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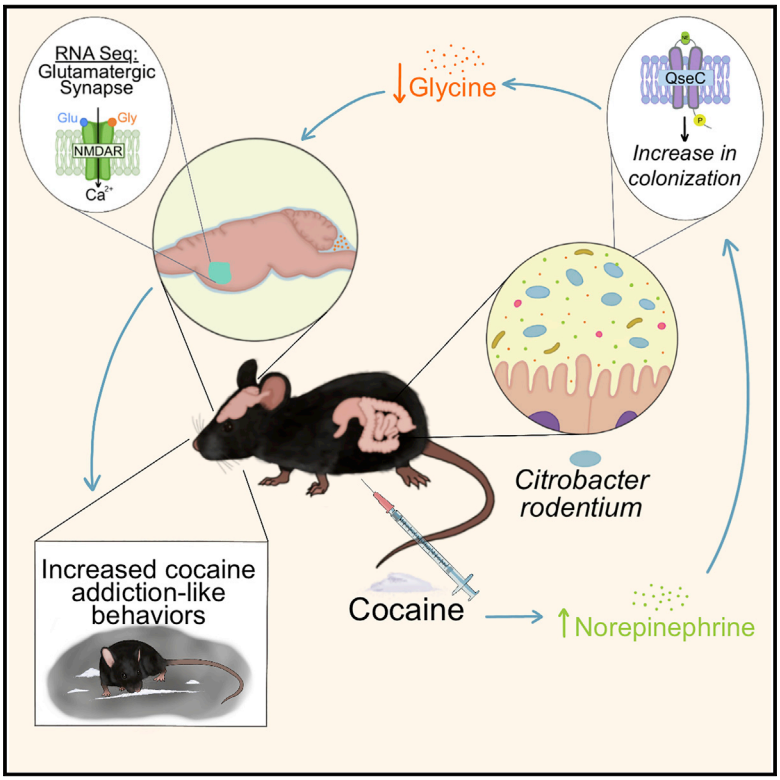
Gut colonization by Proteobacteria alters host metabolism and modulates cocaine neurobehavioral responses

Graphical abstract



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In brief

Cuesta et al. demonstrate in mice that cocaine exposure increases norepinephrine in the gut, thus facilitating Proteobacteria colonization. This gut microbiota shift toward Proteobacteria leads to a depletion of glycine from the host, and this, in turn, facilitates cocaine-induced addiction-like behaviors in mice.

Highlights

- Cocaine raises gut norepinephrine levels facilitating Proteobacteria colonization
- Proteobacteria colonization depletes glycine in gut, blood, and CSF in mice
- Glycine depletion alters cocaine-induced neuroplasticity and drug responses
- Systemic or bacteria-mediated glycine replenishment restores cocaine responses

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Gut colonization by Proteobacteria alters host metabolism and modulates cocaine neurobehavioral responses

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SUMMARY

Gut-microbiota membership is associated with diverse neuropsychological outcomes, including substance use disorders (SUDs). Here, we use mice colonized with *Citrobacter rodentium* or the human γ-Proteobacteria commensal *Escherichia coli* HS as a model to examine the mechanistic interactions between gut microbes and host responses to cocaine. We find that cocaine exposure increases intestinal norepinephrine levels that are sensed through the bacterial adrenergic receptor QseC to promote intestinal colonization of γ-Proteobacteria. Colonized mice show enhanced host cocaine-induced behaviors. The neuroactive metabolite glycine, a bacterial nitrogen source, is depleted in the gut and cerebrospinal fluid of colonized mice. Systemic glycine repletion reversed, and γ-Proteobacteria mutated for glycine uptake did not alter the host response to cocaine. γ-Proteobacteria modulated glycine levels are linked to cocaine-induced transcriptional plasticity in the nucleus accumbens through glutamatergic transmission. The mechanism outline here could potentially be exploited to modulate reward-related brain circuits that contribute to SUDs.

INTRODUCTION

The human gut microbiota contains different bacterial phyla including Bacteroidetes, Firmicutes, and Proteobacteria (Eckburg et al., 2005; Frank et al., 2007; Human Microbiome Project, 2012; Ley et al., 2006). Distinct microbiota compositions have been shown to profoundly impact host homeostasis, physiology, metabolic profile, and vulnerability to disease (Holmes et al., 2012; Li et al., 2008; Nicholson et al., 2012). Reciprocally, host genetics and environmental factors, such as diet, lifestyle, pathogenic infections, and antibiotics usage, shape the establishment of unique microbiota enterotypes (Arumugam et al., 2011; Human Microbiome Project, 2012; Wu et al., 2011), revealing a strong bidirectional crosstalk between the host and its microbiome.

Interkingdom communication has been exploited by different bacteria, such as invading enteric pathogens, which have evolved sensing mechanisms to identify host signals to maximize their colonization and establishment in the gut (Bäumler and Sperandio, 2016). γ-Proteobacteria, including the human enteric pathogen enterohemorrhagic *Escherichia coli* (EHEC) and its murine counterpart *Citrobacter rodentium*, sense the host neurotransmitter norepinephrine (NE) through the QseC bacterial adrenergic receptor to regulate their virulence repertoire and colonize the gut (Clarke et al., 2006; Moreira et al., 2016), leading to a γ-Proteobacteria bloom within the intestine (Lopez et al., 2016; Lupp et al., 2007). Unlike mammalian adrenergic receptors that belong to the G-coupled protein receptor (GPCR) family, QseC is a membrane bound histidine sensor kinase (HK). This receptor directly binds and responds to NE to initiate a complex regulatory cascade that activates the transcription of key virulence and gut colonization genes in γ-Proteobacteria (Clarke et al., 2006; Moreira et al., 2016). *C. rodentium* murine colonization is promoted by host NE within the intestine in a QseC-dependent manner (Moreira et al., 2016) and, consistently, dopamine-β-hydroxylase knockout mice ($DBH^{-/-}$), which do not produce NE and depict less colonization and virulence expression by *C. rodentium* (Moreira et al., 2016).

Recently, many studies have shown that gut microbiota composition, as well as certain gut microbes, can strongly modulate different host social, communicative, stress-related, and cognitive behaviors (Vuong et al., 2017). Furthermore, an altered gut microbiota has been associated with diverse neurological disorders in humans and animal models (Cenit et al., 2017; Lucerne and Kiraly, 2021; Meckel and Kiraly, 2019; Needham et al., 2020; Vuong et al., 2017). However, the studies reporting these findings to date present challenges in determining whether the changes observed in the microbiota are causative, promote, and/or enhance disease or instead are a consequence of an otherwise unrelated pathology. A similar scenario is observed regarding the role of the gut microbiota on addiction and substance use disorders (SUDs). Although it is known that psychostimulants such as cocaine can lead to changes in gut microbiota composition (Lee et al., 2018; Scorza et al., 2019; Volpe et al., 2014; Xu et al., 2017) and that microbiota depletion affects cocaine responses (Kiraly et al., 2016; Lee et al., 2018), the molecular mechanisms behind these interactions are poorly understood.

Psychostimulants, such as cocaine, act systemically blocking the catecholamines transporters, augmenting their signaling (Zimmerman, 2012). This suggests that these recreational drugs could modulate the function of QseC-expressing bacteria in the intestine, potentially impacting host homeostasis and their vulnerability to develop SUDs. Hence, here, we used *C. rodentium* colonization of mice to explore whether and how cocaine-induced increase in catecholamine levels affect intestinal colonization. Then, we evaluated the consequences of γ-Proteobacteria intestinal colonization in cocaine-induced behavioral and neuronal plasticity. We show that cocaine exposure increases intestinal levels of NE, which is sensed through the bacterial adrenergic receptor QseC to increase intestinal colonization of γ-Proteobacteria. Metabolomics and genetic experiments revealed that because γ-Proteobacteria in the gut use glycine as a nitrogen source, this neuroactive metabolite is depleted in in the intestine and also the cerebrospinal fluid (CSF), enhancing host cocaine-induced behaviors. Both systemic repletion of glycine and intestinal colonization with γ-Proteobacteria unable to uptake glycine reversed this response. Transcriptomic studies suggest a role of microbiota-modulated glycine levels in cocaine-induced changes in the nucleus accumbens transcription profile through the glutamatergic transmission, which has been linked to addiction behaviors.

RESULTS

Cocaine exposure enhances murine susceptibility to enteric pathogens

To establish whether cocaine exposure can modulate gut bacterial function, mice were infected with the murine pathogen *C. rodentium* (used extensively as a surrogate model for the human pathogen EHEC (Borenshtein et al., 2008) and treated with cocaine (15 mg/kg i.p.) or saline (vehicle, 1 mL/kg i.p.) (Figure 1A). Cocaine exposure increased pathogen burden in cecum tissues compared with saline-treated infected animals (Figure 1C). Moreover, cocaine-treated mice showed increased markers of intestinal disease in response to *C. rodentium* infection as revealed by increased cecal expression of the proinflammatory cytokines *Nos2*, *Mip*, and *Cxcl1b* (Figures 1F–1H) and by gross pathology in the cecal tissues (Figure S2A). Of note, under these conditions, cocaine treatment in noninfected animals (PBS) did not alter proinflammatory cytokine levels or cecal pathology (Figures 1F–1H and S2A). Tissue colonization of *C. rodentium* is strictly dependent on the locus of enterocyte effacement (LEE)-encoded type three secretion system (T3SS) (McDaniel et al., 1995). T3SSs are molecular syringes employed by pathogens to translocate effector proteins into host-cells that highjack their function, promoting pathogen colonization (Galán and Wolf-Watz, 2006). Transcript levels of the LEE-encoded genes *espA* and *tir* in attached bacteria to the cecum from cocaine-treated infected mice were significantly higher compared with infected animals treated with saline (Figures 1D and 1E). These data demonstrate that cocaine exposure by increasing *C. rodentium* virulence expression leads to more severe intestinal disease.

Cocaine acts by blocking the catecholamines transporter, increasing the levels of these neurotransmitters and consequently their signaling (Zimmerman, 2012). After administration, cocaine is widely distributed in the body (Giroud et al., 2004; (Cocaine); Som et al., 1994), and both the NE transporter (NET) and the dopamine (DA) transporter (DAT) are expressed in cecum tissue (Li et al., 2010, 2006) (Figure S1A). Therefore, to dissect the mechanisms underlying the cocaine-induced increase in virulence, we first assessed whether cocaine itself or DA could directly affect *C. rodentium* growth or virulence. No changes were found in growth or virulence expression (expression of LEE -*ler, tir*, *espA*, and *espB* genes- or proteins) after *in vitro* exposure to cocaine (Figures S1C–S1E) or to DA (Figures S1F–S1H). *C. rodentium* senses NE through the bacterial adrenergic receptor QseC, which leads to activation of LEE-encoded genes promoting pathogen colonization of the gut (Clarke et al., 2006; Moreira et al., 2016) (Figure 1B). Our data suggest a NE-mediated effect of cocaine on *C. rodentium* increased virulence in mice. Congruently, metabolomic determinations revealed that cocaine increased NE levels in the cecum (Figures 2A and 2B) without altering DA (Figure 2C). Furthermore, no changes were observed in the levels of serotonin (Figure 2D), another neurotransmitter whose uptake can be affected by cocaine and that modulates *C. rodentium* infection (Kumar et al., 2020).

To determine whether cocaine-induced increase in *C. rodentium* infection was mediated by NE signaling, we assessed whether preventing the pathogen to sense NE by genetically deleting the QseC receptor (Δ*qseC*) would abrogate this phenotype. *C. rodentium* Δ*qseC* is attenuated for infection (Figures 1 and S1B), and the absence of this receptor prevented cocaine from enhancing bacterial colonization, LEE-encoded gene expression and proinflammatory cytokine expression compared with mice treated with saline (Figures 1A–1H). Furthermore, to functionally confirm the role of NE in the cocaine-induced increase in *C. rodentium* colonization, we used chemical denervation or sympathectomy. Animals were systemically treated with 6-Hydroxydopamine hydrobromide (6-OHDA) to impair noradrenergic nerve terminals in the peripheral nervous system (Kruszewska et al., 1995). As expected, by using this intervention, we were able to block the cocaine-mediated increase in NE levels in the cecum (Figures 2E and 2F). Congruently, 6-OHDA treatment also prevented cocaine-induced increase in *C. rodentium* burdens in colonic tissues (Figures 2G and 2H), supporting a role

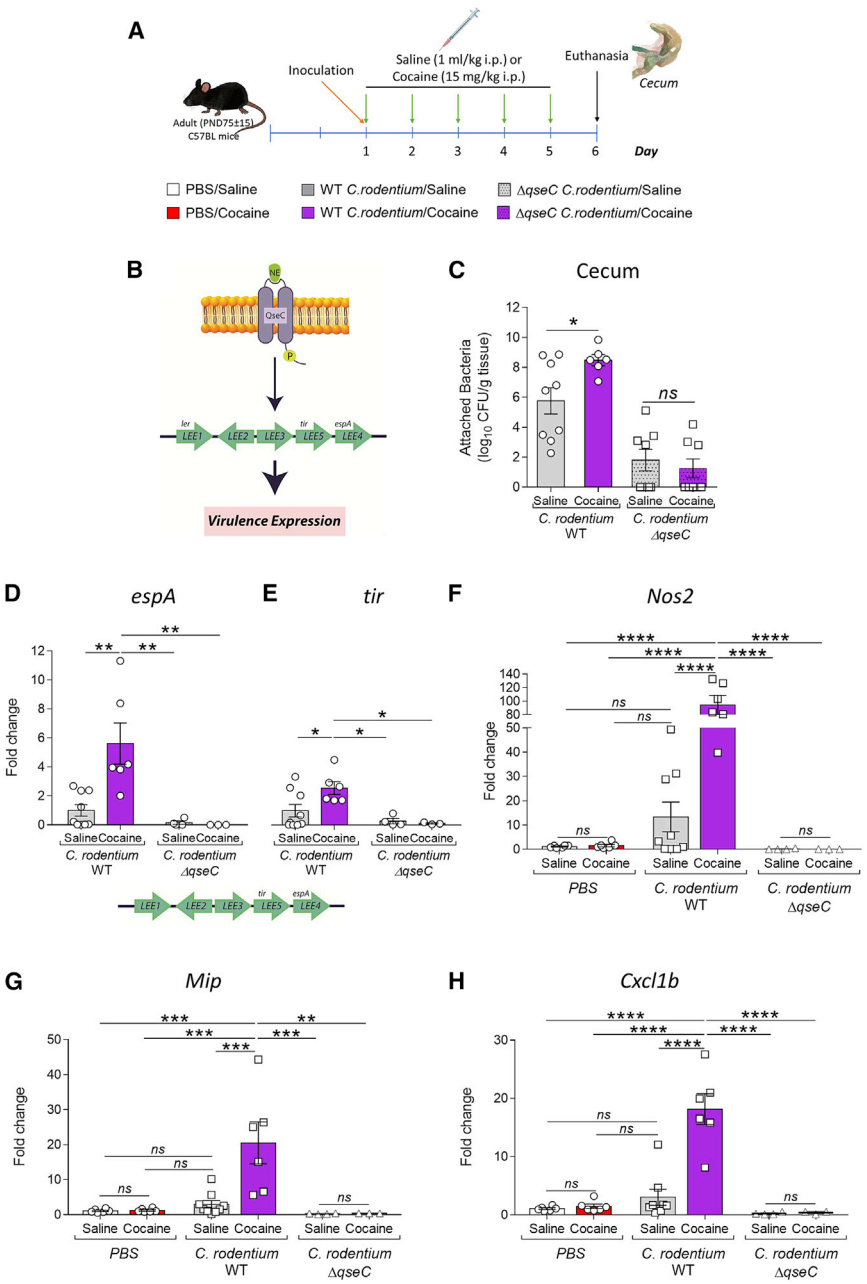


Figure 1. Repeated cocaine exposure increases *C. rodentium* colonization and infectious disease by means of the bacterial norepinephrine sensing QseC receptor

(A) Timeline of treatment and experimental procedures.

(B) QseC signaling receptor senses norepinephrine (NE) to regulate virulence.

(C) *C. rodentium* WT and Δ*qseC* burdens in cecal tissues harvested from saline- or cocaine-treated mice. (*n* = 6–9/2 cohorts per group; two-way ANOVA, with Bonferroni *post hoc* analysis, *ns: nonsignificant*; *p < 0.05).

(D and E) *Top*: relative expression of *C. rodentium* WT and Δ*qseC* LEE-encoded *espA* (D) and *tir* (E) in cecal tissue-attached bacteria recovered from saline- or cocaine-treated mice. (*n* = 3–9/2 cohorts per group; two-way ANOVA, with Bonferroni *post hoc* analysis, *p < 0.05; **p < 0.01). Bottom: Schematic depicting the genetic organization of the locus of enterocyte effacement (LEE) pathogenicity island.

(F–H) Relative cecal transcript levels of *Nos2* (F), of *Mip* (G), and *Cxcl1b* (H) in PBS-treated or *C. rodentium* WT or Δ*qseC* -infected saline- or cocaine-exposed mice. (*n* = 6–9/2 cohorts per group; two-way ANOVA, with Bonferroni *post hoc* analysis, *ns: nonsignificant*; **p < 0.01; ***p < 0.001; ****p < 0.0001).

Data presented as average ± SEM.

for a drug increase in NE levels as a promotor of virulence. Collectively, these data propose a mechanistic model where cocaine exposure acts as a pro-virulence mediator for *C. rodentium* by increasing NE levels in the cecal tissues, which activates QseC signaling promoting the subsequent expression of the LEE and pathogen colonization.

Enhanced behavioral response to cocaine is driven by γ-Proteobacteria colonization and not inflammation

Gut dysbiosis has been corelated with the pathogenesis of SUDs (Lucerne and Kiraly, 2021; Meckel and Kiraly, 2019; Salavrakos et al., 2021). *C. rodentium* infection is known to alter the microbiota composition, promoting an expansion of γ-Proteobacteria (Lopez et al., 2016; Lupp et al., 2007). Therefore, we investigated whether the infection with *C. rodentium* can alter behavioral plastic changes that are observed after repeated exposure to cocaine. We found that *C. rodentium*-infected mice showed a significant increase in the locomotor response to repeated exposure to cocaine in comparison with their PBS-mock-infected counterparts (Figure 3A), an effect that was not observed in infected mice after a saline injection.

High levels of gut inflammation were detected in mice inoculated with *C. rodentium* and treated with cocaine (Figures 1F–1H and S2A). Therefore, to untangle whether this increased response to cocaine is due to the pathogen itself, to the inflammation they cause, or a combination of both, we used a model of chemically induced colitis by treatment with dextran sodium sulfate (DSS). DSS directly damages the epithelium and induces severe colitis in the absence of any bacterial pathogen (Winter et al., 2013) (Figure S2A). No differences were found in the behavioral responses elicited by cocaine in animals exposed to DSS regarding their vehicle counterparts (Figure 3B). These data indicate that gut inflammation and colitis are not mediating the increase in cocaine behavioral responses observed with *C. rodentium* infection.

C. rodentium itself is a γ-Proteobacteria; moreover, its infection promotes expansion of Proteobacteria (specifically,

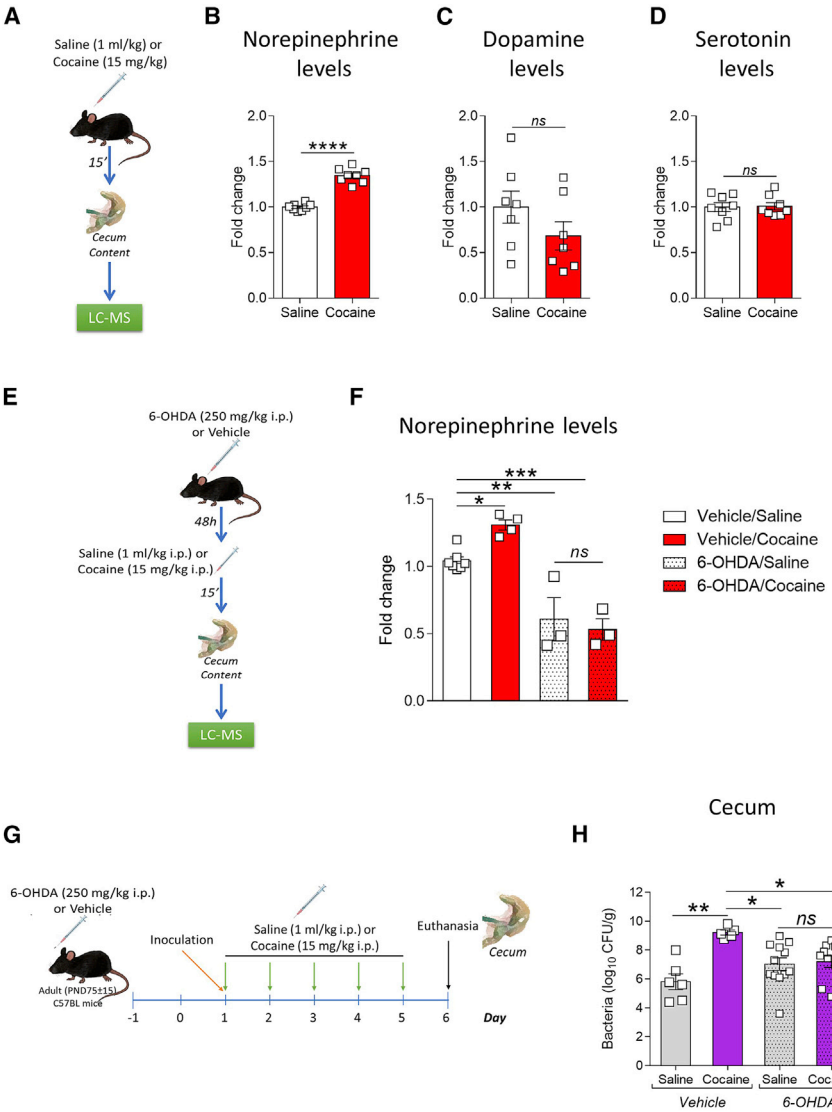


Figure 2. Repeated cocaine exposure increases the levels of norepinephrine in the intestine modulating *C. rodentium* colonization

(A) Timeline of treatment and experimental procedures.

(B–D) Quantification of norepinephrine (B), dopamine (C), and serotonin (D) levels in cecal contents harvested from mice 15 min after an acute saline (1 mL/kg i.p.) or cocaine (15 mg/kg i.p.) injection using liquid chromatography with tandem mass spectrometry (LC-MS) apparatus. (*n = 7–8/2* cohorts per group; Student's t test, *ns: nonsignificant*; ****p < 0.0001).

(E) Timeline of treatment and experimental procedures.

(F) Quantification of norepinephrine levels in cecal contents harvested from 6-hydroxydopamine (6-OHDA)- or vehicle-treated animals 15 min after an acute saline (1 mL/kg i.p.) or cocaine (15 mg/kg i.p.) injection. (*n = 3–7/1–2* cohorts per group; two-way ANOVA with Bonferroni *post hoc* analysis, *ns: nonsignificant*; *p < 0.05; **p < 0.01; ***p < 0.001).

(G) Timeline of treatment and experimental procedures.

(H) *C. rodentium* burdens in cecal tissues harvested from 6-OHDA- or vehicle-treated animals repeatedly exposed to saline (1 mL/kg i.p.) or cocaine (15 mg/kg i.p.). (*n = 5–8/2* cohorts per group, two-way ANOVA with Bonferroni *post hoc* analysis, *ns: nonsignificant*; *p < 0.05; **p < 0.01). Data presented as average ± SEM.

γ-Proteobacteria) within the intestine (Lopez et al., 2016; Lupp et al., 2007). Using metagenomic analysis, we found that overall, in comparison with mock-infected mice, animals infected with *C. rodentium* showed a significant change in microbiota composition (Figures 3C and S2B). We found that although the noninfected mice were γ-Proteobacteria-free (our animals are purchased from Jackson's laboratory, which are free of γ-Proteobacteria), *C. rodentium*-infected animals had a striking increase in Proteobacteria (Figures 3C and S2B). As expected, this increase was mostly due (~93%) to the presence of *C. rodentium*. Therefore, we reasoned that it was the bloom in γ-Proteobacteria driven by *C. rodentium* colonization, rather than the inflammatory process induced by the pathogen in the gut, that was responsible for the increased cocaine behavioral responses. To confirm this hypothesis, we employed a genetically engineered avirulent *C. rodentium* strain, Δ*escN*. EscN is LEE-encoded and is a structural ATPase that is essential for virulence-associated T3SS activity (Zarivach et al., 2007) and colonization of the mouse gut. It has been demonstrated that in the absence of commensal microbiota, Δ*escN* can colonize mice without inducing inflammation (Lopez et al., 2016) (Figures S3 and S6C). Therefore, we depleted the murine microbiota by treating mice with antibiotics (ABXs) (Curtis et al., 2014; Kuss et al., 2011) and then reconstituted them with *C. rodentium* Δ*escN* or with a normal murine microbiota (fecal microbiota transplant (FMT)). The behavioral evaluation revealed a significant increase in cocaine responses in *C. rodentium* Δ*escN* reconstituted mice when compared with their FMT-reconstituted counterparts (Figure 3D). Furthermore, consistent with this hypothesis of a γ-Proteobacteria-rich intestinal microbial environment mediating cocaine-increased behavioral responses, we found that animals reconstituted with the human γ-Proteobacteria commensal *Escherichia coli* HS (*E. coli* HS (Rasko et al., 2008b)) also recapitulated the increase in the cocaine-induced locomotion phenotype (Figure 3D). Altogether, these data indicate that a change in the microbial gut composition, through a bloom in γ-Proteobacteria, mediates alterations in the behavioral plasticity induced by cocaine.

Gut metabolic changes induced by γ-Proteobacteria colonization affect cocaine-induced behaviors and signaling pathways in the brain

Because modifications in the gut-microbial composition impact the host metabolic profile (Holmes et al., 2012; Li et al., 2008;

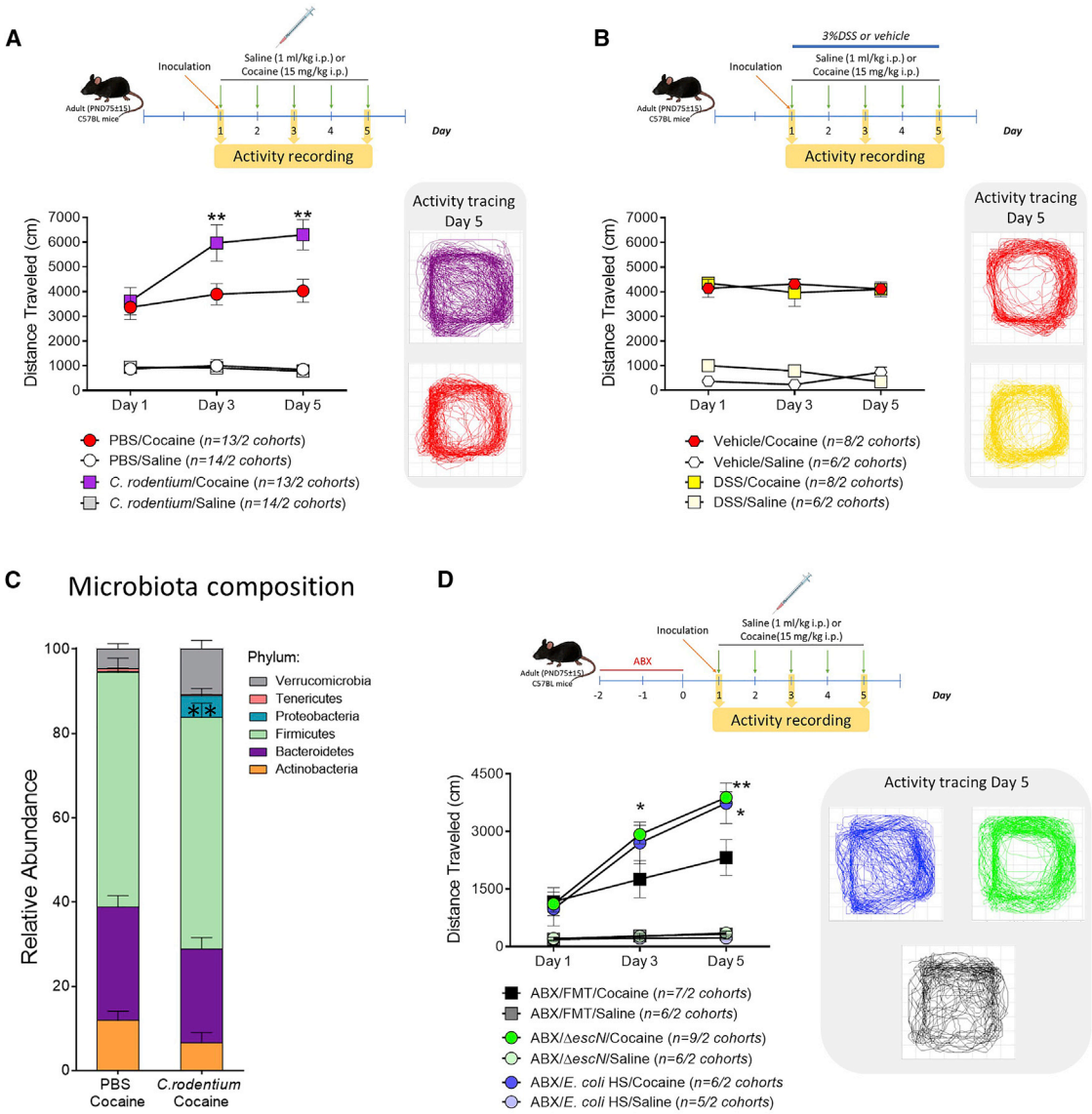


Figure 3. γ-Proteobacteria composition modulates the host behavioral response to repeated cocaine exposure

(A) *Top*: timeline of treatment and experimental procedures. Bottom: Behavioral activity recordings after cocaine or saline injections on days 1, 3 and 5 in PBS-mock- or *C. rodentium*-infected mice. Activity tracing for cocaine-treated mice in day 5 is also shown. (*n* = *13–14*/2 cohorts per group, two-way repeated measures ANOVA, with Bonferroni *post hoc* analysis versus PBS/cocaine, **p < 0.01).

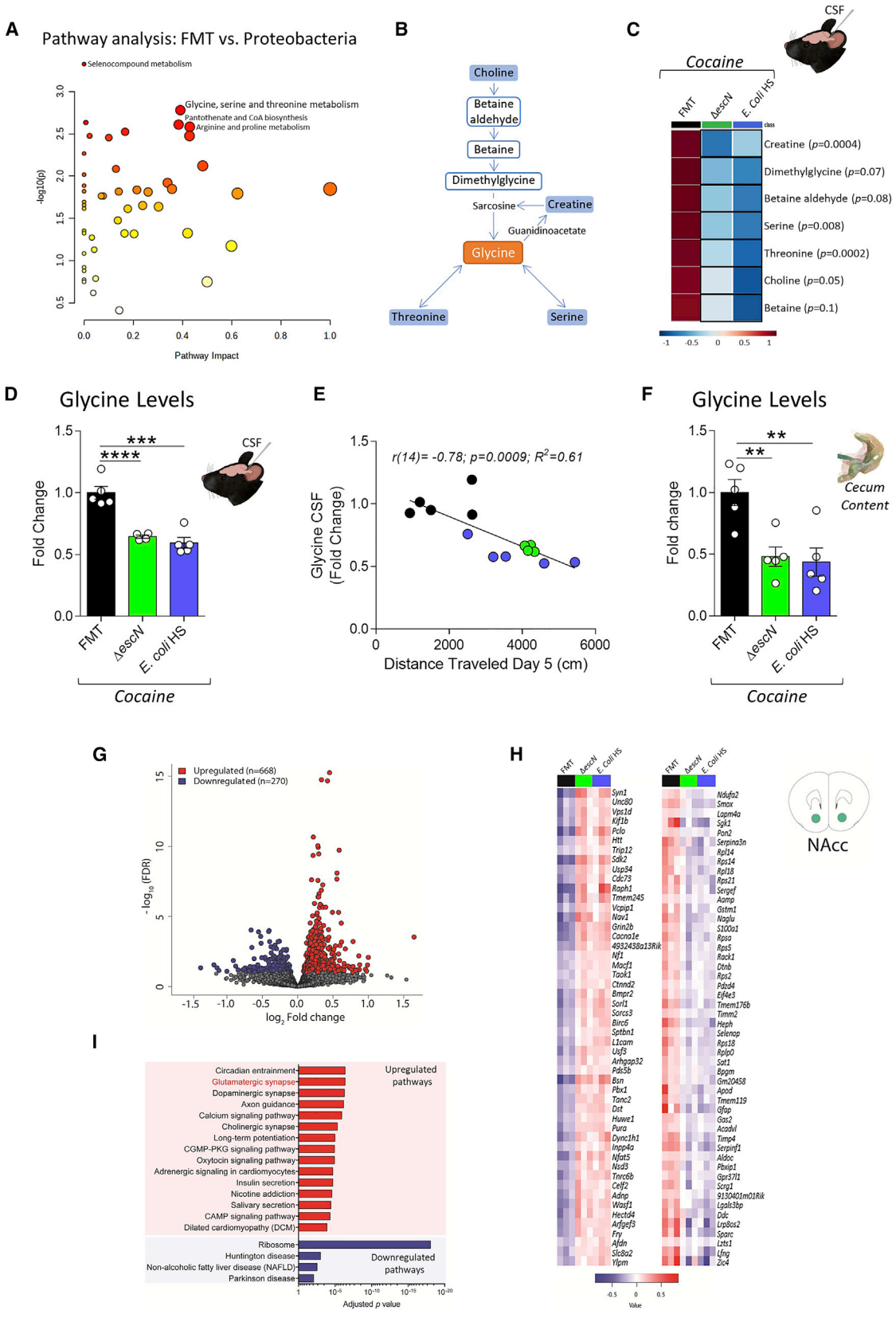
(B) Top: timeline of treatment and experimental procedures. Bottom: Behavioral activity recordings after cocaine or saline injections on days 1, 3, and 5 in mice exposed to 3% dextran sodium sulfate (DSS) or vehicle throughout drinking water. Activity tracing for cocaine-treated mice in day 5 is also shown. (*n* = *6–8*/2 cohorts per group).

(C) Microbiota composition at the phylum level in *C. rodentium*-infected and noninfected mice treated with cocaine. (*n* = 7/3 cohorts per group, two-way ANOVA, with Bonferroni *post hoc* analysis versus same phylum in PBS/cocaine, **p < 0.01).

(D) Top: timeline of treatment and experimental procedures. Bottom: Behavioral activity recordings after cocaine or saline injections on days 1, 3, and 5 in mice antibiotic (ABX)-treated reconstituted with a full microbiota (fecal microbiota transplant, FMT) or with the avirulent *C. rodentium* escN mutant (Δ*escN*) or with the human *E. coli* HS. Activity tracing for cocaine-treated mice in day 5 is also shown. (n = 5–9/2 cohorts per group, two-way repeated measures ANOVA, with Bonferroni *post hoc* analysis versus ABX/FMT/cocaine, *p < 0.05, **p < 0.01).

Data presented as average ± SEM.

Nicholson et al., 2012), we next investigated whether the changes in the behavioral responses to cocaine were associated with metabolite alterations in the *C. rodentium* Δ*escN* and *E. coli* HS colonized mice. We used high-resolution liquid chromatography and mass spectrometry (LC-MS) to perform untargeted comparative metabolomics of cerebrospinal fluid (CSF) obtained from the ABX-treated mice that were reconstituted with FMT, *C. rodentium* Δ*escN* or *E. coli* HS and treated with cocaine. Integrated pathway analyses of the metabolites of the CSF were performed using MetaboAnalyst 5.0 (Pang et al., 2021). No significant



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pathways differences were observed between *C. rodentium* Δ*escN* and *E. coli* HS (Figure S4A). Therefore, we performed an integrated pathway analysis comparing the metabolites of the CSF of FMT-reconstituted mice with the metabolites of the CSF of γ-Proteobacteria-reconstituted mice (*C. rodentium* Δ*escN* and *E. coli* HS combined). This analysis revealed several significantly affected metabolic pathways (Figure 4A). In particular, the *glycine*, *serine*, and *threonine metabolism pathway* was one of most impacted and significantly altered. Notably, of the 11 hits detected by the pathway analysis (Figure S4B), 7 were precursors of the amino acid neurotransmitter and *N*-methyl-D-aspartate (NMDA) receptor agonist, glycine (Figures 4B and S4C), and all of them were downregulated in γ-Proteobacteria-reconstituted mice (Figure 4C). Glycine has been associated with neuropsychiatric disorders such as schizophrenia and drug addiction (Harvey and Yee, 2013; Marques et al., 2020). Furthermore, glycine is consumed at high rates and favors the growth of γ-Proteobacteria (Goldfarb et al., 2011; Han et al., 2002; Zampieri et al., 2019), which can use it as a nitrogen source (Figure S4D). Since glycine was not detected by the LC-MS analysis (not included within the panel of tested metabolites), we directly measured it in CSF samples. A significant reduction in the levels of glycine was observed in the CSF of mice treated with cocaine that were reconstituted with *C. rodentium* Δ*escN* or *E. coli* HS compared with animals reconstituted with FMT (Figure 4D). Moreover, glycine levels in the CSF negatively correlated with the behavioral murine response elicited by cocaine (Figure 4E). In line with the hypothesis that a decrease in host CSF levels of glycine stems from the reduction of this amino acid in the intestine of reconstituted with *C. rodentium* Δ*escN* or *E. coli* HS mice, glycine levels in the cecum were also reduced in mice reconstituted with *C. rodentium* Δ*escN* or *E. coli* HS compared with FMT (Figure 4F).

To further evaluate the hypothesis that an alteration in glycine levels is mediating the changes observed in the behavioral responses to cocaine in *C. rodentium* Δ*escN* and *E. coli* HS-reconstituted mice, we assessed modifications at neuroanatomical levels. Genome-wide transcriptional profiling was performed in the nucleus accumbens (NAcc), an area of the brain highly related to cocaine-induced synaptic plasticity. NAcc tissue dissected from *C. rodentium* Δ*escN* or *E. coli* HS-reconstituted mice treated with cocaine, exhibited comparable transcriptomes with no significant differential expressed genes (DEGs) (Figure S4E). Therefore, as performed with the metabolomic data, we compared the transcriptomes of the NAcc of FMT mice to the γ-Proteobacteria-reconstituted mice (*C. rodentium* Δ*escN* and *E. coli* HS combined) after cocaine exposure. Several significant DEGs were observed between FMT and γ-Proteobacteria-reconstituted animals (Figures 4G and 4H). Consistent with the involvement of glycine, pathway enrichment analysis for DEGs using the Kyoto Encyclopedia of Genes and Genomes (KEGG) showed strong alterations in pathways involved in the glutamatergic synapse, as well as in pathways that regulate the dopaminergic synaptic function (Figure 4I) between FMT and γ-Proteobacteria-reconstituted mice. These findings strongly suggest a role of altered glycine levels in cocaine-induced transcriptional plasticity in the NAcc of γ-Proteobacteria-reconstituted mice.

Glycine levels modulate host susceptibility to cocaine-induced behaviors

To functionally validate the reduction of glycine as a mediator of the increase in cocaine behavioral responses, we directly treated *C. rodentium* Δ*escN*- or *E. coli* HS-reconstituted mice with intraperitoneal (i.p.) glycine before each cocaine injection (Figures 5A and S5). Remarkably, glycine pretreatment prevented the exacerbated behavioral response observed after cocaine in these mice compared with FMT-reconstituted animals. Furthermore, similar results were observed when using the glycine reuptake inhibitor and NMDA partial co-agonist, sarcosine (Zhang et al., 2009a, 2009b) (Figures 5B and S5B). Together, these observations indicate a role for glycine levels in the intestine and the brain, in determining vulnerability to behavioral plastic changes elicited by repeated cocaine exposure.

Proteobacteria-mediated glycine depletion modulate host behavioral responses to cocaine

The cycA gene encodes a permease that mediates glycine transport uptake in different Enterobacteriaceae (Cosloy,

Figure 4. Local and systemic metabolic changes driven by γ-Proteobacteria gut colonization affect cocaine-induced behaviors

(A) Metabolic pathway analysis of cerebrospinal fluid (CSF) samples collected 24 h after the last cocaine treatment injection in mice antibiotic (ABX)-treated reconstituted with a full microbiota (fecal microbiota transplant, FMT) in comparison with mice reconstituted with *C. rodentium* escN deficient mutant (Δ*escN*) or the human commensal *E. coli* HS (evaluated as a single, combined group). (*n = 3–4*/2 cohorts per group).

(B and C) Glycine synthesis pathway (adapted from the Kyoto Encyclopedia of Genes and Genomes, KEGG). Blue boxes indicate downregulated precursors (*blue filled boxes*: statistically significantly downregulated; *blue empty boxes*: non-statistically significantly downregulated) that were identified as *hits* during the partway analysis performed in (A) and which average levels are depicted in (C) (measured using liquid chromatography with tandem mass spectrometry). (n = 3–4/2 cohorts per group, Student's t test, ABX/FMT/Cocaine versus ABX/Proteobacteria/Cocaine).

(D) Glycine levels in CSF collected 24 h after the last cocaine treatment injection in mice ABX-treated reconstituted with FMT, Δ*escN* or *E. coli* HS. (*n = 4–5*/2 cohorts per group, one-way ANOVA with Bonferroni *post hoc* analysis, ***$p < 0.001$; ****$p < 0.0001$).

(E) Negative correlation between glycine levels in CSF and the behavioral response observed in cocaine-treated mice at day 5. The black circles represent FMT-reconstituted, the green ones, Δ*escN*-reconstituted and the blue, *E. coli* HS-reconstituted animals.

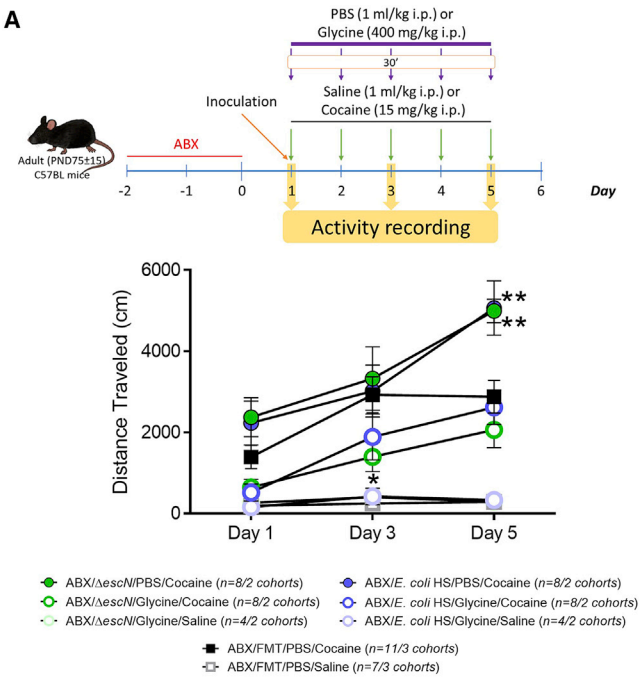
(F) Glycine levels in cecal contents collected 24 h after the last cocaine treatment injection in mice ABX-treated reconstituted with FMT, Δ*escN* or *E. coli* HS. (*n = 5*/2 cohorts per group, one-way ANOVA with Bonferroni *post hoc* analysis, **$p < 0.01$).

(G) Volcano plot of differential expression of genes in the nucleus accumbens in cocaine-exposed mice ABX-treated reconstituted with FMT in comparison with mice reconstituted with Δ*escN* or *E. coli* HS (evaluated as a single, combined group). Mice were euthanized 24 h after the last cocaine treatment injection. Differentially expressed genes (DEGs) between Proteobacteria-reconstituted versus FMT-reconstituted mice are shown as red dots (upregulation) or blue dots (downregulation). (*n= 3*/2 cohorts per group, DESeq2 Wald test, false discovery rate (FDR) < 0.1).

(H) Heat maps showing the top 50 most significantly upregulated or downregulated DEGs.

(I) Significantly enriched KEGG pathways analysis based on all the DEGs.

Data presented as average ± SEM.



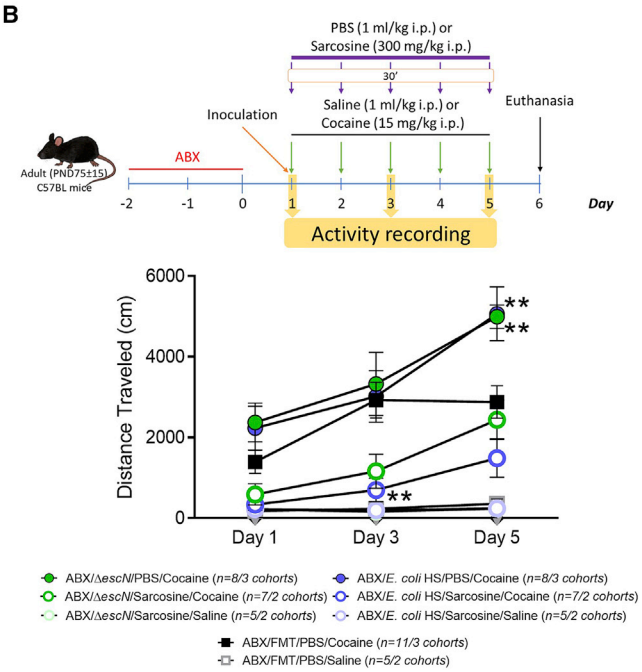


Figure 5. Glycine and sarcosine pretreatment prevents changes in cocaine behavioral responses associated with γ-Proteobacteria gut colonization

(A) *Top*: timeline of treatment and experimental procedures. Bottom: Behavioral activity recordings after cocaine or saline injections on days 1, 3, and 5 of mice antibiotic (ABX)-treated reconstituted with a full microbiota (fecal microbiota transplant, FMT) or with the avirulent *C. rodentium* escN mutant (Δ*escN*) or with the human commensal *E. coli* HS and pretreated with glycine or vehicle. (*n* = *4–11*/2–3 cohorts per group, two-way repeated measures ANOVA, with Bonferroni *post hoc* analysis versus ABX/FMT/PBS/cocaine, *p < 0.05; **p < 0.01).

1973; Ghrist and Stauffer, 1995; Robbins and Oxender, 1973; Wargel et al., 1971, 1970), including *E. coli* HS and *C. rodentium*. Therefore, we next tested whether a *cycA* deletion would reduce bacterial-induced host glycine depletion, reversing the increased vulnerability to cocaine-induced behavioral responses using ABX-treated mice reconstituted with a *E. coli* HS Δ*cycA* mutant (Figures S6A–S6C). Remarkably, the increase in the cocaine-induced locomotor behavioral response was no longer observed in animals reconstituted with *E. coli* HS Δ*cycA*, when compared with FMT-reconstituted mice (Figure 6A). Importantly, most of the precursors of the glycine pathway (Figure 6B), as well as total glycine levels (Figure 6C), were restored to FMT-reconstituted mice levels in CSF samples collected from *E. coli* HS Δ*cycA*. Once again, we observed a significant negative correlation between glycine levels in the CSF and the behavioral response to cocaine on day 5 of the different groups (Figure 6D). As predicted, there were no differences in glycine levels in the cecum of mice reconstituted with *E. coli* HS Δ*cycA* compared with FMT-reconstituted animals (Figure 6E). Finally, we also observed a decrease in glycine in whole blood levels in mice reconstituted with *E. coli* HS WT in comparison with FMT animals, which was not observed when the animals were reconstituted using *E. coli* HS Δ*cycA* (Figure 6F). These data are consistent with the hypothesis that γ-Proteobacteria colonization drives changes in the intestinal metabolome that affects the systemic and CSF metabolic landscape regulating host glycine levels and behavioral responses to cocaine.

Proteobacteria-mediated glycine depletion modulate host rewarding effects of cocaine

To further evaluate the effects of γ-Proteobacteria composition in the rewarding properties of cocaine, we used conditioned place preference (CPP). CPP is a standard preclinical behavioral model largely used to study the rewarding effects of drugs that mimics the association of environmental cues with the drug experience that have been shown to play a critical role in acquiring and maintaining drug-taking behaviors in humans (Bardo and Bevins, 2000; McKendrick and Graziane, 2020; Prus et al., 2009). We found that ABX-treated animals reconstituted with a normal FMT do not demonstrate significant drug-seeking behaviors to 5 mg/kg i.p. dose of cocaine (Figure 7). However, significant cocaine-seeking behavior was observed in animals colonized with *C. rodentium* Δ*escN* or with the commensal *E. coli* HS (Figure 7). Furthermore, similar to FMT, mice reconstituted with the *E. coli* HS Δ*cycA*, which cannot uptake glycine, did not develop significant cocaine-seeking behaviors (Figure 7). These data further support the hypothesis that γ-Proteobacteria-driven glycine depletion can modulate drug-seeking and rewarding responses associated to cocaine exposure.

(B) Top: timeline of treatment and experimental procedures. Bottom: Behavioral activity recordings after cocaine or saline injections on days 1, 3, and 5 of mice antibiotic (ABX)-treated reconstituted with a full microbiota (fecal microbiota transplant, FMT) or with the avirulent *C. rodentium* escN mutant (Δ*escN*) or with the human commensal *E. coli* HS and, pretreated with sarcosine or vehicle. (*n* = *5–11*/2–3 cohorts per group, two-way repeated measures ANOVA, with Bonferroni *post hoc* analysis versus ABX/FMT/PBS/cocaine, **p < 0.01).
Data presented as average ± SEM.

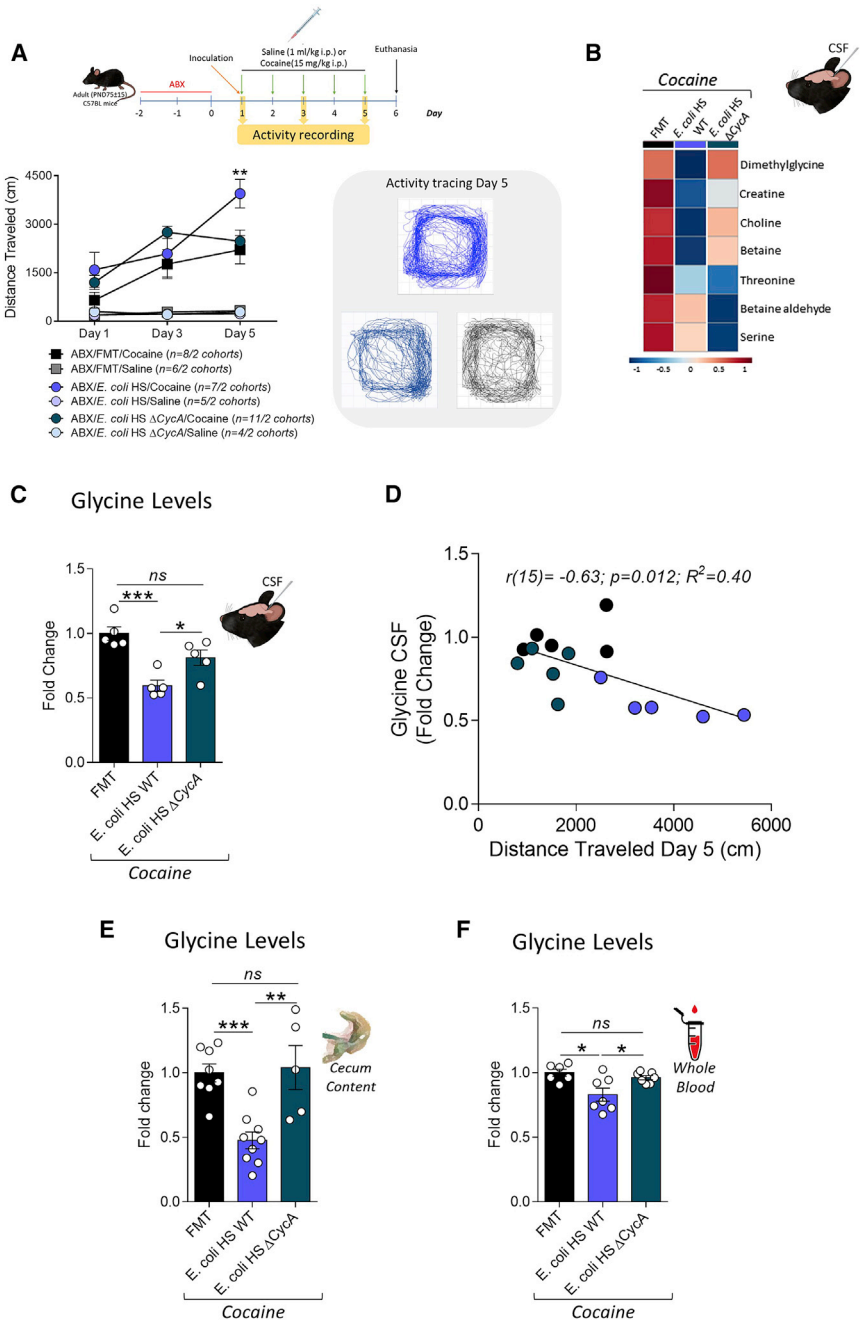


Figure 6. γ-Proteobacteria gut colonization modulates host metabolic glycine levels altering cocaine-induced behavioral plasticity

(A) Top: timeline of treatment and experimental procedures. Bottom: Behavioral activity recordings after cocaine or saline injections on days 1, 3, and 5 in mice antibiotic (ABX)-treated reconstituted with a full microbiota (fecal microbiota transplant, FMT) or with the human commensal *E. coli* HS WT or with *E. coli* HS lacking the glycine transporter (*E. coli* HS Δ*cycA*). Activity tracing for cocaine-treated mice in day 5 is also shown. (*n* = *4–11/2* cohorts per group, two-way repeated measures ANOVA, with Bonferroni *post hoc* analysis versus ABX/FMT/cocaine, $^{**}p < 0.01$).

(B) Glycine synthesis pathway precursors average levels in cerebrospinal fluid (CSF) samples measured by liquid chromatography with tandem mass spectrometry apparatus. (*n* = *3–4/2* cohorts per group).

(C) Glycine levels in CSF collected 24 h after the last cocaine treatment injection in mice ABX-treated reconstituted with FMT, *E. coli* HS WT or Δ*cycA*. (*n* = *5/2* cohorts per group, one-way ANOVA with Bonferroni *post hoc* analysis, *ns: nonsignificant*; $^{*}p < 0.05$; $^{***}p < 0.001$).

(D) Negative correlation between glycine levels in CSF and the behavioral response observed in cocaine-treated mice at day 5. The black circles represent FMT-reconstituted, the teal ones, *E. coli* HS Δ*cycA*-reconstituted and the blue, *E. coli* HS WT-reconstituted mice.

(E) Glycine levels in cecal contents collected 24 h after the last cocaine treatment injection in mice ABX-treated reconstituted with FMT, *E. coli* HS WT or Δ*cycA*. (*n* = *5–9/2–3* cohorts per group, one-way ANOVA with Bonferroni *post hoc* analysis, *ns: nonsignificant*; $^{**}p < 0.01$; $^{***}p < 0.001$).

(F) Glycine levels in whole blood collected 24 h after the last cocaine treatment injection in mice ABX-treated reconstituted with FMT, *E. coli* HS WT or Δ*cycA*. (*n* = *6–7/2* cohorts per group, one-way ANOVA with Bonferroni *post hoc* analysis, *ns: nonsignificant*; $^{*}p < 0.05$).

Data presented as average ± SEM.

DISCUSSION

Growing evidence suggests a bidirectional interaction between the gut microbiome and drug abuse (Meckel and Kiraly, 2019). However, the mechanisms by which abused substances modulate and/or alter microbial composition and the consequences of these changes for cocaine-induced neuroplastic changes remain scarce. Our findings establish that an alteration in the levels of host catecholamines induced by recreational drugs such as cocaine can be sensed by γ-Proteobacteria pathogens. Furthermore, we found that this type of compositional microbial change toward γ-Protobacteria can lead to a depletion of host neuroactive metabolites from the gut and the CSF, affecting cocaine-induced NAcc plasticity and addiction-like behaviors.

Our results support a model of cocaine-induced increase of NE signaling to intestinal pathogens through the activation of the bacterial adrenergic receptor QseC that results in more severe intestinal disease and colonization. Importantly, QseC is also encoded in the core genomes of several other pathogens (Moreira and Sperandio, 2012; Moreira et al., 2010) as well as nonpathogenic γ-Proteobacteria (Rasko et al., 2008a; Rooks et al., 2017). Therefore, it is possible that psychostimulants modulate many additional aspects of bacterial function in both pathogenic and commensal bacteria, increasing their ability to colonize the gut. Indeed, exposure to cocaine and amphetamines has been also reported to increase commensal Protobacteria in mice (Angoa-Pérez et al., 2020; Chivero et al., 2019; Ning et al., 2017), which

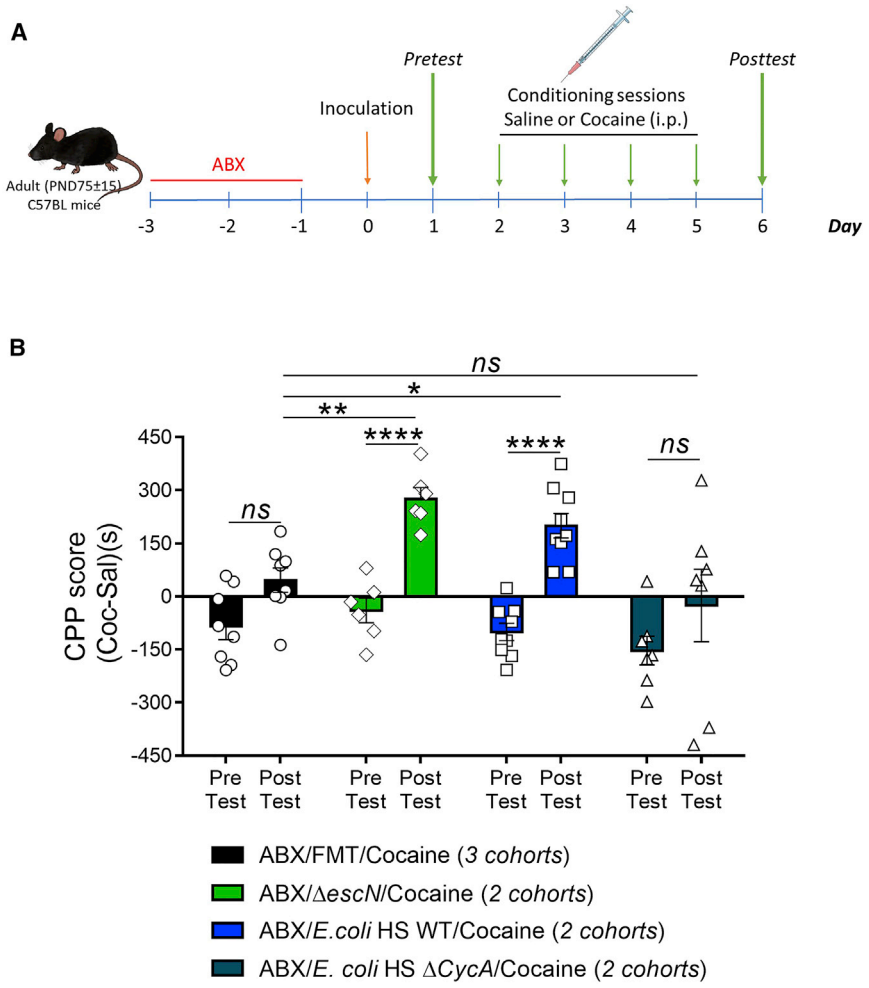


Figure 7. γ-Proteobacteria gut colonization modulates the development of cocaine-induce place preference

(A) Timeline of treatment and experimental procedures for conditioned place preference (CPP).

(B) CPP score for a cocaine dose of 5 mg/kg in mice antibiotic (ABX)-treated reconstituted with a full microbiota (fecal microbiota transplant, FMT) or with the avirulent *C. rodentium* escN mutant (Δ*escN*) or with the human commensal *E. coli* HS WT or with *E. coli* HS lacking the glycine transporter (*E. coli* HS Δ*cycA*). (*n* = 6–9/2–3 cohorts per group, two-way repeated measures ANOVA, with Bonferroni *post hoc* analysis, *ns: nonsignificant;* *p < 0.05; **p < 0.01; ****p < 0.0001).

Data are presented as average ± SEM.

further support a role of host catecholamine levels as modulators of commensal bacteria dynamics.

Changes in microbiota composition impact host homeostasis, physiology, metabolic profile, and vulnerability to disease (Holmes et al., 2012; Li et al., 2008; Nicholson et al., 2012). Specifically, we found that a γ-Proteobacteria expansion (driven by an enteric infection) increases cocaine-induced responses. It is known that microbiota composition can be altered through multiple genetic and environmental cues, including diet, antibiotic usage, and pathogenic insults, among others. Notably, interventions such as high fat diets and oral antibiotic treatment that promote γ-Proteobacteria expansion (Jeong et al., 2019; Kim et al., 2017; Litvak et al., 2017; Rizzatti et al., 2017; Shin et al., 2015) have also be correlated to increased behaviors associated to cocaine exposure (Baladi et al., 2012; Blanco-Gandía et al., 2017a, 2017b, 2018; Blanco-Gandía and Rodríguez-Arias, 2017; Clasen et al., 2020a, 2020b; Kiraly et al., 2016; Serafine et al., 2015, 2016). It is therefore tempting to speculate a potential synergistic contribution of a bloom in γ-Proteobacteria in the gut to these cocaine responses.

Regarding the molecular mechanisms, our data reveal that γ-Proteobacteria colonization cause a significant reduction in glycine levels in the gut, in the blood, and in the CSF leading to an exacerbated response to cocaine. This type of bacterial impact on the host metabolome is not unexpected. Amino acid levels in the gastrointestinal tract are highly affected by the microbiota (Claus et al., 2008; Lahiri et al., 2019; Mardinoglu et al., 2015; Whitt and Demoss, 1975). Glycine, in particular, is considered a conditionally essential amino acid and approximately 50% of its bioavailability depends on dietary uptake in the intestine, (Alves et al., 2019; Li and Wu, 2018) where it can be also highly catabolized by different microbial members. This means that a decrease in glycine bioavailability in the intestine will lead to lower levels of this amino acid to be absorbed by the host, which will reduce its concentration systemically. This modulatory effect of the gut microbiota has been largely observed for many metabolites (e.g., glucose and indole [which is exclusively produced by gut microbes]) and amino acids in many different tissues, including the brain and the CSF (Chu et al., 2019; Kawase et al., 2017; Lahiri et al., 2019; Manca et al., 2020; Mardinoglu et al., 2015; Matsumoto et al., 2013; Velagapudi et al., 2010; Wikoff et al., 2009). Moreover, recently Lai et al. (2021) published metabolomic signatures owing to gut microbiota, yielding hundreds of identified metabolites including 533 altered for feces, 231 for sera, and 58 for brain with numerous significantly enriched pathways involving aromatic amino acids and neurotransmitters. Both *C. rodentium* and *E coli* HS can use glycine as an N source, and indeed, Proteobacteria consume it at high rates during growth (Goldfarb et al., 2011; Han et al., 2002; Zampieri et al., 2019), reducing its availability for the host. Furthermore, reduced circulating glycine levels is consistently reported in patients and murine models of nonalcoholic fatty liver disease (NAFLD), and in line with our data, both, humans and animal models of this disease show consistent increased levels of Proteobacteria in their gut (Kapil et al., 2016; Michail et al., 2015; Rom et al., 2020; Zhu et al., 2013).

Once in the brain, glycine works as the obligatory co-agonist of the NMDA receptor, modulating calcium-dependent neuronal excitation and plasticity affecting glutamatergic and dopaminergic

synapses (Molander and Söderpalm, 2005; Zafra et al., 2017). Congruent with these modulatory effects of glycine, our data show that γ-Proteobacteria-monocolonized mice present transcriptional alterations in both glutamatergic and dopaminergic synapses in the NAcc after cocaine exposure. Furthermore, we demonstrated that this γ-Proteobacteria-mediated glycine reduction is sufficient to modulate cocaine locomotor and rewarding properties. Similar results were previously reported using other glycine agonists, such as D-serine and sarcosine, where the administration of these compounds systemically or intra-NAcc prevented the development of behavioral sensitization as well as the expression of cocaine-induced CPP (Curcio et al., 2013; Liu et al., 2016; Yang et al., 2013). Increases in glycine levels, by using a glycine transporter-1 inhibitor, has also been shown to facilitate cocaine-cue extinction and attenuate reacquisition of cocaine-seeking behaviors (Nic Dhonnchadha et al., 2012). Notably, selectively bred high-responder rats present lower glycine levels in the NAcc after a cocaine injection when compared with their low-responder counterparts (Mabrouk et al., 2018). Combined with our data, these reports support glycine as potent regulator of cocaine responses. Although the specific molecular mechanisms at the level of the NAcc remain to be established, glycine reduction could lead to a hypofunction of the NMDA receptors that could, in turn, alter cocaine-induced plasticity (Curcio et al., 2013; D'Ascenzo et al., 2014; Liu et al., 2016). The RNA-seq data revealed the *Grin2b* as one of the most differentially upregulated genes in the cocaine-treated γ-Proteobacteria-reconstituted group in comparison with animals recolonized with a full microbiota. GluN2B-containing NMDARs have been previously reported to be transiently upregulated in NAcc medium spiny neurons (MSNs). This increase seems to be related to the generation of new silent synapses, a key cellular event that would mediate cocaine-induced neurobehavioral plasticity (Brown et al., 2011; Huang et al., 2009; Neumann et al., 2016). Future studies will be aimed at determining the molecular basis for how microbiota-mediated glycine depletion affect cellular activity in the NAcc.

Drug use and SUDs affect more than 27 million people in the United States, leading to more than $740 billion annually in costs related to crime, lost work productivity, and health care (Birnbaum et al., 2011; Florence et al., 2016; Xu et al., 2015). Unfortunately, despite many years of research, no successful therapeutic targets or evidence-based treatments have been developed. Innovative ideas and more integrative approaches are sorely needed. Microbial composition can strongly modulate the host's metabolic profile and vulnerability to disease. Here, we show a defined mechanism by which a specific microbial compositional shift in the gut modulated behavioral, rewarding, and neuroplastic changes induced by repeated exposure to psychostimulants. Moreover, we show that by manipulating specific bacterial processes, microbial-induced host metabolic alterations can be reversed and thus the increased responses to cocaine. More broadly, our findings demonstrate that intestinal bacteria can serve as a potentially engineered. signaling node within the enteric system that impacts behavioral and molecular targets affected during the course of psychiatric diseases, advancing our understanding of these mental conditions and shifting the current brain-centered paradigm to a more systemic and holistic view in neuropsychiatric research and treatment.

Limitations of the study

Here, we show that different behavioral responses associated with cocaine exposure in mice (behavioral sensitization and drug-seeking behaviors) can be affected by changes in the gut-microbial composition. We acknowledge that there are limitations in our studies. The animal colonization experiments with *C. rodentium* or commensal *E. coli* HS were performed in mice whose microbiota was depleted through antibiotic treatment. One would also consider performing these experiments in germ-free animals. However, it is technically unfeasible to run the behavioral experiments using germ-free mice. The animals are transferred one by one to the behavioral boxes for these tests, and there is no way to have these two different sets of boxes (locomotion and CPP), which are also hooked to a computer for analyses, within germ-free bubbles. Moreover, additional work will be necessary to confirm the translational value of these results to interventions in human SUDs. A major challenge is whether microbiota changes in addicted individuals are the result of cocaine modulating the norepinephrine levels in the gut, or if the often-poor nutrition of these individuals is driving these changes. Finally, it would be valuable to establish a more precise mechanism by which this γ-Proteobacteria-driven glycine depletion impacts the nucleus accumbens and the reward circuitry function.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chom.2022.09.014.

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AUTHOR CONTRIBUTIONS

S.C. designed and conducted experiments and wrote the paper, P.B. analyzed data, A.S. conducted experiments, S.K. supervised experiments and reviewed the manuscript, and V.S. supervised all experiments, analyzed data, and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-EspA-polyclonal, rabbit	UT Southwestern, Vanessa Sperandio	N/A
Anti-EspB-polyclonal, rabbit	UT Southwestern, Vanessa Sperandio	N/A
Anti-rabbit-HRP, goat	BioRad	Cat# 1706515; RRID: AB_2617112
Bacterial and virus strains		
E. coli HS	Center for Vaccine Development at the University of Maryland School of Medicine	N/A
C. rodentium DBS100	Tufts University, John Leong	N/A
Chemicals, peptides, and recombinant proteins		
Dulbecco eagle medium, low glucose	Invitrogen	Cat# 11885-084
Cocaine Hydrochloride	Sigma-Aldrich	Cat#C5776
LB agar, Miller	Fisher Scientific	Cat# BP1425
LB broth, Miller	Fisher Scientific	Cat# BP9723
Ampicillin powder	Sigma-Aldrich	Cat# A9518
Naladixic acid power	Fisher Scientific	Cat# BP908
L-(+)-Arabinose	Sigma-Aldrich	Cat# A3256
Dextran sulfate sodium salt	MP Biomedicals	Cat#021601109
Phusion High fidelity PCR master mix	Thermo Fisher	Cat# F-531L
Random primers	Invitrogen	Cat# 48190-011
Super script II reverse transcriptase	Invitrogen	Cat# 18064-014
Sybr green master mix	Thermo Fisher	Cat# 4309155
Sarcosine	Sigma-Aldrich	Cat#131776
Glycine Hydrochloride	Sigma-Aldrich	Cat# G2879
Trizol reagent	Thermo Fisher	Cat# 1559618
Chloroform	IBM Scientific	Cat# 67-66-3
dNTP mix 10mM	Invitrogen	Cat# 18-427-088
Bovine Serum Albumin	Sigma-Aldrich	Cat# A2153
Dopamine Hydrochloride	Sigma-Aldrich	Cat# H8502
6-hydroxydopamine Hydrochloride	Sigma-Aldrich	Cat# H4381
RNAlater-ICE	Fisher Scientific	Cat# 44-275-75
Protease inhibitor cocktail	Sigma-Aldrich	Cat# P8849-5
Dithiothreitol (DTT)	Fisher Scientific	Cat# BP172-25
Formaldehyde	Fisher Scientific	Cat# BP539
Brain heart infusion agar	Becton Dickinson	Cat# 2418102
Dextran sulfate sodium salt	Fisher Scientific	Cat# 02-160-1109
Metronidazole	MP biomedicals	Cat# 0215571025
Neomycin trisulfate salt hydrate	Sigma-Aldrich	Cat# N5285
Vancomycin hydrochloride	Research Products International	Cat# V06500
Critical Commercial Assays		
RNeasy Power Microbiome	Qiagen	Cat# 26000-50
RNeasy Plus Mini Kit	Qiagen	Cat# 74134
RiboPure RNA isolation Kit	Ambion	Cat# 1405072
GenElute Plasmid Miniprep	Sigma	Cat# PLN350-1KT
QIAquick PCR purification kit	Qiagen	Cat# 28906
Glycine Assay Kit	Sigma-Aldrich	Cat# MAK261

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
RNA sequencing data	https://www.ncbi.nlm.nih.gov/geo/	GSE212421
Experimental models: Organisms/strains		
C57BL/6J mice	The Jackson laboratory	Cat# 000664
Oligonucleotides		
Primers for qRT-PCR used in this study are listed in Table S1	This paper	N/A
Software and algorithms		
Prism 6	GraphPad	N/A
QuantStudio real-time PCR software v1.3	Thermo Fisher	N/A
Image Lab 5.2.1	BioRad	N/A
iDEP.91	http://bioinformatics.sdstate.edu/idep/	N/A
CLC Bio microbial genomics module	https://www.qiagenbioinformatics.com/plugins/clc-microbial-genomics-module/	N/A
MetaboAnalyst 5.0	https://www.metaboanalyst.ca/	N/A
Fiji Image J	https://fiji.sc/	N/A
Fusion 6.5v	Omnitech Electronics Incorporated	N/A
MultiQuant 3.0.3. Software	https://sciex.com/products/software/multiquant-software	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and request for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Vanessa Sperandio (vsperandio@wisc.edu).

Materials availability

All unique reagents generated in this study are available by request made to the lead contact.

Data and code availability

- The raw data of RNA sequencing was deposited at GEO database. The access number is listed in the key resources table.
- This study did not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available upon request of the lead contact.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

C57BL/6 adult (postnatal day, PND, 75±15) male mice were obtained from Jackson Laboratory (arrival weight range: 20±3 g). All mice were housed in specific pathogen free conditions at UT Southwestern, and acclimatized for one week before being used for experiments. All mice were maintained under specific pathogen-free conditions, housed in individual cages with no more than 5 mice per cage, with controlled room temperature (22-25°C) and humidity (45-75%), on a 12-h light-dark cycle, and with autoclaved standard chow and water. All animal protocols were approved by the UT Southwestern Medical Center Institutional Animal Care and Use Committee.

Bacterial strains

Citrobacter rodentium strain DBS100, and *Escherichia coli* HS were grown overnight in Luria bertani (LB) broth at 250 rpm and 37°C prior to subculture for in vitro experiments or mouse infections as described below. All bacteria at stationary phase was mixed with sterile glycerol (25% final concentration) and kept as stocks at -80°C.

Bacterial strains and growth conditions

All the bacterial strains used in this study are listed in Table S1. Unless otherwise indicated, all bacterial strains were grown overnight aerobically in LB prior to subculture for *in vitro* experiments or mouse infections. For *in vitro* studies, *C. rodentium* strains were

subcultured in DMEM with 0.1% glucose (Gibco) and grown in standing cultures (microaerobic) at 37°C (LEE-inducing conditions) or subcultured in minimal medium (MM) and grown microaerobically at 37°C. *E coli* HS strains were subcultured in MM and grown microaerobically at 37°C. Where indicated, bacteria were grown in the presence of dopamine (Sigma) or its vehicle (saline solution + 0.01M HCl), cocaine (Sigma) or its vehicle (saline solution) or Glycine (Sigma).

Isogenic mutants were generated using the lamda red recombinase method as previously described (Datsenko and Wanner, 2000). For growth measurements, bacteria were grown under microaerobic conditions using 96-well microplates.

METHOD DETAILS

Western blot assays for secreted proteins

Secreted proteins were isolated as previously described (Ellermann et al., 2020). Briefly, bovine serum albumin (BSA) was used as a loading control and added to secreted protein samples. Proteins were separated using a gradient 4-15% SDS-PAGE gel, transferred to a polyvinylidene fluoride membrane and blocked with 5% milk in phosphate buffered saline (PBS) with 0.05% Tween. Membranes were probed using anti-EspA or anti-EspB primary antibodies, followed by incubation with secondary antibodies conjugated to streptavidin-horseradish peroxidase. Membranes were exposed with the Bio-Rad ChemiDoc Touch Imaging System with Image Lab 5.2.1 software for image analysis.

C. rodentium infections

Mice were orally infected with 1 x 10^9 CFU of *C. rodentium* DBS100 WT or with QseC deficient mutant, Δ*qseC* or mock-treated with PBS. Fecal pellets were collected throughout the course of infection to enumerate fecal bacteria loads by quantitative culture using selective media for DBS100 (nalidixic acid). At necropsy, cecal tissues were harvested to quantify *C. rodentium* burden, for pathology analysis and for RNA isolation from tissues.

Cocaine treatment

Three hours after infection and for five consecutive days, mice were injected i.p. once a day with either cocaine (15 mg/kg) (Sigma) or saline (1 ml/kg).

RNA isolation and quantitative real-time PCR

For host and bacterial gene expression, RNA was isolated from cecal tissues snap frozen in liquid nitrogen using the TriZol method (ThermoFisher Scientific) according to the manufacturer's instructions. For bacterial expression of LEE genes *in vitro* RNA was extracted using the RiboPure bacterial isolation kit (Ambion) per the manufacturer's instructions. cDNA was synthesized using SuperScript II reverse transcriptase (ThermoFisher Scientific). qPCR was performed in a QuantStudio 6 Flex Instrument (Life Technologies) with Power SYBR Green (Applied Biosystems) using the following PCR conditions: a single hold at 50°C for 2 minutes and at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Each PCR was performed in 10 μL reactions and contained the following: 1x SYBR green mix and 0.25 μM of each primer. Melting curves were assessed to ensure specificity of the PCR products. The relative abundance of mammalian mRNA transcripts was calculated using the delta delta CT method and normalized to *Gapdh* levels. The relative abundance of bacterial mRNA transcripts was calculated using the delta delta CT method and normalized to *rpoA* levels. Table S2 lists qRT-PCR primers to amplify the different transcripts.

Gross pathology and histopathology

Cecal tip was fixed in 10% neutral buffered formalin. Histological inflammation scores (0-20) of hematoxylin and eosin (H&E) stained sections were blindly assessed as previously described (Gibson et al., 2008). Briefly, inflammation was assessed based on the following histopathological features: epithelial hyperplasia (0-4), submucosal edema (0-4), goblet cell depletion (0-4), epithelial integrity (0-4) and polymononuclear (PMN) cell and inflammatory monocyte infiltration (0-4). Data are expressed as the sum of these individual scores (0-20). Histological inflammation scores and representative images corresponding to all the non-antibiotic treated animals were assigned parallel during the same experimental evaluation. The same criterion was applied to the antibiotic-treated groups.

Neurotransmitter measurements in cecal contents

Cecal content samples were collected and frozen immediately at -80°C and then processed as described (Wojnicz et al., 2016). Briefly, the samples were homogenized for protein precipitation in an ice-cold solution containing 75% acetonitrile, 1% formic acid and 0.2 μM of the internal heavy standards (IS) in a 4:1 proportion (solvent:cecum content). The samples were then centrifugated at high speed (~17000g) for 40 min at 4°C. 200 μL of the supernatant were collected, evaporated to dryness at 45°C, resuspended in 100 μL of mobile phase (95:5, 0.2%formic acid:acetonitrile) and submitted to the UT Southwestern Medical Center Metabolomic Core to be evaluated using a Liquid Chromatography with tandem mass spectrometry (LC-MS) apparatus. All the peaks were validated using the pure compound as a control. Peaks representing targeted masses and LC retention times were confirmed manually and the data analyzed using relative quantifications.

16S rRNA sequencing and analysis

For 16S rRNA microbiota profiling, genomic DNA was isolated from feces that were collected at euthanasia, snap frozen and maintained at -80°C and processed as in (Ellermann et al., 2020). Briefly, the hypervariable region V3 & V4 of bacterial 16S rRNA gene were captured using the Illumina Nextera protocol and a single amplicon of about 460 bp was amplified as described in the Illumina protocol. The PCR product were cleaned using Agencourt AmpureXP beads from Beckman Counter Genomics. Illumina adaptor and barcode sequences were ligated to the amplicons in order to attach them to MiSeqDx flow cell and for multiplexing. Quality and quantity of each sequencing library was assessed using Bioanlyzer and picogreen measurements, respectively. About 6 pM of pooled libraries was loaded onto a MiSeqDX flow cell and sequenced using PE300 (Paired end 300 bp) v3 kit. Raw fastq files were demultiplexed based on unique barcodes and assessed for quality. Samples with more than 50 K QC pass sequencing reads were used for downstream 16S operational taxonomic units (OTU) analysis.

Taxonomic classification and OTU abundance analysis were done using CLC Bio microbial genomics module (https://digitalinsights.qiagen.com/plugins/clc-microbial-genomics-module/). Individual sample reads were annotated with the Greengenes database and taxonomic features were determined.

Chemical sympathectomy

6-Hydroxydopamine hydrobromide (6-OHDA, Sigma Chemical Co., St. Louis, MO) was dissolved in sterile saline containing 0.01% of the antioxidant ascorbate and injected i.p. at concentration of 250 mg/kg (Kruszewska et al., 1995). Control animals received the respective vehicle.

Cocaine-induced locomotor activity recordings and analyses

Mice were injected i.p. once a day, for 5 consecutive days with cocaine (15 mg/kg) or saline (1 ml/kg). After the first, the third and the last injection of the treatment, their locomotor activity was measured during 20 min (Delint-Ramirez et al., 2020; Kourrich et al., 2013; Smith et al., 2016) in a SuperFlex open field equipped with infrared photobeams located around the perimeter of the arenas interfaced to a computer running Fusion 6.5v software (Omnitech Electronics Incorporated, OH). Injections in day 2 and 4 were administered in the mice home cages.

Conditioned place preference (CPP)

Mice were evaluated for cocaine place preference using a SuperFlex 3 chambered CPP equipped with infrared photobeams located around the perimeter of the arenas interfaced to a computer running Fusion 6.5v software (Omnitech Electronics Incorporated, OH) using an unbiased design. The two end chambers have distinct visual (vertical or horizontal striped walls) and tactile (small holes *vs.* grid flooring) cues to allow differentiation. During the pretest day mice were allowed to freely explore all three chambers for 20 minutes. Mice that showed significant avoidance for one of the two chambers (<20% of the total time in the chamber) were excluded from further analysis (<10% of animals tested). Groups were then balanced and adjusted to balance out any pre-existing chamber bias. A total of four conditioning sessions were carried out by pairing an injection of saline with one chamber, and one of cocaine (5 mg/kg, i.p.) with the other chamber during four consecutive days. CPP posttest was carried out 24h after the las conditioning session during which mice were again allowed to explore all chambers freely. Place preference scores were taken during the pretest and the posttest as time on the cocaine paired side—time on the saline paired side.

Chemically-induced colitis

Mice were orally administered 3% dextran sulfate sodium (DSS) in their drinking water and allowed to consume ad libitum to damages the epithelium and induces severe colitis (Winter et al., 2013).

Antibiotic treatment and microbiota reconstitution

Mice were orally treated with a four-antibiotic (ABX) cocktail (ampicillin, vancomycin, metronidazole and neomycin) for 3 days to deplete their natural gut microbiota, as previously described (Curtis et al., 2014; Kuss et al., 2011). 24 hrs after the last antibiotic dose, mice were reconstituted with 1 x 10^9 CFU of *C. rodentium* DBS770 Δ*escN*, or with 1 x 10^{10} CFU *E. coli* HS WT or with 1 x 10^{10} CFU *E. coli* HS CycA deficient mutant, Δ*CycA*, or reconstituted with a full microbiota (fecal microbiota transplant, FMT). Cecal contents were collected at euthanasia to enumerate bacteria loads by quantitative culture using selective media in aerobic conditions (LB with chloramphenicol for *C. rodentium* DBS770 Δ*escN*, MacConkey for *E. coli* HS, BHI-Blood for FMT and LB with Kanamycin for *E. coli* HS Δ*CycA*).

Fecal microbiota transplantation (FMT)

Fecal pellets from age matching male controls were collected immediately upon defecation under sterile conditions and resuspended in PBS containing 0.05% of L-cysteine as a reducing agent (~5 pellets/1 ml of buffer), vortexed, briefly spined and transplanted into recipient mice by gavage with 200 μl of the supernatant.

Cerebrospinal fluid collection

Cerebrospinal fluid (CSF) was collected after euthanasia using the modified cisternal puncture method (Šakić, 2019). Samples were snap frozen and maintained at -80°C until processing.

Cerebrospinal fluid untargeted metabolomic

For metabolites extraction, 2 μl of sample were homogenized in an ice-cold solution containing 40% ethanol, 80% acetonitrile, 0.1% formic acid and 0.2 μM of the internal heavy standards (IS) in a 4:1 proportion (solvent:CSF) and maintained at -20°C for 1 hour. The samples were then centrifugated at high speed (~17000g) for 40 min at 4°C, the supernatants were collected, evaporated to dryness at 45°C, resuspended in 75 μL of mobile phase (80:205, acetonitrile:water) and submitted to the UT Southwestern Medical Center Metabolomic Core to be evaluated using a Liquid Chromatography with tandem mass spectrometry (LC-MS) apparatus. MultiQuant software (AB Sciex, Version 2.1) was used for peak analysis of ~110 targeted metabolites, with standards run consecutively. Peaks representing targeted masses and LC retention times were confirmed manually. To perform pathway analysis and create heatmaps, data was analyzed using MetaboAnalyst version 5.0 (Pang et al., 2021).

Glycine levels determination

Glycine levels in cerebrospinal fluid, in cecal contents and in trunk whole blood were determined using the Glycine Assay Kit (MAK261, Sigma-Aldrich, USA) as per manufacturer's protocol.

Glycine and sarcosine pretreatment

Glycine hydrochloride (Sigma) was dissolved in PBS, its pH adjusted to 7.4 using NaOH. A dose of 400 mg/kg was administered i.p. 30min before each cocaine or saline injection (D'Souza et al., 2000; Kanahara et al., 2008; Olsson et al., 2021). Sarcosine (Sigma) was dissolved in PBS and 300 mg/kg were administered i.p. 30min before each cocaine or saline injection (Chan et al., 2012; Fone et al., 2020; Pei et al., 2019). In both cases, PBS injections were used as a vehicle control.

NAcc dissection, RNAseq library preparation, sequencing, and analysis

After euthanasia, mice brains were rapidly removed, snap frozen and stored at -80°C. Before dissection, mice brains were transferred to RNA*later*-ICE® (Ambion) and maintained at -20°C for at least 48hs as per manufacturer's protocol. For NAcc tissue dissection, mice brains were placed in a metal brain mice matrix on ice, razor blades were placed at 1-mm separation, and two bilateral punches per animal were taken containing the NAcc (predominantly the *core* subregion). Total RNA was immediately isolated using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions.

A total of 1 μg from each DNase-treated RNA sample were processed for sequencing by the UT Southwestern Medical Center Next Generation Sequencing Core as described in (Martins et al., 2020). Read counts were generated using feature Counts (Liao et al., 2014) and analyzed and visualized using *iDEP.91* (Ge et al., 2018). Briefly, after ID conversion, low and non-expressed genes (less than 0.5 counts per million reads in more than 70% of all the samples) were omitted from the analysis and data was transformed using the regularized log (rlog) transformation. Differentially expressed genes (DEGs) were selected using the DESeq2 package, with a false discovery rate (FDR) < 0.1. DEGs were then subjected to enrichment analysis based on the hypergeometric distribution using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

All values are presented as means ± SEM. Statistical significance of differences between groups was determined using two-tailed unpaired Student's *t* test when 2 experimental groups were compared. Correlations were calculated using the Pearson correlation coefficient with two-tailed analysis on the fold-change data. Statistical differences between more than two groups were analyzed with one-way, two-way, or two-way repeated-measures ANOVAs as indicated, followed by Bonferroni multiple comparison *post hoc* tests. All enumeration of bacteria by serial dilution and plating was log transformed to normalize the data.

The individual statistic for the Cocaine-induced locomotor activity analysis can be find in the Document S1.