PNAS nexus

PNAS Nexus, 2022, **1**, 1–5
https://doi.org/10.1093/pnasnexus/pgac271
Advance access publication date: 2 December 2022
Brief Report

Human threat learning is associated with gut microbiota composition

Javiera P. Oyarzun [a,b], Thomas M. Kuntz[c], Yoann Stussi[a], Olivia T. Karaman[a], Sophia Vranos[a], Bridget L. Callaghan[d], Curtis Huttenhower [c], Joseph E. LeDoux [b,e] and Elizabeth A. Phelps[a,f,*]

[a]Department of Psychology, Harvard University, Cambridge, MA 02138, USA
[b]Center for Neural Science and Department of Psychology, New York University, New York NY, 10003, USA
[c]Department of Biostatistics, Microbiome Analysis Core, Harvard T.H. Chan School of Public Health, Boston, MA 02115, USA
[d]Department of Psychology, UCLA, Los Angeles, CA 90095, USA
[e]Department of Psychiatry and Department of Child and Adolescent Psychiatry, New York University Langone Medical School, New York, NY 1003, USA
[f]Center for Brain Science, Harvard University, Cambridge, MA, 02138, USA
*To whom correspondence should be addressed: 52 Oxford Street, Cambridge, MA 02138, USA; Email: phelps@fas.harvard.edu
Edited By: Paulo Boggio

Abstract

The ability to learn about threat and safety is critical for survival. Studies in rodent models have shown that the gut microbiota can modulate such behaviors. In humans, evidence showing an association with threat or extinction learning is lacking. Here, we tested whether individual variability in threat and extinction learning was related to gut microbiota composition in healthy adults. We found that threat, but not extinction learning, varies with individuals' microbiome composition. Our results provide evidence that the gut microbiota is associated with excitatory threat learning across species.

Keywords: humans, gut microbiota, threat conditioning, learning, anxiety

Significance Statement:

Learning about threats and safety is critical for survival, and studies in rodent models have shown that the gut microbiota can modulate such behaviors. Although previous literature on humans shows a relationship between emotional circuits and gut microbiota, the evidence linking learning and microbiota is lacking. In a Pavlovian threat conditioning paradigm, we show that patterns of gut microbiota composition in healthy humans relate to their patterns of threat learning, but not safety learning. Our findings suggest one mechanism by which the human gut microbiota may be associated with anxiety-related behaviors.

Introduction

Studies in rodents have demonstrated that the gut microbiota—the collective of all microorganisms that inhabit the host's large intestine—can modulate hippocampal and amygdala-dependent learning as well as anxiety-like behaviors (1). For example, rodents treated with probiotics—live bacteria—show better memory and decreased anxiety-like behaviors compared with non-treated or germ-free groups (mice lacking gut microbiota) (2–5). Furthermore, probiotic administration can have anxiolytic effects and reverse memory impairments observed after protocols of gut infection (through the administration of pathogenic bacteria) or chronic stress (6, 7).

One clinically relevant learning mechanism related to anxiety phenotypes is the ability to form associations with threat and safety outcomes. Studies in rodents have investigated threat and safety learning using Pavlovian threat acquisition and extinction tasks, respectively. Manipulations of gut microbiota through probiotics and heat-killed bacteria facilitate threat acquisition in rodents, as measured by enhanced defensive responses to threatful cues and contexts (3–5). However, for extinction learning, results have been less consistent, with some showing extinction impairment or persistence of defensive responses (3, 5), others facilitation (6, 7), and still others no effect (4) for safety learning after microbiota manipulations.

In humans, evidence linking threat and safety learning and microbiota composition is scarce and indirect. Both threat and safety learning (8) and gut microbiota profiles (9) are altered in anxiety patients compared to healthy individuals, and neuroimaging studies have shown an association between functional and structural brain circuits of threat processing and microbiota composition (10, 11). These previous studies suggest that there might be microbiota patterns associated with threat and safety learning in humans, but they do not directly assess this relationship. In the present study, we sought to test this possibility.

Competing Interest: The authors declare no competing interest.
Received: June 23, 2022. **Accepted:** November 30, 2022

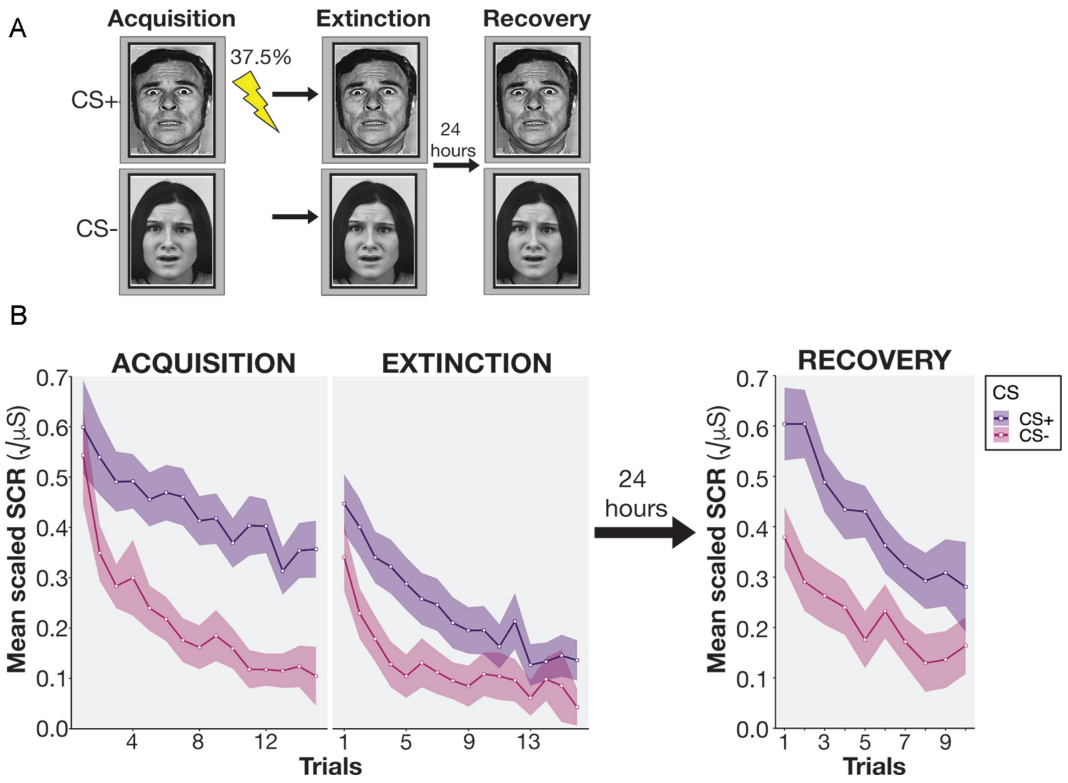

Fig. 1. (A) Threat conditioning task. *Acquisition:* Participants were shown two fear faces. One picture, the conditioned stimulus (CS + no shock, 15 trials) was contingently paired with a mild electric shock to participants' wrist (CS + shock, 9 trials) according to a partial reinforcement schedule, while the other was never paired with shock (CS–, 15 trials). *Extinction:* Immediately following acquisition, participants were presented with the CS+ and CS– (16 trials) without shock. *Recovery:* After 24 hours, CS+ and CS– were presented without shock (10 trials each). (B) Skin conductance responses during threat conditioning for nonshocked CS+ and CS– trials. *faces reprinted with permission from the Paul Ekman Group.

Methods and results

One hundred and twenty-seven healthy individuals (final sample size of 117 participants, see Extended Methods (EM) for inclusion criteria) underwent a Pavlovian threat conditioning paradigm, while skin conductance responses (SCR) were recorded as a read-out of defensive responses (Fig. 1).

After the first session day or the next morning, participants provided a stool sample for 16S ribosomal RNA gene amplicon

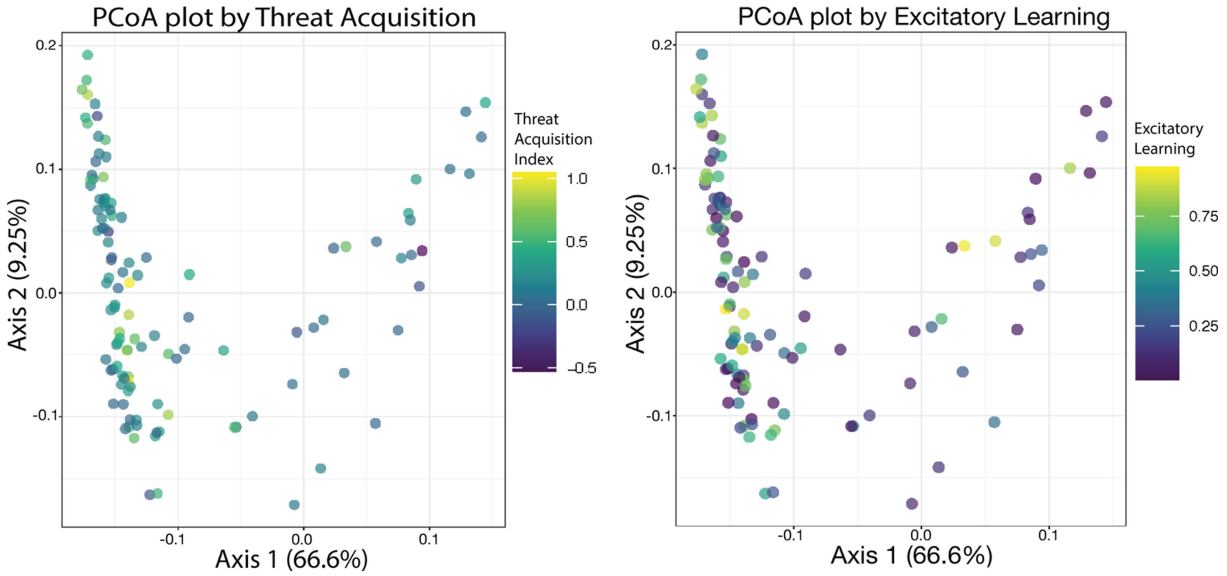

Fig. 2. Principal coordinate analyses for threat acquisition and excitatory learning. Ordinations of all subjects' microbial genera profiles by weighted UniFrac. While neither threat acquisition nor excitatory learning indices were associated with the two major axes of variation, as expected, both showed significant association with overall microbiome composition (threat acquisition: $R^2 = 0.045$, $P = 0.012$; excitatory learning: $R^2 = 0.041$, $P = 0.009$).

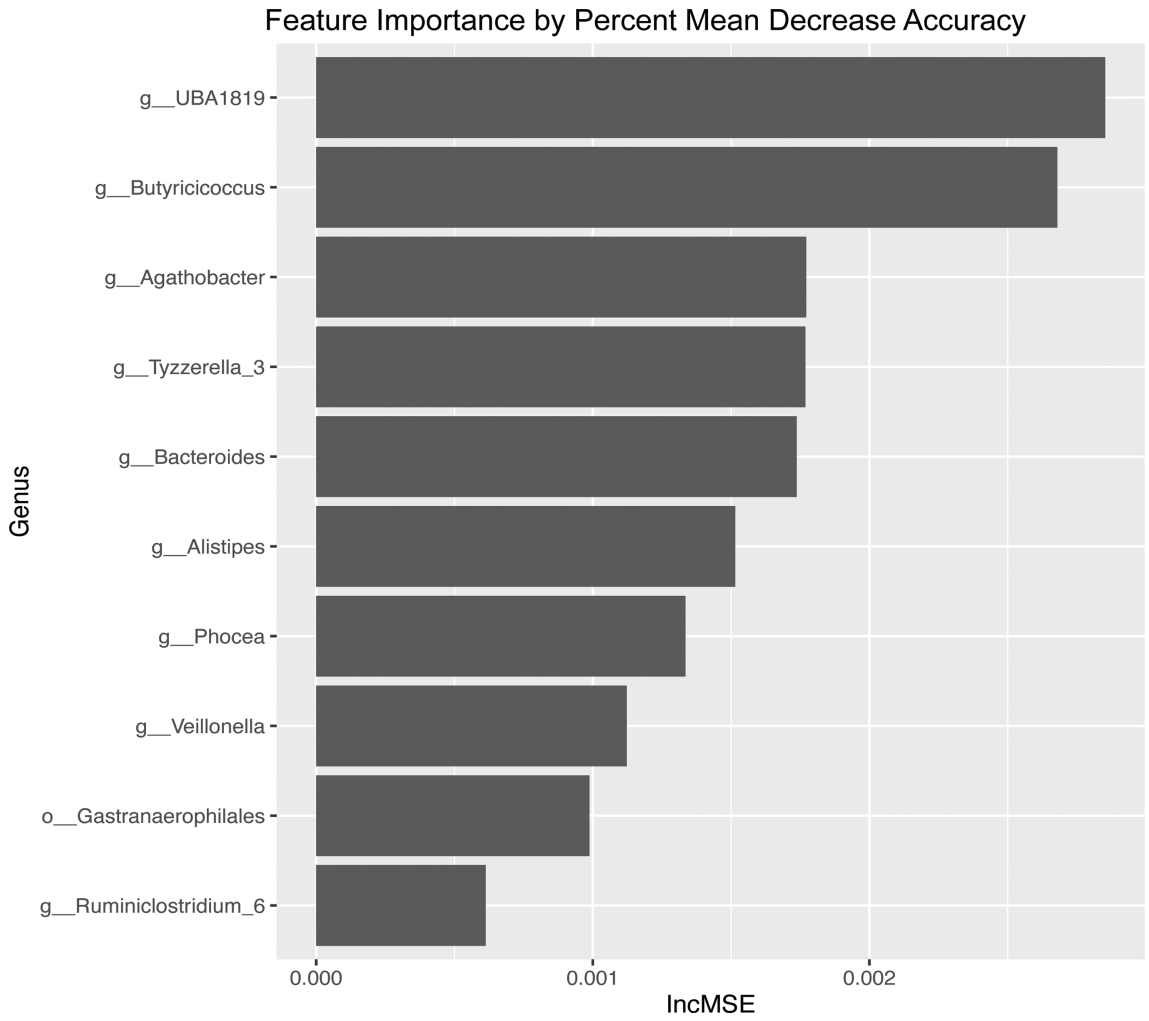

Fig. 3. Feature importance tables for threat acquisition. Tables depict the top 10 features predicting threat acquisition according to random forest model ($R^2 = 0.027$, $P = 0.022$). Importance scores were calculated by % increase in mean square error.

sequencing. Questionnaires were administered to assess factors that have been previously associated with gut microbiota composition, referred to as control variables: age, gender, BMI, human milk feeding versus formula, mode of birth, exercise, current pet cohabitation, childhood pet cohabitation, and diet. These variables were controlled for by adding them to the permutational multivariate analysis of variance (PERMANOVA) and random forest models.

From the normalized SCR data, we calculated *threat acquisition*, *extinction learning*, and *recovery indexes* (see EM). Furthermore, since threat learning paradigms engage a combination of excitatory (i.e., updating due to presence of shock) and inhibitory (i.e., updating due to absence of shock) learning processes, we evaluated individuals' bias to learn from the presence or absence of aversive outcomes. We fit a hybrid reinforcement model to the trial-by-trial SCR data from acquisition and extinction (see EM) and extracted each participant's excitatory and inhibitory learning rates. This allowed us to quantify individual differences in the latent processes that underlie excitatory and inhibitory threat learning.

We performed taxonomic profiling on our sequenced dataset and calculated associated ecological metrics from participants' profiles. There was no association between gut microbiota alpha diversity (species inverse Simpson index) and any threat learning parameters. Next, we performed a PERMANOVA ($n = 999$ permutations) on genus level, weighted UniFrac beta diversities (see EM) in a multivariable model for each threat learning parameter with control variables. We found significant associations for the threat acquisition index (weighted UniFrac $R^2 = 0.045$, $P = 0.012$) and excitatory learning rates (weighted UniFrac $R^2 = 0.041$, $P = 0.009$) (see EM Tables S3a and b). These results suggest that participants who show a different index of threat acquisition and excitatory learning also show different patterns of microbiota composition (Fig. 2).

To identify specific taxa associated with individuals' threat acquisition index and excitatory learning, we used generalized linear models implemented in MaAsLin2 (see EM). Relative abundance of three taxa showed a significant relationship (FDR $q < 0.2$) with participants' acquisition index: UBA1819 (an unclassified *Faecalibacterium* taxon), *Tyzzerela*_3, and *Bacteroides*. However, these did not remain individually significant in a robustness analysis (see EM), and their linear associations were likely outlier-driven.

To test for multivariable associations, we implemented a random forest classifier (see EM) across the same set of genus abundances. In agreement with our PERMANOVA results, the model significantly associated overall microbial community composition with threat acquisition index ($R^2 = 0.027$, $P = 0.022$). The top eight taxa included *Butyricicoccus*, *Agathobacter*, *Alistipes*, *Pho-*

cea, and *Veillonella*, in addition to the three taxa identified univariately above (Fig. 3). This suggests that the combined relative abundance of various taxa could be more important in threat acquisition than the abundance of any single taxon. Alternatively, it could be that interindividual variation in one specific microbial carriage makes univariate tests underpowered in this population.

In contrast to rodent findings, no relationship between gut microbiota composition and extinction learning index, inhibitory learning, or recovery was observed (see EM).

Discussion

The association found between gut microbiota and learned defensive responses aligns with previous research in rodents and humans. In rodents, the absence of gut microbiota and the administration of probiotics alter defensive responses during threat acquisition [2, 7]. In humans, differences in gut microbiota have been associated with structural differences in the amygdala and differences in the functional connectivity in neural networks related to emotional processing and fear [10, 11, 12]. Even though these previous studies were small in sample size, conducted in diverse populations (i.e., infants, adult women, obese adults, or patients with GI symptoms), and used different methods of characterizing gut microbiota composition (10), these findings support the idea that the gut microbiota composition is associated with neural circuits implicated in threat acquisition and its expression.

Anxiety disorders have been associated with both enhanced acquisition of defensive responses to potential threats (excitatory learning) and impaired safety learning (inhibitory learning) (8). Threat acquisition and extinction are mediated by overlapping but distinct, neural circuits (13). The current results suggest that the relation between anxiety and microbiota composition in humans may depend more on variation in the amygdala-dependent acquisition of learned defensive responses than on the ventral prefrontal inhibitory circuits underlying extinction/inhibitory learning and recovery of defensive responses (12).

Although theoretical pathways linking brain circuits of threat acquisition and gut microbiota have been suggested (2), our results do not indicate specific mechanisms by which the gut microbiota might relate to threat circuits or even the specific direction of potential causality. In our dataset, no clear associations were found between individual taxon abundances and skin conductance responses, but rather broad microbiota patterns were indirectly associated. The associations found in our dataset may rely on microbial chemical products or functions shared by multiple taxa or external influences that affect both the microbiome and the threat acquisition. Alternatively, we may have lacked the power to find individual relationships with specific taxa. Larger study populations and the use of methods focused on microbiota functional profiling (metagenomics, transcriptomics, and metabolomics) could help identify potential mechanisms underlying current findings.

Even though our data cannot speak to an underlying mechanism, one possible biological pathway for this interaction is the influence of the gut microbiota on the host's immune system and the effect of systemic inflammation on mood and anxiety symptoms (14). For example, a recent meta-analysis showed that patients with psychiatric illnesses such as depression and anxiety possessed an inflammatory phenotype associated with an enrichment of proinflammatory bacteria and depletion of anti-inflammatory butyrate-producing bacteria when compared with healthy individuals (15). Although some of the taxa identified by our random forest model have been previously associated with anti-inflammatory activity (i.e., *Butyricicoccus*, *Faecalibacterium*, and *Bacteroides*), there is a need for a larger cohort that expands the range of baseline microbial configurations—especially for low prevalence taxa—to confirm (or deny) the associations with defensive responses found here.

The present study found that gut microbiota composition in humans is associated with threat acquisition and excitatory learning using two omnibus microbiome analysis techniques (PERMANOVA and random forest analyses), which controlled for confounding factors associated with microbiota composition (age, gender, BMI, human milk feeding versus formula, mode of birth, exercise, current pet cohabitation, childhood pet cohabitation, and diet).

Although our sample only included healthy adults, the findings suggest that enhanced excitatory threat learning may be one factor contributing to the association between anxiety and gut microbiota. Emerging evidence linking the gut microbiome to mental health harbors the potential for innovative treatment approaches. However, it is necessary to determine the unique and modifiable factors that underlie this relationship. This finding represents a step in this direction by linking a specific learning component of an anxiety phenotype with overall microbiome composition in adult humans.

Acknowledgments

We want to thank Dr. Anne-Catrin Uhlemann and Felix Rozenberg for their insights during stool sample processing and sequencing, and Yasmine Elasmar and Eugenia Zhukovsky for their help during data collection, and Jeremy E. Wilkinson for his support during data analysis.

Supplementary Material

Supplementary material is available at *PNAS Nexus* online (Extended Methods).

Funding

This research has been supported by the Vulnerable Brain Project (vbp.life) to J.E.L. and E.A.P., the James S. McDonnell Foundation to E.A.P., and the National Center for Advancing Translational Science (NCATS) from the National Institute of Health through the grant award number TL1TR001447 to J.P.O. Y.S. was supported by an Early Postdoc Mobility Fellowship from the Swiss National Science Foundation (187911).

Authors' Contributions

J.P.O., E.A.P., B.L.C., and J.E.L.: conception and design; J.P.O.: data acquisition; J.P.O., T.K., O.K., S.V., and Y.S.: data analysis; J.P.O., T.K., Y.S., E.A.P., and C.H.: data interpretation; and J.P.O., T.K., Y.S., E.A.P., and C.H.: writing paper.

Preprints

A preprint of this article is published at https://doi.org/10.1101/2022.06.13.495985.

Data Availability

Demographic information, behavioral data, ASV counts, and taxonomic assignments are available upon request at https://osf.io/rfhxu/?view_only=02023f70035a49e6b56eb969a50ae3ea.

References

1. Luczynski P, *et al.* 2016. Growing up in a bubble: using germ-free animals to assess the influence of the gut microbiota on brain and behavior. Int J Neuropsychopharmacol. 19:pyw020.
2. Malan-Muller S, *et al.* 2017. The gut microbiome and mental health: implications for anxiety- and trauma-related disorders. OMICS J Integr Biol. 22:90–107.
3. Bravo JA, *et al.* 2011. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci USA. 108:16050–16055.
4. Savignac HM, Tramullas M, Kiely B, Dinan TG, Cryan JF. 2015. Bifidobacteria modulate cognitive processes in an anxious mouse strain. Behav Brain Res. 287:59–72.
5. Ishikawa R, Fukushima H, Nakakita Y, Kado H, Kida S. 2019. Dietary heat-killed *Lactobacillus brevis* SBC8803 (SBL88^{TM}) improves hippocampus-dependent memory performance and adult hippocampal neurogenesis. Neuropsychopharmacol Rep. 39:140–145.
6. Cui X, *et al.* 2021. A multispecies probiotic accelerates fear extinction and inhibits relapse in mice: role of microglia. Neuropharmacology. 193:108613.
7. Chu C, *et al.* 2019. The microbiota regulate neuronal function and fear extinction learning. Nature. 574:543–548.
8. Duits P, *et al.* 2015. Updated meta-analysis of classical fear conditioning in the anxiety disorders. Depress Anxiety. 32:239–253.
9. Jiang H-Y, *et al.* 2018. Altered gut microbiota profile in patients with generalized anxiety disorder. J Psychiatr Res. 104:130–136.
10. Tillisch K, *et al.* 2017. Brain structure and response to emotional stimuli as related to gut microbial profiles in healthy women. Psychosom Med. 79:905.
11. Hall CV, *et al.* 2022. Microbiota links to neural dynamics supporting threat processing. Hum Brain Mapp. 43:733–749.
12. Knapska E, *et al.* 2012. Functional anatomy of neural circuits regulating fear and extinction. Proc Natl Acad Sci. 109:17093–17098.
13. Marek R, Sah P. 2018. Neural circuits mediating fear learning and extinction. In: Cheung-Hoi Yu A, Li L, editors. Systems neuroscience. Cham: Springer International Publishing. p. 35–48.
14. Peirce JM, Alviña K. 2019. The role of inflammation and the gut microbiome in depression and anxiety. J Neurosci Res. 97:1223–1241.
15. Nikolova VL, *et al.* 2021. Perturbations in gut microbiota composition in psychiatric disorders: a review and meta-analysis. JAMA Psychiatry. 78:1343–1354.