Bacterial infection causes stress-induced memory dysfunction in mice

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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.

INTRODUCTION
Psychological stress causes intestinal barrier dysfunction,1 disrupted host–microbial interactions2 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 inflammation4 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 hyperplasias.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. Proinflammatory responses to *C. rodentium* infection are well characterised, with both interleukin 22 and adaptive immune cells 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; 109 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).

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. Provided as single strains or in combinations, probiotics can be used for a variety of ailments, including diarrhoea, IBS, inflammatory bowel disease and allergy. Probiotics ameliorate early-life trauma-induced colonic barrier dysfunction in neonatal rats, and prevent stress-induced bacterial translocation and colorectal hypersensitivity in adult rats. In *C. rodentium*-infected mice, probiotics prevent colonic epithelial cell hyperplasia, reduce colonic colonisation of *C. rodentium*, maintain epithelial barrier integrity, and protect neonates from death. Probiotics also change the composition of the colonic microbiota in mice with DSS-induced colitis. 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. 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. 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.

**Behavioural testing**

**Novel object test**

Various strains of mice, including C57BL/6 and Swiss Webster exhibit similar characteristics for spatial working and reference memory. The novel object test represents a test for dorsal hippocampal function based on the tendency of mice to investigate a novel object, rather than a familiar one. To assess memory, a modified version of a novel object test was used.

**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 reintroduced into the cage (referred to now as object #2B) along with a completely new object (object #3, distinguishable from object #1). Memory was assessed as the frequency to explore object #2 during the testing phase (object #2B), compared with the new object #3. An exploration ratio was calculated (exploration ratio = freq. smell #3/(freq. smell #3 + freq. smell #2B)×100). 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. Exploration of objects was defined as orientation towards the object with the nose of the mouse pointed towards the object within 1–2 cm. 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. 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. 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. 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 compartment.
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 (0–5) 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). This combination has been shown previously to ameliorate C. rodentium-induced colitis in mice.24 26 and stress-induced colonic21 22 and brain39 dysfunction in rats. Freeze-dried preparations were rehydrated in sterile water and used as drinking water (changed every 2 days) at a concentration of 10⁹ CFU/ml (final dose: 6×10⁹ 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.31

Corticosterone

HPA axis activation was assessed by measuring levels of serum corticosterone.32 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-immunooassay kit (Assay Designs, Ann Arbor, 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 neurotrophic factor (BDNF) or c-Fos. Briefly, sections were baked overnight at 60°C, deparaffinised, subjected to heat-induced epitope retrieval and blocked for study 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 test32 (B) and the T-maze test36 coupled with light/dark box testing37 (C).

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 test32 (B) and the T-maze test36 coupled with light/dark box testing37 (C).
endogenous peroxidase and bixin. 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 immunodetection 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 Institute of Health, USA) and calculating the intensity relative to control (Sham; set to 100%).\textsuperscript{43} Quantification of c-Fos was performed by counting cells (400×50 μm\textsuperscript{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.

\textbf{Table 1} Primer sequences employed in analysis of the fecal microbiome\textsuperscript{44 45}

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward (5’−3’)</th>
<th>Reverse (5’−3’)</th>
</tr>
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<tbody>
<tr>
<td>Mouse</td>
<td>TATCCCCATCAGCTCCATC</td>
<td>TAGCTGGCTACAAATGCG</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GGCCTTAGTTCCCTCCCTC</td>
<td>CATGCCAGCACTCCTGCCATG</td>
</tr>
<tr>
<td>TNFα</td>
<td>CTAACCTACTACATGTC</td>
<td>CGGACGGCTGATGG</td>
</tr>
<tr>
<td>IFNγ</td>
<td>AGAACTCGCTACCACGATTC</td>
<td>CCACTCTTTGCGACTTCTC</td>
</tr>
<tr>
<td>Bacteria</td>
<td>ATCCCTCCAGGAGGACAGGACT</td>
<td>CTTGCATCACTCACCAG</td>
</tr>
<tr>
<td>Eubacteria</td>
<td>GGGCGGCTGCTAATACGTG</td>
<td>CAGAGGAGGAGGAGGAGGACAGG</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>CTCCATGCGTTAATACGTG</td>
<td>CAGAGGAGGAGGAGGAGGACAGG</td>
</tr>
<tr>
<td>Bacteroides</td>
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<td>CTTGCATCACTCACCAG</td>
</tr>
<tr>
<td>Bacillus</td>
<td>ATCCCTCCAGGAGGACAGGACT</td>
<td>CTTGCATCACTCACCAG</td>
</tr>
<tr>
<td>Enterococci</td>
<td>GTCAGGACGAGGACGACGACGACGAG</td>
<td>CAGAGGAGGAGGAGGAGGACAGG</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>GGCAGCTAATACGGCGATGATG</td>
<td>CAGAGGAGGAGGAGGAGGACAGG</td>
</tr>
<tr>
<td>Lactobacillus/Lactococcus</td>
<td>AGGAGGTGACCTACGACGACGACGAG</td>
<td>CAGAGGAGGAGGAGGAGGACAGG</td>
</tr>
<tr>
<td>Segmented filamentous bacteria</td>
<td>GACGGAGAATGCAAGGACGACGACGAG</td>
<td>CAGAGGAGGAGGAGGAGGACAGG</td>
</tr>
<tr>
<td>H hepatics</td>
<td>GGTTTTGGGAATTTGAGCA</td>
<td>CAGAGGAGGAGGAGGAGGACAGG</td>
</tr>
<tr>
<td>Eubacterium rectale/Clostridium coccoides</td>
<td>GACGGAGAATGCAAGGACGACGACGAG</td>
<td>CAGAGGAGGAGGAGGAGGACAGG</td>
</tr>
<tr>
<td>Salmonella enterica serovar Typhimurium</td>
<td>GACGGAGAATGCAAGGACGACGACGAG</td>
<td>CAGAGGAGGAGGAGGAGGACAGG</td>
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</tbody>
</table>

\textsuperscript{IFNγ}, interferon γ; TNFα, tumour necrosis factor α.
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 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. 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.

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.*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.
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±5.1 mean intensity/100 µm² 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...
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

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**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.

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**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.
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 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.36 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.

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.\textsuperscript{51} Furthermore, the hippocampus is highly responsive to glucocorticoids.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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,\textsuperscript{52–54} and exploration of a T-maze,\textsuperscript{56} together demonstrated that infection with \textit{C. rodentium} does not impair memory. However, exposure to a single session of WAS in \textit{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.\textsuperscript{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.\textsuperscript{56} Previous studies in rats with colitis indicate that altered memory is observed in association with inflammation-induced visceral pain.\textsuperscript{57} In \textit{C. rodentium}-induced colitis, the inflammation observed is significantly less than in chemically induced colitis models.\textsuperscript{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 enteromedullary cell signalling,\textsuperscript{46} have returned to normal. Taken together, these findings suggest that an enteric bacterial infection, via either enteromedullary cell serotonin\textsuperscript{45} or corticotrophin-releasing factor,\textsuperscript{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.\textsuperscript{59} Furthermore, patients with IBS have a bias in recognition memory, similar to subjects with depression.\textsuperscript{60}

Administration of probiotics ameliorates colonic disease resulting from \textit{C. rodentium} infection\textsuperscript{24, 26} and in models of psychological stress.\textsuperscript{10, 52} In this study, treatment with probiotics ameliorated \textit{C. rodentium}-induced changes in the microbiota. Trends towards changes were also seen in the \textit{C. rodentium}-infected, placebo-treated group, suggesting a potential probiotic effect, as has been described previously.\textsuperscript{24} Probiotics also have extra-intestinal effects, such as prevention of myocardial infarction-induced apoptosis in the limbic region of the brain.\textsuperscript{59} 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.\textsuperscript{61} Additionally, NMDA receptors in the CA1 region of the hippocampus participate in non-spatial memory formation.\textsuperscript{62} Recent studies indicate that intraperitoneal administration of lipopolysaccharide in mice leads to decreases in hippocampal BDNF associated with cognitive defects.\textsuperscript{63} Decreases in BDNF levels in \textit{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 \textit{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 \textit{c-fos} is induced by stress\textsuperscript{53} or chronic exposure to corticosterone\textsuperscript{51} in the CA1 region of the hippocampus. \textit{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.\textsuperscript{64} Furthermore, \textit{c-Fos} immunostaining is increased in the vagus nerve of \textit{C. rodentium}-infected mice,\textsuperscript{49} and increased in the hypothalamus following \textit{Campylobacter jejuni} infection.\textsuperscript{65} These observations emphasise that an acute enteric bacterial infection can affect the brain. Immunohistochemistry for \textit{c-Fos} expression in the CA1 region of the hippocampus revealed a decrease in \textit{c-Fos} immunoactivity in \textit{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.\textsuperscript{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 \textit{Escherichia coli}, in contrast to colonised counterparts, which do not develop disease.\textsuperscript{67} Altered stress responses in gnotobiotic mice are accompanied by decreases in hippocampal BDNF staining.\textsuperscript{68} 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,\textsuperscript{10} and in the CA1 region in this study. Germ-free mice also demonstrated a decrease in \textit{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.\textsuperscript{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,\textsuperscript{69} 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.
REFERENCES


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 colonoscopy. 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

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