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Social anxiety disorder-associated gut microbiota increases social fear

Nathaniel L. Ritz[a,b], Marta Brocka[a,1], Mary I. Butler[a,c,1], Caitlin S. M. Cowan[a,1,2], Camila Barrera-Bugueño[a], Christopher J. R. Turkington[a,d], Lorraine A. Draper[a,d], Thomaz F. S. Bastiaanssen[a,b], Valentine Turpin[a], Lorena Morales[a], David Campos[a], Cassandra E. Gheorghe[a,b,c], Anna Ratsika[a,b], Virat Sharma[a,d], Anna V. Golubeva[a], Maria R. Aburto[a,b], Andrey N. Shkoporov[a,d], Gerard M. Moloney[a,b], Colin Hill[a,d], Gerard Clarke[a,c], David A. Slattery[e], Timothy G. Dinan[a,c], and John F. Cryan[a,b,3]

Edited by Tomas Hökfelt, Karolinska Institutet, Stockholm, Sweden; received May 29, 2023; accepted October 5, 2023

Social anxiety disorder (SAD) is a crippling psychiatric disorder characterized by intense fear or anxiety in social situations and their avoidance. However, the underlying biology of SAD is unclear and better treatments are needed. Recently, the gut microbiota has emerged as a key regulator of both brain and behaviour, especially those related to social function. Moreover, increasing data supports a role for immune function and oxytocin signalling in social responses. To investigate whether the gut microbiota plays a causal role in modulating behaviours relevant to SAD, we transplanted the microbiota from SAD patients, which was identified by 16S rRNA sequencing to be of a differential composition compared to healthy controls, to mice. Although the mice that received the SAD microbiota had normal behaviours across a battery of tests designed to assess depression and general anxiety-like behaviours, they had a specific heightened sensitivity to social fear, a model of SAD. This distinct heightened social fear response was coupled with changes in central and peripheral immune function and oxytocin expression in the bed nucleus of the stria terminalis. This work demonstrates an interkingdom basis for social fear responses and posits the microbiome as a potential therapeutic target for SAD.

microbiota-gut-brain axis | social phobia | faecal transplant | microbiome

Significance

Understanding the biological basis of social anxiety disorder (SAD), one of the most disabling of the anxiety disorders, will allow for novel treatment strategies to be developed. Here, we show that gut microbiota may be such a target. Mice that received SAD patient microbiota had a specific heightened sensitivity to social fear without affecting other behaviours tested. This distinct deficit in normal social fear responses was coupled with changes in immunity and the brain.

Social anxiety disorder (SAD) is a common psychiatric disorder characterized by intense fear or anxiety in social scenarios. The onset of SAD typically occurs early in life (childhood or adolescence), and it frequently has a lifelong impact being associated with considerable functional disability and reduced quality of life (1–4). Current pharmacological treatment options for SAD are limited and often ineffective, and new models are necessary to understand the underlying etiology and treatment targets (5). Recent data have implicated the gut microbiota in brain and behavioural function related to social processes across the animal kingdom (6). Intriguingly, we recently showed that patients with SAD have a different microbiota composition than age-matched healthy controls (HC) (7). However, such correlative analysis requires insight to understand whether the compositional changes observed may be mechanistically related to social fear or anxiety. To test causal mechanisms, faecal microbiota transplantation (FMT) studies have emerged as an effective technique to understand how the microbiota can potentially elicit phenotypic changes in the host. Furthermore, our approach adheres to both the recommended practice and to the Guidelines for Reporting on Animal Faecal Transplantation (GRAFT) (8, 9). Previous research in major depression, anxiety comorbid with irritable bowel syndrome and schizophrenia, show that transfer of the microbiota via FMT is sufficient to transfer selective psychological and physiological traits of these disorders, including depression- and anxiety-like behaviour, to the recipient (10–15).

To further elucidate the role of the microbiota in SAD, we designed a translational study to test the hypothesis that the microbiota may play a causal role in promoting host social fear behaviour (Fig. 1*A*). Faecal inocula from individual HC and SAD donors were anaerobically processed for cryogenic storage and later use whereby adult male C57Bl/6J mice received FMT from each donor (Fig. 1*B*). Following antibiotic depletion of the microbiota, mice received the specified donor FMT, and were subsequently tested for social fear, sociability, social cognition, and stress-coping behaviours, as well as gastrointestinal transit and motility (Fig. 1*C*).

Results

Social Anxiety Disorder Leads to Alterations in the Bacteriome. Gut microbiota composition was characterized using 16S rRNA sequencing performed on microbial DNA extracted from stool from individuals with SAD and matched HC (Fig. 1*D*). This analysis

Author contributions: N.L.R., M.B., M.I.B., C.S.M.C., L.A.D., V.T., L.M., M.R.A., A.N.S., G.M.M., C.H., G.C., D.A.S., T.G.D., and J.F.C. designed research; N.L.R., M.B., M.I.B., C.S.M.C., C.B.-B., C.J.R.T., L.A.D., T.F.S.B., V.T., L.M., D.C., C.E.G., A.R., V.S., A.V.G., and G.M.M. performed research; N.L.R., M.B., M.I.B., C.J.R.T., L.A.D., T.F.S.B., D.C., M.R.A., A.N.S., G.M.M., C.H., G.C., and D.A.S. contributed new reagents/analytic tools; N.L.R., M.B., C.J.R.T., L.A.D., T.F.S.B., A.V.G., and G.M.M. analyzed data; and N.L.R. and J.F.C. wrote the paper with contributions made from all authors.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

[1]M.B., M.I.B., and C.S.M.C. contributed equally to this work.

[2]Present address: School of Psychology, University of New South Wales, Sydney 2052, Australia.

[3]To whom correspondence may be addressed. Email: J.Cryan@ucc.ie.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2308706120/-/DCSupplemental.

Published December 26, 2023.

confirmed a distinct compositional difference as noted previously based on a metagenomic analysis ($R^2 = 0.03477$, $P < 0.05$, 1,000 permutations) (7). We selected six SAD and six HC individuals based on host parameters and metrics including microbiota, Liebowitz Social Anxiety Scale score, age, body mass index, sex, and an absence of psychotropic or microbiota medications (i.e., antidepressants, anxiolytics, antibiotics, probiotics, prebiotics, etc.; *SI Appendix*, Table S2). In addition to measuring the bacteriome (the collective bacterial genome) which makes up the majority of the active portion of the microbiome, we also measured the virome (the collective viral genome) which is primarily made up of bacteriophages that specifically infect bacteria (16).

Murine recipient bacteriome beta diversity was significantly altered between SAD and HC groups measured at the end of the study (Fig. 1*E*; $R^2 = 0.02767$, $P < 0.001$, 1,000 permutations). There were three bacterial species that were differentially abundant in the SAD and HC groups at the end of the study, *Bacteroides nordii*, *Bacteroides cellulosiyticus*, and *Phocaeicola massiliensi* (*SI Appendix*, Fig. S1 *A–C*). No group differences were found in alpha diversity metrics at the end of the study (Chao1, Shannon, and Simpson; *SI Appendix*, Fig. S1 *D–F*), but each alpha diversity metric was reduced at the end of study compared to the pretreatment measures (*SI Appendix*, Fig. S1 *G–I*). However, no group differences were observed in virome alpha or beta diversity metrics (*SI Appendix*, Fig. S2). This alteration in bacteriome community composition confirms that the FMT from SAD and HC donors led to differential microbiotas recolonizing and engrafting following antibiotic microbiota depletion.

Social Anxiety Disorder Faecal Microbiota Transplant Specifically Increases Sensitivity to Social Fear. Social fear, sociability, anxiety-like, and stress-coping behaviours were assessed in mice following recolonization with SAD or HC microbiota. Social fear conditioning was applied followed by extinction learning on the next day whereby social behaviour was measured by interaction time during repeated exposure to nonsocial and novel social stimuli (Fig. 1*F*). There was a significant reduction in social interaction over the 6 social stimuli trials ($W(1) = 3.239$, $P < 0.05$) and no difference in nonsocial investigatory behaviour. This reduction in social fear extinction rate provides evidence that the SAD microbiota is capable of increasing sensitivity to social fear stimuli. However, general sociability and social novelty preference were not affected by SAD FMT (Fig. 1 *G* and *H* and *SI Appendix*, Fig. S3 *C* and *D*). Furthermore, other tests of stress-coping behaviours and gastrointestinal transit were similarly unaffected (*SI Appendix*, Fig. S3 *A–F*), demonstrating that social fear sensitivity was specifically transferred by SAD FMT.

Social Anxiety Disorder Faecal Microbiota Transplant Diminishes Stress and Immune Function. The glucocorticoid stress hormone corticosterone was measured in plasma of mice before and after the forced swim test. The basal plasma concentration of corticosterone was found to be significantly reduced by SAD FMT treatment ($t(64) = -1.858$, $P < 0.05$) but no differences were found in corticosterone following stress (Fig. 2 *A* and *B*). Following euthanasia, small intestinal ileal tissue was explanted and stimulated with lipopolysaccharide (LPS), concanavalin A (ConA), T cell receptor clusters of differentiation 3 and 28 (CD3/CD28), or vehicle (buffer) to assess gut immune function in response to paradigmatic antigens between groups. Inflammatory cytokine supernatant concentration from the ileal tissue explants was then assessed. The SAD group displayed reduced IL-17A in response to stimulation by LPS ($t(30) = -2.297$, $P < 0.05$) and ConA ($t(32) = -2.457$, $P < 0.05$) and trends for reduced IL-17A in the vehicle ($t(32) = -1.804$, $P = 0.081$) and CD3/CD28 stimulation ($t(30) = -1.813$, $P = 0.100$) (Fig. 3*A*). Mesenteric lymph nodes (MLNs) and blood were also harvested, and immune cell populations were assessed using flow cytometry. In the MLNs, F4/80+ macrophages were significantly reduced ($t(30) = -2.602$, $P < 0.05$) following SAD FMT while there was a trend for T helper cells to be increased following SAD FMT ($t(34) = 1.953$, $P = 0.079$) (Fig. 3 *B* and *C*). Within the blood immune cell population, there was reduced CD4+ T helper cells in the SAD group ($t(34) = -2.049$, $P < 0.05$) (Fig. 3*D*). These data indicate that the SAD microbiota transfer reduced circulating stress hormone and peripheral immunity.

Next, to discern how central immunity was affected, neuroinflammatory- and blood–brain barrier–related gene expression was measured in the bed nucleus of the stria terminalis (BNST), medial amygdala (MeA), and medial prefrontal cortex (MePFC). Arginase 1 (*Arg1*) expression was depleted across all three regions tested (BNST, MeA, and MePFC) following SAD FMT ($t(43) = -3.927$, $P < 0.001$; $t(43) = -3.241$, $P < 0.01$, $t(42) = -3.55$, $P < 0.001$, respectively) (Fig. 3*E*). In the MeA, tumour necrosis factor alpha (*Tnf-α*; $t(35) = -2.081$, $P < 0.05$), interleukin 10 (*Il-10;* $t(36) = -2.342$, $P < 0.05$), and chemokine C-X-C ligand 15 (*Cxcl15;* $t(38) = -2.137$, $P < 0.05$) were all reduced (Fig. 3*E*). In the MePFC, Toll-like receptor 4 (*Tlr4*) was also reduced ($t(40) = -2.839$, $P < 0.05$) (Fig. 3*E*). Then, blood–brain barrier–related genes were measured and the MeA showed the most significant reductions in tight junction protein 1 (*Tjp1*; $t(43) = -3.033$, $P < 0.01$), claudin 5 (*Cldn5*; $t(41) = -2.601$, $P < 0.05$), and occludin (*Ocln*; $t(42) = -1.811$, $P = 0.08$) (Fig. 3*F*). There was an additional trend for reduced *Ocln* in the BNST ($t(43) = -1.901$, $P = 0.06$) and a reduction of *Tjp1* expression in the MePFC ($t(41) = -2.473$, $P < 0.05$) (Fig. 3*F*). These data show that neuroinflammatory markers are altered and blood–brain barrier function is impaired by SAD FMT. This further demonstrates that the SAD microbiota is capable of far-reaching immunomodulatory effects.

Reduced Neuronal Oxytocin in the Bed Nucleus of the Stria Terminalis and Oxytocin-related Gene Expression in the Medial Amygdala and Prefrontal Cortex Are Associated with Increased Social Fear. Oxytocin, an extensive neuropeptide governing social behaviour, was measured in the paraventricular nucleus (PVN), supraoptic nucleus (SON), and the BNST by immunohistochemistry. These brain regions have been shown to play key roles in directing social behaviour through oxytocin generation and signalling. The regions were stained for oxytocin (Oxt) containing neurons (NeuN) in HC and SAD FMT recipients (representative images of the BNST are shown in Fig. 4 *A* and *B*, PVN *SI Appendix*, Fig. S4 *A* and *B*, and SON *SI Appendix*, Fig. S4 *D* and *E*). No differences were observed in either the PVN or SON (*SI Appendix*, Fig. S4 *C* and *F*), but there was a significant reduction in Oxt neurons in the BNST ($t(17) = -2.369$, $P < 0.05$) (Fig. 3*C*). This result indicates that the BNST is sensitive to the SAD microbiota transfer and is associated with social fear behaviour.

Next, oxytocin- and vasopressin-related gene expression was measured in the BNST, MeA, and MePFC to assess potential transcription level alterations between SAD and HC FMT recipient groups. No statistically significant effects were identified in oxytocin gene (*Oxt*) or receptor expression (*Oxtr*) in the BNST, but there were reductions in both genes in the MeA ($t(34) = -2.455$, $P < 0.05$; $t(40) = -2.143$, $P < 0.05$, respectively) and reduced *Oxtr* in the MePFC ($t(40) = -2.419$, $P < 0.05$). There was reduced vasopressin receptor 1a (*Avpr1a*) in the MePFC ($t(42) = -3.184$, $P < 0.01$) in addition to trends for reduced *Avpr1a* in the MeA ($t(41) = -1.895$, $P = 0.07$) and reduced vasopressin receptor 1b (*Avpr1b*) in the BNST ($t(41) = -2.115$, $P = 0.06$). Taken together,

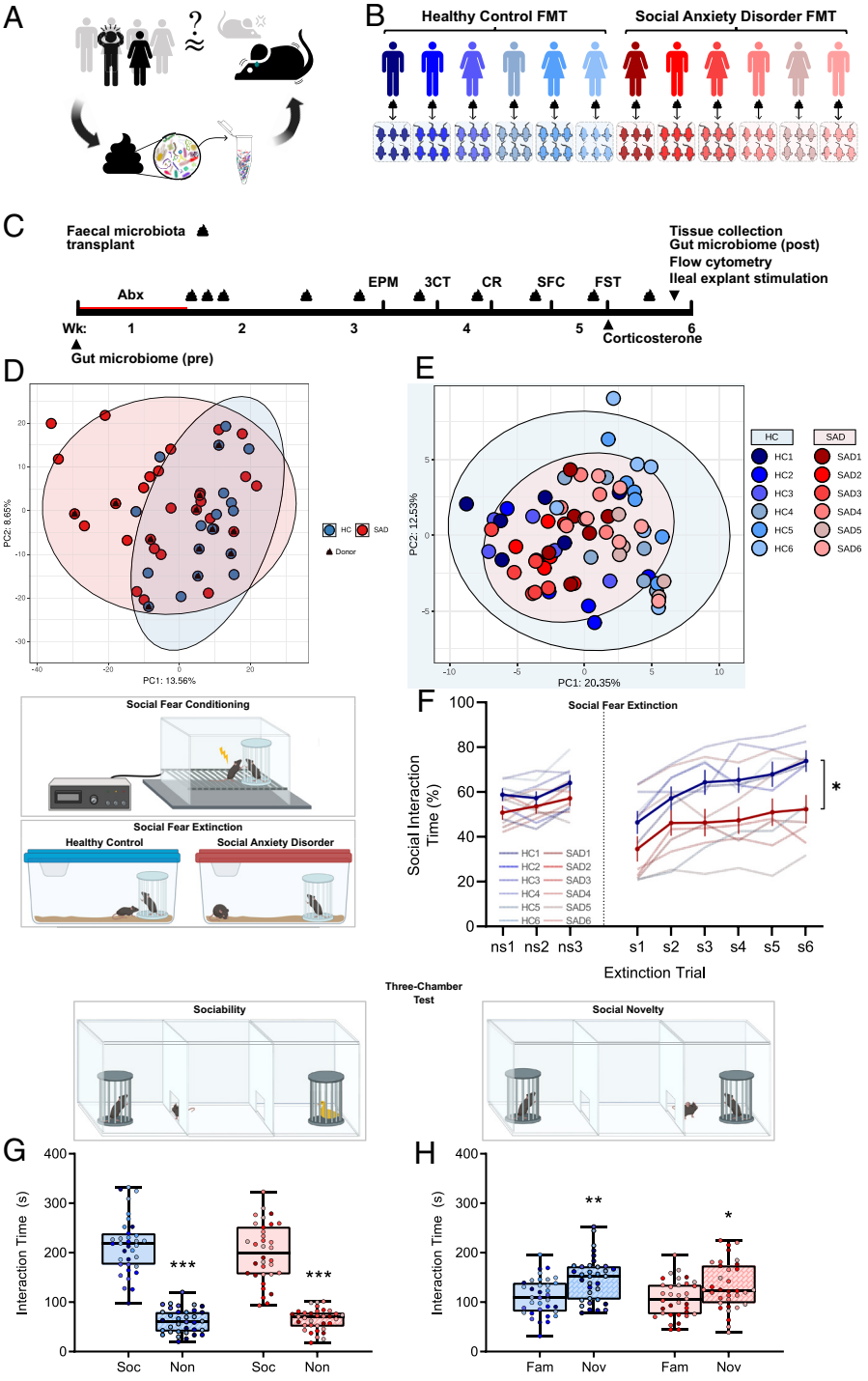

Fig. 1. Human gut microbiota from social anxiety disorder (SAD) promotes social fear behaviour in mice but does not alter other general social behaviours. (*A*) In order to test whether the microbiota plays a causal role in SAD, the microbiota of individuals with SAD was transferred by faecal microbiota transplantation (FMT) to rodent recipients to assess phenotypic characteristics compared to FMT from healthy control. (*B*) Study diagram and human donor and rodent recipient group legend, male (m) and female (f). (*C*) Experimental design: animals first received an antibiotic cocktail (Abx) for 1 wk to deplete the resident microbiota, before receiving FMTs, and behavioural assessment (elevated plus maze—EPM, three-chamber test—3CT, carmine red gastrointestinal transit and motility test—CR, social fear conditioning—SFC, and forced swim test—FST). Faecal samples were collected and microbiota analysed at the beginning and end of study. Corticosterone was measured before and after the FST. At the end of the study animals were killed, tissue was collected; then flow cytometry, and ileal explants were stimulated with lipopolysaccharide, concanavalin A, and CD3/CD28. (*D*) Aitchison beta diversity of SAD human faecal microbiota clustered differentially compared to healthy controls ($R^2 = 0.03477$, $P < 0.05$, 1,000 permutations). Donors (indicated by the faecal symbol) were selected based on microbiota, Liebowitz Social Anxiety Scale score, age, body mass index, sex, and an absence of psychotropic or microbiota medications to treat SAD. (*E*) Murine faecal bacteriome beta diversity was significantly different between SAD and HC groups at the end of the study ($R^2 = 0.02767$, $P < 0.001$, 1,000 permutations). (*F*) Diagram represents social fear conditioning and social fear extinction, which takes place over two days. Social fear behaviour is increased in mice that receive SAD FMT ($W(1) = 3.239$, $P < 0.05$). Social fear extinction data showing interaction percent with either nonsocial stimulus (ns1-3) or six novel social stimuli (s1-6). Transparent lines represent each individual donor group (SAD5 and HC3 $n = 5$; other groups $n = 6$) while bold lines show mean ± SE of the total HC and SAD groups ($n = 35$ per group). (*G*) Diagrams represent the three-chamber social interaction test. Following habituation, sociability was assessed by preference for a novel social stimulus over an inanimate, nonsocial object (rubber duck), then social novelty preference was assessed by preference for a novel conspecific over the now-familiar conspecific used in the sociability phase. There were no significant group differences in sociability; both groups spent significantly more time interacting with the social (Soc) stimulus over the nonsocial (Non) stimulus ($t(68) = 12.01, 11.64$, $P < 0.001$). (*H*) There were no significant group differences in social novelty preference; both groups spent significantly more time interacting with the novel (Nov) over the familiar (Fam) social stimulus ($t(68) = 3.158$, $P < 0.01$; $t(68) = 2.513$, $P < 0.05$) ($n = 35$ per group).

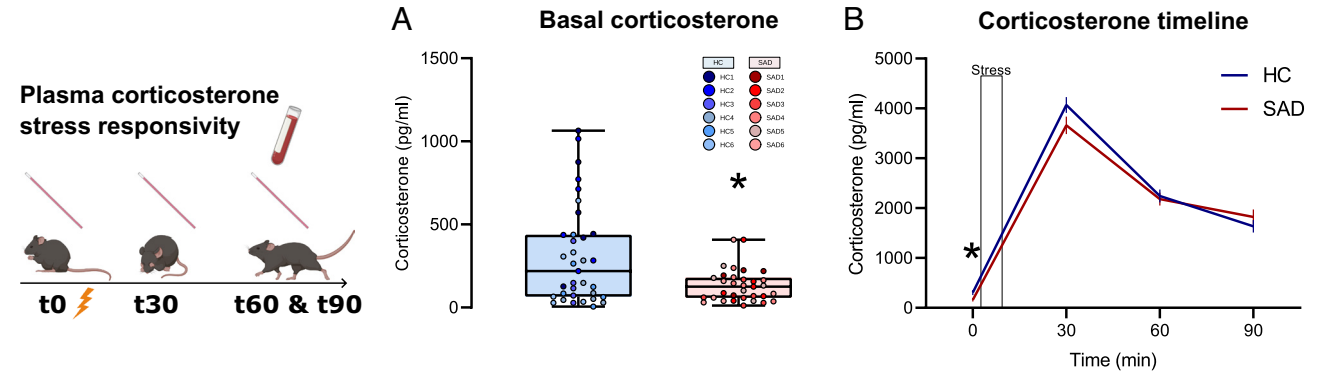

Fig. 2. SAD FMT modulates basal stress hormone corticosterone plasma levels. (*A*) Basal plasma corticosterone is significantly reduced in the SAD group compared to HC (t(64) = −1.858, $P < 0.05$) (n = 35 HC, n = 33 SAD). (*B*) The stress response timeline measured by plasma corticosterone collected at 30-min intervals over 90 min. The forced swim test (stress stimulus) took place from 5 min to 11 min (total duration of stress was 6 min). There was a significantly lower concentration of plasma corticosterone at the basal timepoint (0 min) in the SAD compared to HC group and no significant effects at the 30-, 60-, or 90-min time points.

these data reveal alterations in oxytocin- and vasopressin-related gene expression across these brain regions following SAD microbiota transfer to mice.

Discussion

Understanding the biological basis of complex disorders such as SAD is an important endeavor in psychiatric research. Here, we build on correlational observational studies to causally implicate a role for the microbiome in behaviours relevant to SAD.

At the microbiome level, we show that the beta-diversity microbiota composition alterations observed in human patients with SAD are similar to the beta-diversity found to be different in recipient mice administered SAD or HC faecal microbiota from the selected human donors. Additionally, there were bacterial species that were differentially abundant across the murine groups. *Bacteroides nordii* was reduced in SAD compared to HC. Previously, this species has been negatively correlated with neurodevelopmental behaviour syndromes (18). *Bacteroides cellulosilyticus* was reduced in SAD compared to HC, and accompanying *B. nordii*, higher relative abundances

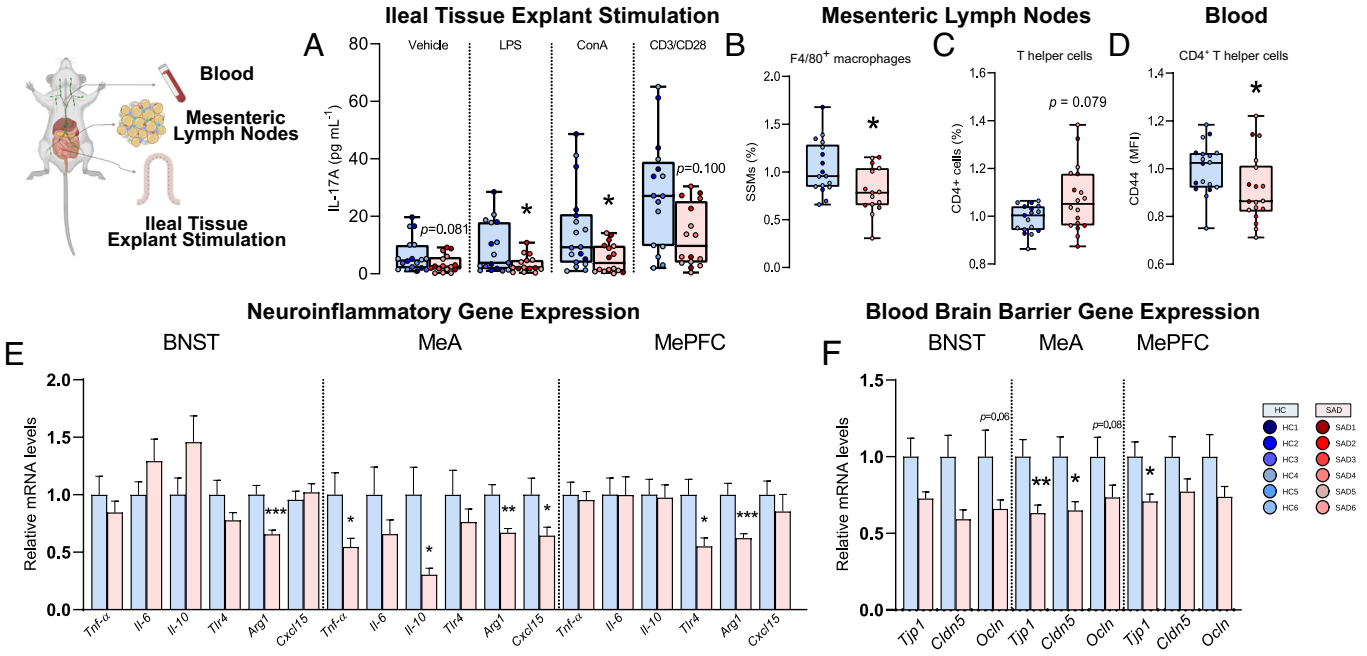

Fig. 3. SAD FMT modulates ileal IL-17A, immune cell population composition, and gene expression markers related to neuroinflammation and blood-brain barrier integrity. (*A*) SAD FMT reduces IL-17A cytokines produced by ileal explants following stimulation with vehicle (buffer; t(32) = −1.804, $P = 0.081$), lipopolysaccharide (LPS; t(30) = −2.297, $P < 0.05$), concanavalin A (ConA; t(32) = −2.457, $P < 0.05$) and CD3/CD28 (t(30) = −1.813, $P = 0.100$) (n = 16–18 per group). (*B*) F4/80$^+$ macrophages in the mesenteric lymph nodes (MLNs) were significantly reduced in the SAD group compared to the HC group (t(30) = −2.602, $P < 0.05$) (n = 17 HC, n = 15 SAD). (*C*) There was a trend for increased CD4$^+$ T helper cells in the MLNs of the SAD group compared to the HC group (t(10) = 1.953, $P = 0.079$) (n = 17 HC, n = 18 SAD). (*D*) The CD44 median fluorescent intensity was reduced in blood CD4$^+$ T helper cells in the SAD group compared to the HC group (t(34) = −2.049, $P < 0.05$) (n = 18 per group). (*E*) Neuroinflammatory- and neuroimmune-related gene expression measured in the bed nucleus of the stria terminalis (BNST), medial amygdala (MeA), and medial prefrontal cortex (MePFC). In the BNST, arginase 1 (*Arg1*) was reduced by SAD microbiota transfer (t(43) = −3.927, $P < 0.001$). In the MeA, there were reductions in expression of tumour necrosis factor alpha (TNF-α; t (35) = −2.081, $P < 0.05$), interleukin 10 (*Il-10*; t(36) = −2.342, $P < 0.05$), *Arg1* (t(43) = −3.241, $P < 0.01$), and chemokine C-X-C ligand 15 (*Cxcl15*; t(38) = −2.137, $P < 0.05$). In the MePFC, there were reductions in Toll-like receptor 4 (*Tlr4*; t(40) = −2.839, $P < 0.05$) and *Arg1* (t(42) = −3.55, $P < 0.001$) following SAD microbiota transfer (n = 17–23 per group). (*F*) Blood–brain barrier–related gene expression measured in the BNST, MeA, and MePFC. In the BNST, there was a trend for reduced occludin (*Ocln*) in the SAD microbiota transfer recipient group (t(43) = −1.901, $P = 0.06$). In the MeA, there were reductions in expression of tight junction protein 1 (*Tjp1*; t(43) = −3.033, $P < 0.01$), claudin 5 (*Cldn5*; t(41) = −2.601, $P < 0.05$), and *Ocln* (t(42) = −1.811, $P = 0.08$). In the MePFC, there was a reduction of expression of *Tjp1* (t(41) = −2.473, $P < 0.05$) following SAD microbiota transfer (n = 20–23 per group).

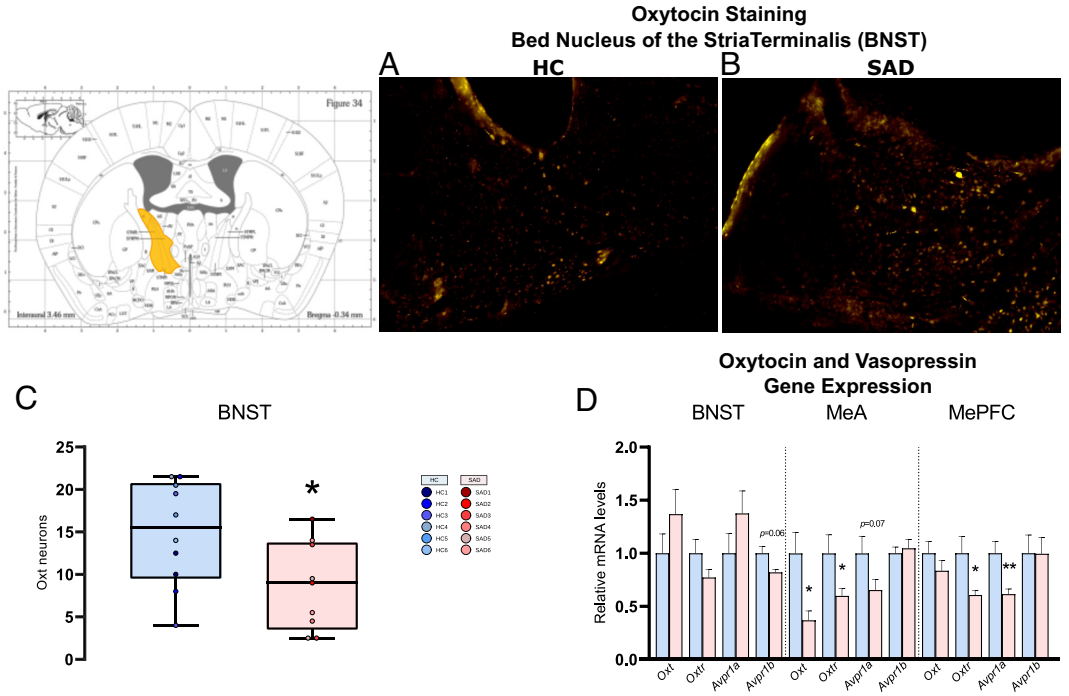

Fig. 4. SAD FMT modulates neurons containing oxytocin in the bed nucleus of the stria terminalis (BNST) and oxytocin- and vasopressin-related genes in the medial amygdala (MeA) and medial prefrontal cortex (MePFC). Representative illustration of the bed nucleus of the stria terminalis (BNST) (17) and immunohistochemistry microscopy images of oxytocin (Oxt) staining in BNST neurons from HC (*A*) and SAD (*B*) FMT recipients (0.02 and -0.08mm from Bregma; NeuN Alexa555, Oxt Alexa488). (*C*) There was a significant reduction in the number of Oxt containing neurons in the BNST of the SAD group compared to the HC group (t(17) = −2.369, $P < 0.05$) (n = 10 HC, n = 9 SAD). (*D*) BNST, MeA, and MePFC expression of oxytocin- and vasopressin-related genes in the HC and SAD microbiota recipient groups. There was a trend for reduced vasopressin receptor 1b (*Avpr1b*) in the BNST (t(41) = −2.115, $P = 0.06$) (n = 22 per group). In the MeA there were reductions in oxytocin (*Oxt*; t(34) = −2.455, $P < 0.05$), oxytocin receptor (*Oxtr*; t(40) = −2.143, $P < 0.05$), and vasopressin receptor 1a (*Avpr1a;* t(41) = −1.895, $P = 0.07$; n = 20–22 per group). In the MePFC there were reductions in *Oxtr* (t(40) = −2.419, $P < 0.05$) and *Avpr1a* (t(42) = −3.184, $P < 0.01$; n = 21–22 per group).

have been associated with fewer symptoms in hyperactivity, impulsivity, and inattention (DSM- HD and AD scores) (19). *Phocaeicola massiliensis* (formerly *Bacteroides massiliensis*) was increased in SAD compared to HC and has been linked with posttraumatic stress disorder (20) and prolonged social isolation stress (21). These microbiota alterations indicate that there are microbial community and specific bacterial taxa differentially transferred to recipient mice by FMT from SAD compared to HC donors, resulting in transfer of a social fear–sensitive phenotype to the recipients.

Social fear conditioning and extinction is a rodent behavioural test specifically designed to model the development and treatment of SAD. Pavlovian conditioning is used to first induce social-specific fear and then measure the rate at which fear diminishes in response to repeated exposure to different social cues (22). Following social fear conditioning, there was a significant reduction in social interaction in the SAD group compared to the HC group (as measured the next day, during social fear extinction), indicating that a SAD microbiota transfer is capable of intensifying the social fear response. These effects are specific to social fear as there was no difference between groups in sociability or social novelty preference measured in the three-chamber test. This fits well with recent research indicating that a substantial proportion of individuals with SAD report normal (or even high) levels of extraversion and novelty-seeking/openness to new experiences, despite their anxiety about social situations (23, 24). Given that the hypothalamic pituitary adrenal (HPA) axis, immune and oxytocin systems have been implicated in social fear responses (25–27), we next assessed whether the SAD recipient group also exhibited changes in these systems as a result of the FMT.

There was a significant reduction in plasma corticosterone measured before stress in the SAD group but no difference in the stress hormone at timepoints following stress. In the SAD patient cohort, there was also a reduction of circulating antiinflammatory cytokine Il-10 compared to HC (28). To assess gut immune function following activation, we stimulated ileal explants with LPS, ConA, and CD3/CD28, the latter two (ConA and CD3/CD28) induce T cell activation. We observed that interleukin-17A (IL-17A) was reduced in the SAD group ileal tissue explants stimulated with LPS and ConA, and there were trends for reduction following vehicle and CD3/CD28 stimulation compared to the HC group. IL-17A is a proinflammatory cytokine that is induced by infection, stress, and trauma (29). Following perturbation, IL-17A is involved in sensory response, T cell activation, and neutrophil mobilisation in various tissues (30–32). Moreover, IL-17A has been shown to rescue sociability deficits in mice exposed to maternal immune activation during embryogenesis (33). In the SAD FMT recipient animal MLNs, there was a significant reduction in $F4/80^+$ macrophages and a trend for an increase in the relative number of $CD4^+$ T helper cells. This fits with the migration of macrophages away from the MLNs previously observed following chronic stress and microbiota perturbation (34). Furthermore, there was a reduction of CD44 receptors on circulating $CD4^+$ T helper cells in the SAD group, indicating inadequate immune memory and activation following SAD microbiota transplant. CD44 is a T cell activation marker indicating memory and effector populations from naïve T cell populations and has been linked with enhanced IL-17 production from T helper cells (35). Taken together, these data indicate that the SAD microbiota drives HPA-axis dysfunction coupled with a reduction of IL-17A and circulating effector and memory T helper cells, leading to diminished immune signalling as a driving contributor to the SAD phenotype.

The neuropeptide oxytocin is a key modulator within the limbic system and has been shown to be critically involved in the circuitry

mediating various social behaviours, including stress-induced defensive and social coping responses (36–38). Oxytocin can elicit diverse effects in various brain regions mediated by circuit-specific actions which are further complicated by differences across sex, species, and models (39–43). Oxytocin receptors in the BNST are activated by PVN and SON projections which have been shown to play a role in fear learning and memory (44). Therefore, using immunohistochemistry, we next investigated the levels of oxytocin within the hypothalamic PVN, SON, as well as the BNST connecting the limbic forebrain to hypothalamic and brainstem regions. No differences in oxytocin-containing neurons were observed in the PVN and SON; however, a significant reduction was observed in the BNST.

In addition to immunohistochemistry, we also measured genes responsible for oxytocin, oxytocin receptor (*Oxtr*), and vasopressin receptors 1a and 1b (*Avpr1a* and *Avpr1b*) in the BNST and other brain areas related to social fear behaviour- the MeA and MePFC. Oxytocin and vasopressin are both well known for playing roles in social behaviour and interestingly, we found a trend for a reduction of *Avpr1b* expression in the BNST. Disruption of Avpr1b has specifically been shown to reduce social memory and aggression in mice (45–47). Furthermore, broad inhibition of Avpr1b with the antagonist SSR149415 partially attenuated the effects of social defeat on social anxiogenic behaviour indicating complex regional responsiveness and effectiveness (48). Since oxytocin and vasopressin have been shown to modulate pro- and antisocial behaviour, respectively, our data suggest that the BNST is a particularly sensitive region to SAD microbiota transfer to mice. However, the BNST is a complex structure that orchestrates emotional and behavioural responses to stress (49) by connecting to other brain regions involved in social circuitry and has been linked to responses to unpredictability that can worsen social anxiety (50). It is a heterogenous brain area with many subregions and thus it is perhaps not unsurprising that there is disconnect between the data on oxytocin at the protein level in slices observed by immunohistochemistry versus the levels of gene expression in relatively crude tissue punch collections.

Importantly, oxytocin released in the MeA has been shown to facilitate social memory formation during social encounters (51). Here, we found reductions in both oxytocin and oxytocin receptor gene expression in the MeA in addition to a trend for reduced *Avpr1a*. Moreover, it has been shown that activation of neurons expressing *Oxtr* in the MePFC have been shown to increase activity of neurons in the BNST (52) indicating region-specific circuitry capable of modulating neural activity. Our MePFC data show reductions in SAD microbiota recipient *Oxtr* and *Avpr1a* receptor gene expression, indicating that oxytocin and vasopressin receptor neural activity may be responsible for regional differences that can lead to increased sensitivity to social fear.

Next, we explored the mechanism by which this gut-derived FMT treatment may be exerting a behavioural effect, as we have observed immune modulation in ileal explants, MLNs, and blood. We thus chose to measure expression of neuroinflammation and blood–brain barrier–related genes in the brain regions (BNST, MeA, and MePFC). Alterations in such genes have been shown with antibiotic or dietary interventions targeting the microbiome previously (53, 54). Again, we noticed regional effects with the only shared gene being significantly reduced in the SAD microbiota recipient group across all regions was arginase 1 (*Arg1*), a marker in the brain of cellular repair activation following inflammation (55, 56). In the MeA, there was a reduction in expression of inflammatory cytokine *Tnf-α* and *Cxcl15* in addition to a reduction in antiinflammatory cytokine *IL-10*, indicating a modulation of the neuroinflammatory response by SAD compared to HC microbiota transfer. Additionally, we observed a reduction in *Tlr4* expression in the MePFC in the SAD FMT recipient group. This receptor is classically known for mediating inflammatory responses following endotoxin activation and knockout of this gene has been shown to increase anxiety-like behaviour and reduce social interaction in mice (57). Additionally, pharmacological blockade of Tlr4 in murine prefrontal cortex has been shown to modulate visceral hypersensitivity, further indicating it as a key marker of microbiota–gut–brain axis activity (58). Last, we measured blood–brain barrier–related gene markers *Tjp1*, *Cldn5*, and *Ocln*. Interestingly, we observed reductions in *Cldn5* in the MeA, and *Tjp1* in both the MeA and MePFC. Previously, it has been shown that social stress can reduce blood–brain barrier integrity and *Cldn5* downregulation in the nucleus accumbens, another region in the limbic system linked with social and emotional behaviours (59, 60). These results indicate that there was a reduction in tight junction network and compromised barrier integrity following SAD microbiota transfer. Overall, these gene expression data provide a picture of neuroinflammatory and immune modulation that was associated with oxytocin and vasopressin alterations.

We next assessed the specificity of the behaviour alterations transferred by SAD FMT. There were no significant changes in anxiety-like behaviour in terms of time spent and entries into the open arms of the elevated plus maze nor were there significant changes in stress-coping behaviour (measured as time spent immobile in the forced swim test). Gastrointestinal transit, measured by timing the duration of passage of carmine red following oral gavage, was also not different between groups. These outcomes further show that the SAD FMT recipients harbored very specific behavioural changes relevant to social fear domains with no distinguishable differences in other distinct social nor stress-coping behaviours, or in gastrointestinal function.

Taken together, our findings provide novel evidence that the microbiota in individuals with SAD can generate increased social fear that is associated with impaired peripheral immune activation and neuronal oxytocin within the BNST in mice. This suggests that the microbiota can play a causal role in heightened social fear responses in the disorder. Moving forward, the microbiota–gut–brain axis is an ideal target for identifying novel therapeutics to improve symptoms in SAD.

Methods Summary

Here, we provide a summary of the methods important to understand our study. Further details are given in *SI Appendix*, *Materials and Methods*.

Participants (FMT Donors). FMT donors were a subset of participants (18–65 y of age, male and female) recruited for our earlier descriptive study of the microbiota in SAD (7). Briefly, the SAD group was recruited through local health practitioners and support groups or via an online website, and a primary clinical diagnosis of SAD was required for inclusion. The HC group were recruited through University College Cork and were required to have no past or current psychiatric diagnosis. For both groups, significant acute or chronic medical illness, other psychiatric illness, presence of any potentially confounding condition or medication, pregnancy or breastfeeding, and strict specific diets were considered exclusion criteria (see for further details ref. (7)). The final subset of FMT donors (n = 6 per group) were selected based on microbiota metrics, Liebowitz Social Anxiety Scale score, age, body mass index, sex, and an absence of psychotropic or microbiota medications (i.e., antibiotics, probiotics, prebiotics, etc.; *SI Appendix*, Table S2). All study procedures involving

human participants were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (Study number APC085) and the study was conducted in accordance with the ICH Guidelines on Good Clinical Practice, and the Declaration of Helsinki. All participants provided written informed consent.

Animals (FMT Recipients). This study used male C57Bl/6J mice (N = 72; 8 wk of age on arrival; Envigo, UK; n = 6 per donor and n = 36 per group). Animals were habituated for 2 wk after which treatments began. The holding room was under a 12-h light/dark cycle (7:00–19:00), with a temperature of 21 °C and humidity of 10%. Standard rodent chow (Teklad Global 18% Protein Rodent Diet, sterilized) and water were available ad libitum throughout the experiments. An examination of the literature, our own publications, and in-house data were carried out to determine probable effect sizes for behavioural readouts as a consequence of social fear conditioning and faecal microbiota transplantation. Comprehensive power analyses ($\alpha = 0.05$, power = 80%) were performed to determine the minimum number of animals required for each of the studies in this project to ensure preclinically and clinically relevant findings. All procedures involving animals were conducted in accordance with the European Directive 2010/63/EC, the requirements of the S.I. No 543 of 2012 and approved by the Animal Experimentation Ethics Committee of University College Cork and the Health Products Regulatory Authority (HPRA AE19130 P118). Procedures are reported in line with the Guidelines for Reporting on Animal Faecal Transplantation (GRAFT) Framework (9).

Antibiotic Treatment. At the start of the study, mice were placed on an antibiotic cocktail consisting of ampicillin (1 g/L), vancomycin (0.5 g/L), imipenem (0.25 g/L), and gentamicin (1 g/L; all from Discovery Fine Chemicals, UK). This was prepared fresh every second day and administered to all animals ad libitum in the drinking water for 7 d to deplete the resident microbiota (see Fig. 1*C* for experimental timeline).

Faecal Microbiota Transplant (FMT). SAD and HC faecal inocula were prepared for FMT within four hours of production. Samples were stored in an airtight container with an AnaeroGen sachet (Oxoid AGS AnaeroGen Compact, Fischer Scientific, Ireland) to maintain an anaerobic environment during transport. Upon arrival, samples were immediately placed within an anaerobic cabinet, mixed with sterile reduced phosphate buffered saline 1% (PBS)/20% glycerol as cryoprotectant (final faecal inocula concentration: 100 mg/mL), and manually passed through a 70-µm stomacher filter to remove large particulates. Then, samples were aliquoted and stored at −80 °C until administered to animals. Animals were randomized to a specific donor and received their respective FMT treatment via oral gavage (100 µL) daily for three successive days followed by two booster FMTs per subsequent week for the remainder of the study (10 total FMTs).

Behavioural Testing. Ten days after the start of FMT treatments, mice began behavioural testing comprising of the elevated plus maze (EPM), three chamber sociability test (3CT), carmine red gastrointestinal transit and motility (CR), social fear conditioning (SFC), and the forced swim test (FST), with a 3 to 4-d interval between each test. Details regarding each behavioural test are given in *SI Appendix, Materials and Methods*. Three days after the end of testing, mice were killed by rapid decapitation (n = 4 per donor) or perfusion (n = 2 per donor, randomly allocated) and tissues were harvested to assess the microbiota, brain, and immune system function (Fig. 1*C*). The allocation of samples was determined by power calculations described above.

Flow Cytometry. Flow cytometry was performed as previously described (61, 62). Trunk blood samples collected during euthanasia were processed on the same day before flow cytometry analysis. Details regarding flow cytometry immune targets, staining, and analysis are given in *SI Appendix, Materials and Methods*.

Immunohistochemistry and Microscopy. The paraventricular nucleus of the hypothalamus (PVN), supraoptic nucleus (SON), and bed nucleus of the stria terminalis (BNST) were sectioned and stained to visualize oxytocin (Oxt) containing neurons (NeuN). Details regarding collection, immunohistochemistry, and microscopy are given in *SI Appendix*, Materials and Methods.

Repeated Blood Sampling for Stress and Immune Measures. Plasma from each animal was sampled by tail tip 5 min prior to the forced swim test, then thirty, sixty, and ninety minutes after the start of the test. The tail was securely restrained, and a diagonal incision was made approximately 1–2 mm from the tip of the tail. Approximately 40 µL of whole blood was collected in an EDTA-lined capillary tube (Fisher Scientific, 749311) per timepoint. Samples were deposited in an Eppendorf and centrifuged for 10 min at 3,500 g at 4 °C. Plasma was collected and stored at −80 °C for later corticosterone quantification.

Corticosterone Stress Response Timeline. Corticosterone quantification of plasma samples (10 µL) collected during FST was performed using a corticosterone ELISA and carried out according to the manufacturer's guidelines (Enzo Life Sciences, ADI-901-097). Samples were analysed in duplicate in a single assay, the threshold detection was less than 32 pg/mL; coefficient of variation limit = 20%; the concentration units are expressed in ng/mL. Light absorbance was read with a multimode plate reader (Synergy HT, BioTek Instruments, Inc.) at 405 nm.

Ileal Explant Stimulations. Ileal tissue explants were collected during tissue harvest, trimmed of fat, and placed in RPMI medium (R8758, supplemented with 10% Foetal Bovine Serum (F7524, Sigma), and 1% penicillin-streptomycin-glutamine 100× (10378-016, Biosciences Ltd.). They were then treated with Red Blood Cell Lysis Buffer (R7757, Sigma), passed through a 70-µm filter, and then resuspended in fresh supplemented RPMI medium. Next, 4 mL of cells were seeded in six-well plates at a concentration of 16 × 106 cells/well. After a 2.5 h rest period, cells were stimulated with lipopolysaccharide (2 µg/mL, tlrl-eblps, Invivogen), concanavalin A (2.5 µg/mL, C5275, Sigma), or CD3/CD28 (CD3 - 100238, CD28 - 102116, Biolegend) for 24 h. Then, cell suspensions were harvested and centrifuged (300 g, 5 min), and supernatants were collected for further analysis.

Inflammatory Cytokine Analysis. Cytokine levels from plasma and splenocyte supernatant samples was collected during euthanasia and were quantified using the V-PLEX Proinflammatory Panel 1 Mouse Kit (K15048D, Meso Scale Discovery, USA) measuring IFN-γ, IL-6, IL-12/IL-23p40, TNF-α, IP-10. Cytokine quantification was carried out according to the manufacturer's guidelines. Samples below the detection limit of each assay were excluded. The lower threshold detection limits for each cytokine were IFN-γ = 0.04 pg/mL, TNF-α = 0.13 pg/mL, IL-1β = 0.11 pg/mL, IL-4 = 0.11pg/mL,IL-5=0.06pg/mL,IL-6=0.61pg/mL,IL-10=0.94pg/mL, IL-13 = 2.7 pg/mL, IL-17A = 0.30 pg/mL, IL-17E/IL-25 = 1.6 pg/mL.

Faecal Microbiome Collection, Sequencing, and Analysis. Human donor and murine recipient faecal microbiotas were analyzed to

assess the differences in community-level composition between SAD and HC. Human donor microbiomes were assessed by 16S rRNA sequencing to identify target candidates to transfer to mice. Murine microbiomes were assessed by full metagenomic sequencing at the beginning of the study before any treatments and at the end of the study the day before euthanasia. Details regarding the collection, sequencing, and analysis of bacteriomes and viromes from human donor and murine recipient faecal microbiotas are given in *SI Appendix, Materials and Methods*.

Bioinformatics and Statistical Analysis of Microbiome Data. Data handling of microbiome measures was undertaken in R (version 4.2.2) using the Rstudio GUI (version 2022.7.2.576) or Python (v3.11.0). Principal component analysis was performed on centred log-ratio-transformed (clr) values as a visual companion to the beta diversity analysis. Alpha-diversity metrics were calculated using scikit-bio (v0.5.8; http://scikit-bio.org/). For all analysis beyond alpha-diversity, taxa with a prevalence of <5% (at the species level for bacteria and vOTU level for viruses) were excluded from analysis as ratios are invariant to subsetting and this study employs compositional data analysis techniques (63, 64). Zero values were then imputed for using the "const" approach (65). Count data were then centred log-ratio (clr) transformed using scikit-bio. Differential abundance estimates were computed using the analysis of composition of microbiomes (ANCOM) (66) method within scikit-bio. Beta diversity was computed in terms of Aitchison distance (Euclidean distance of clr-transformed counts) and differences in beta diversity were assessed using the PERMANOVA implementation from the vegan library using 1,000 permutations (67). Plotting was handled using ggplot2 and the Python implementation of plotly (https://plotly.com/).

Gene Expression of Brain Regions. The BNST, medial amygdala (MeA), and medial prefrontal cortex (MePFC) were dissected from frozen whole brain tissue using mouse brain matrices and a micropunch approach following mouse brain atlas coordinates (17). Total RNA was extracted using the RNeasy® Plus Universal Mini Kit (catalogue no. 73404; Qiagen®, Germany) according to the manufacturer's instructions. RNA concentrations were quantified using a Nanodrop™ spectrophotometer (Thermofisher Scientific®, USA). Complementary DNA (cDNA) was then synthesized using High-Capacity cDNA Reverse Transcription Kit (catalogue no. 4368814; Thermofisher Scientific®, USA).

Genes that were selected for analysis were chosen based on their role in oxytocin signalling, neuroinflammation, and tight junction proteins. Oxytocin signalling genes analysed were for oxytocin generation (*Oxt*), oxytocin receptor (*Oxtr*), vasopressin receptor 1a (*Avpr1a*), and vasopressin receptor 1b (*Avpr1b*). Neuroinflammation genes analysed were tumour necrosis factor alpha (*Tnf-α*), interleukin 6 (*Il-6*), interleukin 10 (*Il-10*), toll-like receptor 4 (*Tlr4*), arginase 1 (*Arg1*), and chemokine C-X-C ligand 15 (*Cxcl15*). Tight junction-related genes selected were tight junction protein 1 (*Tjp1*), claudin 5 (*Cldn5*), and occludin (*Ocln*). Then, RT-qPCR was performed using SYBR® Green real-time PCR on the cDNA samples using SYBR green (KAPA SYBR® FAST Kit; catalogue no. KK4602; Sigma, USA) to evaluate gene expression levels. Gene expression levels were then analysed on an AB7300 system (Applied Biosystems, Thermofisher®, USA). Expression levels were calculated as the average of three replicates for each biological sample from both groups and all donors (n = 23 per group) relative to β-actin expression. Then, fold changes were calculated using the $2^{-\Delta\Delta CT}$ method (68).

Statistics. Behavioural, immune, and gene expression data were assessed for outliers defined as being outside of 2.5× SD of the mean and were subsequently excluded from analysis. Further statistical analysis was handled in R (v4.2.2) using the R Studio GUI (version 2022.7.2.576) and in Python with SciPy (v1.9.3). To account for the nested donor structure, we used the linear mixed-effects modelling framework provided in the lme4 package in R (69), with the following model: feature ~ Treatment + (1|donor_ID). In all cases, an alpha threshold of 0.05 was used to denote significance.

Data, Materials, and Software Availability. Human 16S rRNA sequencing data can be found in European Nucleotide Archive (ENA) under accession PRJEB68191 (70). Murine metagenomic sequencing data can be found on figshare via the following link (https://figshare.com/projects/Social_Anxiety_Disorder-Associated_Gut_Microbiota_Increases_Social_Fear/185134) (71). All data are included in the manuscript and/or *SI Appendix*.

ACKNOWLEDGMENTS. We would like to thank the volunteer donors who participated in the study as well as Drs. C. Fulling, K.E. Guzzetta, A. Lannon, J. Pereira-Cruz, S. J. Leigh, C. M. K. Lynch, M. Segalla, E. Myers, M. Neto, M. van de Wouw, and J. Nagpal for their technical assistance and knowledge sharing with the study, as well as P. Fitzgerald, C. Manley, J. Riley, and A. Markewinski for their assistance with mouse husbandry. We acknowledge the use of the APC Microbiome Ireland Flow Cytometry Platform. APC Microbiome Ireland is a research centre funded by Science Foundation Ireland (SFI), through the Irish Governments' national development plan (grant no. 12/RC/2273_P2). A.N.S. was supported by a Wellcome Trust Research Career Development Fellowship (220646/Z/20/Z), and the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement No 101001684). M.R.A. is supported by ERC_StG_RADIOGUT (project 101040951) and by SFI-IRC Pathway Program SFI21/PATH-S/9424. Illustrations were adapted, in part, using graphics created with http://BioRender.com (DH261LA17M), Servier Medical Art, and Inkscape Project.

Author affiliations: [a]Alimentary Pharmabiotic Centre Microbiome Ireland, University College Cork, Cork T12YT20, Ireland; [b]Department of Anatomy and Neuroscience, University College Cork, Cork T12YT20, Ireland; [c]Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork T12YT20, Ireland; [d]School of Microbiology, University College Cork, Cork T12K8AF, Ireland; and [e]Department of Psychiatry, Psychosomatics and Psychotherapy, University Hospital Frankfurt, Frankfurt 60528, Germany

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