GASTROENTEROLOGY 2010;139:2102–2112

Chronic Gastrointestinal Inflammation Induces Anxiety-Like Behavior and Alters Central Nervous System Biochemistry in Mice

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BACKGROUND & AIMS: Clinical and preclinical studies have associated gastrointestinal inflammation and infection with altered behavior. We investigated whether chronic gut inflammation alters behavior and brain biochemistry and examined underlying mechanisms. **METHODS:** AKR mice were infected with the noninvasive parasite *Trichuris muris* and given etanercept, budesonide, or specific probiotics. Subdiaphragmatic vagotomy was performed in a subgroup of mice before infection. Gastrointestinal inflammation was assessed by histology and quantification of myeloperoxidase activity. Serum proteins were measured by proteomic analysis, circulating cytokines were measured by fluorescence activated cell sorting array, and serum tryptophan and kynurenine were measured by liquid chromatography. Behavior was assessed using light/dark preference and step-down tests. In situ hybridization was used to assess brain-derived neurotrophic factor (BDNF) expression in the brain. **RESULTS:** *T muris* caused mild to moderate colonic inflammation and anxiety-like behavior that was associated with decreased hippocampal BDNF messenger RNA (mRNA). Circulating tumor necrosis factor-α and interferon-γ, as well as the kynurenine and kynurenine/tryptophan ratio, were increased. Proteomic analysis showed altered levels of several proteins related to inflammation and neural function. Administration of etanercept, and to a lesser degree of budesonide, normalized behavior, reduced cytokine and kynurenine levels, but did not influence BDNF expression. The probiotic *Bifidobacterium longum* normalized behavior and BDNF mRNA but did not affect cytokine or kynurenine levels. Anxiety-like behavior was present in infected mice after vagotomy. **CONCLUSIONS: Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry, which can be normalized by inflammation-dependent and -independent mechanisms, neither of which requires the integrity of the vagus nerve.**

Keywords: Inflammation; Colitis; Anxiety; BDNF.

BASIC–ALIMENTARY TRACT

Anxiety and depression are among the most common psychiatric disorders.[1–3] Accumulating evidence suggests that the immune system is involved in the induction and maintenance of these disorders at least in a subset of patients.[4] Depression is increased in patients with chronic illnesses associated with immune activation such as cardiovascular disease,[5] rheumatoid arthritis,[6] chronic obstructive pulmonary disease,[7] and type 1 diabetes.[8] Although in some cases depression is a consequence of the debility caused by the chronic disease, it also is possible that they share a common etiologic basis—inflammation—and that there is interplay between peripheral inflammatory processes and the brain. Clinical studies have shown increased serum inflammatory markers in patients with depression[9–11] as well as a correlation between plasma cytokine levels and the severity of depression,[12] and have described improvement in depression after anti-inflammatory treatment.[13,14] Cytokine-induced sickness behavior is a well-recognized entity that comprises neurovegetative and psychological factors, including depression and anxiety.[15] Behavior and psychiatric changes are well documented during experimental cytokine cancer therapy[16] and are frequent side effects of interferon (IFN) treatment for hepatitis C.[17,18] Although these data suggest that an acute increase in systemic proinflammatory cytokines contribute to depression, the role of low levels of circulating cytokines, such as those observed during chronic inflammation, remains controversial.[19]

Anxiety and depression are frequent comorbidities in gut disorders, including inflammatory bowel disease (IBD).[20,21] Individuals with IBD experience 3 times the rate of depression compared with the general population.[22] Anxiety and depression are estimated to affect 30%

Abbreviations used in this paper: BDNF, brain-derived neurotrophic factor; CNS, central nervous system; IFN, interferon; MPO, myeloperoxidase; mRNA, messenger RNA; MS/MS, tandem mass spectrometry; TNF, tumor necrosis factor.

0016-5085/$36.00
doi:10.1053/j.gastro.2010.06.063

of patients with IBD during periods of remission and as much as 60%–80% of patients during exacerbation of the disease.[23–25] The pathophysiological basis for this association is not understood and it is unclear whether anxiety and depression can be a consequence of the chronic gut disorder. More recently, a link has been proposed between psychiatric disease, inflammation, and exposure to microbes, thus prompting inclusion of psychiatric illnesses in the hygiene hypothesis.[26,27]

Studies in animal models using chemical colitis or *Campylobacter jejuni* infection have shown that inflammation of the gastrointestinal tract can be accompanied by changes in behavior that include anorexia[28] and anxiety-like behavior.[29,30] The latter studies suggested that behavioral changes occurred before the onset of the inflammatory response to infection and that vagal pathways mediated the behavioral response to infection. These studies were based on models of acute inflammation and may not be comparable with the chronic inflammatory conditions encountered in human beings.

The purpose of this study was to determine whether chronic mucosal inflammation in the gut induces anxiety-like behavior in mice. The study focused on a model of chronic noninvasive parasitic infection with *Trichuris muris* in susceptible AKR mice, which results in low-grade colitis. To investigate possible mechanisms, we assessed systemic cytokine and kynurenine levels, screened for changes in serum proteins, examined the role of the vagus nerve, and tested the response to budesonide, etanercept, and specific probiotic bacteria.

Materials and Methods

Animals

Male BALB/c or AKR mice (Harlan, Mississauga, ON, Canada) were purchased at the age of 6–8 weeks and housed in a conventional specific pathogen-free unit at McMaster University Central Animal Facility. All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and received approval from the McMaster University Animal Research Ethics Board. A group of mice (n = 24) underwent subdiaphragmatic vagotomy, as described previously.[31] Briefly, after ketamine/xylazine anesthesia the ventral and dorsal truncal branches of the subdiaphragmatic vagus were cut, and a surgical pyloroplasty was performed. In sham-operated mice (n = 15), vagal trunks similarly were exposed but not cut. All mice were monitored daily for 1 week after surgery and infections, colitis induction, and all interventions, and weekly thereafter, for changes in appearance and body condition.

Probiotic Strains

Lactobacillus rhamnosus NCC4007 and *Bifidobacterium longum* NCC3001 (ATCC BAA-999, initially provided by Morinaga, Tokyo, Japan), obtained from Nestle Culture Collection, Lausanne, Switzerland, were grown anaerobically for 24 hours at 37°C in Man–Rogosa–Sharpe (BioMerieux, Geneva, Switzerland) broth (*B longum* with 0.5% cysteine) and further processed as previously described.[32]

Chronic T muris *Infection*

Male AKR mice (n = 95) were infected with *T muris* (300 eggs/mouse) and compared with uninfected controls (n = 47). Infected mice were gavaged daily with *L rhamnosus* (n = 10), *B longum* (n = 16), and placebo (Man–Rogosa–Sharpe, n = 16) from day 30 for 10 days; uninfected mice (n = 14) were gavaged with placebo. Additional *T muris*–infected mice were either injected intraperitoneally with etanercept (Enbrel; Amgen Canada Inc, Mississauga, ON, Canada; 25 μg/mouse twice weekly for 1 week, n = 16) or saline (n = 7), or were gavaged with budesonide (Entocort Enema, AstraZeneca Canada, Mississauga ON, Canada; 4.3 μg/mouse daily for 1 week, n = 23) or budesonide vehicle (Entocort Enema, containing NaCl and methylparaben propylparaben, n = 7); control uninfected mice received etanercept (n = 7) or saline (n = 10) intraperitoneally, budesonide (n = 6) or saline (n = 10) by gavage. Previously vagotomized mice were gavaged with *T muris* (n = 9) or placebo (n = 12).

Etanercept is a soluble tumor necrosis factor (TNF)-α receptor and budesonide is an anti-inflammatory corticosteroid with a hepatic first-pass metabolism exceeding 90%.

At the end of the treatment period, mice underwent behavioral testing. They were euthanized within 24 hours thereafter, and tissue and blood samples were obtained. Colon samples were fixed in formalin for histology or frozen in liquid nitrogen for myeloperoxidase (MPO) activity determination. Brains were frozen in isopentane (−60°C) and stored for in situ hybridization.

Behavior Assessment

Anxiety-like behavior was assessed in individual mice using a light/dark preference test as described[33] using either an automated system (Med Associates Inc, St. Albans, Vermont) or a custom-built apparatus equipped with a digital video camera. Briefly, each mouse was placed in the center of an illuminated box connected to a smaller dark box, and the mouse's behavior was recorded for 10 minutes. Outcome measures, including total time spent in the light box, latency to re-enter the light box (time spent in the dark box after first entry), total distance traveled, and average velocity, were assessed by a blinded observer. The step-down test was performed as described previously.[34] Briefly, each mouse was placed in the center of an elevated platform (10 cm diameter, 4 cm high) situated at the center of a table top (80 × 60 cm) and latency to step down from the platform was measured (maximum, 5 min).

Intestinal Inflammation

Colon formalin-fixed samples were stained with H&E and examined under light microscopy by a blinded researcher for inflammatory infiltrate as described previously.[35,36] To assess acute intestinal inflammation, MPO assay was performed on frozen tissues as described previously[35] and its activity was expressed in units per milligrams of tissue.

Brain-Derived Neurotrophic Factor Messenger RNA Levels

Expression of brain-derived neurotrophic factor (BDNF) messenger RNA (mRNA) in the hippocampus was assessed by in situ hybridizations using 35S-labeled RNA probes on frozen brain sections as described previously.[37,38] The antisense probe produced from the BDNF complementary DNA template is a 382-bp probe complementary to the coding region of mouse BDNF mRNA (bases 1028–1410, NM_007540). Antisense BDNF ribonucleotide probe was a gift from Dr J. Lauterborn and Dr C. Gall (University of California, Irvine, CA).

Systemic Markers: Proinflammatory Cytokines and Serum Proteins

Plasma levels of proinflammatory cytokines, including IFN-γ and TNF-α, were measured using a BD FACS Array Bioanalyzer and a Mouse Inflammation Kit (BD Biosciences, Mississauga, ON, Canada).

Depletion of high-abundance serum proteins. For each of the 10 mouse serum samples, a total of 25 μL were applied to a Mu-3 MARS spin cartridge (Agilent, Mississauga, ON, Canada) to remove albumin, transferrin, and immunoglobulin (Ig)G. After elution the column buffer was exchanged with lysis buffer (9 mol/L urea, 2 mol/L thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, Tris pH 8.5) using 10-kilodalton cut-off spin concentrators (PALL, Mississauga, ON, Canada).

Two-dimensional difference gel electrophoresis. A total of 50 μg protein from each sample were labeled with 400 pmol of CyDye Fluor minimal dyes (GE Healthcare, Piscataway, NJ) and incubated for 30 minutes. Cy3 and Cy5 were used for samples and paired samples were reverse-labeled to prevent potential dye labeling bias. A pooled fraction containing all the samples in equal amounts also was prepared and labeled with Cy2 to serve as an internal control. The samples were subjected to 2-dimensional gel electrophoresis as described previously.[39] Gels were scanned using a Typhoon 9400 scanner (GE Healthcare). Images were imported into Decyder 6.0 software (GE Healthcare) for analysis. The differential in-gel analysis module was used for spots detection, volume quantification, and volume ratio normalization of different samples loaded on the same gel. The biological variation analysis module was used to analyze spots across gels and to identify protein spots having significant differences. A significance difference was defined as at least a 2.0-fold difference in expression and a P value of less than .05. Preparative gels were run as described earlier with 600 μg of sample mixture pooled from all 10 samples. The gel was stained with Deep Purple (GE Healthcare) and scanned using Typhoon 9400. Proteins of interest were selected for tandem mass spectrometry (MS/MS) analysis using the Ettan Spot Picker (GE Healthcare).

MS/MS analysis. The spots were washed twice with 50 mmol/L ammonium bicarbonate/50% methanol and once with 100% acetonitrile. Plugs then were dried and 20 μL trypsin (Promega, Montreal, QC, Canada) was added. Samples were incubated at 37°C for 2 hours. A total of 12 μL of digested sample were then used to perform MS/MS analysis using the LC/MSD Trap Ultra 6330 Mass Spectrometer (Agilent). The obtained spectra were analyzed using Spectrum Mill MS Proteomics Workbench (Agilent) and run against the mouse subset of the National Center for Biotechnology Information (NCBInr) protein database. Protein identification with at least 2 peptides and the distinct summed MS/MS search score of at least 25 were chosen as true hits.

Tryptophan and Kynurenine Determination

Plasma kynurenine and tryptophan were analyzed by ultra performance liquid chromatography using a Waters Acquity system and MassTrak AAA kit (Waters Corporation, Mississauga, ON, Canada). The kit was used as per the manufacturer's instructions with the addition of kynurenine to the calibration standard at a concentration of 250 umol/L and to the software peak identification table to allow for quantitation as the only modification. Briefly, 10 μL of plasma was mixed with sulfosalicylic acid precipitation reagent containing norvaline internal standard, centrifuged, and the supernatant was derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. A total of 1 μL of derivatized sample (50 nL of plasma) were injected onto a 2.1 $\times$ 150 mm ultra performance liquid chromatography ethylene bridged hybrid C_{18} column with an acetonitrile gradient elution of a complete amino acid profile in less than 45 minutes. Kynurenine and tryptophan have retention times of 27.2 and 30.8 minutes, respectively.

Statistical Analysis

Data are presented as mean $\pm$ standard error. Most data were analyzed using either analysis of variance followed by the Tukey test, or a nonpaired t test as appropriate. Proteomic data were analyzed using principle component analysis. A P value of less than .05 was considered statistically significant.

Results

Assessment of Inflammation

T muris infection induced mild to moderate chronic colitis affecting mainly the cecum and the prox-

imal colon. Macroscopically, the cecum appeared edematous with mild erythema, but no ulcers or erosions were noted. MPO values in infected mice treated with placebo were significantly higher compared with uninfected controls (Figure 1). The chronic inflammatory infiltrate was increased in infected mice compared with controls. Treatment with etanercept or budesonide tended to decrease MPO values and the mononuclear cell infiltrate in *T muris*–infected mice, but this did not achieve statistical significance (Figure 1). Administration of *L rhamnosus* or *B longum* caused a small insignificant decrease in MPO and had no effect at all on the chronic mononuclear infiltrate in *T muris*–infected mice (Figure 1).

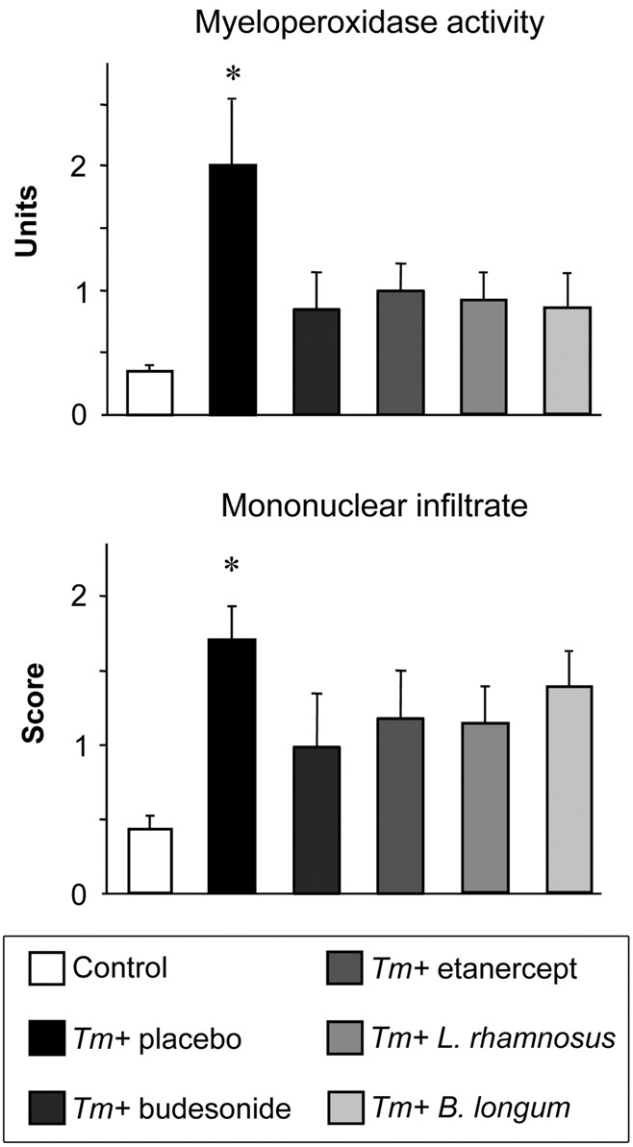

Figure 1. Inflammation in *T muris*–infected mice. MPO activity and the chronic mononuclear infiltrate in the proximal colon of uninfected control and *T muris* (*Tm*)–infected mice, treated with placebo, etanercept, budesonide, and probiotics. (*$P < .01$ vs control).

Behavioral Responses

T muris–infected mice spent significantly less time in the light box, and their latency to re-enter the light box was greater compared with controls (Figure 2*A*). *T muris*–infected mice also displayed a longer latency to step down from an elevated platform compared with control mice. There was no evidence of a general malaise effect of the infection, as reflected by their appearance, body weight, and locomotor activity of infected mice. Infected mice traveled the same distance with a similar average velocity as the control mice (Figure 2*B*). Treatment of *T muris*–infected mice with etanercept, but not with budesonide, normalized total time spent in the light box compared with placebo (Figure 2*A*). Latencies to re-enter the light box and to step-down from the elevated platform were normalized by both etanercept and budesonide. Total locomotor activity was not affected by either drug (Figure 2*B*). Budesonide and etanercept did not affect behavior in control mice, and the data were pooled with placebo-treated mice. Treatment of *T muris*–infected mice with *B longum*, but not with *L rhamnosus*, normalized total time spent in the light box and latency to step down from the elevated platform and decreased latency to re-enter the light box (Figure 2*A*).

BDNF mRNA in the Hippocampus

In situ hybridization revealed lower levels of BDNF mRNA in the hippocampus (CA1 region) in *T muris*–infected mice compared with controls (Figure 3). Treatment with budesonide or etanercept did not affect altered hippocampal BDNF expression in *T muris*–infected mice. In contrast, treatment with *B longum*, but not with *L rhamnosus*, normalized BDNF levels.

Circulating Cytokines

Levels of cytokines were low in general and in many samples were below the level of detection. TNF-α and IFN-γ were higher in *T muris*–infected mice compared with controls (Figure 4*A*). Etanercept, and to a lesser degree budesonide, decreased TNF-α levels in infected mice whereas *B longum* had no effect. Neither treatment affected IFN-γ levels. Levels of interleukin-12p70, monocyte chemotactic protein-1, interleukin-10, and interleukin-6 were not altered by infection or any treatment (data not shown).

Levels of Serum Kynurenine and Tryptophan

Kynurenine levels were higher in *T muris*–infected mice (Figure 4*B*). Treatment with etanercept, and to a lesser degree with budesonide, decreased kynurenine levels. *B longum* administration had no effect on kynurenine levels. Similarly, the kynurenine/tryptophan ratio was higher in *T muris*–infected mice and treatment with etanercept, and to lesser degree with budesonide, decreased this ratio (Figure 4*B*).

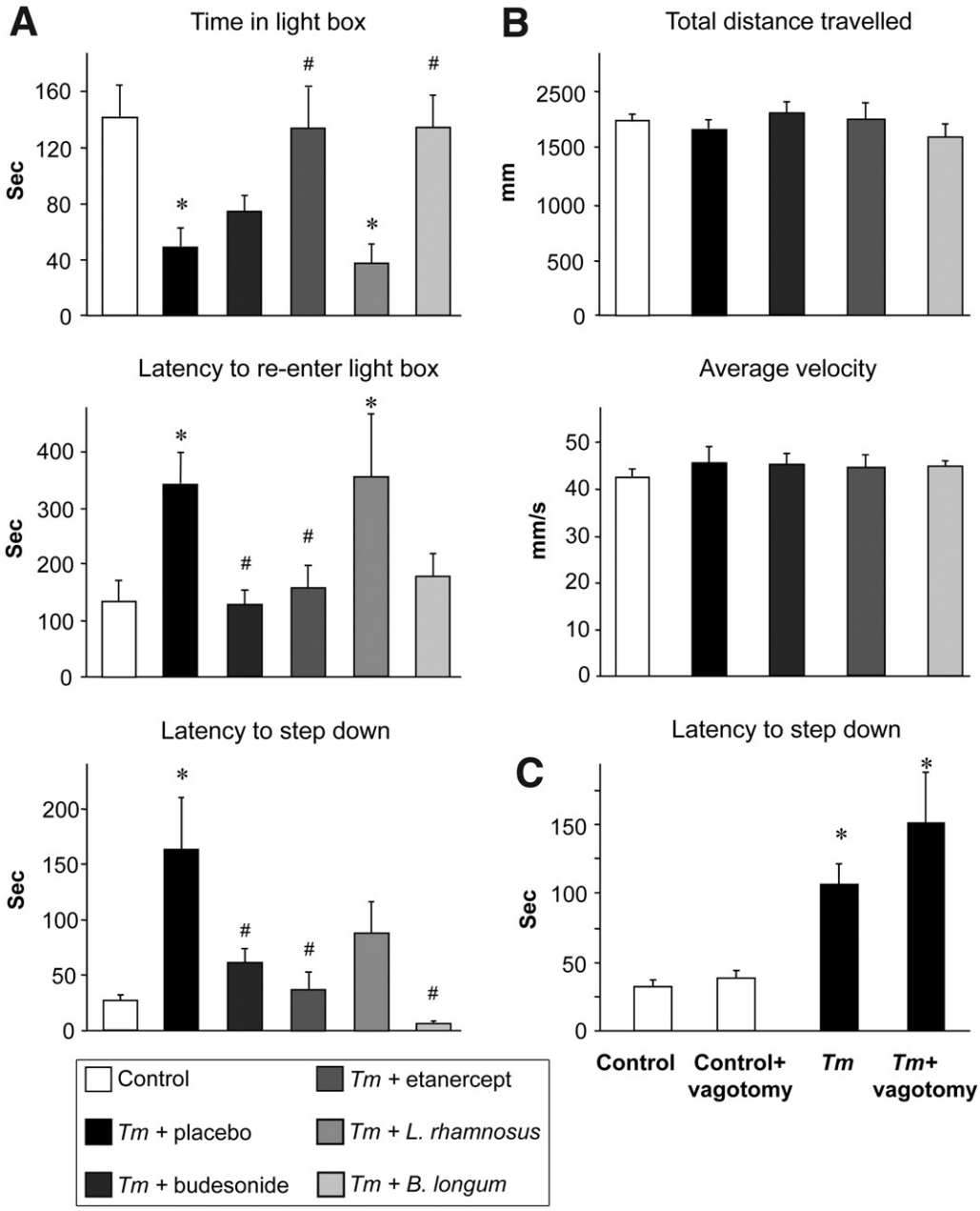

Figure 2. Behavior in *T muris*–infected mice. (*A*) Exploratory behavior in control and *T muris*–infected mice treated with placebo, etanercept, budesonide, and probiotics (*$P < .05$ vs control, #$P < .05$ vs *Tm* + placebo). (*B*) Overall locomotor activity in control and *T muris*–infected mice. (*C*) Effect of vagotomy on control and *T muris*–infected mice (*$P < .01$ vs control).

Effect of Vagotomy on Behavior

Vagotomy did not affect behavior in control mice. Vagotomized mice infected with *T muris* displayed similar anxiety-like behavior as infected mice without vagotomy, stepping down from the elevated platform with longer latency than control mice (Figure 2*C*).

Proteomic Analysis

Analysis of serum samples from control and *T muris*–infected mice revealed altered levels of several proteins. As expected, increased levels of proteins related to inflammation, such as serum amyloid protein and ceruloplasmin, were found in infected mice (Table 1). Other proteins, related to neural function, were decreased during chronic infection, namely serine protease inhibitor A3K, murinoglobulin, afamin, and epidermal growth factor receptor.

The serum protein profile normalized after etanercept and budesonide administration and the levels of inflammation-related and neural-function–related proteins were similar to controls (Figure 5).

Figure 3. BDNF mRNA expression in the hippocampus. (*A*) Representative photographs of BDNF mRNA expression in the hippocampus of different treatment groups. (*B*) Hippocampal BDNF levels in control and *T muris*–infected mice (*$P < .05$ vs control, $^{\#}P < .05$ vs *Tm* + placebo).

Discussion

We show that chronic infection associated with mild gut inflammation caused by the noninvasive parasite *T muris* induces anxiety-like behavior in mice. Chronic inflammation was modest in severity and was not associated with macroscopic tissue damage such as ulceration. We show that abnormal behavior in *T muris* colitis was associated with decreased levels of hippocampal BDNF. Circulating proinflammatory cytokines and kynurenine were mildly, but significantly, increased and proteomic analysis showed alteration of several inflammatory and neural function–related proteins. Behavioral abnormalities were normalized by anti-inflammatory/immunomodulatory treatment with soluble TNF-α–receptor etanercept or budesonide. This was associated with normalization of the serum proteomic profile and kynurenine levels but not of hippocampal BDNF. Altered behavior also was reversed by administration of the probiotic *B longum*, which, in contrast to budesonide and etanercept, normalized BDNF in the hippocampus but did not influence the immune response or kynurenine levels. None of the treatments altered behavior in noninfected control mice.

T muris–infected mice displayed a behavioral profile characterized by more anxious and timid behavior; mice spent less time in the illuminated compartment during the light/dark preference test and displayed a longer latency to re-appear from the dark compartment than healthy controls. *T muris*–infected mice also showed a longer latency to step down from the elevated platform than healthy controls. There was no evidence of a general malaise effect as reflected by changes in appearance or total locomotor activity.

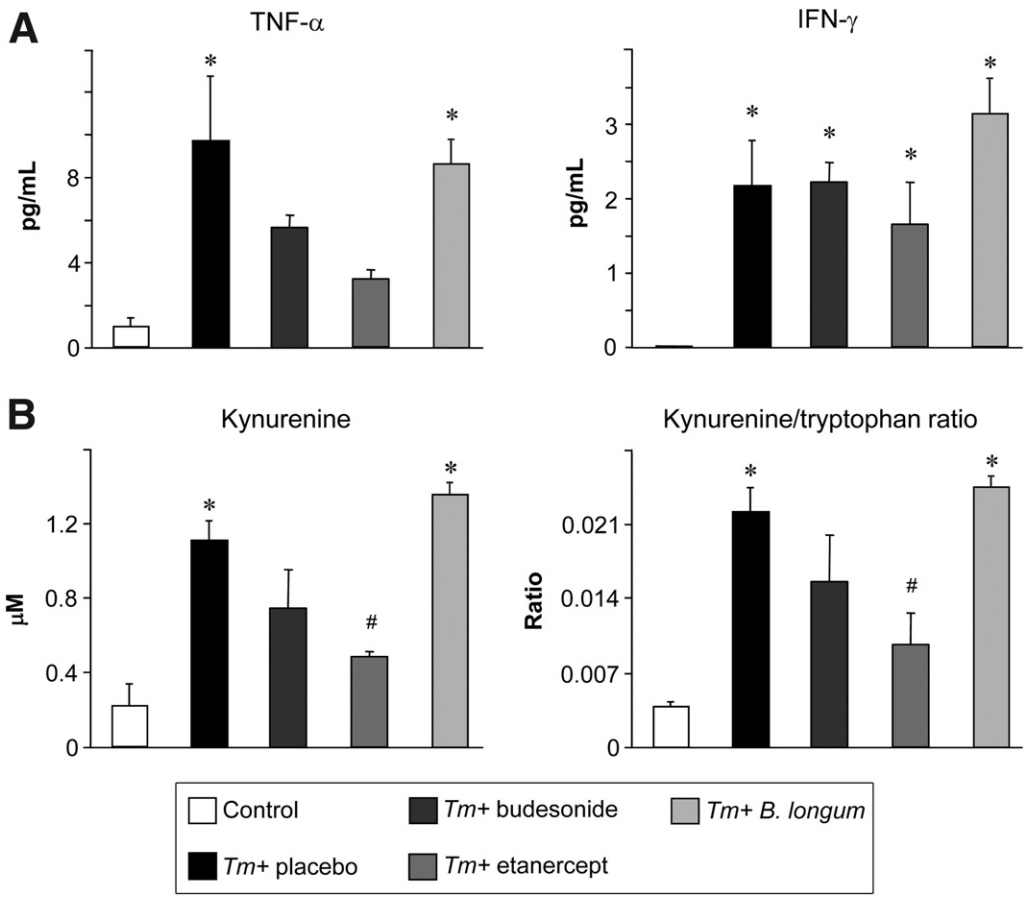

Figure 4. Circulating cytokines and kynurenine/tryptophan ratio in *T muris* infection. (*A*) Serum TNF-α and IFN-γ levels in *T muris*–infected mice and controls (*$P < .05$ vs control). (*B*) Serum kynurenine and kynurenine/tryptophan ratio in control and *T muris*–infected mice (*$P < .05$ vs control, #$P < .05$ vs *Tm* + placebo).

Previous studies on gastrointestinal infection and behavior have focused on the early phase of *C jejuni* infection and suggested that behavioral changes were independent of an inflammatory response[29] but were vagally mediated.[30] In our experiments, prior vagotomy did not alter the behavioral profile of *T muris*–infected mice, indicating that, in contrast to studies on acute infection, behavioral changes induced by chronic infection with *T muris* are not vagally mediated.

Even modest increases in proinflammatory cytokines such as TNF-α can influence central nervous system (CNS) function.[19] Cytokines have been shown to directly activate primary afferent and vagal nerves, but vagotomy did not affect *T muris*–altered behavior in this study. Cytokines also can access the brain via the circumventricular organs, where the brain/blood barrier is more permeable.[15] In this regard, we previously have shown that mice chronically infected with *H pylori* display abnormal feeding behavior together with up-regulated TNFα mRNA in the median eminence of the brain.[38] Another putative mechanism is the altered production or metabolism of neurotransmitters. Recent studies using mice treated intraperitoneally with lipopolysaccharide or systemically infected with *Mycobacterium bovis* have linked depressive-like behavior to cytokine-induced changes in tryptophan metabolism and production of kynurenine.[40,41] We have found significantly increased plasma levels of kynurenine, which normalized after treatment with etanercept and partially improved after budesonide administration, in parallel with improvement in behavior. Our data thus suggest that chronic mild gut inflammation, through production of circulating proinflammatory cytokines, leads to increased levels of kynurenine, which has been shown previously to alter mouse behavior in a dose-dependent fashion.[40]

To investigate alternative communication pathways between the inflamed gut and the brain, we performed a serum proteomic analysis. As expected, we found a significant up-regulation of inflammation-related proteins (ceruloplasmin, kininogen, serum amyloid protein, pregnancy zone protein, and hemopexin) whereas others, such as murinoglobulin, serine protease inhibitor 3, epidermal growth factor, and afamin, were decreased. Interestingly, several of these proteins have been linked to CNS function. Serine proteinase inhibitor 3 and murinoglobulin are potent inhibitors of neuropsin, a protease

Table 1. Altered Serum Proteins in *T muris*–Infected Mice

Protein identification	Known function	Change
Serum amyloid P-component	Acute phase reactant	+2.5
Complement component factor H	Component of the innate immune system	+3.1
Pregnancy zone protein	Involved in the immune system and pregnancy	+2.6
Ceruloplasmin, isoform CRA_c	Acute phase reactant	+2.4
Hemopexin	Heme scavenger	+3.3
Kininogen 1 isoform 3	Cofactor on coagulation, involved in inflammation	+4.1
Apolipoprotein H precursor	Involvement in agglutination, inhibits serotonin release by the platelets	+2.1
Clusterin	Clearance of cellular debris and apoptosis	+2.3
β 2-Glycoprotein I	Involvement in agglutination, inhibits serotonin release by the platelets	+2.7
Vinculin, 117 kD	Linkage of integrin adhesion molecules	−2.4
Predicted: similar to complement component 7	Likely role in inflammation	−2.1
Histidine-rich glycoprotein Hrg	Involved in fibrinolysis and coagulation	−3.0
Afamin	α-Tocopherol transport across the blood-brain barrier	−3.5
mCG9583, isoform CRA_a	Unknown	−2.3
Es 1 protein	Vesicle trafficking of proteins	−3.0
Carboxylesterase precursor	Enzyme for carboxylate production	−3.0
Serine protease inhibitor A3K	Inhibitor of neuropsin (involved in neuronal synaptic plasticity)	−5.9
EGF receptor	Involved in the maintenance of the nervous system	−4.3
mCG114322	Unknown	−2.1
Serine proteinase inhibitor	Inhibitor of neuropsin	−2.8
Serpina1c protein	Inhibition of proteases, involved in coagulation and inflammation	−2.6
Murinoglobulin 1	Plasma proteinase inhibitor, immune modulation, inhibitor of neuropsin	−3.4

NOTE. Altered serum proteins detected by 2D difference in gel electrophoresis were identified in the NCBInr protein database and their function was listed. Their increased or decreased levels were expressed as fold-change compared with uninfected mice.
EGF, epidermal growth factor.

implicated in synaptic plasticity,[42] whereas epidermal growth factor has been suggested to play an essential role in the maintenance of the neural system.[43] Of particular interest is afamin, a protein synthesized by cerebrovascular endothelial cells, responsible for α-tocopherol (vitamin E) transport across the blood-brain barrier.[44] Clinical and experimental studies have suggested an association between vitamin E and depression[45,46] and brain BDNF levels.[47,48] Furthermore, mice with impaired transport of α-tocopherol into the CNS display anxiety-like behavior.[49] In conjunction with these studies, our data suggest that in addition to affecting tryptophan metabolism, chronic peripheral inflammation alters the levels of multiple proteins, which may promote depression/anxiety-like behavior.

To investigate the basis for the induction of changes in brain and behavior, we used 3 interventions with different immunomodulatory capacity: the corticosteroid (budesonide), an anti–TNF-α agent (etanercept), and 2 probiotic strains. Although corticosteroids and biologics commonly are used in patients with IBD, probiotics appear to have only mild anti-inflammatory effects and may serve as an adjunctive treatment.[50] Budesonide acts mainly in the gut, gut-associated lymphoid structures and liver, whereas the effects of etanercept reflect the neutralization of TNF-α. Budesonide, because of its extensive first-pass metabolism in the liver, produces metabolites with minimal systemic activity whereas etanercept is a large molecule containing IgG1 and therefore unable to cross the blood-brain barrier. These compounds are thus unlikely to act directly on the CNS. Treatment with etanercept, and to a lesser degree with budesonide, normalized altered behavior in *T muris*–infected mice. Although inflammatory scores in the colon only partially improved, likely owing to the persistence of the parasite, serum kynurenine and proteomic profile normalized in *T muris*–infected mice treated with etanercept. Treatment with budesonide seemed to be less effective: although the proteomic profile normalized, the effect on the kynurenine was less pronounced. Improvement in behavior by etanercept and budesonide thus can be linked to down-regulation of systemic immune activation and changes in circulating kynurenine levels. It is relevant to point out that treatment with etanercept produced significant improvement in comorbid depression and fatigue in patients with psoriasis.[14] In contrast, the marked anxiolytic action of *B longum* on behavior appears largely independent of an immunomodulatory effect and kynurenine pathway because circulating cytokines and kynurenine remained increased. The determination of the precise mechanisms of action of *B longum* in the gut-brain axis is beyond the scope of the present study but may involve noninflammatory, neural, or metabolic pathways,[51] as suggested by complete normalization of hippocampal BDNF levels.

The neurotrophic hypothesis of depression suggests that its pathophysiology is caused by altered regulation of central neurotrophin signaling, mainly BDNF.[52] Lower hippocampal BDNF has been associated with anxiety and depressive behavior, with BDNF levels normalizing after antidepressant treatment.[53] We found that BDNF levels

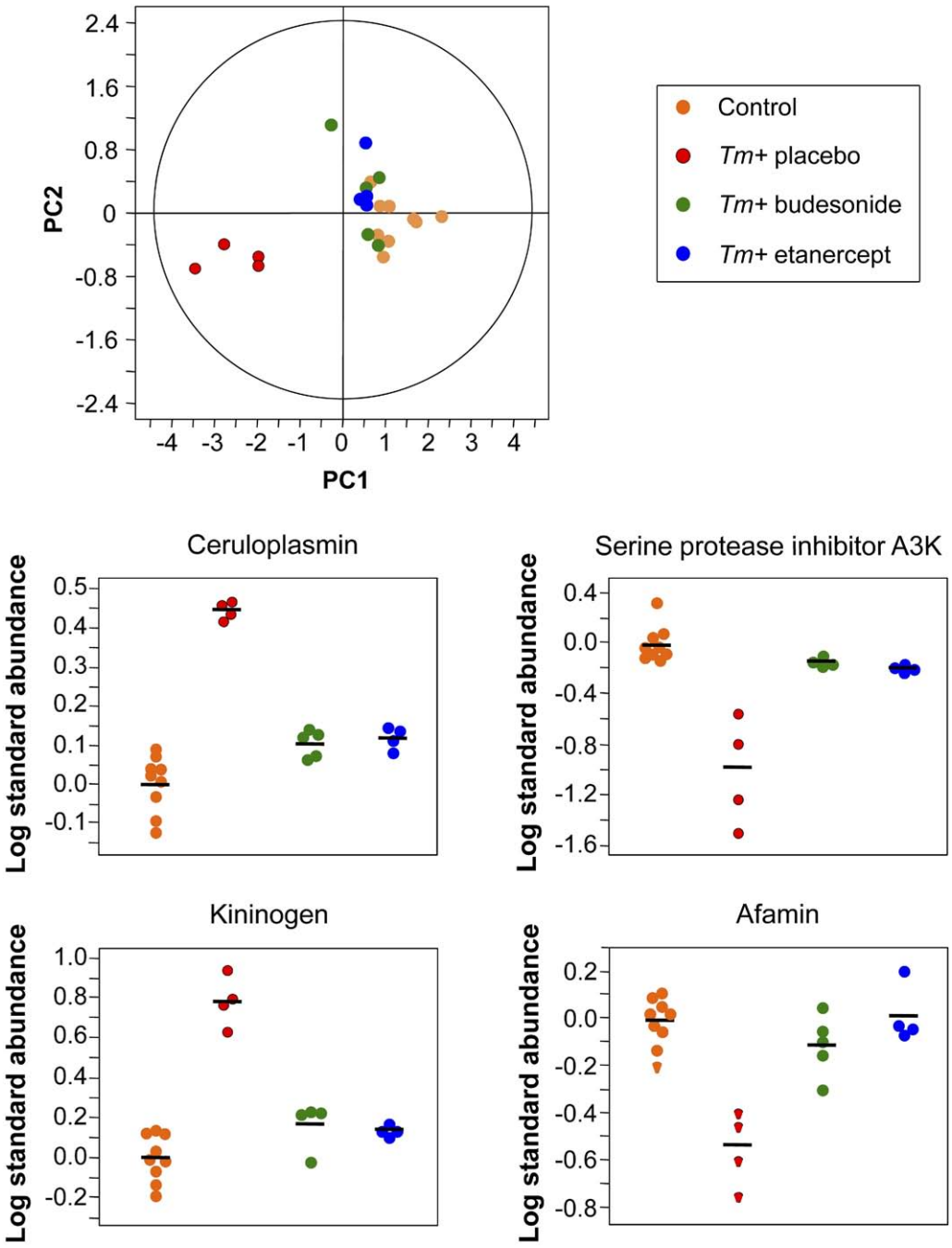

Figure 5. Effect of anti-inflammatory treatment on serum protein profiles. Serum protein profile assessed by principle component analysis in uninfected controls and *T muris*–infected mice. Levels of serum proteins, either those related to inflammation (ceruloplasmin, kininogen) or to neural function (serine protease inhibitor 3 and afamin) in control and *T muris*–infected mice.

in the hippocampus were decreased significantly in *T muris*–infected mice compared with uninfected controls. Interestingly, restoration of normal behavior with *B longum*, but not with etanercept or budesonide, was associated with normalization of hippocampal BDNF expression. The neural circuitry involved in depression and anxiety is complex and different antidepressive treatments have multiple modes of action. It needs to be acknowledged that although etanercept and budesonide did not improve hippocampal BDNF, they may have affected other CNS structures. A recent study has shown that desipramine and fluoxetine administration increased BDNF levels in the frontal cortex but not in the hippocampus, whereas chronic electroconvulsive shock treatment normalized BDNF levels in both areas.[54]

Our results indicate that during chronic gut inflammation, the mechanisms involved in signaling to the brain are complex, with proinflammatory cytokines, tryp-

tophan metabolism, and altered serum proteins playing critical roles. In addition, the ability of the probiotic *B longum* to normalize both behavioral changes and brain biochemistry suggest involvement of BDNF-dependent mechanisms through either metabolic or neural pathways. Because *B longum* did not show a marked anti-inflammatory effect compared with budesonide and etanercept, we conclude that *B longum* may override the influence of chronic inflammation on behavior via a separate, hitherto unidentified mechanism through the microbiota–gut–brain axis.[55] Although it has been speculated that probiotics may be helpful in behavioral disorders,[56,57] we demonstrate that a member of the intestinal microbiota may affect the brain biochemistry and behavior in adult mice.

Previous studies investigating the impact of intestinal inflammation on behavior focused either on the early phase of enteric infection[29,30] or on acute and severe colitis.[28] The focus on chronic inflammation in our study is more reminiscent of human IBD, and provides a clear demonstration of its causal role in producing anxiety/depression-like behavior, which may have bearing on the documented relationship between behavioral illness and the activity of IBD.[25–27] When taken together with our recent work showing that mice with experimentally induced depression/anxiety-like behavior were more susceptible to gut inflammation,[58,59] the current study provides support for a bidirectional relationship between behavior and gut inflammation; depression increases vulnerability to gut inflammation, which, in turn, induces depression/anxiety-like behavior. Thus, these results are relevant to our understanding of the psychiatric comorbidity that occurs in IBD as well as in functional gastrointestinal disorders in which there is an infective or inflammatory basis.

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.2010.06.063.

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Received August 31, 2009. Accepted June 24, 2010.

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Acknowledgments

The authors are grateful to Mrs Jeannette Pearce for the technical support.

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Conflicts of interest

These authors disclose the following: Drs Corthesy-Theulaz, Cherbut, and Bergonzelli are employees of Nestle, Switzerland. The remaining authors disclose no conflicts.

Funding

This work was supported by grants from Canadian Institutes of Health Research (S.M.C., P.B.), Nestle (S.M.C., E.F.V.), Crohn's & Colitis Foundation of Canada (P.B., S.M.C.), and Advanced Foods and Materials Network (S.M.C., P.B., E.F.V.). E. F. Verdu is partially supported by grants from Canadian Association of Gastroenterology/CIHR. P. Bercik and E. F. Verdu hold Internal Career Awards by the Department of Internal Medicine, McMaster University.

Supplementary Materials and Methods

In Situ Hybridization in the CNS

Expression of BDNF in the hippocampus was assessed by in situ hybridizations using 35S-labeled RNA probes on frozen brain sections as described previously.[28] Briefly, brains were removed and rapidly frozen by immersion in 2-methylbutane at −60°C, and stored at −70°C. Cryostat-cut 12-μm–thick coronal sections were thaw-mounted onto gelatin-coated slides, dried, and stored at −35°C. Tissue sections were fixed with 4% formaldehyde, acetylated with 0.25% acetic anhydride in 0.1 mol/L triethanolamine–HCl, pH 8.0, dehydrated, and delipidated with chloroform. Antisense BDNF ribonucleotide probe (a gift from Dr J. Lauterborn and Dr C. Gall, University of California, Irvine, CA) was transcribed from linearized plasmid using the Riboprobe System (Promega Biotech, Burlington, Ontario, Canada) with α-^{35}S–uridine triphosphate (specific activity, >1000 Ci/mmol; Perkin-Elmer, Boston, MA) and T3 and T7 polymerases, respectively. Radiolabeled probe was diluted in a hybridization buffer (0.6 mol/L NaCl, 10 mmol/L Tris pH 8.0, 1 mmol/L ethylenediaminetetraacetic acid pH 8.0, 10% dextran sulfate, 0.01% sheared salmon sperm DNA, 0.05% total yeast RNA, type XI, 0.01% yeast tRNA, 1X Denhardt's solution) and applied to brain sections (approximately 500,000 counts per minute/section). Slides were incubated overnight at 55°C in a humidified chamber. To reduce nonspecific binding of the probe, slides were washed in 20 μg/mL RNase solution for 30 minutes at room temperature, followed by 1 hour each in 2XSSC at 50°C, 0.2XSSC at 55°C and 60°C. Slides were dehydrated and air-dried for autoradiography. Slides and ^{14}C plastic standards were placed in radiograph cassettes, apposed to film (BioMax MR; Eastman Kodak, Rochester, NY) for 5 days, and developed (Kodak Medical X-Ray Processor). Autoradiographic film images of brain sections and standards were digitized with a solid-state camera with a 60-mm Nikon lens (Nikon Canada, Mississauga, ON, Canada) using QCapture software (Qicam; Quorum Technologies, Inc, Guelph, Ontario, Canada) and a computer-based image analysis system with Image software (available: http://rsb.info.nih.gov/nih-image). Light transmittance through the film was measured by outlining the structure on the monitor. The transmittance was converted to radioactivity levels using the Rodbard curve applied to the standards. Illustrations were made directly from the captured images.