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A Gastrointestinal-Specific Antibiotic as an Experimental Treatment for Anxio-Depressive Disorders

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A GASTROINTESTINAL-SPECIFIC ANTIBIOTIC AS AN EXPERIMENTAL TREATMENT FOR ANXIO-DEPRESSIVE DISORDERS

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ABSTRACT

Chronic stress is a predisposing factor for various disease states, including neuropsychiatric disorders, such as depression and anxiety [1]. Stress-related disorders have complicated multifactorial etiologies [2]. Advances in psychotherapeutic and psychotropic treatments have occurred, but anxiety and depressive disorders are still prevalent and remain a burden to our societies [3]. More than 30% of major depressive disorder patients fail to remission despite an FDA-approved medication [4]. Meanwhile, the contribution of microbiota-gut-brain axis signaling in both etiologies and treatment of stress-related disorders is increasingly being recognized [5]. More evidence has shown that the gut microbiota has the potential to alter the host's mental state via neural, endocrine, and immune pathways [6]. Administration of probiotics, fecal microbiota transplantation, and antibiotic supplementation are currently being evaluated as potential strategies to modulate the gut microbiome and assess how such modulation may affect the brain and behavioral function [7]. In this study, we investigate the effects of the supplementation of vancomycin, a non-absorbable antibiotic that doesn't cross the blood-brain barrier on

the contraction of detrimental neurobehavioral effects of chronic unpredictable stress (CUS). Our results indicate that chronic stress-induced anxio-depressive behaviors are attenuated in vancomycin-supplemented mice whereas vancomycin supplementation in non-stressed mice has no substantial effects on anxio-depressive behaviors. Analysis of gut microbiota reveals that the composition changes in gut microbiota in CUS-treated mice match with human patients suffering from mental illnesses. Supplementing with vancomycin reverses these changes. Similarly, we noticed the decreases in serum tryptophan and its related metabolites levels in CUS-treated mice, and vancomycin supplementation reverses the alternations caused by stress. Our quantitative immunoblotting confirms that CUS alters phosphorylation states of CDK5 and PKA signaling pathways in the ventral striatum, which have been linked to neuropsychiatric diseases [8]. Our results support the insights and may help formulate novel therapeutic strategies while advancing our understanding of the basis of stress-related mental illness.

Keywords: antibiotic, depression, stress, gut microbiota, tryptophan, CDK5, PKA

DEDICATION

I dedicate my thesis work to my family, my mentor, committee membranes, my program, and my friends. I am especially grateful to my loving parents, whose words of encouragement and push for tenacity are ever-present in my thoughts. I thank my mentor, Dr. James Bibb, who has supported me throughout the process. His sagacity, patience, and optimistic spirit have encouraged me to move forward in my future academic career. I also give my special thanks to my friends and lab colleagues for the generous gift of their time and thoughtful help. It is thanks to them that I have been able to develop confident technical expertise which I never thought that I might acquire. I will always remember their patience and kindness in allowing me to learn and contribute to their research.

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CHAPTER 1

INTRODUCTION

Stress, anxiety, and major depressive disorder

Stress is a phenomenon that can surround us, but is often too well-known to be understood at the same time [9]. In the early 20th century, Walter Cannon coined the term "homeostasis" and he demonstrated that the sympathoadrenal system was responsible for coordinating the "fight and flight" response to cope with external challenges [10]. Cannon showed that both emotional and physical distributions initiate the same responses in the organism and he proposed that if the homeostatic mechanisms failed, the organism would perish [11]. Lately, Hans Selye started to study the connection between stress and health and proposed three stages of stress coping: (a) general adaptation syndrome as an initial alarm reaction then followed by (b) "fight or flight" response as a stage of adaptation associated with resistance to the stressor. (c) Eventually, exhaustion and organismic death in this stage. These three stages were later proven to associate with the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis[12].

The brain is the organ that senses the effect of stressors and coordinates behavioral and neuroendocrine responses. While these adaptative responses may be effective for a short interval, the alternations can accumulate with wider and wider swings in homeostatic imbalance that have adverse effects over time [13]. More modern concepts view stress as any internal or external stimulus capable of altering physiological homeostasis and the coping responses to stressful situations, a crucial determinant of health state and diseases. Exposure to adverse conditions initiates the sequence of adaptive responses developed evolutionarily to maintain physiological homeostasis and favor an organism's survival. This process is referred to as a "stress response", involving mechanisms that allow the body to make physiological and metabolic adjustments required to adapt to homeostatic changes [14].

Psychological stresses may be divided into two classes, acute and chronic stress based on the duration of stress. Acute stress is encountered as part of our daily lives and covers a spectrum of severity and health implications. The relatively mild acute stressors that we encounter can sharpen mental acuity, raise alertness, and improve performance [15]. However, more traumatic acute stressors or catastrophic events can have a negative impact on mental health resulting in acute stress disorder [16].

Unlike acute stress, chronic stress can involve persistent environmental or psychological conditions. Chronic stress can be further which could be subdivided

into disconnected and persistent psychological stress [17]. Chronic stress can cause consistent immunological changes in the peripheral and central nervous system (CNS), thus causing disproportionate responses to chronic stress [18]. The most common psychiatric response to chronic stress is mood disorders such as anxiety and major depressive disorder (MMD) [19]. The root causes of depression are multifactorial and are not fully understood [20]. An MDD patient shows at least five of the following symptoms: abnormal depressed mood, abnormal loss of interest and pleasure, sleep disturbance, abnormal fatigue or loss of energy, appetite, disturbance in activity (agitation or slowing), abnormal self-reproach, poor concentration or indecisiveness, and morbid thoughts of suicide [21]. MDD not only limits patients' psychosocial function but decreases their quality of life [22]. According to the World Health Organization (WHO), the prevalence of MDD is increasing and will become a major cause of global disability in 2030 [23]. Approximately 10% of the entire U.S. population suffers from depression [24]. While the provenance and social costs of MDD are increasingly being recognized, the mechanistic and genetic causes of this spectrum of disorders remain largely undiscovered [25].

Although the etiology of depression remains unknown, it has been recently recognized as an outcome of harmful social environments [26] or an adaptation to social environments [27]. Recently, studies have focused on stress, as a biological

factor, a major causal factor of depression[28]. Likewise, brain functions, behaviors, and cognition can be affected by stress throughout the lifespan; stress can directly and indirectly, affect multiple brain regions, the endocrine system, and its related receptors expressed in the brain, Stress can also modulate gene expression, memory formation during neuronal development, cognitive functions, behaviors, and aging [29]. On the other hand, many environmental factors, such as personality, intrapersonal conflict, and the presence or absence of support, can regulate the effects of stress; these environmental and stressful factors can, in turn, disrupt the physiological homeostasis state. For example, excessive glucocorticoid release, hypothalamus-pituitary-adrenal axis dysregulation, and changes in the limbic and cortical brain areas. Therefore, the study of depression encompasses a great variety of research fields, including not only psychology and psychiatry but also broader features of biology [30].

Monoamine hypothesis of depression

The monoamine hypothesis of depression suggests a deficiency or imbalance in the monoamine neurotransmitters causes depression. More specifically, depletion of the indoleamine, serotonin (5-hydroxytryptamine, 5-HT) has been causally linked to depression [31]. It stems from a number of significant observations in the 1950s when lysergic acid diethylamide (LSD) and its action were widely researched [32]. LSD

blocks peripheral serotonin receptors and was found to attenuate depressive symptoms [33]. Serotonin was recognized as a major neurotransmitter in the brain and its dysregulation was suggested to contribute to mental disorders [34]. Another piece of evidence was found in reserpine, a drug for hypertension. Patients given reserpine exhibited the symptoms of depression. These patients also showed reduced brain levels of serotonin and increased levels of serotonin metabolites in urine increases [35, 36]. Iproniazid, a specific monoamine oxidase inhibitor-A(MAO-A) inhibitor that degrades serotine [37] and prevents serotonin from being degraded [38] was found to decrease the symptoms in depression patients [39]. These observations indirectly supported the antidepressant effect due to the increase in monoamine levels and also influenced the correlation of serotonin levels with psychiatric symptoms [40].

Subsequently, this discovery in reserpine and iproniazid led to the development of more antidepressants using the mechanism of iproniazid. For example, tricyclic antidepressants (TCAs) regulate the neuronal reuptake of norepinephrine and serotonin [41]. Subsequently, serotonin dysregulation was shown to correlate with the pathophysiology of depression [42] and the development of serotonin-specific re-uptake inhibitors (SSRIs) was based on the correlation between serotonin levels and psychiatric disorders [43]. SSRI quickly became a widely used prescribed antidepressant and then the monoaminergic theories of depression were formed [44].

Although the usage of antidepressants has largely increased in prevalence, anxiety, and depression remain a burden to our society [45]. SSRIs, the most common treatment for major depression, are used for moderate to severe depression patients [46]. Thirty to 50% of patients have residual symptoms of depression after receiving SSRI treatment [47]. Taking prescription antidepressants doesn't effectively prevent depression; studies show that only around 30% of patients successfully avoided relapse after taking it for an extended period of time [48]. Furthermore, the side effects of antidepressants might occur during medications. Sexual dysfunction is the most common side effect of SSRIs, and the side effects are consistent throughout the medication [49]. Nausea and diarrhea may also occur as side effects at the beginning of medications [50]. Similarly, CNS side effects can be observed, including anxiety, insomnia, sedation, and nightmare [51]. As a current alternative treatment, electroconvulsive therapy has higher efficacy compared to antidepressants, but it is still considered as the third-line therapy or the last resort for depression patients [52]. Similarly, low-dose or signal-dose ketamine can also be used as an alternative treatment for severe depression, but the clinical analysis shows ketamine has a limited and less durable effect on major depressive patients [53-55]. As a recent development, psilocybin therapy has shown promise as an effective alternative treatment strategy for depression [56]. In summary, current treatments for depression all have limitations

in their efficacy, underscoring the need to develop more effective treatments. Recently, the interaction between microbiota, intestine, and the brain has been recognized as an important aspect of mental health and for possibly providing a new avenue for treating disorders such as depression [57].

Microbiome-gut-brain axis

The specific interaction between the central nervous system (CNS) and the gastrointestinal (GI) tract has been studied for decades. Termed the “gut-brain axis”, it consists of bidirectional communication between these two organs [58]. The gut-brain axis was first described by St. Martin, who had a fistula in his gut; his record showed the rate of digestion was affected when he became angry, indicating the aspect of the gut-brain axis, host emotional state affected digestion [59]. Multiple studies have shown that the complicated communication between the gut and brain involves the enteric nervous system (ENS), sympathetic and parasympathetic autonomic nervous system (ANS), neuroimmune system, and neuroendocrine signaling pathway. This communication further influences the host’s mental state and behaviors [60] (Figure. 1). To influence the brain, visceral feedback can be sent from the intestines to the spinal cord and solitary tract then the inputs are carried to the higher areas of the brain. In turn, Vagus nerves and spinal autonomic nerves, and control viscera can be

regulated by the cingulate and insular cortex, amygdala, bed nucleus of the stria terminalis, and hypothalamus. These interactions between the viscera and the nervous system provide bi-directional crosstalk of the gut-brain axis [61] and hormones and their signaling molecules contribute to this bidirectional communication [62].

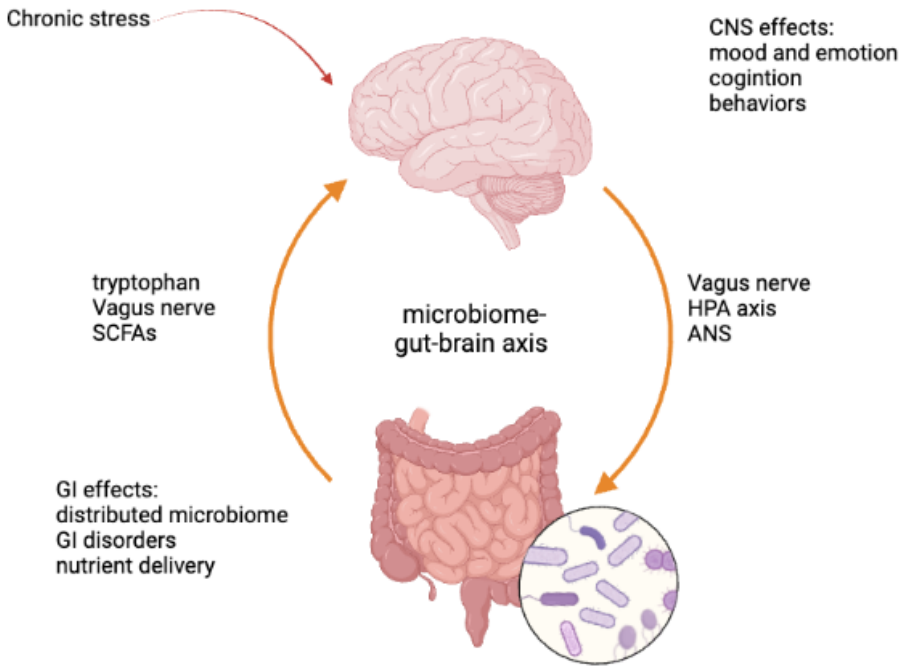

Figure 1. Chronic stress affects the microbiota-gut-brain axis

The research on the microbiome has greatly expanded in the past few years and is revealing its effects on our lives in various ways. The fields of microbiology and neuroscience have become more related and show that microbiota can exert notable influence on host behaviors by regulating the gut-brain axis [63, 64]. Gut microbiota has now been shown to be critical regulators of this pathway, potentially affecting

neuro-immuno-endocrine including afferent and efferent neuronal projection pathways, bi-directional neuroendocrine signaling, immune signaling, intestinal permeability, enteric sensory-motor reflexes, and enteroendocrine signaling [65, 66]. It has become more clear that the composition of gut microbiota is altered by various stressful stimuli [67]. The human gut is dominated by two phyla, *Bacteroidetes* and *Firmicutes*, which account for at least 70% of the gut microbiota [68], and phyla *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia* are in relatively low abundance. All of them contribute to regulating the host's health [69]. 16s rRNA analysis has shown that the composition of gut microbiota in patients with neurological disorders has the phyla *Firmicutes* and *Bacteroidetes* as a larger proportion of the microbiota, while the phyla *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia* accounted for smaller proportions [70]. Similarly, the abundance and diversity of gut microbiota are altered in depression patients. For example, the Genus *Faecalibacterium* and *Ruminococcus* are decreased [71]. A higher proportion of *Bacteroidales* and a lower abundance of *Lachnospiraceae* have been linked to depression [72]. The alternation of gut microbiota composition in depression animal models by stress has also been demonstrated [73]. These investigations suggested that depression is linked to the distribution of certain types of gut microbiota, providing an alternative trajectory of treating and preventing depression

by manipulating gut microbiota [74].

To manipulate gut microbiota, probiotics, fecal microbiota transfer, and antibiotic administration can be used [75]. For example, clinical and meta-analysis studies indicated that probiotics did not reduce depression or anxiety. Despite the high interest in manipulating *Lactobacillus*, this approach did not show an effect on depression. Probiotics are not recommended to replace antidepressants due to their insignificant effects on mental disorders [76-78].

In addition, fecal microbiota transfer (FMT) from healthy donors has been recently reported to have beneficial effects on psychiatric disorders. Autism spectrum disorder patients are known to exhibit a dysbiosis of gut microbiota and GI dysfunction. Abnormal behaviors are improved in post-FMT patients compared to patients without FMT [79]. Two weeks after FMT therapy, depressive patients become less sleepy and more talkative, and also show improvement in their appetite [80]. On the other hand, the most common side effects after FMT are GI disorders [81]. Fecal donor material may be contaminated due to inappropriate donor screening and incorrect analysis and cause serious adverse effects on recipients [82, 83]. Interestingly, the FMT from donors suffering from chronic diseases can possibly induce the same diseases in recipients [84-86].

In contrast, antibiotics can selectively target specific types of bacteria and have

selective toxicity, having minimal or no toxicity to humans. For example, minocycline has been shown to have reduction effects on depressive behaviors in mice and it provides a potential research direction for depression [87]. Moreover, the absorption from the GI tract also needs to be considered to evaluate direct effects on gut microbiota without the neurologic effects of antibiotics [88]. Minocycline is an absorbable antibiotic, which can cross the blood-brain barrier and show neurotoxic consequences under certain circumnutates [89]. Whereas non-absorbable antibiotics such as vancomycin, do not cross the blood-brain barrier and thus its primary effects are localized to the GI tract without directly affecting the CNS [90]. Vancomycin is currently clinically used to treat serious gram-positive bacterial GI infections [91]. It decreases gram-positive bacteria abundance without modifying the number of gram-negative microbiomes; its effect is limited to the GI tract when it's taken orally because it's non-absorbable. [92]. In this research project, vancomycin was used to study whether depressive behaviors can be ameliorated in stress animal models by targeting and modifying the abundance of gram-positive bacteria which have been linked to neurological or psychiatric disorders [70-72].

The linkage between the gut microbiota and brain

Accumulating evidence supports the concept that gut microbiota critically

modulates the gut-brain axis by indirectly or directly regulating the levels of its metabolites [90, 93, 94]. Certain metabolites produced by gut microbiota have been proven to regulate the gut-brain axis. For example, short-chain fatty acids (SCFAs), synthesized by gut microbiota, have been shown to promote memory and synaptic plasticity [95, 96]. Butyrate, an example of a SCFAs, can influence the serotonin release from the intestinal enterochromaffin cells [97]. Likewise, propionate, produced by gut bacteria can protect the BBB from oxidative stress [98] and SCFAs can also modulate the production and recruitment of immune cells to further affect neuro-inflammation [99]. These observations indicate SCFAs as metabolites synthesized by gut microbiota have a neuroprotective effect to regulate CNS functions. In addition, other metabolites produced by gut microbiota can be essential regulatory molecules for the CNS. For example, bacterial tryptophan metabolites modulate CNS functions[100] via the production of serotonin by bacteria Enterococcus and Pseudomonas [101]. It shows that the gut microbiota is critical for the bioavailability of biosynthesis of neurotransmitters [102, 103]. The gut microbiome can metabolize tryptophan for its related metabolites, thereby limiting the host availability of tryptophan [104]. For example, Pseudomonas can synthesize serotonin from tryptophan for its inter-cellular signaling [101]. The distribution of tryptophan levels affects serotonergic neurotransmission and further influences enteric

and central nervous systems [105] and the low serotonin level has been strongly linked to depression or other neurological disorders [106].

Studies show that the composition of gut microbiota affects the gut-brain axis by regulating tryptophan metabolism [107-109]. Tryptophan is an essential amino acid, meaning its only available source is from the diet. Free tryptophan that circulates in the blood contributes to host protein synthesis and it serves as a sole precursor for serotonin biosynthesis and melatonin, which are involved in emotional control, food intake, and sleep [110, 111]. Also, its products and related metabolites, such as serotonin, kynurenines, tryptamine, and indolic compounds, have notable effects on the interaction between the microbiome-gut-brain [112, 113]. However, central serotonin constitutes a small proportion of total serotonin; over 90% of serotonin is located in the GI tract and produced by enterochromaffin cells [114]. These observations show that the physiological effect of tryptophan is regulated by gut microtia and it further influences CNS functions. This research used vancomycin to target gut microbiota to study whether tryptophan and its metabolite levels can be changed back to homeostasis in stress animal models.

Neuronal signaling transduction

Various evidence shows that mood disorders are associated with disruptions of the

brain's reward circuitry [115]. The reward circuitry consists of neurotransmitter signaling pathways between the ventral tegmental area (VTA), nucleus accumbens (NAc), prefrontal cortex (PFC), central (CeA) and basolateral (BLA) amygdala, and the hippocampus. [116]. All of these brain regions are known as brain reward regions and the complicated interaction in brain reward regions responds to aversive stimuli too[117]. Apart from other brain reward regions, the NAc is a major component of the ventral striatum and is a critical structure in the mediation of emotion and motivation and reward modulation [118]. Dopamine (DA), a neuromodulator in the NAc, is essential for depression-related behaviors [119]. The NAc receives great innervation from the VTA, which supplies the NAc with DA, Gamma-Aminobutyric Acid (GABA), and brain-derived neurotrophic factor (BDNF). Animal studies have shown stressful stimuli induce despair and altered response to rewards, which are correlated with symptoms of depressive patients. [120] and also chronic stress reduced reward sensitivity, inducing loss of pleasure or lack of motivation, which is one of the features of depression [121]. Within the reward circuitry, chronic mild stress alters the NAc dopamine receptor sensitivity [122]. Restraint stress increases the NAc D1-like receptors density [123]. Furthermore, dopamine receptor signaling activates cAMP/PKA signaling, which plays a vital role in stress responses [124].

The cAMP/PKA signaling pathway is an important integrator of different signaling

from neuromodulators, regulating CNS functions [125]. Dopamine, a neuromodulator, stimulates G protein-coupled receptors and triggers cAMP synthesis, which can further activate PKA (cAMP-dependent protein kinase) [125]. Cyclin-dependent kinase 5 (CDK5) is associated with the regulation of the cAMP/PKA signaling pathway [126] by directly phosphorylating downstream substrates, including PDE4, dopamine- and cAMP-regulated phosphoprotein, DARPP-32, protein phosphatase 1, and tyrosine hydroxylase [8]. Dysfunction of CDK5 has been linked to neurological and neuropsychiatric disorders [127]. Increases in CDK5 and p35, its activator, levels have been found in the responses to stress stimulations [128, 129]. Moreover, CDK5 provides negative feedback on cAMP/PKA signaling by phosphorylating and then activating PDE4; deletion of CDK5 increases cAMP levels and PKA activity, thus influencing behavioral responses induced by acute and chronic stress [8]. In our studies, we investigated the effect of stress on CDK5 and cAMP/PKA signaling pathways in the absence of the vancomycin [8].

Stress-induced alternations in homeostatic

Preclinical and clinical studies have shown that chronic stress plays a significant role in the onset and progress of psychiatric conditions, including anxiety and MMD [130]. Currently available antidepressants and antipsychotics have limited efficacy

and thus the development of novel antidepressants/anxiolytics is needed [131]. To find an alternative approach, various research has proven that bidirectional interaction between the brain and gut plays an important role in neuropsychiatric and neurological disorders [132]. Exposure to stress can alter this interaction and gut microbiota composition, contributing to CNS disorders [133]. However, the mechanisms by which microbiome and stress modulate vulnerability to neuropsychiatric disorders remain poorly understood. In this study, we investigated the link between stress-induced anxio-depressive behavior, gut microbiota-derived metabolite, and dysregulation of ventral striatal signaling. This research would provide mechanistic insight into the role of microbiota and microbiota-derived metabolites in the pathophysiology of neuropsychiatric disorders and aid in formulating novel therapeutic strategies while advancing our understanding of the basis of stress-related-mental illness

Hypothesis

In this thesis, we hypothesize that oral vancomycin supplementation will ameliorate chronic stress-induced neurobehavioral maladaptation via regulating gut microbiota, metabolites, and neuronal signaling transduction. We proposed to examine the link between stress-induced anxio-depressive behavior, gut microbiota-

derived metabolite, and dysregulation of ventral striatal signaling to better understand stress-related neuropsychiatric alterations. In addition, we determined the effect of vancomycin on chronic stress-induced alternations in behaviors, gut microbiota, serum metabolites, and neuronal signaling pathways. Overall, this study project provided mechanistic insights and helped formulate novel therapeutic strategies.

CHAPTER 2

MATERIALS&METHODS

Animals

6-week-old C57/BL6 male mice were obtained from Jackson Laboratories. Mice were housed 6 animals per cage and maintained on a standard 12-hour light/ 12-hour dark cycle with food and water *ad libitum*. Mice were acclimated for 1 week then they were assigned either stress exposure or control in the absence or presence of vancomycin supplementation in drinking water.

Chronic unpredictable stress (CUS) mouse model

Male C57/BL6 mice were divided into four groups as follows: Group I-control, Group II-vancomycin, Group III-CUS, Group IV-CUS+ vancomycin. CUS is treated in groups III and V and entailed exposure to two stressors for 14 days. Stressors included new and/or adverse environments including a cage without bedding, a cage with a thin layer of water, damp bedding, the change of light cycle, tilted cages, restraint stress for 1 hour, continuous cage shacking for 1 hour, swimming for 5 min,

hot air stream for 5 mins, or tail suspension for 6 min [134] (Figur .2).

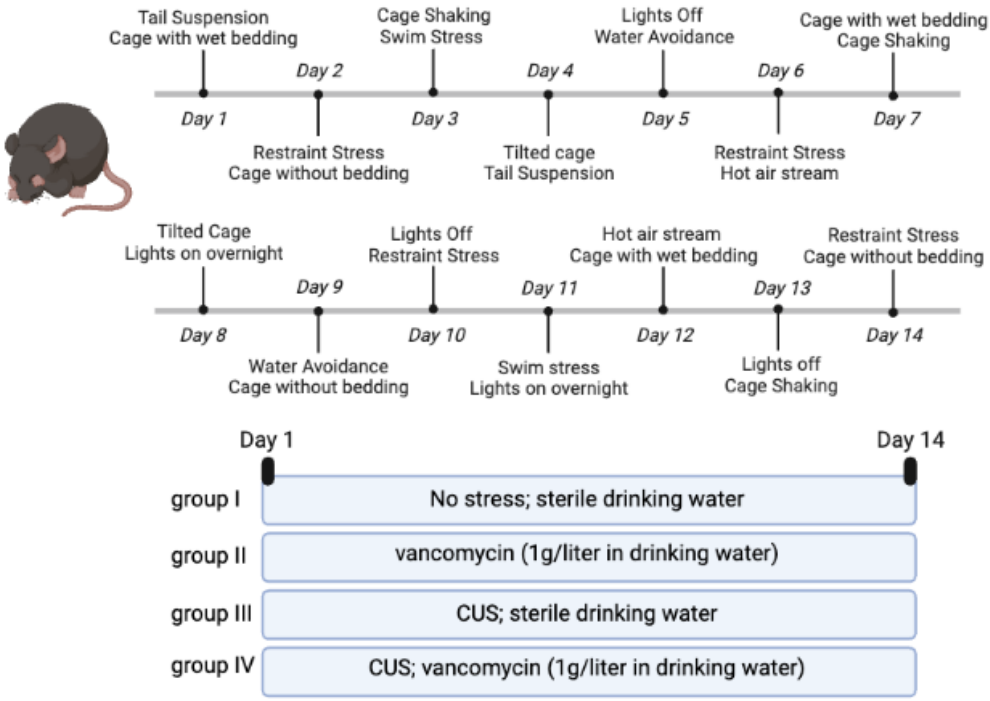

Figure 2. Experimental design for chronic unpredictable stress mouse model

Vancomycin administration

Mice administered CUS were supplemented with vancomycin (1 g/l) (group IV) in drinking water [135] for 14 days and group II mice were supplemented with only vancomycin. On the other hand, group III mice were administered CUS and drinking water, and group I mice were not administered either vancomycin or CUS as a control group. The vancomycin effects on behavior and neuronal signaling were assessed.

Neurobehavioral assessments

The effects of the treatment conditions described above on neurobehavioral

function were assessed using standard protocols by the elevated plus maze (EPM) and open field test (OFT). For EPM, 5 min sessions were analyzed by Ethovision video-tracking. Time in open and closed arms, number of entries, and time in the center were analyzed. Anxiety-like behavior is gauged as a function of unwillingness to venture and spend time in open areas. The OFT also provided locomotor activity and exploratory behavior information. Video-tracking measured locomotor activity within OF boxes. Animals were examined for 5 min in OF chamber and the number of entries and time spent in the central zone were assessed as an indication of anxiety-like behavior [136].

Gut microbiota analysis

Microbiota analysis of fecal samples were carried out at the UAB Microbiome Core facility as described previously [136]. DNA was extracted from caecal content using a commercially available kit (Zymo Research Irvine, CA, United States) following manufacturers specifications. Unique barcoded primers [136] were utilized to amplify DNA coding for the V4 region of the 16S rRNA gene using Polymerase Chain Reaction (PCR) and isolated PCR products were purified by QIAquick Gel Extraction Kit (Qiagen, Germantown, MD). Utilizing Illumina MiSeq NextGen sequencing, PCR products were sequenced corresponding to 250 bp from the V4

region of the 16S rRNA gene. Raw data as FASTQ files will be de-multiplexed, assessed for quality control (FastQ quality control), and used for library construction. Quantitative Insight into Microbial Ecology (QIIME) was used for downstream analysis. Samples were grouped using Uclust and those harboring 97% similarity were segregated into Operational taxonomic units (OTU) and subsequently assigned to different phylogenetic levels.

Serum tryptophan metabolites analysis

Following sacrifice, the serum was separated from blood samples through centrifugation using BD microtainer tubes (Becton Dickinson, USA). For tryptophan analysis, 50 µl mouse serum was combined with 25 ng/ml methanolic tryptophan-d5 internal standard [137]. The serum was expelled into 500 µl of acetonitrile 1.0% formic acid contained within through Phree cartridges (Phenomenex, Torrance, CA). Following a 5 min room temperature incubation, the mixture will be drawn through the sorbent into a borosilicate collection tube. Serum samples are subsequently dried (N2 gas) and reconstituted in 100 µl 0.1% formic acid [137]. Samples were compared against authentic analytical standards prepared in neat solutions. LC-MS was conducted as described [138]. HPLC gradient separation occurred on an Atlantis T3 3 µm 100 x 2.1 mm column (Waters, Milford, MA) at 40˚C. Column waste was diverted

from the mass spectrometer for the first minute of the gradient. MultiQuant 3.0.3 were used for data analysis [137]. Standard curves ranged from 1 – 1,000 ng/ml over 7 points.

Brain Microdissection

Following behavioral analysis, mice were decapitated, brains were rapidly dissected, coronal slices made, NAc punches taken, and snaps frozen.

Quantitative immunoblotting

Quantitative immunoblotting in ventral striatal lysates was conducted as reported [124] for analysis of the changes in the PKA and CDK5 signaling Immunoblots analysis of phosphorylation state-specific antibodies will be normalized to total protein signals from blots. Phosphorylation state-specific was used to analyze and normalized to total protein signals.

Statistical analysis

Data were presented as mean ± Standard error of mean (SEM). All statistical analysis was conducted using Prism 8.0 software (GraphPad Software, San Diego CA). In cases of drawing multiple comparisons, ANOVA analysis with the

Bonferroni *post hoc* test was used. A p-value <0.05 was used to determine statistical significance.

CHAPTER 3

RESULTS

CUS-induced anxio-depressive behaviors are prevented by vancomycin

Behavioral assessments were carried out after two weeks of the CUS regimen to explore how CUS and the administration of vancomycin altered brain functions, following the experimental design shown in Figure 2. Anxiety-liked behaviors were evaluated by EPM and OFT. The EPM is based on the conflict between the natural tendencies of mice to avoid open and bright areas and explore novel environments [139]. CUS mice made fewer entries and spent less time in the open arms compared to control mice (Figure 3A, B). There were no changes in the total distance traveled between CUS and control mice (Figure 3C). It showed that the mice that received CUS shows anxiety-like behaviors. The anxiety-liked effects of the CUS were further assessed by OFT. This test is based on the aversion of mice to novel, bright, open areas [140]. Similar to the effects observed on EPM, mice treated with CUS displayed a significant decrease in the duration of exploration time in the inner zone compared with the control group during the 5 min exploration period (figure 4A). No significant

difference in the total distance traveled between each group (Figure 4B), indicating the stress was not associated with local motor deficits.

Interestingly, vancomycin reversed anxiety-like behaviors caused by CUS. CUS+ vancomycin mice spent more time spent and made more entries in open arms of the elevated plus maze as compared to the CUS group (Figure 3 A, B). It displayed that vancomycin supplementation in stress mice increased the willingness to explore. Meanwhile, the locomotor activities of mice were not affected, when the mice received the vancomycin alone (Figure 3C). This indicated that vancomycin supplementation in non-stress mice had no substantial effects on anxio-depressive behaviors. Likewise, the administration of vancomycin in CUS-treated mice displayed an increase in time spent in the inner zone in OFT (Figure 4A) whereas the locomotor activity was not affected by vancomycin (Figure 5B). These data from EPM and OPF indicated that stress-induced neurobehavioral alterations were attenuated in mice that were supplemented with vancomycin whereas vancomycin supplementation in non-stressed animals had no substantial effects on anxio-depressive behaviors.

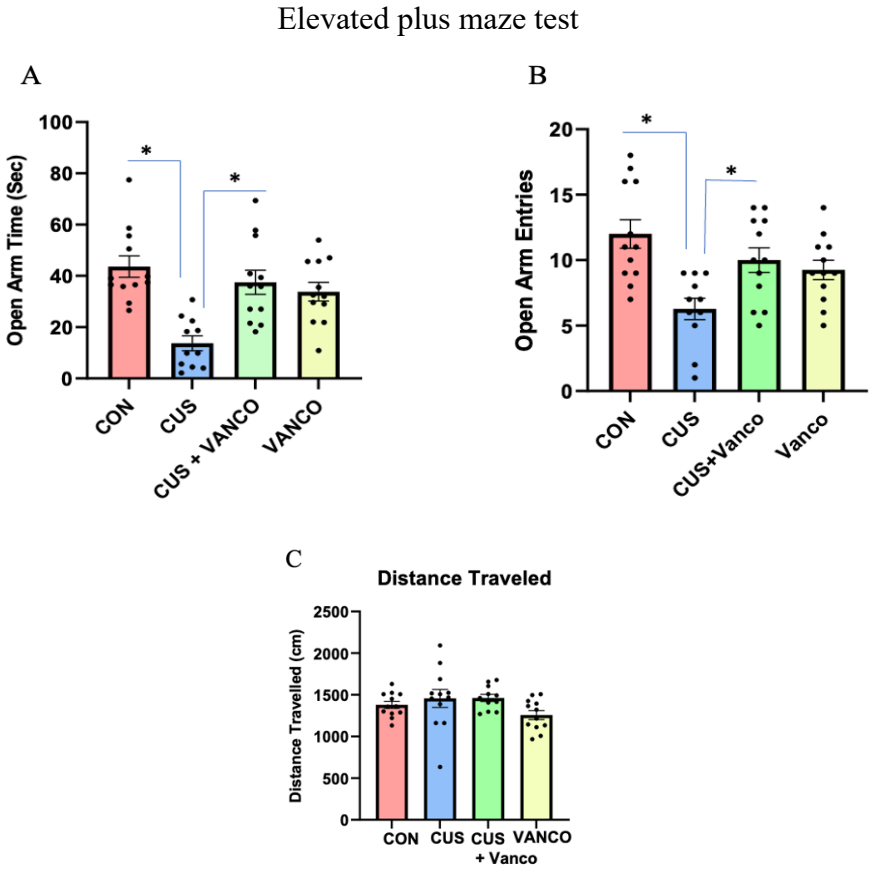

Figure 3. Chronic unpredictable stress -induced anxiety-like behavior in elevated plus maze is reversed by vancomycin. The effects of chronic unpredictable stress and vancomycin on (A) time spent in the open arm (B) entries in open arm (C) distance traveled for 5 min exploration in the elevated plus maze (N=12 *p<0.05 one way-ANOVA follow by post Tukey's test)

Open field test

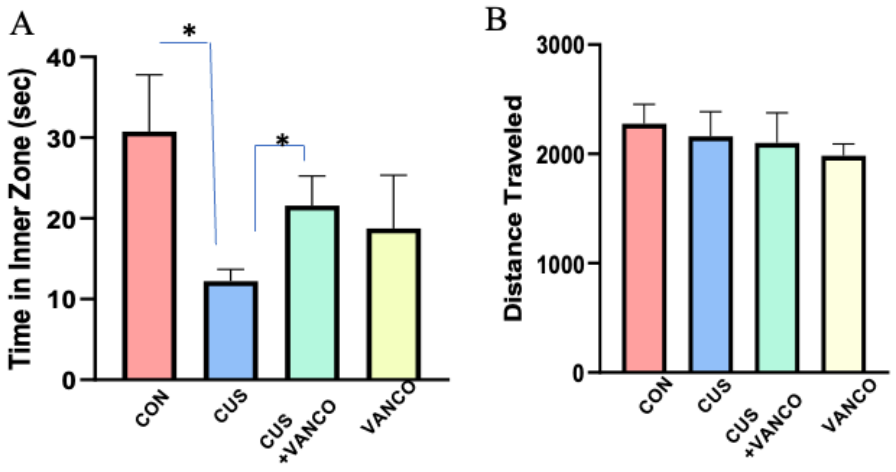

Figure 4. Chronic unpredictable stress -induced anxiety-like behavior in open field test is reversed by vancomycin. The effects of chronic unpredictable stress and vancomycin on (A) time in inner zone. (B) distance travelled for 5 min in the Open filed test. (n=6 per group; *p<0.05 t-test)

CUS alters gut microbiota composition and the effects were attenuated by vancomycin

Alternation in gut microbiota is involved in neurological and psychiatric disorders. To better understand the relationship between CUS-induced behavioral phenotype changes and the gut microbiome, fecal samples were collected at the end of the stress regimen and processed for microbiota analysis. Analysis of the relative abundance of the bacterial populations at the phylum level showed higher abundance in *Firmicutes* (Figure 5A) and decreased abundance in *Verrucomicrobia* (Figure 5B) in CUS mice. At the level of order, the relative abundance of *Clostridales* was increased by CUS (Figure 5C). These alterations in the phylum Firmicutes and Verrucomicrobia and

order Clostridales are associated with MDD, posttraumatic stress disorder, and schizophrenia [141-143]. Likewise, CUS induced a variety of bacterial genera changes, decreases in *Akkermansia* (Figure 5D) and *Parasutterella* (Figure 5E), and increases in *Staphylococcus* (Figure 5F) and *Lactobacillus* (Figure 5G) in CUS mice and changes can be associated with neurological or psychiatric illness [144-147]. Each of the changes could be reversed by supplementation of vancomycin, which rescued the mice's alternative response to CUS (Figure 5), which rescued the mice's alternative behavioral response to CUS. These data demonstrated that exposure to CUS led to an alteration in gut microbiota in a similar way to what has been reported in patients with mood disorders whereas vancomycin supplementation attenuated this effect.

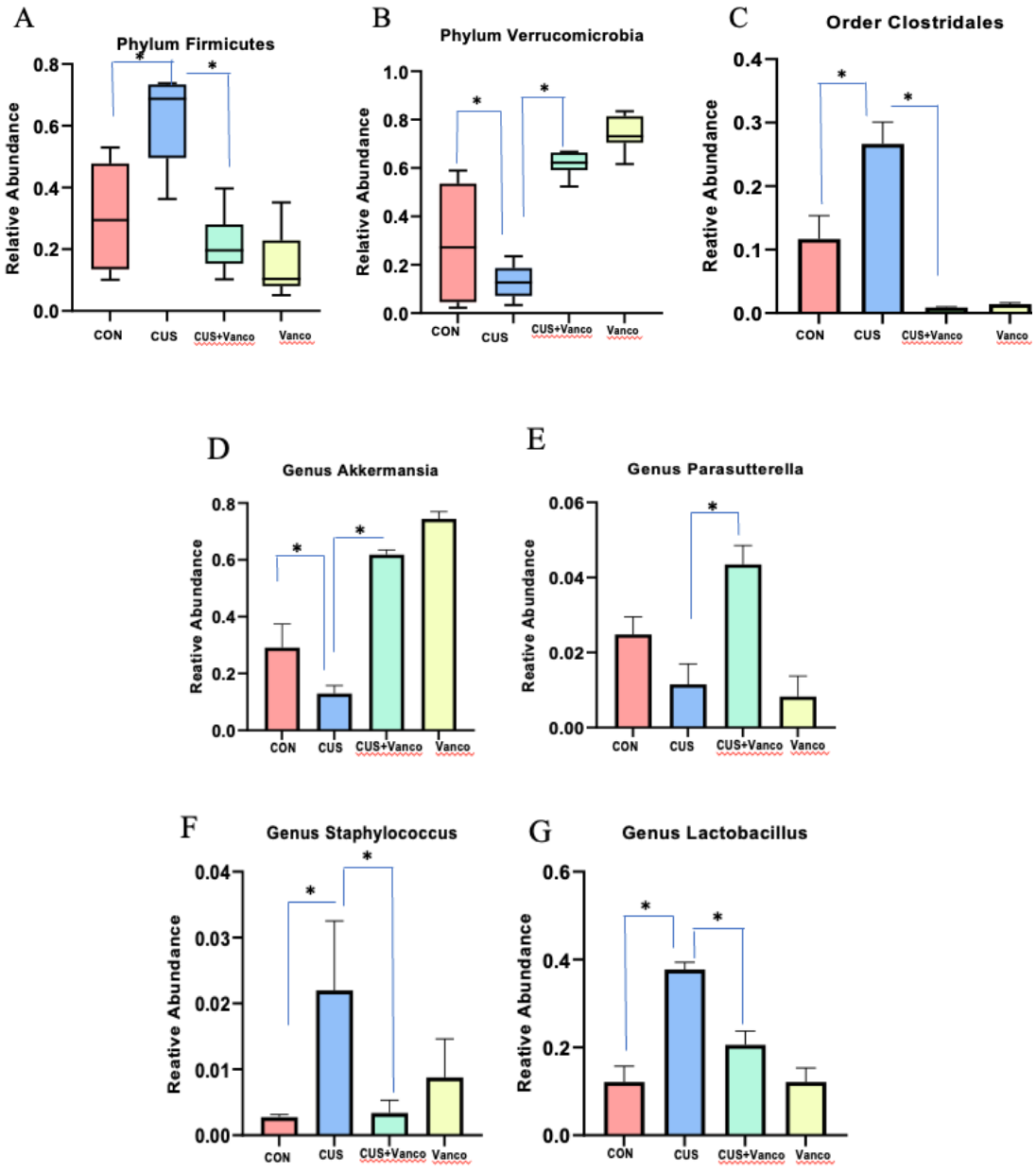

Figure 5. Chronic unpredictable stress altered gut microbiota composition and vancomycin reverses the alterations. The chronic unpredictable stress and vancomycin effects on (A) phylum *Firmicutes* (B) phylum *Verrucomicrobia* (C) order *Clostriadales* (D) genus *AKKermansia* (E) genus *Parasutterella* (F) genus *Staphylococcus* (G) genus *Lactobacillus* (N=6-8 pre group *p<0.05 one way - ANOVA follow by post Tukey's test)

Vancomycin modulates stress-induced alterations in serum tryptophan levels

Peripheral and central tryptophan levels and its metabolites have been considered as the factors in depression, schizophrenia, and other psychiatric disorders [148]. Tryptophan is the sole precursor of serotonin. Quantitative assessment of its metabolites can help to understand the pathophysiological mechanisms of psychiatric disorders. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used to assess the effect of CUS and consumption on serum tryptophan metabolite levels. CUS significantly lowered the tryptophan serum level (Figure 6A) and as well as tryptophan metabolites were significantly reduced in CUS-treated mice including kynurenine (Figure 6B) and picolinic acid (Figure 6F). Administration with vancomycin reversed the reductions (Figure 6A, B, F). In contrast, the serum levels of indole-3-acetate, indole-3-lactate, quinolinic acid, and tryptamine were not significantly different between the CUS-treated and control mice (Figure 6 C, D, E, G) but indole-3-acetate and indole-3-lactate levels were increased in CUS + vancomycin group mice (Figure 6 C, D). These finding demonstrates that chronic stress attenuates the serum levels of neuroactive amino acid, tryptophan, and several of its metabolites, and vancomycin reversed the reductions, which is associated with the effects on the gut microbiota.

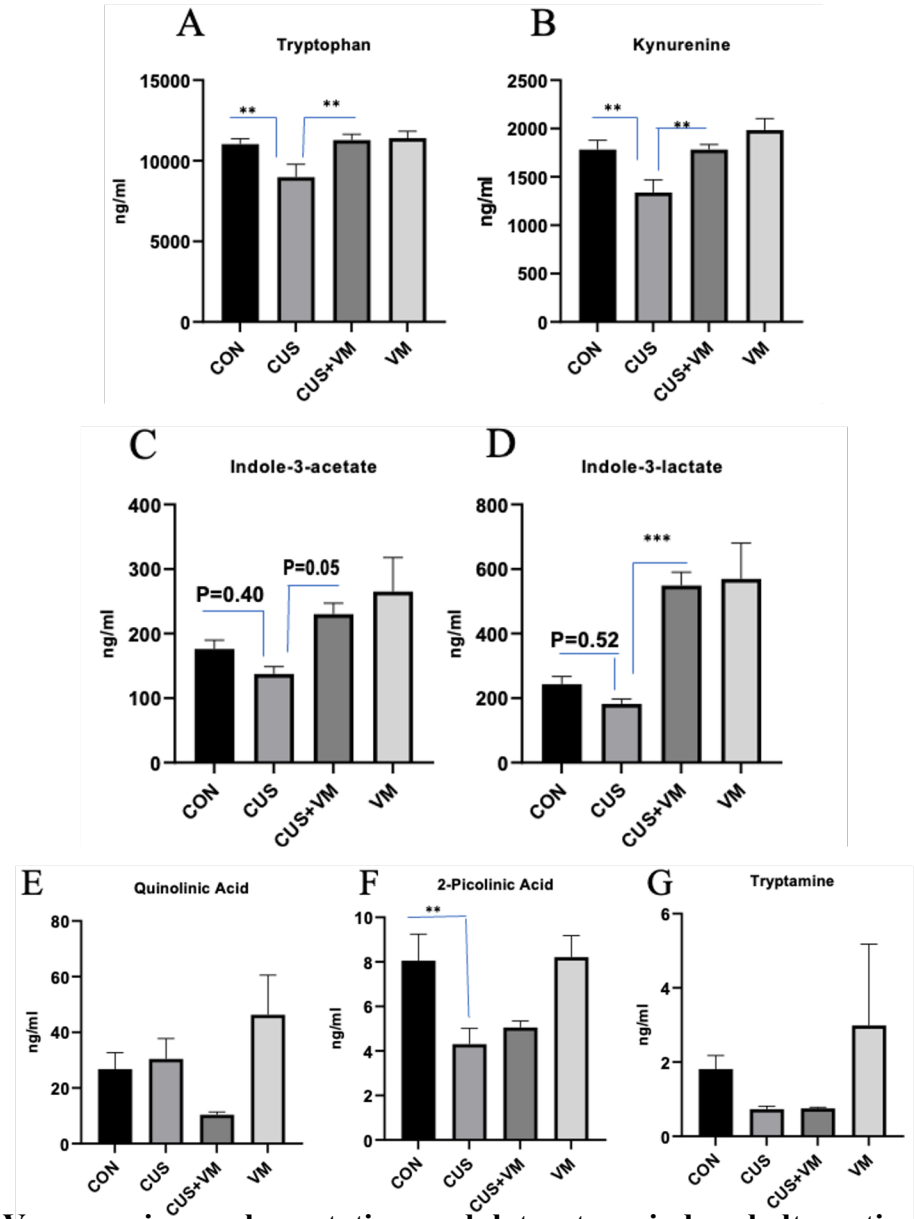

Figure 6. Vancomycin supplementation modulates stress-induced alternations in serum tryptophan levels. The chronic unpredictable stress and vancomycin effects on (A) tryptophan (B) kynurenine (C) indole-3-acetate (D) indole-3-lactate (E) quinolinic acid (F) 2-picolinic acid (G) tryptamine (n=6-8 per group **p<0.01, ***p<0.001 one way -ANOVA follow by post Tukey's test)

CUS alters ventral striatal neuronal signaling and stress-induced downregulation of PKA signaling is reversed by vancomycin

Structural and functional changes in the NAc have been associated with anxiety,

depression, and anhedonia [149]. NAc function is mediated by cellular processes, including neurotransmission input, signaling transductions, and gene expression. ΔFosB, a transcriptional factor, intervenes in reward and aversive response behaviors in NAc [150]. The increase of p35, a cofactor of CDK5, has been shown to correlate with depressive-like behaviors and possibly reverse the antidepressant effect [151]. The distribution of beta-adducin, which regulates actin filaments and actin-spectrin complex, in mice's brains has been associated with behavioral and motor coordination deficits [152]. Studies from our lab suggested a new CDK5 phosphorylation site at Ser 606 ADD2 might be affected by gut inflammation, which further contributes to mood disorder [153]. Anxiety disorders are associated with abnormalities in the neural processing of stress stimuli, regulated by the PKA signaling pathway [154]. Post-mortem studies show the disruptions in PKA in the brain of suicide subjects are modulated by stress[155]. One of the receptors regulated by PKA is AMPA receptors, which mediate excitatory neurotransmission, and phosphorylation of its subunit, GluR1, at Ser 845 by PKA is correlated with synaptic cell surface availability and associated with psychiatric disorders [156].

To determine if the CUS-induced behavioral and metabolic changes in the absence or presence of vancomycin administration affected each signaling pathway, NAc lysates were subjected to quantitative immunoblotting. CUS reduced the

phosphorylation state of the GluR1 subunit of AMPA receptors at Ser845, showing the reduction of PKA activity (Figure 7A). It indicated the reduction of PKA activity and possible alternations in ventral striatal medium spiny neuron excitability. The CUS-induced reduction of the phosphorylation state of GluR1 was reversed by vancomycin (Figure 7A). Moreover, the levels of the transcription factor ΔFosB, which is upregulated during chronic stress [157], were significantly increased in NAc lysates from CUS mice (Figure 8A) that were consistent with other research. Chronic stress increased the phosphorylation of adducin-2 (ADD2) (Figure 8B). p35, a cofactor of CDK5, is affected by CUS but not significantly increased (Figure 8C).

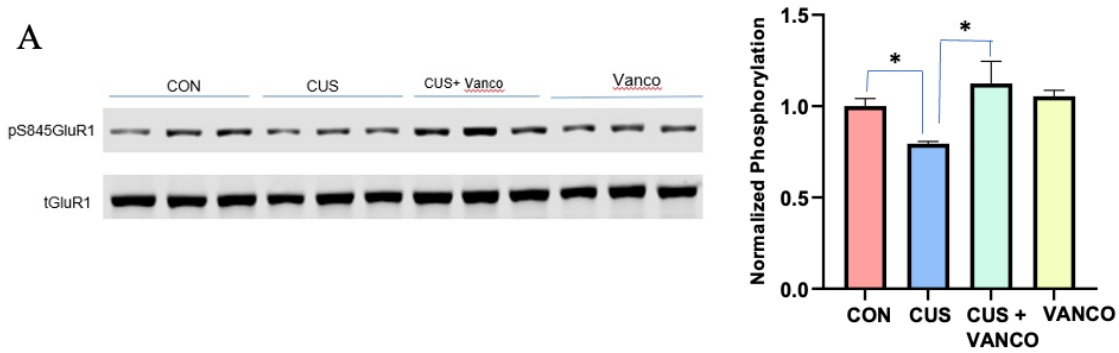

Figure 7. Chronic unpredictable stress-induced downregulation of protein kinase A signaling in Nucleus accumbens is reversed by vancomycin supplementation. The effect of chronic unpredictable stress and vancomycin on (A) pSer845 GluR1 protein levels in ventral striatal lysates of mice following 2 weeks of chronic unpredictable stress and vancomycin. (N=4 *p<0.05 one way -ANOVA follow by post Tukey's test)

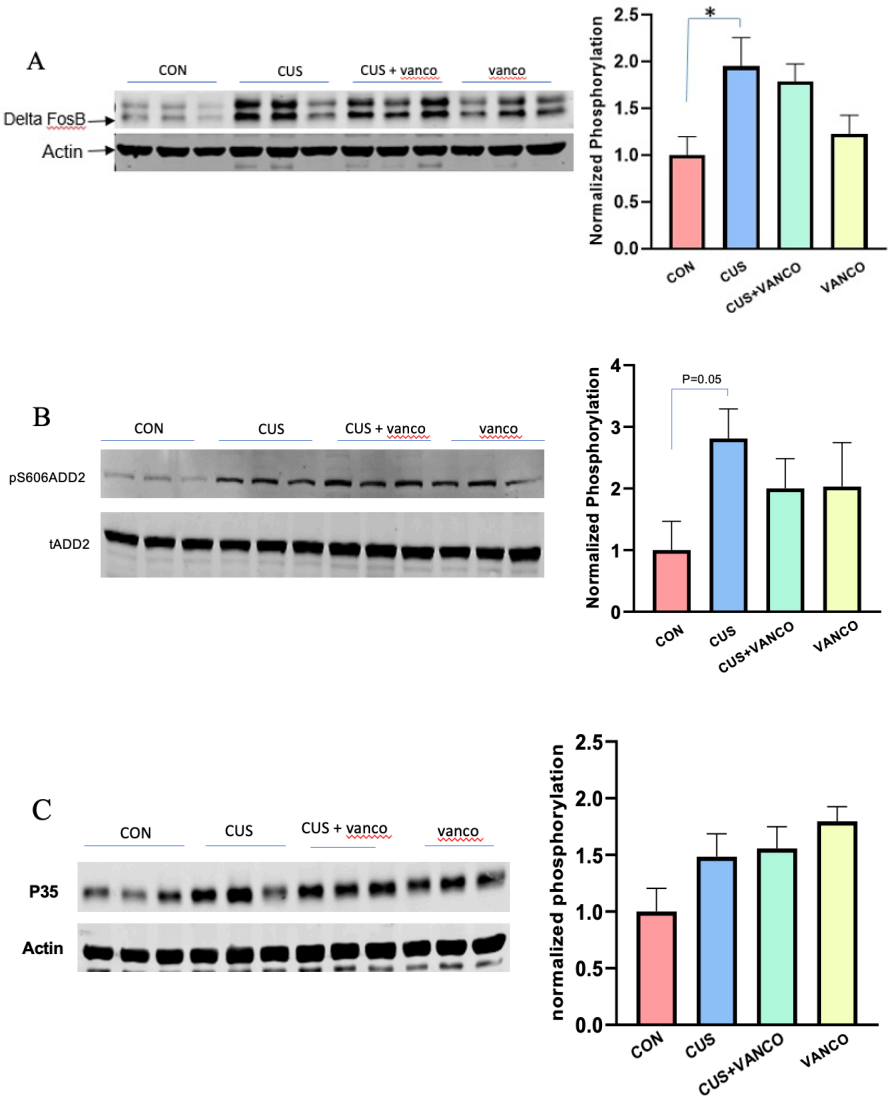

Figure 8. Chronic unpredictable stress alters cyclin-dependent kinase 5 signaling in ventral striatal. The effects of chronic unpredictable stress and vancomycin on (A) ΔFosB (N=4-6 per group; *p<0.05 t-test) (B) phosphor-Ser 606 ADD2 (N=4-6 pre group one way -ANOVA follow by post Tukey's test) (C) p35

CHAPTER 4

DISCUSSION

Stress is an adaptation that is essential for homeostasis, performance, and survival. The stress responses can be induced by physical, physiological, or psychological stimuli and triggered whenever an organism meets with external or internal unpleasant, adverse, or, threatening challenges [158]. Stress is also a leading cause of mental health and neuropsychiatric disorders, affecting not only the life quality of patients but also serving as a major risk factor for disease severity [159]. Chronic stress is increasingly recognized to affect brain functions and neuropsychiatric and neurological disorders progression and also peripheral system [160]. It is associated with GI disorder and further affects the gut microbiota profile [161]. The underlying mechanistic links between stress and the microbiome and its role in the pathogenesis of neuropsychiatric disorders are not well understood. Here we showed that exposure to chronic stress potentiates anxio-depressive behavior, alters gut microbiota profile, dysregulates serum tryptophan metabolism, and alters the phosphorylation state of key neuronal signaling pathways in the ventral striatum that regulates mood and

contributes to anxiety. Administration of the gut-specific antibiotic, vancomycin, attenuated these alternation effects in behaviors, gut microbiota composition, serum tryptophan metabolites profiles, and neural signaling transductions.

Other studies assessing the effects of antibiotic consumption in anxiety animal models have used minocycline in drinking water or injections [162]. However, the GI tract can absorb minocycline and cross the BBB to directly affect neurons and glia cells [163]. The actions of the minocycline on the brain are complicated and haven't been fully understood yet. Studies showed it exerts neuroprotective effects but also has neurotoxicity under certain circumstances [89]. Accordingly, we used vancomycin against gram-positive gut microbiota, which has been linked to neuropsychiatric disorder [70-72]. The advantage of the usage of vancomycin is its actions were limited to the GI tract because it can not be absorbed by the GI tract. Our finding showed that the mice exposed to chronic stress exhibit anxiety-like behaviors in elevated plus maze and open field test, two widely validated tests to assess anxiety in rodents. On the other hand, mice that were given vancomycin did not retain enhanced anxiety even though the mice were exposed to chronic stress. These findings suggested that vancomycin attenuates stress-induced anxiety-like behavior.

An imbalance of intestinal microbiota contributes to the development of psychiatric disorders and gut microbiota can be modified by diet, antibiotics, and a

variety of environmental factors [164]. Our findings showed that the CUS mice had a decreased abundance of bacteria belonging to phylum *Firmicutes*. Studies have reported that MDD patients have suppressed the growth of phylum *Firmicute* compared to the healthy group. [141]. Bacteria belonging to the genus *Akkermansia* has been associated with anxiety and depressive phenotypes in mice [144] and is consistent with our finding of the decreased abundance of *Akkermansia* as well as the anxiety-like behaviors in CUS mice. These alterations in gut microbiota composition correlate with the production of intestinal metabolites, such as phenylalanine, tryptophan, and tyrosine which could affect gut-brain axis signaling [165]. In contrast, vancomycin supplementation showed the reversal effects on the changes in gut microbiota, caused by chronic stress. The observations showed the effects of chronic stress are consistent with the relationship between the gastrointestinal microbiome and neuropsychiatric and neurological diseases; supplementing with vancomycin can reverse the changes in gut microbiota composition.

Serotonergic transmission is important for normal neurological functions and a decrease in serotonin levels can induce pathophysiological abnormalities, which are expressed in dysfunctional behavioral outputs[166]. Serotonin is synthesized from L-tryptophan, an essential amino acid. Mice treated with CUS showed reduced serum levels of tryptophan as well as kynurenine, indole-3-acetate, and indole-3-lactate

(tryptophan-related metabolites). Therefore, the less availability of its precursors might lead to lower brain serotonin levels, which may contribute to the behavioral impairments observed in behavioral analysis. Interestingly, vancomycin supplements reversed the effect of chronic stress on tryptophan and its metabolite levels, which might associate with the decrease in anxiety-like behaviors observed in CUS + vancomycin mice.

NAc is part of cortico-limbic circuits, integrates emotion and behavioral outputs, and intercellular kinase signaling cascades mediate ventral striatal functions [167]. The alternation of homeostatic integration of neurotransmitter and neuromodulator signals in NAc is associated with the pathophysiology of neuropsychiatric disorders [168]. The cyclic adenosine monophosphate (cAMP) / (PKA) pathway plays a critical role in CNS functions by regulating neuronal growth and development, synaptic plasticity, neurogenesis, and memory consolidation. By activating PKA to produce a series of biochemical reactions and physiological effects, extracellular signaling is amplified. [169]. Phosphorylation of PKA at Ser845 GluR1 is important for inserting GluR1 containing AMPAR into the postsynaptic region, which affects AMPAR-mediated excitatory synaptic transmission causing depressive-like behavior in chronic restraint stress mice [170]. In MMD patients' brain samples show lower PKA-related protein expression [171]. We showed the phosphorylation state of Ser845 GluR1 was

decreased by CUS in ventral striatal lysates in our research, consistent with the reduction of PKA activity in other research [172] and indicating that NAc synaptic excitability might be affected. Interestingly, vancomycin reversed the reduction of PKA activity. Besides the PKA signaling pathway, chronic stress increased ventral striatal ΔFosB expression. Prolonged induction of ΔFosB is observed within NAc and other reward-related brain regions in response to chronic stress and may mediate the long-term effects of stress on the brain [157]. Moreover, adducins, actin-capping proteins, stabilize actin filaments and facilitate actin-sceptrin network formation [173]. Our lab recently found a new CDK5 phosphorylation site of ADD2 at Ser 606 that may be affected by gut inflammation. We showed that the ADD-2 phosphorylation could also be increased by CUS. It would help us to find out the interaction of ADD2, CDK5, and stress in future studies. Similarly, p35 regulates CDK5 activity and both of them are correlated with depressive-like behavior in mice [151]. Hyperactivity and/or dysregulation of p35 and CDK5 contribute to excessive anxiety induced by stress [174]. We found chronic stress increased p35 levels in NAc but not significantly, showing that there might be other pathways regulating p35 activity. However, vancomycin supplementation didn't show the effects on these neuronal signaling except for the PKA signaling pathway. It showed that vancomycin might affect other stress-related neuronal signaling pathways, also contributing to

anxiety-liked behavior observed in CUS mice. The effects of chronic stress and vancomycin on ventral striatal signaling provide a mechanism for how its deleterious effects might act on neurobehaviors. The observations also showed the need for a more comprehensive study of the effect of chronic and vancomycin on emotional-related brain circuitry so that new mechanisms could be discovered for formulating alternative treatments.

Our study aimed to observe the interplay between stress and microbiome affects brain function, characterize modes of communication, and assess a realizable therapeutic intervention. Given the array of effects, the next step of this research would be to determine the combined effects of serval factors that cause anxiety in the presence or absence of vancomycin. Diet and stress can be one of the combinations and it is much more associated with reality. It will also be important to incorporate female subjects to exposure to chronic stress and vancomycin to assess any sex-specific susceptibility to chronic stress and vancomycin. Nevertheless, this thesis indicated stress-induced alterations in gut microbiome drive changes in host behavior, and administration of gut-specific antibiotics may serve as a potential therapeutic strategy to counteract such effects and also derive mechanistic insights and help formulate novel therapeutic strategies and advance our understanding of the basis of stress related-mental illness.

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