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Complete List of Authors:	Simpson, Joshua; UNC-Chapel Hill, Chemistry Redinbo, Matthew; University of North Carolina System

Multi-omic Analysis of Host-Microbial Interactions Central to the Gut-Brain Axis

Joshua B. Simpson[1] and Matthew R. Redinbo[1,2,*]

[1]Department of Chemistry, [2]Department of Biochemistry & Biophysics, Department of Microbiology & Immunology, and the Integrated Program in Biological & Genome Sciences
University of North Carolina at Chapel Hill

*Corresponding Author: redinbo@unc.edu

Abstract

The gut microbiota impact numerous aspects of human physiology, including the central nervous system (CNS). Emerging work is now focusing on the microbial factors underlying the bi-directional communication network linking host and microbial systems within the gastrointestinal tract to the CNS, the "gut-brain axis". Neurotransmitters are key coordinators of this network, and their dysregulation has been linked to numerous neurological disease states. As the bioavailability of neurotransmitters is modified by gut microbes, it is critical to unravel the influence of the microbiota on neurotransmitters in the context of the gut-brain axis. Here we review foundational studies that defined molecular relationships between the microbiota, neurotransmitters, and the gut-brain axis. We examine links between the gut microbiome, behavior, and neurological diseases, as well as microbial influences on neurotransmitter bioavailability and physiology. Finally, we review multi-omics technologies uniquely applicable to this area, including high-throughput genetics, modern metabolomics, structure-guided metagenomics, targeted proteomics, and chemogenetics. Interdisciplinary studies will continue to drive the discovery of molecular mechanisms linking the gut microbiota to clinical manifestations of neurobiology.

Keywords

Proteomics, metagenomics, genetics, metabolomics, microbiome, structural biology

Introduction

While bacteria in the human gut encode roughly 200-fold more genes than the human genome, we are only beginning to understand the impact of the microbiota on human physiology.[1] Over the past decade, there has been an increased focus on unraveling the mechanisms by which individual microbes residing in the gut modify xenobiotic compounds. In the early 2010s, gut microbial enzymes were directly linked to chemotherapeutic efficacy and cardiovascular disease.[2,3] The intestinal microbiota have since been shown to act on several other xenobiotics including industrial chemicals, dietary compounds, and numerous small-molecule pharmaceuticals.[4,5,6,7,8,9,10,11] Such microbe-mediated chemical modifications directly influence the bioavailability of xenobiotic compounds, which in turn impact drug efficacy, treatment outcomes and even the risk of disease onset. The actions of intestinal microbial enzymes are not limited to xenobiotics, however. A growing body of research has implicated intestinal bacteria in modulating the structures, and therefore the bioavailability, of endobiotics including hormones and neurotransmitters.[9,12,13,14,15,16]

The monoamine neurotransmitters serotonin (5-hydroxytryptamine; 5-HT) and dopamine (3,4-dihydroxyphenethylamine; DA) are key modulators within the central nervous system (CNS) that regulate behavior and neurological function.[12,17,18,19,20,21,22] Strikingly, these monoamine neurotransmitters are abundant within the gastrointestinal (GI) tract as well, from which they also influence local and global physiology. There are as many 5-HT receptors in the gut as in the brain, and ninety-five percent of 5-HT in the body is stored within the gut and modulates motility, vasoconstriction, and fluid secretion (**Figure 1**).[20,23]

Similarly, DA receptors are abundant in the GI tract and nearly half of the DA in the human body is stored in the gut, where it modulates fluid absorption and, counter to 5-HT, inhibits GI motility (**Figure 1**).[24,25] Beyond fluid balance and peristalsis, the 5-HT and DA neurotransmitters are critical to the "gut-brain-axis", the bidirectional communication ongoing between the central (CNS) and the enteric (ENS) nervous systems.[26] The intricacies of this network are poorly understood relative to the discrete local physiological roles ascribed to 5-HT and DA within the gut.

Neurotransmitter abundance varies substantially between germ-free (GF) and conventional mice, suggesting a role for the microbiota in modulating these levels though the gut microbial enzymes involved .[27,28] While gut microbial sulfatases were recently shown to modulate the abundance of sulfate-conjugated neurotransmitters, such conjugates account for less than ten percent of the total 5-HT or DA residing in the gut.[14,27,28,29] Overall, the actions of the microbiota on neurotransmitters and their derivatives are vastly understudied relative to our rapidly growing understanding of how the microbiota process xenobiotic compounds.[30]

Global analyses of the intestinal microbiota's influence on neurotransmitter bioavailability and on the gut-brain axis would appear to be critical given the impact gut-predominant 5-HT and DA have on physiological and neurological disease states. Here, we explore emerging and foundational studies that begin to unravel the relationships between the intestinal microbiota, neurotransmitters, and the gut-brain axis. We further review key technologies for investigating the gut microbiome, and we propose interdisciplinary approaches for unraveling the molecular mechanisms connecting the gut microbiota to neurological functions beyond local motility and fluid modulating effects in the GI tract.

Gut Microbiota are Intrinsically Linked to Behavior and Neurological Disease

Recent studies have linked specific microbial taxa to behavior and neurological disease. More broadly, this foundational work has demonstrated that interactions between the intestinal microbiota and gut neurotransmitters fundamentally impact both host physiology and psychology.

The abundance of *Lactobacilli* have been connected in clinical studies to behavioral changes including the alleviation of anxiety and symptoms of depression (**Figure 2**).[31,32,33] Animal models have also been employed to examine microbial influences on behavior and manifestations of disease. [12,34,35,36,37] For example, prolonged stress significantly reduced levels of gut *Lactobacilli* in mice compared to controls.[38] When mice are treated with a combination of several strains of probiotic *Lactobacilli,* their memory and learning capabilities are improved compared to control mice, and there is an increased expression of genes regulating cognitive activity in the brain.[39] Microbes from the genes *Bacteroides* have also been linked to favorable behavioral outcomes in mice. For example, in a mouse model of Autism Spectrum Disorder (ASD), colonization with *Bacteroides fragilis* shifted both host- and microbiota-produced serum metabolites, including an increase serotonin, and corrected a wide range of behavioral deficits (**Figure 2**).[40] Similarly, when mice were administered 4-ethylphenylsulfate (4-EPS), a compound linked to a variety of ASD-like behaviors, colonization with *B. fragilis* reduced levels of 4-EPS and corrected many of these behavioral abnormalities.[41] In both studies, these changes were linked to several metabolites thought to be produced by *B. fragilis,* although the enzymes involved were not specified.

Such examples suggest that specific bacteria may directly or indirectly modulate behavior, perhaps in a diet-dependent mechanism. *L. reuteri* has been associated with alterations in diet, changes in gut microbial composition, and subsequent microbiota-dependent influences on host

psychology.[42,43,44,45] A high-fat diet diminishes levels of *L. reuteri* in adult mice and, strikingly, a maternal high-fat diet was sufficient to reduce the abundance of *L. reuteri* in offspring.[46,47] In both instances, lower levels of *L. reuteri* were accompanied by in deficits in social behavior that could be reversed through the direct administration of *L. reuteri.*[46] Similarly, germ-free mice exhibit social deficits that can also be ameliorated by providing *L. reuteri* (**Figure 2**).[46] Intestinal *L. reuteri* was also found in three separate mouse models of ASD to improve abnormalities in sociability and increase preferences for social novelty.[48] Importantly, these behavioral changes were not a result of gut microbial shifts towards homeostasis; instead, the improvements appeared to be driven by increased synaptic plasticity, which were apparently promoted by direct interactions between the metabolites of *L. reuteri* and the vagus nerve.[48]

Commensal microbes can mediate host psychology by modulating the local and systemic bioavailability of endobiotics, including neurotransmitters.[9,37,49,50,51,52] In addition, microbial enzymes that process neurotransmitters also interact with FDA-approved psychiatric medications. Selective serotonin reuptake inhibitors (SSRIs) and other psychotropics have been reported to have off-target interactions with gut microbial proteins that bind to and/or catalytically process neurotransmitters and other compounds in the gut, impacting drug efficacy.[45,53,54] Indeed, drugs commonly prescribed for each of the seven most prevalent psychiatric disorders interact with the gut microbiota (**Table 1)**,[55] although the physiological and psychological consequences of these off-target effects are still largely undefined.[11,49,50,54,55,56,57,58,59,60,61,62,63,64,65] One molecular mechanism that has been described involves the tetracyclic antidepressant amoxapine (brand name Asendin®), which is thought to alleviate major depressive episodes by potently inhibiting norepinephrine and serotonin reuptake receptors.[54,59] Amoxapine was the first psychotropic drug shown to potently inhibit gut microbial β-glucuronidase (GUS) enzymes, which have the potential

to reactivate glucuronidated neurotransmitters in the GI tract.[11,27,28,51,66] Additional SSRIs and related medications have since been found to inhibit these enzymes, suggesting that some benefits of these drugs may involve non-host targets.[11,54,59] Comparable mechanisms may exist for a range of psychotropics (**Table 1**), and such interactions can be explored with the revolutionary advances in multi-omic studies developed over the past decade, as outlined below.[11,49,50,54,55,56,57,58,59,60,61,62,63,64,65]

Microbial Modulation of Neurotransmitter Bioavailability

Intestinal bacteria indirectly promote neurotransmitter production within enterochromaffin (EC) cells. For example, metabolites from spore-forming gut microbes induce EC cell 5-HT biosynthesis for delivery both to the gut lumen and to circulating platelets.[37,67] Similarly, bacterial-derived tryptophan metabolites induce 5-HT secretion in the small intestine, activating cholinergic neurons within ECs.[68] Free 5-HT also promotes the proliferation of spore-forming microbes. One such genus, *Turicibacter,* expresses a sodium symporter-related protein homologous to the eukaryotic 5-HT transporter that enables it to acquire 5-HT.[69] These findings suggest that spore-forming microbes have co-evolved to induce host 5-HT production, and then to attain this compound toward a potential competitive advantage.[69] The microbial production of short-chain fatty acids up-regulates the EC expression of tryptophan hydroxylase 1 (TPH1), which catalyzes the first and rate-limiting step in the synthesis of 5-HT.[70] Furthermore, *Clostridium sporogenes* and *Ruminococcus gnavus* produce a tryptophan decarboxylase that converts tryptophan to tryptamine, which then induces the EC release of 5-HT.[71,72,73]

While these studies highlight enzymes and specific metabolites that impact neurotransmitter regulation, others have shown that intact communities of gut microbiota influence the concentrations of intestinal catecholamine neurotransmitters.[27] For example, the abundance of

DA and norepinephrine (NE) was ~24-fold higher in the gut lumen of specific pathogen-free (SPF) mice compared to GF mice.[27] The same group then showed that the same holds true for 5-HT, with an increase in free intestinal 5-HT in SPF compared to GF mice.[28] These results may be explained by the reactivation of inactive endobiotic conjugates, which are produced by host phase II metabolism and sent to the intestinal lumen for excretion. However, a wide range of gut microbes encode genes for β-glucuronidase (GUS) enzymes that have the capability of reversing this conjugation and reactivating neurotransmitters in the GI tract.[74] The complexity of these interactions highlights the roles multi-omics studies can play in defining molecular mechanisms.

Gut-Derived Neurotransmitters Influence Local and Global Physiology

Since Edith Bulbring first demonstrated that increased gut intraluminal pressure triggers the release of 5-HT to initiate the peristaltic reflex and propulsive motility, it has been clear that neurotransmitters residing in the GI tract play a role in mediating local gut physiology.[75,76,77] 5-HT has since been shown to regulate the maturation of the enteric nervous system (ENS),[78] and catecholamine neurotransmitters have been demonstrated to promote GI motility, microbial biofilm formation, and bacterial virulence.[79] Enteroendocrine cells synapse with vagal neurons, indicating that gut-derived neurotransmitters transduce signals to the brain via the vagus nerve.[80] Indeed, the intestinal lumen connects to the brainstem through a single synapse, enabling the brain to directly sense and respond to gut neurotransmitters within milliseconds.[80]

The impact of intestinal catecholamine neurotransmitters are not limited to actions mediated by the vagus nerve as they are also trafficked systemically via circulating platelets.[19] For example, platelet-derived 5-HT promotes the regeneration of liver tissue, reduces bone cell proliferation, and maintains glucose homeostasis.[22,81,82] Mice deficient in platelet 5-HT exhibited

morphological and cellular aberrations that were indicative of ineffective erythropoiesis.[83] Host transglutaminase enzymes also serotonylate intracellular proteins like GTPases by covalently linking platelet-derived 5-HT to glutamines in a process that facilitates platelet coagulation.[84] In fact, many biological processes are mediated by serotonylation, including regulation of glucose homeostasis, insulin release, contraction of vascular smooth muscle cells, and proliferation of pulmonary artery smooth muscle cells.[85,86,87] Thus, these studies highlight the impact gut neurotransmitters have on systemic physiology via the vagus nerve and circulating platelets.

Multi-omic Analyses of the Gut-Brain Axis

The following multi-omic advances are uniquely positioned to expand our understanding of the roles microbially modulated neurotransmitters play in the regulation of host behavior and physiology (**Figure 3**).

High-Throughput Genetics

Drug-microbiome interactions have been examined toward defining their scope and the environmental requirements to facilitate them.[88] Zimmerman and colleagues measured the ability of 76 distinct bacterial strains to metabolize 271 xenobiotic compounds.[30] Surveying such a diverse "pool" of xenobiotics revealed 20,596 microbe-chemical interactions and that two-thirds were metabolized by at least one bacterial strain. Taxa clustering defined phylum-specific activities on individual drugs and xenobiotics, as well as common chemical modifications suggestive of underlying mechanisms.[30] Then, high-throughput genetics were employed to systematically identify individual microbial gene products responsible for metabolic reactions, establishing links between microbial gene content and substrate modifications, and subsequently enabling the identification of products. Such endeavors could be readily applied towards drugs already linked to the microbiota, including those shown in **Table 1**. Alternatively, tailoring high-throughput

genetics to exploring neurotransmitter modifications would reduce the pool of substrates and improve the identification of the bacterial gene products acting on neurotransmitters. Results from such studies could then be explored through techniques such as structural metagenomics or targeted metaproteomics.

Modern Metabolomics

Wikoff and colleagues were the first to apply mass-spectrometry (MS)-based metabolomics to assess the impact of the gut microbiome on the biochemical profile of serum metabolites.[89] Using untargeted profiling, the metabolomes of GF and conventional mice revealed a range of compounds only present in the sera of animals with intact intestinal microbiota, and showed that >10% of endogenous metabolites varied in concentration by >50% between GF and conventional mice.[89] Notably, 5-HT was nearly 3-fold higher in conventional mice while tryptophan was nearly 2-fold lower.[89] Others have advanced upon this foundational work to further map the apparent ability of the gut microbiome to generate circulating metabolites. By combining a subject-specific culturing system with *ex vivo* metabolomics, Javdan *et al.* developed a screening platform enabling the identification and quantification of substrate-microbiome interactions unique to individuals.[90] Linking their microbiome-derived metabolism (MDM) screen with functional metagenomics revealed microbiome-encoded genes responsible for specific metabolic actions that varied between individuals. While the approach of Javdan and colleagues explored metabolism of orally administered drugs, this approach could be further applied to analyze the metabolism of neurotransmitters by individual human gut microbiomes.[90] Moreover, the structural motifs within the proteins exhibiting unique metabolism within a neurotransmitter-tailored MDM screen could inform the development of structure-oriented metagenomic analyses to explore

enzyme families. Once such proteins are defined across the human gut microbiome, subsequent MDM screens could further tailor the exploration of their actions in a high-throughput fashion.

Structure-Guided Metagenomics

Whole-genome metagenomic sequencing has generated a wealth of data on the abundance of microbial taxa and the genes they encode and has demonstrated that orthologs for a single enzyme family are often structurally and functionally diverse across the gut microbiome.[52,74,91,92] In turn, metagenomics is often not able to initially provide protein-level rationale for molecular processes key to microbial-mammalian interactions, including those involving the gut-brain axis. However, when metagenomic datasets are considered alongside protein structures resolved by crystallography or cryo-electron microscopy, structural motifs unique to specific bacterial clades but essential for differential activities with diverse substrates can be identified. Pollet *et al.* provided such an structural analysis on the β-glucuronidase (GUS) enzymes within the vast number of glycoside hydrolases (GH) present in the human gut microbiome.[74] The group surveyed the Human Microbiome Project (HMP) stool sample database[93] for protein sequences meeting an identity threshold and maintaining the full completement of amino acids that define a GUS,[94] identifying 279 unique GUS enzymes present in 139 individuals, and showing that these proteins can be organized by structure (primary to quaternary) in a manner that informs function.[74,93] These approaches allow researchers to reduce the family of $> 250{,}000$ GH orthologs into a defined set of GUS proteins. Furthermore, by filtering for specific amino acids at active sites defined by structural biology, improper annotations that have occurred can be corrected, for example by identifying GUS rather than beta-galactosidases.[95,96] This approach has been termed structural metagenomics, and has since been used to identify 710 unique microbial GUS enzymes within the larger Integrated Gene Catalog that contains 9,816,533 total protein sequences.[97,98] GUS enzymes

from different bacterial species have been shown to demonstrate unique substrate preferences based upon active site architecture.[99,100,101]

This approach can pinpoint the specific proteins within microbial enzyme families that play broader roles in host metabolism.[91,92,99,100,102,103,104] For example, 728 unique gut microbial sulfatases were identified from the HMP stool sample catalog. [14,93] These enzymes were shown to reactivate a variety of endobiotic compounds including the neurotransmitter DA, however activity efficiencies were dependent on structural features unique to only a subset of sulfatases across the microbiota.[14,93] Once these structural features are identified, however, they can used to refine explorations towards functionally relevant members of an enzyme family. For example, the activity of a panel of enzymes from a given "atlas" of proteins could be reciprocally assessed with an MDM screen tailored to neurotransmitters, helping to concretely define the functional landscape of an enzyme family with a given substrate. Refining a structure-guided rubric for an enzyme family uniquely accounts for the structural and functional diversity across the microbiota and enables meta-analyses, such as targeted metaproteomics, to explore bacterial enzyme families with empirically driven certainty.

Targeted Metaproteomics

Recent advancements in activity-based probes (ABPs) and MS technologies have enabled the identification and quantification of individual proteins responsible for catalyzing specific reactions even from the complexity of human samples.[105] Parasar and colleagues, the first to apply ABPs to the microbiome, profiled variation in activity of gut bacterial bile salt hydrolases (BSHs).[106] Importantly, alterations in BSH activity between individuals were not found to correlate with changes in gene abundance in metagenomic sequencing, demonstrating that next-generation sequencing data alone is insufficient to define molecular pathways in gut microbial samples.[106]

This concept has since been improved upon by Adhikari and colleagues, who used structure-activity relationship studies to optimize an ABP for BSH enzymes around the scaffold of a native bile salt substrate.[107] Similarly, Jariwala and colleagues used ABPs to pinpoint the GUS enzymes responsible for distinct toxic drug reactivation activities between human fecal samples, results that may help to guide clinical treatment regimen.[108,109] While untargeted proteomics can provide broad insight into the total collection of proteins produced by the microbiota, targeted metaproteomics amplifies the signal of the desired proteins to the level where they can be uniquely identified and quantified about the noise. This is achieved by using chemical probes for enrichments, and recent computational advances in protein modeling and docking suites will likely facilitate future probe development.[110] In the future, neurotransmitter-focused targeted proteomics could be integrated with behavioral studies *in vivo* to discretely define the gut microbe-mediated molecular pathways that impact host psychology.[111]

Chemogenetics

Chemogenetics has long been employed to define molecular pathways by engineering proteins to interact with small molecule actuators. In recent years, such approaches have advanced the definition of behavior-specific neural circuits and cellular signaling pathways within the brain.[112,113] Bryan Roth and colleagues used the structure of human muscarinic acetylcholine receptors to engineer hippocampal G-protein coupled receptors (GPCRs) exclusively activated by the inert exogenous ligand clozapine-N-oxide, creating Designer Receptors Exclusively Activated by Designer Drugs (DREADDs).[114] DREADDs were then shown to alter neural activities within the mouse brain and to change behavior.[115] Subsequent DREADDs have been developed to be activated by a range of inert small molecules to modulate a variety of distinct neural pathways, enabling researchers to assess the impact of additional proteins on behavior. Importantly, this

technology has been applied successfully to both excitatory and inhibitory neural pathways, enabling the identification of specific neural circuits underlying motor function, perception, and emotions.[116] While DREADDs to date have only been applied to elucidate neural pathways in the brain, they could be turned to the enteric neurons, investigating, for example, enteric neural pathways in GF vs. mono-associated mice to study how individual microbes impact gut-brain axes communication. Similarly, DREADDs could be integrated with reciprocal MDM screens to explore specific neurotransmitter-metabolizing pathways and their interactions with enteric neuronal function, the brain, and systemic physiology.

Future Directions

Recent improvements in our understanding of the gut-brain axis generated by advancements in multi-omics will help to unravel the mechanisms by which specific proteins produced by individual bacterial species work to modulate the gut-brain axis.[117] Understanding any level of intricacies at play in gut-brain axes of neuromodulation will likely facilitate the development of individualized approaches for using existing drugs to treat psychiatric disorders, and enhance utilization of dietary pre- and/or probiotics[118] or full diets tailored to promote specific bacterial consortiums that maintain optimal host homeostasis and cognitive function. Such breakthroughs will start with studies focused on unraveling the effects of the intestinal microbiome on xeno- and endobiotics that influence the gut-brain axis.

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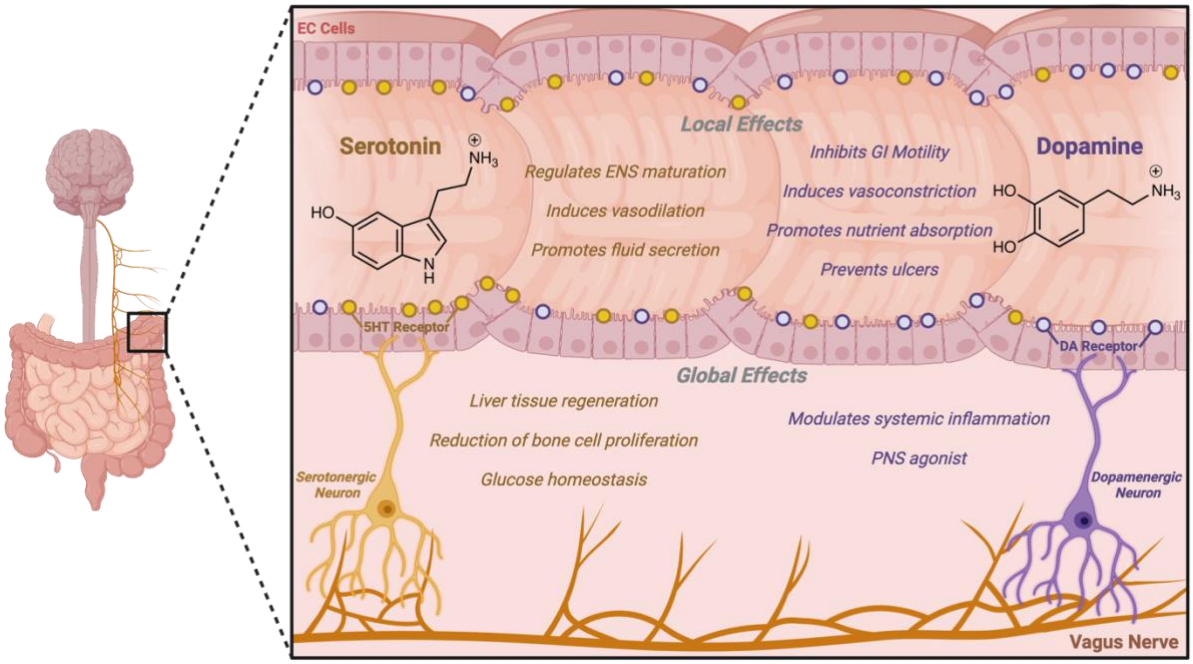

Figure 1. *The monoamine neurotransmitters serotonin (5-HT) and dopamine (DA) exert broad influences on both local and global physiology.*

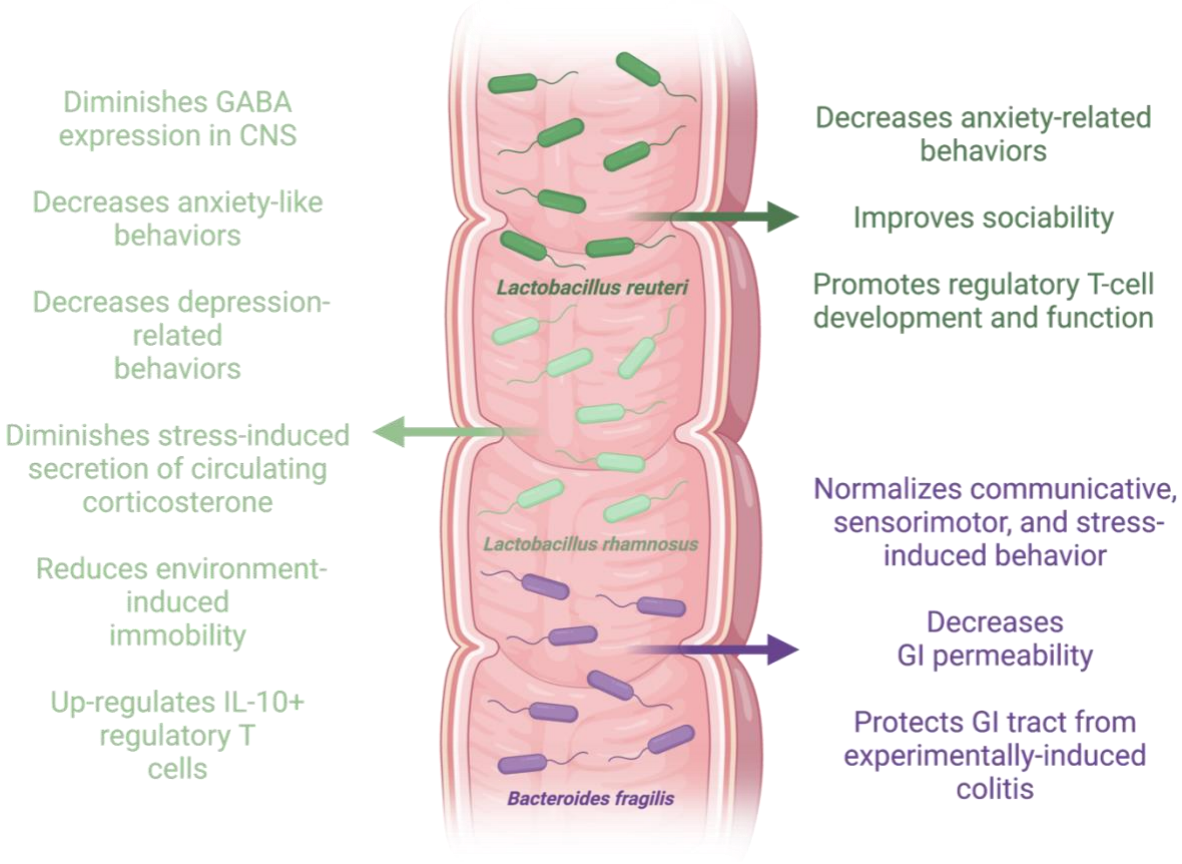

Figure 2. *Individual gut microbial taxa have been linked to discrete behavioral and molecular changes, as shown here for Lactobacillus reuteri, Lactobacillus rhamnosus, and Bacteroides fragilis.*

Psychiatric Disorder	Annual Prevalence (% of US Population)	Prescription	Evidence of Relationship to Microbiota	Citation
Major Depressive Episode	7.2	Amoxapine (Asendin®)	Inhibits bacterial β-glucuronidase enzymes	11, 54, 59
Major Depressive Episode	7.2	Vortioxetine (Trintillex®)	Inhibits bacterial β-glucuronidase enzymes	11
Schizophrenia	< 1	Risperidone (Risperidal®)	Depresses resting metabolic rate; Isoxazole scission of benzisoxazole ring system	56, 57, 58
Bipolar Disorder	2.8	Lamotrigine (Lamictal®)	Inhibits bacterial ribosomal biosynthesis in *E. coli*; anti-microbial against gram-positive prokaryotes	49, 50
Anxiety Disorders	19.1	Clonazepam (Klonopin®)	Bacterial nitroreductase-mediated reduction to 7-amino metabolites	60, 61
Anxiety Disorders	19.1	Nitrazepam (Mogadon®)	Bacterial nitroreductase-mediated reduction to 7-amino metabolites	60, 61
Posttraumatic Stress Disorder	3.6	Sertraline (Zoloft ®)	Hypothesized to inhibit efflux pumps in prokaryotes; affects fungal virulence; broad-spectrum antibiotic activity	62
Obsessive Compulsive Disorder	1.2	Fluoxetine (Prozac®)	Inhibits 5-HT bacterial uptake; Depletes bacteria that induce host 5-HT synthesis	63, 64
Borderline Personality Disorder	1.4	Lithium (Eskalith®)	Increases bacterial richness and diversity	55, 65

Table 1. *Regularly prescribed psychotropics for the seven most common psychiatric disorders are directly linked to alterations in gut microbial composition and/or activity.*

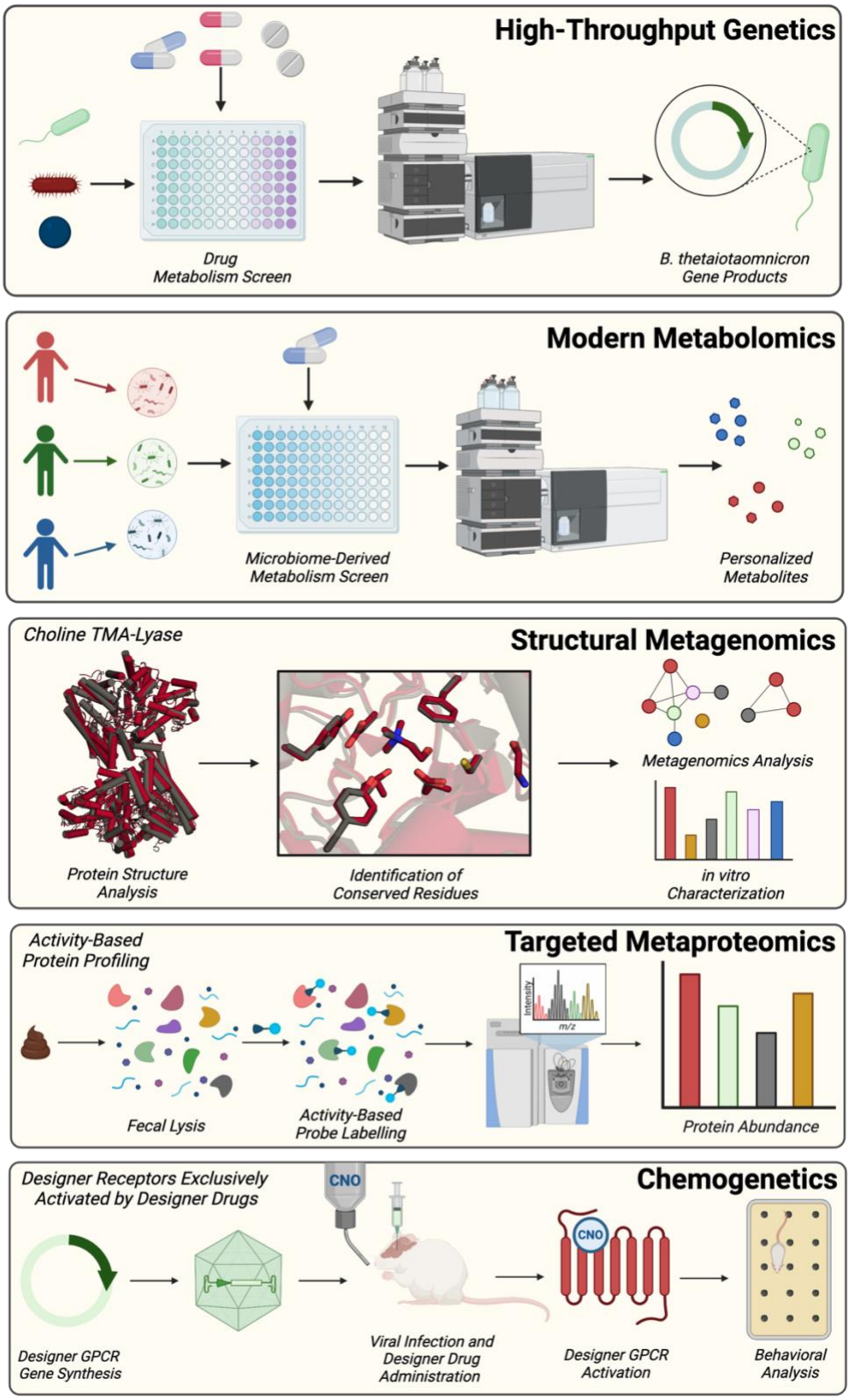

Figure 3. *Multi-omic technologies positioned to be applied towards exploring neurotransmitter metabolism by the gut microbiota.*

Psychiatric Disorder	Annual Prevalence (% of US Population)
Major Depressive Episode	7.2
Major Depressive Episode	7.2
Schizophrenia	< 1
Bipolar Disorder	2.8
Anxiety Disorders	19.1
Anxiety Disorders	19.1
Posttraumatic Stress Disorder	3.6
Obsessive Compulsive Disorder	1.2
Borderline Personality Disorder	1.4

Prescription	Evidence of Relationship to Microbiota
Amoxapine (Asendin®)	Inhibits bacterial β-glucuronidase enzymes
Vortioxetine (Trintillex®)	Inhibits bacterial β-glucuronidase enzymes
Risperidone (Risperidal®)	Depresses resting metabolic rate; Isoxazole scission of benzisoxazole ring system
Lamotrigine (Lamictal®)	Inhibits bacterial ribosomal biosynthesis in *E. coli*; anti-microbial against gram-positive prokaryotes
Clonazepam (Klonopin®)	Bacterial nitroreductase-mediated reduction to 7-amino metabolites
Nitrazepam (Mogadon®)	Bacterial nitroreductase-mediated reduction to 7-amino metabolites
Sertraline (Zoloft®)	Hypothesized to inhibit efflux pumps in prokaryotes; affects fungal virulence; broad-spectrum antibiotic activity
Fluoxetine (Prozac®)	Inhibits 5-HT bacterial uptake; Depletes bacteria that induce host 5-HT synthesis
Lithium (Eskalith®)	Increases bacterial richness and diversity

Citation
11, 54, 59
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56, 57, 58
49, 50
60, 61
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63, 64
55, 65