Determining a role of the gut microbiota on adult limbic BDNF for mood and anxiety regulation

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Background

Disorders of mood and anxiety are common and some of the leading causes of disability worldwide. Major depressive disorder alone has serious costs on individuals and communities, including early termination of education, decreased financial success, and lower work performance (Kessler, 2011). Because of the prevalence of depression and anxiety, these disorders have been widely studied in terms of their symptomatology and contributing causes, both biological and environmental. Symptoms of both anxiety and depression, including feeling sad, loss of energy, and/or heightened fear, are related to specific brain regions, neurochemicals, and the gut microbiome. How the gut microbiome--the bacterial profile naturally present in the digestive tract--interacts with central nervous system is largely unexplored, yet researchers are finding the gut-brain axis to be more influential in nervous system function and regulating behavior than previously thought.

The hippocampus and amygdala, which make up part of the limbic system, are involved in mood and anxiety regulation and are traditionally affected in patients with mood and anxiety disorders. Additionally, while most neurons in the mammalian body arise during fetal development and last throughout the lifetime, the dentate gyrus of the hippocampus and the amygdala are also locations of adult neurogenesis, and are therefore highly plastic, which aids in resiliency against mental disorders. Neurogenesis is the process by which neural precursor stem cells divide, proliferate, and mature into neurons and is important in learning and in reducing vulnerability to mental and neurodegenerative diseases. The process of maturation of neural precursor cells (NPCs) to neurons can be influenced by local and peripheral chemical factors, as well as behavior. In studies of mammals exposed to stress, fewer proliferating granule cells in

the dentate gyrus are observed compared to control animals, indicating a correlation between mood and anxiety regulation and neurogenesis (Gould *et al*., 1997).

During development, endogenous soluble proteins called neurotrophic factors (NTFs) are involved in regulating the survival, growth, morphological plasticity, and protein synthesis for differentiating and mature neurons. Several NTFs are present at different stages of development, including nerve growth factor (NGF), neurotrophin-3 (NT-3), and brain-derived neurotrophic factor (BDNF). These NTFs are also present throughout adulthood, where they foster neurogenesis in neurotrophic regions, like the hippocampus and amygdala. BDNF is a primary neurotrophic factor of adult neurogenesis, and it serves to regulate synapses, with structural and functional effects ranging from short-term to long-lasting, on excitatory or inhibitory synapses, in many brain regions in the adult brain (Lu *et al*., 2014).

Expression level of BDNF in the limbic cortex is correlated with certain mental health disorders, including mood and anxiety disorders, suggesting the molecule's role in regulating mood and anxiety. Rats exposed to chronic mild stress (CMS) displayed memory deficits and anhedonia-like behaviors, which were correlated with reduced expression of central BDNF compared to controls (Fu *et al*., 2016). Further, antidepressants have been shown to upregulate BDNF in the hippocampus after chronic drug treatment, affirming BDNF's role in mood regulation (Nibuya *et al*., 1995). Deficits in BDNF signaling contribute to the pathogenesis of several other major diseases and disorders, such as Huntington's disease, Alzheimer's disease, and major depressive disorder (Lu *et al*., 2014). Manipulating BDNF pathways represents a viable treatment approach to a variety of neurological and psychiatric disorders. Determining the route of influence between BDNF expression in the hippocampus and amygdala and mood and anxiety disorders is crucial in guiding treatment options for these illnesses.

Chronic stress is not the only contributing factor to changes in neurotrophic factor expression post-development and regulation of mood and anxiety. The gut microbiome appears to affect NTF expression in the hippocampus and amygdala. The gut microbiome is a complex environment of diverse bacterial species in the gastrointestinal tract that interacts with the host and aids in digestion and chemical signaling. New light is being shed on the role the gut microbiome plays in nervous system development, function, and pathogenesis. Bacteria have various functions in the microbiome, both beneficial and detrimental to health, and a review by Jameson *et al*. (2018) determined that although there have been preliminary studies of behavioral effects of germ free (GF) mice--mice produced by hysterectomy rederivation, and must be maintained in isolators under very strict handling procedures--our knowledge of the microbiome and how it impacts other physiological systems, such as the brain, is highly unexplored.

However, some findings have been reported in relation to the neurotransmitter and gastric signaling molecule, serotonin. Serotonin, a major mood regulating neurotransmitter, is present in the enteric nervous system (ENS) of the digestive tract, where it serves to regulate gastric motility. Though the ENS acts in an independent network from the autonomic nervous system in the digestive tract, it can be influenced by other nervous systems. ENS serotonin expression increases due to upregulation of Trp1, an enzyme necessary to convert tryptophan, serotonin's precursor molecule, to serotonin. The gut microbiota acts in the ENS and interacts with this signaling pathway, as some bacterial *Clostridial* species in the colon can upregulate Trp1 (Yano *et al.*, 2015). It has not been fully determined how peripheral serotonin of the ENS interacts with central NS serotonin, but there is potential that these molecules cross paths in the body. The complexity of animal physiology suggests that serotonin is likely not the only compound involved regulated by gut bacteria and the CNS.

BDNF expression level in the CNS also change due to the gut microbiota, and these changes are seen directly in the hippocampus. The interaction between gut microbiota and the central nervous system is colloquially referred to as the gut-brain axis. One of the initial studies of the gut-brain axis by Sudo *et al*. (2004) found that in the hippocampus and cortex, BDNF levels of GF mice were lower than in specific-pathogen free (SPF) mice--mice free of a specific list of disease-causing pathogens that can affect mouse health and research outcomes, as well as opportunistic and commensal organisms that typically do not cause illness in normal, healthy animals. Changes in BDNF corresponded to changes in NMDA glutamate receptors as well, due to BDNF's role in promoting NMDA receptor expression. The microbiome was found to decrease gene expression of cortical NR-1 and NR-2a glutamate receptors and hippocampal NR-2a receptors in GF mice compared with SPF mice, indicating ancillary effects of decreased BDNF from GF mice (Sudo *et al*., 2004). Notably, other NTFs including NT-3 and NGF in these brain areas of GF mice were identical to those of SPF mice, suggesting gut bacteria influence BDNF specifically over other neurotrophic factors in the hippocampus. The hypothalamus is not directly involved in mood and anxiety regulation, and BDNF expression between GF and SPF mice in this brain region is not significant (Sudo *et al*., 2004). Likewise, levels of glutamate receptors in the hypothalamus in GF versus SPF mice show no significant differences in expression levels (Sudo *et al*., 2004). The gut microbiota's effects on mood and anxiety regulation could be due to its preferential actions on neurotrophic regions, like the hippocampus and amygdala.

Behavioral studies related to gut microbiome have also been conducted. Jiang *et al*. (2018) found that human patients with generalized anxiety disorder (GAD) had altered gut microbiota profiles compared to healthy controls. GAD resulted in decreased diversity of

bacterial populations in the gut, and this was not reversed when patients achieved remission for GAD, suggesting discrepancies in the microbiome might be intrinsic to the illness (Jiang et al 2018). Opportunistic *Bacteroides* populations were overrepresented in the GAD patients compared with healthy controls (Jiang et al., 2018). Lower prevalence of short chain fatty acid (SCFA)-producing bacterial genera *Faecalibacterium, Eubacterium rectale, Lachnospira, Butyricicoccus* and *Sutterella* was also seen in GAD patients, which in turn causes lower production of SCFAs, causing intestinal barrier dysfunction (Jiang *et al*., 2018). This may relate to signaling changes to the limbic system which effects BDNF synthesis and release in the hippocampus and amygdala. Gut microbiota must be able to communicate with the central nervous system to alter BDNF expression and hence regulate mood.

Preliminary studies using animal models have shed some light on the interactions between the gut-brain axis. One proposed mechanism of communication between these systems is via the vagus nerve (CN X). The vagus nerve contains primarily afferent and some efferent pathways between the digestive tract, heart, and other abdominal organs, and the central nervous system. Besides the parasympathetic effects widely known about the vagus nerve, CN X afferent fibers from the gut play crucial roles in psychiatric conditions, including mood and anxiety disorders (Breit *et al*., 2018). In fact, in patients with depression that has not been effectively treated by psychotherapy or chemical interventions, vagal nerve stimulation (VNS) has been used as a viable treatment option, potentially because increased neuronal activity generally upregulates BDNF and prolongs the molecule's stability (Follesa *et al*., 2007). BDNF mRNA expression and basic fibroblast growth factor (bFGF) increased in the hippocampus and cerebral cortex of rats exposed to VNS, which may be due to increased synaptic activity and firing rate of certain brain stem nuclei (Follesa *et al*., 2007).

These results correspond with antidepressant drug treatment effects that induce upregulation of BDNF in the hippocampus. Moreover, nerve growth factor mRNA decreased in the hippocampus or cerebral cortex following VNS, suggesting vagal nerve stimulation preferentially upregulates BDNF (Follesa *et al*., 2007). It has also been found that the vagus nerve can promote regional induction of *fos* immunoreactivity in the rat brain, which regulates expression levels of the BDNF gene (Follesa *et al*., 2007). VNS therapies increase vagal tone, which is correlated with capacity to regulate stress responses, and inhibit cytokine production, both of which are important mechanisms of mental resiliency (Breit *et al*., 2018).

Some bacteria have a beneficial effect on mood and anxiety, partly by affecting activity of the vagus nerve (Breit *et al*., 2018). For example, the microorganism *Lactobacillus rhamnosus* (strain JB-1) reduced stress-induced corticosterone hormone levels and anxiety-like and depression-like behavior and these changes were not observed in vagotomized mice (Bravo *et al*., 2011).

Under some mechanism, the vagus nerve modifies BDNF expression levels in the hippocampus, and it is plausible that this is due to signaling from the gut microbiome. During adulthood, BDNF in the hippocampus is partially regulated based on neural activity. The vagus nerve communicates with the central nervous system and receives significant input from the GI tract, where bacteria release compounds that likely influence CN X activity, in turn modifying BDNF expression, which influences mood and anxiety regulation. O'Leary *et al*. (2018) sought to determine a pathway of gut and CNS connectivity that specifically modifies BDNF mRNA expression levels in the brain. Her team found that the vagus nerve modulates neurotrophin mRNA expression and neurogenesis in the hippocampus. In mice, subdiaphragmatic vagotomy decreased BDNF mRNA expression in the CA1 and the CA3 regions of the hippocampus

(O'Leary *et al*., 2018). This study also found that vagotomy affects regions of the hippocampus differently. Overall, vagotomy reduced cell proliferation, predominantly in the dorsal hippocampus (dHi) compared to the ventral hippocampus (vHi). Vagotomy affected survival of newly born cells in both the dHi and vHi, however. Vagotomy significantly decreased the number of cells involved in neural proliferation, called DCX-positive cells that had tertiary processes, but not the less mature DCX-positive cells that only had primary or secondary processes; these results were seen in the dHi significantly more than the vHi (O'Leary *et al*., 2018). Though the vHi is more involved in emotional regulation than the dHi, BDNF is expressed in both areas and was impacted by vagotomy.

It is quite likely that the makeup of the gut microbiome is correlated to BDNF expression levels in the hippocampus and that gut-brain axis interaction is different between individuals with mood or anxiety disorders and healthy controls for several reasons. First, because of the significant role BDNF plays in the hippocampus, but its relatively low levels after early neurodevelopment, there must be another mechanisms acting to increase levels of BDNF to maintain neurogenesis in the hippocampus and amygdala. The gut-brain axis could be one of these regulators. Further, due to constant feedback between the brain and the digestive tract via the vagus nerve and enteric nervous system, researchers have shown that gut microbiota have a pathway of interaction via vagus nerve stimulation. It is therefore possible that the gut microbiota act via the vagus nerve to increase BDNF levels in the hippocampus, thus impacting mood and anxiety regulation in mammals.

However, it has not yet been fully determined whether the microbiome impacts BDNF expression in the hippocampus and amygdala or if BDNF levels impact the gut microbiome flora and population densities, which in turn may have compounding effects on mood regulation and

anxiety. More specifically, it is unknown exactly which bacterial species in the microbiome are responsible for healthy brain function and BDNF expression levels in rodents or in humans. It is also possible that bacterial species beneficial to one mammal might not have the same effects on other mammals.

Some animal models have already connected gut microbiome composition and hippocampal BDNF expression levels. GF mice show reduced BDNF expression in the hippocampus compared to SPF mice. However, upon reintroduction of certain bacterial species into the digestive tract, such as *Lactobacillus* spp., BDNF levels in GF mice were restored to levels similar to those of SPF mice, indicating not only does the density of gut bacteria flora play a role in BDNF expression, but the species of bacteria present matters as well.

Due to the high economic cost and personal burden of mood and anxiety disorders, understanding physiological mechanisms involved in regulating mood are essential to reducing these issues. The proposed experiments will aid in our understanding of the role gut microbiota play on mood disorders and anxiety, specifically by analyzing BDNF expression in the hippocampus and amygdala, in hopes to find novel treatment approaches for these mental disorders.

Objectives

To address the impact of the gut microbiome composition on BDNF expression in the hippocampus and amygdala of adult mice and its influence on mood and anxiety regulation, I will:

1. Assess central BDNF expression in germ free (GF) versus specific-pathogen free (SPF) mice.

   a. Assess BDNF mRNA and protein expression in several brain regions, including the hippocampus and amygdala, which are regions associated with mood and anxiety disorders will be conducted. BDNF expression in regions not directly associated with mood and anxiety disorders--the hypothalamus, frontal cortex, and cerebellum--will be assessed as well to determine overall prevalence of central BDNF in adult mice.
   b. Determine the impact of vagotomy on BDNF expression in the hippocampus, amygdala, hypothalamus, frontal cortex, and cerebellum in GF versus SPF mice.

2. Analyze the effect of introducing microbiotic bacteria that promote or reduce BDNF central expression in GF versus SPF mice.
   a. Assess BDNF mRNA and protein expression in the hippocampus, amygdala, hypothalamus, frontal cortex, and cerebellum following introduction of one of three bacterial species.
   b. Assess depressive and anxiety-like behaviors following introduction of bacteria.

Addressing the objectives stated above will aid in our understanding of how the gut-brain axis influences mood and anxiety regulation.

Experimental Approach

This study will assess the role of the gut microbiome on BDNF expression in neurotrophic brain regions and depression- and anxiety-like behaviors of adult mice. Male and female GF and SPF mice fed the same diet and house appropriately will be utilized. Animals will

be allowed to acclimate to housing conditions for at least seven days prior to experimentation. At time of sacrifice, animals will be 10-11 weeks old.

1. Assessment of BDNF mRNA and protein expression in the CNS will be conducted following O'Leary's *et al.* (2018) procedures in the hippocampus and be extended to assessment in the amygdala, hypothalamus, frontal cortex, and cerebellum.
    a. After acclimation to laboratory conditions, one set of GF and SPF animals will be sacrificed and brain tissue analyzed for BDNF mRNA and protein.
    b. After acclimation to laboratory conditions, another set of mice will have the vagus nerve severed, and animals will wait approximately 31 days following vagotomy to be sacrificed. Then BDNF mRNA and protein expression levels will be measured in the hippocampus, amygdala, hypothalamus, frontal cortex, and cerebellum. O'Leary's *et al.* (2018) procedures for vagotomy and imaging will be followed.
2. *Lactobacillus* spp., *Bacteroides* spp., or *Faecalibacterium* spp. will be introduced to both GF and SPF mice following *Lactobacillus* spp. reintroduction procedures from Bravo *et al*. (2011).
    a. BDNF mRNA and protein expression in the hippocampus, amygdala, hypothalamus, frontal cortex, and cerebellum of this cohort of animals will be measured following 28-31 days of treatment.
    b. Depressive- and anxiety-like behaviors of a cohort of GF and SPF mice reintroduced with bacteria and controls will also be analyzed similarly to Bravo's *et al*. (2011) procedures before and after exposure to the bacteria.

Mice will perform the open field test and forced swim test preceding and following ingestion of bacteria.

Anticipated Results and Future Directions

If the gut microbiota communicates with the central nervous system to regulate expression of BDNF in neurotrophic regions of the adult mouse, GF mice will show less BDNF mRNA and protein expression compared to SPF mice. Additionally, if the vagus nerve modulates BDNF expression based upon activation from the gut microbiome, vagotomized GF mice will likely not show changes in BDNF expression compared to vagotomized SPF mice. SPF mice will show decreased BDNF mRNA and protein compared to SPF mice that keep an intact vagus nerve. Further, GF mice will likely show lower BDNF compared to vagotomized SPF mice due to effects of the microbiome early in life of the SPF mice.

Bacterial introduction to GF mice will also produce changes in BDNF expression in neurotrophic regions over other brain regions. Introduction of *Lactobacillus* spp., which have been shown to upregulate BDNF (Breit *et al*., 2018) will increase BDNF mRNA and protein expression in GF mice compared to SPF mice. Introduction of *Bacteroides* spp., which are present in higher levels in patients with generalized anxiety disorder (Jiang *et al*., 2018) will decrease BDNF expression. Introduction of *Faecalibacterium* spp., which were present in lower levels in patients with GAD compared to healthy controls (Jiang *et al*., 2018) will increase BDNF expression. These three genera will provide clues as to how the microbiotic makeup regulates NTF expression in the hippocampus and amygdala

GF mice will likely show greater depression- and anxiety-like behaviors compared to SPF animals, but these behaviors will be improved upon introduction of *Lactobacillus* spp. and

Faecalibacterium spp. Reintroduction of *Bacteroides* spp. will presumably not improve these behaviors.

Overall, we will find support for the gut microbiota's role in regulating mood and anxiety in animals and the physiological markers related to this. From our findings, we can expand experiments to introducing populations of additional and multiple bacterial species to GF mice and elucidate chemical secretions from upregulating BDNF or mood-improving bacteria. This will contribute to the understanding of mood and anxiety disorders and help to determine treatment options for individuals suffering from psychiatric disorders based upon their gut mircobiotic profile.

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