THE RELATIONSHIP BETWEEN GUT MICROBIOTA AND METABOLITES IN THE EXPRESSION OF GENERALIZED ANXIETY DISORDER

By: DEVINNE THRASHER, B.Sc. (Hons)

Supervisor: DR. PREMYSL BERCIK, MD

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AUTHOR: Devinne G. Thrasher, B.Sc. Hons (McMaster University, Hamilton, ON)

SUPERVISOR: Dr. Premysl Bercik, MD

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ABSTRACT

Anxiety disorders are the most prevalent psychiatric conditions within primary care, affecting up to 29% of people across their lifetime**.** Generalized Anxiety disorder (GAD) is frequently comorbid with Major Depressive Disorder (MDD), resulting in greater functional impairment. Gut microbiota have been shown to modulate brain chemistry and function, possibly also playing a role in the genesis of anxiety. Bacteria are also able to produce, or interact with the host metabolism of neuroactive substances, including classical neurotransmitters and trace amines, like octopamine, which although found in trace concentrations in the mammalian brain, can affect CNS function. Specifically, trace amines can affect catecholamine release, reuptake and biosynthesis, and modulate dopamine and serotonin metabolism.

We investigated whether microbiota from patients with GAD with no signs of immune activation can alter behaviour in gnotobiotic mice and whether this is accompanied by changes in metabolites within the gastrointestinal tract.

Germ-free NIH Swiss mice (n=35) were colonized with microbiota from either a GAD patient (n=18) with severe anxiety, comorbid depression, and low serum and fecal octopamine, or an age and sex-matched healthy control (HC) (n=17). Three weeks post-colonization, mouse behaviour was assessed by standard psychometric tests. Emotionality z-scores were calculated to provide a robust integrated behavioural assessment. Microbiota profiles were assessed by 16S rRNA based Illumina, fecal β-defensin-3 level was measured by ELISA. After sacrifice, mouse brain BDNF and GDNF expression was

assessed by immunofluorescence, and gene expression in the hippocampus, amygdala, and olfactory bulbs was assessed by Nanostring. Stool and cecum metabolites were measured in all colonized mice by multisegment injection-capillary electrophoresis-mass spectrometry (MSI-CE-MS).

There were no differences in fecal β-defensin levels between mice colonized with GAD microbiota as compared to mice colonized with HC microbiota. However, GAD mice exhibited greater anxiety and depressive-like behavior compared to HC mice in the digging and tail suspensions tests. Behavioural z-scoring across all six standard psychometric tests showed a significant increase in group emotionality score means of GAD-colonized mice compared to HC-colonized mice. Mice colonized with microbiota from a GAD patient had distinct bacterial profiles from mice colonized with HC microbiota. Compared to HC mice, GAD mice had lower levels of dopamine, octopamine and acetylcholine in cecum contents. Furthermore, GAD mice had higher expression of BDNF in the amygdala, lower expression of BDNF in the hippocampus, and lower expression of GDNF in the midbrain. GAD mice also had lower expression of CCR2 in the hippocampus, higher Cnlp/CAMP in the amygdala and olfactory bulb, and higher Nfkb1 in the olfactory bulb compared to HC mice.

Our results suggest that microbiota from a selected patient with GAD has the ability to induce anxiety and depressive-like behavior, by mechanisms independent of immune system, likely by altered production of biogenic amines and neurotransmitters.

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LIST OF ABBREVIATIONS

ACH	Acetylcholine
BDNF	Brain-derived Neurotrophic Factor
BSA	Bovine Serum Albumin
CAMP	Cathelicidin Antimicrobial Peptide
CCR2	C-C Chemokine Receptor Type 2
CNS	Central Nervous System
CRP	C-reactive Protein
DASS	Depression, Anxiety and Stress Scale
DOP	Dopamine
DSMV	Diagnostic and Statistical Manual, Fifth Edition
ELISA	Enzyme-linked Immunosorbent Assay
FDR	False Discovery Rate
GABA	Gamma-aminobutyric Acid
GAD	Generalized Anxiety Disorder
GDNF	Glial-derived Neurotrophic Factor
GF	Germ Free

GLU	Glutamine
HC	Healthy Control
HEPA	High Efficiency Particulate Air
HIST	Histamine
HPA	Hypothalamic-pituitary-adrenal
IL-1	Interleukin-1
IL-6	Interleukin-6
IQR	Interquartile Range
MAO	Monoamine Oxidase Inhibitors
MDD	Major Depressive Disorder
MSI-CE-MS	Multisegment Injection-Capillary Electrophoresis-Mass Spectrometry
NAc	Nucleus Accumbens
NFKB1	Nuclear Factor Kappa B Subunit 1
OCT	Octopamine
OTU	Operational Taxonomic Unit
PBS	Phosphate-buffered Saline
PEA	Phenylethylamine

SE	Standard Error
SSRIs	Selective Serotonin-Reuptake Inhibitors
TA	Trace Amine
TAAR	Trace Amine Associated Receptor
TBS	Tris-buffered Saline
TNF-α	Tumour necrosis factor-alpha
TRY	Tryptamine
TYR	Tyramine
VTA	Ventral Tegmental Area

DECLARATION OF ACADEMIC ACHIEVEMENT

All experiments for this thesis were conducted by Devinne Thrasher with assistance from Jun Lu, Dr. Shimbori, and Dr. De Palma. Data analysis and its interpretation was performed by Devinne Thrasher under the guidance of Dr. De Palma and supervision of Dr. Bercik, except for Figure 2 which was produced by Dr. De Palma and Figures 18, 19 and 22 which were produced by Dr. Shanmuganathan and Dr. Britz-McKibbin. Insightful direction of this project was provided by Dr. Collins and Dr. Britz-McKibbin, who were members of Devinne's advisory committee.

1. INTRODUCTION

1.1. Generalized Anxiety Disorder

Anxiety disorders are recognized as the most prevalent psychiatric conditions within primary care, affecting approximately 10-29% of people across their lifetime (Swinson *et al.,* 2006) and account for approximately 2% of all health-related disability across Canada (Watterson *et al.,* 2017). Generalized anxiety disorder (GAD) is characterized by a chronic, uncontrollable pattern of worrying that is not restricted to specific circumstances (Lader, 2009). Patients often present with somatic symptoms, such as tachycardia and tremor, as well as frequent, burdensome, psychological symptoms such as fatigue, restlessness, concentration difficulties, irregular sleep patterns, and irritability (Lader, 2009). Abnormal function of serotonin, gamma-aminobutyric acid (GABA), (Stein, 2009), dopamine (Zarrindast & Khakpai, 2015), epinephrine and norepinephrine (Mathew *et al.,* 1980; Bunney *et al.,* 1967; Wyatt *et al.,* 1971; Kopin *et al.,* 1978) have been reported in GAD and in conditions associated with anxiety. Functional imaging data has shown abnormal activity in the amygdala, frontal cortex, basal ganglia (Stein, 2009), and hippocampus (Zarrindast & Khakpai, 2015) in patients with GAD. In addition, GAD is frequently comorbid with major depressive disorder (MDD) and patients with both disorders have considerably more impairment than patients with either disorder alone (Kessler *et al.,* 2001). Unfortunately, despite continuous research, the pathophysiology of anxiety and depression remains unknown, and no single

theory has been sufficiently comprehensive to propose a unitary hypothesis for the development of GAD and other anxiety disorders.

1.2. The intestinal microbiome

Humans are born sterile but rapidly acquire resident microbes at birth. The GI tract harbors a vast microbial ecosystem containing bacteria, yeast and viruses known collectively as the *microbiome* (Collins *et al.*, 2012). Studies with germ-free animals indicate that bacterial colonization of the gut is necessary to develop functional immune and pain pathways, as well as cognitive, emotional and social function of the brain (Collins *et al.*, 2012).

1.3. The gut-brain axis

Recently, there has been an emerging interest in the potential role of intestinal microbiota in the etiology of mood and affective disorders. A recent study observed significant changes in brain activity of regions involved in central processing of emotion and sensation following the administration of specific probiotics (Tillisch *et al.*, 2013). Further, there have been reports of behavioural changes in patients after taking antibiotics, with their mental status returning to normal after discontinuation of drug (Tome, 2011; Mehdi, 2010). Although the underlying communication pathways remain largely unknown, immune, neural, endocrine and metabolic pathways have been

suggested (Jalanka-Tuovinen et al., 2011; Bravo et al., 2011; Bercik et al., 2011; Honda et al., 2009; Uribe et al., 1994; Lee et al., 2011; Berer et al., 2011). Further, products of bacterial metabolism have been shown to gain access to the brain via the bloodstream (Wikoff et al., 2009). Afferent neural pathways have also been suggested as a main communication pathway between the gut and brain (Kunze et al., 2009). Additionally, host stress hormones such as noradrenaline have been suggested to affect gene expression in bacteria or influence the cell-to-cell communication between bacteria (Mawdsley, 2005; Freestone et al., 2008; Kaper & Sperandio, 2005).

1.4 Bacterial metabolism

Together, the host and its microbiota have been shown to coproduce a large array of small molecules through metabolism of food as well as compounds of nonhost origin (Tremaroli & Backhed, 2012). Communication between microbial species and host cells is consistently at play, and this chemical dialogue has been shown to occur via low molecular weight metabolites, peptides, and proteins (Sjogren et al., 2012; Caesar et al., 2012). Many studies have shown the involvement of bacterial genera and species in metabolite production, including but not limited to, neurotransmitters (Strandwitz, 2018). Gut bacteria have also been shown to produce neuroactive substances and affect the metabolism of neurotransmitters involved in the regulation of mood (Barrett *et al.,* 2012; Asano *et al.,* 2012). Certain strains of Escherichia, Streptococus and Bacillus are able to produce serotonin, dopamine and norepinephrine, which are the main neurotransmitters

implicated in anxiety and depression (Shishov *et al.*, 2009; Ozogul, 2011; Tsavkelova *et al.*, 2000). Further, strains of Lactobacilli and Bifidobacteria have been shown to produce GABA, acetylcholine, and histamine (Thomas *et al.*, 2012; Kawashima *et al.*, 2007; Marquardt *et al.*, 1959; Girvin & Stevenson, 1954; Horiuchi *et al.*, 2003; Landete *et al.*, 2008; Coton *et al.*, 1998). Similarly, bacteria have been shown to produce trace amines tyramine, tryptamine, phenylethylamine and octopamine (Davis & Boulton, 1994), which are found in trace concentrations in the mammalian brain and peripheral tissues and can affect CNS function (Burchett & Hicks, 2006). Multiple bacterial strains were shown to produce trace amines, including the genera Lactobacillus, Clostridium, Pseudomonas, and Enterobacteriaceae (Roeder, 1999; Marcobal *et al.*, 2012). The term "trace amine" was coined to reflect the low tissue levels in mammals (<10 ng/g; 100 nM); however, invertebrates have relatively high levels of trace amines that function similar to mammalian adrenergic systems, involved in "fight-or-flight" responses (Berry, 2004). Foods in which anaerobic fermentation is part of their production are enriched in trace amines, such as aged cheeses, fermented meats, red wine, soy products, and chocolate (Berry, 2007). Bacteria E. faecalis and E. faecium have been linked to production of trace amines, tyramine and β-phenylethylamine (Linares *et al*., 2012).

1.5 Trace amines and psychiatric illness

Evolutionarily speaking, trace amines represent early neurotransmitters. In invertebrates, octopamine and tyramine are the major neurotransmitters in the central nervous system and are involved in many vital functions such as movement, feeding, and stress reactions (Roeder, 1999; Sotnikova *et al.*, 2009). Tyramine, tryptamine, phenylethylamine (PEA), and octopamine are established trace amines in the mammalian nervous system (Burchett, 2006) and endogenous concentrations are several hundred-fold below those of the classical neurotransmitters dopamine, norepinephrine and 5-HT ,though they are stored in the same nerve terminals and are released together (Berry, 2004). The trace amines do not appear to alter neuronal excitability, rather they are likely to alter neuronal responsivity (Berry, 2007) and modulate cell-cell signalling through action on the re-uptake of monoamine neurotransmitters and through postsynaptic actions (Branchek & Blackburn, 2003). Trace amines have been implicated in human disorders of affect and cognition, including major depression, anxiety and schizophrenia (Bonham *et al.*, 1980; Baker *et al.*, 1991; Revel *et al.*, 2013). Deficits in the production of octopamine and tyramine have been reported in patients with depression (Sandler *et al.*, 1979) as well as reduced phenylacetic acid levels have been found in urine, plasma, and CSF of depressed patients, with these levels changing following antidepressant treatment (Davin & Boulton, 1994). In addition, urinary PEA excretion has also been found to be increased post stressful events (Berry, 2007), acting as an anxiogen in the elevated plus-maze (Lapin, 1993). Interestingly, clinically effective MAO inhibitor antidepressants are known

to elevate trace amine levels, and elevated PEA levels have been suggested to underlie the antidepressant effects of exercise (Szabo et al., 2001).

1.6 Trace amine associated receptors

The trace amines act through trace amine associated-receptor-1 (TAAR1), a species of G protein-coupled receptor (Miller, 2011; Borowsky *et al.,* 2001). Research has shown that phenylethylamine, the most potent activator of TAAR1 (Pei *et al.,* 2016) is able to alter the serotonin transporter by interacting directly with TAAR1 (Xie & Miller, 2008). The highest concentration of TAARs in mice was found to be within the olfactory sensory neurons in the brain and gastrointestinal tract (Chiellini *et al.,* 2012), with RNA transcript for TAAR1 also found in liver, spleen, and in B lymphocytes (Irsfeld *et al.,* 2013; Babusyte *et al.,* 2013). In humans, the highest expression of TAARs was noted in the amygdala, stomach and intestine (Borowsky *et al.,* 2001). It has been suggested that TAAR1 activation may stabilize maladaptive mood and emotional fluctuations and contribute to the effects of antidepressant and anxiolytics (Pei *et al.,* 2016). Agonists of TAAR1 have been shown to reduce immobility time in a forced-swim test in the rat, and prevent stress-induced hyperthermia in mice, thus suggesting anti-depressant and anxiolytic properties (Pei *et al.,* 2016). With the recent development of TAAR1-selective ligands, particularly the partial agonist RO5203648 (Revel *et al.,* 2013) and selective antagonist EPPTB (Bradaia *et al.,* 2009; Stalder *et al.,* 2011), it has become possible to explore the roles of TAAR1 *in vivo*. In various behavioural paradigms in rodents and monkeys, a selective and potent TAAR1 partial agonist, RO5203648, elicited clear

antidepressant and anxiolytic-like properties and was highly effective in promoting attention, cognitive performance and wakefulness (Revel *et al.*, 2012).

1.7 Inflammation in depression and anxiety

An ample body of evidence suggests that in at least a subset of patients, anxiety and depression are associated with immune activation and elevated inflammatory markers such as IL-1, IL-6, TNF- α and CRP (Dowlati *et al.*, 2010; Valkanova *et al.*, 2013; Howren *et al.*, 2009; Bankier *et al.*, 2008; Hou & Baldwin, 2012; Vogelzangs *et al.*, 2013; Zorilla *et al.*, 2001). Previously, our group has shown that GAD patients (n=71) had significantly higher β-defensin-2 concentrations than healthy controls (n=87) and that transfer of microbiota from a GAD patient with high fecal β-defensin-2 was able to induce anxiety-like behaviour in a murine host, coupled with increased immune activation (*Manuscript in Preparation*). However, it is not known whether elevated inflammation is essential for the expression of anxiety and depression or whether it is strictly epiphenomenal.

1.8 Neurotrophic factors in depression and anxiety

1.8.1. Brain derived neurotrophic factor

The 'neurotrophin hypothesis of depression' postulates that diminished levels of brain-derived neurotrophic factor (BDNF) within the brain may contribute to cell loss within the hippocampus and prefrontal cortex, and that antidepressants may act to increase BDNF expression, thereby reversing this neuronal atrophy. This hypothesis arises from observations that reduced levels of BDNF in the hippocampus are correlated with depressive behaviour and that many classes of antidepressants significantly increase hippocampal BDNF mRNA expression (Duman & Monteggia, 2006). Further, studies have demonstrated that direct infusions of BDNF protein into the hippocampus of rodents can elicit antidepressant effects (Siuciak et al., 1997; Shirayama et al., 2002). Emerging evidence suggests that BDNF is involved in brain stress systems involving the hippocampus and hypothalamic-pituitary-adrenocortical (HPA) axis, as well as in reward systems within the nucleus accumbens (NAc) and the ventral tegmental area (VTA) (Berton et al., 2006; Fisch et al., 2003).

1.8.2. Glial derived neurotrophic factor

Glial derived neurotrophic factor (GDNF) is a neurotrophin derived from glial cell lines that is vital to midbrain dopaminergic neurons (Lin et al., 1993). GDNF has been shown to promote the morphological differentiation and survival of midbrain dopaminergic neurons and increase their affinity to dopamine uptake (Lin et al., 1993).

Studies have revealed that stress-induced impairment of GDNF synthesis leads to an increase in depressive-like behaviours in mice (Uchida et al., 2011). It has been shown that GDNF expression was significantly decreased in both the dorsal and ventral striatum of stressed BALB mice (Uchida et al., 2011)., and that mice who are resistant to social defeat stress exhibit increased expression of GDNF in the ventral tegmental area (VTA) within the midbrain (Krishnan *et al.,* 2007). Similarly, GDNF conditional-knockout mice show reduced exploratory and spontaneous behaviour in the open-field test (Pascual *et al.*, 2008). Taken together, research to date supports the notion that the mesolimbic dopamine system, and particularly GDNF, plays a crucial role in the responsivity and susceptibility to chronic stress.

1.9. Dopaminergic neurotransmission in depression and anxiety

1.9.1. Dopamine

Dopamine (DA) neurons play a fundamental role in pathways of reward processing in the brain. Depression and anxiety have been associated with deficits in systems for reward anticipation (Berridge & Kringelbach, 2008), learning of action-reward contingency (Schultz, 1998), and decisions on whether to expend effort to obtain an expected reward (Salamone et al., 2007), all of which rely heavily on the integrity of dopamine circuits extending from the ventral tegmental area (VTA) and substantia nigra (SN) within the midbrain (Treadway & Zald, 2011). Inhibition of DA neurons has been

shown to elicit the onset of anhedonia, which plays a central role in depressive illness (Dillon et al., 2014; Clark & Watson, 2006; Meehl, 1975), and is characterized by a lack of pleasure upon reward consummation as well as reduced pleasure in anticipation of a reward (Chentsova-Dutton & Hanley, 2010; Sherdell et al., 2012; MdFarland & Klein, 2009).

1.9.2. TAAR-1

Dopamine transmission plays an important role in fear and anxiety circuits, as it has been shown to act as a modulator of the cortical brake that the medial prefrontal cortex exerts on the anxiogenic signals exiting the amygdala. TAAR1, the primary receptor for the trace amines, has been shown to act as a regulator of dopaminergic neurotransmission. TAAR1 knockout mice have been shown to exhibit a supersensitive dopaminergic system, whereas TAAR1 activation results in a reduction of excess dopaminergic activity and a decrease in anxiety-like behaviours (Bradaia et al., 2009).

1.10. Hypotheses and aims

Given the growing experimental evidence on the role of gut microbiota in psychiatric illness, it is hypothesized that a subset of patients with GAD and MDD have abnormal microbiota composition and/or abnormal bacterial metabolites, which is the

driving force for the behavioural alterations observed in GAD. Specifically, the research proposed will aim to examine the following hypotheses:

(a) Microbiota from some patients with GAD and no signs of immune activation will induce brain and behavioural dysfunction in a murine host.

(b) Behavioural alterations in GAD-colonized mice will be accompanied by altered production in at least one of the trace amines: octopamine, tyramine, tryptamine, and/or phenylethylamine.

2. METHODS

2.1. Human donor selection

As outlined previously, experiments in our lab have shown that colonization of germ-free mice with microbiota from a patient with GAD and elevated inflammatory markers, but not with microbiota from a healthy control, was able to induce anxiety and depression-like behaviour in a murine host. Thus, my experiments aimed to address the question whether the immune activation is a requirement for the transfer of the anxious phenotype from human to murine host.

Human stool and blood samples were obtained from a previous clinical study lead by Dr. Rebecca Anglin at McMaster University and were stored at -80 C until the time of analysis. Stool samples were collected from each GAD patient (n= 71) and healthy

control (n= 87) and were used to measure β-defensin-2 concentrations. β-defensin was analyzed as a marker of mucosal innate immune activation triggered by microbiota in patients and in healthy controls. As assessed by the Depression Anxiety Stress Scales (DASS), the selected GAD donor presented with extremely severe anxiety (DASS-A score of 20); moderate depression (DASS-D score of 18); and extremely severe stress (DASS-S score of 42). The anxious donor was female and 35 years of age. An age- and sex-matched healthy human donor was used as a control.

2.2. Colonization of germ-free mice

Germ-free NIH Swiss mice (n=34), obtained from the Axenic Gnotobiotic Unit of McMaster University, were colonized with microbiota from a selected patient with GAD or from a healthy human control. A 1:10 dilution of human fecal sample to saline was prepared in an anaerobic chamber and all mice were gavaged with 200 µl of the diluted sample. Blood was collected by facial bleeding prior to gavage to assess individual baseline metabolomics. Mice were housed for three weeks in sterile racks with air ventilated through a high efficiency particulate absorber (HEPA) filter. All mice were on a 12h:12h light-dark cycle with access to food and water ad libitum. To minimize bacterial contamination, all mice were handled in a Level 2 biosafety hood.

2.3. Mouse biomarker analysis

Inflammatory profiles were assessed by mouse fecal β-defensin-3 levels, which is the homolog of human β-defensin2 (Bals *et al.,*1999; Burd *et al.,* 2002). Full metabolomic analysis of stool and cecum were performed by mass spectrometry. Metabolomic analysis focused primarily on the trace amines octopamine, tyramine, tryptamine, and phenylethylamine as well as monoamines serotonin, dopamine, epinephrine, norepinephrine, glutamine, acetylcholine and GABA.

2.4. Behavioural analysis of colonized mice

Three weeks post-colonization, behaviour was assessed by five standard behavioural tests for anxiety: digging, marble-burying, open field, light preference and step down, as well as one test for depressive behaviour, the tail suspension test. Tests were performed 2-5 days apart to reduce the impact of a previous test on anxious behaviour. In the light preference test, each mouse was placed in the center of an illuminated box connected to a smaller dark box and the mouse's preference to each compartment was captured for ten minutes. Higher preference for the illuminated compartment was indicative of more exploratory and less anxious behaviour. The step-down test measured the latency of each mouse to step down from an elevated platform, in which greater latency to step down was indicative of greater anxiety-like behaviour. The open field test measured the activity of each mouse in an open field over a ten-minute period, in which less time spent in the center of the field, and greater time in the periphery

was suggestive of dampened exploratory behaviour and more anxious-like behaviour. The neophobic aspect of anxiety was captured via the marble-burying test, which measured the total number of marbles buried by each mouse in a 30-minute period. Neophobic and obsessive tendencies were measured in the digging test, which measured the total time spent digging by each mouse over three minutes. Greater time spent digging was indicative of greater anxiety-like behaviour. For measuring depressive-like behaviour in mice, the tail-suspension test was conducted. The tail suspension test assessed learned helplessness by measuring the total time spent immobile when suspended by the tip of the tail for six minutes, in which greater time spent immobile suggested higher depressive-like behaviour.

2.4.1. Integrated behavioural z-scoring

An integrated behavioural z-scoring method was used to analyze behaviour, which combines measures along the same behavioural dimensions in all behaviour tests performed. This reduces the intrinsic variability of a single test and provides a more robust characterization of the underlying "emotionality" of individual mice (Guilloux *et al.*, 2011). Behavioural parameters within and across tests do not necessarily agree across time and within individual animals, as behaviour outcomes in mice can be subject to several factors that contribute to variability such as time of day, recent activity in the colony, and experimenter interaction. As such, mice can vary in emotional states over short periods of time, and comparison of groups exclusively within individual tests may

lack statistical power and is more likely to yield false positive/negative results. This is kin to the characterization of human illness, specifically mood, where the focus is placed on convergent, rather than consistent symptoms, and diagnoses are based on the assessment of different symptoms in varied domains over time.

In this project, results were z-normalized from the raw scores from individual behavioural tests with the aim of assessing an emotionality dimension in mice. Z-normalization yields dimensionless z-scores which allows data between different scales to be compared, thus allowing one to test whether a specific experimental group deviates from average behaviours in converging dimensions across tests and time. The z-score's value is calculated by subtracting the observed mean in a group from an individual raw data value, then dividing it by the difference by the group's standard deviation. Raw data were obtained from each test performed in the same animal in both male and female mice. Normalization of data using z-score method was performed for each parameter using the same-sex healthy control group as the baseline. The z-scores were adjusted for directionality, such that an increased z value reflected increased dimensionality, i.e. anxious behaviour. To minimize weighted effects of locomotion on anxious behaviour, distance ratios (e.g. center/total distance in the open field) were obtained where appropriate and incorporated into the z-score calculation. For example, Z-score in the light preference test (Z_{LP}) was calculated for each animal using normalization of "time in light" (TL) and "distance in dark/total distance ratio" (DR) values:

$$Z_{LP} = \frac{\left(\frac{X-\mu}{\sigma}\right)_{TL} + \left(\frac{X-\mu}{\sigma}\right)_{DR}}{\text{Number of parameters}}$$

Likewise, in the open-field test, Z-scores were obtained by normalizing "time in center" and "distance in periphery/total distance ratio" values. In the digging test, Z_{DIG} was calculated for each animal using normalization of the latency to dig and total time digging, which was also corrected for directionality so that decreased latency to dig and increased total time digging were both captured by increased z-scores and reflected increased emotionality in mice. Similarly, Z-scores in the tail suspension test (Z_{TS}) were calculated by normalizing the latency to immobility and the total time immobile. Finally, in the marble burying and step-down tests, Z_{MB} and Z_{SD} were calculated for each mouse using normalization of the total number of marbles buried, and latency to step down from an elevated platform, respectively. Test Z-values were then calculated by averaging individual mouse z-scores across tests to obtain a single emotionality z-score for each mouse:

$$\textbf{Emotionality score} = \frac{Z_{LP} + Z_{OF} + Z_{DIG} + Z_{MB} + Z_{SD} + Z_{TS}}{\text{Number of tests}}$$

Emotionality score means were then obtained for each group by averaging individual mouse scores within the healthy control and GAD mouse groups. All six behaviour tests were weighted equally, as individual test parameters were averaged in the previous step.

2.5. Microbiota and gene analysis

Genomic DNA was isolated from stool samples of donors and colonized mice and V3 fragments of 16S rRNA gene were amplified and sequenced via the Illumina platform

to assess bacterial diversity and composition, as described previously (De Palma et al., 2017). For the brain gene expression assay, 200 μm coronal sections of the hippocampus and amygdala, as well as whole olfactory bulbs were retrieved and stored in 300 μl of RNase-Free solution. RNA was extracted from brain tissue using an RNeasy Micro Kit (Qiagen, Toronto, ON, Canada) and gene expression was assessed from the extracted RNA using a custom Nanostring code-set

2.6. Immunofluorescent staining

2.6.1. BDNF

Immunofluorescent staining of BDNF was performed according to a protocol established in the lab. Frozen coronal slices (10 um), capturing sections of both the amygdala and hippocampus, were isolated from mouse brains (n=34) and mounted on Aptex coated slides at room temperature. Frozen slides were fixed with cold methanol for ten minutes at room temperature and rinsed with PBS. Slides were then permeabilized in TBS (0.025% Tween-20) for ten minutes and blocked with 10% normal serum with 5% BSA in TBS for two hours to reduce unspecific binding of the antibodies. Anti-BDNF (Novus Bios, Oakville, ON, Canada) diluted 1:500 in TBS with 1% BSA was applied to each section and incubated overnight at 4°C. Slides were rinsed with TBS (0.025% Triton

X-100) and fluorophore conjugated secondary antibody (Invitrogen, Burlington, ON, Canada) diluted 1:1000 in TBS (1% BSA) was applied to each brain section and incubated for one hour at room temperature in the dark and mounted using Fluoroshield mounting medium with DAPI (ThermoFisher Scientific, Mississauga, ON, Canada). Negative control slides received only the fluorophore conjugated secondary antibody. Stained slides were visualized using Nikon fluorescent microscope and Nikon Instrument Software (NIS)-Elements software (Minato, Tokyo, Japan), in which the images were captured at a consistent gain and exposure to achieve an external control. Image intensity was set using pre-determined thresholds across images to maintain consistent settings.

2.6.2. GDNF

Coronal sections containing the anterior cingulate, infralimbic, prelimbic, dorsal and ventral striatum, amygdala, hippocampus, and ventral tegmental areas were obtained from each mouse and mounted on Aptex coated slides. Immunofluorescent staining procedure for GDNF was the same as for BDNF, aside from the primary antibody dilution. Anti-GDNF (Abcam, Toronto, ON, Canada) was applied in 1:250 dilution in in TBS (1% BSA).

2.7. Metabolomics

Biogenic amines and neurotransmitters from cecum and stool samples were quantified by multisegment injection-capillary electrophoresis-mass spectrometry (MSI-CE-MS) in all colonized mice by Dr. Meera Shanmuganathan and Zachary Kroezen from the Dr. Philip Britz-McKibbin Laboratory in the Department of Chemistry and Chemical Biology at McMaster University. All compounds were measured quantitatively (microM or microM/mg for stool) and their identification was confirmed by spiking standards into samples.

2.8. Statistical analysis

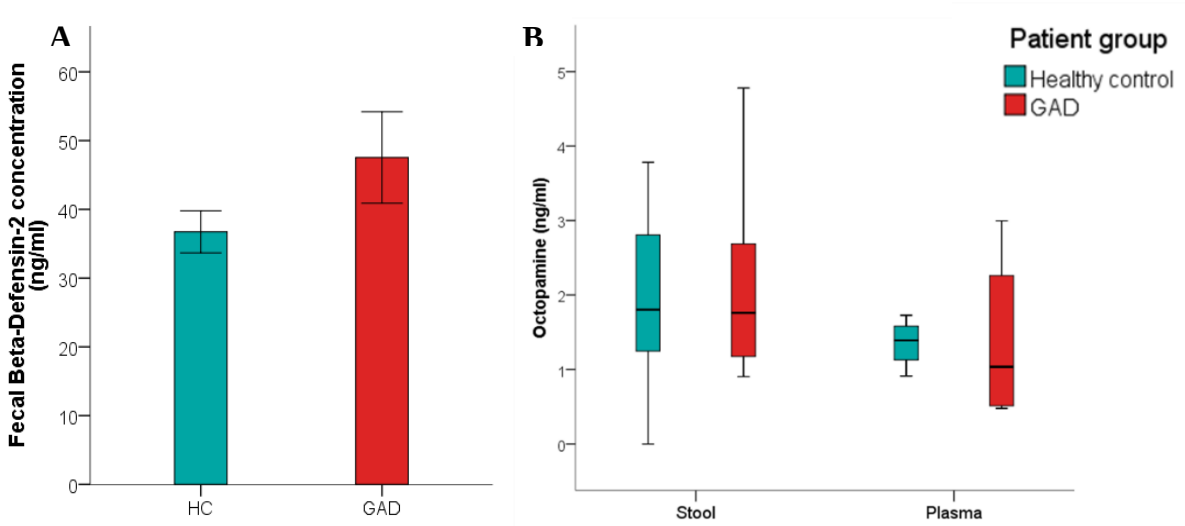
All statistical analysis was performed using IBM SPSS Statistics Version 20. Figures of group comparisons were presented as box plots displaying the data distribution. Two groups comparisons were done with Unpaired Student t-test or Mann-Whitney's test where appropriate. Microbiota β-diversity analysis was conducted using the ANOSIM test on the Bray-Curtis dissimilarity distance matrix. Statistical tests with multiple comparisons were corrected using the Benjamini and Hochberg false discovery rate (FDR) method. A two-tailed significance criterion of $p= 0.05$ was used for all statistical tests conducted.

3. RESULTS

3.1. Human donor biomarkers

A group of patients with GAD (n= 7) along with their age- and sex-matched healthy controls (n= 7) were selected from a cohort of patient samples from a larger clinical study for analysis of octopamine concentrations in plasma and in stool. In the clinical study, it was found that GAD patients (n=69) had greater fecal β-defensin-2 concentrations than healthy controls (n=87) (p= 0.026; Fig. 1A), suggesting that a subset of GAD patients may have greater immune activation which may contribute to the expression of anxiety. In the smaller group selected for extended analysis, all patients were younger than 35 years of age with a primary diagnosis of GAD and with minimal comorbidity. There were no significant differences in octopamine concentrations in plasma (p= 0.669; Fig.1B) or in stool (p= 0.771; Fig.1B) between GAD and HC patients. For the mouse colonization experiments, we chose a GAD donor with normal β-defensin-2 level (GAD donor β-defensin-2 = 38.4 ng/ml; HC donor β-defensin-2 = 3.4 ng/ml) to minimize the role of immune activation in the expression of anxiety, and lowest plasma octopamine levels (GAD donor octopamine = 0.547 ng/ml (plasma), 0.905 ng/ml (stool); HC donor octopamine = 1.437 ng/ml (plasma), 3.781 ng/ml (stool)) to study the role of this trace amine in GAD.

Figure 1. Biomarker Concentrations of GAD Patients and Healthy Controls



A. Fecal beta-defensin-2 concentrations in GAD patients (n= 69) and HC (n= 87). Unpaired t-test, p= 0.03. Error bars represent +/- 1 SE. **B**. Stool and plasma octopamine concentrations in a subset of GAD patients (n= 7) and HC (n=7), depicted in median (IQR) with 5-95% of data distribution. Octopamine stool comparison: Unpaired t- test, p= 0.771. Octopamine plasma comparison: Unpaired t-test, p= 0.669.

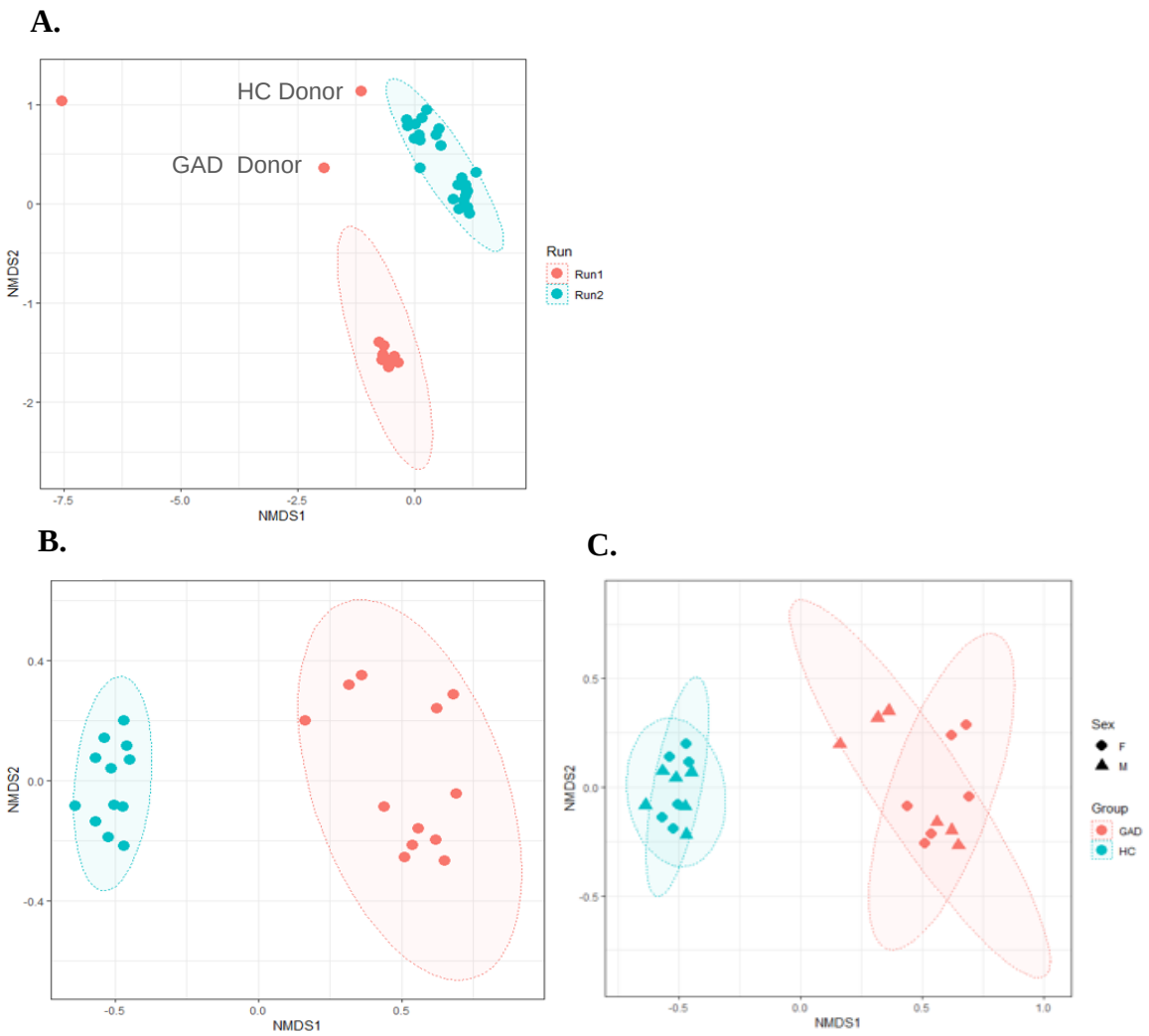
3.2. Mouse microbiota

Following behavioural measurements of the three groups of colonized mice, two 16S rRNA Illumina sequencing runs were performed and principal coordinates analysis (PCoA) plots were generated using the Bray Curtis dissimilarity metric to visualize microbiota profiles. The PCoA plots illustrating beta diversity needed to be separated because the mouse fecal samples clustered by run number (Figure 2A). Illumina run 1 (n=12) did not have enough datapoints to use NMDS scaling. However, in Illumina run 2, GAD mice (n=12) were found to have different microbiota than HC mice (n=11) ($F_{(1,22)} = 11.174$, p= 0.001; Figure 2B). Sex did not influence the clustering of groups

(Figure 2C). Further, out of all operational taxonomic units (OTUs) detected in the samples, 55.6% were shared between the GAD donor and GAD mice, and 65.3% were shared between the HC donor and HC mice.

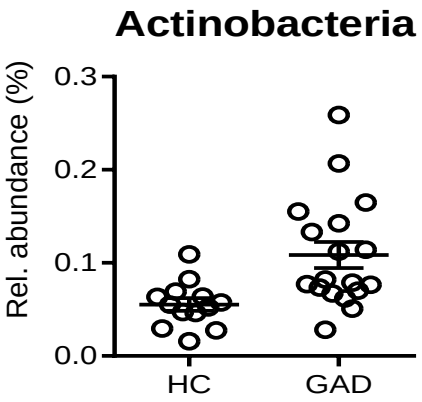
Statistically significant differences between GAD and HC mice fecal microbiota were found at the phylum, family, and genus level. Compared to HC mice, GAD mice had increased relative abundance of Actinobacteria ($p= 0.002$; Figure 3). At the family level, GAD mice were found to have increased relative abundance of Gracilibacteraceae ($p= 0.002$; Figure 4A) and an unknown family of the Clostridiales order ($p= 0.002$) and decreased relative abundance of Barnesiellaceae ($p= 0.0006$) and Porphyromonadeceae ($p= 0.001$) compared to HC mice. Statistically significant differences between groups were also found among 20 different genus (Figure 4B). Compared to HC mice, GAD mice had increased relative abundance of Pseudobutyrivibrio ($p= 0.001$), Odoribacter ($p= 0.001$), Collinsella ($p= 0.002$), Lachnobacterium ($p= 0.004$), Ruminococcus ($p= 0.001$), and 5 unknown genus from the Cyanobacteria phylum ($p= 0.004$), Clostridiales order ($p= 0.001$), Gracilibacteraceae family ($p= 0.003$), Mogibacteriaceae family ($p= 0.003$), Ruminococcaceae family ($p= 0.003$) and decreased relative abundance of Clostridium ($p= 0.004$), Eubacterium ($p= 0.001$), Eggerthella ($p< 0.001$), Parabacteroides ($p< 0.001$), Faecalibacterium ($p= 0.005$), Lachnospira ($p= 0.004$), and 4 unknown genus from the Clostridiales order ($p= 0.001$), Barnesiellaceae family ($p< 0.001$), Clostridiaceae family ($p= 0.001$), and Coriobacteriaceae family ($p= 0.003$).

Figure 2. Microbiota Profiles of Colonized Mice



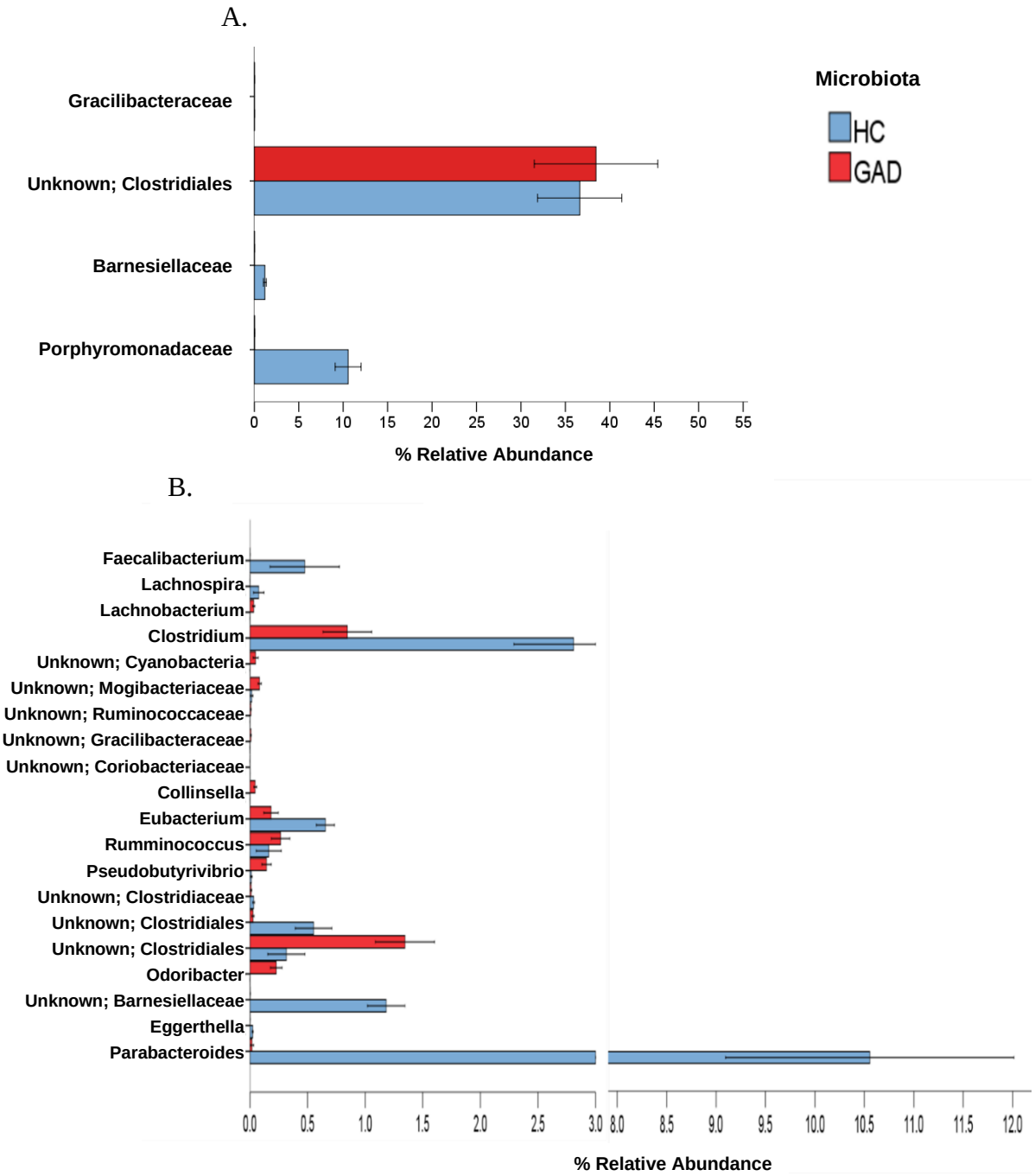
A. Non-multidimensional scale plots constructed on the Bray Curtis dissimilarity matrix using rarefied data. Results are the same with data normalized with relative abundance. No differences in alpha-diversity. Samples clustered by run and colonization (Run 1 contains colonization 1, Run 2 contains colonizations 2-3). Illumina run 1 (n=12) did not have enough datapoints to use NMDS scaling. Plots were separated by run for further analysis. **B.** Principal coordinates analysis (PCoA) plot depicting HC (blue) and GAD (pink) microbiota. GAD mice (n=12) had different microbiota than HC mice (n=11) ($F_{(1,22)} = 11.174$, $p = 0.001$). **C.** PCoA plot illustrating grouping by sex. Sex did not influence the clustering of groups.

Figure 3. Relative Abundance of Actinobacteria in Colonized Mice



Relative abundance of Actinobacteria in fecal pellet of colonized mice. GAD mice (n= 18) had increased relative abundance of Actinobacteria as compared to HC mice (n= 15). p= 0.002; corrected for FDR.

Figure 4. Family and Genus Level Differences in Microbiota of Colonized Mice



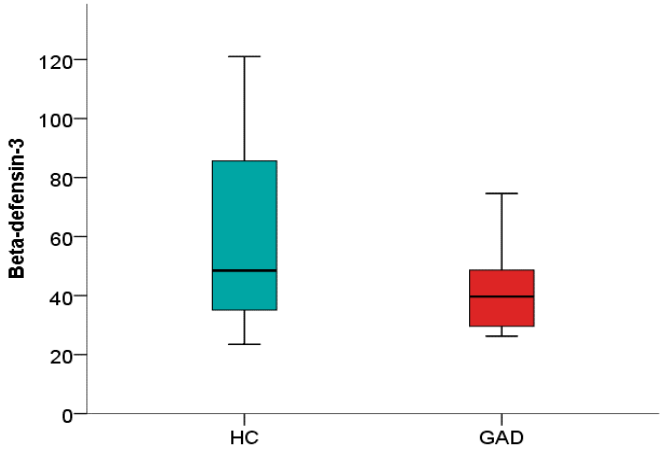
A. Family level differences in GAD microbiota. Compared to HC mice (n= 15), GAD mice (n= 18) had increased relative abundance of Gracilibacteraceae (p= 0.002) and an unknown family of the Clostridiales order (p= 0.002) and decreased relative abundance of Barnesiellaceae (p= 0.0006) and Porphyromonadeceae (p= 0.001). **B.** Genus level

differences in GAD microbiota. Compared to HC mice, GAD mice had increased relative abundance of Pseudobutyrivibrio (p= 0.001), Odoribacter (p= 0.001), Collinsella (p= 0.002), Lachnobacterium (p= 0.004), Ruminococcus (p= 0.001), and 5 unknown genus from the Cyanobacteria phylum (p= 0.004), Clostridiales order (p= 0.001), Gracilibacteraceae family (p= 0.003), Mogibacteriaceae family (p= 0.003), Ruminococcaceae family (p= 0.003) and decreased relative abundance of Clostridium (p= 0.004), Eubacterium (p= 0.001), Eggerthella (p< 0.001), Parabacteroides (p< 0.001), Faecalibacterium (p= 0.005), Lachnospira (p= 0.004), and 4 unknown genus from the Clostridiales order (p= 0.001), Barnesiellaceae family (p< 0.001), Clostridiaceae family (p= 0.001), and Coriobacteriaceae family (p= 0.003). All p-values corrected for FDR. Error bars represent +/- 1 SE.

3.3. Immune profiles of colonized mice

There were no differences in β-defensin3 concentrations between HC mice (n=5) and GAD mice (n=6) (Unpaired t-test, p= 0.578; Figure 5), suggesting there was no major differences in innate immune activation between the groups.

Figure 5. Fecal β-defensin3 in Colonized Mice



Fecal β-defensin3 concentrations of colonized mice (n=11). Mean values between groups were not statistically significant (Unpaired t-test, p= 0.578)

3.4. Behavioural assessment

Compared to HC-microbiota colonized mice, mice with GAD microbiota spent more time digging (Mann Whitney's test, p= 0.04; Figure 6D) and became immobile sooner in the tail-suspension test (Mann Whitney's test, p= 0.02; Figure 6E), which indicates greater anxiety-like and depressive-like behaviour. However, the opposite trend was observed in the light preference test, where GAD mice spent significantly less time in the dark compartment compared to HC mice (Mann Whitney's test, p= 0.03; Figure 6A). GAD and HC- mice behaved similarly in other three psychometric tests, as they spent a similar amount of time in the center in the open field test (Mann Whitney's test, p= 0.26; Figure 6B), stepped down with similar latency from the elevated platform (Mann Whitney's test, p= 0.81; Figure 6H), and buried a similar number of marbles (Mann Whitney's test, p= 0.63; Figure 6G). Further, no differences were observed in locomotor activity or sickness-like behaviour, as measured by the total distance travelled in the open-field test (Mann Whitney's test, p= 0.53; Figure 7). In analysis of test specific z-scores, which transformed absolute values to numbers of standard deviations from the control group means, we found that GAD mice (n=18) exhibited significantly higher emotionality scores than HC mice (n=15) (Unpaired t-test, p=0.02; Figure 8).

Figure 6. Behaviour of Colonized Mice

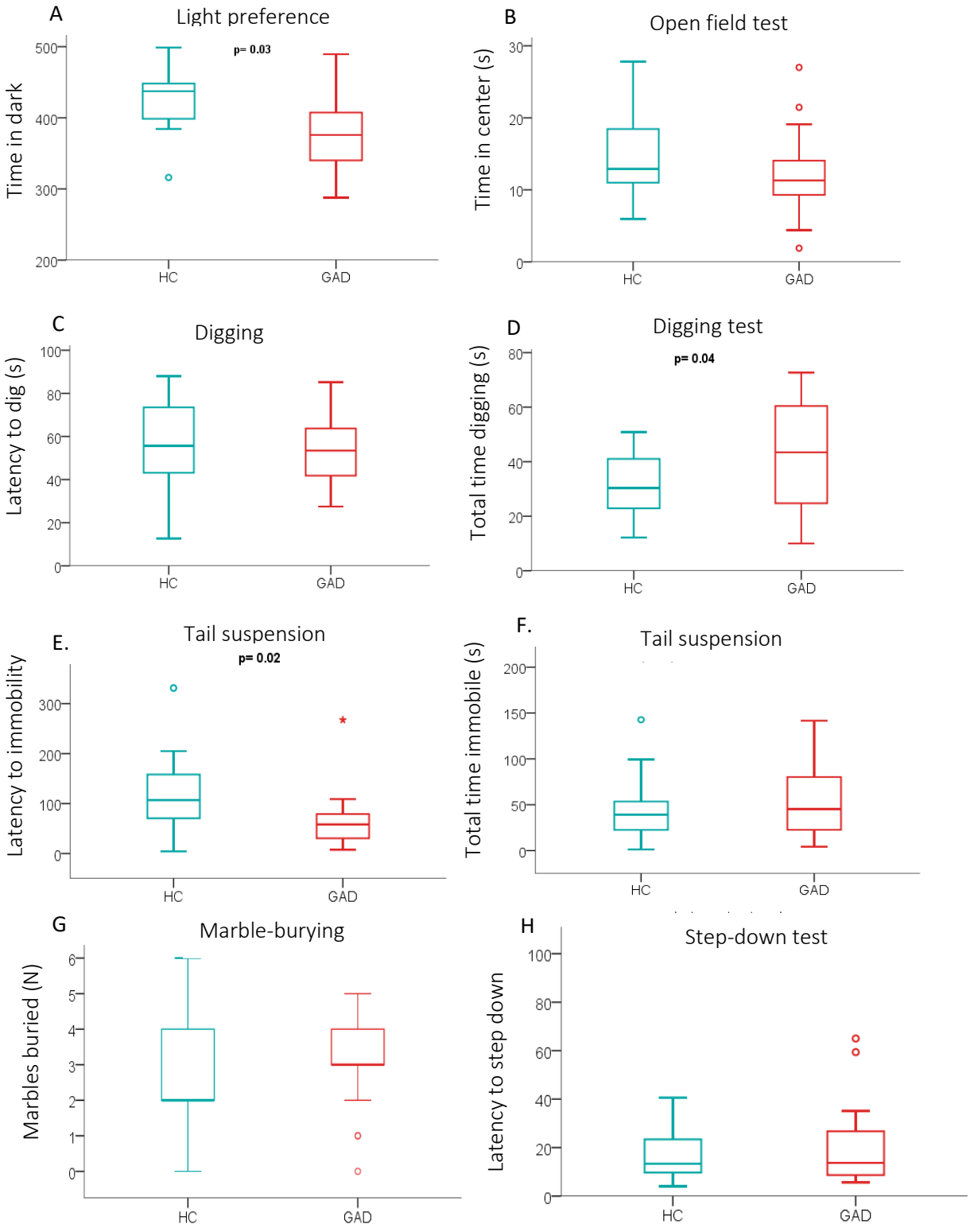
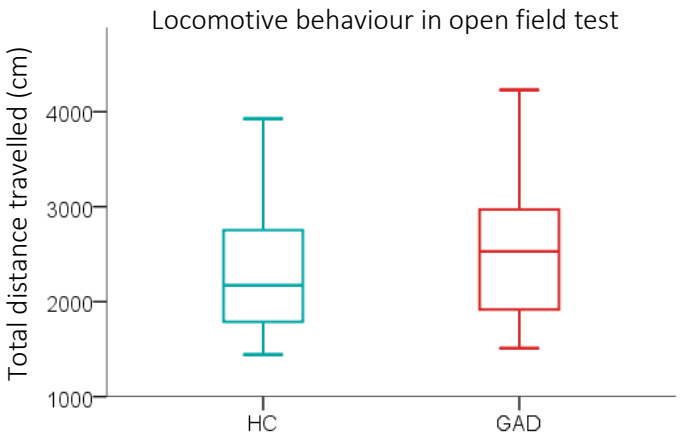
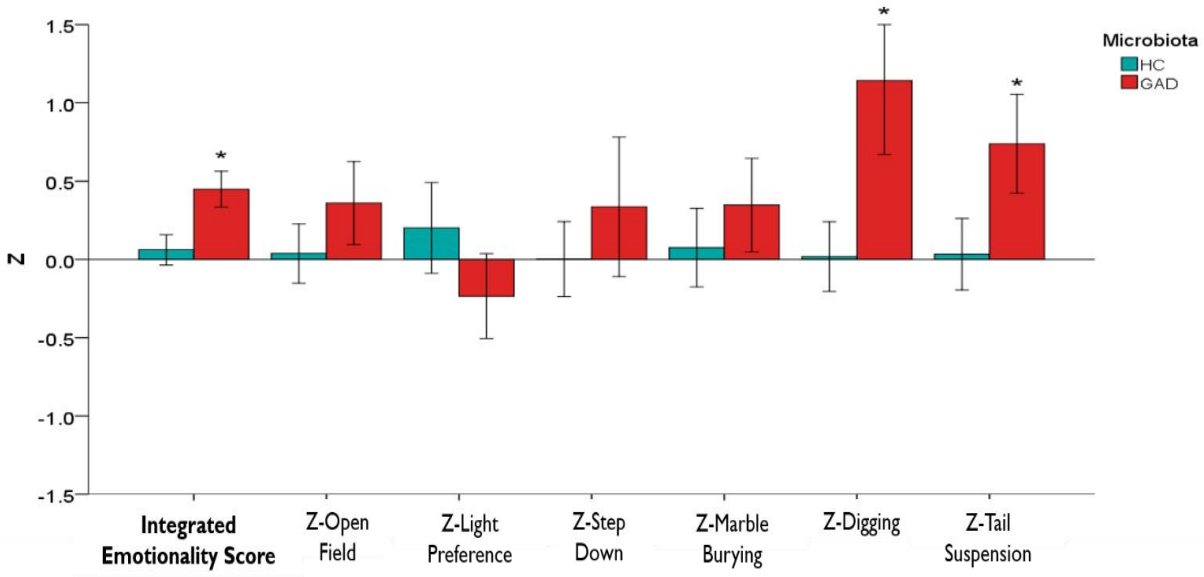


Figure 7. Locomotion of Colonized Mice



Total distance travelled by colonized mice in open field test. GAD (n= 18) and HC mice (n= 15) exhibited no differences in the total distance travelled (Mann Whitney's test, p= 0.53). Data represent mean ± 1 SE.

Figure 8. Integrated Emotionality Scores of Colonized Mice

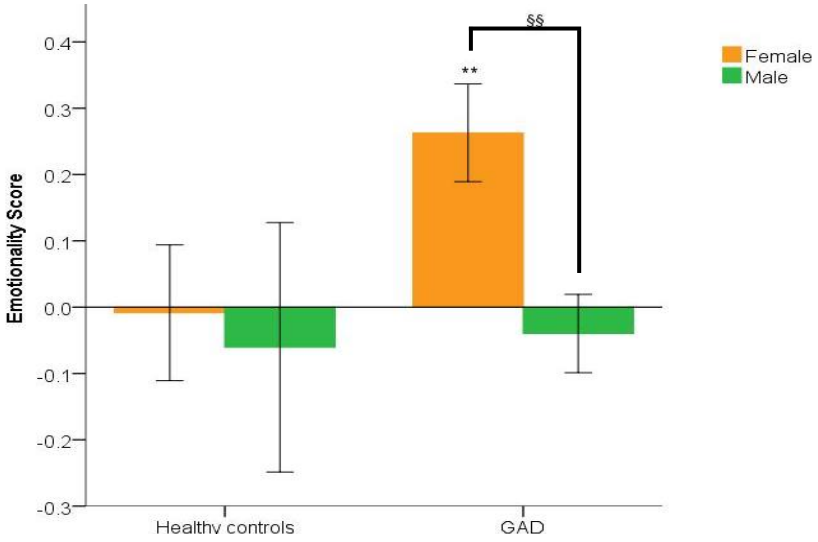


Individual test z-scores (right) and integrated emotionality z-scores (left) of colonized mice. GAD mice (n=18) exhibited significantly higher emotionality scores than HC mice (n=15) (Unpaired t-test, p=0.02). Data represent mean ± 1 SE. * represents p value <0.05.

3.4.1. Sex differences

Integrating emotionality z-scores across all experiments revealed significant sex differences in emotionality following bacterial colonization. Specifically, colonization with GAD microbiota increased emotionality in female mice only (Unpaired t-test, female: n= 16, p= 0.008, male: n= 17, p= 0.51; Figure 9) compared to HC mice, and revealed a higher response to the GAD microbiota in female compared to male mice (Unpaired t-test, p= 0.006; Figure 9). Interestingly, there was no observed difference in emotionality between male and female mice in the healthy control group (Unpaired t-test, p= 0.40; Figure 9).

Figure 9. Emotionality Scores of Male and Female Mice

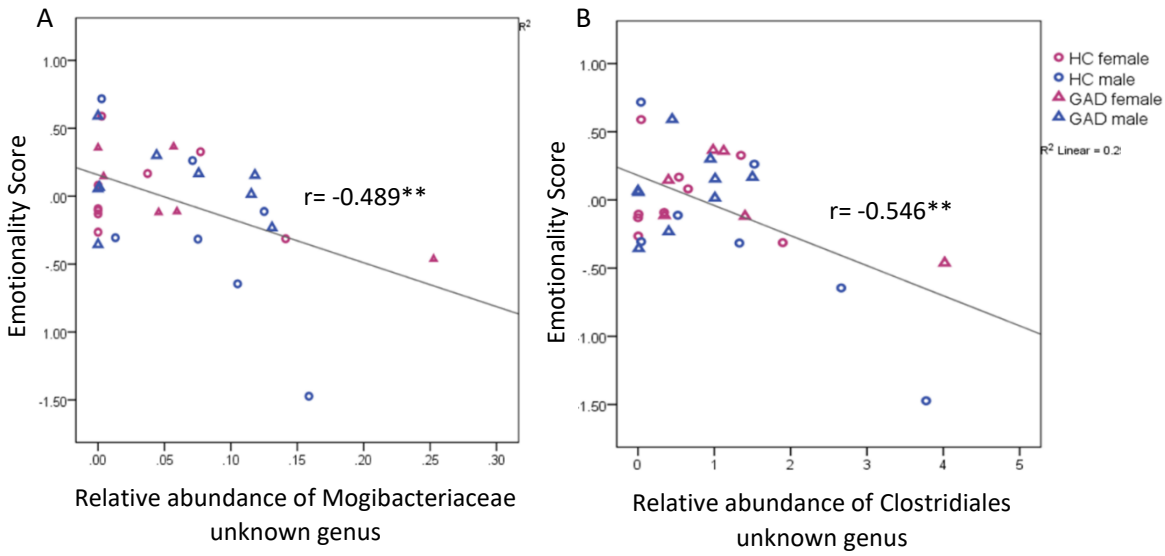


Sex differences in emotionality scores. Colonization with GAD microbiota (right) increased emotionality in female mice only (Unpaired t-test, female: n= 16, p= 0.008, male: n= 17, p= 0.51). GAD female mice also had higher emotionality scores than GAD male mice (Unpaired t-test, p= 0.006). No differences were observed in emotionality between male and female mice in the healthy control group (Unpaired t-test, p= 0.40). Data represent mean ± 1 SE. (** $p<0.01$ for effects of GAD microbiota colonization compared to the sex-matched control group. §§ $p<0.01$ indicates sex differences within groups).

3.4.2. Behaviour-Microbiota Correlations

To further analyze the relationship of gut bacteria and behaviour in mice, correlations between relative abundance of bacteria in stool and emotionality scores of colonized mice (n= 33) were assessed. We found that relative abundances of an unknown genus of Mogibacteriaceae and Clostridiales correlated negatively with emotionality scores (Pearson correlation, r= -0.489, p= 0.005, Fig. 10A; r= -0.546, p= 0.002, Fig. 10B, respectively).

Figure 10. Microbiota-Behaviour Correlations of Colonized Mice



A. Correlation between the relative abundance of an unknown genus in the Mogibacteriaceae family with emotionality scores of mice (Pearson correlation, n= 33, r= -0.489, R^2 linear= 0.239 p= 0.005). **B.** Correlation between the relative abundance of an unknown genus in the Clostridiales order with emotionality scores of mice (Pearson correlation, r= -0.546, R^2 linear= 0.298 p= 0.005).

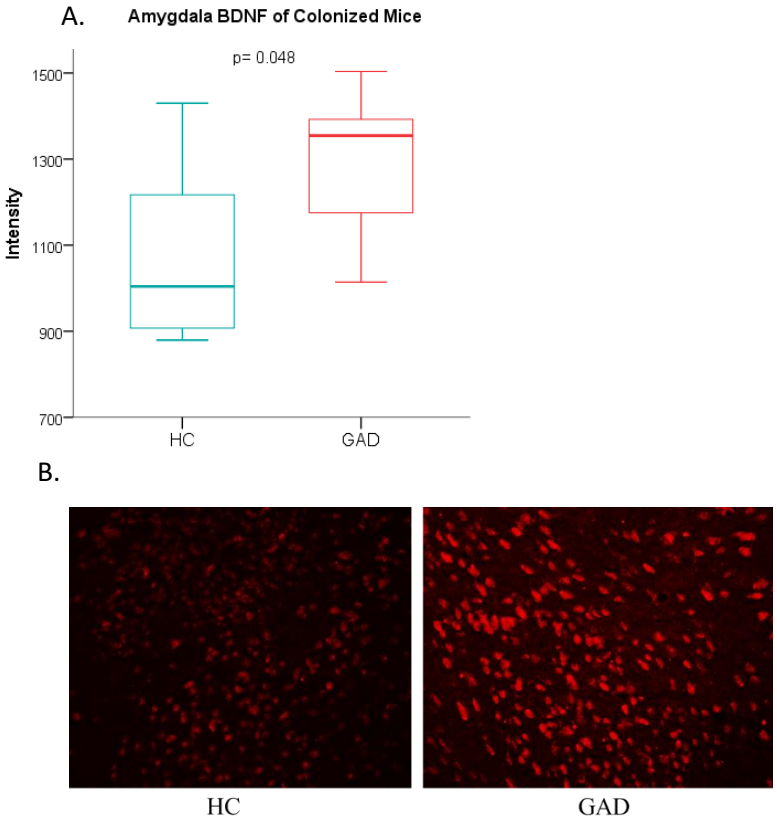
3.5. Immunofluorescent staining

Following sacrifice, brains were harvested from all colonized mice (n= 34). Ten brains from mice with the strongest behavioural phenotypes from each group (GAD, n=5; HC, n=5) were set aside for trace amine analysis. The remaining brains were assessed for differences in central neurotrophins, BDNF and GDNF.

3.5.1. BDNF immunofluorescent staining

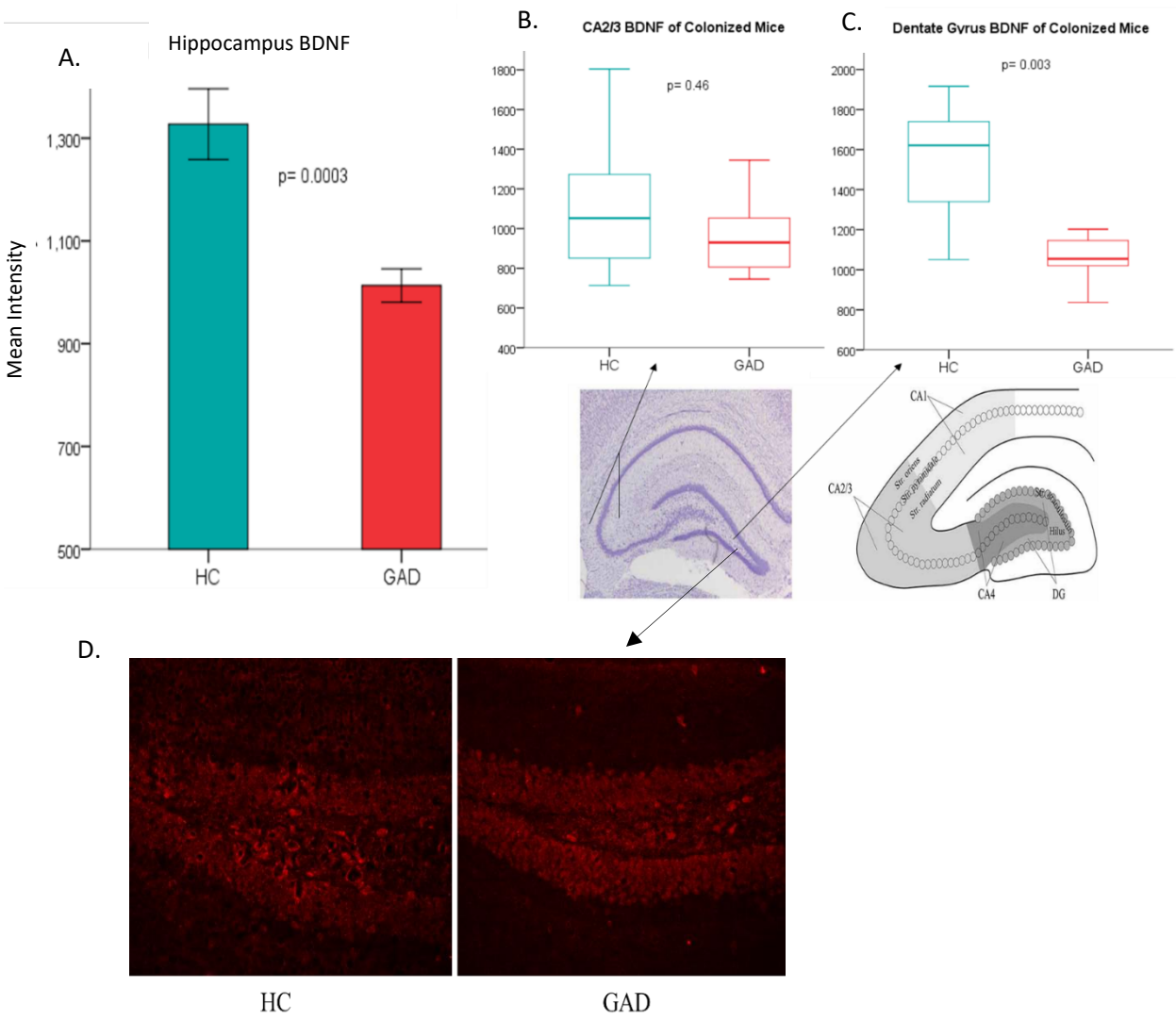
In the amygdala, GAD mice (n=11) showed higher BDNF expression than HC mice (n=8) (Mann-Whitney's test, p= 0.048; Fig. 11A). Two distinct areas of the hippocampus were analyzed for BDNF expression. In the CA2/3 region of the hippocampus, no significant differences were seen between GAD and HC mice (Mann-Whitney's test, p= 0.46; Fig. 12B). However, in the dentate gyrus, GAD mice (n=11) had lower BDNF expression than HC mice (n=8) (Mann-Whitney's test, p= 0.003; Fig. 12C). When combining the CA2/3 region and the dentate gyrus of the hippocampus, GAD mice (n=11) showed significantly lower BDNF expression than HC mice (n=8) (Unpaired t-test, p= 0.0003; Fig. 12A) Together, these results are suggestive of enhanced amygdala neurogenesis and dampened hippocampus neurogenesis in GAD mice.

Figure 11. Amygdala BDNF of Colonized Mice



Immunofluorescent staining of BDNF in the amygdala of colonized mice (n= 19). **A.** GAD mice (n=11) had greater amygdalar BDNF than HC mice (n= 8) (Mann-Whitney's test, p= 0.048). **B.** Images shown were captured at 20x magnification, at consistent gain and exposure with a Nikon fluorescent microscope and Nikon Instrument Software (NIS)-Elements software. Red colour depicts presence of BDNF.

Figure 12. Hippocampal BDNF of Colonized Mice

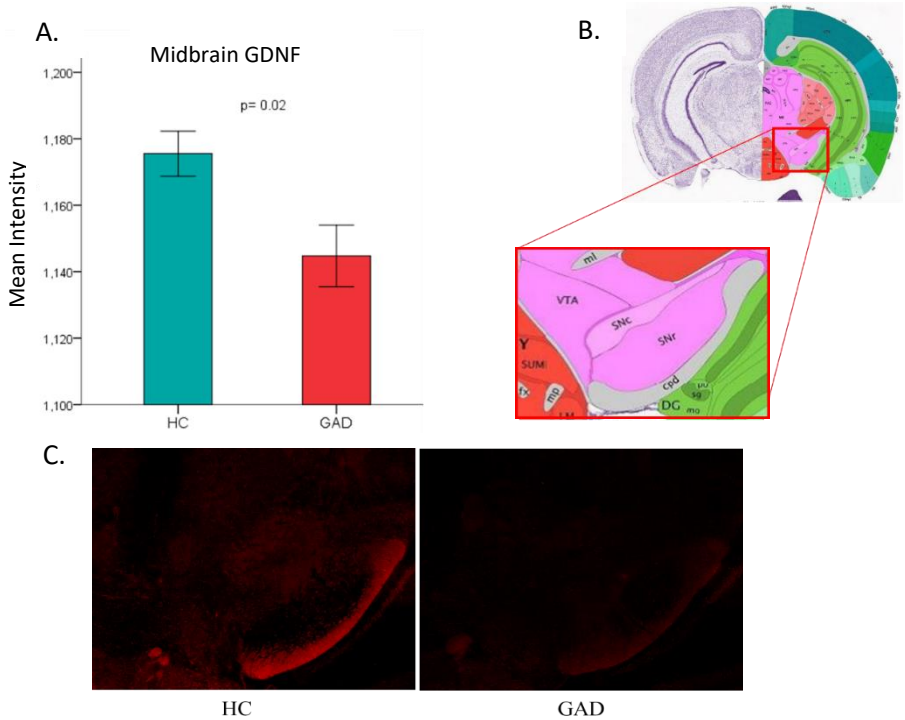


Immunofluorescent staining of BDNF in the hippocampus of colonized mice (n= 19). **A.** GAD mice (n=11) showed significantly lower BDNF expression in the hippocampus than HC mice (n=8) (Unpaired t-test, p= 0.0003). **B.** No significant differences were seen in the CA2/3 region of the hippocampus between GAD and HC mice (Mann-Whitney's test, p= 0.46). **C.** GAD mice had lower BDNF expression than HC mice in the dentate gyrus area of the hippocampus (Mann-Whitney's test, p= 0.003). **D.** Images shown were captured at 20x magnification, at consistent gain and exposure with a Nikon fluorescent microscope and Nikon Instrument Software (NIS)-Elements software. Red colour depicts presence of BDNF.

3.5.2. GDNF immunofluorescent staining

The areas of the midbrain stained for GDNF primarily included the ventral tegmental area (VTA) and the substantia nigra (SN), as depicted in Figure 13B. In the midbrain, GAD mice (n=11) showed significantly lower GDNF expression than HC mice (n=8) (Unpaired t-test, p= 0.02; Fig. 13A). This observation suggests that GAD mice may have impaired dopaminergic neurogenesis, which has been implicated in the pathology of anxiety and depression (Wulff et al., 2010; Dillon et al., 2014).

Figure 13. Midbrain GDNF of Colonized Mice



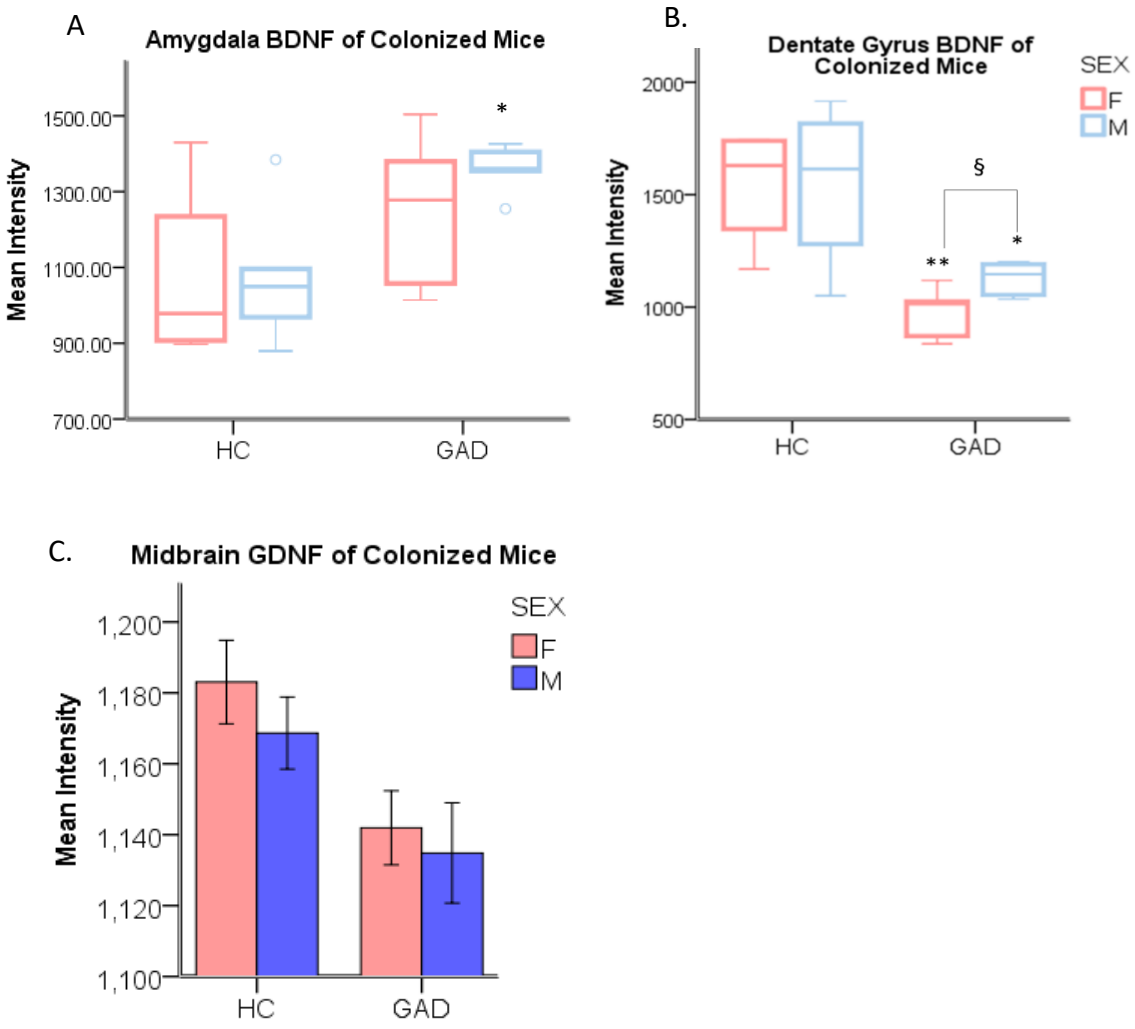
Immunofluorescent staining of GDNF in the midbrain of colonized mice (n= 19). **A.** GAD mice (n=11) showed significantly lower GDNF expression in the midbrain than HC mice (n=8) (Unpaired t-test, p= 0.02). **B.** Graphic representation of the coronal brain slice and area of midbrain stained for GDNF. **C.** Images shown were captured at 20x magnification, at consistent gain and exposure with a Nikon fluorescent microscope and

Nikon Instrument Software (NIS)-Elements software. Red colour depicts presence of BDNF.

3.5.3. Sex Differences

As previously described, male and female mice exhibited marked differences in behaviour following bacterial colonization from a GAD donor. As such, sex differences were also assessed at the level of the brain, specifically, in their individual expression of BDNF and GDNF. In the amygdala, GAD male mice showed higher BDNF expression than HC male mice (Unpaired t-test, $p= 0.01$; Fig. 14A), however there were no differences observed between GAD and HC female mice (Unpaired t-test, $p= 0.29$; Fig. 14A). In the dentate gyrus, both male and female GAD mice had significantly lower BDNF expression than their sex-matched HC groups (Unpaired t-test, $p= 0.02$ (male), $p= 0.004$ (female); Fig. 14B). There were no sex differences observed in dentate gyrus BDNF within the HC group (Unpaired t-test, $p= 0.98$; Fig. 14B), however GAD female mice showed significantly lower BDNF expression than GAD male mice (Unpaired t-test, $p= 0.02$; Fig. 14B). No sex differences were observed in the midbrain (Fig. 14C).

Figure 14. Brain Neurotrophin Expression of Male and Female Mice

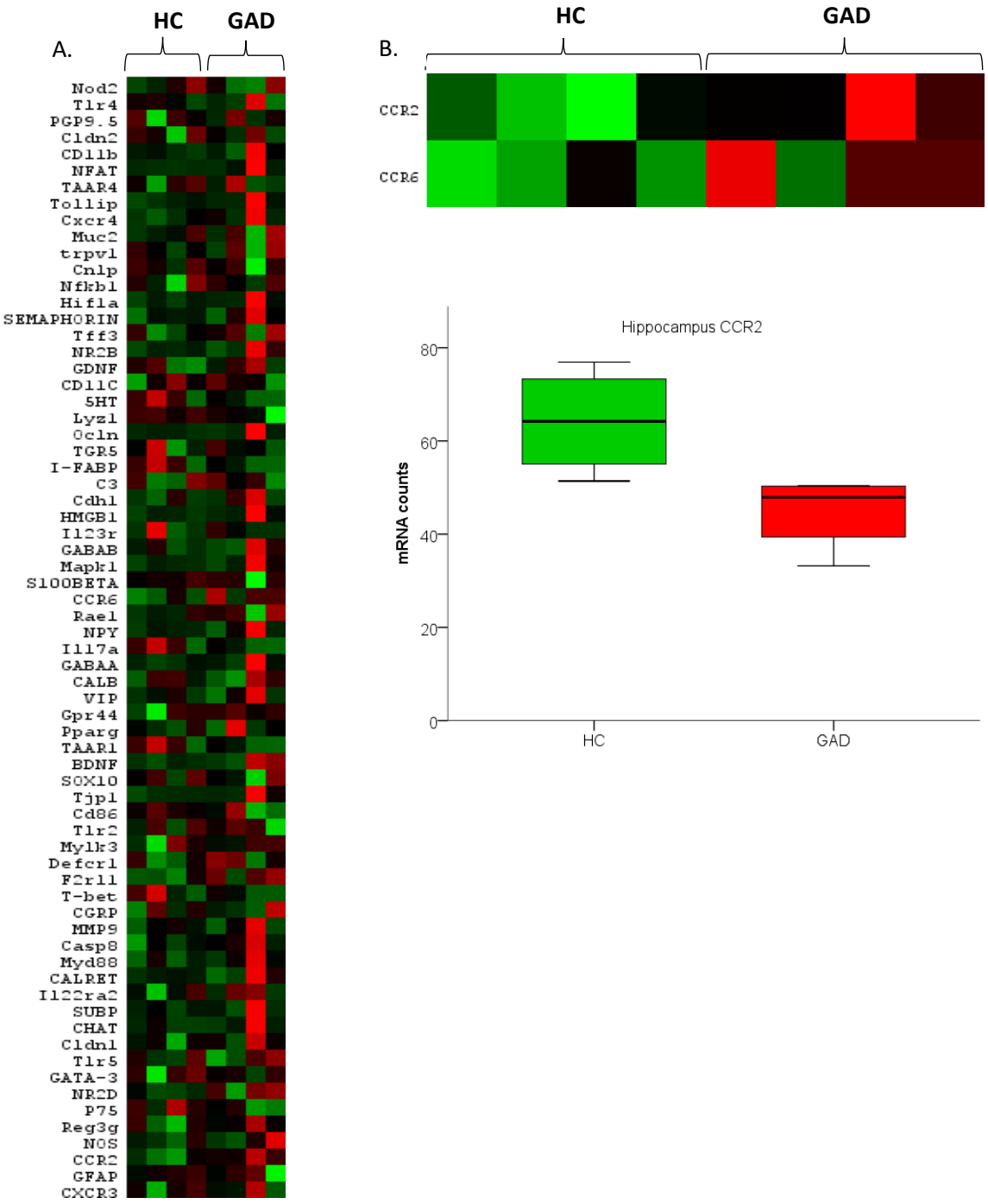


A. GAD male mice showed higher BDNF expression in the amygdala than HC male mice (Unpaired t-test, p= 0.01). No differences were observed between GAD and HC female mice (Unpaired t-test, p= 0.29). **B.** Both male and female GAD mice had significantly lower BDNF expression in the dentate gyrus region of the hippocampus than their sex-matched HC groups (Unpaired t-test, p= 0.02 (male), p= 0.004 (female). No sex differences were observed in dentate gyrus BDNF within the HC group (Unpaired t-test, p= 0.98), however GAD female mice showed significantly lower BDNF expression than GAD male mice (Unpaired t-test, p= 0.02). **C.** No sex differences were observed in the midbrain. (*p<0.05, ** p<0.01 for effects of GAD microbiota colonization compared to the sex-matched healthy control group. § p<0.05 indicates sex differences within groups.)

3.6. Brain gene expression

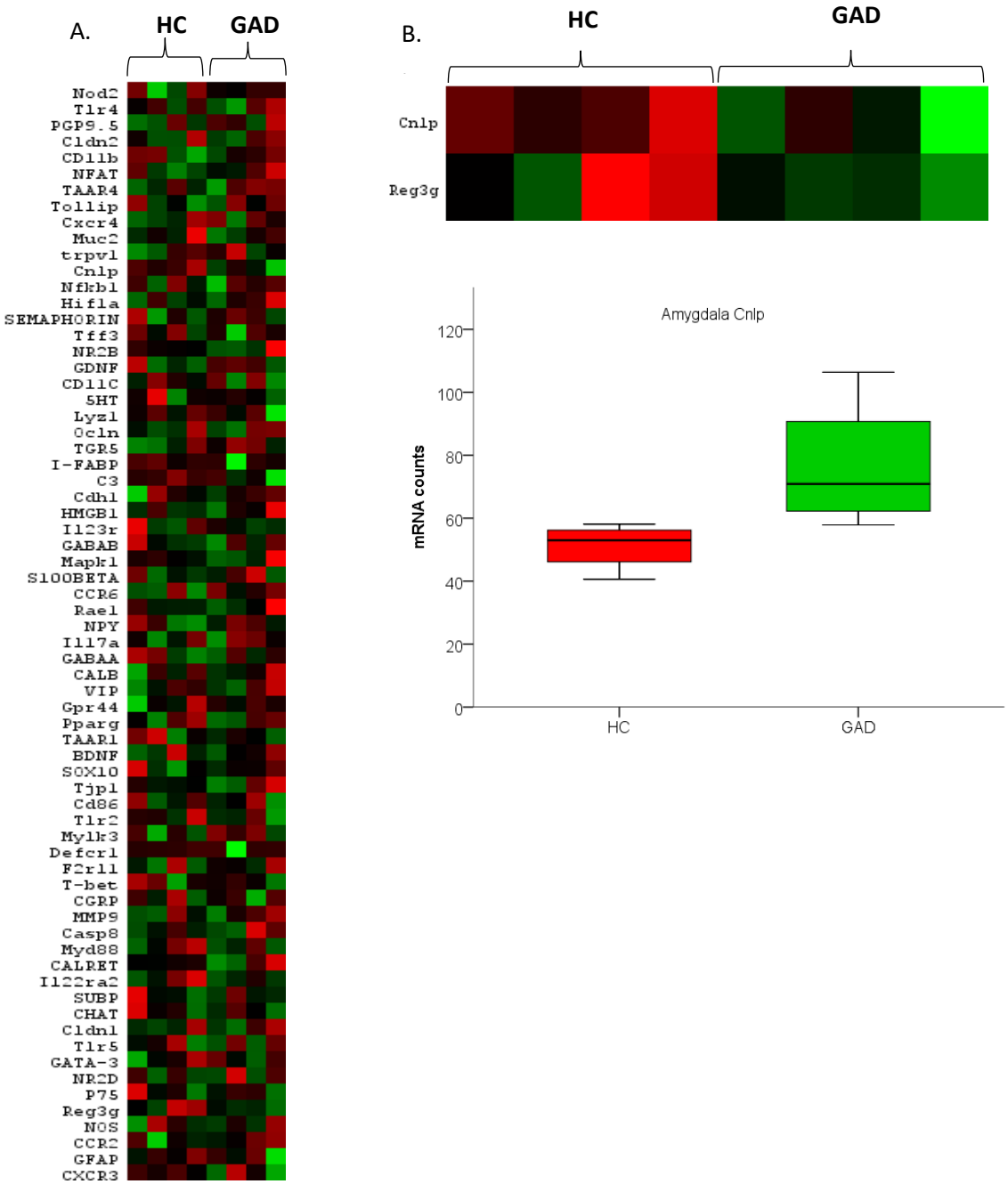
To further assess functional changes in the brain of colonized mice, a brain gene expression assay was conducted. A custom Nanostring assay, which included 72 genes of interest, revealed four genes that were statistically different between GAD (n=4) and HC (n=4) mouse hippocampus (Fig.15), amygdala (Fig.16), and olfactory bulb (Fig.17) samples (each sample consisted of 1 or a mixture of 2 mouse brain samples from each respective group). Of the 72 genes measured in the hippocampus (Fig.15A) GAD mice had downregulated CCR2 (Unpaired t-test, p=0.035; Fig.15B), which has been shown to be involved in neuronal communication (Banisadr et al., 2005). In the amygdala, GAD mice had upregulated Cnlp (Unpaired t-test, p=0.05; Fig. 16B), which has been shown to promote microbial defense (Park et al., 2016). In the olfactory bulb, GAD mice had upregulated Cnlp as well as Nfkb (Unpaired t-test, p= 0.05, p= 0.029; respectively; Fig.17B), which is involved in the maintenance of neurons within the CNS (Asha et al., 2002).

Figure 15. Hippocampal Gene Expression of Colonized Mice



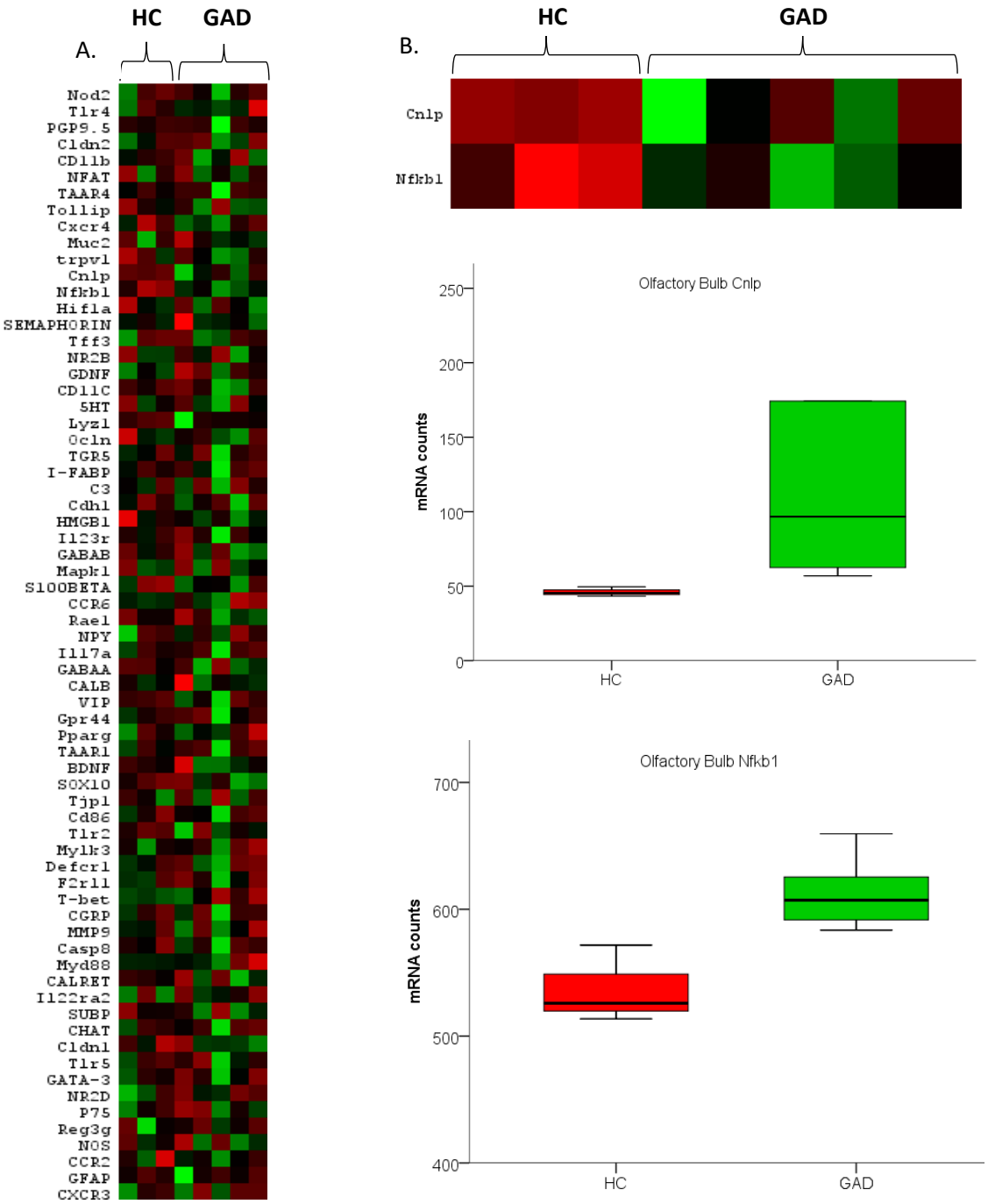
A. Heat map of 72 genes analyzed in the hippocampus of GAD (n=4) and HC (n=4) mouse brain samples. **B.** GAD mice had downregulated CCR2 (Unpaired t-test, p=0.035).

Figure 16. Amygdala Gene Expression of Colonized Mice



A. Heat map of 72 genes analyzed in the amygdala of GAD (n=4) and HC (n=4) mouse brain samples. **B.** GAD mice had upregulated Cnlp (Unpaired t-test, p=0.05).

Figure 17. Olfactory Bulb Gene Expression of Colonized Mice



A. Heat map of 72 genes analyzed in the olfactory bulbs of GAD (n=4) and HC (n=4) mouse brain samples. **B.** GAD mice had upregulated Cnlp as well as Nfkb (Unpaired t-test, p= 0.05, p= 0.029; respectively

3.7. Mouse metabolome

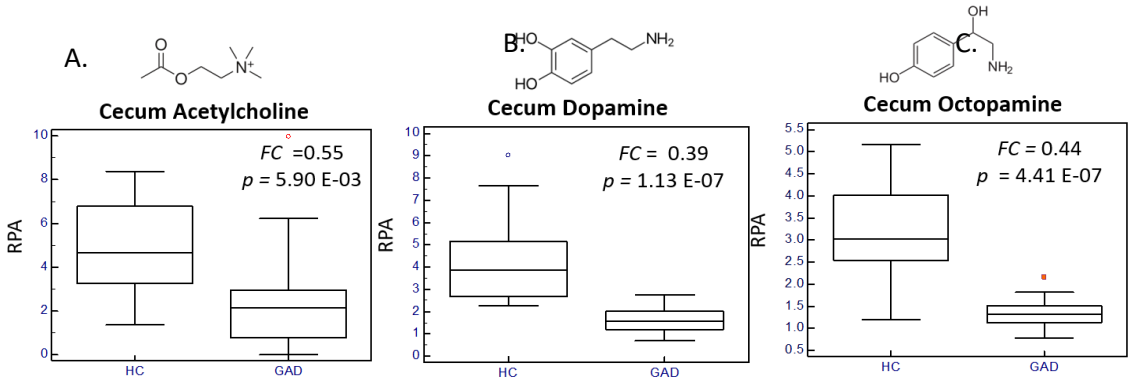
Of the 18 targeted metabolites analyzed, only six compounds were detected consistently in both mice cecum filtrate and stool extract samples, including glutamate, acetylcholine, GABA, histamine, dopamine and octopamine (Table 1). Many of the trace amines were below the method detection limit < 0.2 microM. Of those compounds detected, three compounds were significantly lower in GAD mice (n= 18) as compared to HC mice (n= 15), specifically dopamine (FC = 0.44; p = 1.13 E-7; Fig.18B), octopamine (FC = 0.39; p = 4.41 E-7; Fig. 18C) and acetylcholine (FC = 0.55; p =5.90 E-3; Fig.18A). GABA, glutamate, and histamine did not differ between mouse groups (Table 1). Additionally, both cecum dopamine and octopamine levels were strongly correlated with each other (r = 0.750; Fig.19), which is likely reflective of their similar physiological roles and chemical structures. Interestingly, there were no differences in neuroactive metabolites in stool extracts analyzed (Table 1), likely inferring that cecum provides better assessment of microbiome metabolic activity. Similarly, the concentrations of metabolites measured in stool did not correlate well with the values obtained from cecum. Of all metabolites measured, acetylcholine was the only compound whos values correlated significantly between the stool and cecum extracts (Unpaired t-test, r= 0.679, p= 7.39 E-6; Fig. 20).

Table 1. Stool and Cecum Metabolites Detected in Colonized Mice

Cecum (μM)						
	GABA	Histamine	Acetylcholine	Glutamate	Octopamine	Dopamine
HC†	1.41 ± 0.79	0.31 ± 0.25	4.82 ± 2.21	167 ± 43	3.06 ± 1.10	4.23 ± 1.94
GAD†	1.46 ± 0.60	0.21 ± 0.14	2.64 ± 2.72	178 ± 39	1.34 ± 0.30	1.64 ± 0.55
Fold-change (GAD/HC)	1.03	0.68	0.55	1.07	0.44	0.39
p-value	0.65	0.18	5.90 E-03	0.39	4.41 E-07	1.13 E-07
Stool (μM/mg dried weight)						
	GABA	Histamine	Acetylcholine	Glutamate	Octopamine	Dopamine
HC†	0.012 ± 0.009	0.0062 ± 0.0020	0.039 ± 0.023	0.61 ± 0.28	0.015 ± 0.006	0.023 ± 0.007
GAD†	0.015 ± 0.012	0.0085 ± 0.0028	0.036 ± 0.041	0.74 ± 0.34	0.014 ± 0.005	0.023 ± 0.007
Fold-change (GAD/HC)	1.22	1.20	0.92	1.22	0.86	1.10
p-value	0.29	0.21	0.49	0.33	0.47	0.94

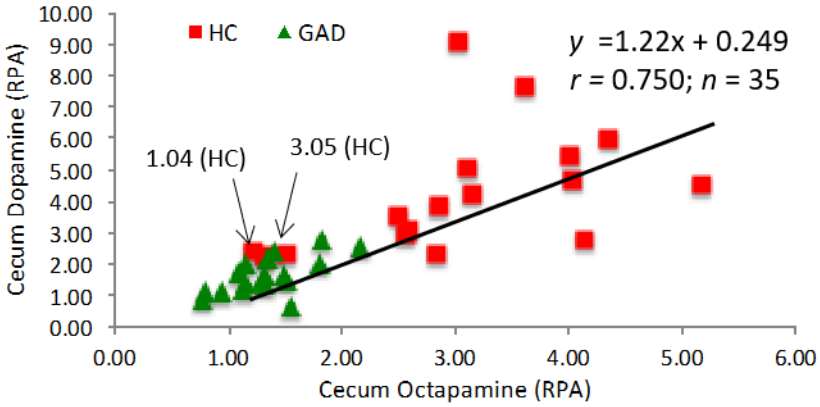
† *Mean concentration (μM) ± std. deviation (n=17-18)*

Figure 18. Acetylcholine, Dopamine and Octopamine in Cecum of Colonized Mice



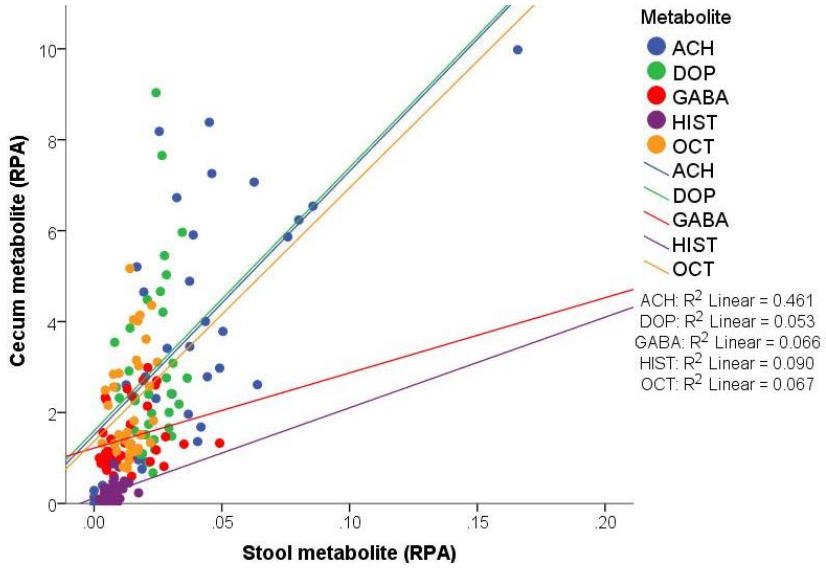
Cecum metabolites of GAD (n= 18) and HC (n= 15) mice as measured by MSI-CE-MS.
A. Acetylcholine was significantly lower in GAD mice (FC = 0.55; p =5.90 E-3).
B. Dopamine was significantly lower in GAD mice (FC = 0.44; p = 1.13 E-7).
C. Octopamine was significantly lower in GAD mice (FC = 0.39; p = 4.41 E-7).

Figure 19. Correlation of Cecum Dopamine and Octopamine in Colonized Mice



Cecum dopamine and octopamine levels strongly correlated with each other (r = 0.750, r^2 = 0.562)

Figure 20. Correlations of Cecum and Stool Metabolites

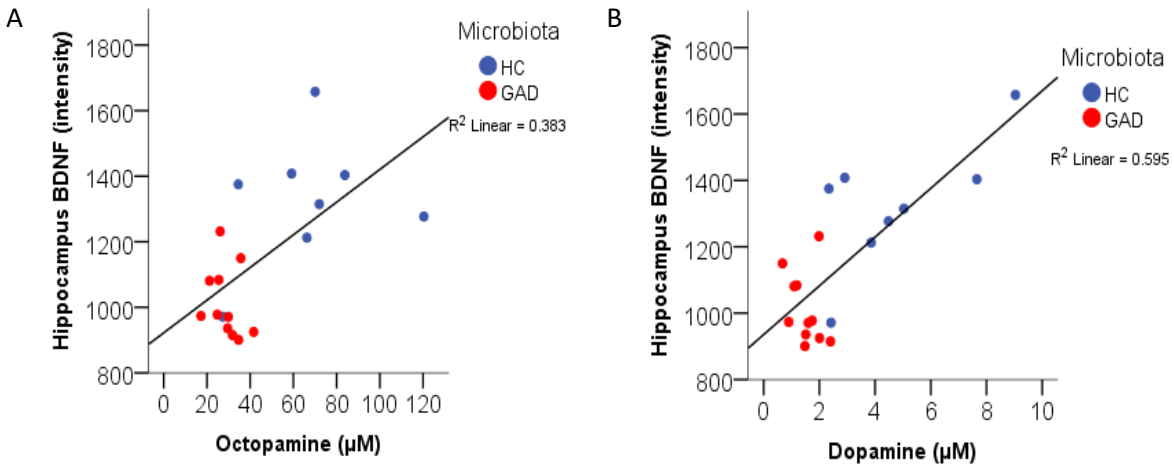


Stool and cecum correlations for each metabolite detected. Acetylcholine (ACH; Unpaired t-test, r= 0.679, r^2= 0.461, p= 7.39 E-6), Dopamine (DOP; r= 0.231, r^2= 0.053, p= 0.183), GABA (r= 0.258, r^2= 0.066, p= 0.135), Histamine (HIST; r= 0.300, r^2= 0.090, p= 0.08), Octopamine (OCT; r= 0.259, p= 0.133), Glutamine (GLU (not shown); r= 0.083, p= 0.684).

3.7.1. Brain neurotrophin correlations with cecum metabolites

Cecum octopamine and dopamine correlated positively with hippocampus BDNF (Spearman correlation, r= 0.619, p= 0.005, Fig.21A; r= 0.772, p= 0.0001, Fig.21B, respectively).

Figure 21. Correlation of Cecum Metabolites and BDNF in Colonized Mice



Cecum dopamine and octopamine levels correlated with hippocampus BDNF. **A.** Octopamine. Spearman correlation, r= 0.619, p= 0.005. **B.** Dopamine. Spearman correlation, r= 0.772, p= 0.0001

3.8. Patient serum metabolome

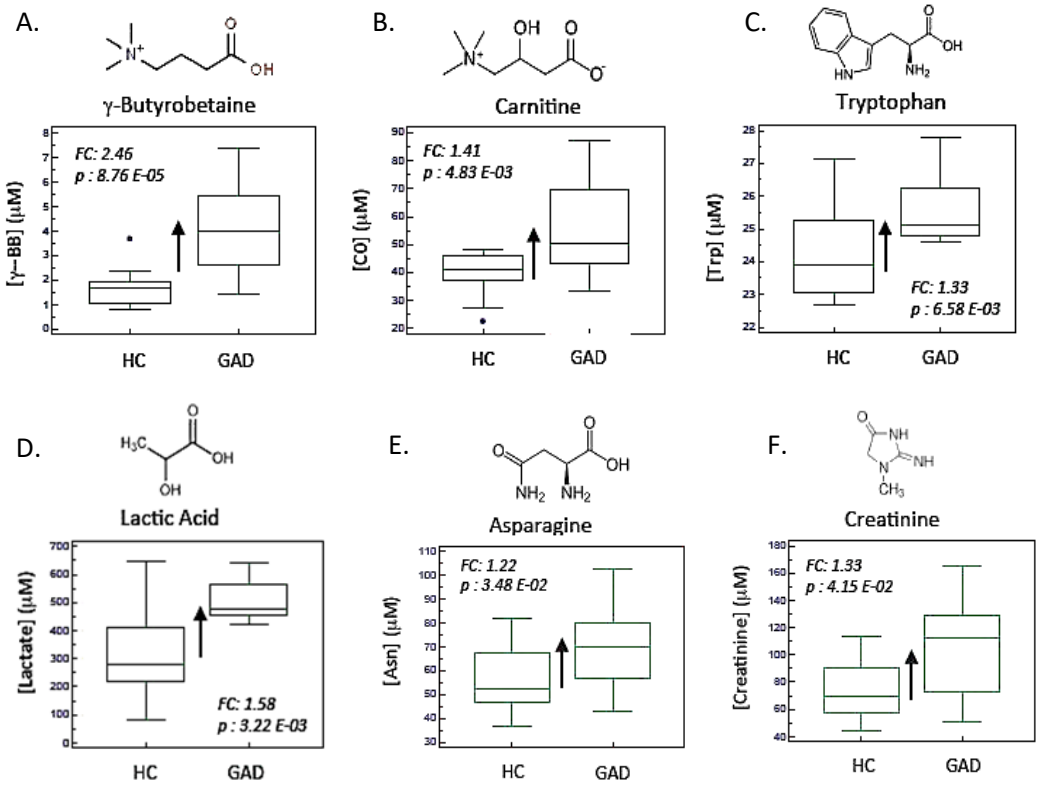
Analysis of the serum metabolome of GAD (n=13) and healthy control (n=12) human samples revealed six metabolites to be elevated in GAD patients as compared to healthy controls. Gamma-butyrobetaine, carnitine, tryptophan, lactic acid, asparagine, and creatinine were all elevated in GAD serum as compared to healthy control serum (p= 8.76

E-05, 0.00483, 0.00658, 0.00322, 0.0348, 0.0415; respectively, Fig.24). Age and sex were largely balanced between groups. Patient BMI values were incomplete and six out of thirteen GAD patients were on antidepressant and/or benzodiazepine medications at time of sample collection.

Table 2. Patient Serum Metabolites

	Plasma (μM)					
	γ-Butyrobetaine	Carnitine	Tryptophan	Lactic acid	Asparagine	Creatinine
HC[†]	1.7 ± 0.8	39.7 ± 8.1	24 ± 34	314 ± 130	56.6 ± 5.7	75 ± 16
GAD[†]	4.1 ± 1.8	56.0 ± 16	33 ± 21	510 ± 47	70.5 ± 8.8	102 ± 28
Fold change (GAD/HC)	2.46	1.41	1.33	1.58	1.22	1.33
p-value	8.76 E-05	0.00483	0.00658	0.00322	0.0348	0.0415

Figure 22. Patient Serum Metabolites



Human serum metabolites as measured by MSI-CE-MS. Gamma-butyrobetaine, carnitine, tryptophan and lactic acid were all significantly higher in GAD serum as compared to healthy control serum. **A.** Gamma-butyrobetaine (FC = 2.46, p= 8.76 E-05). **B.** Carnitine (FC = 1.41, p= 0.00483). **C.** Tryptophan (FC = 1.33, p= 0.00658). **D.** Lactic Acid (FC = 1.58, p= 0.00322). **E.** Asparagine (FC = 1.22, p= 0.0348). **F.** Creatinine (FC = 1.33, p= 0.0415).

4. DISCUSSION

4.1. Gut microbiota effects on host metabolites

Although growing evidence suggests that gut microbiota can affect behaviour of the host, the mechanisms are poorly understood, mainly due to the complexity of microbial communities. However, studies in both humans and mice have shown that gut microbiota can impact host metabolism, both by altering energy yield from food and by altering host metabolic pathways (Tremaroli & Backhed, 2012). In the present study, we have shown that differences in host gut bacterial colonization was associated with altered levels of metabolites in mouse cecum samples. Specifically, acetylcholine, dopamine, and octopamine were significantly lower in mice colonized with microbiota from a patient with GAD, as compared to a healthy donor. This is of particular interest, as these amines are instrumental to CNS function and their deficiencies have been largely implicated in the pathophysiology of anxiety and depression (Yilmazer-Hanke et al., 2003; Lester et al., 2010; Roeder, 2005; Sandler et al., 1979) As such, it is possible that the brain and behavioural changes observed in anxiety and depression originate from the gut, through byproducts of bacterial metabolism and associated neurotransmitter and neuromodulator release. Further studies are required to determine which bacteria are involved in their production, and their mechanisms of relay to the CNS.

Metabolome analysis of GAD and HC human serum samples revealed six metabolites associated with energy metabolism to be elevated in GAD patients as compared to healthy controls. This finding is convergent with many studies which have demonstrated

the presence of metabolic alterations related to energy pathways in psychiatric disorders (Pei & Wallace, 2018; Picard et al, 2015; Andreazza & Nierenberg, 2018; Kim et al., 2019) Notably, the present study revealed carnitine and gamma-butyrobetaine to be increased in GAD patients compared to HC, which are both involved in the oxidative degradation of fatty acids (Strijbis, Vaz & Distel, 2010; Flanagan et al., 2010). Gamma-butyrobetaine is a key player in the process in the synthesis of carnitine, which acts as a transporter of long-chain fatty acids into the mitochondria to be oxidized, and ultimately, produce energy (Tars et al., 2010). Carnitine also acts to stabilize acetyl-CoA, which as previously discussed, is a primary source of energy for cholinergic neurons. Furthermore, several studies have observed mitochondrial, energy metabolism and oxidative stress alterations in highly anxious and stressed individuals and conversely, anxious symptoms in patients afflicted by mitochondrial disorders (de Kloet et al., 2005; Morava & Kozicz, 2013; Filiou et al., 2014). Additionally, recent research has suggested a bidirectional communication between the gut microbiota and mitochondria (Gruber, 2017). These studies have demonstrated that metabolites produced by commensal gut bacteria, such as short-chain fatty acids, might influence mitochondrial processes related to energy production and inflammatory cascades (Circu & Aw, 2012; den Besten et al., 2013). Interestingly, mitochondrial gene polymorphisms have been associated with specific compositions of gut microbiota (Ma et al., 2014). Overall, evidence supports the notion that there is a bidirectional communication between mitochondrial biology, energy metabolism, gut microbiota and anxiety. Prospective studies measuring specific mitochondrial and metabolic states will be needed to consolidate the relationship between

psychosocial stress, microbiota, the resulting neuroendocrine and immune processes, and mitochondrial energetics relevant to psychiatric disorders.

4.2. Gut microbiota effects on behaviour

16S rRNA analysis revealed significant differences in both the diversity and composition of microbiota between HC and GAD mice. Likewise, GAD mice exhibited greater anxiety-like behaviour in the light preference, digging, as well as the tail suspension test, and had significantly higher emotionality scores as compared to HC mice. These results are in agreement with existing literature, as several studies have shown that behavioural traits can be transferred via intestinal microbiota in mice. Previous studies in mice have shown that a single pathogenic bacterium introduced to the gut resulted in the onset of anxiety-like behaviour (Goehler et al., 2005). Further, more recent research has shown that germ-free mice receiving fecal transplant adopt the behavioural phenotype of the donor mouse (Bercik et al., 2011; Collins et al., 2013) and that germ-free mice colonized with stool microbiota from patients with IBS and comorbid anxiety develop anxiety-like behavior (De Palma 2017). Clinical studies have demonstrated that consumption of probiotic enriched fermented milk by healthy females attenuate changes in brain connectivity and activity induced by stress (Tillisch et al., 2013) and that administration of *Bifidobacterium longum* improves depression scores and alters activity in in multiple brain regions in patients with Irritable Bowel Syndrome and comorbid depression (Pinto Sanchez, 2017).

Our study revealed that GAD mice had increased relative abundance of Actinobacteria. This finding is consistent with current literature, which has shown that compared with healthy controls, the relative abundance of Actinobacteria is increased in MDD subjects (Guilloux et al., 2011; Zheng et al., 2016). Actinobacteria has also been found to be correlated with immobility time in the forced swim test in mice, which is indicative of elevated anxiety-like behaviour (Haiyin et al., 2015). Further, acyl-coA synthetases have been found in Actinobacteria (Casabon et al., 2014). Acetyl-CoA, one of the metabolites of acyl-CoA, is a major source of energy production for cholinergic neurons, and is required for their synthesis of acetylcholine in the cytoplasmic compartment (Szutowicz et al., 2013) . For these reasons, maintaining a proper supply of acetyl-CoA in the brain is imperative in attenuating the susceptibility of cholinergic neurons to neurodegeration. In mice, decreases in the density of septal cholinergic neurons was significantly correlated with deterioration on cognitive tests (Wang, Chen & Sun, 2009).

Presently, it is unknown precisely how the microbiota may induce behavioural changes, although many pathways have been suggested, involving neural (Collins & Bercik, 2009; DuPont, 2011), immune (Tracey, 2009), and metabolic pathways (Collins & Bercik, 2009; Freestone et al., 2008). Our experiments performed were thus designed to examine neural aspects through measurement of brain genes and neurotrophins, immune activation through assessment of fecal beta-defensin-3, and metabolic profiles through analysis of trace amines and classical neurotransmitters. This approach was

aimed at further examining the physiological effects of gut microbiota in the expression of anxiety and depression.

4.3. Gut microbiota effects on immune status

Many of the studies that have focused on the relationship between gut microbiota and behaviour have, at least to some degree, shown the involvement of the immune system. However, studies on immune-independent mechanisms by which microbes can influence behaviour have been scarce, though are becoming an area of increased interest (Lyte and Cryan, 2014). Our experiments have shown there to be no major effect of microbiota on immune status, as assessed by fecal beta-defensins in colonized mice. As such, this finding suggests that gut microbiota and the central nervous system may be communicating by pathways that are independent of the immune system, as there were marked differences in brain chemistry and behaviour between mice colonized with GAD microbiota as compared to mice colonized with HC microbiota. It is possible, however, as beta defensins were the only inflammatory markers measured, that alternate inflammatory markers such as CRP may have differed between the groups.

4.4. Gut microbiota effects on brain neurotrophins

The amygdala and the hippocampus were the selected brain areas of interest in assessing BDNF expression. The amygdala is primarily involved in fear recognition and

response and has been shown to have increased amounts of BDNF in anxiety and depression (Guilloux et al., 2011). On the contrary, the hippocampus is mainly responsible for the formation and retrieval of memory as well as spatial navigation (Squire & Zola, 1996; Tulving & Markowitsch, 1998) and has been shown to have decreased levels of BDNF in anxiety and depression (Martinowich et al., 2007). The midbrain was selected to assess GDNF expression, as dysfunctions of midbrain dopaminergic neurons, which underlie brain reward systems, have been frequently reported in MDD and GAD (Wulff et al., 2010).

In our study, microbiota was shown to have effects on central brain neurotrophins BDNF and GDNF, in both limbic and mesolimbic structures of the brain, specifically in the hippocampus, amygdala, and midbrain. Compared to HC mice, GAD mice exhibited higher BDNF expression in the amygdala, lower BDNF expression in the hippocampus, and lower GDNF expression in the midbrain. This is in agreement with a previous study from our group showing that colonization of germ-free NIH Swiss mice with Balb/c microbiota increased anxiety-like behaviour and reduced levels of hippocampal BDNF (Collins & Bercik, 2013). Conversely, colonization of germ-free Balb/c mice with NIH Swiss microbiota resulted in reduced anxiety-like behaviour and elevated levels of BDNF in the hippocampus. As such, from our study and others, it appears that microbiota can impact hippocampal neurogenesis. This finding is reflected in patients with MDD and GAD, as they often report difficulties in working memory and attention (Dalgleish & Watts, 1990; LeMoult & Joormann, 2012; Massimo et al., 2003). Perturbations in microbiota have also been previously shown to affect BDNF levels in amygdala in GF

and antibiotic-treated animals (Bercik et al., 2011; Diaz Heijtz et al., 2011; Hoban et al., 2016; Cowan et al., 2018)

While past research has shown GDNF expression to be reduced in the intestinal smooth muscle cells in microbiota-depleted mice (Obata et al., 2016), our study is the first to show that changes in microbiota can induce alterations in GDNF expression in the mesolimbic structures of the brain. As highlighted in a previous section, GDNF is critical for the maintenance and restoration of midbrain dopaminergic (DAergic) neurons, which have been shown to be impaired in anxiety and depression. Studies have also shown that GDNF injected into the striatum supresses the cell death of dopamine (DA) neurons in the substantia nigra (SN), and conversely, double transgenic mice which overexpress GDNF have increased number of SN DA neurons (Burke, 2006). Similarly, chronic treatment with GDNF increases the amount of synapses of ventral tegmental area (VTA) neurons onto DAergic neurons and enhances DA release (Dorit & Janak, 2005; Yu et al., 1998). The experimental findings we have reported here are thus congruent with an ample body of literature which has demonstrated the importance of GDNF to the functioning of midbrain dopaminergic reward systems in the VTA and SN (Choi-Lundberg et al., 1997; Burke, 2006). As such, considering the reduced levels of GDNF expression observed in the midbrain of GAD mice, it is likely that microbiota alters brain reward systems and motivational behaviours through action on GDNF expression, and consequently, neuronal growth and survival of DA neurons.

4.5. Gut microbiota effects on brain gene expression

Our study has shown differences in multiple gene expression in the hippocampus, amygdala, and olfactory bulbs of mice colonized with microbiota from a GAD donor. Specifically, genes *CCR2*, *Cnlp*, and *Nfkb1* were shown to be markedly different in GAD mice when compared to HC mice.

Earlier research on the physiological role of *CCR2* was mainly suggestive of its role in the trafficking of leukocytes and inflammatory cell recruitment in various disease models (Arai & Charo, 1996; Andjelkovic et al., 2002), however more recent studies suggests its involvement in cholinergic and dopaminergic neurotransmission and related disorders (Banisadr et al., 2002a/2002b; van der Meer et al., 2000). *CCR2* is primarily localized in dopaminergic neurons in the substantia nigra and ventral tegmental area of the midbrain, as well as in cholinergic neurons of the substantia innominate and caudate putamen (Banisadr et al. 2005). In our study, *CCR2* was found to be downregulated in the hippocampus of GAD mice. This is suggestive of altered neuronal communication in the hippocampus, possibly acting through alterations in cholinergic and dopaminergic neurotransmission. This finding coincides with our immunofluorescence staining data which showed reduce expression of GDNF, known to be vital for dopaminergic neurons, in the midbrain of GAD mice. Similarly, mass spectrometry data revealed reductions in acetylcholine and dopamine in the cecum of GAD mice.

The *Cnlp* gene, also known as cathelicidin antimicrobial peptide (*CAMP*), was found to be upregulated in both the amygdala and olfactory bulbs of GAD mice. *CAMP*

plays a crucial role in the defense against microbial infection and is activated in response to ER stress (Park et al., 2016), however the function of *CAMP* in the CNS has received limited attention. Greater understanding of antimicrobial peptides in the brain will shed further light on the mechanisms responsible for psychiatric illnesses and neurological diseases.

In non-neuronal cells, nuclear factor- κB (*NF-κB*) is critical for mediating proinflammatory responses (Hatada et al., 2000; Weih et al., 1994), however its role within the CNS is less clear. Numerous studies revealed that activation of *NF-κB* is highly neuroprotective (Barger et al., 1995; Guo et al., 1998; Lezoualc'h et al., 1998; Maggirwar et al., 1998; Hamanoue et al., 1999; Kaltschmidt et al., 1999), whereas other studies link its activation to neuronal death (Grilli et al., 1996; Post et al., 1998; Schneider et al., 1999). More recent research using a transgenic mouse model has revealed that blocking neuronal *NF-κB* activity *in vivo* results in neuronal death, whereas its activation increases levels of anti-apoptotic proteins, thus demonstrating the requirement of *NF-κB* activity for neuronal survival within the CNS (Bhakar et al., 2002).

4.6. Sex differences

In the comparison of behavioural and neurotrophin data between male and female mice, significant sex differences were revealed. Interestingly, when separated by sex, differences in anxiety-like behaviour between GAD and HC mice reached statistical significance in female mice only. Further, these differences cannot be explained by

increased emotionality of female mice at baseline, as no differences in emotionality scores were observed between male and female HC mice. This is suggestive of an increased behavioural sensitivity and/or responsivity of female mice to the GAD microbiota. This finding is of interest, as women have twice the lifetime rates of depression and most anxiety disorders in comparison to men (Kessler et al., 1994, 2005; Weissman et al., 1996; Gater et al., 1998, Altemus et al., 2014). Similarly, the prevalence of agoraphobia and panic disorder is three times higher in women than in men (Kessler et al., 1994). In many psychiatric disorders, in which sex effect is observed, these differences in prevalence often present themselves at the onset of puberty and adolescence, suggesting that differences in gonadal hormone secretion may have an important role (Altemus et al., 2014). Sex differences in brain structure and function have been abundantly reported and shown to fluctuate through development (Courchesne et al., 2000; Lampl and Jeanty, 2003; Patton et al., 2004; Lenroot et al., 2007; Raznahana et al., 2013; Ingalhalikar et al., 2014). Neuroimaging data have shown that women are consistently more sensitive to threatening social cues and exhibit greater reactivity to social rejection than men (Stroud et al., 2002. McClure et al., 2004). In addition, women with generalized anxiety disorder are more likely to experience somatic symptoms than men, and more often complain of muscle tension, fatigue, and gastrointestinal discomfort (Altemus et al., 2014). Adaptive behavioural sex differences that increase reproductive success and childbearing, such as increased social cognition and empathy (Halpern, 2007; Gur et al., 2012; Thompson and Voyer, 2014), may also put women at heightened risk for mood and anxiety disorders, as they may experience greater sensitivity to rejection and

criticism, marked traits of anxiety and depression (Cyranowski et al., 2000; Taylor et al., 2000; Zahn-Waxler et al., 2008, Martel, 2013). Whether these sex effects represent differences in epidemiology, pathophysiology, or adaptive responses will likely be the greatest challenge in discerning the causal factors in sex differences seen in psychiatric disorders. For these reasons, future research examining host-microbiota interactions focusing on analysis of sex has potential to shed light on the biological underpinnings of sex differences in affective disorders.

4.7. Limitations

Although the present study revealed intriguing results, it bears limitations that must be considered when drawing conclusions. In particular, because this is an animal study, there are inherent difficulties in extrapolating the findings from mice to humans. As such, future clinical studies would carry insight as to whether these changes are also observed in clinical populations. Similarly, due to the constraints in breeding germ-free mice, and in housing large groups of mice, the sample size may not have had adequate statistical power for the differences observed between GAD and HC mice to reach statistical significance. It is likely with a larger sample size, more of the behavioural, brain, and microbiota measurements that fell slightly short of reaching statistical significance between GAD and HC mice, would reach significance with the addition of more mice. Further, only one set of human donors was used in this study. Similarly, the anxious donor was on antidepressant medication. It is therefore possible that the changes

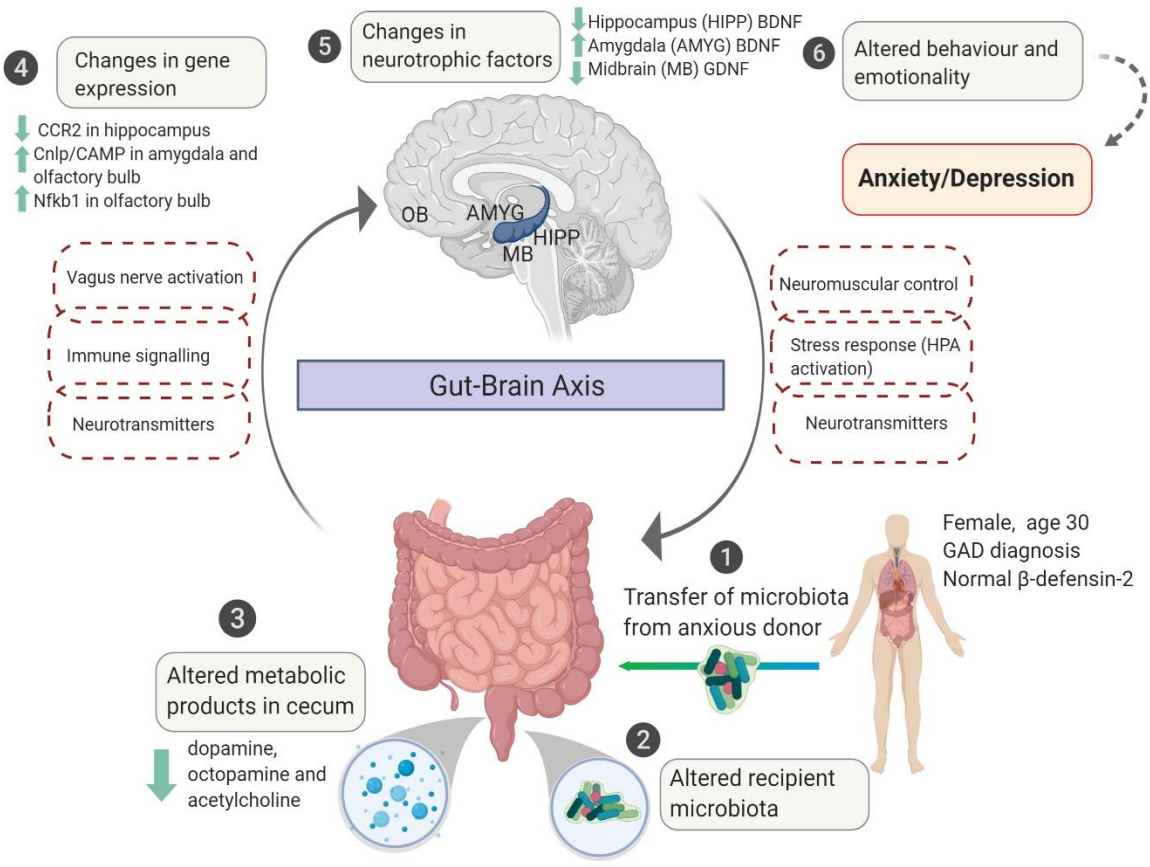
in behavioural and physiological phenotypes observed may be restricted to this specific set of donors used, and/or may be confounded by the effects of the antidepressants on gut microbiota. As such, future perspectives in donor selection for fecal transplants warrant further discussion and continued research on the adoption of behavioural and physiological phenotypes through microbiota transfer.

4.8. Conclusion

Microbiota from a GAD patient with low fecal octopamine and normal β-defensin-2 induced anxiety-like behaviour and altered emotionality scores in a murine host (Fig.23). Mice colonized with microbiota from a GAD patient have distinct bacterial composition and diversity as compared to mice colonized with microbiota from a healthy patient. The changes in behavioural phenotypes in recipient mice were accompanied by changes in cecum metabolites, brain neurotrophic factors, and gene expression in major brain structures. Further, the change in host behaviour is not accompanied by increased immune activation, as assessed by fecal β-defensin-3. Additionally, metabolome analysis of patient serum samples revealed six metabolites associated with energy metabolism to be elevated in GAD patients as compared to healthy controls. These findings suggest that gut microbiota may be able to interact with the central nervous system through systems independent of the immune system. It is hypothesized that this may be achieved through production of neuroactive metabolites such as trace amines and amine-based neurotransmitters. As such, continued investigation on the role of gut microbiota in CNS

disorders and psychiatric illness is warranted. Similarly, further research on bacterial metabolic products and their pathways downstream will be necessary to understand the precise mechanisms by which the intestinal microbiome is influencing the brain and behaviour.

Figure 23. Schematic Summary



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