Title
The role of Nod-like receptors in regulating the microbiota-gut-brain axis

Author
Zhang, Nancy Yingzhen

Publication Date
2016

The role of Nod-like receptors in regulating the microbiota-gut-brain axis

UCSD School of Medicine
Independent Study Project
Yingzhen Nancy Zhang

Abstract

Background: The microbiota-gut-brain axis is central to maintaining normal intestinal physiology. Patients with inflammatory bowel diseases (IBD) can experience stress-induced disease exacerbations, and a strong association with psychiatric comorbidities. Gene mutations in components of innate immunity, such as Nod-like receptors, have been linked to the development of IBD. How an altered intestinal immune response changes behavior, either at baseline or following exposure to stress, is currently unknown.

Hypothesis: We hypothesize that exposure to an acute stressor will dysregulate the microbiota-gut-brain axis in a Nod-dependent manner, with concomitant changes in behavior including anxiety and cognitive deficits.

Methods: Anxiety and recognition memory (assessed via light-dark box and novel object test) in adult (6-8 week old) C57BL/6 mice and Nod1/2 receptor double knockout (NodDKO) mice were evaluated with and without exposure to 1h of acute water avoidance stress (WAS). HPA-axis activation, a measure of acute stress response, between the two strains was assessed by measuring serum corticosterone levels.

Results: No difference was observed in recognition memory between C57BL/6 and NodDKO mice at baseline. However, NodDKO mice demonstrated impaired memory when exposed to acute stress in contrast to C57BL/6 mice. Anxiety-like behavior was observed in NodDKO mice at baseline compared to C57BL/6 mice, which was not further exacerbated following exposure to acute stress. HPA-axis activation was observed via increased serum corticosterone levels in both C57BL/6 mice and NodDKO mice upon exposure to acute stress, however NodDKO mice demonstrated also higher HPA-axis activation at baseline.

Conclusions: Nod-like receptors are associated with behavioral changes, including anxiety-like behavior, at baseline; and stress-induced cognitive defects. This might be in part mediated by changes to the HPA-axis.

BACKGROUND

The microbiota-gut-brain axis is increasingly recognized for its role in maintaining normal health. Patients with inflammatory bowel diseases (IBD), a family of intestinal diseases including Crohn's disease and ulcerative colitis, are found to have altered microbiota compositions and stress-induced disease exacerbations. [4 6] Pathological changes in intestinal physiology can also lead to psychiatric comorbidities[2]. Patients with chronic IBD are at increased risk for developing mood disorders and cognitive defects separate from the potential anxiety and stress experienced in coping with the physical symptoms of disease. [2]

Among the physiological mechanisms that mediate the cross-talk between the gut, brain and microbiota is the HPA-axis. It is activated following exposure to stress and mediated by neurotransmitters and hormones, including corticosterone. [5] HPA-axis activation has been shown to lead to temporary and neurological defects; for example, mouse models of enteric bacterial infection develop memory dysfunction when exposed to acute stress while germ-free (GF) mice without a microbiota lack memory at baseline. [7]

The brain not only influences the course of intestinal disease but also receives input from the gut and its microbial community. Commensal bacteria that colonize the gut play a key role in maintaining intestinal homeostasis: chronic stress can lead to changes in the composition of the microbiota [13], and intestinal dysbiosis induced by infection with the pathogen *Citrobacter rodentium* increased the risk for developing mood disorders following exposure to acute stress. [7]

The mechanisms by which the microbiota interacts with the gut and the brain are complex. Recent studies suggest that Nod-like receptors, a group of intracellular proteins involved in the innate immune response, may play key roles in mediating the MGB axis. Secretions from the crypts of terminal ileum taken from Nod deficient mice show decreased killing of gram-negative and gram-positive bacteria, while mice re-derived in germ-free conditions express fewer Nod receptors. [9]

The importance of Nod receptors in maintaining the microbiota and regulating the immune response in the presence of pathogens have been documented by previous studies. [9] However, the role Nod receptors play in maintaining neurological function through communications between the gut and the brain remains unclear. In this project, we evaluate behavioral changes in Nod deficient mice through tests for anxiety and recognition memory. We hypothesize that the absence of Nod-like receptors is associated with behavioral changes – specifically, anxiety-like behavior – at baseline. Nod deficient mice may also demonstrate increased susceptibility to developing memory defects upon exposure to acute psychological stress.

MATERIALS AND METHODS

Animals

Nod double-knockout (NodDKO; bred on a C57BL/6 background) mice were provided by Dr. Dana Philpott, University of Toronto, and bred in the UCSD medical teaching facility building vivarium. C57BL/6 mice were used as controls. Both male and female mice were studied. Mice were housed under standard 12-h light-dark conditions with ad libitum access to food and water. All behavioral experiments were performed in a biosafety cabinet. All animal procedures and protocols were approved by the University of California San Diego Institutional Animal Care and Use Committee (IACUC protocol #S12068).

Water Avoidance Stress

Exposure to WAS induces acute psychological stress in mice by exploiting their innate fear of water. [11] Mice are placed on a small platform surrounded by shallow (1-2cm), room temperature water in a covered plastic clear mouse cage for 1 hour. Previous studies have found HPA-axis activation and altered colonic physiology (increased secretions and mucosal barrier dysfunction) that last up to 4 hours after WAS. [1]

Serum corticosterone

Blood was collected at euthanasia by cardiac puncture and serum isolated by centrifugation. Serum corticosterone was evaluated by commercial enzyme immunoassay [EIA].

Behavioral Testing

Light/dark box test - This test measures anxiety using a light/dark box setup. A non-heating fluorescent bulb illuminates 2/3 of an open cage while the other 1/3 area is made dark by a box fitted inside the cage. The mouse may transit freely between the light and dark box through an entryway into the dark box. This test utilizes the mouse's natural tendency to explore new areas with its innate preference for the safety of the dark to assess its level of anxiety. Increased time spent in the dark suggests an increased level of anxiety which leads the mouse to seek security over exploration. During the test, the mouse is placed in the light area and videotaped for 10 minutes. The amount of time (seconds) spent in the light area and the number of transitions from the dark to the light area are tabulated. Decreased time in the light alongside increased number of transitions indicate anxiety-like behavior.

Novel object recognition (NOR) task - This behavioral test assesses for recognition memory and serves as a measure of dorsal hippocampal function. Mice have an inherent tendency to explore novel objects more frequently than familiar ones in their environment. After 1 hour habituation in individual cages with clean bedding, mice are presented with two distinct objects and their behavior recorded on video camera for 5 minutes. A subset of mice will undergo WAS following habituation, then immediately proceed to the NOR task. The objects used are a solid napkin ring (A), a star-shaped cookie cutter (B), and a checkered napkin ring (C). Preliminary studies established equal preference for the objects used. [7]

The test consists of two parts: a training phase and a testing phase. During the training phase, mice are exposed to a solid napkin ring (object A) and a star-shaped cookie cutter (object B) placed in opposite corners of an open cage for 5 minutes. Touching or pointing the nose at the object in order to smell it (within 2cm) counts as an exploratory bout. Each bout must be separated by a period during which the mouse suspends its exploration of the object in favor of other activities. The objects are removed after 5 minutes.

The mouse is allowed to rest for 20 minutes in individual cages before proceeding to the testing phase. During testing, the star-shaped cookie cutter from training phase (now object B2) and the checkered napkin ring (object C) are placed in opposite corners. An exploration ratio is calculated in order to assess for the degree of preference, if any, the mouse exhibits for the novel

object C over the familiar object B2: freq. smell C/(freq. smell C + freq. smell B2) * 100%. A ratio of 50% indicates that the mouse does not distinguish between the familiar and the novel object and suggests impaired memory.

Study Design

Mice (C57BL/6, NodDKO) were tested for behavior. Behavioral tests were performed in the same mice on the same day with a rest period between tests. The light/dark box was performed first, followed by habituation and then the NOR task. Mice undergoing WAS were subjected to this stressor immediately prior to behavior testing. For the NOR task, mice were subjected to WAS immediately after habituation.

Statistics

Results are presented as means ± SE. Differences of $P < 0.05$ were considered as significant.

RESULTS

NodDKO mice have an overactive HPA-axis that is further enhanced by exposure to WAS.

NodDKO mice show elevated baseline serum corticosterone levels compared to B6 mice. Exposure to acute stress (WAS) led to increased serum corticosterone over baseline for NodDKO and B6 mice (FIGURE 1).

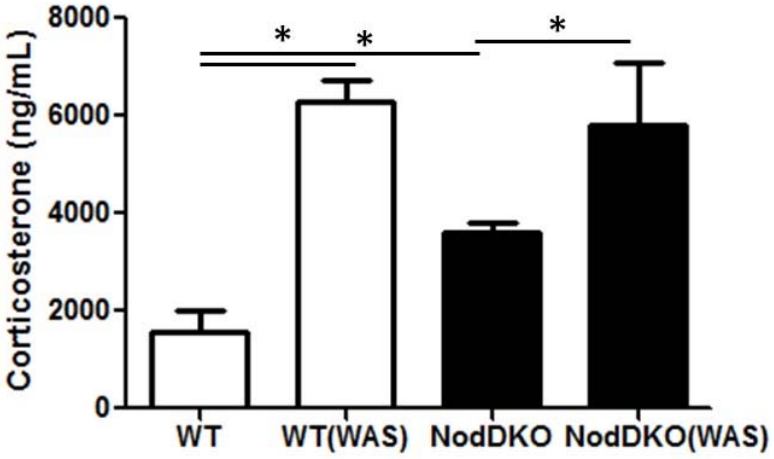

Figure 1. **The HPA-axis is increased at baseline in NodDKO mice.** Exposure to acute stress (WAS) led to increased serum corticosterone over baseline for NodDKO and B6 mice (N= 21; *p<0.05). The difference in serum corticosterone between the two groups at baseline was abrogated after WAS.

NodDKO mice display a stress-induced cognitive deficit.

No difference was observed in exploration ratio (using the NOR task) between B6 and NodDKO mice at baseline. However, NodDKO mice demonstrated impaired memory when exposed to acute stress (WAS) in contrast to B6 mice (FIGURE 2).

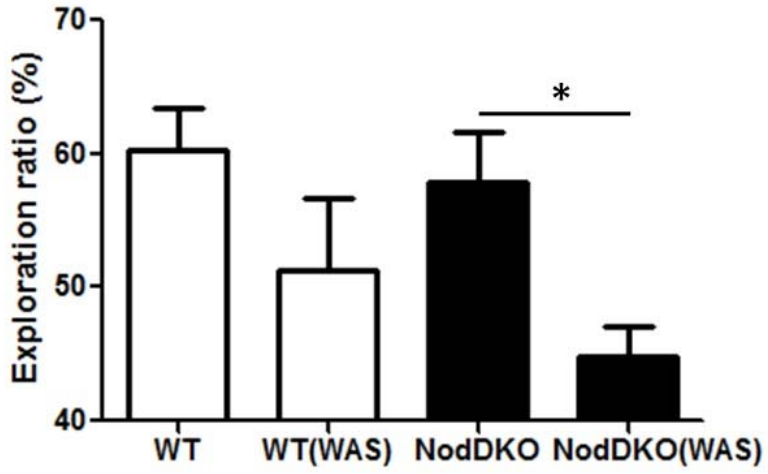

Figure 2. Non-spatial memory is altered in mice with defects in innate immunity. While the exploration ratio between B6 and NodDKO mice did not differ at baseline, NodDKO mice displayed susceptibility to memory impairment upon exposure to acute stress (WAS) in contrast to B6 mice (N=10; *$p<0.05$).

NodDKO mice exhibit increased anxiety-like behavior.

Anxiety-like behavior was observed in NodDKO mice at baseline compared to B6 mice as demonstrated by the decreased time spent in the light box in the light/dark box test (FIGURE 3). Exploratory behavior was also reduced as demonstrated by the decreased frequency of transition from the light/dark compartment. Exposure to WAS did not further impact the increased anxiety-like behavior or the decreased exploratory behavior seen in NodDKO mice.

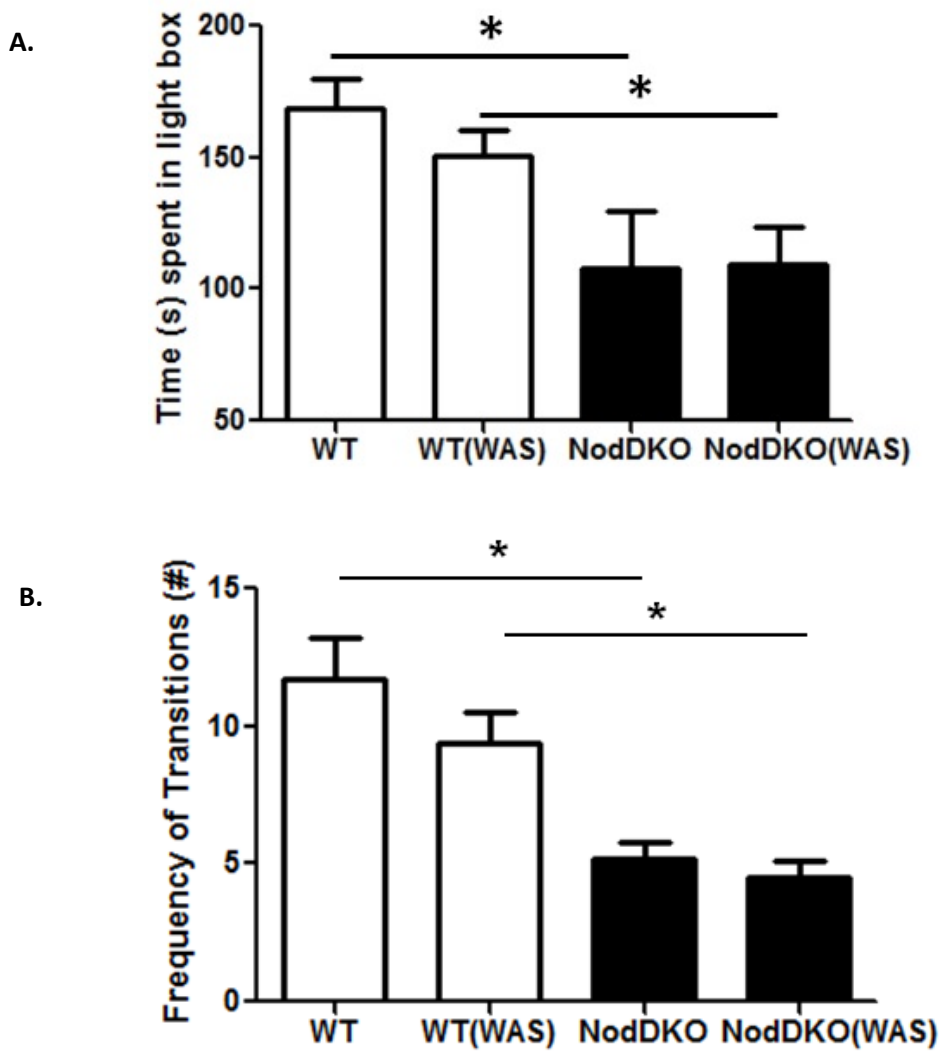

Figure 3. Anxiety-like behavior is present in NodDKO mice. At baseline, NodDKO mice were observed to exhibit anxiety-like behavior compared to B6 mice as demonstrated by the decreased time spent in the light box **(A).** Frequency of transition between the dark and light box, a measure of exploratory behavior, was also reduced **(B).** Exposure to WAS did not significantly alter the increased anxiety-like behavior or the decreased exploratory behavior seen in NodDKO mice (N= 10; *$p<0.05$).

DISCUSSION

Multiple genetic and environmental factors contribute to the pathogenesis and pathophysiology of IBD. This project seeks to better understand how the MGB axis contributes to disease establishment and exacerbation in a genetically susceptible host. Though Nod receptor deficiency in this study was not associated with an appreciable defect in recognition memory at

baseline, NodDKO mice developed significant memory defects upon exposure to acute stressors. The absence of Nod-like receptor function also results in an overactive HPA-axis that is further enhanced by exposure to acute stress. These behavioral effects in NodDKO mice may be mediated, at least in part, by constitutive over-activity of the HPA-axis.

The results of this study support the role of Nod receptors as a potential signaling platform for communication between the brain and gut through the innate immune system. Previous studies have demonstrated no significant differences in the relative abundance of bacterial groups in the gut between wild type and Nod-deficient mice, thus suggesting that Nod-associated immune differences alone do not promote dysbiosis[14]. Housing conditions were also found to influence gut microbiota composition and may confound studies data from wild type and Nod-deficient mice, thus requiring that future studies minimize environmental sources of variation[14]. Further explorations of the relationship between Nod receptor signaling and the brain-gut axis may begin with establishing baseline conditions for gut microbiota homeostasis in Nod-deficient mice in order to understand the microbial dysbiosis associated with defective innate immunity and IBD.

References

1. Cameron, Heather L., and Mary H. Perdue. "Stress impairs murine intestinal barrier function: improvement by glucagon-like peptide-2." *Journal of Pharmacology and Experimental Therapeutics* 314.1 (2005): 214-220.

2. Capuron, Lucile, and Andrew H. Miller. "Immune system to brain signaling: neuropsychopharmacological implications." *Pharmacology & therapeutics* 130.2 (2011): 226-238.

3. Clarke, Thomas B., et al. "Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity." *Nature medicine* 16.2 (2010): 228-231.

4. Farrokhyar, Forough, et al. "Functional gastrointestinal disorders and mood disorders in patients with inactive inflammatory bowel disease: prevalence and impact on health." *Inflammatory bowel diseases* 12.1 (2006): 38-46.

5. Gareau, Mélanie G., et al. "Neonatal maternal separation causes colonic dysfunction in rat pups including impaired host resistance." *Pediatric research* 59.1 (2006): 83-88.

6. Gareau, Mélanie G., Philip M. Sherman, and W. Allan Walker. "Probiotics and the gut microbiota in intestinal health and disease." *Nature Reviews Gastroenterology and Hepatology* 7.9 (2010): 503-514.

7. Gareau, Mélanie G., et al. "Bacterial infection causes stress-induced memory dysfunction in mice." *Gut* 60.3 (2011): 307-317.

8. Hasegawa, Mizuho, et al. "Nucleotide-binding oligomerization domain 1 mediates recognition of Clostridium difficile and induces neutrophil recruitment and protection against the pathogen." *The Journal of Immunology* 186.8 (2011): 4872-4880.

9. Petnicki-Ocwieja, Tanja, et al. "Nod2 is required for the regulation of commensal microbiota in the intestine." *Proceedings of the National Academy of Sciences* 106.37 (2009): 15813-15818.

10. Robertson, Susan J., and Stephen E. Girardin. "Nod-like receptors in intestinal host defense: controlling pathogens, the microbiota, or both?." *Current opinion in gastroenterology* 29.1 (2013): 15-22.

11. Saunders, Paul R., et al. "Physical and psychological stress in rats enhances colonic epithelial permeability via peripheral CRH." *Digestive diseases and sciences* 47.1 (2002): 208-215.

12. Silberman, Dafne Magalí, Miriam Wald, and Ana María Genaro. "Effects of chronic mild stress on lymphocyte proliferative response. Participation of serum thyroid hormones and corticosterone." *International immunopharmacology* 2.4 (2002): 487-497.

13. Zareie, Mehri, et al. "Probiotics prevent bacterial translocation and improve intestinal barrier function in rats following chronic psychological stress." *Gut* 55.11 (2006): 1553-1560.

14. Robertson, SJ, et al. “Nod1 and Nod2 signaling does not alter the composition of intestinal bacterial communities at homeostasis.” *Gut Microbes* 4.3 (2013): 222-231.