The Intestinal Microbiota Affect Central Levels of Brain-Derived Neurotropic Factor and Behavior in Mice

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BACKGROUND & AIMS: Alterations in the microbial composition of the gastrointestinal tract (dysbiosis) are believed to contribute to inflammatory and functional bowel disorders and psychiatric comorbidities. We examined whether the intestinal microbiota affects behavior and brain biochemistry in mice. **METHODS:** Specific pathogen–free (SPF) BALB/c mice, with or without subdiaphragmatic vagotomy or chemical sympathectomy, or germ-free BALB/c mice received a mixture of nonabsorbable antimicrobials (neomycin, bacitracin, and pimaricin) in their drinking water for 7 days. Germ-free BALB/c and NIH Swiss mice were colonized with microbiota from SPF NIH Swiss or BALB/c mice. Behavior was evaluated using step-down and light preference tests. Gastrointestinal microbiota were assessed using denaturing gradient gel electrophoresis and sequencing. Gut samples were analyzed by histologic, myeloperoxidase, and cytokine analyses; levels of serotonin, noradrenaline, dopamine, and brain-derived neurotropic factor (BDNF) were assessed by enzyme-linked immunosorbent assay. **RESULTS:** Administration of oral antimicrobials to SPF mice transiently altered the composition of the microbiota and increased exploratory behavior and hippocampal expression of BDNF. These changes were independent of inflammatory activity, changes in levels of gastrointestinal neurotransmitters, and vagal or sympathetic integrity. Intraperitoneal administration of antimicrobials to SPF mice or oral administration to germ-free mice did not affect behavior. Colonization of germ-free BALB/c mice with microbiota from NIH Swiss mice increased exploratory behavior and hippocampal levels of BDNF, whereas colonization of germ-free NIH Swiss mice with BALB/c microbiota reduced exploratory behavior. **CONCLUSIONS: The intestinal microbiota influences brain chemistry and behavior independently of the autonomic nervous system, gastrointestinal-specific neurotransmitters, or inflammation. Intestinal dysbiosis might contribute to psychiatric disorders in patients with bowel disorders.**

Keywords: Host–Bacterial Interactions; Gut–Brain Axis; Commensal Bacteria; Inflammatory Bowel Disease.

The intestinal microbiota is a vast ecosystem that shapes a wide variety of host functions, both within and outside the gastrointestinal tract.[1] Within the gut, colonization of germ-free mice with the human and mouse commensal *Bacteroides thetaiotaomicron* affects the expression of messenger RNAs that encode for immune and smooth muscle function, epithelial cell permeability, and enteric neurotransmission.[2] Examples of the extensive impact of the microbiota on host function beyond the gut include the regulation of body weight[3] and cutaneous pain perception.[4]

In health, the intestinal microbiota shows stability and diversity but in chronic intestinal conditions such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) the microbiota has less diversity and its composition is unstable over time.[5–7] It is generally accepted that the intestinal microbiota is critical for the expression of IBD[8] and diversion of the fecal stream results in healing of the inflamed gut.[8] Changes in the microbiota now have been described in IBS[6–9] and there is experimental evidence that perturbation of a stable microbiota results in changes in gut function reminiscent of those associated with IBS.[10] Depression and anxiety are common in IBD and are associated with a more active disease course.[11–13] Up to 50% to 90% of patients with IBS show psychiatric comorbidity.[14] The question arises as to whether behavioral changes are secondary to the disability imposed by chronic gastrointestinal symptoms, or whether they are a direct manifestation of the underlying pathophysiology, which includes alterations in the intestinal microbiota.

Studies in young germ-free mice indicate that the intestinal microbiota influences the postnatal development of the hypothalamic–pituitary response to stress.[15] Observations that include the well-established benefit of oral antibiotics in the treatment of hepatic encephalopathy[16] and induction of anxiety-like behavior after introduction of pathogenic bacteria into the

Abbreviations used in this paper: ATM, antimicrobial; BDNF, brain-derived neurotropic factor; DGGE, denaturing gradient gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; IBS, irritable bowel syndrome; IL, interleukin; SPF, specific pathogen-free.

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gut[17] suggest that intestinal microbiota affects behavior.

The purpose of this study was to determine whether gut commensal bacteria influence brain neurochemistry and behavior. We used 2 strategies. First, we perturbed the microbiota in adult mice by oral administration of antimicrobials (ATM), which have been shown previously to alter the bacterial composition of the gut and change function in the enteric nervous system in mice.[10] Second, we exploited established differences in behavior and microbiota profiles between mouse strains and attempted to modify the behavior of germ-free recipient mice after colonization with commensal bacteria from a strain of mouse with a behavioral phenotype that is different from the recipient mouse. The results of each approach support the existence of a microbiota–gut–brain axis, which influences behavior and hippocampal expression of brain-derived neurotrophic factor (BDNF). Changes in microbiota composition thus may contribute to behavioral changes that frequently accompany functional and inflammatory bowel conditions.[9]

Material and Methods

Animals

Male BALB/c mice (8–10 weeks old) were purchased from Harlan (Indianapolis, IN) and maintained under specific pathogen–free (SPF) conditions. Germ-free NIH Swiss and BALB/c mice (7–9 weeks old), obtained from the Farncombe Gnotobiotic Unit of McMaster University, were colonized by gavaging fresh cecal contents from SPF BALB/C and NIH Swiss donors (obtained from the Central Animal Facility of McMaster University). They were housed in ultraclean conditions using ventilated racks. All mice were handled only in the level II biosafety hood to prevent bacterial contamination. The experiments were approved by the McMaster University animal ethics committee.

ATM Treatment

BALB/C mice received a mixture of nonabsorbable ATMs (neomycin 5 mg/mL, bacitracin 5 mg/mL, and pimaricin 1.25 μg/mL) in drinking water for 7 days. Control mice received sterile water. Additional mice received ATMs (1% of daily dose) or saline by intraperitoneal (IP) injections daily for 7 days. Mice were killed thereafter and tissue samples were taken.

Subdiaphragmatic Vagotomy

A group of mice underwent subdiaphragmatic vagotomy, as described previously.[18] Briefly, after ketamine/xylazine anesthesia, the ventral and dorsal truncal branches of the subdiaphragmatic vagus nerve were cut and a surgical pyloroplasty was performed. In sham-operated mice, vagal trunks were similarly exposed but not cut, and the pyloroplasty was performed. All mice were monitored daily for 1 week after surgery.

Chemical Sympathectomy

A group of mice underwent chemical sympathectomy, as described previously.[19] Briefly, mice received 2 IP injections of the selective adrenergic neurotoxin 6-hydroxydopamine (100 mg/kg/body weight); control mice received saline IP. The success of sympathectomy was confirmed using immunofluorescent staining for the adrenergic nerve marker tyrosine hydroxylase.

Microbiota Determination

Culture-based analysis. Cecal contents were serially diluted in pre-reduced peptone saline containing 0.5 g/L cysteine/HCl l21 (pH 6.3) (Sigma, Oakville, Ontario, Canada), and plated on blood agar medium (BD, Sparks, MD) under anaerobic (AnaeroGen; Oxoid, Basingstoke, England) and aerobic conditions at 37°C for 24–48 hours. The colonies grown from ATM-treated mice were checked for ATM resistance by foot printing on a blood agar medium complemented with the ATM mixture at the same concentration as the drinking water.

DNA extraction and polymerase chain reaction–denaturing gradient gel electrophoresis. Bacterial DNA/RNA was extracted from biological samples as previously described.[20] RNA or DNA concentrations were determined spectrophotometrically. The hypervariable V4 region of the bacterial 16S ribosomal DNA gene was amplified using polymerase chain reaction or reverse-transcription polymerase chain reaction with universal bacterial primers (HDA1-GC, HDA-2; Mobixlab, McMaster University core facility, Hamilton, Ontario, Canada) as described.[21] Denaturing gradient gel electrophoresis (DGGE) was performed using a DCode universal mutation system (Bio-Rad, Mississauga, Ontario, Canada). Electrophoresis was conducted at 130 V, 60°C for 4.5 hours. Gels were stained with SYBR green I (Sigma) and viewed by ultraviolet transillumination. A scanned image of an electrophoretic gel was used to measure the staining intensity of the fragments using Quantity One software (version 4-2; Bio-Rad Laboratories). The intensity of fragments is expressed as a proportion (%) of the sum of all fragments in the same lane of the gel. Identification of bacterial phylogenies from DNA bands or bacterial colonies was performed as previously described.[22] Polymerase chain reaction products were first checked by DGGE and then sequenced using the method of Sanger et al[23] on an ABI 3730 automated sequencing system. The retrieved sequences were compared with the RDP-II and NCBI GenBank databases using the maximum likelihood algorithm.

Behavioral Testing

The light/dark preference test was performed as described[24] using commercial automated apparatus and analysis software (Med Associates, Inc, St Albans, VT). Briefly, each mouse was placed in the center of an illuminated box connected with a darker box, and its behavior was monitored for 10 minutes. Total time spent in the illuminated compartment, number of transitions between compartments (zone entries), total distance, and average velocity were assessed. The step-down test was performed as described previously.[25] Briefly, each mouse was placed in the center of an elevated platform, and latency to step down from the pedestal was measured (maximum duration, 5 min).

Assessment of Inflammation

Small intestine and colon samples were formalin-fixed and stained with H&E. The slides were examined under light microscopy to grade for acute and chronic inflammatory infiltrate as described.[26] A myeloperoxidase assay was performed on frozen tissues, and its activity was expressed in units per mg of tissue.[26]