

Role of gut microbiota in brain function and stress-related pathology

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Gut microbiota are responsible for a variety of metabolic activities including food digestion and production of biologically active substances. Moreover, several recent works, including our own, have also shown that gut microbiota play an important role not only in the development of brain function but also in the pathology of stress-related diseases and neurodevelopmental disorders. In this review, we focus on the interaction between gut microbes and the brain-gut axis and introduce some basic concepts and recent developments in this area of research.

Key words: behavior, gut microbiota, microglia, resilience, short-chain fatty acids, stress response

INTRODUCTION

Remarkable advances have been achieved in the past decade in the field of gut microbiota research [1–3]. In particular, a great deal of attention has been paid to the role of gut microbes in the function and pathology of the brain [4–6].

In this paper, a brief outline about this theme is provided based on recent studies, including those of our group.

Gut microbiota affect host responses to stress

Upon exposure to harmful stress stimuli, the hypothalamic-pituitary-adrenal axis (HPA axis) and the sympathetic nervous system mammals are activated in mammals, including humans, to maintain homeostasis in the body [7]. Interestingly, the HPA axis, the activation of which is a major component of stress responses, is known to be affected not only by genetic determinants but also by postnatal environmental factors during infancy. For example, the responsiveness of the HPA axis in adults is substantially influenced by maternal behaviors such as licking and grooming during the early stages of life [8, 9]. Because gut microbes are an important environmental factor, we hypothesized that the gut microbiota play a role in host stress responses. Therefore, we examined this speculation using gnotobiotic animal models.

The degree of plasma adrenocorticotropin hormone (ACTH) and corticosterone elevation in response to a 1-hour restraint stress was higher in germ-free (GF) mice than in specific pathogen free (SPF) mice [10]. During an *in vivo* glucocorticoid feedback sensitivity test, a bolus injection of

corticosterone reduced plasma ACTH levels in response to restraint stress in a dose-dependent manner, a decrease that occurred to a significantly lesser extent in GF mice than in SPF mice [11]. These results suggest that GF mice have low sensitivity to the inhibitory effect of glucocorticoids on the HPA response. Furthermore, as summarized in Table 1 [10], monoassociation with *Bifidobacterium infantis*, a representative inhabitant of the neonate gut, decreased the HPA stress response to SPF, while monoassociation with *Bacteroides vulgatus* had no effect. The hormonal stress response in the rabbit-derived enteropathogenic *Escherichia coli* (EPEC)-monoassociated mice was substantially higher than that in the GF mice, although no such exaggerated response was found in the mice reconstituted with an EPEC mutant strain, Δ Tir [12], which was not internalized due to defects in the translocated intimin receptor.

Table 1. Plasma ACTH and corticosterone elevation upon exposure to restraint stress (RS) in the gnotobiotic mice¹

| | ACTH (pg/ml) | | Corticosterone (ng/ml) | |
|--------------------|--------------|-------------|------------------------|-------------|
| | Basal | 1 hr RS | Basal | 1 hr RS |
| GF | 66 ± 12 | 188 ± 16 | 19 ± 3.9 | 131 ± 12 |
| SPF | 54 ± 6.1 | 106 ± 20*** | 21 ± 6.5 | 86 ± 9.9*** |
| <i>B. infantis</i> | 60 ± 9.8 | 113 ± 15*** | 21 ± 5.2 | 79 ± 9.5*** |
| <i>B. vulgatus</i> | 63 ± 9.9 | 166 ± 14 | 17 ± 6.8 | 140 ± 14 |
| EPEC | 49 ± 15 | 243 ± 22* | 19 ± 6.6 | 172 ± 20* |
| Δ Tir | 60 ± 9.5 | 153 ± 25 | 15 ± 3.6 | 102 ± 17 |

¹Plasma ACTH and corticosterone levels were measured at before or immediately after 1 hr RS in germ-free (GF), SPF, and gnotobiotic mice reconstituted with a single strain with *Bifidobacterium infantis* (*B. infantis*), *Bacteroides vulgatus* (*B. vulgatus*), rabbit-derived enteropathogenic *E. coli* (EPEC), or EPEC mutant strain (Δ Tir) at 9 wks of age. ***Significantly different from the GF value (p<0.001). *Significantly different from the GF value (p<0.05).

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Table 2. Normal gut microbiota render the host less active and anxious¹

| | | 7 wks of age | 10 wks of age | 16 wks of age |
|-------|------------|----------------|---------------|---------------|
| GF | OFT (DT30) | 62.7 ± 12.2*** | 63.7 ± 9.4** | 66.4 ± 21.4* |
| | MBT (NBM) | 14.3 ± 5.3 | 15.9 ± 5.9* | 14.6 ± 6.2* |
| EX-GF | OFT (DT30) | 46.5 ± 7.1 | 54.0 ± 8.9 | 53.5 ± 11.3 |
| | MBT (NBM) | 12.5 ± 5.8 | 12.4 ± 4.5 | 9.5 ± 5.5 |

¹To make EX-GF mice, the parent germ-free (GF) mice were orally given stools of SPF mice, and their offsprings were used as EX-GF mice. GF and EX-GF mice at 7, 10, and 16 wks of age were subjected to an open-field test (OFT) and marble-burying test (MBT). Total distance travelled for 30 min (DT30; meters) was automatically calculated as spontaneous locomotor activity. The number of buried marbles in 30 min (NBM) was counted as a parameter of anxiety-like behavior. All data are expressed as the mean ± SD. *Significantly different from the corresponding EX-GF values (p<0.05). **Significantly different from the corresponding EX-GF values (p<0.01). ***Significantly different from the corresponding EX-GF values (p<0.001).

These results indicate that gut microbiota can play a critical role in the development and regulation of the HPA response to stressors.

Commensal microbes influence host behavior [13]

Findings such as those reported above raise an interesting question as to whether gut bacteria can affect host behavior. This question has been addressed, with animal studies performed by several independent groups, including our own, showing the commensal microbiota to be a crucial factor in the modulation of the host's behavioral profile [14–19]. Some of our findings are shown below.

We first developed a reliable method to accurately analyze the behavior of GF mice maintained in an isolator [17]. This method enabled us to evaluate GF animal behavior without the risk of exposure to contamination. Using this system, GF mice were found to be more active and anxious than EX-GF mice reconstituted with a normal SPF microbiota, based on open field and marble-burying tests (Table 2). Colonization with *B. infantis* decreased the locomotor activity to the EX-GF level but had little effect on the levels of anxiety. In contrast, monoassociation with *Clostridium coccoides* reduced the anxiety levels; however, it did not affect the locomotor activity [17].

Recently, an elegant study by Schretter and co-workers [20] found that *Drosophila* are more active under GF conditions than when in possession of with a normal gut microbiome, mirroring the observations described above in GF mice. Interestingly, the hyperactivity found in GF *Drosophila* was normalized by colonization with *L. brevis*, a normal component of *Drosophila*'s gut microbiota. Moreover, they also revealed that the enzyme xylose produced by *L. brevis* is key to this phenomenon.

Taken together, these results indicate that gut microbiota can exert a substantial effect on behavioral phenotype, and that this may be mediated by molecules of bacterial origin.

Microbe-induced behavioral manipulation

Behavioral changes caused by microorganisms have been observed in different host species [16] and in special

situations. For example, it is well known that *Toxoplasma* infection can alter mouse behavior [21, 22]. In fact, mice infected with *Toxoplasma* become insensitive to the smell of cats (the parasite's end host) and are consequently more easily and rapidly preyed upon. This phenomenon enables the parasites to reach the end host more efficiently, with the series of processes involved, referred to as "behavioral manipulation" [23] or "mind control" [6] elicited by bacteria.

Recently, Schnorr *et al.* [24] compared gut bacteria and metabolites in Hadza hunter-gatherers with those of Westerners. The results revealed that bacterial diversity in gut microorganisms is richer in the Hadza than in Westerners. Their results suggested that alteration in diet and lifestyle induced a drastic change in enteric bacteria as societies transitioned from a hunter-gatherer to an agricultural lifestyle during the Neolithic era. The associated shift in the composition of the gut microbiome may have altered the "carnivorous character" in humans, which is characterized by a high level of novelty seeking and aggressiveness, into a "herbivorous character" that is more cooperative and passive.

The fact that indigenous bacteria are involved in the development of host behavioral characteristics in various animals ranging from *Drosophila* to mammals may implicate the possible role of microorganisms in the evolution of living organisms [25, 26].

Possible pathways and molecules involved in the microbiota-brain-gut axis

How can information derived from intestinal bacteria be transmitted to the brain? Many substances and pathways are presumably involved; however, we will focus on some of recent topics, as described below.

Neural pathways

Many afferent nerves, including the vagus nerve and spinal afferent nerve, are distributed in the intestinal tract and are thought to transmit information from the intestinal lumen to the central nervous system (CNS). As we previously demonstrated [10], when *B. infantis* was orally administered to GF mice, c-fos expression in the hypothalamus was enhanced immediately after administration. Interestingly, this response

was partially suppressed not only by treating neonatal GF mice with capsaicin [27], which can destroy vagal afferent nerve fibers, but also by pretreatment with granisetron, an antagonist of serotonin type 3 receptors [28]. These results indicate that serotonin released from enterochromaffin cells upon exposure to gut microbes acts on serotonin type 3 receptors on capsaicin-sensitive afferent nerve endings, thereby transferring the information generated in the gut to the brain. Moreover, Bravo and colleagues [29] also found that orally administered *Lactobacillus rhamnosus* JB1 attenuates stress-induced anxiety or depression, although this attenuation effect is absent in mice pretreated with vagotomy. Taken together, these findings indicate that the JB1-induced anti-stress effect is mediated through the vagus nerve.

More recently, Han *et al.* [30] demonstrated that gut-innervating vagal sensory neurons are an essential component of the neuronal reward pathway, linking sensory neurons in the upper gut to striatal dopamine release. Furthermore, Kaelberer *et al.* [31] have shown that a subset of mouse enteroendocrine cells marked by cholecystokinin and peptide YY expression form direct synaptic connections with vagal and spinal neurons. Infusing sucrose or table sugar into the gut causes vagus nerve activation in an enteroendocrine cell-dependent manner.

These exciting findings clearly indicate that vagal and spinal afferent neurons can play a central role in the gut-brain signaling and exert a profound effect on brain function, including the neuronal reward system.

Short-chain fatty acids (SCFAs) and microglia

Some gut bacteria metabolize indigestible dietary fiber or oligosaccharides and produce SCFAs [32]. The majority of SCFAs are absorbed from colonic mucosa and used as energy sources for epithelial proliferation, mucus secretion, and water and mineral absorption [33]. Recently, together with the identification of specific receptors for SCFAs, much focus has been placed on the novel and important functions of SCFAs. Here, we describe the influence of butyric acid (BA), a type of SCFA, on the CNS. Furthermore, microglia, a type of CNS glial cell, will be presented as an important mediator connecting the gut and the brain.

BA is mainly produced by *Clostridium* spp. and has been demonstrated to have antidepressive action in animal experiments [34], possibly through the histone deacetylase inhibitory action of BA itself. In fact, brain-derived neurotrophic factor concentrations in the hippocampus and frontal lobe increase following BA treatment [35]. Nonetheless, it remains uncertain whether physiological concentrations of BA produced under normal circumstances are capable of affecting the CNS.

Microglial cells are extremely sensitive not only to damage in the CNS but also to environmental challenges such as psychological stress [36, 37]. A recent study conducted by Erny and co-workers [38] demonstrated that gut microbiota influence the CNS immune system by regulating microglial cell activation and homeostasis. RNA sequencing showed striking differences between the transcriptional profiles of microglia

isolated from GF and SPF young adult mice. Notably, DNA damage-inducible transcript 4 (*Ddit4*), the product of which regulates cell growth, proliferation, and survival, was elevated in microglia from GF mice in comparison with those from SPF mice. Other genes significantly upregulated in microglia from GF mice were *Sfp1* (encoding Pu.1) and *Csf1r*, both of which are highly expressed in developing microglia [36, 37], while several genes involved in cell activation were downregulated. Interestingly, further increasing microbiota complexity by housing partially recolonized animals with normal SPF animals normalized microglial numbers and morphology, as well as *Ddit4* levels. Moreover, when the GF mice were given a mixture of SCFAs in their drinking water, the microglial numbers, *Ddit4* mRNA levels, microglial morphology, and microglial expression of CSF1R were normalized, mirroring what was seen in SPF animals. Thus, SCFAs appear to be important to the regulation of microglial maturation; however, the precise molecular mechanism by which SCFAs render microglia mature remains unknown.

Collectively, these findings clearly show that microglia are a critical link between microbiota and the brain.

Tryptophan and its metabolites

Tryptophan, an essential amino acid, is a precursor in the biosynthesis of serotonin, a representative neurotransmitter. More than 95% of free tryptophan enters the kynurenine pathway that is mainly controlled by indolamine 2, 3-dioxygenase (IDO), a rate-determining enzyme [39]. In general, inflammatory cytokines upregulate IDO and promote this tryptophan-kynurenine route. The IDO is expressed in both astrocytes and microglia in the brain. In addition, kynurenic acid, glutamic acid, and quinolinic acid, all of which are metabolic products of kynurenine, are known to exert various effects on the functions of glial and neural cells [40].

Another important pathway is mediated by gut microorganisms [41]. Tryptophan is broken down by a variety of gut bacteria that possess tryptophanase, resulting in the production of indole-related molecules. In fact, high concentrations of indoles ranging from 250 to 1,100 μM are routinely found in the human intestinal tract [42]. In plants, indoles and their related metabolites play an important role as signal molecules during growth and defend the host against noxious insects [43]. Some mosquitoes and butterflies perceive indoles through their olfactory receptors and alter their behavior in response [44]. The mechanism by which indoles affect animal behavior is becoming an important research topic in recent years, with an increasing number of related studies demonstrating involvement of astrocytes [45, 46]. Moreover, whether indole-induced behavioral manipulation occurs in humans is an important question for future studies.

Role of microbiota in mental health and stress-related pathology in humans

Whether or not gut bacteria can influence stress responses or other behavioral characteristics in humans is an open question. Unfortunately, clinical evidence thus far remains

limited; however, a variety of trials aimed at addressing this question are presently being carried out globally. Here, we provide a brief summary of this topic.

In the beginning of the 20th century, prior to the development of antidepressant drugs, Phillips [47] treated 18 melancholia patients with lactic acid bacilli. As a result, 11 patients recovered fully, 2 patients improved, 4 patients exhibited no change in condition, and 1 patient died. All of the patients displayed a decrease in constipation and body weight gain. Although this report is merely a case report without a control group, it is still worth noting that there were some researchers who expressed interest in the relationship between gut microbes and mental illness at that time.

In the 21st century, Benton and colleagues [48] conducted randomized controlled trials in order to examine the effects of dairy products containing *L. casei* Shirota on mood and cognitive function in 132 healthy volunteers. Statistical analysis failed to show any significant difference between the intervention group and the placebo group; however, in a sub-analysis of participants showing high levels of depression at baseline, a significant improvement of depressed mood was found in the intervention group when compared with the control group. In a recent clinical study [49], healthy volunteers were given either a placebo or a combination of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 in a double-blind, randomized parallel-group study for 30 days and were assessed using the Hopkins Symptom Checklist (HSCL-90), the Hospital Anxiety and Depression Scale (HADS), the Perceived Stress Scale, and the Coping Checklist (CCL) and by analyzing 24 hr urinary free cortisol (UFC). Daily administration of probiotics to volunteers was found to alleviate psychological distress, including global severity index, somatization, depression, and anger-hostility, as measured by the HSCL-90 scale, HADS, and CCL (problem solving), as well as UFC levels. In a study examining the effects of prebiotics, 45 healthy volunteers received one of two prebiotics (fructooligosaccharides, FOS, or Bimuno[®] galactooligosaccharides, B-GOS) or a placebo (maltodextrin) daily for 3 weeks [50]. As a result, the salivary cortisol awakening response was significantly lower following B-GOS intake than with the placebo. Participants also showed decreased attentional vigilance to negative versus positive information in a dot-probe task after administration of B-GOS. No effects were found after the administration of FOS. These results are consistent with previous observations concerning the endocrine and anxiolytic effects of microbiota proliferation. More recently, an interesting study has been reported from Japan [51]. Medical students were randomly assigned to the following two groups: an intervention group, the participants of which ingested dairy products containing *Lactobacillus casei* Shirota and a control group whose participants consumed a placebo. Abdominal symptoms and salivary cortisol concentrations were lower in the intervention group than in the placebo group. Furthermore, the relative occupancy rate of *Bacteroidaceae* in the intervention group was lower than that observed in the placebo group. These

results suggest that probiotic intake may ameliorate stress-relevant symptoms through the modulation of gut microbes.

The remarkable recent advances in neuroimaging techniques have shed light on the mechanisms by which gut microbes can interact with the gut-brain axis. Using functional magnetic resonance imaging (fMRI), Tillisch *et al.* [52] investigated whether four weeks of fermented milk product supplementation can affect brain activity in response to emotional stimulation. They showed that probiotic supplementation is associated with decreased brain responses in affective, viscerosensory, and somatosensory brain regions in response to emotional tasks. Moreover, Pinto-Sanchez *et al.* [53] demonstrated that probiotic administration results in a decrease in depressive complaints associated with irritable bowel syndrome. This was found to be connected with reduced brain limbic reactivity to negative emotional stimuli. More recently, a Dutch research group [54] investigated the effects of a multispecies probiotic on specific neurocognitive measures of emotion reactivity, emotion regulation, and cognitive control using fMRI in a double blind, randomized, placebo-controlled, between-subjects intervention study. Although probiotics failed to affect any parameters without stress induction, the probiotics group exhibited a significant stress-related increase in working memory performance. Furthermore, this change was associated with intervention-related neural changes in the frontal cortex during cognitive control, an effect observed in the probiotics group exclusively.

Thus, accumulating high-quality evidence based on animal studies clearly shows a substantial crosstalk between gut microbes and brain functions; in contrast, clinical studies are still limited. Well-controlled clinical trials with a large sample size are needed to conclusively demonstrate the anti-stress effects of probiotics in humans.

Stress resilience and gut microbes

In the fields of psychiatry and neuroscience, researchers are expressing increasing interest in “stress resilience (SR)”. This is defined as “the process of adapting well in the face of adversity, trauma, tragedy, threats or even significant sources of stress” [55] and is sometimes used as a synonym for “stress tolerance”.

The following factors are known to affect the development and regulation of SR:

- (a) Enriched environment [56]: when rodents are bred in a socially “rich” environment, they generally show reduced anxiety as well as improved learning ability later in life, compared with mice raised in isolation.
- (b) Maternal care [9]: pups that benefit from high levels of maternal behaviors such as licking and grooming as neonates become more resistant to stress stimuli as adults.
- (c) Stress immunization [57, 58]: when animals are repeatedly exposed to “mild” or “manageable” stress during juvenile development, they usually exhibit phenotypes resistant to stressors later in life, compared with animals without such early stress exposure.

The above findings suggest that five senses, such as vision, hearing, touch, taste, and smell, could stimulate the development of a neural network that controls stress response and provides the host with the ability to deal with a variety of stresses encountered later in life. In addition, a “sixth sense” originating from visceral organs is suggested to play an important role in regulation of the stress-related neural networks. For example, Goehler and coworkers [59, 60] showed that oral administration of *Campylobacter jejuni* to mice activates the amygdala, prefrontal cortex, and hypothalamus, which comprise the limbic system, without causing a systemic inflammatory reaction. Activation of these brain areas can occur not only in response to pathogenic bacteria but also upon exposure to indigenous bacteria such as *Bifidobacterium*, as we previously reported [10]. Interestingly, the brain areas activated by gut microbes are similar to the sites activated by “stress immunization”, indicating that a signal derived from intestinal bacteria activates brain areas regulating stress response. This promotes the development of stress-resilient systems in the prefrontal cortex, as stress immunization does affect the brain. Gut bacteria are of course one of the external stressors; however, they repeatedly act on the host as “mild stressors” and render it resilient to stress stimuli, which is useful for coping with survival competition later in life. In this sense, gut microbiota can be regarded as a “eustressor” [61, 62], a term first coined by Hans Selye when referring to a positive aspect of “stressors”.

CONCLUSION AND PERSPECTIVES

From the 19th to the early 20th century, a minority of scientists postulated that psychiatric diseases might result from “auto-intoxication”, meaning that waste products or toxins generated in the gut can lead to depression, anxiety, and even psychosis [63–65]. The concept of auto-intoxication was regarded as an “unscientific” theory and was largely forgotten until recently; however, it has reemerged as an attractive research area and is currently being extensively studied. Further developments in this field could provide a strong rationale for the application of probiotics in the treatment of mental health and diseases.

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