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Title	Gutsy moves: the amygdala as a critical node in microbiota to brain signaling
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Publication date	2017-11-17
Original Citation	Cowan, C. S. M., Hoban, A. E., Ventura-Silva, A. P., Dinan, T. G., Clarke, G. and Cryan, J. F. 'Gutsy Moves: The Amygdala as a Critical Node in Microbiota to Brain Signaling', BioEssays, 1700172 (12pp), In Press. doi:10.1002/bies.201700172
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1002/bies.201700172
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Download date	2026-11-06 21:33:07
Item downloaded from	https://hdl.handle.net/10468/5116

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Gutsy Moves: The Amygdala as a Critical Node in Microbiota to Brain Signaling

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The amygdala is a key brain area regulating responses to stress and emotional stimuli, so improving our understanding of how it is regulated could offer novel strategies for treating disturbances in emotion regulation. As we review here, a growing body of evidence indicates that the gut microbiota may contribute to a range of amygdala-dependent brain functions from pain sensitivity to social behavior, emotion regulation, and therefore, psychiatric health. In addition, it appears that the microbiota is necessary for normal development of the amygdala at both the structural and functional levels. While further investigations are needed to elucidate the exact mechanisms of microbiota-to-amygdala communication, ultimately, this work raises the intriguing possibility that the gut microbiota may become a viable treatment target in disorders associated with amygdala dysregulation, including visceral pain, post-traumatic stress disorder, and beyond.

1. Introduction: The Microbiota-Gut-Brain Axis

Scientific perspective on the regulation of neural and psychological processes is now expanding beyond the brain to incorporate an understanding of the brain's interactions with other systems. In particular, a recent surge of interest in the gut-brain axis, and its regulation by the microbiome, has produced an ever-growing evidence base supporting the idea that the gut microbiota can alter both brain and behavior.[1–5] In this review, we will focus on the impact of the gut microbiota on the amygdala because this small, almond-shaped brain region is a key regulator of many of the functions found to be altered by microbiota-gut-brain axis perturbations. While the investigation of the relationship between the microbiota and the amygdala is still relatively in its infancy, the early evidence suggests that both the structure and function of the amygdala can be influenced by the gut microbiota.

In daily life, we use common phrases such as "butterflies in my stomach" or "I have a gut feeling" to articulate our intuitions, the first warning signs when something is wrong. This concept that the gut can reveal, or even predict, our thoughts and feelings is one that is ingrained in our psyche and is now gaining the attention it deserves in the scientific literature. In this context, the term "gut-brain axis" was coined to describe the bidirectional communication links between the gastrointestinal tract and the brain. Over the last two decades, this concept has been expanded to become the microbiota-gut-brain axis, in recognition of the accumulating preclinical data implicating the gut microbiota (the trillions of microorganisms within our gut) in this bidirectional communication.[1] Indeed, current evidence points to an influence of the gut microbiota in central nervous system (CNS) function, appropriate brain development, as well as psychological and behavioral outcomes.[1–5]

There are many approaches available to chart the impact of the gut microbiome on CNS function albeit many are used much more in preclinical than human studies.[1,6] These include dietary manipulations, administration of specific strains of bacteria (e.g., probiotics), interventions which support the growth of beneficial bacteria (e.g., prebiotics), germ free (microbiota deficient) mice, antibiotic treatments, C-section delivery, and fecal microbiota transplants. To date, the investigation of the effects of these microbiota manipulations on brain function has been difficult to map to specific brain regions. In particular, germ-free facilities impose substantial logistical restrictions on experimental protocols and access to specialized behavioral equipment within the germ free environment.[7] Thus far, the use of microbial manipulations to test for brain region-specific effects has largely focused on the hippocampus and the prefrontal cortex.[8–12] However, we are

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/bies.201700172.

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DOI: 10.1002/bies.201700172

now beginning to understand that the same microbial manipulations may also have implications for the amygdala.

2. The Amygdala

The amygdala derives its name from its small, almond-like shape. Its small size belies its influence: it is considered to be one of the most important regions within the limbic system and there is a rich history of scientific investigation into its role in emotion processing and behavior modulation.[13,14] It is also surprisingly complex in structure, being composed of several networked regions that have different connectivities, neurochemical characteristics, and cyto-architecture.[15] There is some debate over exactly how to define these regions but it is generally agreed that there are at least 13 sub-nuclei that can be grouped, according to their embryological origins, into the centromedial (central [CeA] & medial [MeA]), basolateral (basal/basolateral [BLA] & lateral [LA]), and cortical nuclei (CoA; see **Figure 1**).[15]

From its central location in the temporal lobe, the amygdaloid complex is highly connected with several brain regions. It receives sensory inputs from the thalamus and cortical areas as well as extensive inputs from other regions in the limbic system, including the prefrontal cortex and hippocampus,[15] both of which are markedly altered in germ-free (GF) animals.[10,12,16] In fact, the connectivity between these three major regions, not just the function of the individual structures, is fundamental to appropriate emotional responses.[17] Although tracer studies have shown afferents reaching all amygdala nuclei, sensory inputs often reach the amygdala through the LA while inputs from other brain regions target primarily the LA and BLA. From here, numerous and complex intra-amygdaloid projections allow substantial communication between subdivisions, often following a lateral to medial direction until signals reach the CeA, from which the majority of efferent projections originate.[15] These efferent projections connect the amygdala to a wide variety of brain areas such as the brainstem, cortical areas, and subcortical nuclei including the hypothalamus and bed nucleus of the stria terminalis.[15,17]

The amygdaloid complex undergoes ongoing development well into postnatal life. Rodent studies have demonstrated that the soma size and number of dendritic spines on BLA neurons reach adult levels by early adolescence, while there are ongoing changes in dendritic morphology into adulthood that parallel electrophysiological maturation of neurons in this region.[18] In humans, imaging studies have shown that total amygdala volume peaks in preadolescence (9–11 years of age).[19] The complex connectivity of the amygdala with other brain regions also continues to develop and mature at least into early adulthood.[20] This ongoing postnatal development, in combination with the high density of glucocorticoid receptors in the amygdala, renders it vulnerable to the effects of stress, particularly during early development and aging.[21] Of note, these timeframes coincide with periods of low diversity and instability in the microbiota.[6,22,23] Although there are differences between humans and rodents in the specific taxonomic changes that contribute to age-related shifts in the microbiota,[22,23] the parallel periods of vulnerability in the amygdala and microbiota are observed across species, suggesting that these are key times for these structures to be mutually influential (see **Figure 2**).

2.1. Amygdala Dysfunction Is a Characteristic Feature of Stress-Related Psychiatric Disorders

2.1.1. The Amygdala Is a Critical Regulator of Fear, Stress, and Anxiety Disorders

Fear and anxiety are overlapping, threat-related emotions that are mediated by similar neurocircuits.[13] The basic components of this fear circuitry are well preserved across species and center

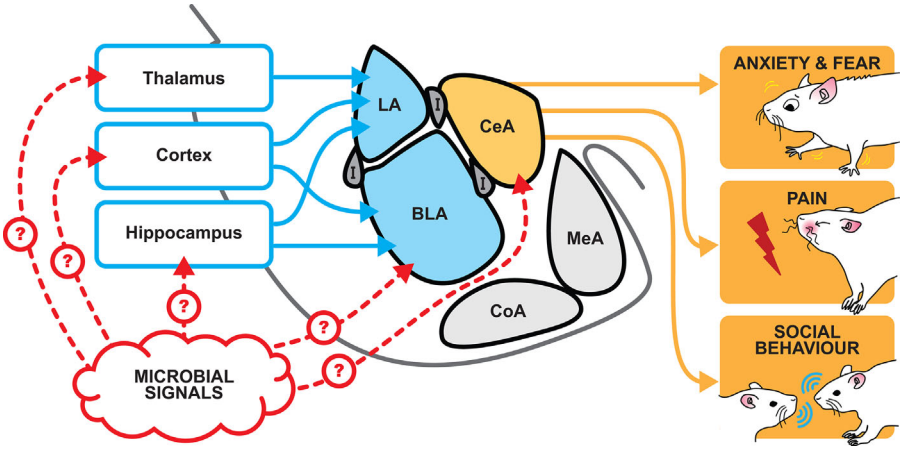


Figure 1. Schematic of the basic structure of the amygdaloid complex in the rat showing major nuclei: lateral (LA), basal/basolateral (BLA), central (CeA), medial (MeA), and cortical (CoA) nuclei, and the intercalated cells (I). Major afferent signals reach the amygdala predominantly through the BLA and LA, while efferent signals mediating behavioral outputs tend to originate from the CeA. It is currently unknown how signals from the microbiota (such as microbial metabolites, including tryptophan/serotonin derivatives, peptidoglycans, immune mediators, and short-chain fatty acids) might reach the amygdala but there are several possible pathways, as highlighted here.

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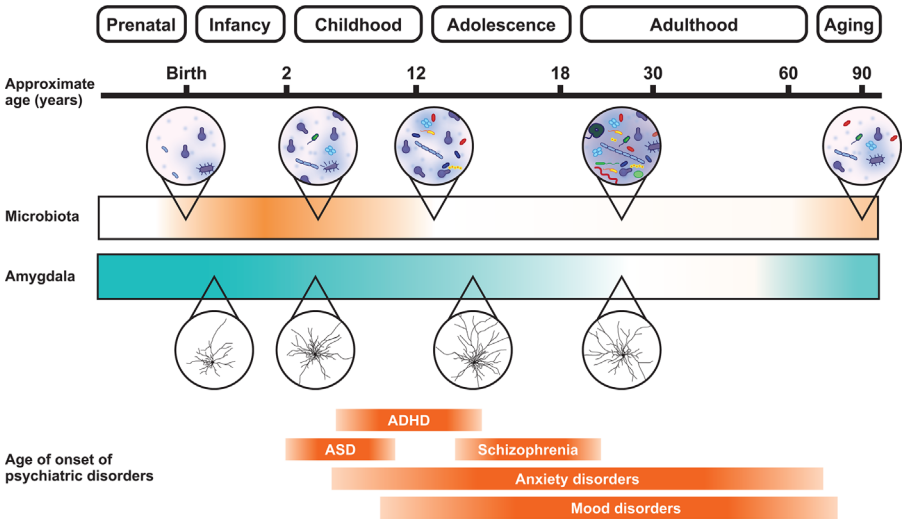


Figure 2. Overlapping timelines of sensitivity to environmental insult or intervention for the amygdala and gut microbiota across the lifespan. The composition of the microbiota is both simple and unstable early in life. During childhood, the microbiota stabilizes and becomes more complex over development, until it begins to destabilize once more during the aging process. Similarly, the amygdala undergoes ongoing development in the postnatal period, extending into late adolescence or early adulthood, and is vulnerable during both early and late life. Early-life vulnerability of the microbiota and amygdala also overlaps with the typical age of onset of many psychiatric disorders.

around the amygdala.[13,14] Different nuclei within the amygdala appear to process diverse aspects of learned fear. For example, the BLA is critical for most learned fear tasks (although perhaps not contextual fear conditioning), while the CeA is considered essential for retrieval of learned fear and the MeA is necessary for learning of aversive associations.[15] The amygdala, and particularly the BLA, is also involved in the development of anxiety-like behavior.[13,17] Imaging studies of clinical populations show that patients with post-traumatic stress disorder (PTSD) and other anxiety disorders exhibit increased activation of the amygdala [24] but reduced resting state connectivity between the amygdala and the prefrontal cortex.[17] Amygdalar expression of microRNAs also appears to be involved in the modulation of anxiety-like behavior.[25]

Fear and anxiety are commonly exacerbated by chronic stress.[21] The amygdala appears to have a role in mediating this relationship, and lesions of the central amygdala prevent the manifestation of stress-related anxiety.[26] Chronic stress upregulates amygdala activity in rodents and this excitatory state is linked with dendritic remodeling, including a persistent expansion of basolateral dendrites.[27] Hypertrophy of the amygdala is also observed in humans and non-human primates that have experienced prolonged exposure to stress.[28,29] Such changes in amygdala structure are likely to compound the effects of ongoing stress because the amygdala has an important role in regulating the body's hormonal stress response via the hypothalamic-pituitary-adrenal axis and the autonomic nervous system.[27]

2.1.2. Mood Disorders and Amygdala Function

While traditionally the amygdala has been mostly associated with fear and anxiety, it also plays an important role in other stress-related disorders. Studies using rodent models of depression indicate that the amygdala undergoes dendritic remodeling and alterations in synaptic plasticity in depressed animals.[30] Clinically, increased activity in the amygdala, which is also seen in GF animals (see section below), has been found in bipolar disorder and major depression.[31] This relationship between amygdala activity and mood begins early in life; amygdala reactivity to negative facial expressions has been correlated with higher severity of depression in pre-schoolers.[32] Additionally, reduced amygdala-frontal connectivity has been associated with depression in both children[33] and adult women.[34]

2.1.3. Role of Amygdala in Neurodevelopmental Disorders and Social Behavior

Many neurodevelopmental disorders are also associated with amygdala abnormalities. Alterations in the volume, activity, and/or connectivity of the amygdala (features influenced by the gut microbiota)[16,35–37] have been reported in individuals diagnosed with schizophrenia,[38,39] attention deficit hyperactivity disorder (ADHD),[40] and autism spectrum disorders (ASD).[41,42] In particular, functional dysregulation of the amygdala is considered central to the social deficits observed in individuals with ASD.[43] Although this "amygdala theory of autism" has been challenged by some,[44] there is certainly evidence that the amygdala has relevance for the development of social behavior and socio-emotional processing across species. Amygdala lesions in humans and non-human primates seem to diminish social inhibition and limit social perception, increasing social interaction and interfering with adherence to social norms.[45,46] In ASD, enlarged amygdala volumes during infancy have been associated with the severity of social and communicative

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impairments.[47] Functionally, hyperactivity of the amygdala has been reported in ASD populations during social tasks.[48] Unfortunately, very few imaging studies in humans have examined the amygdala at the level of its subdivisions, but one investigation of adults with ASD revealed complex patterns of atypical functional connectivity between various sub-regions of the amygdala and other brain regions.[42]

2.1.4. Amygdala Regulates Affective Responses in Pain and Irritable Bowel Syndrome

The amygdala also plays a role in the experience of pain, and particularly in the emotional-affective dimensions of pain perception.[49] In fact, the laterocapsular division of the central nucleus (CeA) has been labeled by some as the "nociceptive amygdala" because of its role in integrating nociceptive inputs from the brainstem and thalamus with information about the emotional relevance of pain.[49] Neuronal activity and synaptic transmission in the CeA become elevated in response to chronic pain, which has been suggested to enhance nociception and contribute to the heightened anxiety that often accompanies persistent pain conditions.[49] Consistent with this interpretation, experimental activation of the amygdala has been shown to exacerbate visceral pain in rats.[50]

Given the affective role of the amygdala in pain perception, it is unsurprising that this region has also been implicated in irritable bowel syndrome (IBS), a disorder associated with microbiota alterations[51] and characterized by gastrointestinal discomfort, visceral pain, and frequent comorbidity with affective dysfunction.[52] IBS patients exhibit hyperactivity in the amygdala and connected brain regions both at baseline and in response to visceral stimulation.[53,54] In some studies at least, sex and genetic differences have been identified in IBS-induced amygdala hyperactivity, and stronger effects are observed in males[55] and those with a particular polymorphism of the serotonin receptor gene, HTR3A.[56] Overall, amygdala hyperactivity seems to be related to chronic visceral hypersensitivity, pointing to another important link between the gut and the amygdala.

3. The Microbiota Has Many Potential Routes of Communication to the Amygdala

Our current understanding of the microbiota-gut-brain axis suggests that there are a number of plausible routes of communication from the microbiota to the brain.[1,57] It is not yet known how microbial signals might travel across the gut-brain axis to reach the amygdala specifically, however there are some strong candidate pathways including the vagus nerve, spinal cord, tryptophan metabolism, and immune modulation (see **Figure 3**).

3.1. Microbiota Interacts with the Enteric Nervous System and Vagus Nerve

The gastrointestinal tract is home not only to a large ecosystem of microorganisms, it also contains approximately 50,000

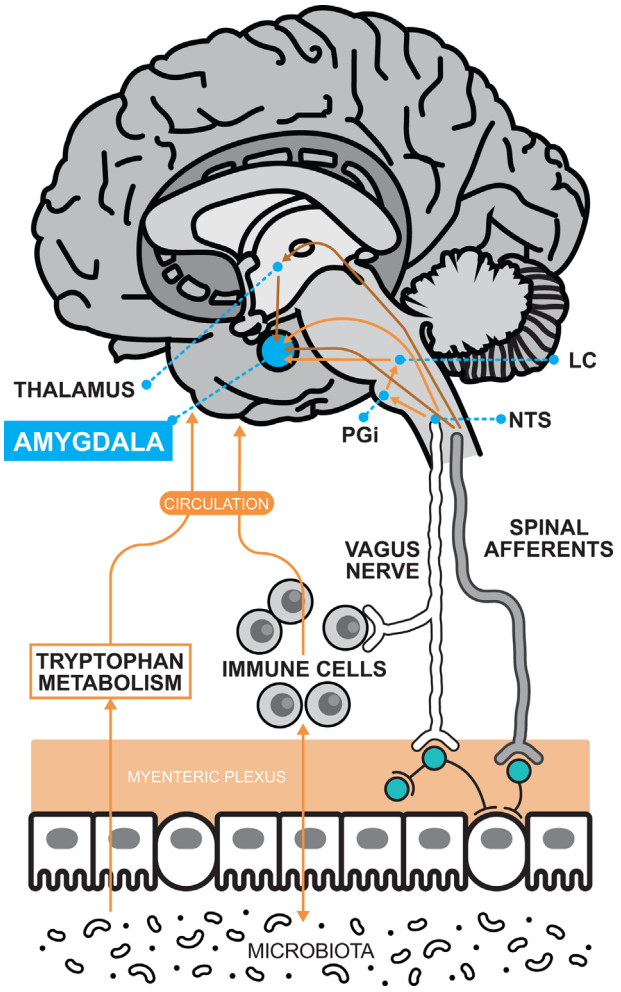


Figure 3. Potential pathways for microbiota-to-amygdala communication. There are a number of established pathways of communication between the microbiota and the brain, which include the circulation (via microbial metabolites, e.g., tryptophan/serotonin derivatives, and immune cells), the vagus nerve (via the immune and enteric nervous systems), and the spinal cord. The vagus nerve and spinal cord terminate in the brain stem, from which signals may be relayed to the amygdala via different pathways, as shown here. LC, locus coeruleus; NTS, nucleus tractus solitarius; PGi, nucleus paragigantocellularis.

extrinsic and 100 million intrinsic sensory afferent neurons in the enteric nervous system (ENS).[58] As reviewed elsewhere,[58] the microbiota can alter neuronal activity within the ENS both directly and indirectly. Axonal processes on intrinsic primary afferent neurons extend into the gut mucosa, where they can interact directly with microbial cell components (e.g., lipopolysaccharide) and microbial metabolites (e.g., serotonin) in the gut lumen, while indirect microbial communication with the ENS may occur through enteroendocrine cells, including enterochromaffin cells.[59,60]

Signals produced by these microbial-neural interactions may then reach the brain via the vagus nerve; vagal afferents from the gastrointestinal tract terminate in the nucleus tractus solitarius (NTS).[61] Confirming this pathway, the behavioral and neural

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actions of certain probiotic strains are abolished if the vagus nerve is severed (a procedure known as a vagotomy).[4,62] From the NTS, there are both direct and indirect noradrenergic projections to the amygdala (see Figure 3).[61] Thus, visceral information collected by the vagus nerve may ultimately influence amygdala activity. Indeed, vagus nerve stimulation has been shown to stimulate the release of norepinephrine in the amygdala[63] and to modulate amygdala-prefrontal cortex connectivity while enhancing behavioral outcomes (i.e., reduced fear/depressive symptoms) in both preclinical models of fear extinction[64] and clinical investigations of major depressive disorder.[65] Conversely, disruption of vagal communication by subdiaphragmatic deafferentation of the vagus nerve has been shown to impair fear extinction but reduce anxiety-like behavior in rats.[66]

3.2. Spinal Cord Transmission of Microbiota to Amygdala Signals

Microbial signals may also be communicated to the CNS through the spinal cord. This pathway is thought to be particularly important for the perception of visceral pain signals.[57] There are a number of different types of afferent spinal nerve endings that innervate the gastrointestinal tract, where they may detect the release of neuroactive compounds.[67] Signals from these neurons may then reach the amygdala via the brainstem (spinoparabrachial pathway) and the thalamus (spinothalamic pathway).[68] Importantly, it is now known that the gut microbiota can directly produce many neurotransmitters used for communication in the ENS and CNS (e.g., gamma-aminobutyric acid [GABA], norepinephrine, dopamine, acetylcholine, serotonin) and stimulate the production of others.[69] Bacterial modulation of spinal cord responses has been demonstrated using probiotic species; the presence of live *Lactobacillus reuteri* reduces spinal nerve firing in response to the introduction of painful visceral stimulation.[70]

3.3. Microbiota Regulates Tryptophan Metabolism

One key pathway for neurotransmitter production that is highly influenced by the microbiota is the metabolism of tryptophan. Tryptophan is an essential amino acid that is obtained from dietary proteins and is metabolized primarily into kynurenine or serotonin.[71] Serotonin is well-known as a neurotransmitter involved in mood and cognition, and disruption of the serotonergic system is a key feature of anxiety and mood disorders, for which the first-line therapeutics are selective serotonin receptor inhibitors (SSRIs). In addition to its mood regulatory function, serotonin has an important role in gastrointestinal function and is thought to facilitate communication throughout the gut-brain axis.[71] In fact, the vast majority of the body's serotonin is produced in the gastrointestinal tract by enterochromaffin cells.[71]

Recent studies highlight the role of the microbiota in modulating both serotonin secretion and tryptophan metabolism in humans and rodents.[72–75] To this end, GF animals exhibit striking alterations in circulating tryptophan levels and serotonergic neurotransmission, at least in the hippocampus.[10,76] Similarly, alterations in the microbiota in a murine model of autism are associated with deficient tryptophan metabolism and gastrointestinal dysfunction.[77] Prebiotic and probiotic treatments have been shown to alter central levels of serotonin or serotonin receptors in the hippocampus and prefrontal cortex, both regions that are highly connected to the amygdala.[11,73] This could have an important influence on the amygdala, where serotonin signaling regulates activity and synaptic plasticity, guiding the structure's response to emotional stimuli.[78] Modulation of peripheral serotonin availability via dietary tryptophan depletion has been shown to increase amygdala activation during emotion recognition tasks in humans.[79] Furthermore, dietary tryptophan depletion enhances functional connectivity between the amygdala and the prefrontal cortex.[80]

3.4. Immune Modulation Is a Key Communication Pathway in the Microbiota-Gut-Brain Axis

The microbiota is crucial for the establishment and development of the immune system.[81] When the microbiota is depleted (either in GF or antibiotic-treated animals), the immune response is profoundly altered.[7,81] At least some of these immune irregularities in GF mice can be restored by colonization with a normal microbiota[82] or with specific probiotic strains.[83] Additionally, some probiotics have been shown to attenuate pro-inflammatory profiles in cases of pathogen exposure or clinical disease states.[84,85] This microbial modulation of inflammation has implications for the amygdala, which is responsive to peripheral immune activation. For instance, systemic inflammation stimulates cytokine production and neuronal activity in the amygdala.[86] Further, diet-induced obesity is associated with a neuroinflammatory profile across a range of brain regions, including the amygdala, and it has been hypothesized that one mechanism for this diet-induced neuroinflammation is through alterations in the microbiota.[87] Overall, it is clear that there are many routes by which the microbiota might influence the amygdala.

4. Gut Microbiota Plays a Key Role in Establishing and Modulating Amygdala Function

4.1. Microbiota Modulates Amygdala-Dependent Behavior

For many of the behaviors and conditions described above as amygdala-dependent, there is also evidence of regulation by the microbiota (see **Table 1** for a summary). These behavioral findings gave the first indications that there may be correspondence between amygdala and microbiota functioning, alluding to the possibility that the microbiota-gut-brain axis may extend beyond visceral perception to influence higher order brain structures including the amygdala.

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Table 1. Effects of microbiota manipulation on amygdala-dependent behaviors.

	Germ-free	Antibiotic	Prebiotic	Probiotic	FMT
Anxiety	Diminished anxiety in mice (open field, elevated-plus maze, light-dark box) and zebrafish (thigmotaxis)[5,9,10,88,90] Heightened anxiety in rats (open field)[89]	Anxiolytic effects, sometimes sex-specific, in mice (light-dark box, elevated-plus maze)[72,113]	Anxiolytic effects in mice (open field, defensive marble burying, elevated-plus maze, light-dark box)[91,93]	Strain-specific anxiolytic effects in humans (e.g., Beck Anxiety Index, Hospital Anxiety & Depression Scale) and rodents (defensive marble burying, elevated-plus maze, open field)[11,62,133,134]	Transplant from humans with depression or comorbid IBS and anxiety increases anxiety-like behavior in mice (open field, step-down test, light-dark box)[96,125]
Depression	Increased depressive-like behavior in mice (forced swim)[90]	Increased depressive-like behavior in rats (forced swim)[94]	Antidepressant effects in mice and rats (forced swim, tail suspension, learned helplessness after inescapable shock)[91,92]	Strain-specific antidepressant effects in humans (e.g. Beck Depression Inventory, Hospital Anxiety & Depression Scale) and rodents (tail suspension test, forced swim, sucrose preference)[11,97,98,133,134]	Transplant from depressed human donors induces depressive-like behavior in mice (sucrose preference, forced swim [varied results], tail suspension test)[90,96]
Learned fear	Impaired fear recall in adult mice[101]	Acute administration enhances fear extinction in rodents and exposure therapy in humans,[103,104] reduces fear recall in humans[102]		Enhanced fear learning and memory or slow fear extinction in adult rats[62,105] Reversal of stress effects on fear recall and extinction in infant rats[107,108]	
Social behavior	Social deficits in rats and mice (less interest in social interaction, poor memory for social partners.)[107,108] (Stilling et al., unpublished observations)	Social deficits in mice (less interest in social interaction, poor memory for social partners)[110,113]		Reversal of social deficits in murine models of ASD[111,115] Reversal of antibiotic-induced social deficits in mice[113] Reduced risk for ASD[116], reduced symptom severity in children with ASD (open pilot)[117]	Transplant of standardized human gut microbiota to children with ASD improved ASD symptoms in open-label pilot[118]
Pain	Visceral hypersensitivity in mice[119]	Visceral hypersensitivity in healthy mice[94,120,135] Reversal of visceral hypersensitivity in stressed mice[122] Increased risk for IBS in humans[51] Positive effects on pain in IBS patients[127]	Reversal of stress-induced visceral hypersensitivity in rats[121]	Reversal of antibiotic-induced visceral hypersensitivity in mice[135] Positive effects on IBS symptoms in humans[127]	Transplant from IBS donors increases GI symptoms (accelerated gastrointestinal transit, increased intestinal permeability) in mice[125,126] Transplant from healthy donors reduces GI symptoms (constipation, diarrhea, indigestion, abdominal pain) in children with ASD (open-label pilot)[118]

4.1.1. Anxiety- and Depression-Like Behavior are Regulated by Gut Microbiota

Perhaps the most consistent finding in this regard has been for anxiety-related behaviors.[2] Diminished anxiety-like behavior has been reported in GF mice[5,9] and GF zebrafish.[88] Interestingly, there seems to be a divergence between behavioral and hormonal measures of the stress response in GF mice, which exhibit heightened corticosterone levels following exposure to a stressor despite their low-anxiety behavioral profile.[8,10] However, in agreement with the neuroendocrine profile, heightened anxiety-like behavior has been observed in GF rats.[89] GF animals also exhibit increased depressive-like behavior.[90] Additional preclinical studies have shown that anxiety- and/or depression-like behaviors are also altered by certain probiotic treatments,[62] prebiotics,[91–93] and antibiotics.[94]

In humans, alterations in the microbiota have been observed across a variety of amygdala-related clinical disorders, including depression.[90] In addition, the composition of the microbiota is associated with differences in affective responses to unpleasant emotional images in healthy females.[95] Supporting these correlational studies, fecal microbiota transfer of samples from depressed individuals has been shown to alter the behavioral profile of recipient microbiota-deficient mice and rats, increasing anxiety- and depression-like behaviors.[90,96] Finally, different probiotic strains have been shown to reduce symptoms of anxiety and depression across several studies in clinical and healthy populations.[97,98] While there have also

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been several studies reporting no effect of probiotics on these measures,[99] these disparate results may be explained by strain-dependent effects or a moderating effect of symptom severity because it has been reported that the beneficial effects are greatest for those with the most negative symptoms at baseline.[100]

4.1.2. Microbiota Alters Fear-Related Behavior

One key difficulty in using anxiety- and depression-related behavioral tests or symptom scores to index amygdala dysfunction is that these factors are not specifically or exclusively dependent on the amygdala.[24,31] A recent investigation from our group aimed to bridge this gap by examining cued fear conditioning, which is considered a more specific behavioral read-out of amygdala function.[101] Using a modification of the classical conditioning protocol, it was demonstrated for the first time that amygdala-dependent fear memory is compromised in GF mice.[101] That is, the GF individuals displayed impaired short-term fear recall in adulthood as compared to conventionally raised adults or ex-GF adults (i.e., mice raised in a GF environment but re-housed with conventional animals at weaning to restore the microbiota). In this same study, a number of differences in amygdala gene expression were also observed in the GF animals, suggestive of baseline hyperactivity and differential responding to the fear stimulus, which may have contributed to these behavioral changes (discussed in detail in a later section).

The impaired fear recall observed in GF animals has interesting parallels to a recent study showing that administration of doxycycline during a fear conditioning procedure can reduce later expression of fear recall in humans.[102] While the authors investigated doxycycline due to its properties as an inhibitor of extracellular matrix metalloproteinase 9, it is also a broad-spectrum antibiotic. The N-methyl-D-aspartate (NMDA) receptor partial agonist D-cycloserine (DCS) is another broad-spectrum antibiotic with fear-modulating properties. Specifically, it has been shown to enhance fear extinction in rodents and exposure therapy in clinical anxiety disorders.[103,104] Although it is unclear whether a microbial mechanism is at play in these cases of acute antibiotic administration, it is certainly a hypothesis worth considering.

It has also been shown that probiotic treatments can alter learned fear expression. Administration of probiotic *Bifidobacteria* strains has been shown to enhance both contextual and cued fear conditioning as well as contextual fear recall.[105] The effects of probiotics on extinction of learned fear responses have been more mixed in adult rodents. The *Bifidobacteria* strains had no effect on fear extinction,[105] whereas a *Lactobacillus* strain slowed extinction learning in adult mice although anxiety-like behavior was reduced following treatment.[62] Most recently, it has been shown that immunization with a heat-killed bacterium (*Mycobacterium vaccae*) accelerates extinction learning in adult rats.[106] In developing rats, a probiotic formulation of two *Lactobacillus* strains has been shown to protect against the effects of early-life stress on learned fear behavior.[107,108] Specifically, maternally separated male rats exhibit unusually persistent fear memories and higher rates of fear relapse after extinction during infancy. However, this "high-fear" profile can be prevented by administration of probiotics during the period of stress.[108] The same probiotic treatment also prevented generational transmission of the high-fear profile to the offspring of maternally separated males.[106,107] It remains to be seen whether these findings will translate to humans, but together these studies suggest that the microbiota could become a valuable tool in regulating amygdala-dependent fear expression.

4.1.3. Microbiota is Critical for Social Behavior

It has been hypothesized that the co-evolution of humans with our microbiota may have been a driving force in our sociability.[109] Notably, high levels of social interaction allow greater transfer of microbial species between hosts. This creates a mutually beneficial situation whereby the micro-organisms have a greater chance of survival while the host is exposed to greater diversity in the microbiota, which has positive implications for pathogen resistance and dietary metabolism.[109] In line with this hypothesis, investigations of GF mice have illustrated that the microbiota is key for normal social interactions. Like humans, rodents are naturally social animals, but GF rats and mice exhibit deficits in social behaviors such that they show less interest in social interaction and poorer memory for social partners.[89,110–112] Deficits in social behavior are also observed following antibiotic-induced depletion of the microbiota.[72,113]

The microbiota also plays a role in murine models of ASD, where treatment with specific bacteria has been shown to reverse many of the social deficits observed in these animals.[111,114] There is some promising early evidence that this approach may translate to clinical settings. Individuals with ASD often exhibit gastrointestinal problems and alterations in the microbiota.[115] Accordingly, preliminary pilot studies show that probiotics may reduce risk for ASD onset if administered perinatally[116] and reduce symptom severity in children already diagnosed with ASD.[117] In addition, significant improvements in gastrointestinal and behavioral symptoms were observed in an open pilot of faecal microbiota transplants for children with ASD.[118]

4.1.4. Microbial Signals Modulate Visceral Pain

As was the case for other key amygdala-dependent behaviors, visceral pain perception is also regulated by the gut microbiota.[57] For example, microbiota depletion has been shown to induce visceral hypersensitivity in adulthood for both GF mice[119] and mice exposed to antibiotics early in life.[120] On the other hand, stress-induced visceral hypersensitivity can be reversed by treatment with either probiotics[121] or antibiotics.[122] Microbiota modulation of visceral pain perception is one reason that the microbiota has been posited to play an important role in the etiology and maintenance of IBS.[51] Indeed, IBS is associated with altered microbiota composition, at least in a substantial subset of patients.[123,124] Furthermore, the microbiota has been causally implicated in IBS in two studies utilising

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fecal microbiota transplant.[125,126] By transferring the microbiota from IBS patients into healthy mice, characteristic symptoms of IBS gastrointestinal dysfunction can be induced, including accelerated gastrointestinal transit and increased intestinal permeability. In addition, both probiotic and antibiotic treatments have been shown to have positive effects on pain symptoms in clinical studies of IBS patients.[127]

4.2. Microbiota Modulates Amygdala Structure and Function

Building on observations that amygdala-dependent behavior is altered by the microbiota, there are an accumulating number of studies suggesting that the amygdala itself is sensitive to microbiota manipulations (see **Table 2** for a summary). Most convincingly, converging evidence from GF mice indicates that the amygdala transcriptome is hyperactive in the absence of a microbiota.[36,37] This hyperactive state is consistent with the altered phenotype of GF mice in regards to both social and fear-related behaviors,[101,110] pain sensitivity,[119] as well as elevated stress-induced HPA axis responses (see **Figure 4**).[8,10] More broadly, the studies outlined below suggest that microbiota-to-amygdala signalling may result in functionally relevant changes in the amygdala.

4.2.1. Amygdala Morphology and Gut Microbiota

Using GF mice, it has been demonstrated that the microbiota influences amygdala morphology.[16] It was observed that the amygdala is enlarged in GF adult mice, reminiscent of findings in clinical studies of children with ASD albeit that differences in amygdala size typically resolve by adulthood in clinical samples, and dendritic morphology in amygdala neurons is also altered. Specifically, dendritic length was extended, the number of branch points on interneurons was increased, and spine density on pyramidal neurons was increased in the amygdalae of GF mice. Although two studies failed to find a relationship between overall microbiota composition and amygdala volume in IBS patients and healthy women,[95,123] another identified a particular bacterial phylum that was associated with microstructure of this region.[128] That is, abundance of Actinobacteria correlated with increased fractional anisotropy in the amygdala. The specificity of these findings is likely reflective of the fact that differences in microbiota composition across human participants are far more subtle and varied than the stark contrast to be expected between conventionally raised and GF mice.[7]

4.2.2. Microbiota Influences Amygdala Activity and Connectivity in Functional Brain Imaging Studies

Recent functional magnetic resonance imaging (fMRI) studies in humans suggest that the microbiota can also alter amygdala connectivity. Preliminary data indicate that the composition of the microbiota is associated with altered functional connectivity between the amygdala and anterior insula in very young children, which is also predictive of cognitive performance at 2 years of age.[129] In healthy women, it has been shown that different microbiota profiles are associated with differences in the patterns of structural white-matter connectivity.[95] The main distinguishing feature was an alteration in the connectivity between an emotion regulation network (including the amygdala) and other brain regions involved in attention and sensory perception. In a separate study by the same group,[35] 4 weeks of a probiotic treatment was sufficient to alter functional network responses to emotional faces in healthy females. In particular, amygdala activity was dampened during the task and resting state connectivity of a network that included the

Table 2. Relationships between the microbiota and amygdala structure and function.

	Germ-free	Antibiotic	Probiotic	Observational
Morphology	Enlarged volume, increases in dendritic length, branch points and spine density in mice[16]			No correlation between microbiota composition and amygdala volume in healthy women or IBS patients[95,123] Actinobacteria correlated with enhanced microstructure (increased fractional anisotropy)[128]
Activity and connectivity			Dampened activity during emotional faces task, altered resting state connectivity with distributed network including hippocampus and thalamus[35]	Microbiota profile associated with structural white-matter connectivity in healthy women and with functional connectivity in young children[95,129]
Gene expression	Specific mRNA expression: NGFI-A, BDNF decreased, NR2B decreased in CeA of mice[5,9] Whole genome sequencing: broad alterations suggestive of upregulation of neuronal activity, differential gene regulation in response to fear and social stimuli [36,101](Stilling et al., unpublished observations) Broad dysregulation of miRNA in mice [37]	mRNA expression: Nr3c1, CRH-R1 decreased, BDNF increased in rats[94] Protein expression: BDNF decreased in mice[4]	*Lactobacillus rhamnosus* (JB-1) decreased $GABA_{B1b}$ mRNA in BLA and CeA of mice[62]	

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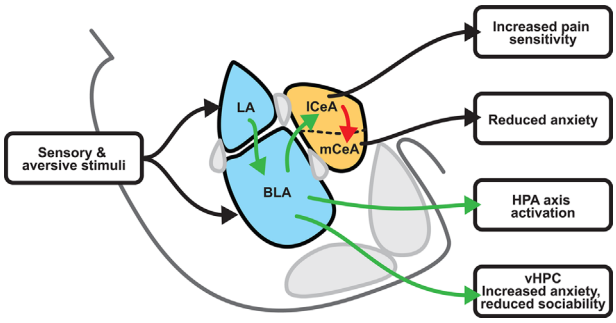


Figure 4. Hypothesised activation and inhibition of amgydalar circuits can explain the behavioral and physiological profile of germ-free mice. Specifically, hyperactivation of the lateral (LA) and basolateral (BLA) nuclei as well as the lateral division of the central nucleus (lCeA) would promote HPA axis and ventral hippocampus (vHPC) activation but inhibition of the medial division of the central nucleus (mCeA). The behavioral consequences of these functional changes include increased pain sensitivity, increased stress responsivity, changes in anxiety, and dampened sociability.

amygdala was altered for the probiotic-treated participants. Essentially, these data provide the first evidence of a clinical link between the microbiota and functional connectivity of the amygdala.

4.2.3. Microbial Regulation of Gene Expression in the Amygdala

A variety of changes in mRNA and protein expression have been observed in the amygdala of GF and antibiotic-treated animals. These include frequently reported alterations in levels of BDNF,[4,5,94] which has critical roles in CNS development and neuronal plasticity and has been implicated in a range of psychiatric disorders.[130] Further, microbiota-depletion has been shown to alter expression of mRNA for a number of genes related to anxiety and mood disorders, stress responses, and fear learning and extinction, such as the glucocorticoid receptor Nr3c1, CRH receptor 1, and the NMDA receptor NR2B.[9,94] The probiotic *Lactobacillus rhamnosus (JB-1)* has also been shown to alter GABA receptor mRNA levels in the rodent amygdala in a vagus-dependent manner.[62] However, this particular strain did not seem to have any effect on healthy human subjects, highlighting the importance of conducting translational studies in this field.[131]

Whole genome sequencing studies in GF mice have demonstrated that the absence of a microbiota broadly alters the amygdala at a transcriptional level.[36,37,101] Multiple complementary analyses produced converging evidence that, under basal conditions, GF mice exhibit significant up-regulation of genes related to synaptic plasticity, transcription, and neuronal activity.[36] This included upregulation of the immediate early response genes *Fos*, *Fosb*, *Egr2*, and *Nr4a1*, while network analysis of dysregulated genes identified interaction partners belonging to the cAMP responsive element binding protein (CREB) and MAP-Kinase pathways (both of which are heavily implicated in the regulation of neuronal plasticity).[30,132] Similar increases in genes related to neuronal activity, synaptic plasticity, synaptic transmission, and neuron development were later observed in a different GF mouse strain.[101] In both strains of mice, these changes in gene expression were partially reversed by post-weaning colonisation in ex-GF animals.[36,101]

The amygdala transcriptome has also been measured in GF mice following exposure to a fear-conditioned stimulus.[101] As expected, increased expression of the immediate early genes *Fos*, *Fosb*, and *Egr2* was observed in the conventional mice following fear expression, indicating task-related activation of the amygdala. However, the expression of these genes was similar for naïve and fear conditioned GF animals, likely due to the elevated levels of expression in naïve GF animals, possibly due to differential baseline priming of the amygdala. More recently, further transcriptomic studies from our group, this time in response to social interaction stimuli, have shown similar dysregulation of the amygdalar transcriptional response, including a lack of social interaction-induced gene expression for many of the genes upregulated in conventionally raised mice (Stilling et al., unpublished observations). Taken together, these studies reveal that the transcriptional landscape of the amygdala is contingent on the host microbiota. Further, it appears that elevated basal activity in GF animals may prevent appropriate upregulation of neuronal activation in response to amygdala-dependent tasks.

To complement the above findings, we have investigated whether the altered transcriptional pathways in the amygdala are also regulated by the microbiota at the post-transcriptional level.[36,37] We found a large dysregulated network of miRNA expression in GF mice, which may be associated with the observed behavioral changes. Using an in silico approach, it was noted that many of the altered transcriptional pathways in GF mice could indeed be regulated by miRNAs. Thus, it seems likely that microbes may influence not only transcriptional but also post-transcriptional mechanisms in GF mice. Overall, these data support the concept that the microbiome can have a direct role in the regulation of behavior, particularly for amygdala-dependent responses.

5. Conclusions and Future Perspectives

There is a growing literature to support the notion that changes in the microbiota can not only be communicated to the amygdala but can also induce functionally relevant alterations in this brain region. This highlights the potential of the microbiota as a target to produce changes in amygdala-dependent behaviors, particularly in cases of amygdala dysfunction. Although preliminary in nature, there is some promising but largely preclinical evidence to suggest that microbiota modulation may be a useful tool in the treatment of amygdala-related clinical disorders. These findings warrant replication to establish the robustness of microbiota effects on amygdala-dependent outcomes. In addition, further research is needed to test hypotheses regarding the mechanisms for current findings and the translational value of preclinical research in this area.

As we delve further into the mechanisms for microbial interactions with the brain, and more specifically with the

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amygdala, we will be able to define the conditions under which the microbiota contributes to amygdala function. This will require creative solutions to the problems posed by the study of amygdala-specific behaviors in preclinical research on the microbiota, particularly in germ-free settings. It will also be crucial for clinical research to move beyond observational studies in order to assess the translational value of current findings in rodents. These goals, while challenging, are certainly worth pursuing because the reward for such research could be invaluable insights into the potential applications of microbiota manipulations for the treatment of amygdala-related disorders.

Abbreviations

ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; BLA, basolateral amygdala; CeA, central amygdala; CNS, central nervous system; CoA, cortical nucleus of the amygdala; DCS, D-cycloserine; ENS, enteric nervous system; fMRI, functional magnetic resonance imaging; GABA, gamma-aminobutyric acid; GF, germ-free; IBS, irritable bowel syndrome; LA, lateral amygdala; MeA, medial amygdala; NMDA, N-methyl-D-aspartate; NTS, nucleus tractus solitarius; PTSD, post-traumatic stress disorder; SSRI, selective serotonin reuptake inhibitor.

Acknowledgements

This work was supported through the JPI-HDHL on "Biomarkers for Nutrition and Health," "HEALTHMARK" by Science Foundation Ireland (SFI), Ireland (16/ERA-HDHL/3362). The APC Microbiome Institute is funded by SFI, through the Irish Government's National Development Plan. JFC is supported by SFI (Grant 12/RC/2273). The authors thank Ryan Carceller for his assistance with the figures.

Conflict of Interest

The APC Microbiome Institute has conducted research funded by many Pharmaceutical and Food Companies. TGD has been an invited speaker at meetings organized by Servier, Lundbeck, Janssen, and AstraZeneca and has received research funding from Mead Johnson, Cremo, Suntory Wellness, Nutricia, and 4D Pharma. JFC has been an invited speaker at meetings organized by Mead Johnson, Yakult, Alkermes and Janssen and has received research funding from Mead Johnson, Cremo, Suntory Wellness, Nutricia, and 4D Pharma.

Keywords

anxiety, fear, gut-brain axis, limbic system, pain, social behavior, stress

Received: September 19, 2017
Revised: October 23, 2017
Published online:

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