Available online at www.aexpbio.com

ISSN : 2348-1935

RESEARCH ARTICLE

Annals of Experimental Biology
2017, 5(1):1-6

Small Intestinal Bacterial Overgrowth Accelerates Completion of Maze Task in Mice

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ABSTRACT

Perturbations to the gut microbiome (by antibiotics, infection, stress, etc.) are capable of disrupting the gut bacterial community leading to a state of dysbiosis. Small intestinal bacterial overgrowth (SIBO), characterized by excessive density of resident bacteria in the small intestine, is a common example of dysbiosis. Pathogenic bacteria in the gut induce anxiety-like behaviour and stress, accelerating completion of learning maze performance. However, it is not known whether an overgrowth of resident bacteria such as that seen in SIBO would have the same effect. A diet containing raw red kidney bean (RRKB) is capable of inducing SIBO. The aim of this study was to test the hypothesis that SIBO induced by an RRKB-supplemented diet might accelerate completion of learning maze performance during radial 8-arm maze testing. Twelve mice were tested in a radial 8-arm maze while on either standard rodent chow (Control group) or an RRKB-supplemented rodent chow (RRKB group) for 24 hrs. To test for bacterial overgrowth, DNA was extracted from small intestinal tissues, qPCR was performed, and universal 16S rRNA data was transformed into fold change compared to the Control group using the $2^{-\Delta\Delta Ct}$ *method. The RRKB group had 37.4+/-12.1 fold higher bacterial density in the mid-small intestine than control mice ($P<0.05$). The maze run time was shorter for RRKB group (124.8+/-28.7 s) than Controls (251.8+/-72.8 s) ($P<0.05$). The RRKB group had less time spent in arms (97.5+/-53.5 s) than Controls group (212.2+/-53.8 s) ($P<0.01$). A diet of rodent chow supplemented with raw red kidney beans accelerated the completion of radial 8-arm maze test. This observed behaviour change may be an acute effect on the host by the overgrowth of resident bacteria in the small intestine.*

Keywords: Microbiome, Microbiota, Memory, Red kidney bean, SIBO, phytohemagglutinin

INTRODUCTION

Changes in the gut bacterial community structure can significantly alter host physiology and behaviour through the bidirectional communication pathway known as the gut-brain axis. Gut microbes communicate with the brain via the autonomic nervous system, enteric nervous system, neuroendocrine system, and immune system which can modulate mood and trigger stress and anxiety. Disruptors to the microbiome (e.g., antibiotics, infection, diet, and stress) can destabilize the gut bacterial community inducing a state of microbial imbalance termed "dysbiosis." Dysbiosis is associated with obesity, cardiovascular disease, inflammatory bowel disease, asthma, and type II diabetes, as well as several neurological disorders [1-6].

Small intestinal bacterial overgrowth (SIBO) is a specific form of dysbiosis characterized by increased bacterial density in the small intestine and is implicated as a possible etiology for irritable bowel syndrome [7]. Excess colonization of this normally sparsely populated region of the gastrointestinal tract is associated with increased intestinal permeability, which can lead to bacterial translocation across the gut barrier and can trigger an immune response [8-10]. The immune response includes the production of pro-inflammatory cytokines which, in turn, activate the sympathetic nervous system and adrenal glands to release stress mediators including corticosteroids and catecholamines [11]. Stress mediators heighten anxiety which has been shown to improve performance of rodents in a number of maze models, as represented by faster completion of their maze task [12-14].

Rodent behaviour is altered when the gut is exposed to probiotics [15], antibiotics [16], or pathogenic bacteria [17]. Treatments in rodents that disrupt the gut microbiome have variable effects on cognitive behaviour and maze

performance [18,19]. Challenge with the pathogenic microbes *Campylobacter jejuni* or *Citrobacter rodentium* [20,21] increased anxiety-like behaviour in mice, whereas antimicrobial treatment led to decreased anxiety [22]. In the *C. rodentium* study, induction of anxiety-like behaviour occurred through the activation of sensory or afferent nerves, specifically via the vagal pathways communicating from the gut to the CNS [21]. Bercik et al. provided strong evidence on the vagal afferent nerve pathway as an avenue by which host behaviour can be modified by intestinal microbes [15]. Communication from the gut to the brain and from the brain to the gut can take place via sympathetic pathways [23].

Although various forms of dysbiosis have been shown to directly affect rodent behaviour [17,20-24], the effects of SIBO on maze performance are not known. Raw red kidney bean (RRKB) containing the lectin phytohemagglutinin has been used to experimentally induce SIBO in a rodent model [10]. In this study, we tested the hypothesis that completion of maze performance may be accelerated by RRKB-induced small intestinal bacterial overgrowth.

MATERIALS AND METHODS

Animals

Five week old, female C57BL/6 mice (20-25 g) were purchased from the Charles River Lab (Wilmington, DE). Upon arrival the mice were housed in groups of 3 in polypropylene cages, placed on a 12-hour light/dark cycle, and kept on a standard rodent diet (Harlan Teklad Laboratory Diets). After a one-week acclimatization period, mice began training in the radial 8-arm maze. Once training was completed, the mice were tested and euthanized. Mice (n=6 per group) were fed *ad libitum* either a standard rodent pellet chow (Control group) or a RRKB-supplemented chow (RRKB group) for 24 hrs, fasted for 4 hrs, then tested in the radial 8-arm maze. The procedures were in compliance with the 8th Edition of the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the New Mexico VA Health Care System.

Food preparation

The standard rodent pellet chow (Harlan 8604 Rodent Diet) used for the Control group, was pulverized into a powder and mixed with ground RRKB and turned into a paste from which new pellets were made for the RRKB group (74% Harlan 8604 Rodent Diet/26% RRKB). Both batches of feed were kept at room temperature in air-tight containers before use.

DNA isolation and qPCR

Following completion of maze learning assessments, bacterial density in the small intestine was measured by qPCR in all 12 mice. DNA was extracted from tissue samples obtained from the mid-1/3 of the small intestine. DNA was extracted by DNeasy Blood & Tissue Kit 69506 (QIAGEN, Valencia, CA) following manufacturer's instructions. DNA concentrations were normalized across all samples and 60 µl of each of the samples were then subjected to qPCR to assess the bacterial density by targeting 16S rRNA gene. Additional qPCR was performed using mouse actin primers for normalizing 16S data relative to sample size. Data were compared by paired T-test and universal 16S rRNA data for the RRKB group was represented as fold change compared to the control group using the $2^{-\Delta\Delta Ct}$ method (mean+/-SE).

Maze performance analysis

Mice were tested in a radial 8-arm maze (Figure 1; TSE System, Chesterfield, MO). The maze tested the ability of mice to collect food pellets from the end of all 8 arms. The use of the maze has been well validated for the reproducible assessment of spatial memory. The maze setup is constructed as a large central platform with 8 arms that branch out at 45 degree angles. Each arm has infrared motion sensors at the beginning of the arm to track movement from the central platform onto the specific arm, and another infrared motion sensor at the end of the arm that records the removal of the bait. A small (0.01 g) piece of their standard chow was used as bait. The bait was held in a raised food dish at the end of each of the 8 arms so that it would not be visible from the central platform; additional baits were scattered around the outside of the maze to deter the mice from using smell to determine if bait was present in individual arms. As the animal must use visual cues and working memory to avoid an arm that no longer held bait, this setup allows working memory to be tested rather than sight or smell. A perfect run is defined as one in which the mouse enters each arm only once, recovers the bait at the end of the arm before going on to the next arm, i.e., entering 8 arms to recover 8 baits.

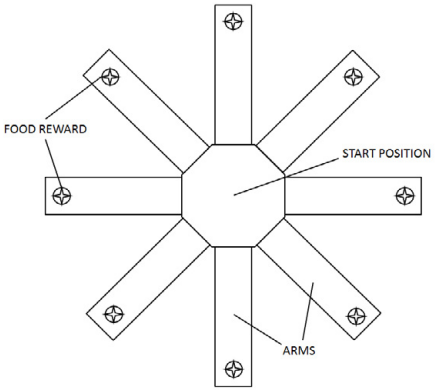

Figure 1: Radial 8-arm maze diagram.

The outcome measurements were measured as the total time taken to collect all the baits from all 8 arms (TT), the total number of errors made (TE), the time spent in the arms (AT), and the time spent in the central platform of the maze (CT). Data were presented as time in seconds (mean+/-SE) and compared by paired T-test. All mice were fasted for 4 h before maze runs to increase motivation for collecting bait. Mice were trained with 2 trials per day until each animal was able to complete the maze perfectly and repeatedly. Twenty-four hours before the final maze run, the diet of the RRKB group was switched to the RRKB-supplemented diet while the diet of the Control group was not changed.

RESULTS

Bacterial concentration

There was a significant difference between groups. The bacterial density in the mid-1/3 of the small intestine of the RRKB group was different than the Control group ($P<0.05$). The bacterial density of the RRKB group was 37.4+/-12.1 fold greater than the Control group (Figure 2). This was therefore a significant increase in bacterial density in the mid-1/3 of the small intestine in animals fed the RRKB-supplemented diet confirming small intestinal bacterial overgrowth.

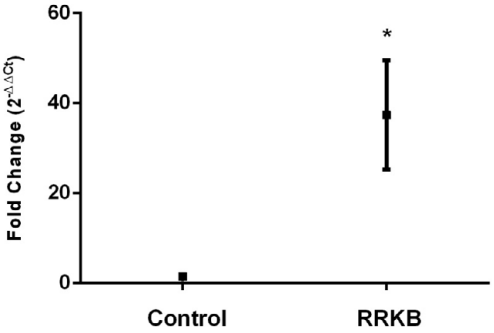

Figure 2: 16S Universal rRNA gene copy fold change difference in mid small intestine between control and raw red kidney bean fed (RRKB) mice, $^{*}P<0.05$.

Maze learning behaviour

There was a significant difference between groups. The total maze run time (TT) was shorter for the RRKB group (124.8+/-28.7 s) compared to the Control group (251.8+/-72.8 s) (Figure 3; $P<0.05$). The RRKB group spent less time in arms (AT) (97.5+/-53.5 s) than the Control group (212.2+/-53.8 s) (Figure 4; $P<0.01$). However, there was no significant difference in centre time (CT) between the RRKB group (18.0+/-8.8 s) and the Control group (35.7+/-22.2 s) ($P=0.08$). There was also no significant difference in total number of errors (TE) between the RRKB group (3.0+/-3.1) and the Control group (8.7+/-8.6) ($P=0.24$).

DISCUSSION

In this study, we induced dysbiosis in the form of small intestinal bacterial overgrowth (SIBO) in mice by disrupting the gut microbiome with RRKB-supplemented chow. Our examination of the universal 16S rRNA gene from the

mid-section of the small intestine found increased bacterial density in the RRKB-treated group compared to controls thereby confirming SIBO, a finding consistent with previous studies [10]. Using radial arm maze performance as a behavioural test for spatial memory function, we found that feeding RRKB-supplemented chow was associated with a faster maze run time compared to controls that had remained on a standard diet.

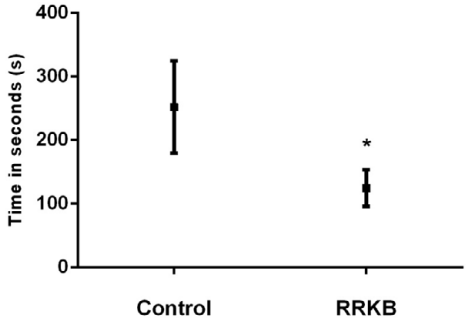

Figure 3: Total time taken to collect all baits in radial 8-arm maze for control and raw red kidney bean fed (RRKB) mice, $^{*}P<0.05$.

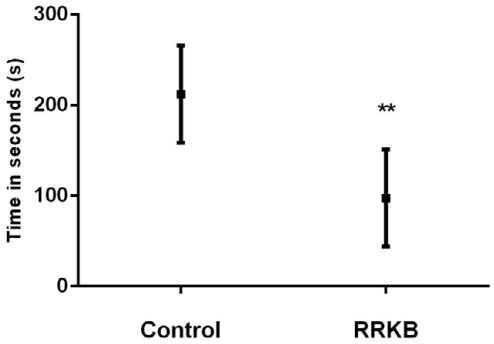

Figure 4: Time spent in arms of radial 8-arm maze for control and raw red kidney bean fed (RRKB) mice, $^{**}P<0.01$.

The faster maze performance observed in the RRKB group is suggestive of attention hypervigilance, a symptom of heightened stress and anxiety. Hypervigilance is a central feature of post-traumatic stress disorder, anxiety, and social phobias [25-28]. The behavioural-social components of hypervigilance in humans have been extensively studied, as reviewed by Bögels and Mansell, and are characterized by an abnormally active avoidance to novel stimuli or perceived threats [25]. These findings are consistent across rodent models showing that hypervigilant avoidance behaviour is a common response to both acute and chronic stress [29,30]. Physiologically, stress disorders are associated with increased activation of the sympathetic nervous system and, in turn, the adrenal glands, which release stress mediators such as catecholamines and cortisol [31]. In the context of the radial arm maze, heightened stress and elevated stress hormones lead to faster maze run time [32,33], these findings are similar to the results from the present study.

Our novel finding that SIBO accompanies hypervigilance further supports an association between gut dysbiosis and anxiety [15,17,20,21,24]. Since SIBO is one of the primary causes of bacterial translocation [34], resulting from increased intestinal permeability [10], it is highly plausible that the movement of resident gut bacteria across the gastrointestinal epithelium triggers a host response that is responsible for the development of the abnormal maze performance. Bacteria and bacterial products (e.g., endotoxins or gram-positive cell walls) activate the immune system to produce pro-inflammatory cytokines, which then increases plasma levels of stress mediators such as cortisol and catecholamines (e.g., norepinephrine, epinephrine, and dopamine) [35-38] via activation of sympathoadrenal system [11]. The activation of this pathway has also been documented in humans after direct administration of lipopolysaccharide, which leads to a rise in circulating cytokines and a state of heightened anxiety, hastened reaction time, and increased plasma cortisol and norepinephrine levels [39]. Cytokine-induced production of stress mediators can affect cognition and behaviour via pathway activation of the HPA axis [40,41] and the CNS [38].

The vagus nerve serves as the afferent limb of this response while the sympathetic nerve serves as the efferent limb of this response [18,42,43]. This evidence, along with the substantial body of research on the relationship between gut microbiome composition and behaviour [1,18,44], suggests that dysbiosis in the gut microbiome can have anxiogenic properties. The potential for the gut microbiome to modulate stress and anxiety is further evidenced by the anxiolytic

properties of some probiotics, which have been observed to lessen anxious behaviour and decrease levels of serum cortisol in mice and humans [22,42]. Measurement of stress mediators in the test animals was beyond the scope of this study but is of interest for future experiments.

In line with the bi-directionality of the gut-brain axis, psychological stress can have reciprocal effects on the gut microbiome. Stress in mice can signal production of cytokines and catecholamines, leading to compositional changes in the gut microbiome such as the overgrowth of resident microbes [45]. The gastrointestinal tract is densely innervated by catecholaminergic neurons, and evidence suggests a substantial proportion of luminal norepinephrine may also be of bacterial origin [46]. Thus, catecholamines serve as a signalling molecule by both host and bacteria. Catocholamines have also been documented to promote growth and virulence in numerous types of enteric bacteria [47-53]. It follows that the increased circulation of stress mediators, whether as a result of bacterial translocation or psychological stress, will further promote the overgrowth of intestinal bacteria.

The bidirectional pathway between psychological stress and bacterial overgrowth can form a positive feedback loop, giving SIBO the potential to be a self-perpetuating disease state. This provides an explanation for the anxiety seen so frequently in patients with SIBO-associated disorders, such as irritable bowel syndrome (IBS) [54]. IBS, in particular, is a condition highly relevant to our study since the findings of IBS include heightened psychological stress, elevated pro-inflammatory cytokines and stress mediators, and SIBO [55-57]. Further investigations should focus on the precise mechanism responsible for the outcomes observed in this study, particularly in the context of IBS.

ACKNOWLEDGEMENTS

Nathaniel Ritz helped design, perform, and author the experiment. Derek Lin provided writing and proof assistance. Larry Barton helped design the experiment. Henry Lin helped design and author the study.

FUNDING

This work was supported, in part, by the Winkler Bacterial Overgrowth Research Fund.

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