Chapter 8
Microbiome, HPA Axis and Production of Endocrine Hormones in the Gut

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Abstract Recent accumulating evidence indicates that the gut microbiome can affect the development and regulation of the hypothalamic-pituitary-adrenal axis and behavior, with central integrative systems being crucial in the successful physiological adaptation of the organism to external stressor. In contrast, host-derived hormones increase the bacterial proliferative capacity and pathogenicity. In the gut lumen, this type of cross-talk between microorganisms and the host is presumed to be performed continually through various kinds of luminal molecules, as numerous types of bacteria and host cells are in close proximity in the gastrointestinal tract of mammals.

We herein focus on bidirectional signaling between the gut microbiome and the host in terms of commensal microbiota affecting the hypothalamic-pituitary-adrenal HPA axis response and behaviors and further discuss the role of gut luminal catecholamines and γ-aminobutyric acid, both of which are presumed to be involved in this signaling.

Abbreviations

ACTH	Adrenocorticotropin hormone
CA	Catecholamines
CRH	Corticotrophin-releasing hormone
DA	Dopamine
E	Epinephrine
EHEC	Enterohemorrhagic *Escherichia coli*
GABA	γ-Aminobutyric acid
GAD	Glutamic acid decarboxylase

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GC	Glucocorticoids
GUS	β-Glucuronidase
HPA	Hypothalamic-pituitary-adrenal
NE	Norepinephrine
Tir	Translocated intimin receptor

Introduction

Gut microbiota have an estimated mass of 1–2 kg, numbering 100 trillion [1] and together possessing 100 times the number of genes in the human genome [2]. These bacteria not only play a principal role in the postnatal maturation of the mammalian immune system [3], but also aid in the digestion and absorption of macromolecules and act as a barrier to gut pathogens by blocking attachment to gut binding sites [4]. Moreover, it is also rapidly becoming apparent that the gut microbiome plays a major role in the development and regulation of the hypothalamic-pituitary-adrenal (HPA) axis [5] and behavior [6–11].

In contrast, host hormones can signal commensal microbial cells via converging pathways directed to bacterial signaling molecules. Lyte and colleagues first demonstrated in their pioneering studies conducted in the 1990s that some species of pathogens can recognize exogenous catecholamines (CA) in vitro and that such recognition increases the bacterial proliferative capacity [12–15]. Sperandio and colleagues subsequently showed that enterohemorrhagic *Escherichia coli* (EHEC) virulence increases upon exposure to epinephrine (E) and norepinephrine (NE) and that E binds and signals through the QseC receptor [16, 17]. This type of bidirectional communication is called "microbial endocrinology" [15] or "interkingdom signaling" [17, 18], which mediates the symbiotic and pathogenic relationships between the bacteria and mammalian host. Since numerous kinds of bacteria and host cells are in close proximity in the gastrointestinal tract of mammals, interkingdom signaling via various kinds of luminal molecules is presumed to be performed continually in the gut lumen [19] and to participate in the regulation of various pathophysiological functions.

We herein focus on the bidirectional signaling between the gut microbiome and the host in terms of commensal microbiota affecting the HPA axis response and behavior and further discuss the possible involvement of some gut luminal molecules in this signaling.

Gut Microbiota and the Stress Response of the Host

The HPA axis is considered to be a central integrative system, being crucial in the successful physiological adaptation of the organism to stress. During stress, corticotrophin-releasing hormone (CRH) and arginine vasopressin, the principal

hypothalamic regulators of the HPA axis, are released. CRH stimulates the secretion of the adrenocorticotropin hormone (ACTH) from the anterior pituitary into the hypophyseal portal system via collateral fibers in the systemic circulation. ACTH induces the secretion of glucocorticoids (GCs; cortisol in humans and corticosterone in rodents) from the adrenal cortex, the main target of ACTH. GCs regulate multiple bodily functions and prepare the individual to cope with the demands of metabolic, physical and psychological stressors [20].

Critical Role of the Gut Microbiota in Determining the Set Point of the HPA Axis

It is well known that the HPA axis is susceptible to environmental influences, particularly early in life [21, 22]. Since indigenous microbiota constitutes a major environmental force affecting the host physiology, we examined whether these bacteria can alter the development of the HPA response using gnotobiotic mice.

As shown in Fig. 8.1A, the degree of plasma ACTH and corticosterone elevation in response to a 1-h restraint stress was substantially higher in the GF mice than in the SPF mice. When the mice were exposed to ether stimulus, no significant differences in the plasma ACTH or corticosterone response were found in either group of animals (Fig. 8.1B). Monoassociation with *Bifidobacterium infantis*, a representative inhabitant of the neonate gut, lessened the HPA stress response to SPF (Fig. 8.2). The hormonal stress response in the rabbit-derived EPEC-monoassociated mice was substantially higher than that observed in the GF mice, although no such exaggerated response was found in the mice reconstituted with an EPEC mutant strain, ΔTir [23], which is not internalized due to defects in the translocated intimin receptor.

Interestingly, the enhanced HPA stress response of the GF mice was partially corrected at 3 weeks after reconstitution of SPF feces at an early stage of development (Fig. 8.3A), while no such correction was found following reconstitution at a later stage (Fig. 8.3B). Therefore, the microbe-induced reversal of the HPA axis set point extended into adulthood, but only if bacterial colonization occurred before the animals reached 6 weeks of age. Colonization of the adults was ineffective, which suggests a critical window of susceptibility to the effects of bacteria-host interactions.

Recently, animal studies performed by several independent groups have shown the commensal microbiota to be a crucial factor modulating the host behavioral profile [6–9, 11]. In our recent study performed under a strictly contamination-free environment, EX-GF mice, gnotobiotic mice reconstituted with a normal specific pathogen-free microbiota, were less anxious and active than the GF mice based on open field and marble-burying tests [11]. Monoassociation with *Clostridium* (*Brautia*) *coccoides* reduced the anxiety levels; however, it did not affect the

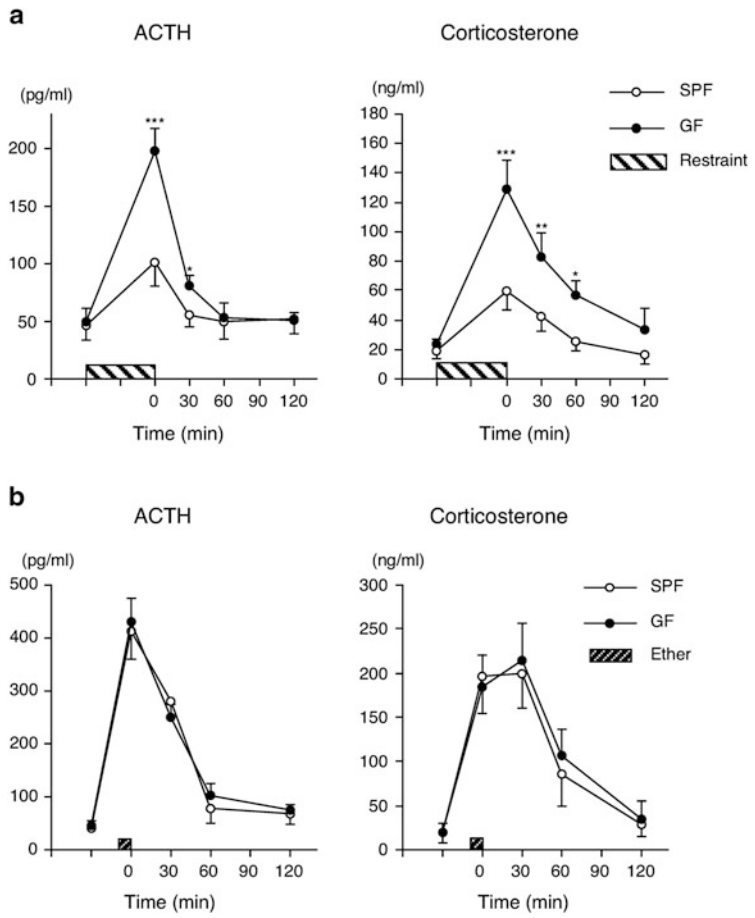

Fig. 8.1 Increased plasma ACTH and corticosterone responses to restraint stress but not to ether exposure in GF mice. **Panel A**: The mice were subjected to a 1-h period of restraint stress (GF, n = 6–11 per each time-point; SPF, n = 6–11 per each time-point). The baseline data were obtained via cardiac puncture in mice killed using cervical dislocation before stress exposure. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ in a post hoc Dunnett's test between GF and SPF. **Panel B**: The GF and SPF mice failed to show any differences in the HPA response to ether exposure (n = 6 per each time point)

locomotor activity. In contrast, colonization with *B. infantis* decreased the locomotor activity while having little effect on the anxiety level.

Therefore, the commensal gut microbiota affects the development and regulation of the biobehavioral stress response of the host.

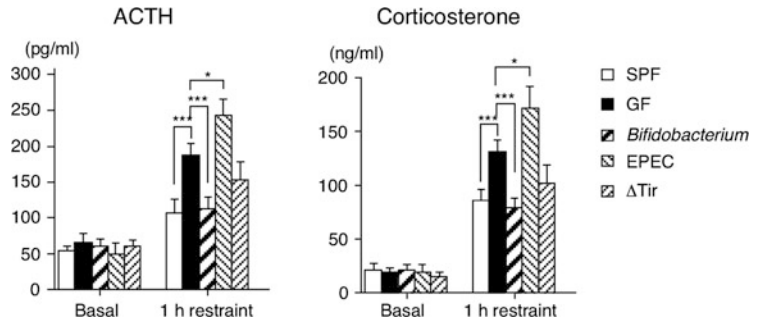

Fig. 8.2 Effects of restraint stress on the plasma ACTH and corticosterone levels in the gnotobiotic mice. The plasma ACTH and corticosterone levels were measured before or immediately after the 1-h restraint test in the GF (n = 20), SPF (n = 18) and monoassociated mice (n = 18–24 per group) at 9 weeks of age. $***P < 0.001$, $*P < 0.05$ according to Dunnett's test. *Bifidobacterium*, EPEC and ΔTir indicate *Bifidobacterium infantis*-, enteropathogenic *E. coli*- and EPEC mutant strain deficient of Tir (translocated intimin receptor)-associated mice, respectively

Microbiota and Stress Resilience

Recently, there has been increasing interest in the individual's response to managing adverse events and stressors [24, 25]. Such an ability to recover from adverse changes, known as "stress resilience," includes psychological and biological processes that allow an individual to avoid or reduce the harmful consequences of extreme stress. Resilient individuals encountering chronic psychosocial stress minimize pathophysiological outcomes, such as extended or exaggerated HPA axis activity [26, 27], that can precipitate stress-related diseases, such as post-traumatic stress disorder, anxiety and major depression [28, 29]. In addition to genetic factors, a broad range of environmental factors contribute to resilience. In fact, a recent elegant study conducted by Lehmann and Herkenham [30] showed that enriched environmental housing (environmental enrichment) confers stress resilience through an infralimbic cortex-dependent neuroanatomical pathway in a mouse model of social defeat stress. Taken together, these findings lead us to the following interesting hypothesis: newborn babies are likely to recognize colonizing bacteria as a stressor when encountering them for the first time because the babies have little capability to discern whether a novel stimulation from the external environment is good or bad. This is supported by the fact that colonization of GF mice by a nonpathogenic bacterium induces a small and transient increase in the plasma corticosterone and IL-6 levels in addition to hypothalamic c-fos activation without eliciting any apparent inflammation of the gut [5]. Such colonizing microbes, however, are not harmful to the host, but rather offer beneficial stimulation for enhancing host resistance to future severe stressors. Selye called this type of stressor "eustress," a positive form of stress usually related to desirable events in a person's life [31]. Therefore, the commensal microbiota may be a "eustress" that

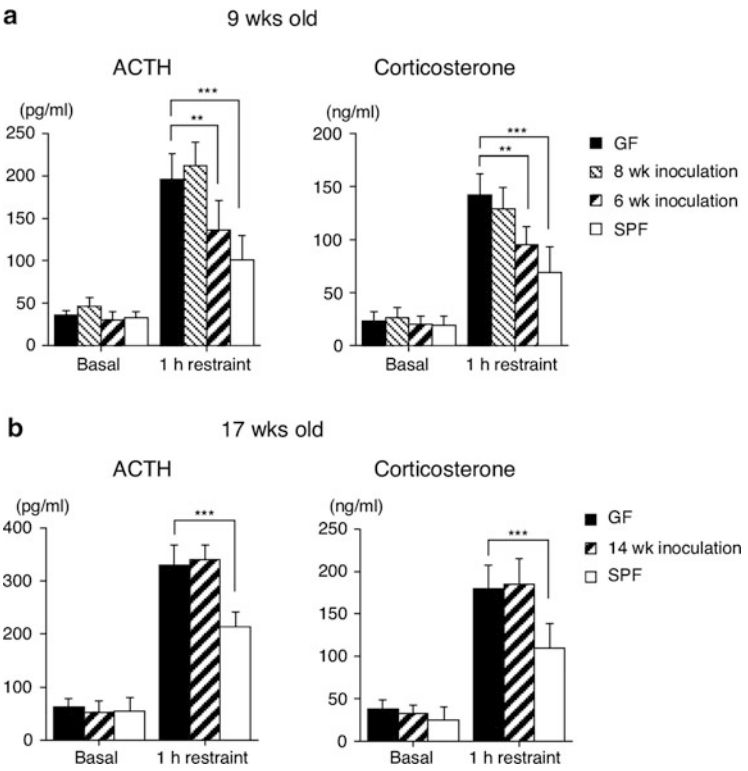

Fig. 8.3 Effects of restraint stress on the plasma ACTH and corticosterone levels in the mice reconstituted with SPF feces. SPF flora-reconstituted mice were established by orally introducing fresh SPF murine feces into the GF mice at either 1 or 3 weeks before being subjected to the stress protocol. Restraint stress was applied to the reconstituted mice at 9 (**panel A**) and 17 (**panel B**) weeks of age (n = 18–24 per group). $***P < 0.001$, $**P < 0.01$ according to Dunnett's test

plays an important role against the development of stress-related disorders, such as anxiety and depression, by providing the host with "stress resilience," similar to environmental enrichment.

Possible Luminal Molecules Mediating Gut Microbe-Host Interactions

The exact mechanisms whereby commensal bacteria interact with the host in the gut and what molecules are involved in this interaction remain to be elucidated. Vagal afferent nerves have been shown to play a role in the signaling from gut microbes to the central nervous system [32]; however, the underlying pathways and molecules are highly complex, and it is unlikely that only one common pathway or

series of molecules is involved. However, we herein pay particular attention to CA and γ-aminobutyric acid (GABA) present in the gut lumen.

CA as an Interkingdom Signal in the Gut Lumen

CA, such as NE and dopamine (DA), are utilized in the central and peripheral nervous systems, which regulate various types of body functions, such as cognitive abilities, mood and gut motility [33]. In addition to the well-established roles of CA, recent accumulating evidence suggests CA to be important interkingdom signal molecules in the gut.

CA Exist in a Biologically Free Form in the Lumen of the Gastrointestinal Tract

Our recent work [34] showed that free NE and DA are present in the lumen of the ileum, cecum and colon. The NE and DA levels in the lumen are the highest in the colon among the three parts of the gut (Fig. 8.4). In contrast, there are no significant differences in the tissue NE or DA levels between the ileum, cecum and colon. Since a large proportion of peripheral CA in the blood and urine exist in a conjugated form that is biologically inactive [35, 36], we investigated the free and glucuronide- and sulfate-conjugated forms of CA in the lumen of the ileum, cecum and colon.

Figure 8.5 shows that almost all of the NE and DA molecules were present in a biologically free form in the lumen of the cecum and colon of the SPF mice, although substantial amounts of glucuronide-conjugated NE and DA were present in the ileum.

Crucial Role of Bacterial β−Glucuronidase in the Generation of a Biologically Active Free Form of CA

The β-glucuronidase (GUS) from *E. coli* is a 290-kDa tetrameric protein that is essentially free of sulfatase activity [37]. Its optimal pH is 6.8, while that of the tissue-type GUS is 4.5 [38]. Since the mean pH in the intestinal lumen ranges from 6.5–7.9 (upper segment of the small intestine) to 6.8–8.0 (colon) in rodents [39–41], we hypothesized that free CA present in the lumen of the cecum and colon are generated via deconjugation by bacterial GUS derived from the gut microbiota. As shown in Fig. 8.6, the luminal free NE and DA levels were lower in the GF mice than in the SPF mice. In addition, more than 90 % of the DA in the GF mice was in the glucuronide-conjugated form in all parts of the digestive tracts examined (Fig. 8.7A), while approximately 40 % of the NE was in the glucuronide-conjugated

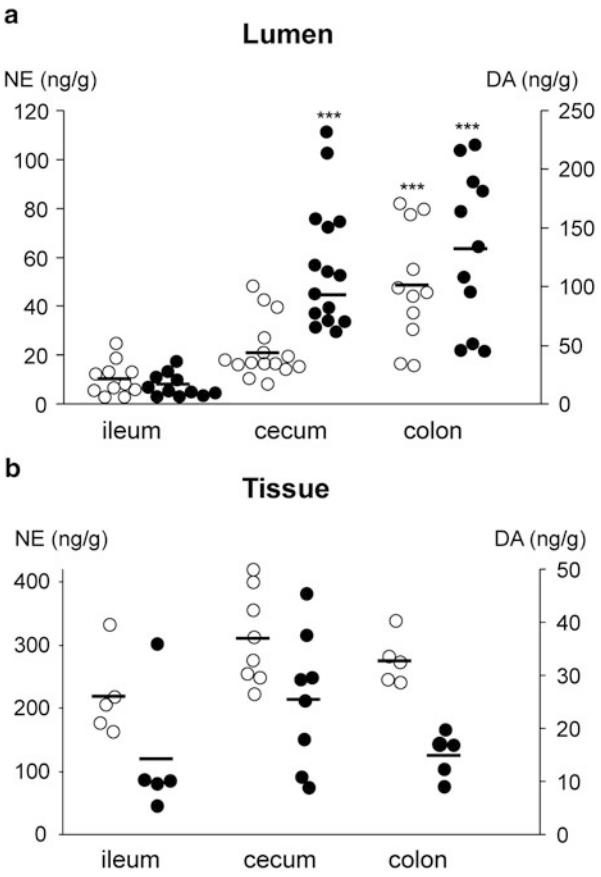

Fig. 8.4 Luminal and tissue CA concentrations in the gastrointestinal tract in the SPF mice. The luminal (n = 11–15, **panel A**) and tissue (n = 5–8, **panel B**) CA levels are shown. The *open* and *closed circles* indicate the NE (*left vertical axis*) and DA (*right vertical axis*) levels, respectively. ***$P < 0.001$ was significantly higher than the corresponding ileum value

form (Fig. 8.7B). The critical role of bacterial GUS in the production of free CA was further verified in additional experiments using gnotobiotic mice.

Association with either a mixture of 46 *Clostridia* species (*Clostridia*) or fecal flora from SPF mice (EX-GF) showed a drastic elevation of the free NE and DA levels (Fig. 8.8). In another set of experiments, the changes in the luminal CA levels were examined in the cecum after GF mice were colonized with either an *E. coli* mutant strain lacking the GUS-encoded gene, *uidA* (JW1609: ΔGUS), or its parent *E. coli* strain (BW25113). Figure 8.9 shows that 70 % of the DA remained in the glucuronide-conjugated form even after the association with ΔGUS, although 25 % of the total DA was in the free form. In contrast, two-thirds of the DA was converted into the free form, while 29 % of the total DA remained conjugated after the association with BW25113. The conjugated form of NE accounted for 29 % of the total following inoculation with ΔGUS, while representing 15 % of the total following inoculation with BW25113. The GUS activity in the cecal lumen of the ΔGUS-gnotobiotic mice was only marginally detectable and almost identical to the GF value (n = 5 per each group, ΔGUS 6.7 ± 0.4 μg PheP/h/mg protein,

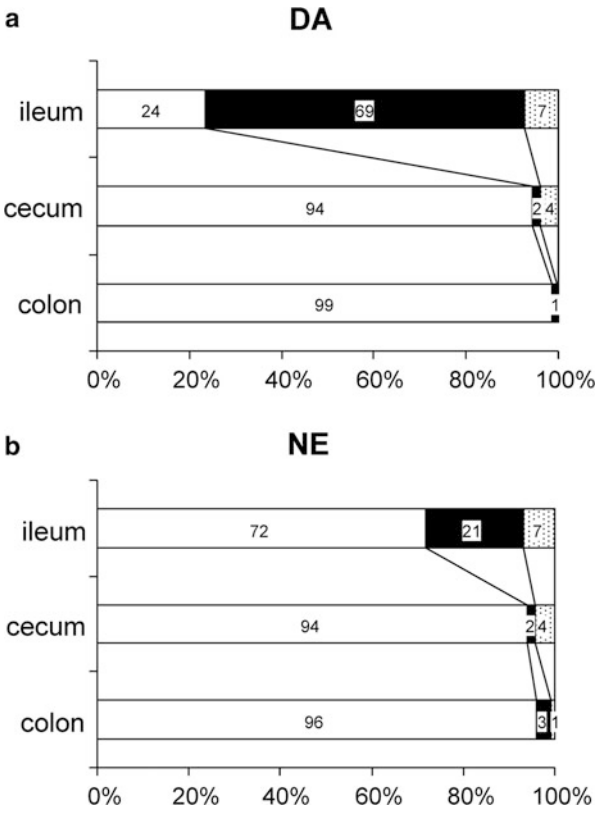

Fig. 8.5 Free and glucuronide- and sulfate-conjugated CA in the gut lumen in the SPF mice. The luminal glucuronide- and sulfate-conjugated DA (n = 6, **panel A**) and NE (n = 6, **panel B**) levels in the ileum, cecum and colon were analyzed with post column HPLC using diphenylethylenediamine as a fluorogenic reagent. The mean value of each form of CA is expressed as the percentage of the total (free CA + conjugated CA). The *open*, *closed* and *dotted bars* indicate the free, glucuronide-conjugated and sulfate-conjugated CA, respectively

GF 6.4 ± 0.3 μg PheP/h/mg protein). On the other hand, the BW25113 *E. coli*-gnotobiotic mice exhibited a small but significant increase in the GUS activity in the cecal lumen in comparison with that observed in the ΔGUS-gnotobiotic mice (n = 4, 11.6 ± 1.3** μg PheP/h/mg protein, $^{**}P < 0.01$ vs. ΔGUS value). Interestingly, the GUS activity in the cecal wall was significantly increased upon exposure to ΔGUS to a comparable level found upon exposure to the BW25113 strain (n = 5 per each group, ΔGUS 3.00 ± 0.25** μg PheP/h/mg protein, BW25113 2.72 ± 0.18* μg PheP/h/mg protein, GF 1.93 ± 0.06 μg PheP/h/mg protein; $^{**}P < 0.01$ and $^{*}P < 0.05$ vs. GF value).

Collectively, these results indicate that the gut microbiota plays a critical role in the generation of luminal free CA via GUS. The bacteria-induced increase in the tissue GUS activity may be involved in this phenomenon.

Can Commensal Bacteria Themselves Produce CA In Vivo?

Russian researchers [42, 43] have reported that some species of microorganisms produce CA in an in vitro culture system. In fact, transcripts that have some

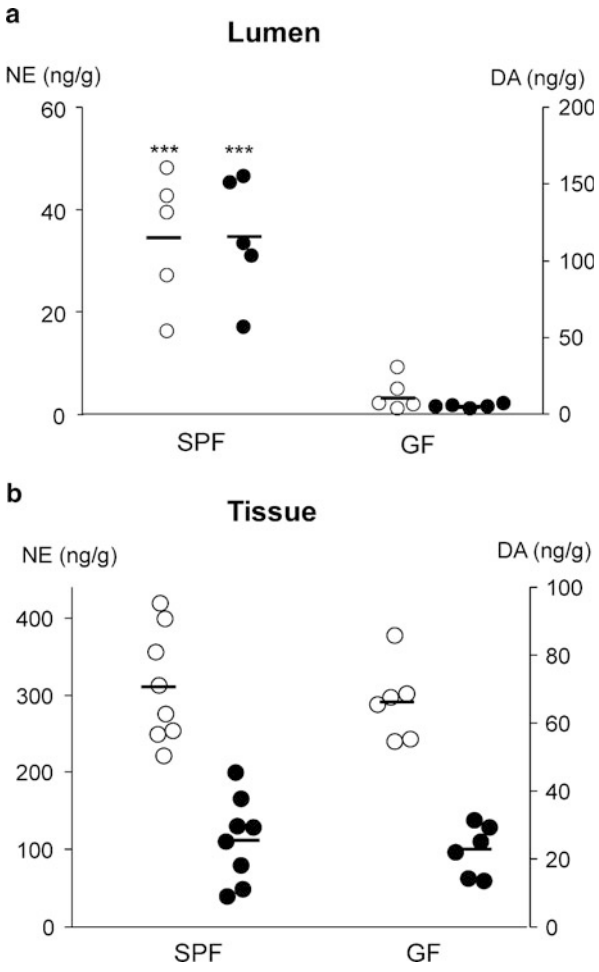

Fig. 8.6 Cecal free CA levels in the lumen and tissue of the SPF and GF mice. The luminal (n = 6–10, **panel A**) and tissue (n = 6–8, **panel B**) free CA levels in the SPF and GF mice are shown. The luminal NE levels (*left vertical axis*) in the SPF and GF mice were 35 ± 5*** ng/g and 3.8 ± 1.3 ng/g, respectively, and the luminal DA levels (*right vertical axis*) in the SPF and GF mice were 115 ± 14*** ng/g and 5.0 ± 0.5 ng/g, respectively. ***$P < 0.001$ was significantly higher than the corresponding GF value

similarity with mammalian tyrosine hydroxylase, a rate-limiting enzyme, are found in some species of bacteria [44, 45]. In our study, no significant differences were observed in the total DA levels (free + conjugated types) of the cecal content between the GF and SPF mice; however, the total NE levels of the cecal and colonic content were substantially higher in the SPF mice than in the GF mice (n = 5 per each group, cecum, GF 7.4 ± 1.7, SPF 36.4 ± 5.6***; colon, GF 6.4 ± 1.3, SPF 62.6 ± 6.7***; ***$P < 0.001$ vs. GF value). These results suggest that gut microbes are a likely source of gut luminal NE. In addition, gut bacteria enriched from murine feces actually contain substantial amounts of NE and a lesser amount of DA (Fig. 8.10). Therefore, it is possible to speculate that gut microbes are an important source of luminal NE. However, some species of bacteria, including *E. coli*, have a functional transporter for CA, such as the bacterial neurotransmitter sodium symporter family member, Leu T [46]. Therefore, there is thus far

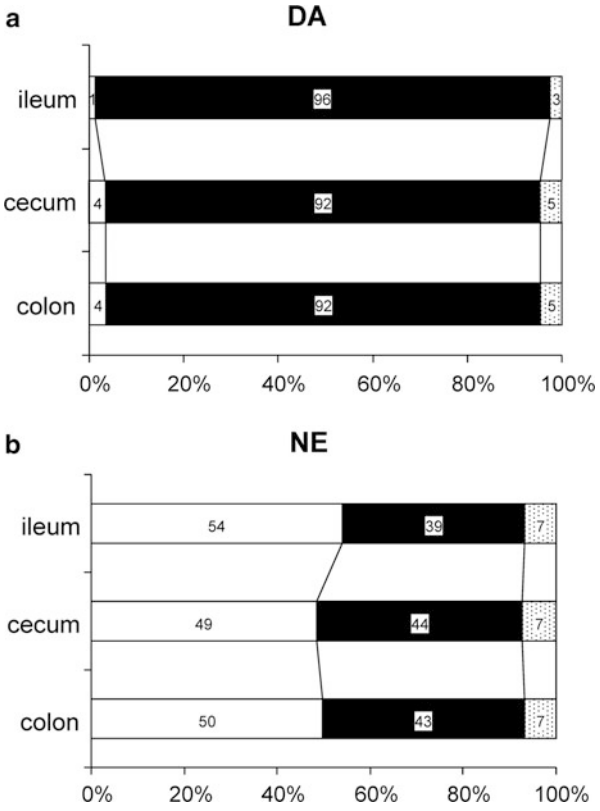

Fig. 8.7 Free and glucuronide- and sulfate-conjugated CA in the gut lumen in the GF mice. The luminal glucuronide- and sulfate-conjugated DA (**panel A**) and NE (**panel B**) levels in the ileum, cecum and colon are shown. The mean value of each form of CA is expressed as the percentage of the total (free CA + conjugated CA). The *open*, *closed* and *dotted bars* indicate free, glucuronide-conjugated and sulfate-conjugated CA, respectively

insufficient evidence to determine whether the NE and DA found in gut microbes originate from bacterial production by tyrosine hydroxylase-like enzyme or if they are obtained from the gut lumen via a Leu T-like transporter.

CA Receptors on Gut Epithelial Cells and Their Functions

DA 1A receptors are identified in the cells at the base of the intestinal crypts of the rat small intestine [47]. Alpha 2-adrenergic receptors are also reported to be present on gut epithelial cells [48]. These findings suggest the physiological importance of luminal CA. In fact, the luminal administration of DA stimulates active ileal ion absorption via α2-adrenergic or dopaminergic receptor activation, demonstrating the role of luminal DA as a proabsorptive modulator of ion and water transport [49, 50]. These results were also confirmed by our recent findings using an in vivo colon loop model in which the injection of ten micromoles of DA into the loop was found to induce a 30 % increase in water absorption out of the gut lumen in comparison to the injection of vehicle without DA (vehicle 55 ± 5 μl/30 min/cm, DA 72 ± 4* μl/30 min/cm; $*P < 0.05$).

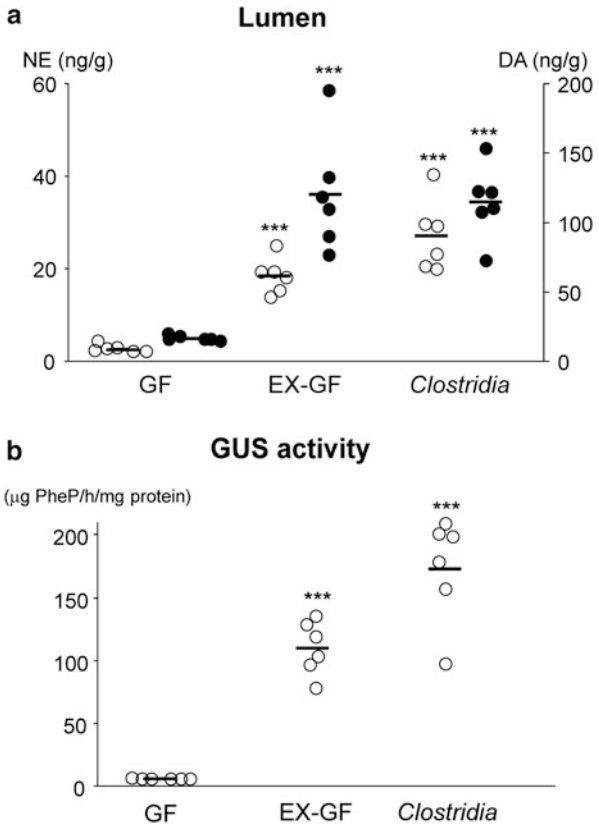

Fig. 8.8 Luminal free CA levels and the GUS activity in the cecum in the gnotobiotic mice. **Panel A**: The cecal luminal contents obtained from EX-GF (n = 6) and *Clostridia* (n = 6)-associated mice were processed for free NE and DA measurement. ***$P < 0.001$ was significantly higher than the corresponding GF value. **Panel B**: The cecal luminal contents of GF, EX-GF and *Clostridia*-associated mice were subjected to measurement of the GUS activity. ***$P < 0.001$ was significantly higher than the corresponding GF value

It is conceivable that luminal CA are involved not only in proabsorptive functions and increases in bacterial pathogenicity, but also a variety of physiological and pathological functions, such as gut motility [51] and modulation of immune reactions [52, 53]. Clarifying such unidentified functions will further support the notion that CA are important molecules mediating between gut microorganisms and the host under pathophysiological conditions.

GABA as an Interkingdom Signal in the Gut

GABA is a four carbon, nonprotein amino acid conserved from bacteria to vertebrates. Organisms have the ability to synthesize GABA from glutamate in a reaction catalyzed by the cytosolic enzyme L-glutamic acid decarboxylase (GAD). Several researchers have found that some bacteria, including *E. coli* and members of the *Lactobacillus* genus, possess a GAD activity, allowing them to

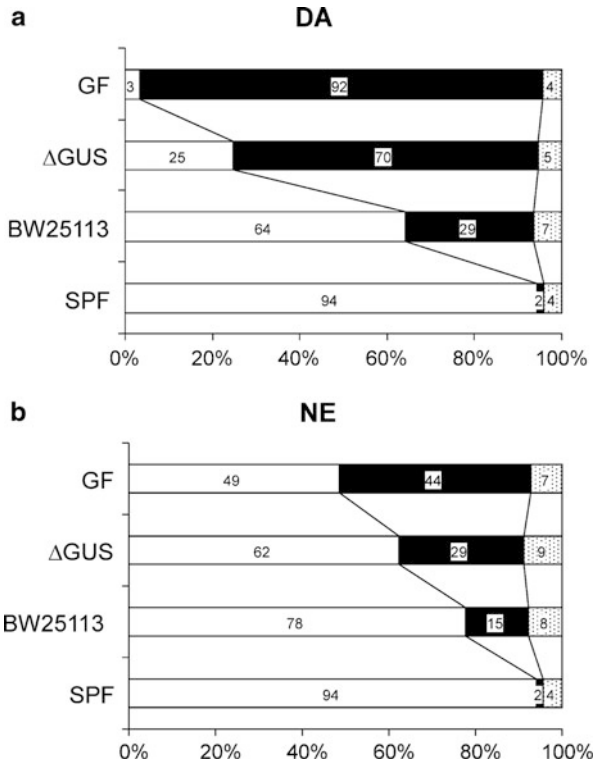

Fig. 8.9 Free and glucuronide- and sulfate-conjugated CA in the cecal lumen in the mice colonized with the *E. coli* mutant strain JW1609 or its parent strain BW25113. The cecal contents were subjected to measurement of the free and glucuronide- and sulfate-conjugated NE (**panel A**) and DA (**panel B**) levels 4 weeks after exposure to *E. coli* mutant strain devoid of GUS (n = 5, JW1609: ΔGUS) or its parent strain (n = 5, BW25113). The mean value of each form of CA is expressed as the percentage of the total (free CA + conjugated CA). The *open*, *closed* and *dotted bars* indicate free, glucuronide-conjugated and sulfate-conjugated CA, respectively

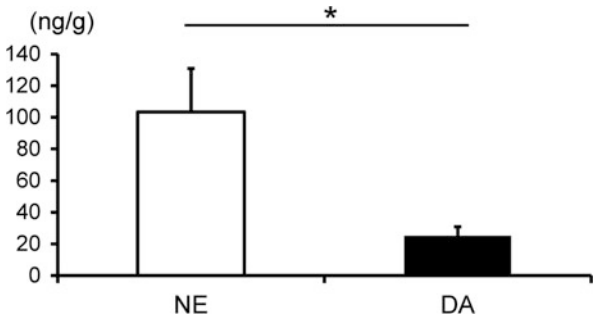

Fig. 8.10 Bacteria-rich fractions enriched from the cecal contents contain NE and DA. Bacteria-rich fractions were enriched from the cecal contents of the SPF mice according to a previously reported method [68]. The enriched fractions were thoroughly disrupted using repeated sonication then processed for CA measurement. A microscopic test revealed that the enriched bacteria had no contaminants, such as epithelia or debris. The NE and DA levels (mean ± standard error) were 103 ± 27 and 25 ± 6 ng/g, respectively (n = 6). $*P < 0.05$ indicates a significant difference between the NE and DA values

convert glutamic acid to GABA [54–56]. The production of GABA by bacteria appears to naturally occur under physiological conditions, as a recent study using metabolomics showed that the gut luminal GABA levels in Ex-GF mice are considerably higher than those observed in GF mice [57]. It is well known that plant-derived GABA mediates communication between organisms belonging to different kingdoms [58]; therefore, the GABA locally produced by the resident microbiota may play an important role as an interkingdom signal in the gut. In fact, a recent publication by Li and colleagues [59] showed that gut epithelial cells express several types of GABA receptors, including β2/3- and π-subunits, on their surface. The authors also demonstrated that endogenous autocrine GABAergic signaling in the mammalian intestinal epithelium upregulates intestinal fluid secretion and becomes intensified in mice with allergic diarrhea. To date, there is no direct evidence demonstrating that gut luminal GABA is actually involved in signaling from the gut to the brain. However, neural and/or humoral interactions between the intestinal GABA system and the brain GABA system comprise a fascinating research theme, as the JB-1 strain of *Lactobacillus rhamnosus* reduces stress-induced anxiety- and depression-related behavior, accompanied by an altered GABAAα2 receptor mRNA expression in the brain [32].

Other Hormones in Microbial Cells

Hormones and hormone-binding proteins with homology to those of vertebrates are reported to be present in fungi, yeast and bacteria [60, 61]. In particular, insulin and insulin-like materials contained in microbes have been the most extensively studied [62–64]. Corticotropin [65] and somatostatin [66] have also been identified in a unicellular organism (*Tetrahymena pyriformis*) and *Bacillus subtilis*, respectively. In this regard, Iyer and colleagues [67] proposed the interesting theory that the evolutionary history of prokaryotic genes encoding many of the enzymes involved in the synthetic and metabolic pathways of CA, histamine, acetylcholine and GABA is best described by scenarios that include late horizontal gene transfer from bacteria. This concept is substantiated by the growing body of evidence showing that bacteria produce small molecules that are formally involved in bacteria–bacteria communication and have now become involved in bacteria-host communication.

While we emphasize the role of CA or GABA in this context, this is but one of many examples of the consequences of the bacterial synthesis of neuroactive molecules that remain to be explored.

Conclusion and Perspectives

We are living in a bacterial world. Bacterial signaling helps us maintain homeostasis, keeping us healthy and happy. Given that the gut microbiome plays a crucial role in the development of the HPA axis and behaviors, gut microbes may play a critical role against the development of stress-related disorders, such as anxiety and depression, by providing the host with the "stress resilience" necessary to adapt to a changing external environment.

Clearly, further studies are called for; however, the recent findings described herein provide strong evidence in this rapidly developing field of research. We foresee a day when a comprehensive view regarding the interactions and pathways involved in the "microbiota-gut-brain axis" will be unraveled.

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