東海大学大学院令和３年度博士論文

Effect of gut microbiota early in life on aggressive behavior in mice （マウスにおける神経発達早期の腸内細菌叢が攻撃行動特性に与える影響に関する研究）

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1. Summary

Recent reports have indicated that gut microbiota modulates the responses to stress through the microbiota-gut-brain axis in mice, suggesting a connection between gut microbiota and brain function. We hypothesized that the gut microbiota early in life would have an effect on aggressiveness, and examined how gut microbiota affect aggressive behaviors in mice. BALB/c mice were housed in germ-free (GF) and ex-germ-free (Ex-GF) isolators. An aggression test was performed between castrated and a non-castrated mice at 8 weeks of age; the mice were allowed to confront each other for 10 min in strictly contamination-free environments. To evaluate aggressive behavior related to gut microbiota, we orally administered diluted Ex-GF mouse feces to the offspring of GF mice at 0, 6, and 10 weeks. GF mice showed more aggression than Ex-GF mice. Furthermore, GF mice who were administered feces of the Ex-GF group at 0-week-old were less aggressive than the GF mice. These findings suggested that the gut microbiota in the early stages of development was likely to have an effect on aggressiveness. Maintenance of healthy gut microbiota early in life can affect the mitigation of aggressive behavioral characteristics throughout the lifetime.

2. Background and Introduction

The gut microbiota plays an extremely important role in the production and absorption of energy and in the defense of the body through gastrointestinal immunity. In particular, the gut microbiota during the neonatal period is thought to regulate the gene expression of host epidermal cells, and it determines the development of the desired immune system. In recent years, advances in neuroscience and molecular biology have revealed that the central nervous system and peripheral organs form a bidirectional network through common signal transmitters and receptors, such as the nervous system and fluid factors. It was also revealed that the intestinal microbiota influences stress responses and behavioral characteristics.

The gut and the brain transmit information bidirectionally through humoral factors such as hormones and cytokines and through the autonomic nervous system; this link is commonly called the "gut-brain axis." The gut microbiota has recently been attracting increasing attention as a new component in this communication network, with the relationship between the gut and the brain also known as the "microbiota-gut-brain axis" (Collins and Bercik, 2009; Bercik et al., 2011). Recent research has revealed that the gut microbiota is involved in pathways of the nervous system, such as the connections of the vagus nerve, and those of the immune system through immunocompetent cells (Collins et al., 2012; Hand et al., 2016; Zheng et al., 2016). Furthermore, the relationship between the gut microbiota and mental development is also becoming clearer, including effects of physiologically active substances derived from the microbiota on stress response, emotions, and behavior (Neufeld et al., 2011; Vuong et al., 2017).

In humans, the hypothalamic-pituitary-adrenal axis (HPA axis) and the sympathetic nervous system are activated predominantly to respond rapidly to changes in the external environment, particularly exposures to harmful stimuli. In a previous study using specific pathogen-free (SPF) and germ-free (GF) mice, we discovered that, in addition to genetic factors, the gut microbiota immediately after birth was also intimately involved with the development and growth of the HPA axis. Our study demonstrated that the stress response at maturity can differ depending on the makeup of the gut microbiota immediately after birth (Sudo et al., 2004). These results formed the impetus for subsequent work to verify the effects of the gut microbiota on mental activity. Focusing on the gut microbiota in the early stages of development using GF and Ex-GF mice, we found that the gut microbiota in early development affects hyperactivity and anxiety symptoms in the host (Nishino et al., 2013). However, there is still little information about how the gut microbiota in the early stages of development affects behavioral and mental activities.

Based on the above findings, we have since focused on problematic behaviors in early development, such as aggression and impulsivity, and hypothesized that the gut microbiota in the early stages of development would affect the aggressiveness of the host. This study aimed to clarify the effects of gut microbiota early in life on host aggression through a comparison of GF and Ex-GF mice in a strictly contamination-free environment.

3. Discussion

This study demonstrated that, in a strictly bacterial contamination-free environment, Ex-GF mice with commensal gut microbiota had significantly lower levels of aggression-related behavior than GF mice. The absence of aggressive wrestling behaviors between mice was likely due to the low aggressiveness of the opponent castrated mice. We also found that when GF mice were conventionalized by the administration of feces from Ex-GF mice, groups administered feces at 0 and 6 weeks of age had less frequent aggression behaviors than the normal GF mice.

There has been a gradual increase of studies using GF and Ex-GF mice to study the relationship between gut microbiota and the behavioral characteristics of the host (Cryan and Dinan, 2012). We have also evaluated the behaviors of GF and Ex-GF mice using the OF method and marble-burying tests to investigate effects of the gut microbiota on the mental activity and behavior of the host. Those studies revealed that the gut microbiota was associated with decreased hyperactivity and anxiety. The current study, an extension of our previous work, suggests that the presence of gut microbiota affected not only mental activities such as anxiety, but also behavioral aspects such as hyperactivity and aggression. Gut microbiota is known to enhance resilience to stress and affect responsiveness to stress (McEwen, 2007; Gareau et al., 2011; Lehmann and Herkenham, 2011; De Palma et al., 2015; Pearson-Leary et al., 2020), and our results may reflect reduced aggression associated with the stress of encountering an unknown individual.

To observe changes in aggressive behavior, we orally administered diluted Ex-GF mouse feces to 0-, 6-, and 10 week-old offspring of GF mice at to establish the same gut microbiota of Ex-GF mice. Among these mice conventionalized with Ex-GF feces, both the aggressive behavior rates of CVL0 and CVL6 mice were significantly lower than that of the CVL10 mice. On the other hand, in the CVL6 and CVL10 groups, rates of aggressive behavior were comparable to those of the untreated GF mice, but in the CVL0 group, the rate of aggression was significantly reduced compared to GF mice. Given these results, the earlier (by weeks of age) the conventionalization is performed, the greater aggression is decreased. This suggested maintenance of a healthy gut microbiota at an early age influences neurodevelopment and can affect the hosts' aggression later in life.

The brain-gut-microbiome axis and HPA axis were considered to determine how the gut microbiota affects the behavioral characteristics of the host. In this study, we observed the changes in behavioral characteristics by transplanting commensal microbiota. There are reports that *B. fragilis* is protectively involved in autism-related behavior and that anxiety is reduced by monoassociation (Hata et al., 2019). However, although it was not possible to speculate which single bacterium contributed to aggression in this study, it is assumed that the network that coexists with the gut microbiota interacts with neurodevelopment.

Changes in monoamines in the brain may have been affected by gut microbiota, resulting in changes in behavioral characteristics (Clarke et al., 2013; Hata et al., 2019). A variety of neurotransmitters have been found to be involved in aggressive behavior. Cerebral DA transmission is thought to be a crucial modulator for the development and occurrence of aggressive behavior (Schlüter et al., 2013). In addition, there is also an established dysfunction in the brain DA systems in attention-deficit/hyperactivity disorder (ADHD) (Del Campo et al., 2011). Aggressive behavior is a characteristic of individuals with ADHD. In our previous study, GF mice were shown to be hyperactive compared to Ex-GF mice, and that phenomenon was related to dopamine metabolism (Nisino et al., 2013). Therefore, among all neurotransmitters, we focused on dopamine. In our study, DA levels differed between GF and Ex-GF mice immediately after exposure to intruder stimulation. The high DA and low DA metabolite levels we measured in GF mice after the aggression test could be due to inherently high DA levels or poor metabolism of monoamines in the brain, resulting in different behavioral characteristics in response to environmental changes (in particular, aggressive behaviors). Notably, there was no difference in DA and DOPAC levels between the GF mice that showed aggression and those that did not. Hence GF mice may overexpress dopamine relative to other mice.

Innate anxiety, hyperactivity, and aggression are common features noted in patients with neurodevelopmental disorders. Patients with autism spectrum disorder (ASD), a common neurodevelopmental disorder, present with problems in communication, socialization, and imagination and have innate behavioral characteristics such as anxiety, hyperactivity, and impulsivity. ASD typically becomes apparent early in a child's development. A recent study of mice that received feces from human patients with ASD developed the ASD-specific behavioral characteristics of anxiety, hyperactivity, and attachment (Finegold et al., 2002; Parracho et al., 2005; Diaz Heijtz et al., 2011; Hsiao et al., 2013; Jiang-Xie et al., 2014; Mayer et al., 2014; Sharon et al., 2019). A key contribution of the present study is that it focused on aggression, which has not been evaluated in previous work.

It is known that testosterone has an effect on aggression, but testosterone in the testes was lower in GF mice when compared to SPF mice (Al-Asmakh et al., 2014).

On the other hand, considering the viewpoint of intestinal microflora, the analysis of intestinal microflora has recently been elaborated by molecular biological methods using 16S ribosomal RNA as an indicator. Focusing on specific bacteria, it has been suggested that the intestinal microbiota of ASD differs from that of the regularly developing intestinal microbiota, such as in terms of a high percentage of Clostridium and Bacteroides, and low frequency of Bifidobacterium (Finegold et al., 2002; Parracho et al., 2005)]. If the original gut microbiota formation in ASD is different from that in normal development, it suggests that the relationship between the gut microbiota and the nervous system is bidirectional and the brain and gut show correlation, considering that stress is one of the factors that affects the composition of the gut microbiota. This study suggests that healthy gut microbiota immediately after birth may contribute to the prevention of the negative effects seen in mental activity, cognitive characteristics, and behavioral characteristics in cases of developmental disorders. In addition, it was found that maternal intestinal bifidobacteria influenced the formation of fetal intestinal bifidobacteria flora in our study, and that intestinal microflora were vertically transmitted between mother and child (Mikami et al., 2009). In other words, for the healthy intestinal microflora of newborns, the intestinal microflora of the pregnant mother needs to be stable, which could contribute to the prevention of the core symptoms and secondary disorders in developmentally disabled children, especially if the intestinal microflora of developmentally disabled neonates can be adjusted at the stage of gestation itself.

The following limitations must be acknowledged. The experiments in this study were conducted in an aseptic environment using an isolator. To maintain sterility when observing the aggressive behavioral characteristics, castrated GF littermates were put with GF mice and likewise, castrated Ex-GF littermates were put with Ex-GF mice. Use of different opponents may have influenced the comparison of behavioral characteristics of GF and Ex-GF mice. We believe that it made little or no difference because none of the castrated mice from either group showed aggressive behavioral characteristics.

4. Conclusion

In conclusion, we found that the gut microbiota present in the early stages of development affects aggressive behavioral traits. This study strongly supports the hypothesis that gut microbiota affect brain development and behaviors of the host through the microbiota-gut-brain axis. Maintenance of a healthy gut microbiota early in life can affect the mitigation of aggressive behavioral characteristics throughout the lifetime.