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Bacterial infection causes stress-induced memory dysfunction in mice

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ABSTRACT

Background The brain—gut axis is a key regulator of normal intestinal physiology; for example, psychological stress is linked to altered gut barrier function, development of food allergies and changes in behaviour. Whether intestinal events, such as enteric bacterial infections and bacterial colonisation, exert a reciprocal effect on stress-associated behaviour is not well established.
Objective To determine the effects of either acute enteric infection or absence of gut microbiota on behaviour, including anxiety and non-spatial memory formation.
Methods Behaviour was assessed following infection with the non-invasive enteric pathogen, *Citrobacter rodentium* in both C57BL/6 mice and germ-free Swiss-Webster mice, in the presence or absence of acute water avoidance stress. Whether daily treatment with probiotics normalised behaviour was assessed, and potential mechanisms of action evaluated.
Results No behavioural abnormalities were observed, either at the height of infection (10 days) or following bacterial clearance (30 days), in *C rodentium*-infected C57BL/6 mice. When infected mice were exposed to acute stress, however, memory dysfunction was apparent after infection (10 days and 30 days). Memory dysfunction was prevented by daily treatment of infected mice with probiotics. Memory was impaired in germ-free mice, with or without exposure to stress, in contrast to conventionally reared, control Swiss-Webster mice with an intact intestinal microbiota.
Conclusions The intestinal microbiota influences the ability to form memory. Memory dysfunction occurs in infected mice exposed to acute stress, while in the germ-free setting memory is altered at baseline.

Significance of this study

What is already known about this subject?
▶ *Citrobacter rodentium* infection causes anxiety in mice at 8 h after infection.
▶ Diet-induced changes in the intestinal microflora alter memory in mice.
▶ Germ-free mice have an altered hypothalamic-pituitary-adrenal axis, resulting in an exaggerated stress response.
▶ Non-spatial memory defects are linked to expression of brain-derived neurotropic factor and c-Fos in the CA1 region of the hippocampus.

What are the new findings?
▶ Infection with *C rodentium* causes stress-induced memory dysfunction in mice at 10 and 30 days after infection.
▶ Probiotics administered before and during the infection prevent memory dysfunction.
▶ Germ-free mice lack memory even in the absence of stress.

How might they impact on clinical practice in the foreseeable future?
▶ Probiotics could provide benefit in relation to behavioural abnormalities in patients with irritable bowel syndrome.
▶ A role for the intestinal microflora in mediating hippocampal-dependent memory formation in mice is highlighted, which could translate to a similar phenomenon in humans.

INTRODUCTION

Psychological stress causes intestinal barrier dysfunction,[1] disrupted host—microbial interactions[2] and an enhanced risk of developing food allergy.[3] While the effect of the brain on the gut is increasingly characterised, there remains a relative paucity of data assessing whether the gut microbiota influences the brain; particularly with respect to changes in behaviour.

Infection with enteric bacterial pathogens causes acute mucosal inflammation[4] and is a risk factor for the development of chronic postinfectious irritable bowel syndrome (IBS).[5 6] However, whether these short- and long-term intestinal changes affect behaviour is not well defined. For example, patients with major depressive disorders have elevated levels of immunoglobulins A and M directed against bacterial lipopolysaccharide, suggesting an association between translocation of bacteria and effects

on the brain and behaviour.[7] In rodents, neonatal maternal separation is associated with hypothalamic-pituitary-adrenal (HPA) axis alterations, colonic dysfunction and altered intestinal microbiota in neonates, and stress-induced colonic dysfunction and behavioural changes later during adulthood.[8 9] Similarly, germ-free animals have an altered HPA axis, compared with gut colonised controls.[10]

Citrobacter rodentium is an attaching and effacing Gram-negative pathogenic bacterium that colonises the colon and causes a transient colitis in mice.[11 12] Multiple strains of adult mice, including C57BL/6 and Swiss Webster, are susceptible to *C rodentium*, which induces attaching and effacing lesions and colonic epithelial cell hyperplasia.[12 13] *C rodentium* infection is self-limited in immune-sufficient animals.[14] Infection with *C rodentium* causes community-wide responses in the host microbiota with increases in *Enterobacteriaceae*, owing to

colonisation by *C rodentium*, and reductions in *Lactobacillus* species.[15] Proinflammatory responses to *C rodentium* infection are well characterised,[14] with both interleukin 22[16] and adaptive immune cells[17] indispensable for clearance of the infection and animal survival. However, colonic mucosal inflammation is less severe than with other colitis models, such as dextran sodium sulphate (DSS) colitis—as reflected by moderate myeloperoxidase scores in *C rodentium* (twofold increase; 10^9 CFU (colony forming units) killed at 14 days after infection) compared with DSS (40-fold increase compared with controls; 3% DSS killed at 10 days following initiation of the chemical challenge).[18]

Probiotics are live micro-organisms, which confer beneficial effects on the host when provided in adequate amounts and administered chronically, owing to their transient colonisation of the gut.[19] Provided as single strains or in combinations, probiotics can be used for a variety of ailments, including diarrhoea, IBS, inflammatory bowel disease and allergy.[20] Probiotics ameliorate early-life trauma-induced colonic barrier dysfunction in neonatal rats,[21] and prevent stress-induced bacterial translocation[22] and colorectal hypersensitivity[23] in adult rats. In *C rodentium*-infected mice, probiotics prevent colonic epithelial cell hyperplasia, reduce colonic colonisation of *C rodentium*,[24][25] maintain epithelial barrier integrity[25] and protect neonates from death.[26] Probiotics also change the composition of the colonic microbiota in mice with DSS-induced colitis.[27] The role of modifications in the intestinal microbiota, such as infection and probiotics, in mediating stress-induced changes in behaviour, however, has not been characterised.

The goals of this study, therefore, were to determine whether *C rodentium* infection resulted in changes in anxiety-like behaviour or hippocampal-dependent memory in adult C57BL/6 mice and whether administration of probiotics would provide protection. Germ-free mice were used to study the absence of a microbiota and behaviour.

METHODS
Animals
Female SPF C57BL/6 mice (5–6 weeks; Charles River Laboratories, St-Constant, Quebec, Canada), female germ-free Swiss-Webster mice (5–6 weeks; Taconic Farms, Germantown, New York, USA) and age-matched, female SPF Swiss-Webster controls (Charles River) were used. As in our previous studies, female mice were tested, owing to the higher frequency of IBS in female than male mice.[24] Mice were housed in cages lined with chip bedding on a 12 h light/dark cycle (lights on at 08:00) with free access to food and water. Animals were kept in the containment unit of the animal facility and all experiments were performed in a biosafety cabinet. All procedures and protocols were reviewed and approved by the Hospital for Sick Children's Animal Care Committee.

Water avoidance stress (WAS)
Mice were placed onto a small platform surrounded by shallow, room temperature water in a covered mouse cage for 1 h.[2][8][28] Mice avoided the water, an aversive stimulus, by remaining on the platform for the duration of the procedure. WAS is a well-established model of psychological stress, showing altered colonic physiology after only 1 h of exposure.[2][29]

Behavioural testing
Novel object test
Various strains of mice, including C57BL/6 and Swiss Webster exhibit similar characteristics for spatial working and reference memory.[30] The novel object test represents a test for dorsal hippocampal function based on the tendency of mice to investigate a novel object, rather then a familiar one.[31] To assess memory, a modified version of a novel object test[32] was used. After habituation or WAS, mice were exposed to two objects.[33][34] Exploratory behaviour was recorded by video camera (Sony, Toronto, Ontario, Canada) and analysed for frequency to smell each object. Objects used included: small smooth napkin rings (#1), large checkered napkin rings (#2) and a star-shaped cookie cutter (#3). To avoid object bias, preliminary studies tested objects for equal preference. Behavioural assessment consisted of distinct training and testing phases.

Training (or familiarisation) phase
Objects #1 and #2 were placed in opposite corners of the cage. Behaviour was videotaped for 5 min, after which the objects were removed. Mice were allowed a 20 min rest period before testing.

Testing phase
After resting, mice were re-exposed to object #2, which was re-introduced into the cage (referred to now as object #2B) along with a completely new object (object #3, distinguishable from object #1).[33][34]

Memory was assessed as the frequency to explore object #2 during the testing phase (object #2B), compared with the new object #3.[34] An exploration ratio was calculated (exploration ratio = freq. smell #3/(freq. smell #3 + freq. smell #2B)× 100).[35] This ratio represents the proportion of smelling bouts associated with the new object versus the old object. A ratio of 50% represents no discrimination between the two objects and impaired preference.[35] Exploration of objects was defined as orientation towards the object with the nose of the mouse pointed towards the object within 1–2 cm.[33] The training phase showed no preference between either object #1 or #2.

T-maze
The T-maze test uses the natural tendency of mice to explore a novel environment, rather than choose a familiar one. Spontaneous alternation or exploration detects hippocampal dysfunction, and represents a model of working memory.[36] Mice are placed at the base start arm of the 'T'-shaped maze, which contains a central partition into the start arm, and two goal arms. The mouse is allowed to proceed into the maze and choose either the left or right goal arm. After a short exploration period, the mouse is removed and replaced at the start arm for a second trial.[36] Generally, six trials were performed, allowing the mouse to explore the maze without loss of interest. Memory was assessed as a ratio of left versus right turns, with equal amounts constituting 100% spontaneous exploration rating and selection of a single arm in all trials considered as a 0% exploration ratio.

Light/dark box
Anxiety was assessed using light preference, as measured by a light/dark box.[37] This test is based on the aversion of mice to a bright area when presented with the option to remain in a dark enclosure. Coupled with the inherent tendency to explore novel environments, the light/dark box measures anxiety as an increased tendency to remain in the dark box, rather than explore. Mice were placed in the larger light compartment (2:1) and behaviour recorded for 10 min. Measurements of total time spent in the light versus dark compartments and the number of transitions from one compartment to the other were taken. Although exploratory behaviour varies between strains, mice on average spend approximately 60% of the time in the dark

chamber.[37] Increased transitions coupled with reduced time spent in the light box are associated with anxiety.[37]

Study design

C57BL/6 mice were infected with *C rodentium* or sham-infected (LB broth—controls) after 1 week acclimatisation in the animal facility (figure 1). Bacteria (10^8 CFU in LB broth) were administered by oral gavage.[24] Mice were tested for behaviour on 10 days or 30 days after infection. For the novel object test, mice were placed individually in clean cages and allowed to habituate to this environment for 1 h.[8] Transfer of rodents to a new cage results in an increase in serum corticosterone within 15 min and normalisation by 45 min.[38] In a subset of experiments, mice were then subjected to WAS. Immediately following either habituation or WAS, animals were administered the novel object test.[32–34] For the T-maze, mice were tested directly from their home cage (or following WAS) and the test duration was a maximum of 5 min/mouse. After a rest period, mice were subsequently tested for anxiety in the light/dark box (T-maze alternation can be interleaved before subsequent tests).[36] No habituation was performed for these two tests, because the novelty of the maze and box drives exploratory behaviour. Behaviour was scored by two independent observers. Within 20 min of behavioural testing, mice were killed without anaesthetic by cervical dislocation.

A subset of mice was treated with probiotics or placebo daily via their drinking water following the acclimatisation period.[24 39] Placebo-treated mice received carrier alone, containing maltodextrin, without viable bacteria. One week after starting treatment, mice were infected with *C rodentium*. Treatment with either probiotics or placebo was continued for the remainder of the protocol, until sacrifice.

Germ-free mice, used to study the impact of the microbiota on memory and stress, were maintained in their sterile micro-isolators for 72—96 h before use (preliminary studies confirmed that stress from shipping was normalised by this time). Sterility was confirmed by plating of caecal contents onto sheep blood agar plates at sacrifice (samples collected aseptically; incubated aerobically for 24 h at 37°C). Habituation and behavioural testing were performed, as described above, and mice then killed and samples collected immediately after testing. Colonised Swiss-Webster mice purchased from Taconic behaved similarly to those obtained from Charles River (data not shown).

Bacteria

C rodentium, strain DBS100, was provided by the late Dr David Schauer (Massachusetts Institute of Technology).[40] Bacteria were grown under aerobic conditions on Luria-Bertani (LB) plates overnight at 37°C followed by overnight culture in 10 ml LB broth at 37°C. Colonisation was confirmed at 10 days after infection by swabbing fecal samples onto MacConkey agar. Plates were scored (0—3) depending on the thickness of the resulting colonies.

A commercially available probiotic combination was employed in this study: *L rhamnosus* (R0011) + *L helveticus* (R0052) (Lacidofil) (Institut Rosell-Lallemand, Montreal, Quebec, Canada).[24] This combination has been shown previously to ameliorate *C rodentium*-induced colitis in mice[24 26] and stress-induced colonic[21 22] and brain[39] dysfunction in rats. Freeze-dried preparations were rehydrated in sterile water and used as drinking water (changed every 2 days) at a concentration of 10^9 CFU/ml (final dose: 6×10^9 CFU/mouse/day based on consumption of ~6 ml per mouse per day).[24] This approach, rather than orogastric gavage, reduces stress in the animals and provides probiotics to the mouse throughout the day.[41]

Corticosterone

HPA axis activation was assessed by measuring levels of serum corticosterone.[42] Blood was collected by cardiac puncture at sacrifice, immediately placed on ice, centrifuged and serum isolated and frozen at −20°C, until further analysis. A commercial enzyme-immunoassay kit (Assay Designs, Ann Arbour, Michigan, USA) was used to measure levels of corticosterone, and samples were read using a fluorescent plate reader (Perkin Elmer, Toronto, Ontario, Canada). Results are presented as ng/ml.

Histology

Sections of distal colon were collected and fixed in 10% neutral-buffered formalin. Tissues were embedded in paraffin, 5 μm sections cut and placed onto glass slides. Slides were stained for histology with haematoxylin and eosin. Colonic epithelial cell hyperplasia was assessed by measuring crypt depth on coded slides under light-field microscopy (Leica NEWDM 4500BR microscope, Willowdale, Ontario, Canada) with commercial software (Leica Application Suite, Leica). Measurement of 20-well-oriented crypts for each colonic sample was performed, with results presented in micrometres.[24]

Immunohistochemistry

Sections of formalin-fixed, paraffin embedded brain tissue were cut, and stained for brain-derived neurotropic factor (BDNF) or c-Fos. Briefly, sections were baked overnight at 60°C, deparaffinised, subjected to heat-induced epitope retrieval and blocked for

Figure 1 Flow diagram of experimental design employed in these studies. (A) Mice were infected with *C rodentium* in the absence or presence of probiotics provided for one week before and after orogastric challenge with either bacteria or LB broth. Behaviour was then studied at 10 days after infection in the absence or presence of water avoidance stress (WAS). Behavioural testing consisted of the novel object test [32] (B) and the T-maze test[36] coupled with light/dark box testing[37] (C).
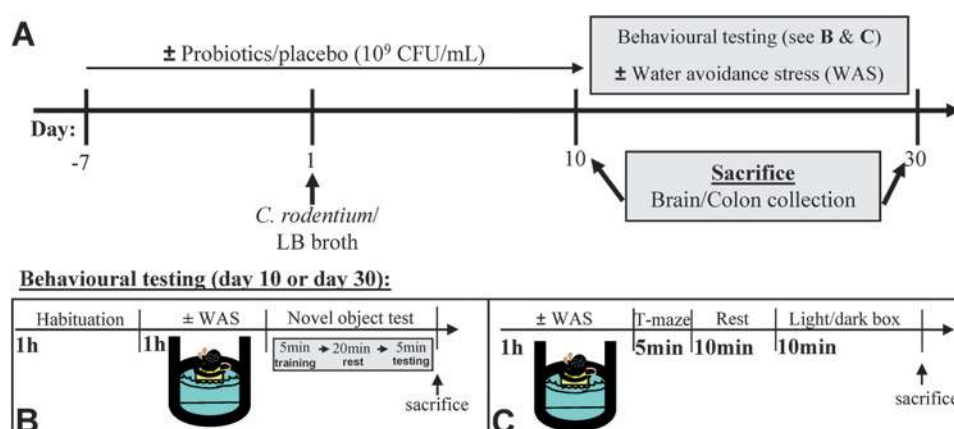
endogenous peroxidase and biotin. Incubation of polyclonal sheep anti-BDNF (Affinity Bioreagents, Colorado, USA; 1:100) or polyclonal rabbit anti-c-Fos (Abcam, Cambridge, Massachusetts, USA; 1:300) was performed at room temperature for 1 h. Subsequent immuno-detection was performed using the Vector species-specific biotinylated anti-sheep/anti-rabbit Ig, and the Vector Elite ABC detection system (Vector Laboratories, Burlington, Ontario, Canada), as recommended by the manufacturer. Colour visualisation was performed using 3-3'- diaminobenzidine (Sigma, St Louis, Maryland, USA) or NovaRed (Vector Labs) as the chromagen substrate and counterstained with haematoxylin. Quantification of BDNF was performed by calculating the intensity of BDNF staining in the CA1 region of the hippocampus relative to the background (ImageJ, National Institutes of Health, USA) and calculating the intensity relative to control (Sham; set to 100%).[43] Quantification of c-Fos was performed by counting cells ($400 \times 50\ \mu m^2$ area) in the CA1 region. A ratio was calculated and results presented as levels of expression (positive/total cells) and normalised to controls. Quantification was performed by a blinded observer.

qPCR

Colonic tissues were collected at sacrifice, frozen at $-80^\circ C$ and homogenised in Trizol (Invitrogen). RNA was isolated according to the manufacturer's instructions (Invitrogen), treated with DNase 1 (Invitrogen) and transcribed into cDNA (BioRad, Mississauga, Ontario, Canada). qPCR for tumour necrosis factor α (TNFα), interferon γ (IFNγ) and β-actin was performed using SYBR green and CFX96 C1000 Thermal Cycler (BioRad), with values presented as $\Delta\Delta CT$.

Intracolonic fecal samples were collected at sacrifice and frozen at $-20^\circ C$. Bacterial DNA was extracted using a stool kit (Qiagen, Mississauga, Ontario, Canada), following the manufacturer's instructions. DNA was amplified by qPCR using SYBR and primer sets (table 1) designed for 16S rRNA of bacterial species,[44 45] including Eubacteria (all bacteria; housekeeping gene), *Bacillus*, *Bacteroides*, *Enterobacteriaceae*, *Firmicutes*, *Lactobacillus*/*Lactococcus*, segmented filamentous bacteria, *Salmonella enterica* (serovar Typhimurium), *Helicobacter hepaticus* and *Eubacterium rectale* to compare overall colonisation patterns between treatment groups. Primers were validated previously to exclude cross reactivity.[44 45] Results are presented as percentage expression of each species relative to total bacteria.

Statistics

Results are expressed as means±standard error (SE). Groups were compared using the Student t-test, analysis of variance (one- or two-way, where applicable), or Mann–Whitney test (non-parametric). Post hoc analyses (Bonferroni or Student–Newman–Keuls) were performed, as indicated. Analyses were performed using InStat3 and Prism4 (GraphPad, San Diego, California, USA). Differences of $p<0.05$ were considered as significant.

RESULTS

C rodentium infection causes a mild injury in mice

As in our previous studies,[24] changes in body weight and colonic inflammation following *C rodentium* infection were minimal (online supplementary figure 1A,B). Furthermore, treatment with probiotics did not affect fecal colonisation levels of *C rodentium* (supplementary figure 1C).

C rodentium infection is not associated with anxiety-like behaviour

C57BL/6 mice infected with *C rodentium* did not demonstrate anxiety-like behaviour compared with controls, as shown by a similar frequency of transitions from the dark to light compartment (figure 2A) and equal time spent in the light zone (figure 2B) between groups during light/dark box testing.[37] Exposure to WAS did not result in a measurable change in anxiety-like behaviour in either sham or *C rodentium*-infected mice (figure 2A,B).

C rodentium infection causes WAS-induced memory impairment, which is maintained at 30 days after infection

Normal learning and memory was apparent in control mice and *C rodentium*-infected mice, as demonstrated by high exploration ratios in both groups (figure 3A). Exposure to WAS decreased non-spatial memory, indicated by exploration ratios of approximately 50%, in *C rodentium*-infected mice at 10 days after infection, but not unstressed, infected controls (figure 3A). A significant *C rodentium*–stress interaction was observed ($p<0.05$; two-way analysis of variance, with Bonferroni correction).

At 30 days after infection, after resolution of the bacterial infection,[46] altered memory was not observed, with recognition of the new object independent of infection status. However, after exposure to 1 h WAS, absence of non-spatial memory was

Table 1 Primer sequences employed in analysis of the fecal microbiome[44 45]

Target	Forward (5'–3')	Reverse (5'–3')
Mouse		
β-Actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
TNFα	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
IFNγ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
Bacteria		
Eubacteria	ACTCCTACGGGAGGCAGCAGT	ATTACCGCGGCTGCTGGC
Bacillus	GCGGCGTGCCTAATACATGC	CTTCATCACTCACGCGGCGT
Bacteroides	GAGAGGAAGGTCCCCCAC	CGCTACTTGGCTGGTTCAG
Enterobacteriaceae	GTGCCAGCMGCCGCGGTAA	GCCTCAAGGGCACAACCTCCAAG
Firmicutes	GCTGCTAATACCGCATGATATGTC	CAGACGCGAGTCCATCTCAGA
Lactobacillus/Lactococcus	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG
Segmented filamentous bacteria	GACGCTGAGGCATGAGAGCAT	GACGGCACGGATTGTTATTCA
H hepaticus	GCATTTGAAACTGTTACTCTG	CTGTTTTCAAGCTCCCCGAAG
Eubacterium rectale/Clostridium coccoides	ACTCCTACGGGAGGCAGC	GCTTCTTAGTCAGGTACCGTCAT
Salmonella enterica serovar Typhimurium	TGTTGTGGTTAATAACCGCA	GACTACCAGGGTATCTAATCC

IFNγ, interferon γ; TNFα, tumour necrosis factor α.

Figure 2 Anxiety-like behaviour is not induced in *C rodentium*-infected mice at both 10 and 30 days after infection. A light/dark box was used to measure anxiety.[37] The frequency of transitions from the dark to the light box (A) and time spent in the light box (B) were measured in *C rodentium*-infected mice, compared with controls, both in the absence or presence of water avoidance stress. White bar histograms denote sham-infected mice and the black bars *C rodentium*-infected animals. There were no statistical differences between groups of infected and sham-infected animals. N=6 mice/group.
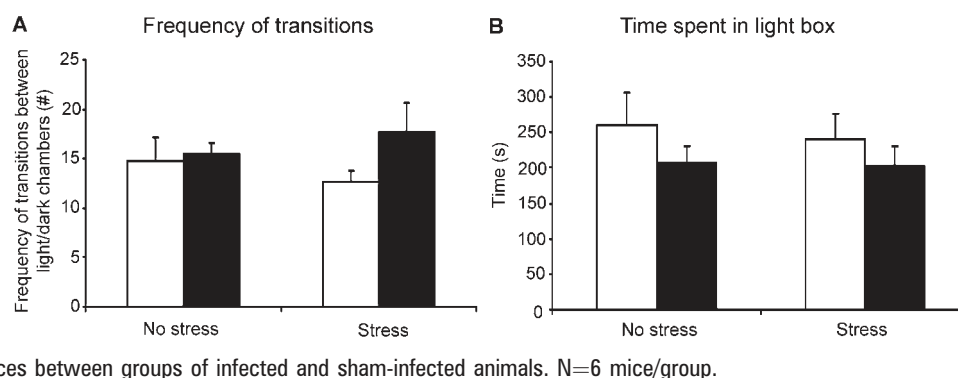
still observed in *C rodentium*-infected mice (figure 3B), with a reduced exploratory ratio, not evident in uninfected mice (*p<0.05; unpaired t-test compared with unstressed, *C rodentium*-infected mice).

To confirm memory dysfunction, the T-maze test for working memory was employed. Exposure to WAS resulted in a decrease in spontaneous exploration in *C rodentium*-infected mice, compared with the unstressed infected group (figure 4A). By contrast, no change in memory was observed in sham-infected controls exposed to WAS.
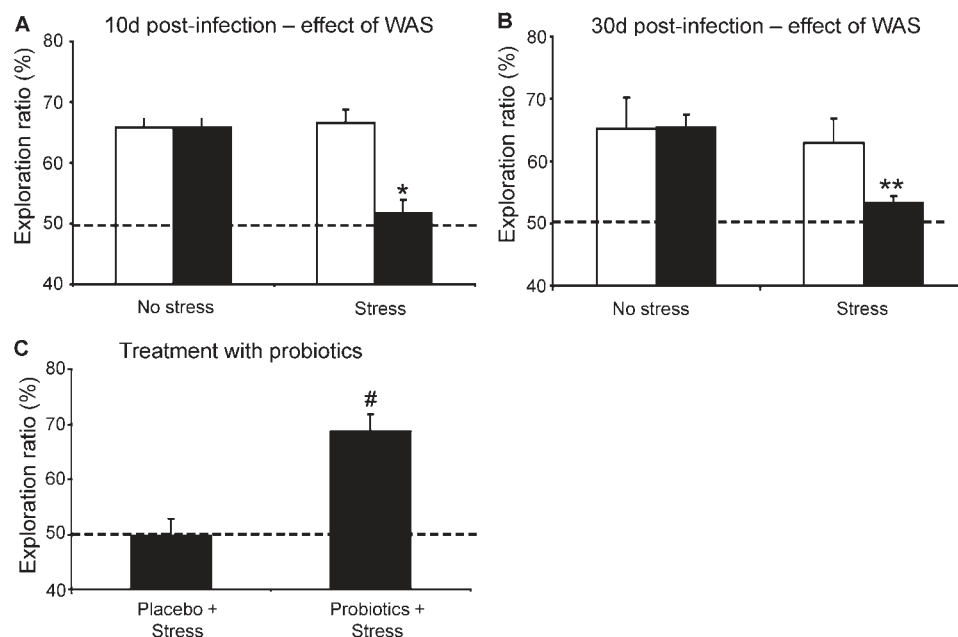
Hippocampal-dependent memory is restored by treatment with probiotics

Daily treatment with probiotics prevented stress-induced memory deficits in *C rodentium*-infected mice, compared with the placebo group (figure 3C and 4B). Mice treated with probiotics had normal ability to discriminate the novel object, as indicated by a high exploration ratio. The effect of probiotics on memory was confirmed by the T-maze, which showed increased exploration ratios in infected mice treated with probiotics, compared with placebo (figure 4B).

Stress-induced increases in serum corticosterone are ameliorated by treatment with probiotics

Exposure to WAS caused a significant increase in serum corticosterone levels, regardless of infection with *C rodentium*.

Treatment with probiotics significantly ameliorated WAS-induced elevations in serum corticosterone levels, compared with placebo (figure 5A).

Colonic epithelial cell hyperplasia is ameliorated by treatment with probiotics

Infection with *C rodentium* caused a significant increase in colonic epithelial crypt depth compared with control mice (358±47 vs 192±5 μm; *p<0.05). This effect was prevented only in the probiotics-treated group (238±4 μm; #p<0.05; compared with placebo), in contrast to the placebo-treated group (316±3 μm) (figure 5B).

Colonic proinflammatory cytokine levels are ameliorated by treatment with probiotics, irrespective of exposure to WAS

C rodentium infection resulted in an increase in colonic IFNγ (figure 5C) and TNFα (figure 5D) mRNA expression, compared with control mice. Treatment with probiotics ameliorated IFNγ, but not TNFα levels. Exposure to 1 h WAS did not influence cytokine mRNA expression.

C rodentium infection changes the microbiota, which is normalised by probiotics

Using 16S rRNA qPCR to quantify expression of bacterial species, no differences were observed in the fecal microbiota of mice exposed to WAS versus non-stressed animals; therefore, the

Figure 3 Exposure to water avoidance stress (WAS) alters non-spatial memory in *C rodentium*-infected mice at 10 days after infection, which is prevented by pretreatment with probiotics. The effect of exposure to 1 h of WAS on non-spatial hippocampal-dependent memory at 10 days (A) and at 30 days (B) after *C rodentium* infection was compared with unstressed, infected mice using the novel object test. Pretreatment with probiotics and placebo (C) were also used to study non-spatial memory in mice infected with *C rodentium* and then challenged with WAS. White bar histograms denote sham-infected mice and the black bars *C rodentium*-infected animals. Dashed line indicates a 50% ratio, indicative of an absence of memory.[35] *p<0.05, two-way analysis of variance with Bonferroni post-test correction; **p<0.05, Student t-test compared with *C rodentium*-infected, unstressed mice; #p<0.05, Student's t-test; N=10—14 mice/group.

Gut microbiota

Figure 4 Exposure to water avoidance stress (WAS) prevents working memory in *C rodentium*-infected mice that is normalised by treatment with probiotics. The effect of exposure to 1 h of WAS on working hippocampal-dependent memory at 10 days after infection was compared with unstressed, *C rodentium*-infected mice (A). Pretreatment with probiotics and placebo (B) were also used to study working memory in mice infected with *C rodentium* and then exposed to acute WAS. (A) White bar histograms denote sham-infected mice and the black bars *C rodentium*-infected animals. (B) White bar histograms denote placebo provided to *C rodentium*-infected animals and the black bars probiotics given to *C rodentium*-infected mice. *p<0.05, Mann—Whitney test; #p<0.05 Student's t-test; N=8—12 mice/group.
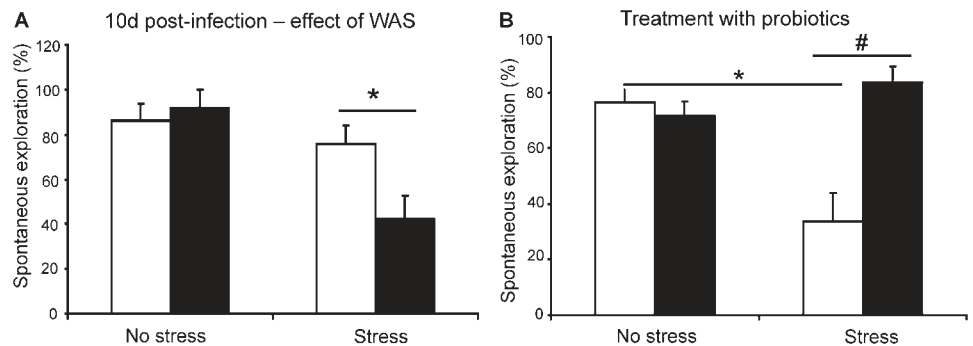
data were combined for each treatment group. *C rodentium* infection resulted in an increase in *Firmicutes* (figure 6A), *Enterobacteriaceae* (figure 6B) and *Eubacteria rectale* (figure 6C), which were all ameliorated after treatment with probiotics. In contrast, elevations in *Bacteroides* (figure 6D) were not normalised by probiotics. Although *Lactobacillus* levels (figure 6E) were not reduced after *C rodentium* infection, levels were increased by probiotics. Levels of *Bacillus* (figure 6F) were unchanged and expression of segmented filamentous bacteria, *Salmonella enterica* (serovar Typhimurium) and *Helicobacter hepaticus* were all below detection limits.

Hippocampal BDNF and c-Fos expression is reduced by *C rodentium* infection and exposure to WAS, with amelioration after probiotic treatment

BDNF expression levels at 10 days after infection following exposure to WAS were decreased in the CA1 region of the

hippocampus in *C rodentium*-infected mice, compared with controls (figure 7A). Such a decrease did not occur in mice pretreated with probiotics. Hippocampal c-Fos expression was decreased in the CA1 region of *C rodentium*-infected, WAS exposed mice, compared with controls (figure 7B). Similar to BDNF levels, exposure to probiotics prevented the drop in c-Fos expression. Both c-Fos and BDNF levels were unaffected by infection with *C rodentium* in the absence of WAS (online supplementary figure 2). WAS alone did not cause a change in the relative intensity of BDNF expression in the hippocampus (29.1 ± 3.1 mean intensity/100 μm^2 in controls versus 34.8 ± 1.1 control+WAS; p=NS; n=4—6).

Germ-free mice are not anxious, but lack memory

To further assess the role of microbes in mediating memory, behaviour was studied in the absence of a gut microbiome. Using the light/dark box test, no indication of anxiety was

Figure 5 Treatment with probiotics ameliorates serum corticosterone levels, colonic epithelial cell hyperplasia, and colonic interferon γ (IFNγ) mRNA. Activation of the hypothalamic-pituitary-adrenal axis was assessed by measuring levels of corticosterone in serum of both *C rodentium*-challenged mice at 10 days after infection and in sham-infected animals subject to 1 h water avoidance stress (WAS) (A). Pretreatment with probiotics and placebo was also compared. *p<0.05 compared with control; #p<0.05 compared with placebo; one-way analysis of variance with Newman—Keuls post-test; N=8 mice/group. Colonic epithelial crypt cell hyperplasia was measured at 10 days after infection with *C rodentium* compared with sham-infected animals and animals pretreated with probiotics or placebo (B). *p<0.05 compared with control; #p<0.05 compared with placebo; one-way analysis of variance with Newman—Keuls post-test; N=8 mice/group. Levels of IFNγ (C) and tumour necrosis factor α (TNFα) (D) mRNA were measured following infection with *C rodentium* (with and without probiotic/placebo) and compared with exposure to WAS (hatched bars). *p<0.05, Student t-test; N=5 mice/group.
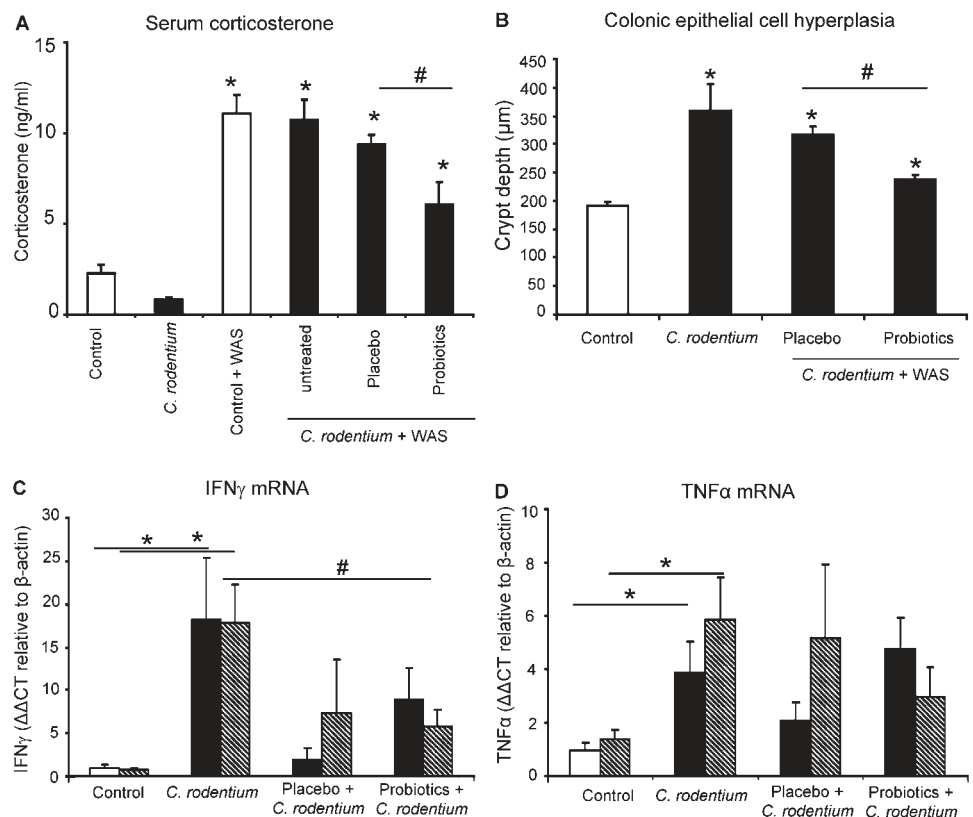
Figure 6 Infection with *C rodentium* changes the fecal microbiota, which is normalised by treatment with probiotics. Using 16S rRNA qPCR, quantification of expression of bacterial species was determined. *Firmicutes* (A), *Enterobacteriaceae* (B), *Eubacteria rectale* (C), *Bacteroides* (D), *Lactobacillus* (E) and *Bacillus* levels (F) were assessed following *C rodentium* infection, and treatment with probiotics. *p<0.05 compared with control; #p<0.05 compared with *C rodentium*, one-way analysis of variance with Bonferroni post hoc testing; N=8–10 mice/group.
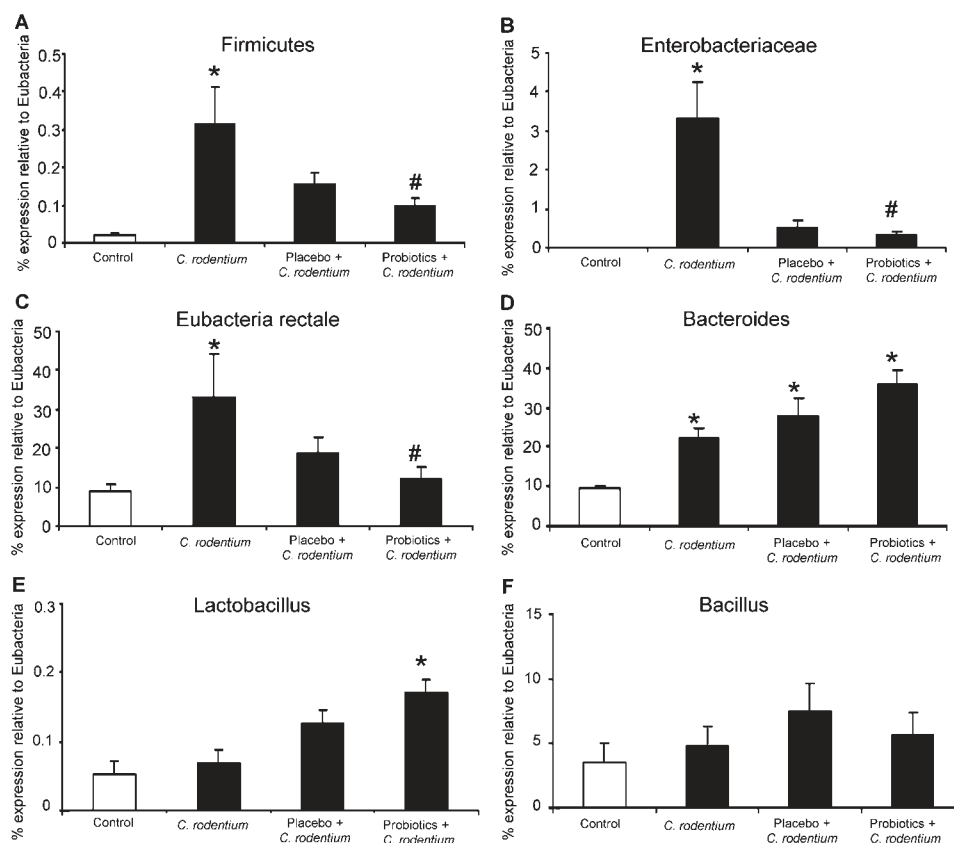
observed in germ-free mice compared with colonised controls, with stress having no impact on levels of anxiety (figure 8A,B). In contrast, germ-free mice displayed no indication of non-spatial or working memory in response to exposure to either the novel object or T-maze, with low exploration ratios and low spontaneous exploration in either the presence or absence of stress (figure 8C,D). This finding was in contrast to gut colonised Swiss-Webster mice, which demonstrate behaviour comparable to SPF C57BL/6 mice, and high exploration ratios and high spontaneous exploration (figure 8C,D).

BDNF immunohistochemistry showed a decrease in CA1 hippocampal expression in germ-free mice, compared with gut colonised controls after exposure to WAS (figure 9A). Similarly, a decrease in c-Fos positive cells in the hippocampus was observed in germ-free mice, compared with controls, after WAS (figure 9B).

Exposure to WAS increased serum corticosterone in gut colonised Swiss-Webster mice (6.5 ± 1.0 ng/ml; n=15), compared with non-stressed animals (3.9 ± 0.6 ng/ml; $p<0.05$; n=14). An increase in basal serum corticosterone levels was observed in

Figure 7 Decreased expression of brain-derived neurotropic factor (BDNF) and c-Fos in the hippocampus mediate altered non-spatial memory caused by *C rodentium* infection. Immunohistochemistry to assess BDNF expression in the CA1 region of the hippocampus (black box) of *C rodentium*-infected mice at 10 days after infection was performed and compared with results for uninfected mice following exposure to 1 h water avoidance stress (WAS). Immunohistochemistry for c-Fos was also performed to assess hippocampal levels in *C rodentium*-infected mice (arrows), compared with sham-infected controls (B). Representative images were taken from mice in each study group. Quantification of BDNF and c-Fos was performed and the results are presented on the right-hand side of panels A and B, respectively. *p<0.05 compared with sham; #p<0.05 compared with *C rodentium* infection; one-way analysis of variance with Bonferroni post hoc testing; N=6–10 mice/group.
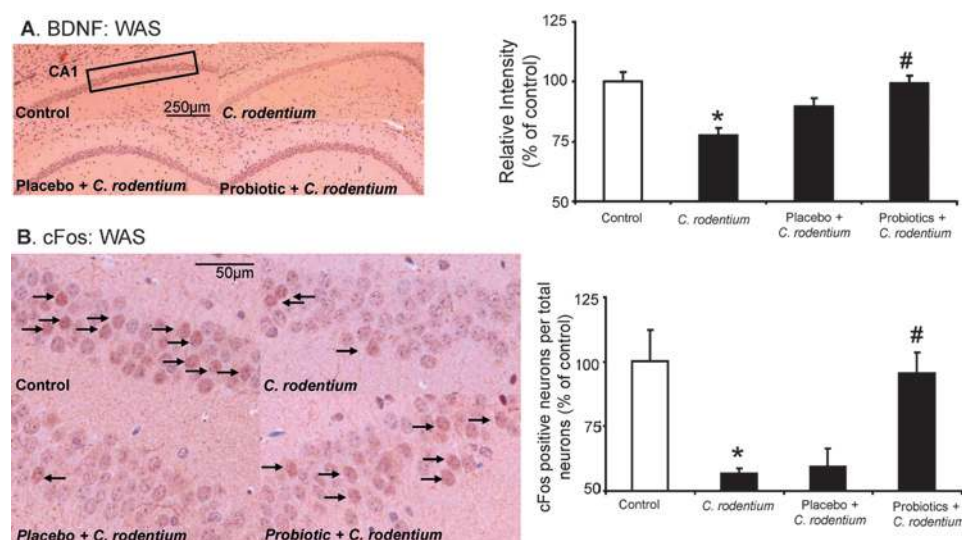
Figure 8 Germ-free (GF) mice display normal anxiety levels, but lack memory. GF Swiss-Webster mice were exposed to the light/dark box and assessed for anxiety levels compared with their colonised (specific-pathogen-free (SPF) counterparts by assessing the frequency to transition between the light and dark compartments (A; n=5—6), and the time spent in the light box (B; n=5—6) with and without 1 h water avoidance stress (WAS). GF mice were also subject to either the novel object test (C; n=3—6) or the T-maze (D; n=7—8), with or without prior exposure to WAS, and assessed for non-spatial memory or working memory, by measuring exploration ratios and spontaneous exploration. Dashed horizontal line in C represent 50% exploration—indication of absence of memory.[35] White bar histograms denote gut colonised, SPF Swiss-Webster mice and black bars indicate GF animals. *p<0.05 compared with SPF, one-way analysis of variance with Newman—Keuls post-test.
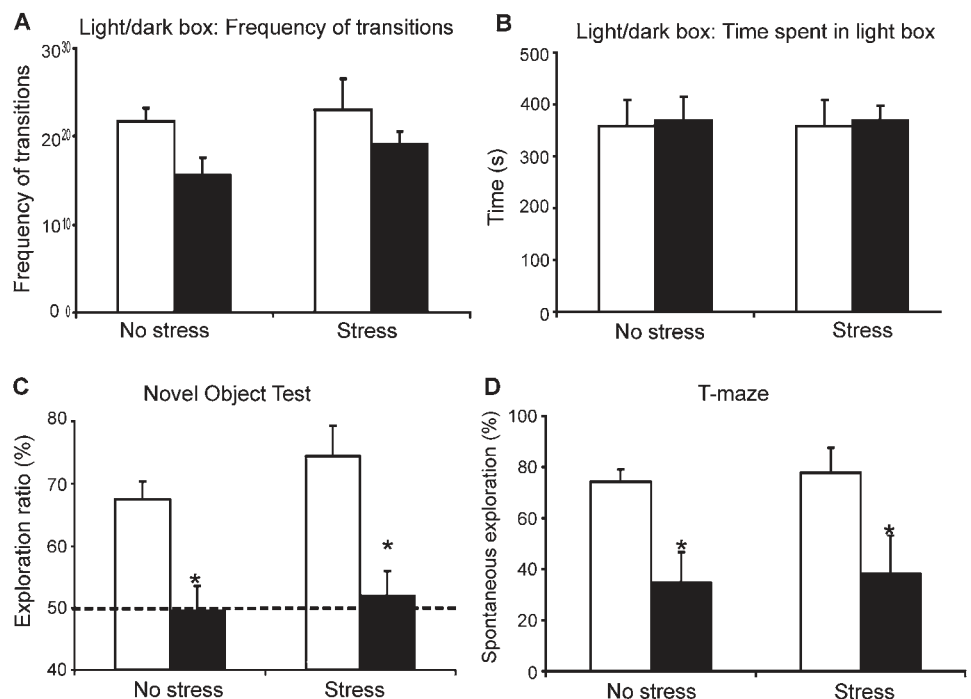
germ-free animals (8.7±1.4 ng/ml; p<0.05; n=7) compared with gut colonised controls, confirming an altered HPA axis in these mice.[10]

DISCUSSION

This study demonstrates, for the first time, that an enteric bacterial infection in combination with psychological stress is associated with impaired learning and memory. *C rodentium* infection causes stress-induced impairment in memory, persisting after bacterial clearance and resolution of intestinal injury. Pretreatment of *C rodentium*-infected mice with a combination of *Lactobacillus*-containing probiotics prevented stress-induced memory deficits, ameliorated serum corticosterone levels and colonic epithelial crypt hyperplasia, compared with placebo treatment. This beneficial effect was associated, at least in part, with restoration of hippocampal BDNF and c-Fos expression in the context of a normalised microbiota. Germ-free mice displayed absence of non-spatial and working memory, indicating the requirement for a commensal gut microbiota in memory.

The brain—gut axis is important for maintaining normal gut function, with alterations in the axis implicated in both IBS and chronic inflammatory bowel diseases.[47 48] Bidirectional effects, including bacterial influence on the HPA axis,[21] have prompted the study of the modulating effect of infections on behaviour. Behavioural responses to *C rodentium* were recently described in mice: at 8 h after infection, anxiety was observed in the absence of a rise in proinflammatory cytokines.[49] However, behaviour at the peak of host response to the pathogen was not determined. Therefore, our study assessed the time of maximal inflammation (10 days), using a light/dark box as a reproducible indicator of anxiety-like behaviour.[37] At 10 days after infection, there were no changes in anxiety-like behaviour in *C rodentium*-infected mice compared with controls, independent of exposure to WAS.

Corticosterone levels and brain expression of glucocorticoid and mineralocorticoid receptors are essential for learning and memory,[50] with alterations in HPA-axis function resulting in impaired hippocampal memory. Administration of a single dose of corticosterone, mimicking acute stress, enhances exploratory behaviour and decreases freezing reactions in a

Figure 9 Germ-free (GF) mice have altered brain-derived neurotropic factor (BDNF) and c-Fos expression in the hippocampus. Specific pathogen-free (SPF) Swiss-Webster and GF animals had expression of BDNF in the CA1 region of the hippocampus compared (black box; A). Expression of c-Fos was also compared in GF mice (arrows) versus animals with an intact colonic microbiota (SPF; B). Quantification of BDNF and c-Fos was performed and the results are presented on the right-hand side of panels A and B, respectively. *p<0.05; unpaired Student t-test; N=7—12 (BDNF) or N=4—6 (c-Fos) mice/group.
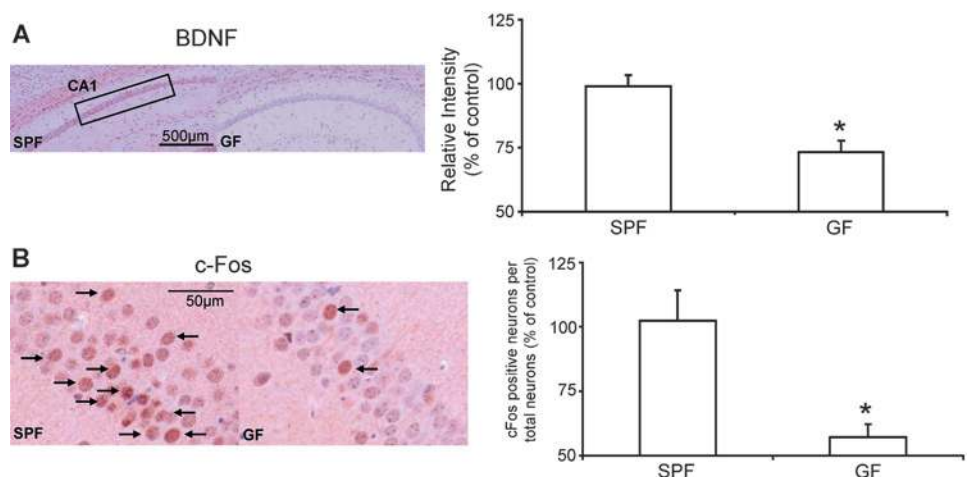
conditioned fear test.[51] Furthermore, the hippocampus is highly responsive to glucocorticoids.[52] Hormone binding to receptors in the CA1 region of the hippocampus leads to multiple cellular responses, including changes in synaptic function and, in excess, neuronal injury.[53] Morris water maze training causes an increase in corticosterone levels, but hippocampal BDNF is resistant to these changes—suggesting that stress alone is not sufficient to cause altered learning and memory.[54] Thus, exposure to stressors in the presence of another stimulus, such as infection, could have an impact on hippocampal-dependent memory.

Use of a novel object recognition test,[32–34] and exploration of a T-maze,[36] together demonstrated that infection with *C rodentium* does not impair memory. However, exposure to a single session of WAS in *C rodentium*-infected mice prevented non-spatial and working memory, a feature not observed in uninfected animals. Lack of behavioural changes in the absence of WAS implies that these features occur independently of sickness behaviour.[55] Similarly, administration of bacterial endotoxin in neonatal rats results in altered anxiety-like behaviour in adult rats, but only after exposure to restraint stress.[56] Previous studies in rats with colitis indicate that altered memory is observed in association with inflammation-induced visceral pain.[57] In *C rodentium*-induced colitis, the inflammation observed is significantly less than in chemically induced colitis models.[18] These findings highlight the compounding roles of stress and infection on behaviour.

Stress-induced memory deficits at 30 days after infection are novel and unexpected findings, because the pathogen has cleared and all colonic parameters, except enteroendocrine cell signalling,[46] have returned to normal. Taken together, these findings suggest that an enteric bacterial infection, via either enteroendocrine cell serotonin[46] or corticotrophin-releasing factor,[58] primes the HPA axis so that exposure to a mild stressor is sufficient to elicit an abnormal behavioural response, resulting in altered memory. Altered memory persists well after resolution of the infectious insult, which may parallel findings observed with stress and postinfectious IBS in humans. After a bout of acute enteritis, patients with comorbid stress are at increased risk of developing IBS, compared with controls.[59] Furthermore, patients with IBS have a bias in recognition memory, similar to subjects with depression.[60]

Administration of probiotics ameliorates colonic disease resulting from *C rodentium* infection[24 26] and in models of psychological stress.[21 22] In this study, treatment with probiotics ameliorated *C rodentium*-induced changes in the microbiota. Trends towards changes were also seen in the *C rodentium*-infected, placebo-treated group, suggesting a potential prebiotic effect, as has been described previously.[26] Probiotics also have extra-intestinal effects, such as prevention of myocardial infarct-induced apoptosis in the limbic region of the brain.[39] To determine the mechanism of action of microbe-induced memory deficits, the hippocampus was studied. Hippocampus-specific deletion of BDNF causes impaired spatial and object recognition.[61] Additionally, NMDA receptors in the CA1 region of the hippocampus participate in non-spatial memory formation.[62] Recent studies indicate that intraperitoneal administration of lipopolysaccharide in mice leads to decreases in hippocampal BDNF associated with cognitive defects.[63] Decreases in BDNF levels in *C rodentium*-infected mouse hippocampi were observed in this study, suggesting that an enteric bacterial infection impairs memory via reduced hippocampal BDNF. Recovery in BDNF expression was observed in the probiotic-treated group, but not in the placebo-treated group. Thus, memory impairment following infection with *C rodentium* and exposure to WAS is probably mediated, at least in part, by a reduction in BDNF in the hippocampus.

The immediate-early gene *c-fos* is induced by stress[53] or chronic exposure to corticosterone[51] in the CA1 region of the hippocampus. c-Fos expression is necessary for the consolidation of non-spatial hippocampus-dependent memory, as demonstrated in rats using a socially transmitted food preference model.[64] Furthermore, c-Fos immunostaining is increased in the vagus nerve of *C rodentium*-infected mice,[49] and increased in the hypothalamus following *Campylobacter jejuni* infection.[65] These observations emphasise that an acute enteric bacterial infection can affect the brain. Immunohistochemistry for c-Fos expression in the CA1 region of the hippocampus revealed a decrease in c-Fos immunoreactivity in *C rodentium*-infected mice, compared with controls, which normalised in probiotic-treated infected mice.

Memory and learning behaviour are associated with diet-induced changes in the intestinal microbiota.[66] To test for a role of gut microbes in mediating non-spatial memory, germ-free mice were used. These mice, maintained under normal conditions in a sterile environment, are capable of responding to a pathogenic bacterial infection like enterohaemorrhagic *Escherichia coli*, in contrast to colonised counterparts, which do not develop disease.[67] Altered stress responses in gnotobiotic mice are accompanied by decreases in hippocampal BDNF staining.[10] In these studies, compared with colonised animals, germ-free mice displayed an absence of non-spatial and working memory. The presence of microbes is, therefore, crucial for the development of hippocampus-dependent memory, possibly owing to reduced expression of BDNF, as observed in the cortex in previous studies,[10] and in the CA1 region in this study. Germ-free mice also demonstrated a decrease in c-Fos expression, compared with colonised animals. In addition, an increase in baseline corticosterone, but with an absence of a hyper-responsive HPA-axis response in germ-free mice was observed, a finding which may relate to the strain of mouse and stressor type used in various studies.[10] Caution must be taken in interpreting these results, however, given that germ-free mice have altered immunological and physiological features compared with colonised controls. Since rearing in different facilities can also alter the gut microbiome of genetically identical mice,[68] it is likely that the presence of a gut microbiome which regulates memory, and not the specific composition of the microbiota, that is the crucial determining factor.

Taken together, these results emphasise that alterations in the composition of the intestinal microbiota exert a measurable impact on certain aspects of animal behaviour, and that normalisation of the microbiota can prevent behavioural abnormalities. Future studies should delineate the breadth of behaviours that are influenced by alterations in the intestinal microbiota.

Gut microbiota

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Editor's quiz: GI snapshot

Refractory iron-deficiency anaemia in a child with portal cavernoma

CLINICAL PRESENTATION

We present the case of a 9-year-old boy affected by portal hypertension due to a portal cavernoma which developed after umbilical vein catheterisation in the neonatal period.

Figure 1 View of the mid-ileum: venous ectasias, non-specific edema and erythema.

Figure 2 View of the mid-ileum: granularity and pseudo-polypoid lesions.

Figure 3 View of the mid-ileum: cherry-red spot.

When our patient was 3 years of age splenomegaly developed and investigations revealed portal hypertension. During the following years he developed third degree oesophageal varices, treated with sclerotherapy.

At 6 years of age he developed severe iron-deficiency anaemia refractory to iron replacement, but no haemorrhagic lesions were found in the oesophagus, stomach, duodenum or colon on standard endoscopy and colonscopy. Clotting tests were normal. A wireless capsule endoscopy was performed (figures 1, 2 and 3).

QUESTION

What is the cause of the lesions?
See page 377 for the answer

Serena Pastore, Margherita Londero, Gabriele Cont, Grazia Di Leo, Alessandro Ventura

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Bacterial infection causes stress-induced memory dysfunction in mice

Mélanie G Gareau, Eytan Wine, David M Rodrigues, et al.

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