Life Sciences 223 (2019) 54–61

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Sema3A - mediated modulation of NR1D1 expression may be involved in the regulation of axonal guidance signaling by the microbiota

Xunzhong Qi[a,c,e,1], Guowei Wang[k,1], Xiaogang Zhong[d,e,f,1], Benhua Zeng[j,1], Jianjun Chen[g], Li Zeng[i], Shunjie Bai[h], Shaohua Xu[b,d,e], Wei Wang[a,c,e], Ke Cheng[a,d,e], Zhi Chen[b], Hong Wei[j,*], Peng Xie[a,b,c,d,e,**]

[a] Department of Neurology, the First Affiliated Hospital of Chongqing Medical University, Chongqing, China
[b] Department of Neurology, Yongchuan Hospital of Chongqing Medical University, Chongqing, China
[c] Institute of Neuroscience, Chongqing Medical University, Chongqing, China,
[d] Institute of Neuroscience and the Collaborative Innovation Center for Brain Science, Chongqing Medical University, Chongqing, China
[e] Chongqing Key Laboratory of Neurobiology, Chongqing Medical University, Chongqing, China
[f] School of Public Health and Management, Chongqing Medical University, Chongqing, China
[g] Institute of Life Sciences, Chongqing Medical University, Chongqing, China
[h] Department of Laboratory Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China
[i] Department of Nephrology, Second Affiliated Hospital of Chongqing Medical University, Chongqing, China
[j] Department of Laboratory Animal Science, College of Basic Medical Sciences, Third Military Medical University, Chongqing, China
[k] Ning Xia Medical University, 750004, NO804, Road Shengli, Yinchuan, Ningxia, China

ARTICLE INFO

Keywords:
Microbiota
Axon guidance signaling
Sema3A
NR1D1
RhoA/ROCK pathway

ABSTRACT

Aims: The microbiota has a profound impact on host development and function. Axon guidance is essential for the formation of neural circuits and plays an important role in neurological diseases and behavioral disorders. However, the impact of the microbiota on axon guidance signaling is unclear.

Main methods: Gnotobiotic models-germ free mice were applied to explore behavioral phenotypes and possible molecular mechanisms that were evaluated by Realtime-PCR and western blot analysis. Primary cultures of mouse cortical neurons were performed to demonstrate the role of Sema3A on NR1D1 expression.

Key findings: The results showed that the microbiota modulates host behavior, and that colonization is not sufficient to normalize behavioral alterations in germ-free (GF) mice. Five genes, *Sema3A*, *Sema3E, EphB2*, *Slit3* and *Robo1*, were differentially expressed in GF and specific pathogen-free (SPF) mice. Furthermore, colonization did not completely reverse the differential expression, which was consistent with the behavioral phenotypes in colonization germ-free (CGF) mice. The transcript and protein levels of Sema3A, and of its membrane-bound co-receptor NRP1, were increased in GF mice. Interestingly, Sema3A inhibited the expression of NR1D1, which was blocked by a RhoA/ROCK pathway agonist in primary cortical neurons. The NR1D1 and ROCK2 expression levels were reduced in GF and CGF mice compared with SPF mice, consistent with the increased expression of Sema3A.

Significance: Our findings suggest that the microbiota regulates axon guidance signaling in the prefrontal cortex. Furthermore, this effect appears to involve the inhibition of NR1D1 expression by Sema3A through the RhoA/ROCK pathway.

Abbreviations: PFC, Prefrontal cortex; GF, Germ-free; SPF, Specific-pathogen-free; CGF, Colonization germ-free; Sema3A, Semaphorin 3A; Sema3E, Semaphorin 3E; EphB2, Ephrin type-B receptor 2; Slit3, Slit homolog 3; Robo1, Roundabout 1; NRP1, Neuropilin-1; NR1D1, Nuclear receptor subfamily 1, group D, member 1; OFT, Open field test; FST, Forced swimming test; S + N, Sema3A + Narciclasine; S + U, Sema3A + U46619

* Corresponding author.
** Correspondence to: P. Xie, Department of Neurology, the First Affiliated Hospital of Chongqing Medical University, Chongqing, China.
E-mail addresses: weihong63528@163.com (H. Wei), xiepeng@cqmu.edu.cn (P. Xie).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.lfs.2019.03.023
Received 10 December 2018; Received in revised form 2 March 2019; Accepted 9 March 2019
Available online 11 March 2019
0024-3205/ © 2019 Published by Elsevier Inc.

1. Introduction

The microbiota has a profound impact on the host's development and function [1–4]. Over the past few years, the effects of the microbiota on host systemic immune function and bone physiology [5,6] have been well described, and emerging evidence demonstrates that the microbiota influences host behavior [7]; the presence of the microbiota increases motor activity and reduces anxiety-like behavior in rodents. Early research findings by our group have confirmed this phenomenon [8], and these findings may have wider implications when considering psychiatric disorders in humans. However, the mechanisms by which the microbiota modulates host development and behavior are unclear.

As the basic structural units and functional units of the central nervous system (CNS), connect to each other via axon and dendrites to form neural networks and neural circuits, neurons regulate basic physiological functions, such as blood pressure and respiration, and advanced functions, such as learning, memory, and behavior. Abnormal neural circuitry and network structure may lead to neurological diseases, such as autism [9], depression [10], schizophrenia [11] and alcohol dependence [12]. At the apical tip of the developing axon, an enlarged structure called the growth cone has the ability to receive environmental stimuli and control axon growth and orientation. Axon guidance signals [13], such as netrins [14], slits [15], ephrins [16] and semaphorins [17], play a key role in this process, especially semaphorin 3A (Sema3A) [18]. The axon guidance function of the growth cone is essential for the formation of neural circuits and may play an important role in neurological diseases and behavioral disorders.

The prefrontal cortex (PFC) is an important brain region associated with fear and anxiety processes, and dysfunction of this region has been implicated in a range of neuropsychiatric disorders such as depression and schizophrenia [19–23]. The effect of the microbiota on the PFC may involve modulation of the mRNA expression levels of *NGFI-A* [24] and myelin component genes in germ-free (GF) mice, which appear to be associated with the hypermyelination of axons [25]. Recently, Luczynski et al. [26] have shown that the microbiota influences the neurite structure and the morphology of the amygdala and hippocampus in mice. However, the effect of the microbiota on axon guidance in the PFC, which may be a regulatory mechanism for behavioral disorders, remains unknown. Therefore, studies investigating how the microbiota influences axon development in the PFC are needed. This research was carried out to investigate the influence of the microbiota on axon guidance in the PFC and to test the hypothesis that the microbiota is an integral part of normal axon development.

2. Experimental procedures

2.1. Animals

GF and specific pathogen-free (SPF) Kunming male mice (aged 6–8 weeks) were obtained from the Department of Laboratory Animal Science of the Third Military Medical University (Chongqing, China). GF mice were housed in flexible film gnotobiotic isolators under a strict 12-h light/dark cycle, and sterilized water and food were provided to avoid contamination. All experiments followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23), revised in 1996. The Animal Ethics Committee of Chongqing Medical University approved all experimental procedures. For colonization experiments, 4–5-week-old GF mice were transferred to cages with bedding and faecal matter from SPF mice and housed next to SPF mice to allow microbes present in the environment to colonize[hereafter referred to as colonization germ-free (CGF) mice], a protocol that has previously been demonstrated to be effective for restoring normal microbiota [27].

2.2. Open field test (OFT)

On the day of testing, GF and CGF mice were transported in sterile filtered cages to the testing room with SPF mice and allowed to rest for 1 h to acclimate to the conditions before testing. The apparatus was cleaned with 70% alcohol to eliminate odors and then with 70% alcohol and water after each individual test session. At the beginning of the test, animals were individually placed at the center of the open field arena (45 × 45 × 45 cm) and allowed to explore for 6 min. The total distance traveled and distance moved in the inner zone was recorded by a tracking software system and analyzed using SMART 2.5 software. The spontaneous activity of the mice was measured in the last 5 min.

2.3. Forced swimming test (FST)

Twenty-four hours before the test swim, all animals performed a pre-test (15 min) swim. On the day of testing, all experimental animals were habituated to the testing room 1 h prior to testing. Each animal was individually placed in a Plexiglas cylinder (30 cm height, 15 cm diameter) filled with 18 cm of water (23–25 °C). All FST experiments were recorded by a tracking software system and analyzed using SMART 2.5 software by surface change rate between sequential images of the animal shape.

2.4. Quantitative real-time PCR (qRT-PCR) analysis

For PFC samples, RNA obtained by TRIzol (Thermo Fisher Scientific, Rockford IL, USA) extraction was reverse-transcribed using the PrimeScript RT Master Mix kit (Takara, Otsu, Japan). qRT-PCR was performed on a LightCycler 96 System (Roche, Basel, Switzerland) using SYBR Premix Ex Taq II (Takara, Otsu, Japan). Transcript levels were calculated relative to β-actin. Fold change was calculated using the ΔΔCt method. The qRT-PCR primer sequences are listed in Appendix Table 1.

2.5. Western blotting

Protein expression in PFC lysates was assessed as previously described [28]. Briefly, the PFC was dissected from mice, immediately frozen on dry ice, and stored at −80 °C. Brain tissues were homogenized in ice-cold RIPA lysis buffer in the presence of protease and phosphatase inhibitors and centrifuged at 4 °C to extract total protein. Primary antibodies used for western blotting analysis included Sema3A (1:1000, Abcam; Cambridge, MA, USA), NRP1 (1:1000, Abcam), NR1D1 (1:700, Abcam) and ROCK2 (1:10,000, Abcam). Enhanced chemiluminescence was used to detect immunoreactive signals, and GAPDH (1:10,000, Abcam) was used as a loading control.

2.6. Primary cultures of mouse cortical neurons

Cultures of cortical neurons from neonatal mice were prepared as described previously [29]. Briefly, cortical neurons were enzymatically isolated from the cortex of 1 to 4-day-old C57 mice using 0.125% (w/v) Trypsin-EDTA (Gibco, Glasgow, UK). Cells were cultured in serum-free Neurobasal medium containing 2% B27 supplement, 1% glutamine and 1% penicillin/streptomycin (all from Gibco), and plated at a density of 0.5–0.7 × 10^5/cm^2 on 6-well plates pre-coated with 100 μg/mL poly-D-lysine (Sigma Aldrich, St Louis, MO, USA). After 2 days in culture, 1 nM recombinant mouse Sema3A [30] (R&D Systems, Abingdon, UK), 1 nM U46619 [31] (Sigma Aldrich), 25 nM Narciclasine [32,33] (MCE, Monmouth Junction, USA) or 10 μM fasudil (Selleck Chemicals, Houston, TX, USA) was added and incubated for 48 h to regulate axon development.

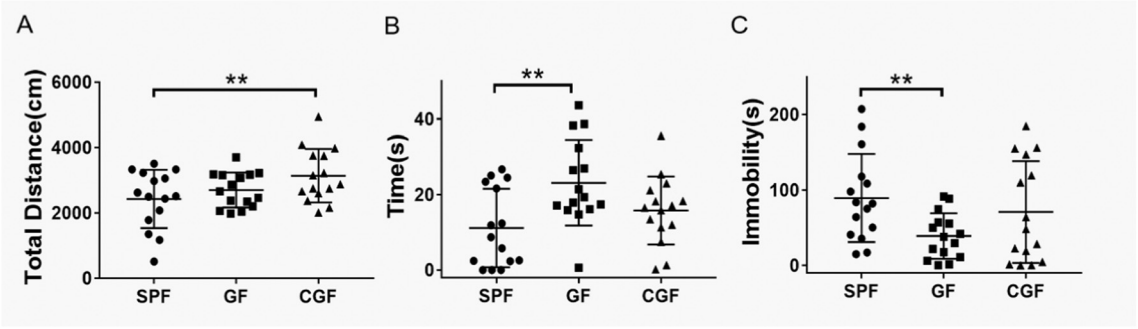

Fig. 1. Alteration of the gut microbiota changes host behaviors. A: Total distance traveled in the OFT. B: Time spent in the center in the OFT. C: Immobility time ($n = 15$) *$P < 0.05$; **$P < 0.01$.(Fig. 1A,B,C Assessed by one-way ANOVA with the LSD post-hoc test).

2.7. Statistical analysis

All data are reported as mean ± SEM. Differences between two groups were assessed by Student's *t*-test, and differences among more than two groups were assessed by one-way ANOVA with the LSD post-hoc test, as appropriate. $P < 0.05$ was considered statistically significant.

3. Results

Colonization is not sufficient to normalize anxiety-related and passive behavioral alterations.

In the first set of experiments, tests for exploratory activity and Anxiety were carried out on SPF, GF and CGF mice. Compared with SPF mice, GF mice showed more exploration of the center of the open field in the OFT ($F2,42 = 5.157$, $P = 0.003$; Fig. 1B) and less time of immobility in the FST ($F2,42 = 3.295$, $P = 0.015$; Fig. 1C). However, CGF mice showed only greater total distance traveled compared with SPF mice ($F2,42 = 3.317$, $P = 0.014$; Fig. 1A). These results show that the microbiota can modulate host behavior, but colonization is not sufficient to normalize anxiety-related and passive behavioral alterations in GF mice.

Microbiota influences genes expression of axon guidance signaling in the PFC.

To determine the influence of the microbiota on axon guidance signaling, the expression levels of 22 important genes involved in axon guidance and dendritic morphogenesis were measured in the PFC of GF and SPF mice, including netrins, slits, ephrins and semaphorins. Analysis of mRNA transcript levels in the PFC of GF and SPF mice revealed increased levels of *Sema3A* ($F1,8 = 6.913$, $P = 0.008$) and *Sema3E* ($F1,8 = 2.627$, $P = 0.024$) (Fig. 2A). Moreover, a number of genes involved in axon guidance were downregulated in GF mice, including *ephrin type-B receptor 2* (*EphB2*; $F1,8 = 0.027$, $P = 0.029$; Fig. 2C), *slit homolog 3* (*Slit3*; $F1,8 = 0.444$, $P = 0.035$; Fig. 2E) and *roundabout 1* (*Robo1*; $F1,8 = 0.075$, $P = 0.004$; Fig. 2F). These results suggest that the microbiota can influence axon guidance signaling in the PFC. (The data in Fig. 2 is presented as a heat map in the Supplementary Information. Fold changes in gene expression shown in Fig. 2 has been added in the Supplementary Information. Fig. R2).

(Fig. 2A-G: Assessed by Student's *t*-test; H: Assessed by one-way ANOVA with the LSD post-hoc test).

Colonization is not sufficient to normalize differentially expressed genes involved in axonal guidance.

To further investigate the effect of the microbiota on axonal guidance signaling, a colonization experiment was carried out. The mRNA levels of five differentially expressed genes between SPF and GF mice were measured in SPF, GF and CGF mice. qRT-PCR revealed no significant differences in the expression of *Sema3A* ($F2,15 = 12.973$, $P = 0.365$), *Sema3E* ($F2,15 = 4.876$, $P = 0.075$), *EphB2* ($F2,15 = 3.077$, $P = 0.639$), *Slit3* ($F2,15 = 5.298$, $P = 0.601$) or *Robo1* ($F2,15 = 5.397$, $P = 0.067$) between GF and CGF mice (Fig. 2 H). These data suggest that colonization of the microbiota carried out after maturity was not sufficient to normalize differentially expressed genes involved in axonal guidance.

Absence of microbiota results in changes in Sema3A/NRP1 signaling in the PFC.

To analyze the mechanisms by which the microbiota regulates brain development and behavior, we examined the Sema3A signaling, which is closely associated with mental illnesses such as depression, [34–37]. The transcription levels of six Sema3A membrane-bound co-receptors were determined by qRT-PCT; Compared with SPF mice, the transcription level of *NRP1* was upregulated in the PFC of GF ($F1,10 = 0.672$, $P = 0.026$; Fig. 2G) and CGF mice ($F2,15 = 0.672$, $P = 0.026$; Fig. 2H),but there were no significant differences between GF and CGF mice. The protein levels of Sema3A ($F2,6 = 28.876$, $P = 0.003$) and NRP1 ($F2,6 = 7.823$, $P = 0.030$) were both increased in GF mice compared with SPF mice. Furthermore, an upregulation of Sema3A ($F2,6 = 28.876$, $P = 0.034$) and downregulation of NRP1 ($F2,6 = 7.823$, $P = 0.009$) were found in the PFC of CGF mice (Fig. 3A, B) compared with GF mice. These results indicate that the microbiota regulates the Sema3A/NRP1 signaling in the PFC.

A, B: Effect of microbiota on the protein levels of Sema3A and NRP1 in the PFC ($n = 3$).*$P < 0.05$, **$P < 0.01$. (Fig. 3A,B: Assessed by one-way ANOVA with the LSD post-hoc test).

Sema3A inhibits NR1D1 expression via the RhoA/ROCK pathway in primary cortical neurons.

To further analyze the mechanism of Sema3A regulation of neuronal development, changes in the Sema3A downstream genes were assessed using in vitro experiments. The development of the cerebral cortex in mice is associated with changes in NR1D1 signaling. Therefore, we examined the effects of Sema3A on *NR1D1* expression by qRT-PCR. The results showed that Sema3A inhibited *NR1D1* gene expression ($F1, 4 = 7.525$, $P = 0.018$; Fig. 4A), similar to the RhoA/ROCK pathway inhibitor fasudil ($F1, 4 = 0.017$, $P = 0.007$, Fig. 4B). As previous reports have shown that Sema3A downregulates ROCK2, this result indicates that the Sema3A regulation of NR1D1 expression is mediated by inhibition of the RhoA/ROCK pathway.

To further elucidate the role of the RhoA/ROCK pathway in the regulation of NR1D1 expression by Sema3A, Narciclasine and U46619, two RhoA/ROCK pathway agonists, were individually used to treat primary cortical neurons in combination with recombinant Sema3A protein. Compared with the Sema3A group, the Narciclasine + Sema3A group and U46619 + Sema3A group showed increased NR1D1 expression of NR1D1 both at the gene (S + N: $F3,8 = 54.032$, $P < 0.0001$; S + U: $F3,8 = 17.413$, $P = 0.004$; Fig. 4C, D) and protein levels (S + N: $F3,8 = 18.414$, $P = 0.024$; S + U: $F3,8 = 23.788$, $P = 0.046$; Fig. 4E, F). These results also suggest that both RhoA/ROCK pathway agonists can reverse the inhibitory effect of Sema3A on NR1D1 mRNA and protein expression. Together, these results suggest that Sema3A inhibits NR1D1 expression via the RhoA/ROCK pathway in

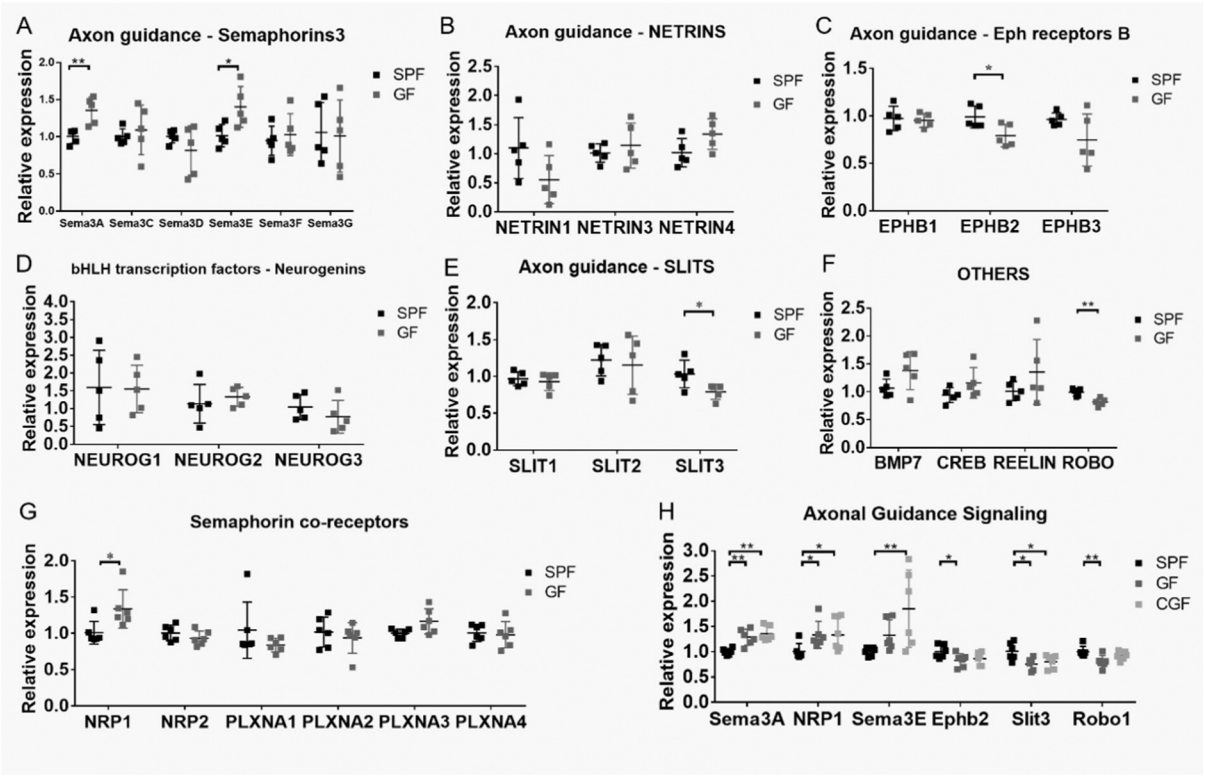

Fig. 2. Altering the microbiota affects host axonal guidance signaling-related gene expression in the prefrontal cortex. A–F: mRNA expression of axon guidance and dendritic morphogenesis-related molecules. (n = 5). G: Effect of microbiota on the mRNA expression of Sema3A membrane-bound co-receptors in the PFC (n = 6). H: Effect of microbiota colonization on the differential-genes expression involved in axonal guidance signaling. (n = 6). *P < 0.05; **P < 0.01.

primary cortical neurons.

Microbiota regulate the NR1D1 and RhoA/ROCK pathways in vivo.

To further analyze the regulation mechanism of the microbiota in brain development, we next examined the changes in the levels of Sema3A downstream effectors in vivo. NR1D1 transcript ($F2,15 = 18.716$, $P = 0.002$; Fig. 5A) and protein levels ($F2,6 = 26.651$, $P = 0.030$; Fig. 5B) were considerably low in GF mice compared with SPF mice. However, compared with GF mice, the CGF mice showed lower expression of NR1D1 both in transcript ($F2,15 = 18.716$, $P = 0.032$; Fig. 5A) and protein levels ($F2,6 = 26.651$, $P = 0.004$; Fig. 5B). RhoA/ROCK pathway-related genes were investigated for potential expression changes in the PFC. Compared with SPF mice, GF mice showed decreased ROCK2 transcription ($F2,15 = 7.757$, $P = 0.002$; Fig. 5C), and protein expression ($F2,6 = 26.675$, $P = 0.004$; Fig. 5D), which was consistent with the changes in transcription levels in GF mice. After colonization, decreased ROCK2 protein expression was detected in CGF mice compared with GF mice ($F2,6 = 26.675$, $P < 0.001$; Fig. 5D). The results showed that the variation in expression trends of NR1D1 and ROCK2 in vivo were opposite to those of Sema3A, which suggests that Sema3A-mediated modulation of NR1D1 expression may be involve in the regulation of axon development and behavior by microbiota.

4. Discussion

Using multi-gene analysis and gnotobiotic models, we demonstrated

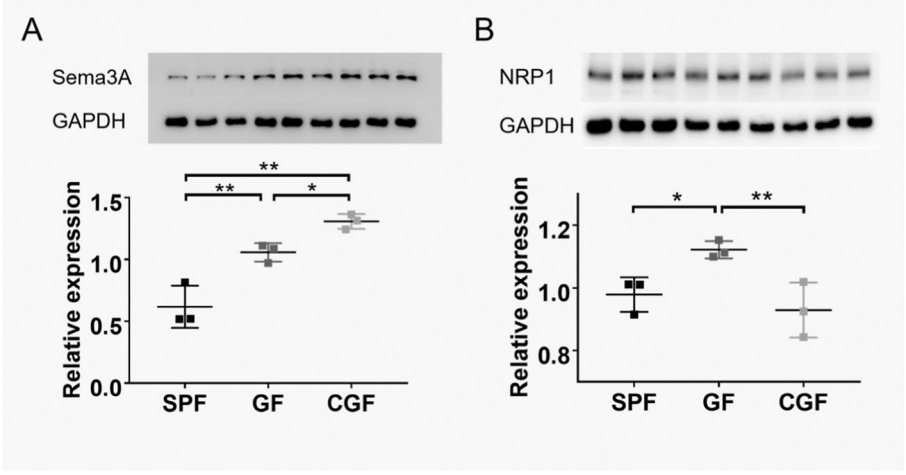

Fig. 3. Absence of microbiota affects Sema3A/NRP1 signaling in the PFC.

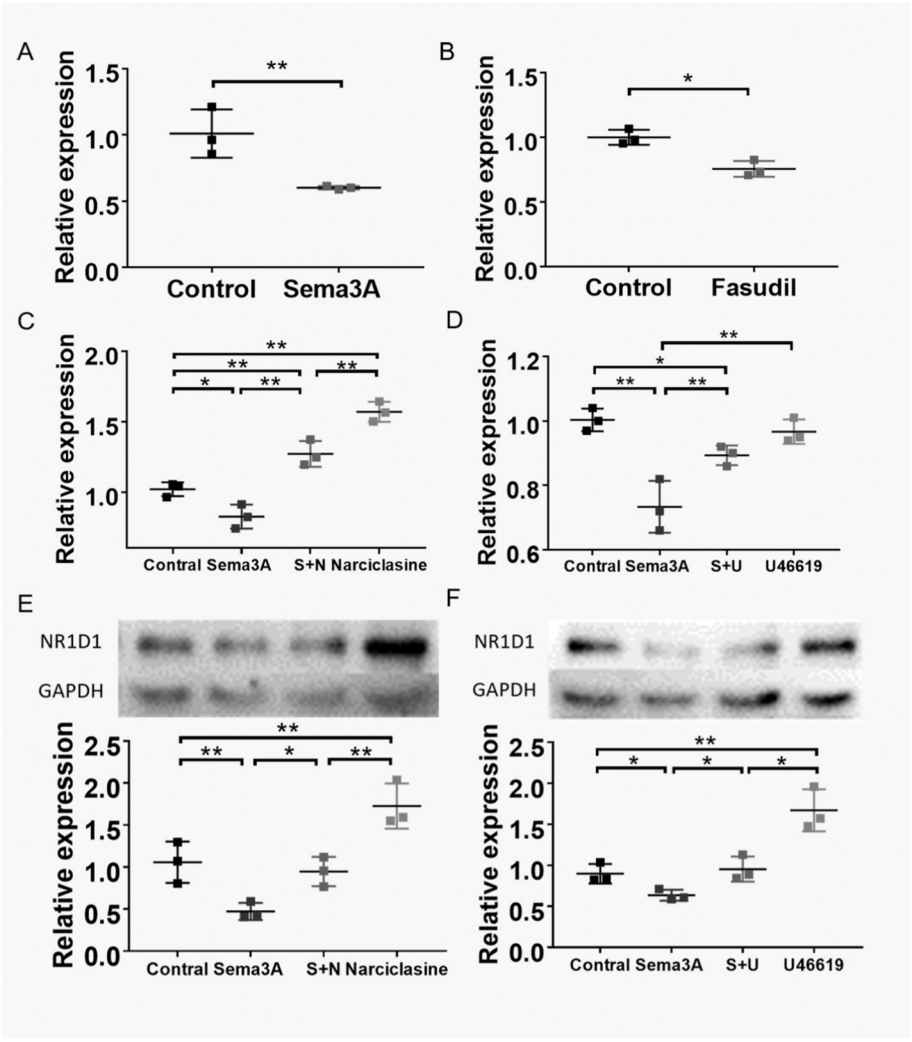

Fig. 4. Sema3A inhibits NR1D1 expression via the RhoA/ROCK pathway in primary cortical neurons. (A) NR1D1 mRNA expression in primary neurons is inhibited by recombinant mouse Sema3A ($n = 3$). (B) qRT-PCR analysis of *NR1D1* mRNA levels in primary neurons showed that the expression was inhibited by fasudil (n = 3); (C,E) The mRNA and protein levels of NR1D1 after treatment with Sema3A and/or Narciclasine (n = 3). (D, F) The mRNA and protein levels of NR1D1 after treatment with Sema3A and/or U46619 (n = 3). S, Sema3A; N, Narciclasine; U, U46619. $^{*}P < 0.05$, $^{**}P < 0.01$. (Fig. 4A,B, Assessed by Student's *t*-test; Fig. 4C-F Assessed by one-way ANOVA with the LSD post-hoc test).

here that the microbiota modulates the expression of axonal guidance signaling molecules in the PFC, including EphB2, Slit3, Robo1, Sema3E, Sema3A and NRP1.EphB2, a receptor tyrosine kinase that binds transmembrane ephrin-B family ligands, plays a critical role in axon guidance in the mammalian CNS. During brain development, EphB2 is involved in the regulation of dendritic spine development. Knockout of EphB2 leads to abnormal projection of specific axons [38,39]. Slit3 and its interaction with Robo homolog receptors are important for the correct targeting of basal vomeronasal organ axons [40]. During brain development, Sema3E has a gating function in the assembly of forebrain neuronal circuits. In the postnatal spinal cord, astrocyte-produced Sema3A is required for proper motor neuron and sensory neuron circuit organization [44]. Furthermore, Sema3A is upregulated in the hippocampus of a neurodevelopmental disorder mouse model [45]. In this study, Sema3A and its membrane-bound receptor NRP1 were increased at both the gene and protein levels in the PFC of GF mice. Along with the other four differential genes detected above, this differential gene expression of axonal guidance signaling molecules suggests that the absence of the microbiota in GF mice may cause changes in axonal development in the PFC.

The role of the microbiota in host development is not yet clear and its essential role in the host immune system seems to be a key indirect communication route between the microbiota and the CNS. The loss of microbiota significantly impaired the development and function of microglia in adult mice that maintained a more juvenile state and reduced the response to pathogen exposure. The use of short-chain fatty acids completely restored this damage [46]. During brain development, microglia participate in synaptic pruning to ensure proper neural network connections [47]. However, probiotics can increase the infiltration of specific immune cells, e. g: Ly6Chi monocytes, which rescued hippocampal neurogenesis upon antimicrobial treatment [48]. These discoveries sheds light on the link between microbiota, host immune system and neurodevelopment, indicating that the microbiota produces microbial-associated molecular patterns and metabolites that can stimulate the host immune system and eventually influence neurodevelopment, such as axonal guidance signaling, and behavior [49–52].

To further investigate the effect of the microbiota on axonal guidance signaling, we carried out a colonization experiment and found that there was a lack of differential expression of axon guidance genes between GF and CGF mice, which was consistent with the behavioral phenotypes of CGF mice. These results indicate that colonization commencing after the critical period of axon development may not be sufficient to reverse the changes in axon guidance signaling. During the complex process of brain development after birth, especially during the critical periods, environmental stimuli play a fundamental role in refining neuronal circuits [53,54]. The modulation of axonal development occurs during critical periods and continues into adulthood in a brain region-specific manner. Absence of the microbiota during those periods may lead to anomalous remodeling of cortical and subcortical structures, thereby affecting brain development and behavior in GF mice. However colonization commencing after the critical period may not be able to reverse these changes. Therefore, we concluded that in

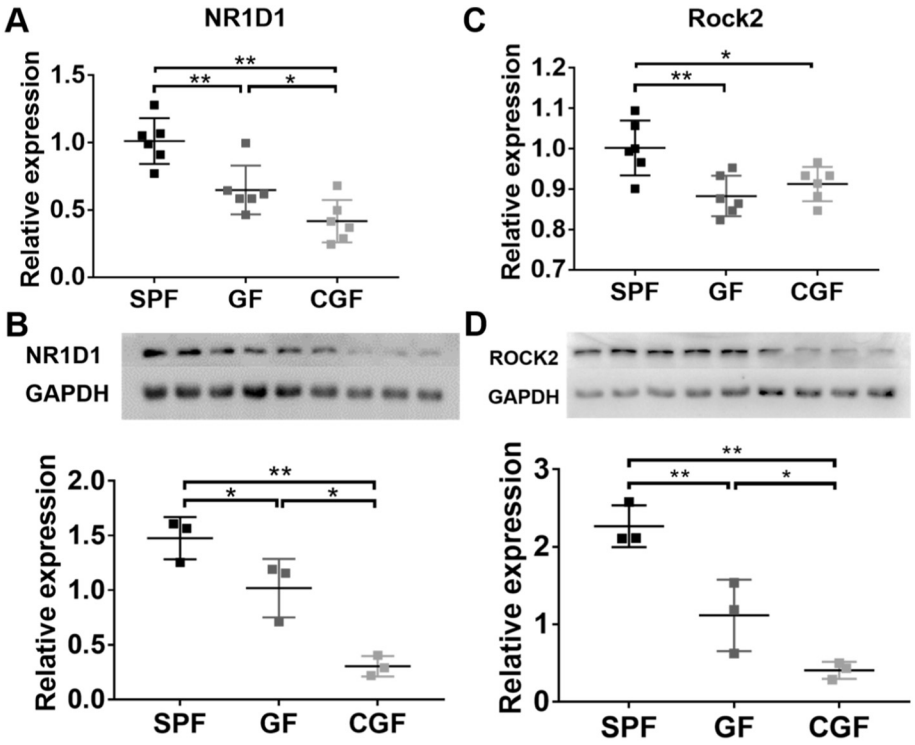

Fig. 5. Absence of microbiota alters NR1D1 and Rho/ROCK signaling in the PFC. (A) Effect of microbiota on NR1D1 transcript levels ($n = 6$). (B) NR1D1 protein levels ($n = 3$). (C) The expression of the Rho/ROCK pathway gene, Rock2 (n = 6). (D) Effect of microbiota on the protein levels of ROCK2 (n = 3). Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.
(Fig. 5A-D, Assessed by one-way ANOVA with the LSD post-hoc test).

the early stages of brain development, the microbiota maybe indispensable for normal axon development.

In the study, GF mice displayed more exploration of the center of the open field in the OFT and less immobility time in the FST. However, colonization was not sufficient to normalize anxiety-related and passive behavioral alterations in CGF mice. These results were consistent with the changes in axonal guidance signaling in GF and CGF mice [28]. Axonal guidance signaling is an essential factor in the formation of neural circuits that are associated with behavioral disorders [9–12]; thus, changes in signaling may be involved in alteration of behavior in GF and CGF mice.

Of the differential axonal guidance-associated genes mentioned above, Sema3A has been reported to be linked to behavioral disorder diseases such as depression [36,55].In African-Americans, Sema3A may contribute to susceptibility to comorbid alcohol dependence and major depression [34]. Sema3A is a secreted protein that is vital for the collapse of growth cones and the growth of apical dendrites. In vitro experiment indicated that Sema3A treatment increased the dendrite length but reduced the axon length of neurons [56].Sema3A overexpression in neurons disrupted nerve projections and prevented oriented growth of axons in vivo [57,58]. Furthermore, Sema3A-Npn1 signaling is involved in this mechanism. By signaling through plexinA/NRP1 complexes, Sema3A plays a key role in neuronal development [41–43].

In this study, Sema3A and NRP1, were upregulated at both the transcript and protein levels in the PFC of GF mice. These results suggest that Sema3A/NRP1 signaling may participate in the microbiota regulation of axon development. In the PFC of CGF mice, *Sema3A* and *NRP1* transcript levels were unchanged, but the Sema3A protein level was increased, compared with those in SPF and GF mice. Additionally, NRP1 expression was decreased in CGF mice. These findings suggest that after the critical period, colonization of microbiota only affects the protein levels of effectors in the axon guidance pathway. This effect may be associated with changes in protein degradation pathways.

We also showed here that Sema3A inhibited NR1D1 expression in primary cultured neurons. NR1D1, also known as rev-ErbA alpha, is a transcriptional repressor that coordinates the circadian rhythm by repressing the expression of core clock components [59–63]. It also controls mitochondrial biogenesis and respiration by regulating the STK11 pathway [64]. By recruiting the corepressor NCOR, NR1D1 mediates transcriptional repression and activation of histone deacetylase. In the developing mouse brain, Nr1d1 participated in neuronal architecture and function via interaction with GTPase-activating protein OPHN1 [67].Knockdown of NR1D1 inhibits axonal extension and dendritic arbor formation in cortical neurons [65], which is similar to the effect of increased Sema3A expression. In this study, the transcript and protein levels of NR1D1 were significantly decreased, concomitant with the increased expression of Sema3A in GF mice. In our in vitro experiment, we demonstrated that Sema3A inhibits NR1D1 expression at both the transcript and protein levels in primary cultured neurons. These results suggest that NR1D1 may be involved in the role of Sema3A in the regulation of axon guidance and development.

It has been reported that dopaminergic systems have central roles in the regulation of anxiety-like behaviors and dopamine depletion can induce anxiety and depression-like behavior which could be alleviated by L-DOPA treatment [70–75]. However,Nr1d1 knockout mice display marked hyperactivity consistent with the behavioral phenotypes of CGF mice in this study [66] and exhibit up-regulation of dopaminergic gene expression and higher dopamine turnover which associated with anxiety-like behavior [68,69]. NR1D1 participation in the regulation of axonal development and behavior changes of GF mice is a potential mechanism, however further research is required.

Sema3A signals through the downstream RhoA/ROCK pathway, which regulates cytoskeletal dynamics and actomyosin contractility. Sema3A downregulates ROCK2, and alters cell migration and spreading through a ROCK-dependent mechanism [76–78]. In this study, the inhibitory effect of Sema3A on NR1D1 in primary cultured neurons was consistent with the effect of fasudil, an inhibitor of ROCK, which affects axonal growth and prevents chronic restraint stress-induced depressive-like behaviors [79,80]. Therefore, the inhibitory effect of Sema3A on NR1D1 may occur through the RhoA/ROCK pathway. To test this hypothesis, two agonists of the RhoA/ROCK pathway, U46619 [81] and Narciclasine, were used to interfere with the expression of NR1D1. When incubated with Sema3A, both agonists lowered the inhibitory

effect of Sema3A on NR1D1 expression and regulated the expression of NR1D1 at an intermediate level. Because these two inhibitors are not specific inhibitors of the RhoA/ROCK pathway, they may have other targets that could be involved in the effect. However, activation of the RhoA/ROCK pathway is a common characteristic of these agonists. Therefore, this effect of the two agonists on NR1D1 expression inhibited by Sema3A suggests that Sema3A inhibits NR1D1 expression by inhibiting the activity of the RhoA/ROCK pathway.

We also found that the protein expression levels of ROCK2 were significantly decreased, concomitant with the increased expression of Sema3A, in GF mice. Taken together, the in vitro and in vivo results suggest that Sema3A-mediated modulation of NR1D1 expression through the RhoA/ROCK pathway may be involved in the regulation of axonal development and behavior by the microbiota. As a member of the semaphorin family, Sema3A signals through heterocomplexes of neuropilins and Class A Plexins. Corresponding evidence that Nrp1 is a receptor for Sema3A comes from overexpression studies. Moreover, the presence of Plxna1 increased the affinity of Nrp1 for Sema3A [82]. In this study, the expression of NRP1 and ROCK2 concomitant with Sema3A indicates that Sema3A regulates the RhoA/ROCK pathways by binding to NRP1 in GF mice, however Plxna1 and other Class A Plexins, which did not differ between SPF and GF mice, are not involved in this process. Therefore the microbiota may regulate axonal development and behavioral phenotypes by regulating this pathway. In particular, in the CGF group, colonization did not reverse the changes in Sema3A, ROCK2 and NR1D1 expression, in contrast to the GF group. These results are consistent with behavioral changes in GF and CGF mice.

5. Conclusion

Our findings suggest that the microbiota influences the behavioral phenotype of the host and the gene expression of axonal guidance signaling molecules in the PFC (Appendix Fig. R1). Colonization was not sufficient to normalize differentially expressed genes involved in axonal guidance signaling; Sema3A-mediated modulation of NR1D1 expression through the RhoA/ROCK pathway may be involved in the behavior regulation by the microbiota.

Acknowledgements

Funding: This work was supported by the National Key R&D Program of China (Grant numbers:2017YFA0505700) ; The National Natural Science Foundation of China(Grant numbers: 81701360, 81701361) ; The Chongqing Science & Technology Commission (Grant number: cstc2017jcyjA0207) and Chongqing Post-doctoral Science Foundation Project (Grant number: Xm2017026).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.03.023.

References

[1] Bäckhed, F., Ley, RE., Sonnenburg, JL., et al., Host-bacterial mutualism in the human intestine. Science, 307(5717) (2005), 1915–1920.
[2] L.V. Hooper, Bacterial contributions to mammalian gut development.[J], Trends Microbiol. 12 (3) (2004) 129–134.
[3] Velagapudi V R, Hezaveh R, Reigstad C S, et al. The gut microbiota modulates host energy and lipid metabolism in mice[J]. J. Lipid Res., 2010, 51(5):1101–1112.
[4] Nicholson J K, Holmes E, Kinross J, et al. Host-gut microbiota metabolic interactions.[J]. Science, 2012, 336(6086):1262–1267.
[5] Yan J, Herzog J W, Tsang K, et al. Gut microbiota induce IGF-1 and promote bone formation and growth[J]. Proc. Natl. Acad. Sci., 2016:2016 07235.
[6] Schwarzer M, Makki K, Storelli G, et al. Lactobacillus plantarum strain maintains growth of infant mice during chronic undernutrition[J]. Science, 2016, 351(6275):854–857.
[7] Heijtz R D, Wang S, Anuar F, et al. Normal gut microbiota modulates brain development and behavior[J]. Proc. Natl. Acad. Sci., 2011, 108(7):3047–3052.
[8] Huo R, Zeng B, Li Z, et al. Microbiota modulate anxiety-like behavior and endocrine abnormalities in hypothalamic-pituitary-adrenal axis[J]. Front. Cell. Infect. Microbiol., 2017, 7:489.
[9] Suda S, Iwata K, Shimmura C, et al. Decreased expression of axon-guidance receptors in the anterior cingulate cortex in autism[J]. Mol. Autism, 2011, 2(1):14–14.
[10] Shansky R M, Hamo C, Hof P R, et al. Stress-induced dendritic remodeling in the prefrontal cortex is circuit specific[J]. Cereb. Cortex, 2009, 19(10):2479–2484.
[11] T.U. Woo, Alterations in chandelier neuron axon terminals in the prefrontal cortex of schizophrenic subjects.[J], Am. J. Psychiatry 156 (11) (1999) 1709–1719.
[12] Taoka, T., Emura, T., Sakamoto, M. et al. Tract-specific diffusion tensor analysis of limbic fibers and tract-based spatial statistics (TBSS) of diffusion tensor image in the cases of Alzheimer disease: characteristics and agreement of the methods. Radiological Society of North America 2010 Scientific Assembly and Meeting (2010).
[13] A. Pittman, M.C. Law, Pathfinding in a large vertebrate axon tract: isotypic interactions guide retinotectal axons at multiple choice points.[J], Development 135 (17) (2008) 2865–2871.
[14] María J. Barallobre, Pascual M, José A. Del Río, et al. The netrin family of guidance factors: emphasis on Netrin-1 signalling[J]. Brain Res. Rev., 2005, 49(1):0–47.
[15] Erskine L, Williams S E, Brose K, et al. Retinal ganglion cell axon guidance in the mouse optic chiasm: expression and function of Robos and slits[J]. J. Neurosci., 2000, 20(13):4975.
[16] J. Egea, Rüdiger Klein, Bidirectional Eph–ephrin signaling during axon guidance[J], Trends Cell Biol. 17 (5) (2007) 230–238.
[17] Janssen B J C, Malinauskas T, Weir G A, et al. Neuropilins lock secreted semaphorins onto plexins in a ternary signaling complex[J]. Nat. Struct. Mol. Biol., 2012, 19(12):1293–1299.
[18] A.L. Kolodkin, D.J. Matthes, C.S. Goodman, The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules.[J], Cell 75 (7) (1993) 1389–1399.
[19] M.A. Morgan, L.M. Romanski, J.E. Ledoux, Extinction of emotional learning: contribution of medial prefrontal cortex[J], Neurosci. Lett. 163 (1) (1993) 109–113.
[20] R.M. Sullivan, A. Gratton, Prefrontal cortical regulation of hypothalamic–pituitary–adrenal function in the rat and implications for psychopathology: side matters[J], Psychoneuroendocrinology 27 (1–2) (2002) 99–114.
[21] R.J. Davidson, Anxiety and affective style: role of prefrontal cortex and amygdala [J], Biol. Psychiatry 51 (1) (2002) 68–80.
[22] Y. Goto, C.R. Yang, Functional and dysfunctional synaptic plasticity in prefrontal cortex: roles in psychiatric disorders.[J], Biol. Psychiatry 67 (3) (2010) 199–207.
[23] D.G. Amaral, C.M. Schumann, C.W. Nordahl, Neuroanatomy of autism[J], Trends Neurosci. 31 (3) (2008) 0–145.
[24] Heijtz R D, Wang S, Anuar F, et al. Normal gut microbiota modulates brain development and behavior[J]. Proc. Natl. Acad. Sci., 2011, 108(7):3047–3052.
[25] Hoban A E, Stilling R M, Ryan F J, et al. Regulation of prefrontal cortex myelination by the microbiota[J]. Transl. Psychiatry, 2016, 6(4):e774.
[26] Luczynski P, Whelan S O, O'Sullivan C, et al. Adult microbiota-deficient mice have distinct dendritic morphological changes: differential effects in the amygdala and hippocampus[J]. Eur. J. Neurosci., 2016, 44(9):2654–2666.
[27] Clarke G, Grenham S, Scully P, et al. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner.[J]. Mol. Psychiatry, 2013, 18(6):666–673.
[28] Li Z, Zeng B, Wang H, et al. Microbiota modulates behavior and protein kinase C mediated cAMP response element-binding protein signaling:[J]. Sci. Rep., 2016, 6:29998.
[29] Beaudoin G M J, Lee S H, Singh D, et al. Culturing pyramidal neurons from the early postnatal mouse hippocampus and cortex[J]. Nat. Protoc., 2012, 7(9):1741–1754.
[30] Kobayashi H, Saito T, Sato K, et al. Phosphorylation of cyclin-dependent kinase 5 (Cdk5) at Tyr-15 is inhibited by Cdk5 activators and does not contribute to the activation of Cdk5[J]. J. Biol. Chem., 2014, 289(28):19627–36.
[31] Singh J, Kumar S, Krishna C V, et al. Aging-associated oxidative stress leads to decrease in IAS tone via RhoA/ROCK downregulation[J]. Am. J. Physiol. Gastrointest. Liver Physiol., 2014, 306(11):983–91.
[32] Wahyuni D S, Van d K F, Klinkhamer P G, et al. The use of bio-guided fractionation to explore the use of leftover biomass in Dutch flower bulb production as allelochemicals against weeds.[J]. Molecules, 2013, 18(4):4510–4525.
[33] Lefranc F, Sébastien Sauvage, Goietsenoven G V, et al. Narciclasine, a plant growth modulator, activates rho and stress fibers in glioblastoma cells[J]. Mol. Cancer Ther., 2009, 8(7):1739–1750.
[34] Zhou H, Polimanti R, Yang B Z, et al. Genetic risk variants associated with comorbid alcohol dependence and major depression.[J]. JAMA Psychiat., 2017, 74(12):1234.
[35] de Anda F C, Rosario A L, Durak O, et al. Autism spectrum disorder susceptibility gene TAOK2 affects basal dendrite formation in the neocortex[J]. Nat. Neurosci., 2014, 17(12):1022–1031.
[36] Eastwood S L, Law A J, Everall I P, et al. The axonal chemorepellant semaphorin 3A is increased in the cerebellum in schizophrenia and may contribute to its synaptic pathology[J]. Mol. Psychiatry, 2003, 8(2):148–155.
[37] Josef G, Linda K, Catherine R, et al. Semaphorin and plexin gene expression is altered in the prefrontal cortex of schizophrenia patients with and without auditory hallucinations[J]. Psychiatry Res., 2015, 229(3):850–857.
[38] Noraz N, Jaaoini I, Charoy C, et al. Syk kinases are required for spinal commissural axon repulsion at the midline via ephrin/Eph pathway[J]. Development, 2016:(dev. 128629).
[39] Cowan C A, Yokoyama N, Bianchi L M, et al. EphB2 guides axons at the midline and is necessary for normal vestibular function.[J]. Neuron, 2000, 26(2):417–430.
[40] B. Knoll, On the topographic targeting of basal vomeronasal axons through slit-

mediated chemorepulsion[J], Development 130 (21) (2003) 5073–5082.
[41] Messersmith E K, Leonardo E D, Shatz C J, et al. Sernaphorin III Can Function as a Selective Chemorepellent to Pattern Sensory Projections in the Spinal Cord[J].
[42] Kolodkin A L, Levengood D V, Rowe E G, et al. Neuropilin is a semaphorin III receptor.[J]. Cell, 1997, 90(4):753–62.
[43] R.J. Pasterkamp, R.J. Giger, Semaphorin function in neural plasticity and disease. [J], Curr. Opin. Neurobiol. 19 (3) (2009) 263–274.
[44] Molofsky A V, Kelley K W, Tsai H H, et al. Astrocyte-encoded positional cues maintain sensorimotor circuit integrity[J]. Nature, 2014, 509(7499):189.
[45] Fatemi S H, Reutiman T J, Folsom T D, et al. Maternal infection leads to abnormal gene regulation and brain atrophy in mouse offspring: implications for genesis of neurodevelopmental disorders[J]. Schizophr. Res., 2008, 99(1):56–70.
[46] Erny D, Hrabě de Angelis, Anna Lena, Jaitin D, et al. Host microbiota constantly control maturation and function of microglia in the CNS[J]. Nat. Neurosci., 2015, 18(7):965–977.
[47] Paolicelli R C, Bolasco G, Pagani F, et al. Synaptic pruning by microglia is necessary for Normal brain development[J]. Science, 2011, 333(6048):1456–1458.
[48] Möhle L, Mattei D, Heimesaat M, et al. Ly6Chi monocytes provide a link between antibiotic-induced changes in gut microbiota and adult hippocampal neurogenesis [J]. Cell Rep., 2016:S2211124716305186.
[49] M. Chunlong, Y. Yuxiang, Z. Weiyun, Gut microbiota: the brain peacekeeper[J], Front. Microbiol. 7 (2016).
[50] Zheng P, Zeng B, Zhou C, et al. Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism[J]. Mol. Psychiatry, 2016, 21(6):786–796.
[51] Chen JJ, Xie J, Zeng L, et al. Urinary metabolite signature in bipolar disorder patients during depressive episode. Aging (Albany NY). 2019; 11(3):1008–1018.
[52] Chen J, Bai S J, Li W, et al. Urinary biomarker panel for diagnosing patients with depression and anxiety disorders. Transl. Psychiatry. 2018; 8(1): 192.
[53] J. Stiles, T.L. Jernigan, The basics of brain development[J], Neuropsychol. Rev. 20 (4) (2010) 327–348.
[54] Eric I. Knudsen, Sensitive periods in the development of the brain and behavior[J], J. Cogn. Neurosci. 16 (8) (2004) 1412–1425.
[55] Josef G, Linda K, Catherine R, et al. Semaphorin and plexin gene expression is altered in the prefrontal cortex of schizophrenia patients with and without auditory hallucinations[J]. Psychiatry Res., 2015, 229(3):850–857.
[56] Shelly M, Cancedda L, Lim B K, et al. Semaphorin3A regulates neuronal polarization by suppressing axon formation and promoting dendrite growth.[J]. Neuron, 2011, 71(3):381–382.
[57] Hong Zhao, Takuro Maruyama, Yuki Hattori, et al. A molecular mechanism that regulates medially oriented axonal growth of upper layer neurons in the developing neocortex[J]. J. Comp. Neurol., 2011, 519(5):0–0.
[58] Moret F, Renaudot C, Bozon M, et al. Semaphorin and neuropilin co-expression in motoneurons sets axon sensitivity to environmental semaphorin sources during motor axon pathfinding[J]. Development, 2007, 134(24):4491–4501.
[59] Lazar M A, Hodin R A, Cardona G,, et al. Gene expression from the c-erbA alpha/rev-ErbA alpha genomic locus. Potential regulation of alternative splicing by opposite strand transcription[J]. J. Biol. Chem., 1990, 265(22):12859.
[60] Preitner N, Damiola F, Luis LopezMolina, et al. The orphan nuclear receptor REV-ERBα controls circadian transcription within the positive limb of the mammalian circadian oscillator[J]. Cell, 2002, 110(2):251–260.
[61] Gérard Triqueneaux, Thenot S, Kakizawa T, et al. The orphan receptor rev-erb gene is a target of the circadian clock pacemaker[J]. J. Mol. Endocrinol., 2005, 33(3):585–608.
[62] Vieira E, Marroquí, Laura, Batista T M, et al. The clock gene\r, rev-erb\r, α regulates pancreatic β-cell function: modulation by leptin and high-fat diet[J]. Endocrinology, 2012, 153(2):592–601.
[63] Hélène Duez, Veen J N V D, Duhem C, et al. Regulation of bile acid synthesis by the nuclear receptor rev-erbα[J]. Gastroenterology, 2008, 135(2):689–698.e5.
[64] Woldt E, Sebti Y, Solt L A, et al. Rev-erb-[alpha] modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy[J]. Nat. Med., 2013, 19(8):1039–1046.
[65] Goto M, Mizuno M, Matsumoto A, et al. Role of a circadian-relevant gene NR1D1 in brain development: possible involvement in the pathophysiology of autism spectrum disorders[J]. Sci. Rep., 2017, 7:43945.
[66] Jager J, O'Brien W T, Manlove J, et al. Behavioral changes and dopaminergic dysregulation in mice lacking the nuclear receptor rev-erbα.[J]. Mol. Endocrinol., 2014, 28(4):490–8.
[67] Valnegri, P. et al. A circadian clock in hippocampus is regulated by interaction between oligophrenin-1 and rev-erbα. Nat. Neurosci. 28, 1293–1301, doi:https://doi.org/10.1038/nn.2911 (2011).
[68] Jager J, O'Brien W T, Manlove J, et al. Behavioral changes and dopaminergic dysregulation in mice lacking the nuclear receptor rev-erbα.[J]. Mol. Endocrinol., 2014, 28(4):490–8.
[69] M. Zarrindast, F. Khakpai, The modulatory role of dopamine in anxiety-like behavior[J], Arch. Iran. Med. 18 (9) (2015) 591–603.
[70] M. Bonomaully, T. Khong, M. Fotriadou, J. Tully, Anxiety and depression related to elevated dopamine in a patient with multiple mediastinal paragangliomas, Gen. Hosp. Psychiatry 36 (4) (2014) e447–e448.
[71] K.L. Eskow Jaunarajs, J.A. George, C. Bishop, L–DOPA–induced dysregulation of extrastriatal dopamine and serotonin and affective symptoms in a bilateral rat model of Parkinson's disease, Neuroscience. 218 (2012) 243–256.
[72] L.M. Broersen, F. Abbate, M.G. Feenstra, J.P. De Bruin, R.P. Heinsbroek, B. Olivier, Prefrontal dopamine is directly involved in the anxiogenic interoceptive cue of pentylenetetrazol but not in the interoceptive cue of chlordiazepoxide in the rat, Psychopharmacology 149 (4) (2000) 366–376.
[73] P.M. Wall, R.J. Blanchard, C. Markham, M. Yang, D.C. Blanchard, Infralimbic D1 receptor agonist effects on spontaneous novelty exploration and anxiety–like defensive responding in CD–1 mice, Behav. Brain Res. 152 (1) (2004) 67–79.
[74] P.M. Wall, C. Messier, Concurrent modulation of anxiety and memory, Behav. Brain Res. 109 (2) (2000) 229–241.
[75] N.M. Lauzon, M. Bechard, T. Ahmad, S.R. Laviolette, Supra–normal stimulation of dopamine D1 receptors in the prelimbic cortex blocks behavioral expression of both aversive and rewarding associative memories through a cyclic–AMP–dependent signaling pathway, Neuropharmacology 67 (2013) 104–114.
[76] V.D. Dontchev, P.C. Letourneau, Growth cones integrate signaling from multiple guidance cues.[J], J. Histochem. Cytochem. 51 (4) (2003) 435–444.
[77] Gehler S, Compere F V, Miller A M. Semaphorin 3A increases FAK phosphorylation at focal adhesions to modulate MDA-MB-231 cell migration and spreading on different substratum concentrations[J]. Int. J Breast Cancer, 2017(6):1–16.
[78] Lins M P, Da S E, Da S G, et al. Association between biomechanical alterations and migratory ability of semaphorin-3A-treated thymocytes[J]. Biochim. Biophys. Acta Gen. Subj., 2018, 1862(4).
[79] W.D. Xiao, A.X. Yu, D.L. Liu, Fasudil hydrochloride could promote axonal growth through inhibiting the activity of ROCK.[J], Int. J. Clin. Exp. Pathol. 7 (9) (2014) 5564–5568.
[80] Gonzalo García-Rojo, Cristóbal Fresno, Vilches N, et al. The ROCK inhibitor Fasudil prevents chronic restraint stress-induced depressive-like behaviors and dendritic spine loss in rat hippocampus[J]. Int. J. Neuropsychopharmacol., 2017, 20(4):336–345.
[81] Huzoor A, Duan X, Saima S, et al. RhoA and Rac1 GTPases differentially regulate agonist-receptor mediated reactive oxygen species generation in platelets:[J]. PLoS One, 2016, 11(9):e0163227.
[82] Takahashi T, Fournier A, Nakamura F, et al. Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors.[J]. Cell, 1999, 99(1):59–69.