACSF: artificial cerebrospinal fluid
ASD: autism spectrum disorder
Asp: aspartic acid
DA: dopamine
DOPAC: dihydroxy-phenyl acetic acid
D1R: dopamine D1 receptor
D2R: dopamine D2 receptor
E/I imbalance: excitation/inhibition imbalance
EP: epinephrine
FITC: fluorescein isothiocyanate
FMT: fecal microbiota transplantation
GABA: gamma-aminobutyric acid
GI: gastrointestinal
Glu: glutamic acid
Gly: glycine
Gln: glutamine
HPLC: high performance liquid chromatography
5-HT: 5-hydroxytryptamine
mPFC: medial prefrontal cortex
NE: norepinephrine
PLP: pyridoxal 5'-phosphate
PM: pyridoxamine
PN: pyridoxine
VB6: vitamin B6
VTA: ventral tegmental area
Ser: serine
sEPSCs: spontaneous excitatory postsynaptic currents
sIPSCs: spontaneous inhibitory postsynaptic currents
Th: tyrosine hydroxylase

Declarations

Ethics approval and consent to participate

All procedures treated with mice were in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals in China. All procedures treated with mice were approved by the Institutional Animal Care and Use Committee of the Sun Yat-Sen University (approval ID SYXK-2017-0081).

Consent for publication

Not applicable.

Availability of data and material

The whole data in this work are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that there are no conflicts of interest.

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**Authors’ contributions**

Jian-Ming Li and Tian-Ming Gao contributed to study concept; Jian-Ming Li, Tian-Ming Gao, Ying-Li, Jian-Ming Yang and Tong Shen contributed to study design; Ying-Li, Zheng-Yi Luo, Yu-Ying Hu, Yue-Wei Bi and Ming-An Liu contributed to data acquisition and analysis of behavioral and molecular studies; Zheng-Yi Luo and Ming-An Liu contributed to data acquisition and analysis of electrophysiological studies; Wen-Jun Zou and Yun-Long Song conducted the stereotactic surgery; Shu-Ji Li contributed to data acquisition of bacterial culture; Ying-Li and Lang-Huang conducted the HPLC; Shi-Li and Ai-Jun Zhou conducted the HE staining; Jian-Ming Li, Tian-Ming Gao, Ying-Li, Zheng-Yi Luo, Jian-Ming Yang and Yu-Ying Hu contributed to data analysis and interpretation; Jian-Ming Li, Tian-Ming Gao, Ying-Li and Yue-Wei Bi contributed to manuscript drafting; Jian-Ming Li contributed to funding obtaining and study supervision.

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Figures
Deletion of EphB6 led to autism-like behavior and gut microbial disturbance in mice
Transplantation of fecal microbiota from EphB6-deficient mice caused autism-like behavior in 3-week-old SPF C57BL/6j mice
Fecal microbiota transplantation from EphB6-deficient mice induced social deficits in 6-week-old SPF C57BL/6j mice
Figure 4

Transplantation of fecal microbiota from wild-type mice ameliorated autism-like behavior in adult EphB6-deficient mice
Gut microbiota-mediated vitamin B6 metabolism regulated social behavior in EphB6-deficient mice
Figure 6

The modulated dopamine by gut microbiota-mediated vitamin B6 regulated social behavior of EphB6-deficient mice
Gut microbiota and dopamine modulated E/I balance in mPFC of EphB6-deficient mice
Figure 8

Working model of the modulated social deficits in EphB6-deficient mice by gut microbiota

Supplementary Files

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