Probiotics normalize the gut-brain-microbiota axis in immunodeficient mice

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The gut-brain-microbiota axis is increasingly recognized as an integral network for regulation of numerous physiological systems (12). This bidirectional route of communication between the emotional and cognitive centers in the brain and the gastrointestinal (GI) tract is important for mediating numerous functions, including responses to stress (14). By way of this axis, exposure to psychological stress can result in intestinal barrier dysfunction (25), alter the composition of the gut microbiota and regulate interactions between the host and these microbes (18, 29). Immune defense effectors include lymphocytes, antimicrobial peptides, mucus secretion, cytokine production, and secretory immunoglobulin A (IgA) (29). Rag1 knockout mice (Rag1−/−) have shown that probiotics can overcome this immune-mediated deficit in the gut-brain-microbiota axis.

Probiotics are live microorganisms defined as being capable of providing benefits to their host when they are given in sufficient quantities and administered continuously, beyond any inherent nutritional value (42). Probiotics have demonstrated beneficial effects in patients with infectious diarrhea, allergies as well as subsets of IBS and IBD patients (20), making them increasingly used as alternative treatment options (24). In rat models, administration of probiotics prevented chronic stress-induced bacterial translocation (46), colorectal hypersensitivity (19) and restored intestinal barrier dysfunction induced by maternal separation in both neonates and adults (22). In mice, probiotics ameliorated infection with the bacterial pathogen Citrobacter rodentium (30), including prevention of C. rodentium-induced death in infected neonates (26). Probiotics also exerted a beneficial effect on the gut-brain-microbiota axis, preventing hypothalamic-pituitary-adrenal (HPA) axis hyperactivation following exposure to maternal separation (22), and reducing anxiety-like behavior in a chronic dextran sulfate sodium model (3) as well as stress-induced memory dysfunction following C. rodentium infection in adult mice (27). Whether probiotics mediate their effects on the gut-brain-microbiota axis via lymphocytes is currently unknown.
Therefore, the aims of this study were to determine, by using Rag1−/− mice, whether both behavior and intestinal function were modulated by the adaptive immune system and whether these changes were affected by psychological stress. We also assessed whether probiotic treatment could normalize any observed defects.

MATERIALS AND METHODS

Animals

Rag1−/− and wild-type C57BL/6 mice (male and female, 6–8 wk old; bred in house, originally from Jackson Laboratories, Bar Harbor, ME) were used in this study. Mice were housed under standard 12-h light-dark conditions with ad libitum access to food and water. All behavioral experiments were performed in a biosafety cabinet. All animal procedures and protocols were approved by the University of California San Diego Institutional Animal Care and Use Committee (IACUC).

Water Avoidance Stress

Mice were placed on small platforms located in the center of a mouse cage filled with shallow (1–2 cm) room-temperature water for 1 h. As previously described, water avoidance stress (WAS) is a well-established model of psychological stress, resulting in activation of the HPA axis after a single 1-h exposure to this aversive stimulus (22).

Behavioral Testing

Novel object test. The novel object test was used to analyze nonspatial recognition memory. It reflects dorsal hippocampal function, based on the mouse’s ability to recognize a familiar object (17, 27). This test is based on the inherent tendency of mice to investigate an unfamiliar object, when presented with the option of exploring either a familiar or a novel object (27). Mice were first habituated in a clean cage and then subjected to a training phase followed by a testing phase, as described previously (27). Exploratory behavior was recorded by video camera (Sony) and the video recordings were analyzed by a single observer (C. J. Smith).

Memory was determined on the basis of the frequency of exploration of the novel object relative to the familiar one. An exploratory action or investigation of an object was counted when the mouse approached the object and moved its nose within 1–2 cm of the object. A 50% exploration ratio indicates there was no preference for either object and thus indicates the inability to recognize the novel object. A mouse with an unimpaired memory is expected to favor the new object, with an exploration ratio greater than 50% (27).

Light/dark box test. The light/dark box test was used to assess anxiety levels in mice, as described previously (27). Briefly, a large polycarbonate cage was fitted with a dark compartment (1/3 of the area) and a light compartment (2/3 of the area) allowing the mouse free access to both environments, and behavior was videotaped for 10 min. The light/dark box test relies on the inherent preference of mice for a dark area over a well-lit one, balanced against their innate inclination to explore an unfamiliar environment. Video recordings were analyzed to determine the total amount of time spent in the light compartment, along with the number of transitions from one compartment to the other. Anxiety is reflected by an increased tendency to remain in the dark compartment of the cage (27). An increase in transitions is an indicator of increased exploratory behavior. There was no habituation period for the light/dark box test because the novelty of the environment was used to drive exploratory behavior.

Probiotics

A commercial combination of probiotics (Lacidofil; Institut Rosell-Lallemand, Montreal, Quebec, Canada) was used in these studies. The probiotic mixture was composed of Lactobacillus rhamnosus (R0011) and L. helveticus (R0052) in maltodextrin. This mixture has previously been shown to ameliorate C. rodentium-induced colitis (30) and to prevent abnormal behavior in infected mice (27). Lyophilized probiotic powder or maltodextrin alone (placebo) was dissolved in sterile water at 10⁷ CFU/ml and provided in drinking water starting at weaning (4 wk). Drinking water was changed every 2 days until the end of the experiment (8 wk of age). Viability of probiotics in the drinking water has been confirmed in previous studies by plating rehydrated bacteria on de Man, Rogosa, Sharpe (MRS) plates (30). The final dose of probiotics consisted of 6 × 10⁷ CFU per mouse per day based on plating studies and consumption of ~6 ml per mouse per day (30).

Study Design

Mice (C57BL/6, Rag1−/− ± probiotics/placebo) were tested for behavior at 6–8 wk of age. Behavioral tests were performed in the same mice on the same day with a rest period between tests. The light/dark box was performed first, followed by habituation and then the novel object test. Mice subjected to WAS were tested for behavior (novel object test or light/dark box) immediately after exposure to this stressor. For the novel object test, mice were exposed to WAS immediately after habituation followed by the novel object test. Within 20 min of test completion, mice were euthanized by CO₂ asphyxiation, followed by cervical dislocation and sample collection (blood, brain, colon, fecal).

Corticosterone

Serum corticosterone levels were used as a measure of HPA-axis activation (27). Blood was collected via cardiac puncture into serum separator tubes and placed on ice. The samples were centrifuged (5,000 rpm; 10 min) and serum frozen at −80°C until analysis using a commercial enzyme immunoassay kit (Enzo Life Sciences, Plymouth Meeting, PA). Concentrations were determined by use of a fluorescent plate reader and comparison with a standard curve (Victr V, Perkin Elmer).

Ussing Chamber Studies

Segments of distal colon obtained from wild-type (WT) and Rag1−/− mice were collected and mounted into Ussing chambers. Full-thickness tissues (0.09 cm² in area) were exposed to oxygenated Ringer solution (in mM: 140 Na⁺, 5.2 K⁺, 1.2 Ca²⁺, 0.8 Mg²⁺, 120 Cl⁻, 25 HCO₃⁻, 2.4 H₂PO₄⁻, 0.4 HPO₄²⁻, 10 glucose). Tissues were voltage clamped to zero potential difference by the application of short-circuit current (Isc). Under these conditions, Isc values reflect electrogenic chloride secretion and/or sodium absorption. Baseline measurements were taken after a 15-min equilibration period, after which FITC-labeled dextran (4 kDa: 110 mg/ml) was added to the mucosal chamber. Flux of this tracer to the serosal chamber was assessed for 2 h, with samples taken at 30-min intervals and measured for fluorescence by use of a plate reader (Victor V).

Immunohistochemistry

Brains were isolated, fixed in 10% formalin for 48 h, and embedded in paraffin, and 5-μm sections were cut and placed onto glass slides. Rehydrated sections were subjected to heat-induced epitope retrieval using citrate buffer, blocked for endogenous peroxidase (Bloxall, Vector Labs), blocked for nonspecific binding (5% normal goat serum, Jackson Immune Research), and exposed to primary anti-c-Fos antibody (1:200, Abcam) overnight. Detection was performed with an ABC kit and developed with impact-DAB (Vector) as substrate.

Microbiome Analysis

Fecal samples were collected from the colon and frozen at −80°C until use. Bacterial DNA was extracted by using a stool extraction kit (Qiagen) according to the manufacturer’s instructions. qPCR for the
16S gene was performed with SYBR green and primers [details previously published (27)]. PCR conditions were as follows: 1 cycle at 95°C (2 min), 1 cycle at 50°C (1 min) and 39 cycles at 95°C (15 s) and 60°C (1 min). Copy threshold values were calculated and data expressed as % of total bacteria as determined with use of a Eubac-
tera universal primer (Table 1) (27).

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
</tr>
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<tr>
<td>Eubacteria</td>
<td>ACTCTACGGGAAGGCAAGCGGT</td>
<td>ATTACGGGCTGCTGCGG</td>
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<tr>
<td>Bacillus</td>
<td>GCGGCGGCTGCTGCTAATTGAGTC</td>
<td>CTTACTCATCATAACGCGGCT</td>
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<td>Bacteroides</td>
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<tr>
<td>Enterobacteriaceae</td>
<td>GTGCCAGCMGCCGCGGTAA</td>
<td>GCCCGAAAGGACACACCTGCAA</td>
</tr>
<tr>
<td>Lactobacillus/Lactococcus</td>
<td>GCAGTACGGGAATCTTCCA</td>
<td>CACCGCTCACATGGAG</td>
</tr>
<tr>
<td>Segmentated filamentous bacteria</td>
<td>GACGGTGAGGATGAGAGGAT</td>
<td>GACGCGAGCAGATTGTATTTCA</td>
</tr>
<tr>
<td>Eubacterium rectale/Clostridium coccoides</td>
<td>ACTCTACGGGAGGCGGC</td>
<td>GCTTTACTGAGGATCCTGAGGTGAT</td>
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The reduced nonspatial memory in Rag1−/− mice was also accompanied by anxiety-like behavior compared with WT controls. In the light/dark box test, Rag1−/− mice spent significantly less time in the light compartment compared with WT control mice (Fig. 1B). Exposure to WAS did not alter time spent in the light compartment in WT control or Rag1−/− mice (Fig. 1B). Decreased exploratory behavior was also observed in Rag1−/− mice compared with WT controls, as demonstrated by a decrease in the number of transitions made between the light and dark compartments (Fig. 1C). Again, exposure to WAS did not further decrease exploratory behavior in Rag1−/− mice, whereas exploratory behavior was reduced (albeit not significantly so) by WAS in WT mice (Fig. 1C). Therefore, both nonspatial memory defects and anxiety-like behavior are observed at baseline in Rag1−/− mice, which are not further exacerbated by exposure to stress.

**Results**

**Nonspatial Memory Is Impaired and Anxiety-Like Behavior Is Present in Rag1−/− Mice**

Testing of nonspatial memory was performed by using the novel object test in WT C57BL/6 and Rag1−/− mice. Significantly decreased nonspatial memory was observed in Rag1−/− compared with WT mice as indicated by lower exploration ratios (Fig. 1A). Exposure to the acute psychological stressor WAS did not induce further dysfunction in Rag1−/− mice, nor did it affect WT control mice.

A possible role for activation of the HPA axis in modulating the gut-brain-microbiota axis in Rag1−/− mice was assessed by measuring serum corticosterone levels. Stress-induced HPA-

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**Table 1. Primer sequences employed in analysis of the fecal microbiome**

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**Fig. 1. Behavioral dysfunction is observed in immunodeficient mice. A: nonspatial memory was assessed in wild-type (WT) C57BL/6 and Rag1−/− mice by the novel object test, with or without exposure to water avoidance stress (WAS), with memory determined as the exploration ratio. B and C: anxiety-like behavior was studied in the same groups of mice by using a light/dark box, with anxiety determined as total time spent in the light (B) and exploratory behavior determined as the frequency of transitions between the light and dark compartments (C). Means ± SE; **P < 0.01; 1-way ANOVA, Newman-Keuls post hoc; N = 6–8 mice/group.**

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Statistics

Results are presented as means ± SE. Groups were compared by the Student’s t-test or ANOVA followed by the Newman-Keuls post hoc test as appropriate. The analysis was performed using Prism5 (GraphPad, San Diego, CA). Differences of P < 0.05 were considered as significant.
axis activation was reflected by a significant increase in serum corticosterone in WT mice exposed to WAS. In contrast, Rag1/H11002/H11002 mice exhibited increased serum corticosterone at baseline compared with the WT controls (Fig. 2), which was not further enhanced by exposure to WAS (Fig. 2).

**Colonic Physiology Is Disrupted in Rag1/H11002/H11002 Mice**

Since baseline colonic permeability is elevated in weanling Rag1/H11002/H11002 mice compared with WT controls (26), and barrier dysfunction can lead to altered gut-brain signaling, colonic physiology was assessed with Ussing chambers. Baseline electrogenic ion transport ($I_{sc}$) and permeability (measured both as conductance and as permeability to 4 kDa FITC-dextran) were assessed in WT and Rag1/H11002/H11002 mice, with and without exposure to WAS. As expected, 1 h of WAS significantly increased basal ion transport in WT mice (Fig. 3A) (7). In contrast, baseline $I_{sc}$ was significantly increased in Rag1/H11002/H11002 mice compared with unstressed WT controls and was not further enhanced by WAS (Fig. 3A). No difference in transepithelial conductance was observed in any of the groups compared with naive WT (Fig. 3B), whereas macromolecular permeability to FITC-labeled dextran was increased following exposure to WAS in both WT and Rag1/H11002/H11002 mice (Fig. 3C).

**Nonspatial Memory, Anxiety-Like Behavior, and Baseline $I_{sc}$ Were Ameliorated in Rag1/H11002/H11002 Mice Following Treatment with Probiotics**

Administration of probiotics was previously demonstrated to restore stress-induced behavioral defects associated with an enteric bacterial infection in mice (27). To determine whether cognitive impairments and altered colonic physiology in Rag1/H11002/H11002 mice could similarly be normalized, probiotics were provided in the drinking water starting at weaning and continuing until euthanasia. Nonspatial memory defects present in Rag1/H11002/H11002 mice were restored compared with those mice given placebo (Fig. 4A), as demonstrated by increased exploration. However, this beneficial effect of probiotics was abrogated in probiotic-treated mice that were also exposed to WAS (Fig. 4A). Similarly, in the light/dark box test, Rag1/H11002/H11002 mice administered probiotics spent more time in the light box compared with placebo-treated controls; however, this beneficial effect was maintained in the presence of WAS (Fig. 4B).
Administration of probiotics also normalized baseline colonic ion transport in Rag1−/− mice (Fig. 4C), in contrast to placebo, but only in the absence of WAS. Probiotics had no significant effect on elevated serum corticosterone levels in these mice in either the presence or absence of WAS (Fig. 4D).

Probiotics Modulate the Composition of the Microbiota

Changes in the composition of the fecal microbiota in Rag1−/− mice were assessed by qPCR of the 16S gene. Compared with WT controls, Rag1−/− mice demonstrated shifts in the composition of the microbiota, including increased levels of segmented filamentous bacteria (SFB) and Eubacterium rectale, along with decreased levels of Bacteroides, Enterobacteriaceae, and Firmicutes (Fig. 5). No changes were observed in Bacillus or Lactobacillus species in Rag1−/− mice. Administration of probiotics to Rag1−/− mice normalized levels of Bacteroides, Enterobacteriaceae and Firmicutes compared with administration of placebo, but had no effect on E. rectale levels. In contrast, administration of either probiotics or placebo was capable of reducing elevated SFB levels seen in Rag1−/− mice.

Hippocampal c-Fos Expression Is Modulated by Probiotics

Quantification of c-Fos expression in the CA1 region of the hippocampus by immunohistochemistry revealed a significant decrease in c-Fos-positive neurons in Rag1−/− mice vs. WT controls (Fig. 6). Administration of probiotics, but not placebo, restored c-Fos expression in Rag1−/− mice.

DISCUSSION

The gut-brain-microbiota axis is critical for establishing normal intestinal function, with disruptions in the axis observed in patients with both IBD and IBS (43). In mouse models of IBD, as well as following enteric bacterial infection, behavioral dysfunction can be demonstrated (2, 27). The complex interrelationships between the immune system, the brain, and the gut microbiota prompted the present investigation of the role of the adaptive immune system in modulating behavioral changes induced by way of the gut-brain-microbiota axis.

The results of our study demonstrate that an aberrant adaptive immune system is associated with impaired learning and memory as well as anxiety-like behavior in mice. Rag1−/− mice displayed behavioral dysfunction coupled with increased HPA-axis activation in the context of increased colonic ion transport and an altered microbiota. Changes in behavior and colonic physiology observed in Rag1−/− mice were ameliorated by treatment with a Lactobacillus-containing probiotic preparation. The beneficial effects of probiotics, however, were in some cases lost upon concomitant exposure of Rag1−/− mice to psychological stress, suggesting only a partial recovery in gut-brain-microbiota axis function, incapable of overcoming a subsequent additional challenge. Taken together, these results demonstrate that lymphocytes significantly influence behavior...
and cognition, and beneficial microbes are capable of overcoming the behavioral changes observed in the absence of adaptive immunity, likely by way of the gut-brain axis.

The precise mechanism of how adaptive immune cells, or lack of these cells, contribute to behavior remains to be elucidated. Reduced nonspatial memory and anxiety-like behaviors in Rag1−/− mice are in keeping with previous reports showing that lymphocytes play an important protective role in the development of the central nervous system (CNS) by supporting hippocampal neurogenesis and spatial memory formation (41). In the absence of self-specific T cells, brain-derived neurotropic factor (BDNF) expression is reduced, further supporting a role for the adaptive immune system in maintaining hippocampal plasticity (47). This contribution of hippocampal BDNF in dictating behavioral outcomes is further supported by increased anxiolytic behavior following hippocampal BDNF injections (15). Development, maintenance, and consolidation of nonspatial hippocampal-dependent memory are complex processes that require neuronal activation (11). This activation, indicated by nuclear localization of c-Fos, was significantly decreased in the CA1 region of the hippocampus in Rag1−/− vs. WT mice and could be normal-
alyzed by administration of probiotics. These data suggest that cognitive defects in Rag1−/− mice could be due to the aberrant intestinal microbiota, and components of these bacteria can regulate CNS neuronal protein expression. Alternatively, the primary effect may be on the epithelium with an indirect effect on the CNS; this remains to be elucidated in future studies.

Learning and memory are influenced by the presence of corticosterone and the expression of glucocorticoid and mineralocorticoid receptors (13). The hippocampus is highly sensitive to glucocorticoids (34), and hormone binding to receptors in the CA1 region leads to cellular responses including altered synaptic function and, in extreme cases, neuronal injury (9). Stress induced by Morris water maze testing increases corticosterone levels without altering hippocampal BDNF, indicating that stress alone cannot cause learning and memory dysfunction (39). However, we have previously demonstrated that acute stress coupled with C. rodentium infection impairs hippocampal-dependent memory in mice (27). This deficit in recognition memory was only present following exposure to both infection and WAS, which correlated with stress-induced increases in serum corticosterone (27). Administration of probiotics can reduce stress-induced increases in serum corticosterone levels but does not affect baseline levels (5). In the present study, Rag1−/− mice exhibited a significant increase in baseline serum corticosterone, which was not further enhanced by exposure to WAS. In both this and our prior study, increased serum corticosterone correlated with nonspatial memory dysfunction. Taken together, these findings emphasize the prominent link between HPA axis activation and behavioral dysfunction.

Since intestinal physiology is modulated by the gut-brain-microbiota axis (22) and can be affected by changing the composition of the microbiota by administration of probiotics (22), we wanted to assess potential changes in intestinal physiology in the absence of adaptive immune cells. Increased baseline ion transport was observed in Rag1−/− mice, which
was not further enhanced by exposure to WAS. In contrast, baseline paracellular and macromolecular permeability were not altered in Rag1−/− mice. Nevertheless, these changes were not accompanied by overt diarrhea in Rag1−/− mice. These findings demonstrate that increased ion transport is present in the absence of mucosal barrier dysfunction, supporting a role for lymphocytes in the maintenance of fluid and electrolyte homeostasis and development of intestinal physiology. Whether this altered ion transport was mediated by increases in chloride secretion or sodium absorption, or both, is not currently known. Furthermore, the presence of elevated ion transport correlates in part with hyperactivation of the HPA axis, which are known to occur together in conditions of psychological stress. In contrast, permeability is not always correlated with corticosterone levels. For example, transcellular but not paracellular permeability is increased under conditions of early life stress (23) whereas the corticotropin-releasing factor (CRF) receptor antagonist, α-helical CRF, is unable to restore colonic paracellular permeability following exposure to acute stress (16). The failure of stress to change either ion transport or serum corticosterone in Rag1−/− mice in the present study suggests that maximal HPA-axis activation is present at baseline and may be the cause of the changes in physiology and behavior.

Probiotics are known to restore intestinal homeostasis in mice with enteric inflammation (37) and to normalize gut-brain-microbiota axis-induced behavioral changes in this setting (27). Lactobacillus-containing probiotics have previously been demonstrated to exert beneficial effects both in the gut and the brain. L. rhamnosus (JB-1) acts by way of the vagus nerve to normalize GABA expression in the brain (decrease in the prefrontal cortex and increase in the hippocampus) along with reducing corticosterone levels, anxiety-like behavior and depression-related behavior in mice (5). In the present study, recognition memory and anxiety-like behavior were ameliorated in Rag1−/− mice following treatment with Lactobacillus-containing probiotics. This finding suggests that the beneficial effects of probiotics on colonic physiology and behavior may be mediated by alteration of the composition of the microbiota. The benefits of probiotic treatment were lost in part, however, in Rag1−/− mice exposed to WAS. Stress is known to be detrimental to intestinal health and is involved in both the initiation and relapse of colitis (4). Exposure to stress decreases efferent vagus nerve signaling and increases sympathetic outflow to the enteric nervous system (45). Such alterations in the gut-brain axis could explain the inability of probiotics to normalize behavioral dysfunction in stressed Rag1−/− mice, although further studies are needed to confirm the specific mechanisms of action involved. Thus, in our present findings, although stress did not induce additional dysfunction in the colon or in behavior, it was sufficient to prevent the beneficial effect of administration of probiotics in the absence of lymphocytes.

Dysbiosis, or an alteration to the microbial community that may be maladaptive to the host, is present in numerous disease states, including IBD. Patients with ileal Crohn’s disease have been reported to have a dysbiosis characterized by a decrease in Firmicutes species, particularly Faecalibacterium prausnitzii (8), accompanied by a rise in proteobacteria (35). In our present findings, Rag1−/− mice also display decreased Firmicutes compared with WT controls, which could be restored by probiotic administration. Levels of SFB were also elevated in Rag1−/− mice and lowered by administration of either probiotics or placebo. SFB are known to play an important role in the establishment of Th17 immunity, without causing pathology, despite intimate attachment to the intestinal epithelium (40). Although SFB has not been studied in the context of a lack of B and T cells, such as in Rag1−/− mice, knockout mice that lack the activation-induced cytokine deaminase, and consequently IgA, show an increase in SFB colonization (44). In addition, mice fed cyclophosphamide to render them immune-compromised resulted in an increase in colonization by SFB, which was prevented by administration of a Lactobacillus-containing probiotic (21). The fact that placebo administration restored SFB in Rag1−/− mice suggests perhaps a probiotic effect on Lactobacillus or Bifidobacteria, rather than a probiotic effect, was mediating this change due to the presence of maltodextrin in the placebo preparation. Maltodextrin can be fermented by gut bacteria, promoting the growth of Lactobacilli and Bifidobacteria, although to a lesser extent than dextran and oligodextran (38). The cyclophosphamide study described above was not placebo controlled (21), therefore a direct comparison cannot be made between the two studies. Although probiotics changed the overall composition of the fecal microbiota, the exact mechanism by which they normalized behavior and intestinal physiology remains unknown.

In conclusion, this study demonstrates that lymphocyte-deficient Rag1−/− mice display altered memory and increased anxiety, coupled with a hyperactive HPA axis and increased baseline colonic ion transport. Probiotics can normalize alterations in both behavior and colonic physiology in Rag1−/− mice when treatment commences at weaning, but only in the absence of concomitant stress. Taken together, our findings implicate an important role for the adaptive immune system in aiding the normal development of the gut-brain-microbiota axis, by mediating signaling between the gut and the central nervous system.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


