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]

Cytokines and Neurotransmitters in the Small Intestine and Colon

Tissue samples were homogenized in Tris-HCl buffer containing protease inhibitors, centrifuged, and the supernatants were stored at −80°C. Cytokine levels (interleukin [IL]-10, IL-6, IL-4, transforming growth factor-β, tumor necrosis factor-α, interferon-γ, IL-1β, IL-12, and IL-17) were determined using an enzyme-linked immunosorbent assay (ELISA) (Quantikine; R&D Systems, Minneapolis, MN). Because noradrenaline, dopamine, and serotonin have been proposed to be involved in the pathogenesis of anxiety, depression, and mood control,[27] their levels were measured using the 3-CAT and serotonin ELISA (LDN, Nordhorn, Germany). The protein concentration in each sample was measured using a BCA protein assay kit (Bio-Rad, Mississauga, Ontario, Canada).

BDNF Analysis

After death, brains were collected and frozen in cooled 2-methylbutane (Sigma) and stored at −80°C. Coronal sections were prepared using cryostat, and the hippocampus and amygdala regions were excised. Protein extraction was performed as described.[28] BDNF was measured using 2-site ELISA (BDNF Emax immunoassay system; Promega, Madison, WI). The protein concentration in each sample was

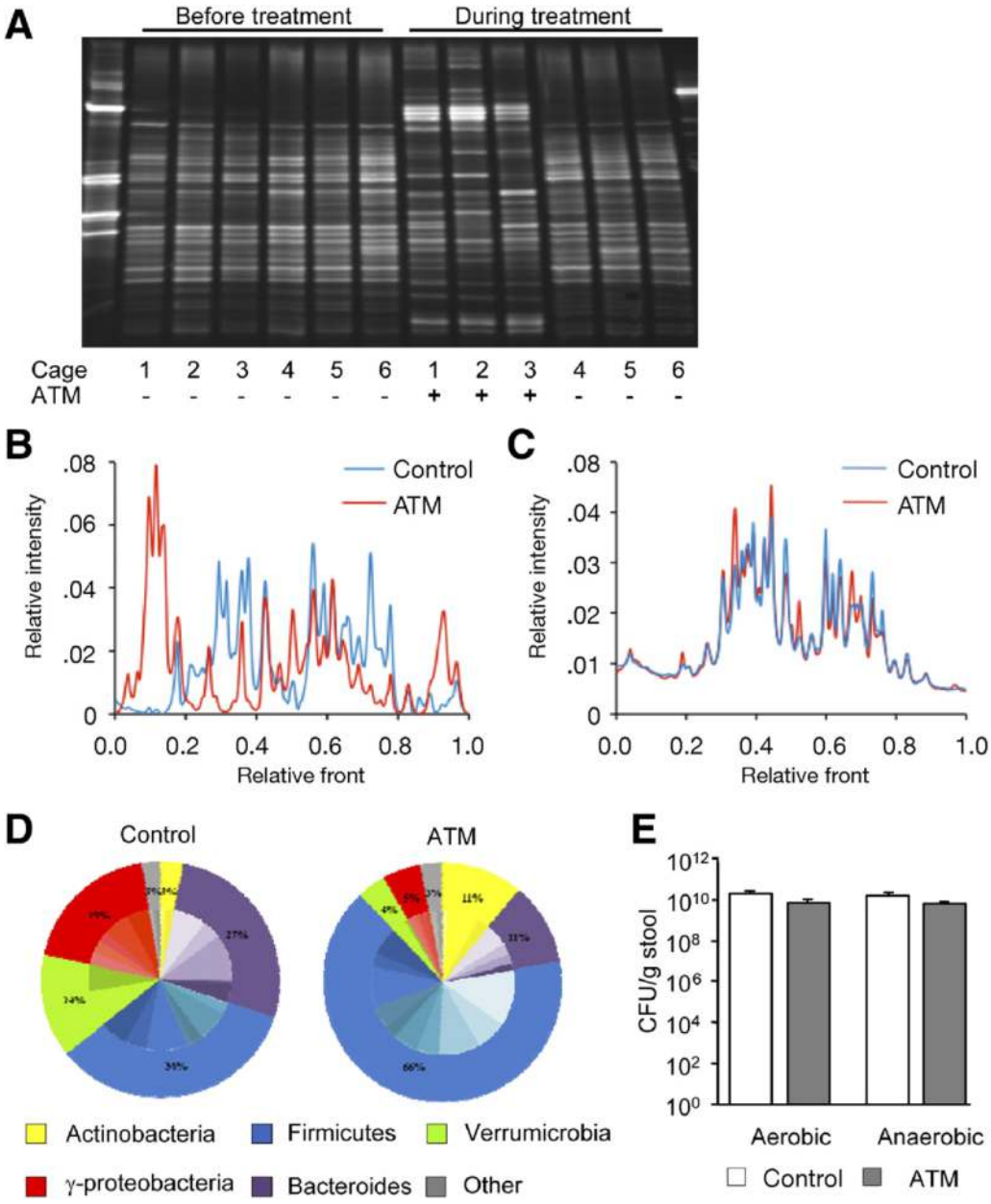

Figure 1. ATM treatment alters the composition of intestinal microbiota. (*A*) Representative DGGE gel of fecal microbiota from control and ATM-treated mice, before and during the treatment. Samples were pooled from each cage (5 mice per cage). (*B*) Mean DGGE profiles of cecal microbiota from control (n = 17) and ATM-treated (n = 20) mice. (*C*) Mean DGGE profiles of controls (n = 15) and mice at 2 weeks after ATM treatment (n = 19). (*D*) Detailed analysis of the microbiota by sequencing single excised DNA bands from DGGE gel from control and ATM-treated mice. Shades within the inner ring represent specific bacterial species. (*E*) Total number of cultivable bacteria using blood agar media. All data are mean ± standard error of the mean.

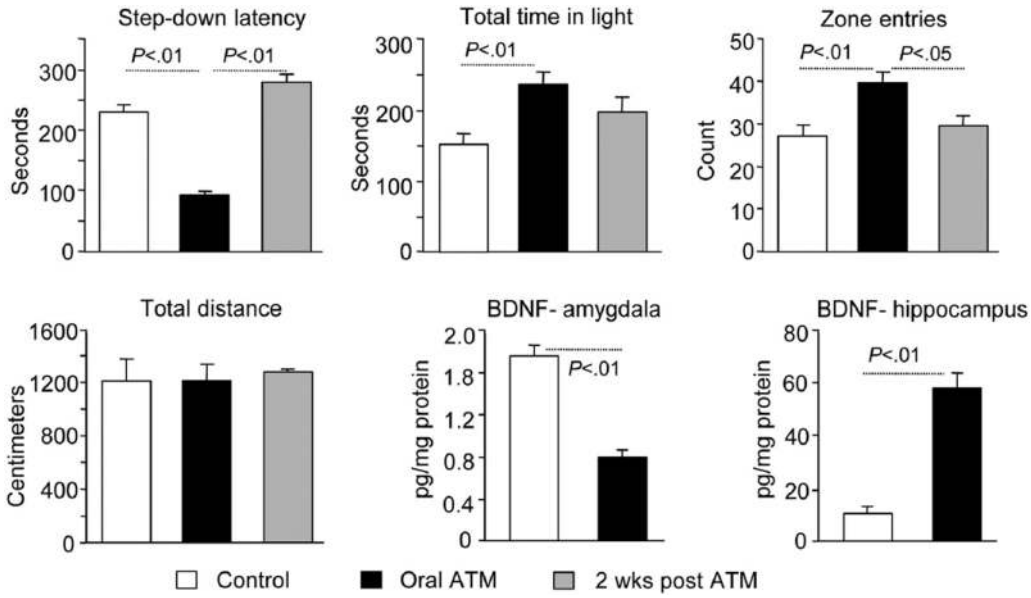

Figure 2. Oral ATM treatment alters mouse behavior promoting exploration. Results of step-down and light/dark preference tests in orally ATM-treated mice (n = 39), mice 2 weeks after ATM treatment (n = 19), and control mice (n = 47). BDNF protein levels measured by ELISA in hippocampus and amygdala of control (n = 17) and ATM-treated (n = 20) mice.

measured using a BCA protein assay kit (Bio-Rad, Mississauga, Ontario, Canada).

Statistical Analysis

Data are presented as means ± standard error of the mean. Statistical analysis was performed using analysis of variance (ANOVA) followed by the Tukey test, or the nonpaired *t* test as appropriate. A *P* value of less than .05 was considered significant. To compare the intestinal microbiota, the similarity between DGGE profiles was calculated using the Dice similarity coefficient.

Results

Antimicrobial Treatment Induces Changes in the Gut Microbiota

To test whether altering the established intestinal microbiota alters mouse behavior, we administered a mixture of nonabsorbable ATMs or sterile water for 7 days to BALB/c SPF mice. A combination of culture and molecular-based approaches were used to identify changes in the intestinal microbiota. Because many bacteria cannot be cultured, 16S ribosomal RNA targeted polymerase chain reaction–DGGE and sequencing was used to examine microbiota composition. Before treatment, DGGE profiles were similar (74% ± 11%) between groups, as well as within groups (71% ± 9% and 77% ± 6% within ATM and control groups, respectively). ATM administration induced a significant perturbation of microbiota composition (Figure 1*A* and *B*) with a similarity index of only 39% ± 7% between control and ATM groups, in the absence of changes in total cultivable bacteria counts (Figure 1*E*). Sequence analysis using excised DGGE bands and dominant cultivable bacteria showed that ATM treatment increased the proportion of Lactobacilli (dominant species: *Lactobacillus intestinalis*, *L johnsonii/gasseri*, and *L plantarum*) and Actinobacteria populations (Figure 1*D*, Supplementary Table 1). At the same time, there was a decrease in the γ-proteobacteria (dominant genus: *Shigella/Klebsiella*) and Bacteroidetes populations (dominant genus: *Bacteroides*).

Perturbation of the Gut Microbiota Increases Exploratory Behavior

Mouse behavior was assessed on day 7 of ATM treatment using standard techniques of step-down and light/dark preference tests, which have been used previously by others to evaluate the effects of anxiogenic and anxiolytic drugs.[24,25] ATM-treated mice showed more exploratory and less apprehensive behavior than controls (Figure 2). Specifically, they stepped down faster from the elevated platform, spent more time in the illuminated compartment of the apparatus, and displayed an increased number of zone entries between the dark and light compartments. However, their overall locomotor activity, assessed by total distance covered or average velocity, was not affected.

ATM-Induced Changes in Gut Microbiota and Behavior Are Reversible

After a 2-week wash-out period, microbiota profiles of ATM-treated and control mice showed a similar relative quantitative and qualitative distribution of the bacterial populations (Figure 1*C*) with a profile similarity of 67% ± 15%. The intestinal microbiota was also stable within groups with a similarity profile of 73% ± 11% and 78% ± 8% in the ATM and control groups, respectively. At 2 weeks after treatment, mice previously treated with ATM displayed similar behavior as controls when assessed by step-down and light/dark preference tests (Figure 2). Thus, the overall microbiota disruption, characterized by

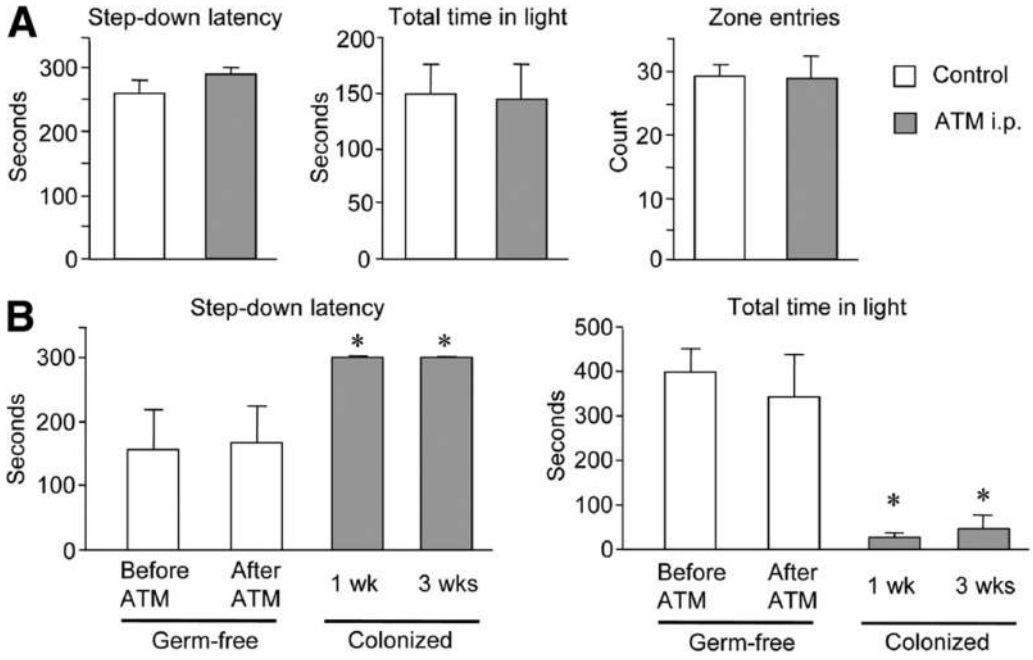

Figure 3. ATM effect on behavior is mediated by intestinal microbiota. (*A*) Effect of IP administration of ATMs (1% of daily oral dose for 7 days, n = 15) on mouse behavior compared with controls (n = 15). (*B*) Behavior in germ-free BALB/c (n = 7) before and after ATM administration, and after colonization with SPF BALB/c microbiota.

a dominance of *Firmicutes* bacteria, was transient and normalized at 2 weeks after ATM, which correlated with a return to normal behavior.

IP ATM Administration Does Not Alter Mouse Behavior or Gut Microbiota

Although previous studies using this regimen have shown that less than 0.05% of the oral dose is absorbed,[29] we administered 1% of the oral dose of ATM or saline IP daily for 7 days to rule out a possible systemic effect of ATM. No difference was observed in behavior (Figure 3*A*) or microbiota profiles between IP ATM-treated mice and controls (Supplementary Figure 1).

Oral ATM Administration Does Not Alter Mouse Behavior on Germ-Free Mice

To confirm the role of microbiota in the observed behavior, we administered oral ATM to germ-free mice and found no differences in behavior before or after ATM administration (Figure 3*B*). However, there was a marked change in behavior when germ-free mice were colonized with microbiota from SPF BALB/c mice.

ATM-Induced Changes in Gut Microbiota Alter Levels of Central BDNF

To correlate ATM-induced changes in behavior with possible alteration in brain biochemistry we measured BDNF protein levels using ELISA. We found that BDNF levels in ATM-treated mice were greatly higher in the hippocampus and lower in the amygdala compared with control mice (Figure 2), which was consistent with the observed behavioral changes.

ATM-Altered Behavior Is Not Accompanied by Gut Inflammation or Changes in Specific Enteric Neurotransmitters, and Is Not Autonomically Mediated

To investigate mechanisms involved in the microbiota–gut–brain axis communication we assessed gut inflammation and specific neurotransmitters. ATM treatment did not induce any significant changes in intestinal morphology or myeloperoxidase activity in the small intestine or colon (Figure 4*A*). Furthermore, no differences in tumor necrosis factor-α, IL-1β, IL-4, IL-6, IL-12, interferon-γ, transforming growth factor-β, IL-10, or IL-17 levels measured by ELISA were observed in colon and small intestinal tissues of ATM-treated and control mice. Thus, ATM-induced changes in the intestinal microbiota were not associated with overt inflammation in the gut.

Similarly, we did not find alterations in serotonin, dopamine, or noradrenalin levels, in either small intestine or colon, as assessed by ELISA (Figure 4*B*). This suggests that changes in the enteric nervous system are not the major determinant of behavioral abnormalities in mice with ATM-induced changes in the intestinal microbiota.

ATM administered orally 2 weeks after vagotomy induced similar behavior in controls, mice with pyloroplasty alone, or vagotomy (Figure 4*C*). Similarly, ATM administered orally 2 weeks after sympathectomy showed similar behavior in sympathectomized and control mice (Figure 4*D*). This indicates that neither parasympathetic nor sympathetic pathways are involved in the behavioral alterations that accompanied ATM treatment.

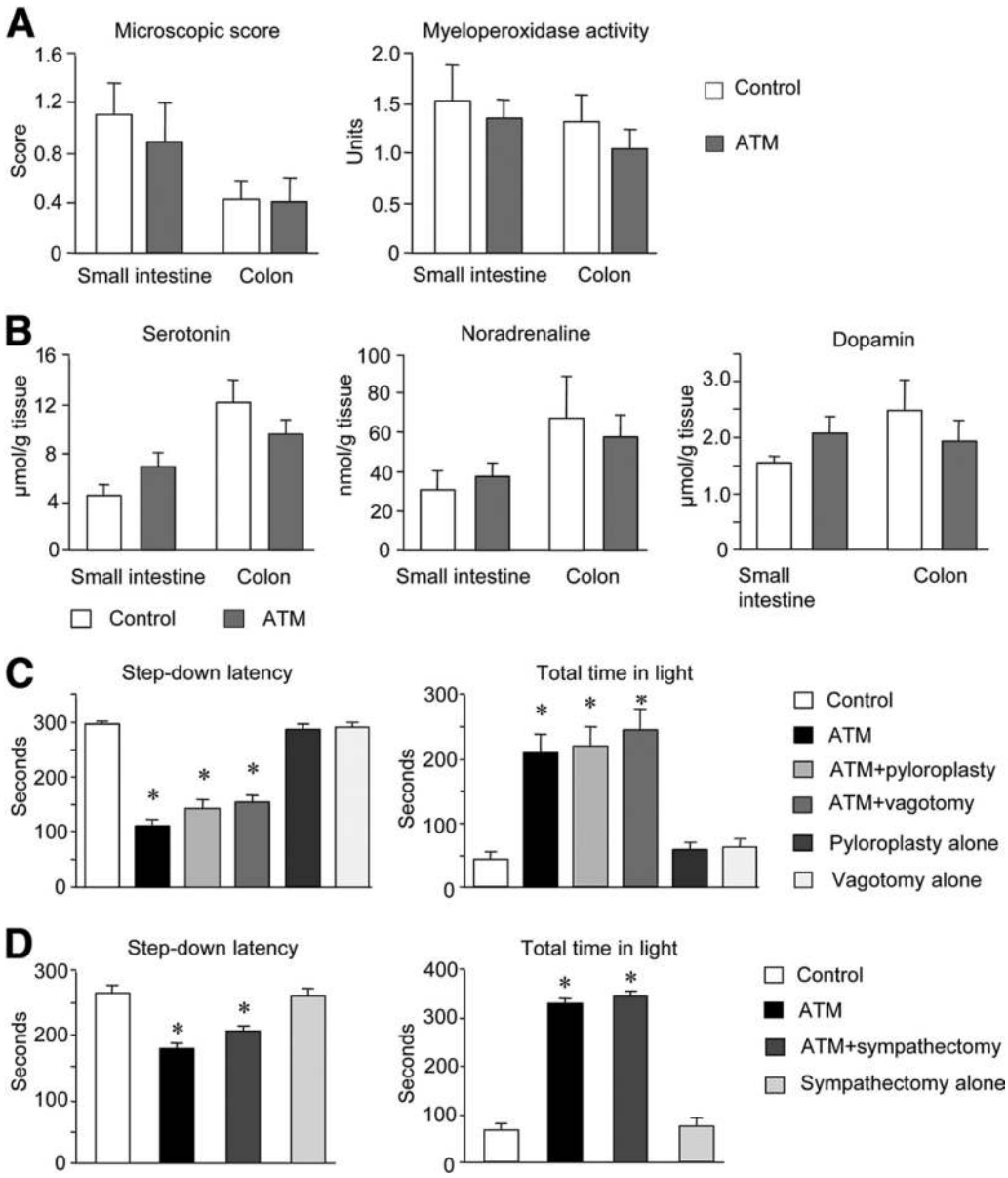

Figure 4. Oral ATMs do not induce gut inflammation or changes in enteric neurotransmitters, and do not act through the autonomic nervous system. (*A*) Microscopic scores and myeloperoxidase activity of small intestine and colon from control (n = 15) and ATM-treated (n = 19) mice. (*B*) Levels of serotonin, dopamine, and noradrenalin as assessed by ELISA in control (n = 15) and ATM-treated (n = 19) mice, both in the small intestine and colon. (*C*) Behavior in controls (n = 15), ATM-treated mice (n = 15), ATM-treated mice with pyloroplasty alone (n = 15), ATM-treated mice with vagotomy and pyloroplasty (n = 15), mice with pyloroplasty only (n = 9), and vagotomy only (n = 12). (*D*) Behavior in controls (n = 15), ATM-treated mice (n = 15), ATM-treated mice with sympathectomy (n = 15), and mice with sympathectomy only (n = 15). Data are mean ± standard error of the mean, statistics by ANOVA and the Tukey test.

Colonization of Germ-Free Mice With Microbiota From Different Mouse Strains Alters Exploratory Behavior

In the second part of the study we exploited well-documented differences in behavior between mouse strains,[30,31] with BALB/c mice displaying more timid and anxious behavior compared with other strains such as NIH Swiss. We investigated whether the behavioral phenotype of a mouse is altered by transferring intestinal microbiota from another mouse strain with a different behavioral profile. DGGE analysis showed that the microbiota profile under SPF conditions was different in BALB/c and NIH Swiss mice (Figure 5*A* and *B*). We then gavaged germ-free NIH Swiss and BALB/c mice (6–8 weeks old) with fresh cecal contents from adult SPF BALB/c or NIH Swiss mice. Three weeks later, germ-free NIH Swiss mice colonized with BALB/c microbiota displayed substantially less exploratory behavior than those colonized with NIH Swiss microbiota (Figure 5*C*). In contrast, germ-free BALB/c mice colonized with NIH microbiota displayed markedly more exploratory behavior than those with BALB/c microbiota. DGGE analysis showed that 96%–100% bacterial strains from donor mice were transferred, but their relative proportions were altered, likely owing to host genetic pressures, resulting in similarity profiles

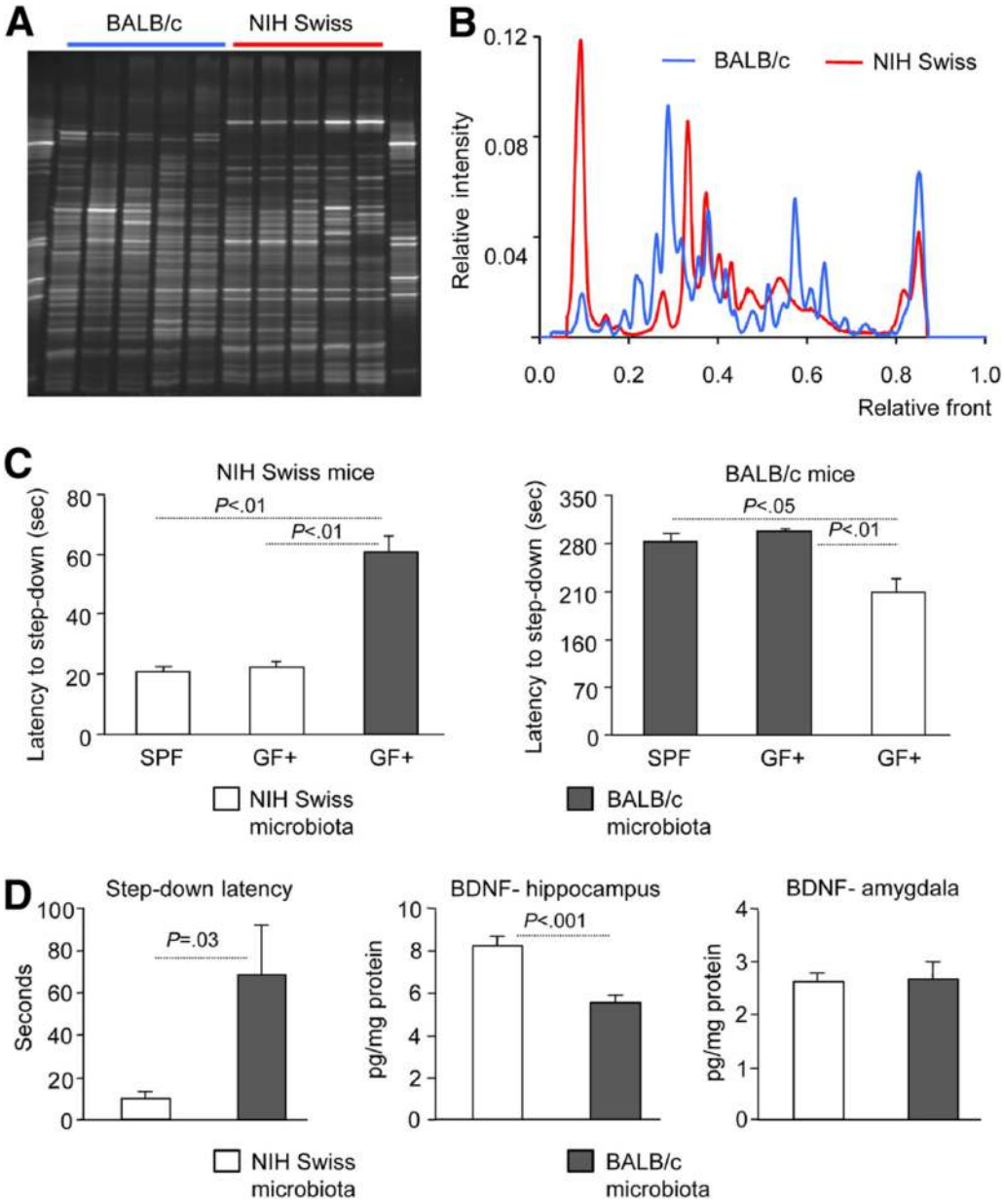

Figure 5. Intestinal microbiota transfer differentially affects behavior of recipient mice. (*A*) Representative DGGE gel of fecal microbiota from SPF BALB/c and NIH Swiss mice (pooled samples, 5 mice per cage). (*B*) Mean DGGE profiles of cecal microbiota from SPF BALB/c and NIH Swiss mice (n = 8 per group). (*C*) Step-down test in NIH Swiss and BALB/c mice at 3 weeks after colonization. SPF NIH Swiss (n = 22), and germ-free (GF) NIH Swiss mice colonized with either NIH Swiss (n = 43) or BALB/c (n = 41) microbiota (*left panel*). SPF BALB/c (n = 15), or GF BALB/c mice colonized with either BALB/c (n = 15) or NIH Swiss (n = 15) microbiota (*right panel*). (*D*) Step-down test in NIH Swiss mice colonized for 1 week with either BALB/c (n = 12) or NIH Swiss (n = 11) microbiota. BDNF levels in hippocampus and amygdala in NIH Swiss mice colonized with BALB/c (n = 7) or NIH Swiss (n = 7) microbiota for 1 week. Data are mean ± standard error of the mean, statistics by ANOVA and the Tukey test.

ranging from 59% to 84% compared with the respective SPF mice.

Hippocampal BDNF Levels Are Altered During the Early Phase of Bacterial Colonization

At 3 weeks after microbiota transfer, BDNF levels in the amygdala and hippocampus were similar in NIH Swiss and BALB/c mice, irrespective of their specific microbiota (Supplementary Figure 2). However, in studies performed 1 week after transfer (Figure 5*D*), we found that mice colonized with BALB/c microbiota had decreased levels of hippocampal BDNF and delayed latency to step-down, compared with mice with NIH Swiss microbiota. This suggests that central neurotrophin-dependent mechanisms are involved in the induction, but not in the maintenance, of altered behavior during gut microbial colonization.

Specific Murine Microbiota Did Not Alter Levels of Cytokines or Gut Neurotransmitters

Levels of circulating cytokines (tumor necrosis factor-α, interferon-γ, and IL-1β) were low and similar between

recipient mice colonized with BALB/c or NIH Swiss microbiota (Figure 6*A*), indicating that the observed behavioral changes were not cytokine-mediated and did not reflect a malaise effect. Similarly, the levels of serotonin and dopamine in the colon and the small intestine were not affected by microbiota composition, but were lower in the colon of NIH Swiss mice compared with BALB/c mice (Figure 6*B*).

Discussion

The results of this study provide strong evidence for a microbiota–gut–brain axis that influences brain biochemistry and modulates behavior in adult mice. This is supported by several lines of evidence. First, transient perturbation of the microbiota increased hippocampal BDNF and exploratory behavior. Second, these changes were reversible upon normalization of the microbiota after withdrawal of the ATM. Third, ATM administration did not alter behavior in germ-free mice. Fourth, we showed that colonization of germ-free mice with an SPF flora alters behavior. Last, we were able to modify the behavioral phenotype and brain BDNF in germ-free mice receiving cecal commensals from a mouse strain with a different behavioral phenotype.

A 7-day course of ATM resulted in a significant increase in Firmicutes and Actinobacteria, and a decrease in γ-proteobacteria and Bacteroidetes. We believe that these changes in bacterial composition of the colon were responsible for the documented changes in brain BDNF levels and in behavior. This is supported by the observa-

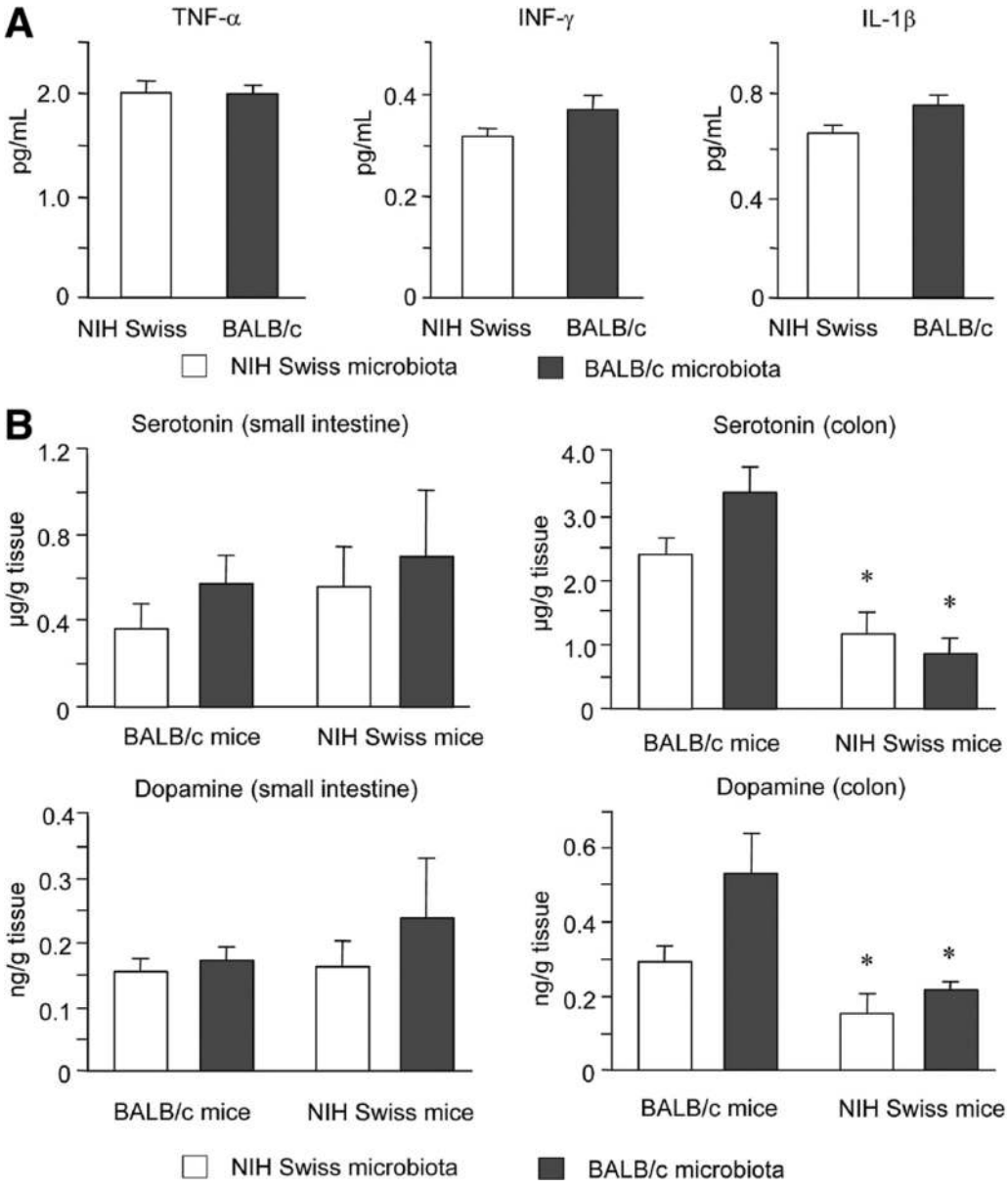

Figure 6. Intestinal microbiota does not affect circulating cytokines or gut neurotransmitters. (*A*) Levels of serum tumor necrosis factor-α, interferon-γ, and IL-1β in germ-free recipients who received either NIH Swiss (n = 21) or BALB/c (n = 20) microbiota. Data are mean ± standard error of the mean, statistics by *t* test. (*B*) Serotonin and dopamine levels in both small intestine and colon in mice colonized with NIH Swiss or BALB/c microbiota (n = 6 per group). Data are mean ± standard error of the mean, statistics by *t* test. *$P < .05$ vs BALB/c mice.

tion that the administered ATMs, which are poorly absorbed from the gastrointestinal tract, failed to influence the behavioral parameters under study when applied in smaller dosage by IP injection. Furthermore, a toxic effect of the ATM would be expected to produce a malaise effect associated with a decrease in overall locomotor activity, rather than the increase in exploratory behavior observed in our study. Furthermore, administration of ATM did not alter behavior in germ-free mice, which points to the crucial role of microbiota, effectively excluding a direct effect of ATM on the gut or the central nervous system.

In the hippocampus, BDNF is associated with memory and learning, but recent evidence indicates that increases in hippocampal BDNF are associated with anxiolytic and antidepressant behavior.[32] The increase in hippocampal BDNF seen in the ATM-treated mice is therefore consistent with their gregarious behavior. The amygdala also is associated with memory and mood disorders; a recent study has shown increased BDNF expression in the amygdala during fear learning.[33] Overactivation of the amygdala also has been implicated in depression and anxiety.[34] Lower levels of BDNF in the amygdala of ATM-treated mice are therefore consistent with the observed increase in exploratory behavior.

A comparison between this and a previous study[10] using the same ATMs indicates that the impact of a given ATM combination on the gut microbiota differs among mouse strains and the ATM regimens. In the present study using BALB/c mice, we found no changes in total cultivable bacteria, but we showed a significant shift in bacterial composition. This was not accompanied by evidence of gut inflammation, in contrast to the study by Verdú et al[10] in which NIH Swiss mice received ATMs in a higher dose and for a longer duration. In the present study, perturbation of the microbiota did not alter myeloperoxidase activity, histologic appearance, or cytokine profile of the colon or small intestine. Similarly, no differences in serotonin, dopamine, or noradrenaline content in the small intestine or colon of ATM-treated mice were observed, suggesting that these neurotransmitters are not involved in mediating the behavioral changes observed in the model. However, we cannot rule out the possibility that other enteric neuromediators are involved in the observed behavioral changes, and further studies are therefore needed.

A recent study showed that during early phases of enteric *Campylobacter jejuni* infection, mice displayed anxiety-like behavior, which was mediated vagally.[35] The effect of ATMs on behavior was present in previously vagotomized and sympathectomized mice, suggesting that autonomic pathways are not required for the induction of ATM-induced behavioral changes. Taken together, these observations indicate that ATM-induced changes in the intestinal microbiota alter behavior and brain biochemistry through mechanisms that are not accompanied by a discernible increase in inflammatory activity or changes in specific enteric neurotransmitters. The alteration in behavior was independent of the autonomic nervous system, and thus is likely to involve substances produced by gut bacteria acting directly or indirectly on the central nervous system. Although not explored in this study, Toll-like receptor signaling could be involved in the altered behavior induced by intestinal dysbiosis. Toll-like receptors are critical in microbial recognition and regulation of intestinal homeostasis, and work to date has implicated these receptors in illness-like behavior during inflammation, as well as in addictive behavior.[36,37]

Our results are supported by a recent article by Li et al,[38] who used different dietary supplementation to perturb the intestinal microbiota in very young mice and assessed memory and learning in adulthood. Mice fed a beef-enriched diet for 3 months displayed improved working and reference memory compared with those fed with standard rodent chow. However, the observed differences in behavior in that study may have been at least partially attributable to different dietary components acting directly on the brain, and independently of diet-induced changes in the microbial composition of the gut.[39–41]

The adoptive transfer strategy used in our study to confirm the ability of the microbiota to influence behavior previously has been used successfully to show the role of commensal bacteria in obesity.[42] Here, we exploited the well-established differences in the behavior of commonly used mouse strains[30,31] and the fact that they have different microbiota. BALB/c mice are more timid than NIH Swiss mice. The transfer of cecal bacteria from BALB/c to NIH Swiss mice resulted in greater hesitancy in the step-down test in the recipient mice. In contrast, transfer of cecal contents from NIH Swiss to BALB/c mice resulted in shorter latency to step-down in this normally hesitant mouse strain. The increased hesitancy seen in NIH Swiss recipients colonized with BALB/c microbiota possibly could reflect a malaise effect, although we did not observe any increase in malaise-inducing cytokines in the recipient mice. However, changes in cytokines would be unlikely to explain decreased hesitancy observed in BALB/c recipients colonized with NIH Swiss microbiota. As in the ATM experiments, we did not detect changes in gut neurotransmitters associated with different microbiota, although NIH Swiss mice have a lower content of serotonin and dopamine in the colon, which likely is determined genetically. Microbiota transfer experiments showed changes in central BDNF levels at 1 week after colonization, but 2 weeks later their levels normalized. Modulation of central neurotrophin expression, therefore, may play a role in the induction of behavior changes in both models.

In summary, using two experimental strategies, we show that the intestinal microbiota can influence the central nervous system in the absence of discernible changes in local or circulating cytokines or specific gut neurotransmitter levels. Elucidation of the precise pathway(s) of communication underlying the microbiota–gut–brain axis likely will require vast interdisciplinary efforts because our results suggest the pathway may involve production of neurally active substances by commensals. Examples of this include the production of benzodiazepine ligands in a rat model of encephalopathy[43] or butyrate acting as a histone D-acetylesterase that recently was shown to have an antidepressant effect.[44]

The establishment of a gut–microbiota–brain axis has implications for the understanding and treatment of chronic

gastrointestinal diseases, such as IBD and IBS, which often show psychiatric comorbidity and alterations in the intestinal microbiota.[45] It was not the purpose of our study to identify a microbiota signature associated with an altered behavioral phenotype. Instead, our results show that disruption of a previously stable microbiota in healthy mice results in changes in brain chemistry and behavior, and the role of the microbiota was confirmed in our adoptive transfer experiments. This finding is relevant to conditions such as IBS, in which the bacterial composition of the gut is unstable over time, and raises the possibility that intestinal dysbiosis contributes to the behavioral phenotype of these patients. This has therapeutic implications because we recently have shown that treatment with the specific probiotic strain *Bifidobacterium* can normalize both altered behavior and brain biochemistry of mice with chronic mild to moderate gut inflammation.[46] In conclusion, the results of this study indicate that, in addition to determining immune and metabolic function of the host,[1] intestinal commensals play a critical role in behavior and central neurotrophin expression.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.04.052.

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Conflicts of interest

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Supplementary Table 1. Phylogenetic Groups in Control and ATM-Treated Mice

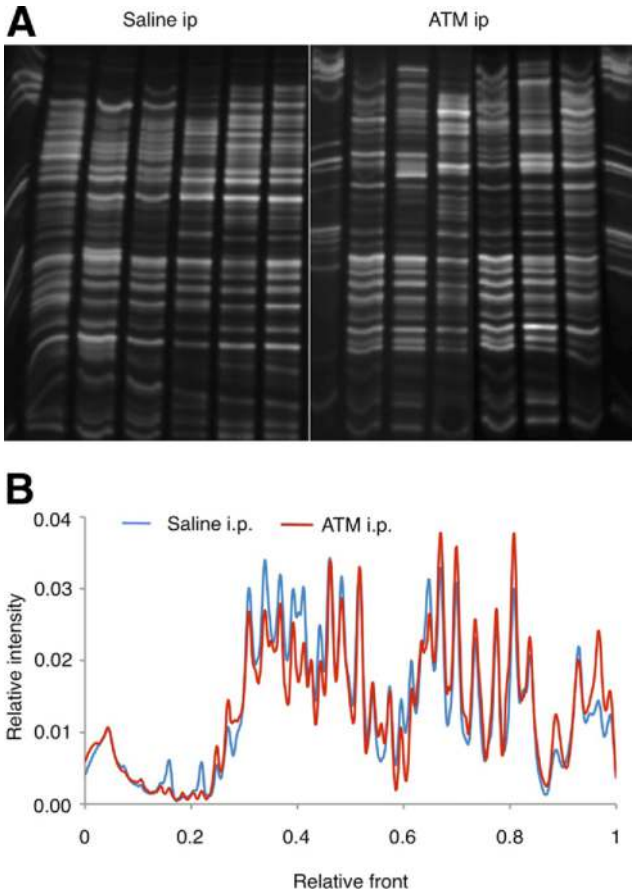
RF	Sequence v4 16S rDNA	Order/genus/species	Phylum	Control (RI)	ATB (RI)
0.885	TACGGGAGGCAGCAGTGGGGAATATTGCACAATG GGCGCAAGCCTGATGCAGCGACGCCGCGTG AGGGATGGACCTTCGGGTTGTAAACCTCTTT	*Actinomyces/ Bifidobacterium*	*Actinobacteria*	0.007	0.012
0.925	GAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCC TGANTGCAGCGACGCCGCGTGCGGGATGGAGGCC NTTCGGGTTGTANAACCGCTTTNNC	*Bifidobacterium*	*Actinobacteria*	0.007	0.073
0.962	GAGGCAGCAGTGGGGAATATTGCACAATGGGCG CAAGCCTGATGCAGCGACGCCGCGTGGGGGATG ACGGCCTTCGGGTTGTAAACCTCTTT	*Corynebacterium/ Mycobacterium*	*Actinobacteria*	0.018	0.028
0.655	CCAGCCAAGTAGCGTGAAGGATGACTGCCCTATGGGTT GTAAACTTCTTTTATAAAGGAATAAAGTCGGGTATG CATACCCGTTTGCATGTACTTTATGAATAA GGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC	*Bacteroides*	*Bacteroidetes*	0.076	0.051
0.688	GCAGTGAGGAATATTGGTCAATGGGCGCTAGCCTGAACC AGCCAAGTAGCGTGAAGGATGAAGGCTCTATGGGTCGTAAA CTTCTTTTATATAAGAATAAAGTGCAGTATGTATACT GTTTTGTATGTATTATATGAATAAGGATCGGCTAACTCCG TGCCAGCAGC	*Bacteroides*	*Bacteroidetes*	0.043	0.025
0.721	CTACGGGAGGCAGCAGTGAGGAATATTGGTCAATGGACGGGAGTC TGAACCAGCCAAGTAGCGTGAAGGATGACTGCCCTATG GGTTGTAAACTTCTTTTATATGGGAATAAAGTGATCCACG TGTGGAATTTTGTATGTACCATATGAATAAGGATCGGCTAA CTCCGTGCCAGC	*Bacteroides*	*Bacteroidetes*	0.108	0.023
0.747	CTACGGGAGGCAGCAGTGAGGAATATTGGTCAATGGACG AAAGTCTGAACCAGCCAAATCGCGTGAAGGAAGAAGGTA TTATGTATCGTAAACTTCTTTTGTAAGAGAGTAAAGTG CACTACGTGTAGTGTATTGCAAGTACCTTACGAATAAGCATC GGCTAATTCCGTGCCAGCAGCCGCGGTAATAC	*Porphyromonadaceae*	*Bacteroidetes*	0.007	0.001
0.777	GGCAGCAGTGAGGAATATTGGTCAATGGGCGTAAGCCTGAACC AGCCAAGTCGCGTGAGGGATGAAGGTTCTATGGATCGTAAA CCTCTTTTATAAGGGAATAAAGTGCGGGACGT GTCCCGTTTTGTATGTACCTTATGAATAAGGATC GGCTAACTCCGTGCCAGCAGCCGCGGTAANNN	*Parabacteroides*	*Bacteroidetes*	0.045	0.014
0.094	CGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG TTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGA TAGAGGTAAGTCACGGCTAACTACGTGCCAG CAGCCGCGGTAATAC	*Lactobacillus intestinalis*	*Firmicutes*	0.000	0.126
0.116	ACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAA GGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAA CATATCTGAGAGTAACTGTTCAGGTATTAGAAAGCCAC GGCTAACTACGTGCCAGCAGCCGCGGTAATAC	*Lactobacillus plantarum*	*Firmicutes*	0.001	0.069
0.133	CTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGC TCGTAAAGCTCTGTTGGTAGTGAAGAAAGATAGAGGTAGTAA CTGGCCTTTATTTGACGGTAATTACTTAGAAAGTCACGGC TAACTACGTGCCAGCAGCCGCGGTAATAC	*Lactobacillus johnsonii/gasseri*	*Firmicutes*	0.001	0.093
0.174	CTGATGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGG ATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACGAGAGGG NNTGCTCGTACNCTTGACGGTACCTTGNCGAGAAAGCC ACGGCTAACTACGT	*Exiguobacterium*	*Firmicutes*	0.025	0.051
0.424	AGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAG CAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCT CTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAA AGTTCACACTGTGACGGTAGCTTACCAGAAA GGGACGGCTAACTACGTGCCAGCAGCCGCGGT	*Streptococcus*	*Firmicutes*	0.073	0.058
0.463	AGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGA GCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAA CTCTGTTATTAGGGAAGAACATATGTGTAAGTAACT GTGCACATCTTGACGGTACCTAATCAGAAAGCCACG GCTAACTACGTGCCAGCAGCCGCGGTAATAC	*Staphylococcus*	*Firmicutes*	0.025	0.029

Supplementary Table 1. Continued

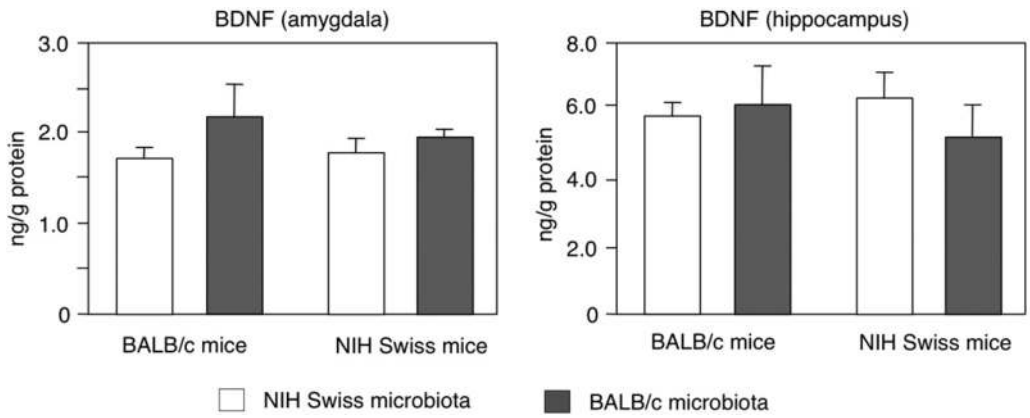
RF	Sequence v4 16S rDNA	Order/genus/ species	Phylum	Control (RI)	ATB (RI)
0.502	AGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGG AGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGT AAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTT GAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGC CACGGCTAACTACGTGCCAGCAGCCGGGTAATAC	*Bacillus*	*Firmicutes*	0.015	0.051
0.559	TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAA GCCTGATGCAGCGACGCCGCGTGAGCGAAGAAGTATTT CGGTATGTAAAGCTCTATCAGCAGGGAAGATAATGA CGGTACCTGACTAAGAAGCACCGGCTAAATACG	*Pseudobutyrivibrio*	*Firmicutes*	0.095	0.093
0.589	ACAATGGGCGAAAGCCTGATNCAGCAACGCC GCGTGAGTGATGAAGGCCTTCGGGTCGTA	*Clostridiales*	*Firmicutes*	0.049	0.030
0.614	AAACTCTGTCCTCAAGGAAGATAATGACGGTACT TNNGGAGGAAGCCCCGGCTAACTACGTGC	*Peptostreptococcaceae Incertae Sedis*	*Firmicutes*	0.065	0.065
0.359	CTACGGGTGGCAGCAGTCGAGAATCATTCACAATGGGGG AAACCCTGATGGTGCGACGCCGCGTGGGGGAATGAAGG TCTTCGGATTGTAAACCCCTGTCATGTGGGAGCAAATT AAAAAGATAGTACCACAAGAGGAAGAGACGGC TAACTCTGTGCCAGCAGCCGCGGTAATACAG	*Akkermansia*	*Verrucomicrobia*	0.083	0.033
0.378	GGGGCAGCTGGAGATATTCCAATGGGGCGAAACCCTGA TGGTGCGACGCCGCGTGGGGGAATGAAGGTCTTCGGATT GTAAACCCCTGTCATGTGGGAGCAAATTAAAAA GATAGTACCACAAGAGGAAGAGACGGCTAACTCTG TGCCAGCAGCCGCGGTAATACA	*Verrucomicrobiales*	*Verrucomicrobia*	0.061	0.008
0.212	GCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGG CCTTAGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTG ATAAGGTTAATACCCTTGTCAATTGACGTTACCCGCAGAA GAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG	*Proteus*	*γ-proteobacteria*	0.029	0.015
0.242	CGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCA GCGGGNNGGAAGGCGANTAAGGTTAATAACCTTGACGA TTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCG TGCCAGCAGCCGCGGTAATA	*Klebsiella*	*γ-proteobacteria*	0.030	0.011
0.264	AATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC GCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGG NNGGAAGGCGANTAAGGTTAATAACCTTGNCG ATTGACGTTACCCGCAGAAGAAGCACCGGCT AACTCCGTGCCAGCAGCCGCGGTAATA	*Klebsiella/ Enterobacter*	*γ-proteobacteria*	0.024	0.019
0.293	AGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCC ATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAA GTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATA CCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACC GGCTAACTCCGTGCCAGCAGCCGCGGTAATA	*γ-proteobacteria*	*γ-proteobacteria*	0.062	0.007
0.315	TTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGT GTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGG AGGAAGGGGGAAAGGTTAATAACCTTTTTCATTGA CGTTACCCGCAGAAGAAGCACCGGCTAACTCC GTGCCAGCAGCCGCGGTAATA	*Erwinia*	*γ-proteobacteria*	0.053	0.002
0.035	CNNNGNGGNNCTANGNNGNNNAGTGGGGAATATTGGAC AATGGGCGAAAGCCTGANCNNCCTGCCGCGTGTGTGAAGAA GGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGG AAGGGCAGTAAGTTAATACC	Pseudomonas	*γ-proteobacteria*	0.008	0.016
0.827	NTTGGATAGTGGACGTTACTCGCAGAATAAGCACCGG CTAACTCTGTGCCAGCAGCCGCGGTAATACA	Acinetobacter	*γ-proteobacteria*	0.010	0.012
0.856	CTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGG GGGAAACCCTGACGCAGCAATGCCACGTGAATGATGAA GGCCTTCGGGTTGTAAAGTTCTTTTAGTAGGGAA GATAGTGACGGTACCTACAGAAAAAGCTCCGG CTAACTCCGTGCCAGCAGCCGCGGTAATAC	Other	Other	0.008	0.002

NOTE. Phylogenic analysis of Sanger-sequenced amplified V4 region of bacterial 16S ribosomal RNA was based on comparison with the sequences of the NCBI/RDP II databases (cut-off levels: RDP classifier confidence higher than 99%). The relative intensity of peaks referenced to their relative front (normalized to the migration front) was calculated as the area under the curve of the selected sequenced peaks from the electrophoregram shown in Figure 1.

RF, relative front; RI, relative intensity.



Supplementary Figure 1. IP administered ATMs do not affect intestinal microbiota. (*A*) Representative DGGE gels of cecal microbiota from individual mice treated with saline or ATMs IP. (*B*) Mean DGGE profiles of cecal microbiota from IP saline (n = 15) and IP ATM-treated (n = 15) mice.



Supplementary Figure 2. Intestinal microbiota does not affect brain BDNF at 3 weeks after colonization. BDNF levels in amygdala and hippocampus after 3-week colonization with NIH Swiss or BALB/c microbiota (n = 6 per group).