Behavioural Processes 96 (2013) 27–35

Contents lists available at SciVerse ScienceDirect

Behavioural Processes

journal homepage: www.elsevier.com/locate/behavproc

Ingestion of *Mycobacterium vaccae* decreases anxiety-related behavior and improves learning in mice

Dorothy M. Matthews[a,*], Susan M. Jenks[b]

[a] Department of Biology, The Sage Colleges, Troy, NY 12208, USA
[b] Departments of Biology & Psychology, The Sage Colleges, Troy, NY 12208, USA

ARTICLE INFO

Article history:
Received 5 January 2013
Accepted 18 February 2013

Keywords:
Mycobacterium vaccae
Serotonin
Learning
Memory
Anxiety-related behaviors
Old friends hypothesis

ABSTRACT

Coevolution of microbes and their hosts has resulted in the formation of symbiotic relationships that enable animals to adapt to their environments and protect themselves against pathogens. Recent studies show that contact with tolerogenic microbes is important for the proper functioning of immunoregulatory circuits affecting behavior, emotionality and health. Few studies have examined the potential influence of ambient bacteria, such as *Mycobacterium vaccae* on the gut–brain–microbiota axis. In this preliminary research, we show that mice fed live *M. vaccae* prior to and during a maze learning task demonstrated a reduction in anxiety-related behaviors and maze completion time, when tested at three maze difficulty levels over 12 trials for four weeks. Treated mice given *M. vaccae* in their reward completed the maze twice as fast as controls, and with reduced anxiety-related behaviors. In a consecutive set of 12 maze trials without *M. vaccae* exposure, treated mice continued to run the maze faster for the first three trials, and with fewer errors overall, suggesting a treatment persistence of about one week. Following a three-week hiatus, a final maze run revealed no differences between the experimentals and controls. Additionally, *M. vaccae*-treated mice showed more exploratory head-dip behavior in a zero maze, and *M. vaccae* treatment did not appear to affect overall activity levels as measured by activity wheel usage. Collectively, our results suggest a beneficial effect of naturally delivered, live *M. vaccae* on anxiety-related behaviors and maze performance, supporting a positive role for ambient microbes in the immunomodulation of animal behavior.

Coevolution of microbes, macrobiotic organisms and their animal hosts over the past 500 million years has resulted in the development of some symbiotic relationships that enable animals to adapt to the ambient environment and protect themselves against pathogens (Strachan, 1989; Chakrabarty, 2003; Tlaskalova-Hogenova et al., 2011; Rook, 2012). Such relationships involve bidirectional signaling between the gastrointestinal tract and the brain via neural, hormonal and immune interactions (Grenham et al., 2011). Recent work on communication between the brain–gut–microbiota axis using rodents (Berick et al., 2011; Grenham et al., 2011; Bravo et al., 2012), monkeys (Bailey et al., 2004), pigs (Barnes et al., 2012) and humans (Knowles et al., 2008; Khani et al., 2012) has deepened our understanding of how such symbiotic relationships can influence animal behavior. Studies with germ-free animals allow evaluation of the effects of the microbiota on the CNS; antibiotic studies provide insight on how use of broad-spectrum antibiotics can modulate the microbiome and affect behavior; infection studies show that enteric pathogens can induce anxiety-like behaviors in animals; probiotic studies show beneficial effects on the intestinal tract and improved behaviors associated with anxiety-related conditions (see Bravo et al., 2012). For example, Li and colleagues (2009) reported that alterations in the diversity of enteric bacteria influence memory and learning in mice, Clarke et al. (2012) found sex-specific regulation of hippocampal serotonin associated with anxiety using germ-free mice and Bravo et al. (2011) further demonstrated that *Lactobacillus rhamnosus* influences emotional behavior in mice through the GI tract with involvement of the vagus nerve and gamma-amminobutyric acid (GABA) system. This research provides evidence about how changes in the gut microbiota can lead to modification in CNS function with ramifications for behavior.

Homeostatic function and behavior, however, can be influenced not only by normal and disrupted enteric microbiota associations, but by organisms present in the ambient environment as well. Rook and Brunet (2002) have proposed and championed the "old friends" hypothesis as a way to explain the explosion of allergic, chronic inflammatory and autoimmune disorders present among people living in developed nations. They suggest that contemporary urban

* Corresponding author at: Department of Biology, The Sage Colleges, 63 First Street, Troy, NY 12208, USA. Tel.: +1 518 925 4958; fax: +1 518 244 3174.
E-mail addresses: matthd@sage.edu, dorothymatthews1@gmail.com (D.M. Matthews).

0376-6357/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.beproc.2013.02.007

lifestyles have disrupted long established relationships during prenatal, neonatal and adulthood with coevolved organisms such as helminths, soil and water microbes, farm animals and pets that are typically recognized as harmless by the innate immune system and induce an anti-inflammatory response. Since allergies are mediated by T helper (T_H2) lymphocytes, and autoimmunity is mediated by T helper (T_H1/T_H17) lymphocytes, the immune dysregulation caused by lack of exposure to "old friends" likely involves disruptions not only in innate immunity but to the adaptive immune system as well. Such dysregulation of immunoregulatory circuits of the immune system may also potentially affect mood, cognitive function and behavior (Rook et al., 2003, 2012; Raison et al., 2010; Rook, 2012). These same homeostatic processes are likely important to the behavioral ecology of all mammals.

A microbe that has been the subject of several studies investigating the hypothesis that extant nonpathogenic organisms can improve behavioral health outcomes is *Mycobacterium vaccae*. *M. vaccae* is an aerobic bacterium found in temperate environments and animals are likely exposed to it through contact with water, soil and vegetation (Sneath et al., 1986; Gomez et al., 2001; Kazda et al., 2009). As an aerobe, it cannot colonize the anaerobic GI tract of animals and is thought of as a transient commensal (Rook and Brunet, 2005). *M. vaccae* was used in clinical trials in which terminal lung cancer patients were inoculated with heat-killed *M. vaccae*. Treated patients showed improved emotional health and general cognitive function (O'Brien et al., 2004). These findings led to speculation that an immune response to *M. vaccae* antigens might involve a ubiquitous neurotransmitter such as serotonin that plays a role in mood, arousal and learning (Leussis and Bolivar, 2006; Cools et al., 2007; Hohmann et al., 2007; Cifariello et al., 2008). Thus, an immune response to this microbe might positively impact behavior influenced by emotionality.

Examining this idea in a mouse model, Lowry et al. (2007) tested the hypothesis that peripheral exposure to *M. vaccae* antigen causes a T helper cell response that activates brain serotonergic systems in mice. Their research demonstrated that mice injected with heat-killed *M. vaccae* antigen experienced (1) a T_H1 and T regulatory cell biased immune activation of a subset of serotonergic neurons located in the dorsal raphae nucleus (DRI) of the brainstem and that project to the hippocampus and other forebrain regions, (2) elevated serotonin metabolism in the ventromedial prefrontal cortex, and (3) a reduction in stress-related emotional behavior in the forced swim test. Prior to this, Hunt et al. (2005) showed that heat-killed *M. vaccae* could influence immunocompetence through GI tract interaction in mice after administration by gavage.

Several researchers document the effect of immunomodulation on cognition and psychiatric disorders (Brynskikh et al., 2008; Miller, 2010; Yirmiya and Goshen, 2011). Integration of Lowry et al.'s (2007) findings and recent research on the nature of brain–gut–enteric microbiota interactions encouraged us to ask: Could ingestion of *M. vaccae* alter anxiety behavior and influence learning in mice? We hypothesized that if *M. vaccae* decreases stress response through an immune system activation of serotonin pathways, then mice that ingest *M. vaccae* may show superior complex maze performance and fewer anxiety-related behaviors than control mice.

1. General methods: all experiments

1.1. Animals

For all experiments, male, BALB/c specific pathogen free mice were obtained from Charles River Laboratories when they were about 38 days old, housed individually in an isolated animal room under a 12 h light/dark cycle and at a constant 25 °C temperature, and fed Carolina Biological Supply Company Mazuri rodent pellets (5663) (ad libitum). This mouse strain was used to maintain consistency with the mice used by Lowry et al. (2007). Each mouse was placed in an individual polycarbonate cage with a wire bar lid used to hold the water bottle and feed. Carefresh Natural Premium pet bedding, obtained from Carolina Biological Supply Company, was placed directly into the cage allowing the absorption of urine and the animal to burrow and/or den. To allow the mice to become acclimated to their new setting, the experiments were started when mice were 52 days old, and weighted approximately 21–25 g.

1.2. Ethical note

All animal experiments were conducted in accordance with the 2010 US Animal Welfare Act under animal use protocols (#01-2010 and #01-2011) and animal husbandry standard operating procedures approved by the Sage Colleges Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering. At the completion of each study animals were humanely euthanized using CO_2.

1.3. M. vaccae

M. vaccae (15,483) was purchased from the American Tissue Cell Culture (ATCC) and stored at 5 °C until reconstituted. *M. vaccae* was grown in nutrient broth for four days at 37 °C and stored in a refrigerator until needed. Aliquots of 0.1 mL, containing approximately 4.5×10^6 CFU/mL (determined by a standard plate count) was applied to the food vehicle of treatment mice, as appropriate.

1.4. Food vehicles

All mice were denied food for 24 h before administration of food vehicles. The food vehicle given the experimental mice consisted of a piece of white *Wonder* bread (produced by Hostess Brands), approximately 1 cm × 1 cm, onto which 0.1 mL of *M. vaccae* was aseptically pipetted. The bread was coated on the same side with a thin layer of store brand creamy peanut butter to increase palatability. Control mice received a food vehicle like that given the experimental animals (1 cm × 1 cm square of white bread coated with peanut butter), but which lacked *M. vaccae*. Treatment mice in experiments 2 and 3 received a food vehicle identical to that given the control mice, i.e. it lacked *M. vaccae*.

2. Experiments 1–3: Complex maze experiments

2.1. Methods

2.1.1. Sample

In experiments 1–3, ten mice constituted the treatment group and eight mice constituted the control group. The same mice were used through the progression from experiments 1 to 3.

2.1.2. Complex maze

A Hebb–Williams style complex maze was used in this study (Fig. 1). This type of maze is widely used in measuring spatial learning tasks and working memory with rodents (Shore et al., 2001; Parle et al., 2006). This maze operates on appetitive rather than aversive principles.

The mice were tested in a maze free of bedding or other materials. The maze was a square Plexiglas box (14 cm high, 45 cm × 45 cm) consisting of five rows, 9 cm wide, with five door openings, 8 cm wide. The start box was 9 cm × 13 cm in size. Eight turns are required to reach the end point of the unobstructed maze.

Three levels of maze difficulty were used in experiment 1. Each successive level involved additional turns and openings and longer