A Study of the Gut-Brain Axis in Stress-Induced Depression

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Doctoral Thesis

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Chapter 1: Introduction to and History of Depression and its Treatments

Depression is known to impact over 16% of the world's population and is estimated to cost society over $200 billion annually, making it one of the most prevalent and costly health concerns across the globe[1]. Given the widespread impacts on society and individual health, much effort has been dedicated to understanding the causes of and finding treatments for this disorder. However, after centuries of study dedicated to depression, methods for treatment remain only partially effective and much question surrounds the mechanisms behind the disease.

The difficulty in treating depression may arise from the numerous forms that the disease can take -- currently, there are more than seven distinct types of depression recognized by clinicians[2]. Most of these subsets present the same "core" symptoms, but arise in unique situations or are accompanied by other distinguishing features[2]. The onset of post-partum depression, for example, is triggered by pregnancy and the birth of a child. The most common form of depression, however, is major depressive disorder (MDD). This "classic" type of depression will be the focus of this thesis and will be referred to simply as depression from this point on.

Depression has numerous symptoms, ranging from changes in sleep patterns, to persistent feelings of despair and suicidal thoughts, to anhedonia—the loss of interest in enjoyed activities [3]. While many of these symptoms occur transiently in most people, a clinical diagnosis of depression requires these indicators to be experienced consistently for at least two weeks at a time[3]. The modern approach to diagnosing and treating depression in humans, however, is far

from straightforward for most physicians. Once a diagnosis is reached, treatment often involves the arbitrary and gradual modification of prescriptions until symptoms improve, making for a long drawn out and often expensive process.

As the understanding of depression has progressed, medical techniques have likewise advanced to provide more sophisticated methods of treatment. Despite this increased sophistication, there is much work left to be done and numerous research avenues that warrant exploration. Here, the history, etiology, and current and prospective treatment options for depression will be highlighted.

History of Depression

Depression, or "melancholia" as it was formerly known, has been observed in humans since ancient times. Considered to be a "spiritual disorder" attributable to demonic possession, it wasn't until the fifth century that the disorder was understood to have a physical cause[4]. Hippocrates is credited as the first to associate depression with an imbalance within the physical body, rather than a spiritual ailment. The earliest proposed treatments for depression were centered around expelling excess "black bile" from the liver through various means: blood-letting, warm baths, or even lifestyle modifications, such as changes in diet and exercise[5].

As scientific understanding progressed, hypotheses surrounding the causes of depression evolved. By the 1800s, ailments of the brain were favored to be the main cause of depression. In 1817, a surgeon by the name of James Foy tracked a patient that was exhibiting signs of melancholia through the patient's eventual suicide. Dr. Foy then performed an autopsy in hopes

of discovering the root cause of the patient's feelings of despair. The autopsy revealed an abnormal bone growth in the brain, leading Dr. Foy to posit that this protrusion, and the inflammation it caused, may have contributed to the melancholia[6]. The association between abnormal physiology and melancholia was also demonstrated in 1867, when a patient displaying a depressed state was believed to have Addison's disease[7], a disorder involving the adrenal cortex under-producing hormones[8]. These observations established some of the first connections between depression, hormonal imbalances, and inflammation, all critical to future research and treatments.

In the late 19th and early 20th centuries, physicians began to subdivide the broad term of "melancholia" into different disorder types. This was when the term "depression" started to appear in the literature, and when its causes began to be studied in earnest [9]. By the early 1930s, physicians believed that the sympathetic nervous system played a role in the presentation of depression, and suggested that hormones served an important role in the onset and persistence of depressive states[10]. Several documented instances demonstrated that hormones such as the "thyroid gland substance" seemed to benefit depressive patients, but these positive results were not observed in all cases[11]. Encouraged by these results, researchers continued to search for treatment methods that would be effective for all patients who exhibited depressive tendencies. Electroconvulsive therapy (ECT) became the next popular management plan: it was the first line of treatment from the late 1930s until the 1960s. ECT was first conceptualized by observing that patients who suffered from both epilepsy and mental illness experienced improved mental health outcomes after a seizure. In accordance with these observations, those suffering from depression also showed symptomatic improvement after ECT sessions. Although the mechanism

of action was unknown, the technique spread to nearly every hospital in the United States by the early 1940s[12]. However, as mental health reforms gained traction[13], the ethical concerns over ECT grew - its use steadily declined throughout the 1960s and 1970s. In its place, clinicians began to prescribe mood stabilizers such as lithium or Imipramine – drugs which are still in use today, though less frequently than newer classes of drugs[14, 15].

Although these pharmaceuticals led to signs of improvement in patients, not much was understood about how these mood stabilizing drugs worked. The success of these treatments, however, prompted researchers to investigate the changes in neurotransmitters that guide our practices today[16, 17]. Over the last several decades, research has led to a better understanding of depression, as well as improved management of its symptoms. These prevailing mechanistic hypotheses, mostly surround changes in neurotransmitters, will be outlined below.

Monoamines and Depression

Monoamine theories suggest that depression is, at least in part, caused by an imbalance of the neurotransmitters known as monoamines -- primarily serotonin, norepinephrine, and dopamine. These theories are the foundation of modern therapeutic design, and drugs that target these deficiencies are the most frequently prescribed to treat depression[18]. This section will cover the rise and fall of various monoamine theories over the last several decades, as well as the prescribed treatments associated with them.

Serotonin (5-Hydroxytriptamine, 5-HT) was first discovered in 1948[19]. Most serotonergic neurons are expressed in the gut or the brain[20, 21], and serotonin is associated with many

different functions. The brain stem houses most of the serotonergic nuclei in the central nervous system (CNS), but projections from these neurons terminate in areas associated with mood: the hypothalamus, amygdala, hippocampus, striatum, and cortex[22]. While serotonin is primarily discussed in connection to mental health disorders such as depression, it is worth noting that it has also been observed to have effects on other anatomical functions, including cardiovascular regulation, respiration, and thermoregulation[22, 23].

Another monoamine that is frequently associated with depression and its symptoms is norepinephrine (NE). First characterized in 1946[24], CNS NE is found primarily in the brainstem [25, 26]. Noradrenergic neurons also project to the hippocampus and cortex, and is associated with many behavioral functions including mood, arousal, appetite and general homeostasis[26, 27]. NE is uniquely associated with the activation of anxiety and fear related circuitry, and is believed to play a role in these symptoms of depression[28]. Unlike 5-HT, NE is thought to act as a behavioral stimulant rather than a constraint – outside the context of depression, NE is known for stimulating the body's fight-or-flight response[22].

The monoamine that is thought to have the least significant role in the onset of depression is dopamine (DA), which was first attributed to the brain in 1957[29]. While discussed below primarily as a minor drug target, the recent developments of DA in depression are important to note. As mentioned earlier, anhedonia – the loss of pleasure in enjoyed activities – is one of the main symptoms of depression. Research suggests this can be attributed to a deficit in motivation and reward seeking circuits that are associated with changes in DA [30, 31]. DA is a signaling hormone that is integral to motivation: it communicates how much a reward is liked, which is then translated into the will to act[32]. Neuroimaging and electrophysiological studies

have investigated the role of DA in depression, but few treatments impact dopamine specifically, and none target it exclusively[33].

Monoamine Oxidase Inhibitors and Monoamine Re-uptake Inhibitors

Although the first literature proposing the importance of specific monoamines was published in the late 1960's[16, 17], non-specific monoamine oxidase inhibitors (MAOIs) such as Iproniazid had been used to treat depression since at least 1957[34]. Iproniazid was originally used to treat tuberculosis, but was observed to significantly improve outcomes of patients with affective disorders. Iproniazid causes a non-reversible blockage of Monoamine Oxidase, an intra-mitochondrial enzyme that catalyzes the breakdown of monoamines like serotonin and norepinephrine (see Figure 1)[34, 35]. This blockage allows for monoamines to be present in the brain for longer, allowing for more neuronal signaling: a result that is thought to aid in alleviating depressive symptoms. Unfortunately, as the prescription of MAOIs as anti-depressants rose, the prevalence of harsh side effects became more common. Both hepatotoxicity and hypertensive crises were reported in patients that took MAOIs, leading to the swift removal of these drugs from treatment plans[36].

The next iteration of antidepressant drugs that attempted to prolong the presence of monoamines in the brain were monoamine reuptake inhibitors (MAUIs), also known as tricyclic antidepressants (TCAs). The prototype of this drug class was Imipramine, and early research into the mechanisms of action demonstrated numerous possible pharmacological effects. TCAs mainly act by preventing cellular uptake, which subsequently inactivates serotonin and

norepinephrine. While the mechanism of action was not entirely understood at the time, it was known that TCAs had a range of other interaction sites. These areas of interaction proved to be anticholinergic, antihistaminergic, serotonergic and noradrenergic (see Figure 1)[36-38]. Unfortunately, the prevalence of these off-target interactions caused detrimental side effects such as increased heart rate, decreases in blood pressure, and urine retention[39] -- which posed significant obstacles to their continued prescription[38].

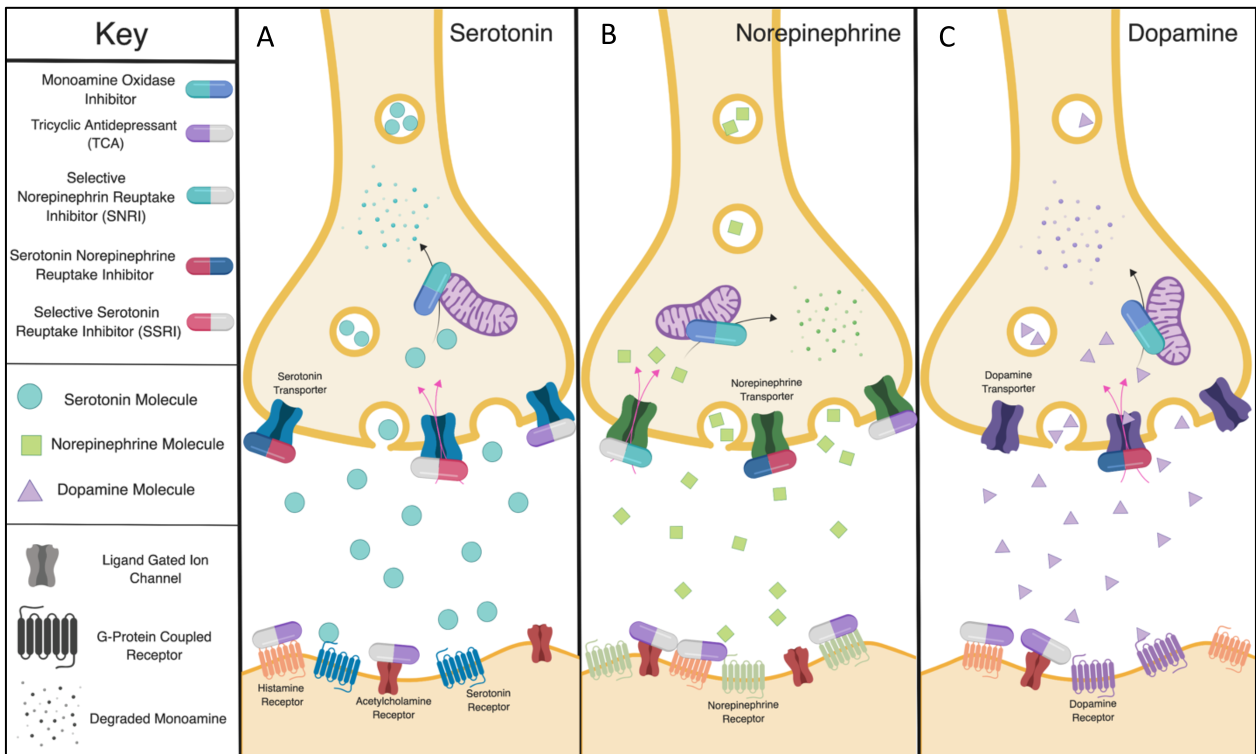

Figure 1: Main Mechanism of Action for Antidepressant Drugs- A. In a serotonergic neuron, monoamine oxidase inhibitors act by blocking the mitochondrial-dependent degradation of serotonin allowing for more molecules to be present in the brain. Tricyclic antidepressants are thought to improve mood by blocking the re-uptake of serotonin through its reuptake transporter, while also blocking post-synaptic histamine and acetylcholine receptors. SSRIs prevent the pre-synaptic reuptake of serotonin through the serotonin reuptake transporter (SERT). Serotonin norepinephrine reuptake inhibitors have high affinity for the SERT and block the uptake of serotonin. **B.** In a noradrenergic neuron, monoamine oxidase inhibitors act by blocking the mitochondrial-dependent degradation of NE, allowing for more molecules to be present in the brain. Tricyclic antidepressants block the re-uptake of norepinephrine through its reuptake transporter, while also blocking post-synaptic histamine, acetylcholine, and adrenergic receptors. In the noradrenergic neuron, selective NE reuptake inhibitors block the reuptake of NE at the pre-synaptic neuron**.** Serotonin norepinephrine reuptake inhibitors have a relatively high affinity for the NE transporter and block the re-uptake of norepinephrine. **C.** In a dopaminergic neuron, monoamine oxidase inhibitors act by blocking the mitochondrial-dependent degradation of DA, allowing for more DA to be present in the brain. Tricyclic antidepressants block post-synaptic histamine and acetylcholine uptake. Finally, in the dopaminergic neuron, serotonin norepinephrine reuptake inhibitors have a low affinity for the DA transporter and partially block the re-uptake of DA.

In hopes of creating a more tolerable drug, researchers looked closely at Imipramine to understand which of its properties was mediating the reversal of depression symptoms. One observation that propelled this research forward was that Imipramine seemed to take several hours to reverse the effects of depressive agents like reserpine. Due to this delay of therapeutic effect, it was hypothesized that a metabolite of imipramine may be responsible for the desired effects. This metabolite was found to be desmethylimipramine (DMI) [40, 41]. DMI was later demonstrated to prevent the re-uptake of norepinephrine, and to a lesser extent, dopamine and serotonin [42, 43]. This finding led researchers to hypothesize that NE was the main catecholamine responsible for the chemical imbalances in depression. Focus then shifted to improving the effects of MAUIs by developing drugs that *selectively* inhibited the re-uptake of norepinephrine and other monoamines thought to be involved in depression.

Norepinephrine Reuptake Inhibitors

Research into both amphetamine and other norepinephrine inhibiting agents added to the evidence from MAUI studies to suggest that norepinephrine depletion could be the key factor in the onset of depressive symptoms. Amphetamine, a strong central nervous system stimulant that impacts NE release, had been used to treat depression in the early 20th century. However, after receiving large doses of amphetamine, patients with no history of diagnosed depression were often observed to fall into a transient "depressive-like" state[17]. To reconcile this seemingly counterintuitive observation, Glowinski et al. demonstrated that large doses of amphetamines appeared to cause the release of significant quantities of NE into the synaptic cleft, while

simultaneously inhibiting the NE reuptake on the post-synaptic side [44]. This suggests that significant stimulation, and the subsequent release of NE from nerve endings, results in a shortage of NE in the brain. These results indicated that the transient nature of these depressive-like symptoms could be explained by the time it takes for the brain to replenish NE, allowing for its signaling once again[17, 27]. Lastly, α-methyl-tyrosine, a potent inhibitor of tyrosine hydroxylase, the rate-limiting step in the creation of norepinephrine, appeared to induce depression-like symptoms in animals[45]. In 1964, Spector et al. demonstrated that interfering with tyrosine hydroxylase significantly reduced levels of NE in both the brain and heart of guinea pigs, inducing transient depressive states for as long as the drug was active[45]. This work highlighting the importance of NE in depression, when combined with the low efficacy and harsh side effects from MAOIs and MAUIs, led to the desire for more selective drugs capable of preventing norepinephrine re-uptake in the brain.

The early to mid 1970s saw the production of several selective norepinephrine re-uptake inhibitors (NRI)[46] that had varying degrees of potency [47]. These NRIs appeared to be inhibiting the re-uptake of NE by altering the binding capacity of α-adrenoceptors in the brain, thus prolonging the life of NE in the synapse (see Figure 1) [41].

Despite the optimism surrounding this new class of drugs and the initial studies in animal models that showed promising reversals of reserpine and tetrabenazine induced depressive effects[48], clinical trials revealed no improvement in symptoms over the MAUI imipramine [49]. Development of this drug class continued for the next several years, but none of the drugs created were marketed widely for depression treatment. This failure to provide clinical relief in depressed patients caused a major shift in the understanding of depression treatments. Research

quickly moved away from focusing on NE and spawned new efforts to understand the true etiology of the disorder.

Selective Serotonin Reuptake Inhibitors

The limited success of NRIs thrust serotonin into the spotlight for depression treatment once again. While much of the early 1970s was dedicated to NRI development, many researchers remained convinced that serotonin was the monoamine responsible for depressive symptoms[16]. Though the prevailing theory that NE was the dysregulated molecule in depression remained, evidence challenging this dogma continued to accumulate

In 1967, Alec Coppen suggested two primary reasons that serotonin may be the metabolite responsible for depression: the antidepressive effects of tryptophan and a lack of improvement in symptoms after DOPA administration. Tryptophan is a serotonin precursor, while DOPA is metabolized to NE and other catecholamines – the ineffectiveness of DOPA suggested to Coppen that the dysregulated molecule in depression was serotonin and not NE[16].

Later, physicians observed that patients that had been successfully treated for depression relapsed when small amounts of parachlorophenylalanine, a serotonin synthesis inhibitor, were administered[50]. As scientific techniques progressed and NRIs continued to prove ineffective for the treatment of depression, the anti-depressive effects of imipramine were re-examined. In 1980, two influential papers demonstrated that imipramine showed high affinity for serotonin binding sites in both the rat hypothalamus and in human platelets, redefining the mechanism of action of the monoamine reuptake inhibitor (MAUI)[51, 52].

Selective Serotonin Reuptake Inhibitor (SSRI) development began shortly after the identification of serotonin as a key player in depression. The first SSRI, fluoxetine, was developed by Eli Lilly and approved by the Food and Drug Administration (FDA) in 1987. Fluoxetine appeared on the market in 1988 under the name Prozac[53, 54]. The marketing efforts of Prozac and other SSRI's were extremely successful, and a 2003 study demonstrated that SSRIs and therapy, alone or in conjunction, were the most frequently used depression management tools between 1991 and 1996[55].

SSRIs work by enhancing the synaptic levels of serotonin through the inhibition of the serotonin transporter[56] (see Figure 1). Initially, this inhibition causes the over-activation of 5-HT neurons, and a decrease in their firing. Over time, neurons become desensitized to the over-abundance of 5-HT, and their fire rates return to normal. When firing rates have returned to baseline, there is still an abundance of serotonin in the synapse -- this accumulation and increased prevalence is thought to help alleviate some depression symptoms[57].

Interestingly, despite an increase in the rate at which SSRIs were being prescribed, their effectiveness in managing symptoms did not provide complete relief -- they were in fact often outperformed by MAUIs[36]. While SSRIs represented a definite improvement over NRIs with fewer harsh side effects and lower risks for overdose than MAUIs, the SSRI response rate ranged from 53-64% and only reduced a subset of depression symptoms[58]. Today, SSRIs remain the most prescribed anti-depressants[59, 60]. While the success of SSRIs cannot be denied, this class of drug has not proven to be the "cure-all" many had hoped for.

One potential reason that SSRIs are not entirely effective is their impacts on other neuron subtypes, and subsequent monoamines. Both NE and DA neurons were found to be significantly

inhibited during SSRI treatment, having a reduction in firing as great as 50%[61-63]. It is possible that the retention of some depression symptoms could be related to these disruptions within other monoamine neuronal subsets. This possibility circles back to the understanding of multiple monoamines likely being important to depression treatment, and the idea that symptoms are more complex than originally presumed.

Multi-Receptor Drugs

Over decades of research, many teams have attempted to provide evidence supporting either catecholamine or serotonin loss in depression. While some of these efforts have tried to integrate both lines of inquiry, most suggested different forms of depression with separate pathologies [64, 65]. Evidence from SSRI research hinted at the importance of optimizing different monoamines at once. Efforts to provide a one-stop drug with high response rates that could alleviate depressive symptoms regardless of monoamine deficiency began, and were relatively fruitful. These drugs, while similar to MAOIs and TCAs, had different mechanisms of action and provided alternatives to avoid the common and dangerous side effects of their predecessors.

Serotonin Noradrenergic Reuptake Inhibitors (SNRIs) were first introduced into the US in 1991[66] and approved by the FDA in 1993[53]. This new class of drugs acted by inhibiting the reuptake of 5-HT, NE and DA -- with decreasing affinities, respectively. Importantly, SNRIs did not have inhibitory effects on monoamine oxidase, and showed no pharmacological activity for adrenergic, histamine, muscarinic, dopamine or post-synaptic serotonin receptors (see Figure 1)[53, 67]. These findings suggested a therapeutic potential for depression treatment without many of the side effects found in TCAs [67]. Along with a reduction in side-effects, SNRIs appeared to

improve clinical outcomes in depression patients. In a double-blind trial, 72% of patients given Venlafaxine, the first SNRI, had a meaningful clinical outcome; this was compared to only 60% of patients having a meaningful clinical outcome on the SSRI Fluoxetine[67].

In addition to SNRIs, several other "atypical" anti-depressants have been brought to market over the last several years. Though they still strive to change monoamine levels in the brain, the effects of these newer drugs are not well understood. Mirtazapine is classified as a "noradrenergic and specific serotonergic antidepressant" and is thought to work by blocking subtypes of serotonin receptors, while simultaneously blocking α_2-adrenergic receptors. These blockades result in increases in general noradrenergic and specific serotonergic activity[38]. Again, while a proposed mechanism for the action of Miratazapine has been reported, its true function remains poorly understood.

Trazodone and its newer cousin, Nefazaodone, act as serotonin agonist and re-uptake inhibitors. Trazodone acts by inhibiting SERT and two other serotonin receptor family members simultaneously: 5-HT2A and 5-HT2C. In addition to these functions, trazodone has anti-histaminergic and anti-alpha-1-adrenergic effects[68]. While the full signaling ability of trazadone is still not well understood, it is known to have potent sedative effects and is therefore only useful in very specific cases [38, 68]. Similarly, lithium has been used as a mood stabilizer to treat depression for nearly 70 years [69] while the mechanism of action remains unknown. The prevailing theories regarding lithium as an anti-depressant range from dampening excitatory and increasing inhibitory neuronal signaling in the brain, to neuroprotective effects through a reduction in reactive oxidative species[70]. Lithium has proven to be effective in many patients,

but it remains clear that there are many possible routes of therapeutic potential -- not all of which include monoamine rebalancing

The drugs mentioned above represent just a few of many that target multiple monoamines in the brain in cases of depression. Different combinations of receptors and different levels of potency continue to be experimented with in new drug development, without much success. While drug development in the monoamine field will undoubtedly continue, it is time that the research community acknowledge that monoamine levels in the brain may not fully represent depression etiology. Inconsistencies with the monoamine hypothesis include the incomplete reversal of depression, the high rate of treatment-resistant depression, and the considerable latency for the drugs to take effect. Often taking weeks before signs of clinical improvement, this lag has been difficult to for researchers to explain. As many of the drugs targeting monoamines can change molecular availability on shorter time scales, this discrepancy remains an open question in the field[71].

While much time and effort have been dedicated to monoamine theories over the last few decades, new lines of inquiry examining other possible causes for depression or reasons for its augmentation are gaining traction. These new avenues have already provided interesting results and thought-provoking new mechanisms to provide insight into depressive states.

1.3 Neurogenesis and Neuroplasticity in Depression

Originating from several clinical and experimental observations involving stress and glucocorticoids, two relatively recent hypotheses regarding the cause of depression involve the

generation and continued plasticity of neurons. Stress is known to have a causal relationship with the onset of depression and represents one of the most important risk factors to depression[72]. One of the most important components of the human stress response is activation of the hypothalamus-pituitary-adrenal (HPA) axis [73]. Stress induced activation of the HPA axis results in the stimulation of the hypothalamus to produce corticotrophin-releasing hormone (CRH). CRH then stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH), which travels through the blood to induce cortisol or corticosterone release from the adrenal gland [74]. It has been observed that cortisol and other glucocorticoids can reduce hippocampal volume, a phenotype also observed in cases of depression [75, 76]. After this discovery, it became a priority for many researchers to better understand how exactly stress and glucocorticoids reduced hippocampal volume, and why this reduction is correlated with depression. Although many questions remain, two potential explanations have emerged: the neurogenesis and neuroplasticity hypotheses.

1.3.A. Neurogenesis Hypothesis

The neurogenesis hypothesis states that stress -- and the subsequent prevalence of glucocorticoids -- reduces the rate at which neurons are produced in the hippocampus (see Figure 2)[71, 77, 78]. This decline in neuron generation is thought to be responsible for the loss of total hippocampal volume in depression. While still unclear, it is believed that this loss in total neuron number could influence hippocampal circuitry and overall signaling capability[79]. Current understanding of signaling changes in the brain suggest that newly differentiated neurons can

integrate into a mature circuit and redistribute synapses to alter overall connectivity, essentially reprogramming the network[80, 81]. How this reprogramming may impact relief of depressive symptoms remains under investigation. While evidence supporting the neurogenesis is plentiful, others remain skeptical[79, 82].

If aberrant neurogenesis is indeed the root of depression symptoms, we would expect that monoamine manipulating antidepressants would impact the ability of the hippocampus to produce new neurons. Malberg et al. demonstrated that treatment with SSRIs, MAOIs, or NRIs was found to increase adult neurogenesis in the rat hippocampus[83]. This same group also demonstrated that stress induced loss of hippocampal neurogenesis could be reversed with the application of fluoxetine, an SSRI[84]. Importantly, this study demonstrated that even after stress has occurred, depression treatments can still be effective in improving the hippocampal generation of neurons, a finding that may have far reaching therapeutic consequences.

Other, more modern, types of depression treatment have also been found to impact neurogenesis. The brief retirement of electroconvulsive therapy in the mid 20th century led researchers to explore ways to make electricity-based treatment more humane and effective. In the late 1980s, modern ECT was developed with patient safety in mind. The modified version still used electricity to induce seizures, but also incorporated muscle relaxants and anesthetics. These were used to protect against pain and other potential dangers of ECT induced seizures, including cardiac arrhythmias, hemorrhage, and joint dislocation[85, 86]. While the mechanism of action of ECT is still not entirely known[87], it has been shown to increase neurogenesis in the dentate gyrus of the rat hippocampus[88]. ECT has also been observed to increase the volume of areas in the human brain including the hippocampus and areas of the prefrontal cortex[89]. In addition to this

modified version of ECT, other forms of brain stimulation have shown effectiveness in the treatment of depression. These include deep brain stimulation (DBS), transcranial direct current stimulation (tDCS), Vagus nerve stimulation (VNS), and transcranial magnetic stimulation (tMS) – all of which stimulate different areas of the brain to help treat depression and other mood disorders[87, 90]. As was observed with ECT, these other forms of brain stimulation have also been demonstrated to increase neurogenesis[90-94].

New pharmaceutical treatment methods have also shown to aid in neurogenesis based depression management[95]. Ketamine has demonstrated a rapid reversal of depression symptoms -- within hours of infusion [95] – leading to thorough investigations into which of the drug's characteristics could be responsible for its anti-depressive effects. One property of ketamine is to increase the rate of neuronal maturation in the hippocampus[96]. Ketamine's proposed influence on the rate of neuronal maturation supports the hypothesis that total hippocampal volume-- and neurogenesis-- may be critical to the onset of depressive states. However, some of the same studies demonstrating the maturational properties of ketamine also claim that anti-depressive effects can be observed independent of adult neurogenesis and neuronal maturation, suggesting other potential roles for this treatment[96]. Ketamine also antagonizes the N-methyl-D-aspartate (NMDA) receptor, which binds glutamate. Animal models have demonstrated that this antagonization may also have anti-depressive potential [97, 98]. While the question of how ketamine is acting as a depression treatment has been extensively researched, insight into the most critical anti-depressive qualities of ketamine remains elusive. Several data-supported hypotheses have been proposed: NMDA antagonism, increasing brain-

derived neurotrophic factor (BDNF), the increasing maturation of neurons in the hippocampus, and inhibition of the kynurenine pathway, a metabolite associated with depression [96, 99].

These ambiguous findings cast doubt upon a hypothesis that seemed to answer many outstanding depression related questions. It is important to note that the mechanisms behind these modern treatments are still not entirely understood. While ketamine's immediate anti-depressive effects may not be reliant on neuronal maturation, its sustained success might[100]. Considering the conflicting evidence presented above, it remains possible that neurogenesis is a

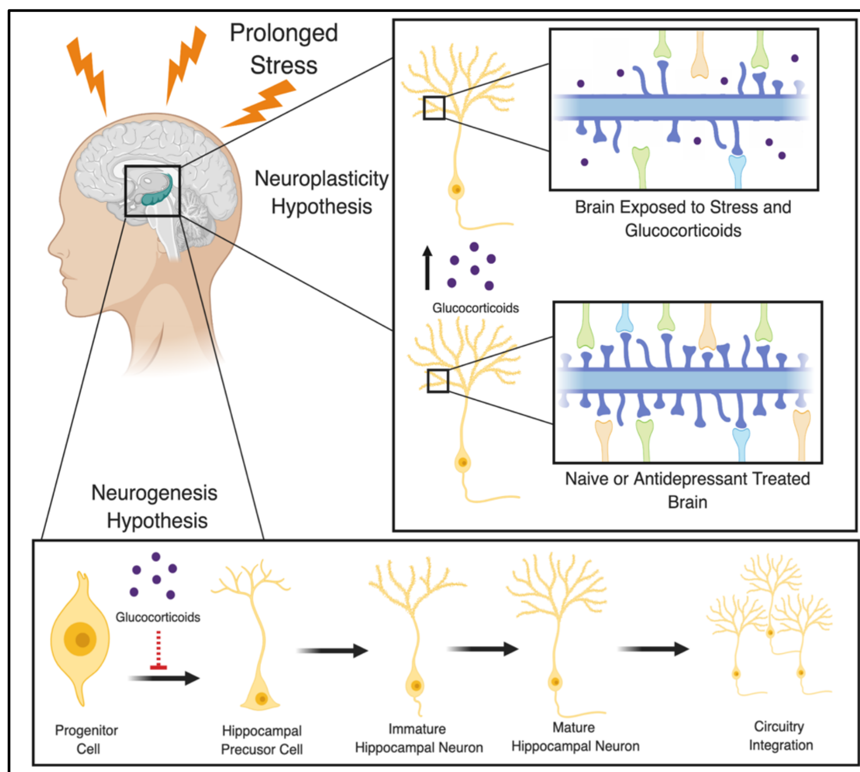

Figure 2: Graphical Representation of the Neurogenesis and Neuroplasticity Hypotheses. 1. Neurogenesis Hypothesis: Glucocorticoids inhibit the maturation of hippocampal progenitor cells. The inability of these cells to mature and form new neurons is believed to be the cause of hippocampal volume loss in the neurogenesis hypothesis. This inhibition also is believed to prevent newly mature cells from integrating into the neural circuit and changing overall synaptic signaling rates, potentially impacting mood and depression. **2. Neuroplasticity Hypothesis:** Glucocorticoids inhibit the formation of dendritic spines. This loss of spinal volume is thought to be representative of total hippocampal volume loss observed in depression. Additionally, the loss of dendritic spines is suggested to change the overall connectivity of hippocampal neurons, limiting the number of neuronal substrates, potentially creating a depressive-like state.

side effect of depression treatment and not a key component – leaving the door open to alternative hypotheses.

1.3.B Neuroplasticity Hypothesis

The neuroplasticity hypothesis also attempts to draw an association between reduction in hippocampal volume and depressive states, but with a slightly different mechanism than that described above. The neuroplasticity hypothesis posits that stress and other depressive factors may be damaging mature neurons, rather than eliminating them entirely. This damage and the subsequent decrease in dendritic spines would reduce the total neuronal volume in the hippocampus, impacting mood and overall depression pathology (see Figure 2)[71]. Indeed, research in the early 90's demonstrated that stress and corticosterone induced damage and a reduction in total volume of hippocampal neurons in both humans and animal models of depression[101-103].

When the impacts of stress on neuronal structure were investigated, it was found that glucocorticoids reduced the amount of brain-derived neurotrophic factor (BDNF), a molecule previously observed as promoting neuronal survival[104, 105]. BDNF is also thought to increase dendritic spine formation, both *in vitro* and *in vivo* [106, 107]. Reduction in dendritic spines is believed to reduce the amount of glutamatergic or monoaminergic innervation a neuron can experience, potentially reducing important signaling mechanisms in the brain[108]. This BDNF dependent mechanism of dendritic spine formation is thought of as the main mechanism underlying synaptic plasticity in depression.

These findings are crucial to the neurogenesis hypothesis, as both reduced levels of BDNF and dendritic spine alterations have been demonstrated in animal models of depression [109, 110]. Reduced levels of BDNF have also been found post-mortem in brains of suicide victims[111]. Interestingly, another way to increase BDNF levels and enhance dendritic spine formation is exercise, a recommendation for depression treatment that has been prescribed since the days of Hippocrates[110, 112]. It has also been shown that fluoxetine (SSRI), imipramine (TCA), and ketamine all can increase dendritic spine formation in a BDNF dependent manner within the hippocampus[113-117]. In the last few years, research has focused on demonstrating that changes in dendritic spine production are required for the anti-depressive effects of known anti-depressants. Though these changes in production may be required for long term effects of anti-depressants, in a mouse model that involved the use of ketamine, behavioral changes were found to precede any physical differences in their dendritic spines[117]. This suggests that while neuroplasticity may be critical for the sustained remission of depression, there may be a more acute mechanism of action for depression treatment that is not reliant on synaptic remodeling.

In addition to the pharmaceutical options discussed above, changes in neuroplasticity have also been observed from brain stimulation. Deep brain stimulation, Vagus nerve stimulation, and electroconvulsive therapy have all been demonstrated to enhance dendritic spine formation *in vivo*[118-120]. While it is challenging to measure dendritic spine changes in humans, increases in spine formation have been inferred from ECT trials demonstrating increased BDNF levels in MDD patients[121].

While significant evidence has accumulated to show that changes in neuronal plasticity and dendritic spine formation can influence depressive states, questions remain about the

temporal resolution of these effects. Additionally, while current treatments seem to indicate that dendritic plasticity is critical to maintain a non-depressive state, treatments that increase spine formation are not fully effective in all patients. Despite the above hypothesis and the evidence supporting it, we are still left with the possibility that neuronal plasticity in the brain is not solely responsible for depression. Further investigation is required to fully understand the mechanistic properties and consequences of synaptic changes, but alternatives must be pursued to ensure novel therapies continue to be developed. One such alternative approach to the study of depression is to explore the possible interplay between the brain and other systems within the body.

Chapter 2: Introduction to the Immune System in Depression

The immune system is the body's main defense against infection and foreign entities. It activates in response to danger signals from the body known as pathogen-associated molecular patterns (PAMPs),[122] and danger-associated molecular patterns (DAMPS, or alarmins)[123], representing foreign and non-foreign entities respectively. These danger signals trigger biological cascades that help propel the body towards homeostasis as quickly and efficiently as possible. The immune system is most often modulated by infectious agents, though it also reacts to psychological stimulants such as stress[124]. Immune cells express both adrenergic[125] and glucocorticoid receptors,[126] demonstrating their ability to "read" signals from the CNS. Finally, glucocorticoids have been used to treat immunological illnesses since the late 1940's [127], highlighting that the clinical implications of the neuro-immune axis.

Dysregulation of the immune system has been closely linked to depression. There is extensive evidence that in times of stress or depression, people are more susceptible to illness – implying that a certain amount of system cross-talk has occurred[128]. An activated immune system is often characterized by increases in immune cell prevalence, as well as inflammation. Inflammation is characterized by red, swollen, and warm areas of tissue -- symptoms that are produced by pro-inflammatory mediators which allow for easier immune cell entry and faster repair[129]. These mediators are small secreted molecules, called cytokines, that influence the actions and communication between many cell types [130]. In the context of inflammation, cytokines can be pro- or anti-inflammatory, and are secreted by many types of immune cells within the body [126]. Acting as the hormones of the immune system, these molecules signal to immune cells the location, timing, and strength of the required immune response.

Cytokines have been directly linked to changes in behavior. Animals treated with cytokines such as interleukin 1 alpha(IL-1α) and beta(IL-1β) have been known to exhibit "sickness-like behavior"[131], which mimics many depressive states and may be related to clinical depression[132]. Additionally, human studies have demonstrated that treatment with pro-inflammatory cytokines – such as interferon alpha (IFN-α) – results in lasting depression-like side effects during and after treatment[133]. Moreover, one of the critical mediators of corticosterone and cortisol production, known as corticotrophin-releasing factor, was increased after IL-1α and IL-1β exposure [134]. This is indicative of another feedback mechanism between the immune system and the brain. Lastly, a 1993 meta-analysis discussed noticeable increases in immune cell populations amongst patients with depression [135]. These connections were unified in 1995 with the creation of an entirely new field of research called psychoneuroimmunology: the study of the interactions

between immunological function, neuronal outputs, endocrine activity, and behavior[136]. One of the primary objectives of psychoneuroimmunology is to better understand the relationship between the immune system and the central nervous system. Further understanding is needed to know how this relationship affects the behavior and symptoms of depression. Despite the outstanding unknowns, it can be said with certainty that the immune system influences the brain, and therefore has implications for depression therapeutics. Highlighter below are some of the most relevant and convincing data that demonstrate exactly how the immune system can influence depressive states.

The Cytokine Hypothesis

First proposed in 1991 as the "Macrophage Theory of Depression", this hypothesis suggests that some of the pro-inflammatory cytokines produced from macrophages (IL-1, IL-6, and Tumor Necrosis Factor Alpha (TNF- α,)) are involved in depression. This idea was supported by several pieces of evidence: the onset of depression symptoms in human volunteers given cytokines; the observation of higher rates of depression in groups where macrophage activation is observed; the ability of microglia, the brain's resident macrophage, to secrete cytokines [137]. More recently, the cytokine hypothesis has been adapted to allow for the inclusion of cytokines produced from cells other than macrophages as contributors to depression[138]. Further investigation is required to understand the complicated role cytokines play in interactions between the immune system and CNS. Highlighted below are some cytokines that are hypothesized to play a more significant role in depression.

Interleukin 1

Interleukin 1 (IL-1) was the first cytokine implicated in depression (see Figure 3)[137]. There two distinct forms of IL-1: alpha and beta, collectively referred to as IL-1. These two forms are distantly related but both have IL-1-like pro-inflammatory activity[139]. IL-1α and IL-1β are both generally produced by macrophages and monocytes[140-143], but have also been demonstrated to be released from other cell types, such as endothelial cells [144, 145] and fibroblasts[146, 147]. The main function of these cytokines is to control proinflammatory responses after danger signals from the body are received[148]. This control is achieved primarily by recruiting other immune cell types, such as neutrophils [149], to help clear the alarmins and return the body to homeostasis. IL-1α is primarily expressed within epithelial cells, the small intestine and microglia[150, 151], while IL-1β is mainly expressed by myeloid cells[152]. Importantly, while both are regulators of inflammation and activate the same receptor (IL-1R), though these forms of IL-1 have different downstream mediators and may serve different functions in depression[153].

IL-1α and IL-1β were first associated with the onset of sickness behavior[131, 132, 154, 155]. However, due to the close resemblance between sickness behavior and depressive states, researchers began looking for general IL-1 expression in mood disorders. Elevated levels of IL-1β have been found in depressed patients[137, 156], but this finding remains controversial as others have claimed no correlation between this cytokine and depression [157]. As methods used to detect IL-1β or IL-1α may not be sensitive enough to detect changes in depressed populations, many still believe this molecule has a role in depression.

In support of this idea, IL-1β has been used to induce depression in animal models [134, 158, 159]. Additionally, both IL-1α and IL-1β have been demonstrated to directly stimulate both the release of adrenocorticotropic hormone (ACTH), as well as an increase in glucocorticoid production [134, 160]-- associating the cytokine with stress, a pathway known to influence depressive states. The discovery of this feedback mechanism proved both exciting and enigmatic to researchers when first discovered. It was unclear how increased levels of the IL-1 forms could lead to a more inflammatory environment while simultaneously stimulating glucocorticoids (GC), as increased GC prevalence is generally immunosuppressive [127]. In 1993, it was discovered that prolonged stimulation of the HPA axis by pro-inflammatory cytokines appears to blunt ACTH responses over time[161, 162]. This response is thought to have two primary implications in depression. First, prolonged GC exposure desensitizes the HPA axis to the negative GC feedback loop, allowing it to stay more activated. Second, it sensitizes the animal to stress responses: because of the higher amount of GC in the system, less stress is needed to provoke a response[163]. Additionally, the brain can respond to the presence of GCs differently, depending on the kind of stressor used on the animal. In a rat model of chronic stress, researchers found that instead of HPA desensitization to a negative feedback loop, neurons were more excitable, allowing for a stronger response to smaller stressors. It should be noted, however, that the chronic stress used in this experiment was not sufficient to induce depressive phenotypes -- potentially influencing the relevance to depression models[163]. This IL-1 dependent dysregulation in the HPA-immune axis is thought to be one of the ways IL-1α and IL-1β can propagate depression symptoms.

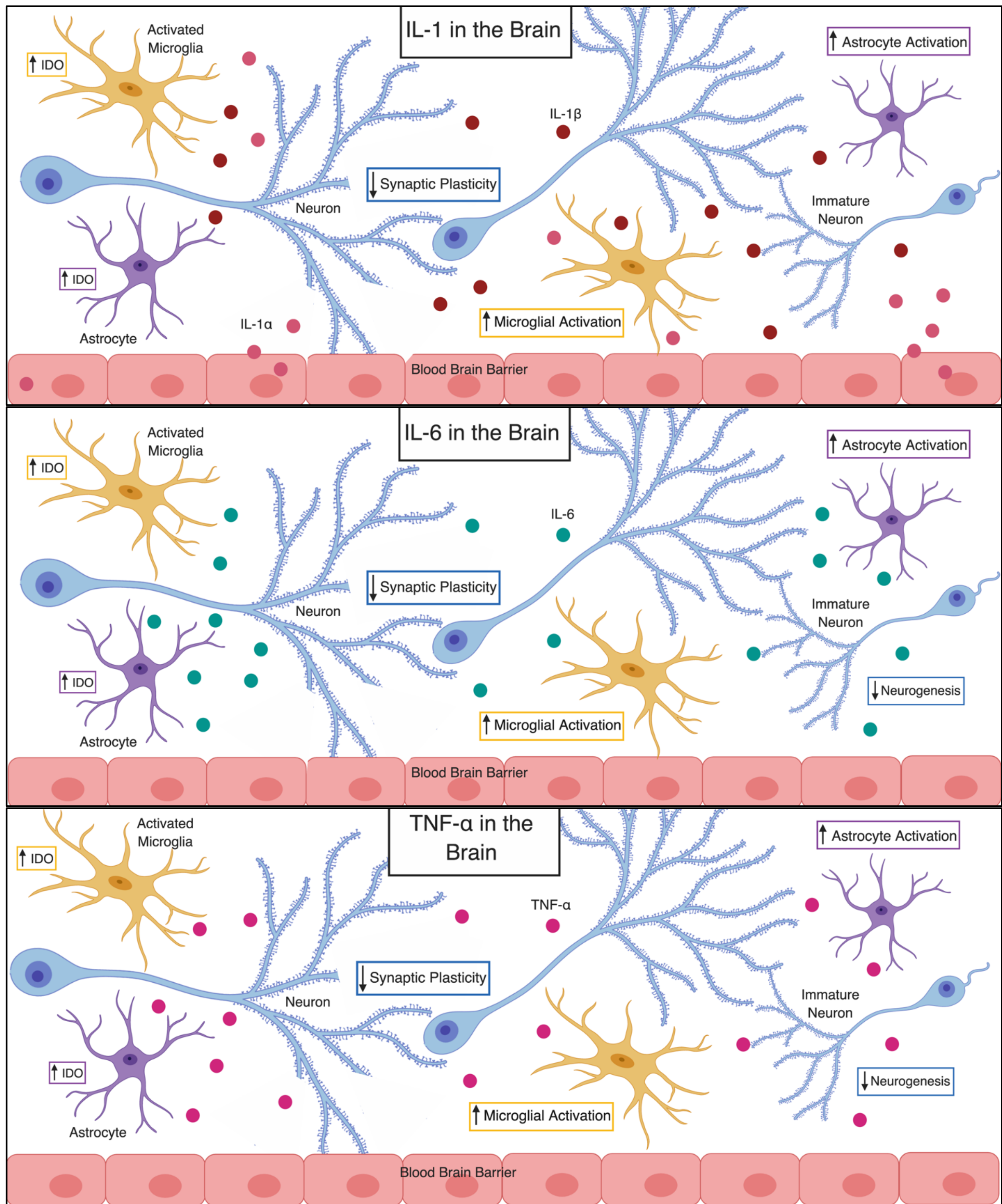

Figure 3: IL-1, IL-6 and TNF-α on Brain Resident Cell Types during Stress and Depression: Cartoon representation of the actions of IL-1α and β, IL-6 and TNF-α on glia and neurons during stress-induced depressive states. Top: IL-1α (light red) and β (dark red) are released by microglia, endothelial cells mainly release IL-1α, and astrocytes can produce IL-1β in stressful states. IL-1α and β can then further activate more microglia and astrocytes, leading to further inflammation. IL-1β is also thought to influence neurons directly by decreasing synaptic plasticity in the hippocampus during stress-induced depression. Middle and Bottom: IL-6 and TNF--α are both mainly produced by astrocytes, but are also released from microglia. These cytokines also further activate both microglia and astrocytes, potentiating the inflammatory response. In addition to acting on glial cells, IL-6 and TNF-α also act on neurons by decreasing synaptic plasticity and neurogenesis.

IL-1β is also thought to influence the kynurenine pathway (see Figure 4). Kynurenine, a tryptophan metabolite, has been associated with depression and other inflammatory conditions [164, 165]. IL-1β is believed to induce expression of indoleamine 2,3-dioxygenase (IDO), the enzyme responsible for converting tryptophan to kynurenine which appears elevated in depressive states [166]. Importantly, blocking IDO directly with 1-methyltryptophan or indirectly with anti-inflammatory minocycline alleviates depression in mice. This suggests that this pathway is critical for depressive phenotypes[166]. Kynurenine was originally thought to be correlated with depression because it depletes tryptophan levels in the body, reducing the essential amino acid's availability for conversion to serotonin[167]. Recently, however, it has been suggested that kynurenine metabolites are more important for the onset of depression[168]. Once converted from tryptophan by IDO, kynurenine can be further degraded into two neurotoxic agents, 3-hydroxykynurenine (3-HK) and quinolinic acid (QA)[169]. 3-HK has been shown to produce reactive oxidative species (ROS), leading to oxidative stress and neuronal death[170]. While mostly associated with schizophrenia, 3-HK may also lead to depressive states in this same manner [29, 164, 170]. Quinolinic acid is known to be neurotoxic and gliotoxic, to induce oxidative stress, to change BBB permeability, and to influence pro-inflammatory cytokine expression -- all actions implicated in depression[171, 172]. Additionally, QA has been demonstrated to be excitotoxic to hippocampal neurons, suggesting there may be a role for this toxin in hippocampal volume changes[173]. Lastly, QA is an NMDA receptor agonist[174]. As NMDA over stimulation has been implicated in depression, increases of QA may also be changing overall neuronal signaling and influencing mood circuits[175]. In support of this, it has been shown that hippocampal and cortical

neurons are most sensitive to QA-induced signaling changes, areas both known to be impacted by depression[174].

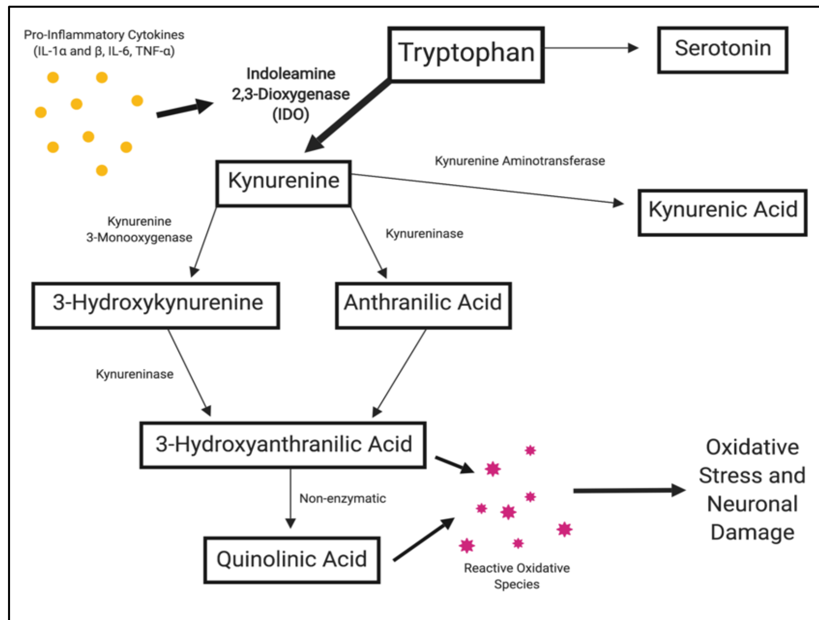

Figure 4: Tryptophan-Kynurenine Pathway.
Tryptophan is converted to Kynurenine by IDO. IDO expression is increased by the presence of pro-inflammatory cytokines, such as IL-1α and β, IL-6, TNF- α. Kynurenine can then be further converted to 3-hydroxykynurenine and anthranilic acid by kynurenine 3-monooxygenase and kynureninase, respectively. Both 3-hydroxyanthranilic acid and anthranilic acid are converted to 3-hydroxyanthranilic acid, which is then spontaneously transformed into quinolinic acid. Both quinolinic acid and 3-hydroxyanthranilic acid are producers of reactive oxidative species (ROS). Increased ROS production leads to oxidative stress and neuronal death, contributing to depressive phenotypes.

Another way in which IL-1 may be impacting depression is through microglial activation. Microglia express IL-1R and can be activated by both IL-1α and IL-1β[176]. In the brain, microglia act as the CNS resident macrophage and are critical for recruiting other immune cells to relieve danger [177]**.** Microglia "activate" in response to danger signals, for which they are thought to be constantly sampling their environment[178]. This activation results in the production of pro-

inflammatory cytokines, including both IL-1α and IL-1β, that signal when an immune response is necessary [178, 179]. Interestingly, microglial activation potentiates sickness behavior in animal models[180], and microglia can be activated by stress[181]. A recent human study utilizing positron emission tomography (PET) scans has also demonstrated that the level of activation of microglia was highly associated with the severity of depression [179]. During inflammatory events, microglia are one of largest producers of IL-1 in the brain, which form of IL-1 is produced, however, depends on the inflammatory stimulus[182, 183]. In addition to pro-inflammatory cytokines, activated microglia also produce the above-mentioned QA and 3-HK, potentially leading to further damage within the CNS [184].

Lastly, IL-1β has recently been found to act on neurons directly. In animal models, IL-1β decreases synaptic plasticity, and causes damage to existing dendrites [185-187]. While not yet associated with hippocampal volume loss, existing lines of evidence suggest that these research findings could play an important role in cytokine induced depression.

Interleukin 6 and Tumor Necrosis Factor Alpha

Interleukin 6 (IL-6) and Tumor Necrosis Factor Alpha (TNF- α) are also believed to play a role in depression pathology (see Figure 3). While many of the actions of these cytokines parallel the two forms of IL-1, they are more strongly correlated with the onset of depression[157, 188]. IL-6 was discovered in 1968, and plays a key role in inflammatory signaling, myeloid cell precursor development, and the induction of thymocyte cell (T cell) differentiation[189]. TNF- α was first discovered in 1968, and is released by lymphocytes [190]. Aptly named for its tumor killing

properties [191], TNF- α was quickly understood to be an important player in the immune system. Like IL-1α, IL-1β and IL-6, TNF- α can recruit immune cells to sites of inflammation. Additionally, when too much signaling occurs, TNF- α can induce apoptotic pathways in cells, resulting in cell death[192].

IL-6 has been associated with depression since the 1990's [138, 188, 193], while TNF- α was first associated with depressive symptoms in 2000[194]. Meta-analyses and human correlative studies have found a positive relationship between serum levels of TNF- α and IL-6 and depression, rendering these cytokines more consistent pathological biomarkers than IL-1β[157, 195-198]. These findings may be attributed to the sensitivity of the assays used, the relationship between IL-1 and sickness behavior, or the time from onset of depression; further investigation is required to determine if any of these cytokines can be considered a true biomarker for depressive symptoms. Both IL-6 and TNF- α are found to be elevated in the serum of depressed patient[197, 199]. Additionally, IL-6 and TNF- α can produce the same inflammatory effects related to depression that IL-1α and β do. As discussed in detail above, these effects include changing glucocorticoid feedback mechanisms and regulation in the brain[200, 201], activating microglia[202, 203], influencing the kynurenine pathway via increased IDO expression[204, 205], and influencing synaptic function[206, 207].

IL-6 and TNF- α could also be influencing depression through astrocytes. Astrocytes represent the most abundant glial cell type in the brain, and are critical for maintaining homeostasis. Some of the major roles for this cell type include BBB maintenance, supporting neuronal growth and synapse formation, and immune cell trafficking[208, 209]. With each of these functions playing a potential role in depressive phenotypes, it follows that astrocytes may be

important for disease pathology [210]. Astrocytes are major producers of both IL-6 and TNF- α within the brain, and have been known to produce IL-1β with certain stimulation[211]. Although IL-1β can be produced by astrocytes, the primary producers of this cytokine appear to be microglia[212, 213]. Additionally, astrocytes can be activated by all three of the cytokines mentioned above[214, 215]. Astrocytic production of IL-6 and TNF- α has also been shown to reduce hippocampal neurogenesis and synaptic connectivity[206, 216]. The mechanism behind this reduction is still not understood, but it is speculated that IL-6 may be able to influence the fate of neuronal progenitors, potentially skewing them to a non-neuronal lineage[216]. In models of stress induced depression, the number of astrocytes were found to be reduced while microglial numbers increased, suggesting a possible protective role for astrocytes in depressive states. Mechanistically, it was suggested that this reduction in astrocytes negatively impacted synaptic plasticity by reducing expression of synaptic vesicle proteins in the brain[217]. Further research is needed to elucidate the exact roles of astrocytes in depression, as evidence has suggested both protective and toxic effects. Given the reported astrocyte plasticity, it is entirely possible that, depending on the stressor or route of inflammation, astrocytes could be activated in different ways and consequently perform different functions.

The mechanisms listed above provide some clues into the roles inflammatory cytokines may play in depression pathology. Although there has been relatively little research done on this line of inquiry, its potential is significant: in 2013, the Diagnostic and Statistical Manual of Mental Health Disorders listed elevated cytokines and inflammation as a diagnostic factor in depression [218].

T Cells in Depression

Another promising area of research regarding the immune system and depression centers around T cells. Within the immune system, there are two cell types that originate from lymphocytes – B cells and T cells. While B cells represent the humoral immune response, T cells arise from thymocytes, and differentiate in the thymus rather than the bone marrow[219]. Once activated by cytokines, T cells differentiate into CD4+ cells, known as T helper cells, or CD8+ cells, cytotoxic (killer) cells. CD4+ helper cells exert control over the immune response by releasing cytokines to instruct other immune cells. The main function of CD8+ killer cells, as their name implies, is to induce death in the infected cell. There are several types of CD4+ T cells that are identifiable by the cytokines they produce. These include the T helper varieties: Th1, Th2, Th17, and Treg (T regulatory) cells. Th1 cells mainly produce IFN-γ, while Th2, Th17s and Tregs mostly produce IL-4, IL-17 and TGFβ respectively[220]. Differentiation of T cells depends on cytokine exposure and transcription factor activation after stimulation[221]. Typically, Treg cells are considered anti-inflammatory, while Th1, Th2 and Th17 cells are believed to be pro-inflammatory. Proper immune responses involve a balance between both anti- and pro-inflammatory cells. Too many Treg cells would result in the immune response not clearing a pathogen effectively; too much Th1, Th2 and/or Th17 activation, and tissue damage may occur from excess inflammation.

There are many examples of dysregulation between pro- and anti-inflammatory cells. One such example is auto-immunity. Interestingly, as many as 50% of patients with diagnosed auto-immune disorders also show signs of depression[222, 223]. Auto-immunity is the loss of the ability

of the immune system to recognize the difference between self and foreign entities[224]. This recognition leads to an understanding of when to attack a pathogen, versus when not to attack a cell produced by the body. There are several ways to "teach" the immune system what is a self-produced cell and what is not. One such way is by inactivating T cells that are auto-reactive before they are fully developed in the thymus [225]. However, routine monitoring of auto-reactive cells must occur in order to maintain auto-immunity throughout life. This monitoring is mainly carried out by Treg cells. In 2005, it was demonstrated that dysregulation of Treg development resulted in tolerance breaking and the subsequent development of auto-immune disease[226]. It has also been suggested that the balance between Tregs and pro-inflammatory Th17s is critical for auto-immune propagation and pathology[227]. This balance was one of the first pieces of evidence that connected T cells, specifically Th17s, to depression. Th17s have been implicated in the worsening pathology of many auto-immune diseases including: rheumatoid arthritis, psoriasis, multiple sclerosis, and inflammatory bowel disease, among others[228-231]. Additionally, IL-1 and IL-6 are important to Th17 differentiation, bolstering the credibility of evidence linking Th17s to depression[229, 232]. These associations have led many researchers to hypothesize that this type of T cell could be playing a major role in disease pathology, and has opened several novel research avenues.

Despite the evidence provided above, many of the specifics regarding the role of Th17s in depression remain unclear. Many believe that Th17s are pathogenetic in depression, as they are in auto-immune diseases[233, 234]. Animal studies have demonstrated that Th17 cells are elevated in the brain during the elevated foot shock stressor, and that the adoptive transfer of Th17s from stressed mice into naïve mice produces depression-like behavior. This same study

found that blocking either the primary cytokine produced by Th17, IL-17, or its master transcription factor, RORγT, prevented the onset of depressive behavior [235]. In addition to animal studies, some researchers have reported finding increased levels of IL-17 in the serum of depressed human patients[223, 236, 237]. However, this finding remains controversial: others have reported no correlation between serum levels of IL-17 and depressive symptoms[238, 239]. It has also been reported that IL-17 can have beneficial effects within the CNS. For example, microglia upregulate neuronal growth factors NGF, BDNF, and GDNF, which promote neuronal growth, connectivity, and health, in response to IL-17 exposure[240]. Furthermore, IL-17 has been implicated in corneal nerve regeneration. In these sets of experiments, the authors demonstrated that IL-17 was necessary to recruit neutrophils and platelets to sites of injury or infection. This recruitment brought with it vascular endothelial growth factor (VEGF), a protein that supports neurite growth, suggesting that IL-17 may play a role in trying to recruit cells that can support neuronal health[241]. Together, these data suggest that IL-17 warrants closer examination.

Chapter 3: RORγT+ Th17s and their Role in Depressive-like Behaviors

Given the conflicting data surrounding the role of Th17s and their master transcription factor, IL-17 in depression, I sought to investigate the role of this cell type in stress-induced depression in mice. To address this gap in knowledge I developed a novel mouse model which lacked RORγt in CD4+ cells, eliminating the Th17 compartment in mice. The following chapter is taken from my

co-first author publication "Stress-induced despair behavior develops independently of the Ahr-RORγt axis in CD4+ cells.

Stress Induced Depression Occurs Independently of CD4+ Th17s

Abstract

Current treatments for major depressive disorder are limited to neuropharmacological approaches and are ineffective for large numbers of patients. Recently, alternative means have been explored to understand the etiology of depression. Specifically, changes in the immune system have been observed in both clinical settings and in mouse models. As inflammatory RORγt+ CD4+ Th17 T cells and their primary cytokine IL-17 have been implicated in the development of stress-induced depression, the connection between stress, Th17s, and depression remains critical to disease understanding. Here, we utilized a CD4-specific RAR Related Orphan Receptor C (*Rorc)* knockout line to disrupt the production of Th17s. Mice lacking *Rorc* produced IL-17 did not show any differences in behavior before or after stress when compared to controls. Additionally, we utilize an unsupervised machine learning system to examine minute differences in behavior that could not be observed by traditional behavioral assays. Our data demonstrate that CD4 specific *Rorc* is not necessary for the development of stress-induced anxiety- or depressive-like behaviors. These data suggest that research approaches should focus on other sources or sites of IL-17 production in stress-induced depression.

Introduction:

In the United States, an estimated 17.3 million adults are diagnosed with major depressive disorder (MDD). With over 60% struggling with severe impairments, MDD is the number one cause of disability in the U.S.[242]. Dogma states that depression and other mood disorders are caused by an imbalance in neurotransmitters[36, 64, 243]. As such, the majority of existing treatments target neurotransmitter uptake (SSRIs, SNRIs, etc)[57]. However, a significant number of patients do not benefit from these therapeutics, suggesting alternate etiologies for MDD[57].

The immune system has been suspected to play a role in depression since the early 1990s when the macrophage hypothesis of depression was first posited[137]. While many aspects of the the immune system could be playing a role in the onset and maintenance of depression, T helper 17 cells (Th17s) have become a main focus of research. Th17s and their main cytokine, IL-17, have been shown to contribute to depression- and anxiety-like behaviors[235, 244]. Studies have shown that the number of Th17s in the gut associated lymphoid tissue (GALT) increases in response to stress and correlates with depressive-like behavior[245]. Additionally, the transfer of Th17 cells into mice has been found to induce depressive-like behaviors[235]. Administration of IL-17A blocking antibodies has also been found to reduce learned helplessness behaviors in mice[235]. However, the evidence for the role of IL-17 in human depression remains controversial, with some reports claiming a positive correlation between the inflammatory cytokine and depression and others reporting no differences in IL-17 levels between those with the disease and

controls[198, 223, 236-239]. These conflicting results highlight the need for further investigation into the mechanism and role of increased inflammatory Th17 cells in depression.

Here, for the first time, we use genetic tools and artificial intelligence behavior analysis to assess the contribution of T cell specific IL-17 to the development of anxiety- and depressive-like behaviors in a murine model of stress. While our data confim that stressed mice present with a larger number of Th17 cells in the gut, our behavioral analyses show that the deletion of RORγT in T cells does not impact anxiety- or depressive-like behaviors in mice. Taken together our data suggest that RORγT induced IL-17 in T cells is not necessary for the pathological development of depressive-like behaviors induced by unpredicalble chronic restraint stress.

Results:

Stress Induces Increases in the Th17 compartment in Gut Associated Lymphoid Tissues

Work from Andrea Merchak in our lab has investigated the role of the Aryl Hydrocarbon Receptor on T cell skewing in stress-induced depression. Through her work, she investigated how *Ahr* knockout (*Ahr* KO) impacts changes in T cell number in the intestines after unpredictable chronic mild stress (UCMRS) exposure. She subjected both wildtype and *Ahr* KO animals to 3 weeks of UCMRS and examined the immune compartment in various tissues in the body. However, she observed no Ahr driven differences between groups (Fig. 5A). Interestingly, both the *Ahr* KO animals and controls showed an increase in the number of CD4+RORγT+ Th17 cells in the GALT that was significantly impacted by stress (Fig. 5A). She also observed a genotype independent decrease in Th17s in the inguinal lymphnodes. We believe this is due to cells

migrating out of the lymphnodes and into the GALT. These data suggest that the microbiome changes and increases in Th17 cells observed in response to stress are not mechanistically linked by Ahr activation in CD4+ cells. Thus, I pursued the role of Th17s in stress-induced depression.

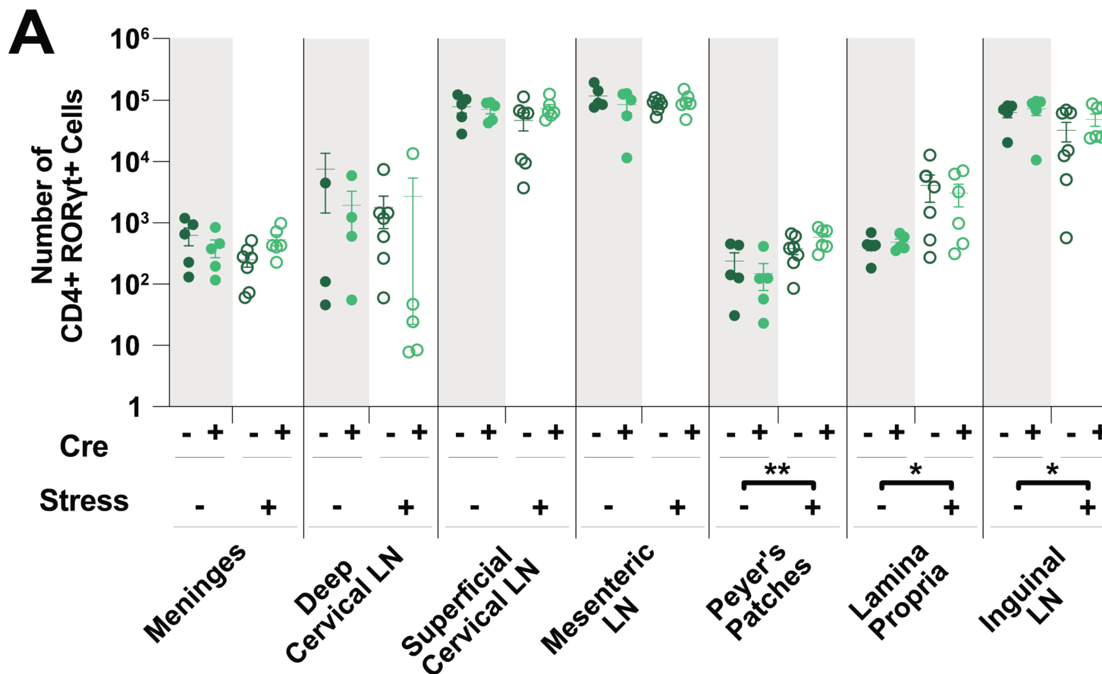

Figure 5. Th17s are increased in Gut Associated Lymphoid Tissue after UCMRS Exposure: (A) Number of CD4+ RORγT+ cells in various immune tissues between stressed and naïve *Ahr* KO and littermate controls (n= 5-7/group). Two-way ANOVA (Peyer's Patches: p= 0.0026, Lamina Propria: p= 0.0359, Inguinal LN: p= 0.0440), N=1, male mice. LN= lymphnode.

Deletion of *Rorc* in T cells does not Induce Spontaneous Anxiety- or Depressive-like Behaviors:

To explore the role Th17 cells in anxiety- and depressive-like behaviors, *Cd4 Cre Rorc$^{flox/flox}$* mice were generated (*Rorc* KO). Knock down of RORγT was confirmed using *in vitro* derived Th17s by examining levels of *Il17* and RAR Related Orphan Receptor (*Rorc)* by qPCR (Fig. 6A) and IL-17 secretion by ELISA quantification (Fig. 6B). Immunophenotyping on spleen revealed the absence of CD4+RORγT+ T cells *in vivo* (6C-E). After model validation, baseline behaviors were compared between male *Rorc* KO mice and controls. No differences between the *Rorc* KO mice or age

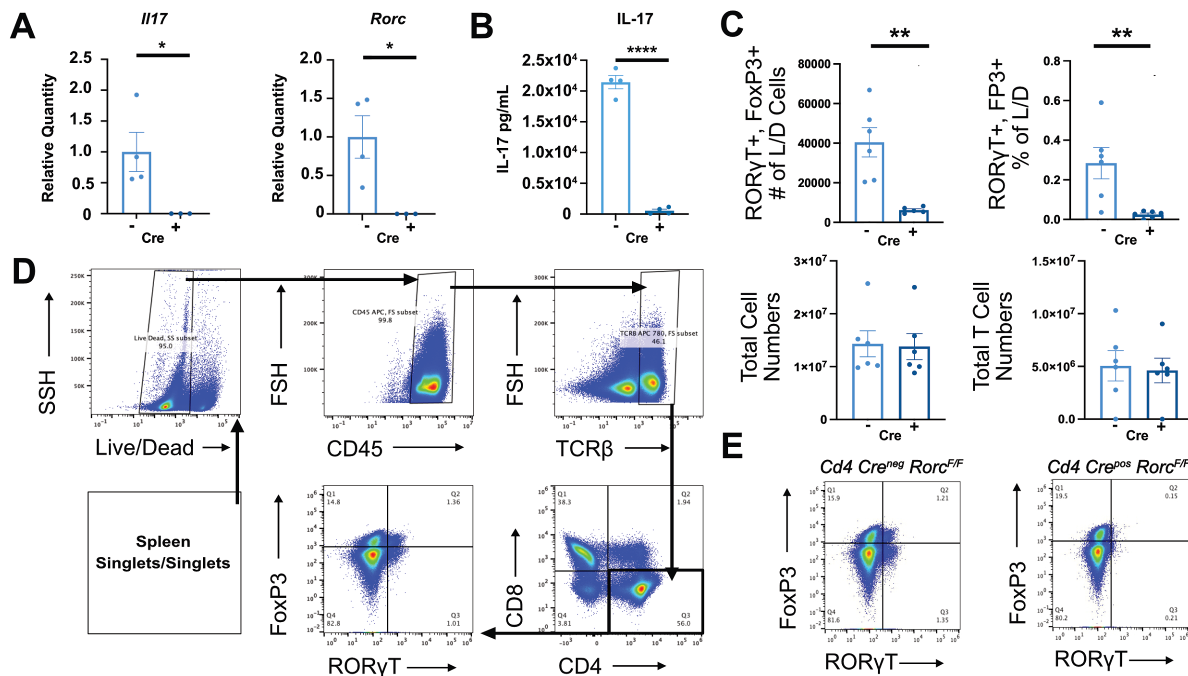

Figure 6: Validation of CD4 Driven *Rorc* Knockout Line: (A) Loss of gene expression of both *Il17* and *Rorc* in *in vitro* skewed Th17s from *Rorc* KO mice and littermate controls by qPCR (n=3-4/group). T tests (*il17*: p= 0.0450, *rorc*: p= 0.0281). (B) ELISA representing loss of IL-17 in *in vitro* skewed Th17s from *Rorc* KO animals (n= 3-4/group). T test (p= <0.0001). (C) Quantification of the number and percent of mesenteric RORγT+ and FoxP3+ cells and total cell and T cell numbers between *Rorc* KO and littermate controls (n=5-6/group). T tests (Number of RORgT, FP3+ cells: p= 0.0048, RORgt, FoxP3+ % of L/D: p= 0.0085). (D) Representative gating strategy for spleen Foxp3 and RORγT positive cells between *Rorc* KO and control animals. (E) Representative flow cytometry quadrants for FoxP3 and RORγT+ cells between *Rorc* KO and littermate controls.

matched controls were observed in tasks used to assess depressive- (Fig. 7A) or anxiety- like behaviors (Fig. 7D). As Th17 cells are also known to contribute to autism-like behaviors in male mice[244], we analyzed the marble burying (Fig. 7C), social preference (Fig. 7C) and novel object recognition (Fig. 7E) tests at baseline. No differences between genotypes were observed in these behavior tests. While classic assays may detect strong phenotypic differences, they may not detect more subtle behavioral changes. To circumvent this limitation, we applied an unsupervised machine learning approach to analyze behaviors in both genotypes using DeepLabCut[246]. First, we validated this computational approach using a preclinical model of multiple sclerosis (MS) known as experimental autoimmune encephalomyelitis (EAE). EAE produces significant locomotor changes and should produce detectable motor differences

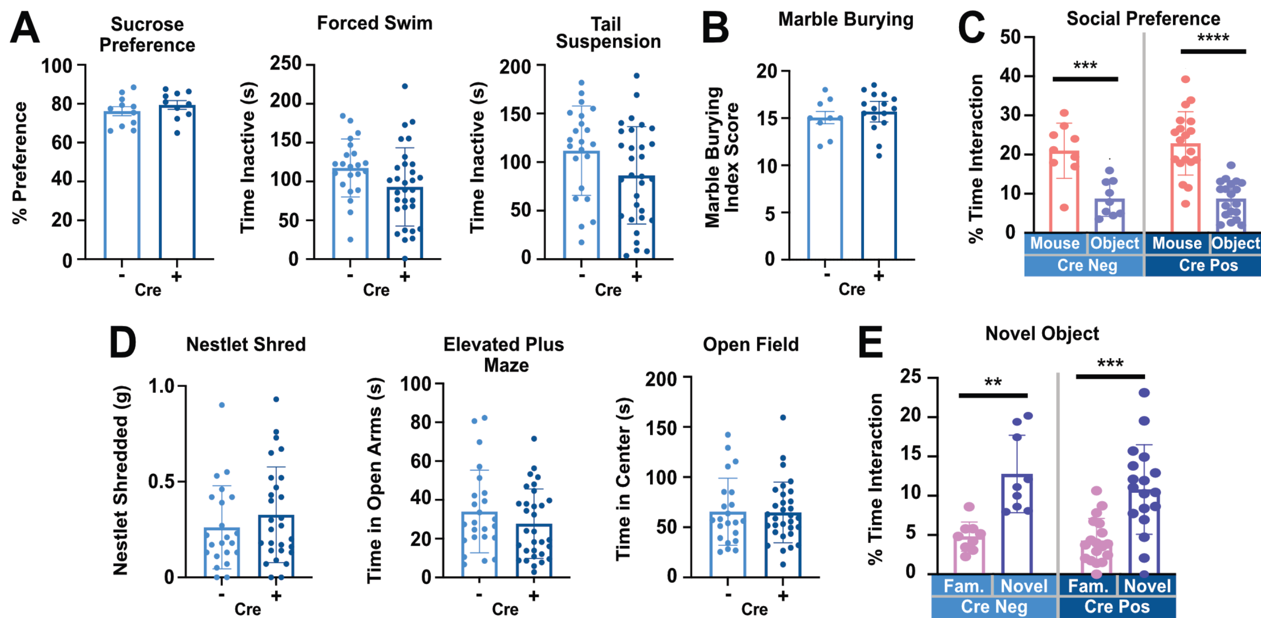

Figure 7: Baseline Behavioral Analysis in *Rorc* Knockout Animals: (A) Baseline depressive-like behaviors (sucrose preference: n=9/group, forced swim and tail suspension: n= 22 or 37/group), (B) baseline marble burying (n= 9 or 16/group), (C) social preference (n= 9 or 19/group), (D) anxiety-like (nestlet: n=22 or 28/group, elevated plus maze: n= 24 or 31/group, open field: n= 22 or 31/group), and (E) novel object recognition (n= 9 or 19/group) behaviors in *Rorc* KO mice vs littermate controls. T tests used in A and D. Two-way ANOVA used in E (Cre Neg: p= 0.0010, Cre Pos: p<0.0001) and E (Cre Neg: p= 0.0014, Cre Pos: p=0.0002).

between groups to act as a positive control. DeepLabCut was able to detect robust behavioral differences between groups in nearly 40% of behavioral motifs characterized by the software (Fig.8 A-F). After validation of the DeepLabCut model, we examined behavioral changes between our *Rorc* KO mice and controls. The Kullback-Leibler Divergence, a measure quantifying variance within and between groups, plot showed individual variance had a larger impact on behavior than genotype effects, suggesting that individual mice have more impacts on behavior than the genotype groups (Fig. 9A and D). Similarly, the PCA plot showed large overlap between groups (Fig. 9B). Additionally, only 3 of 35 behavioral motifs demonstrated significant differences between groups after DeepLabCut analysis (Fig. 9C and E). Together, these data suggest that at baseline, a lack of *Rorc* from development does not impact autism-, depression-, or anxiety-like behaviors in male mice.

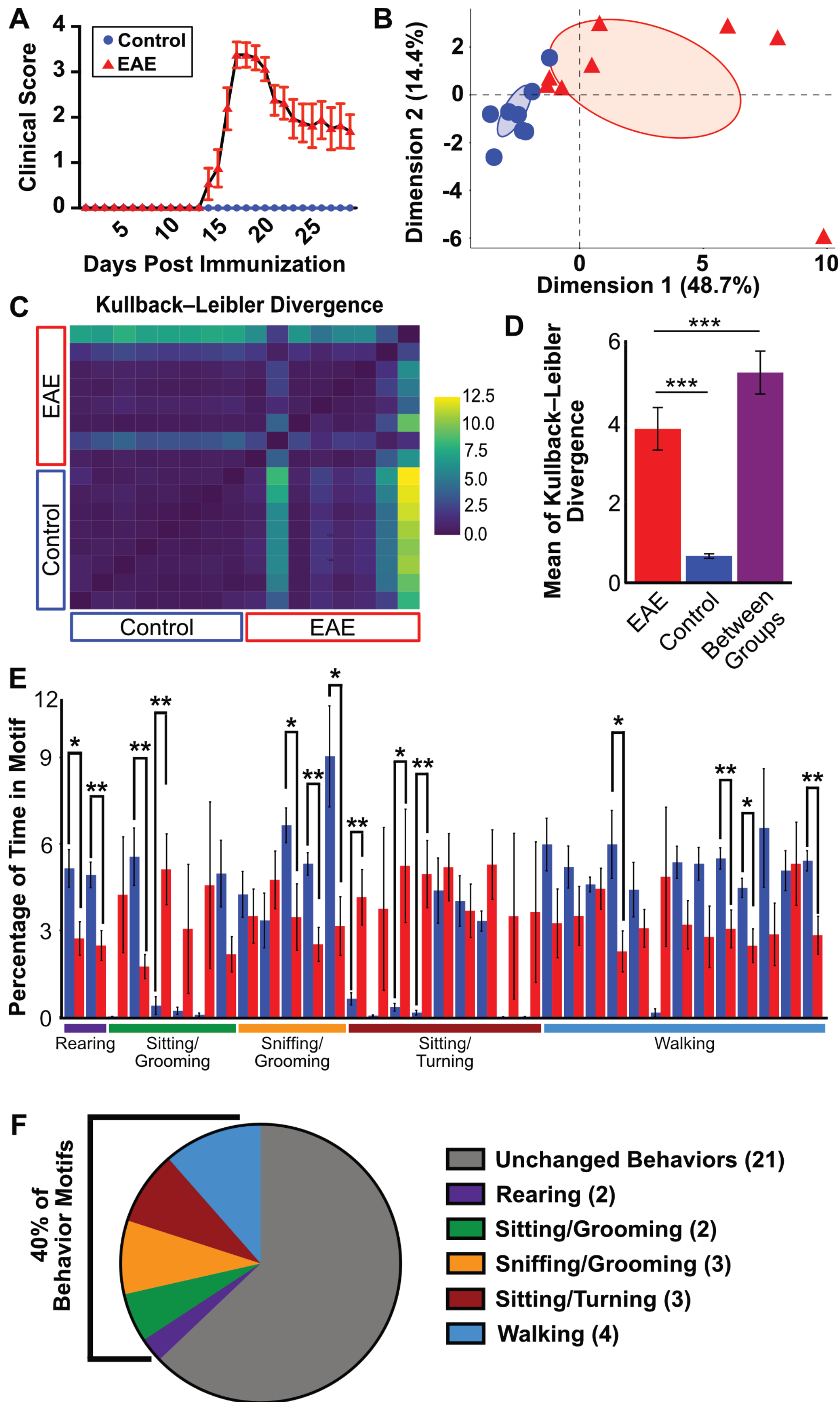

Figure 8: Validation of DeepLabCut in EAE Mice. (A) Average clinical scores of EAE and control mice (n=8/group). Mann-Whitney U Test (p= <0.0001) . (B) PCA and (C) Kullback-Leibler Divergence plots representing differences between EAE and control animals (n=8/group). (D) Means of Kullback-Leibler Divergence scores in control mice, EAE mice, and between groups. Multiple T tests (p= <0.0001). (E) Percentage and grouping of motif usage by EAE vs control mice in DeepLabCut analyzed videos (n=8/group). T tests (Supplemental Table 1). (F) Quantification and grouping of significantly changed motifs (by % usage) in EAE vs control mice. N=1, All female mice.

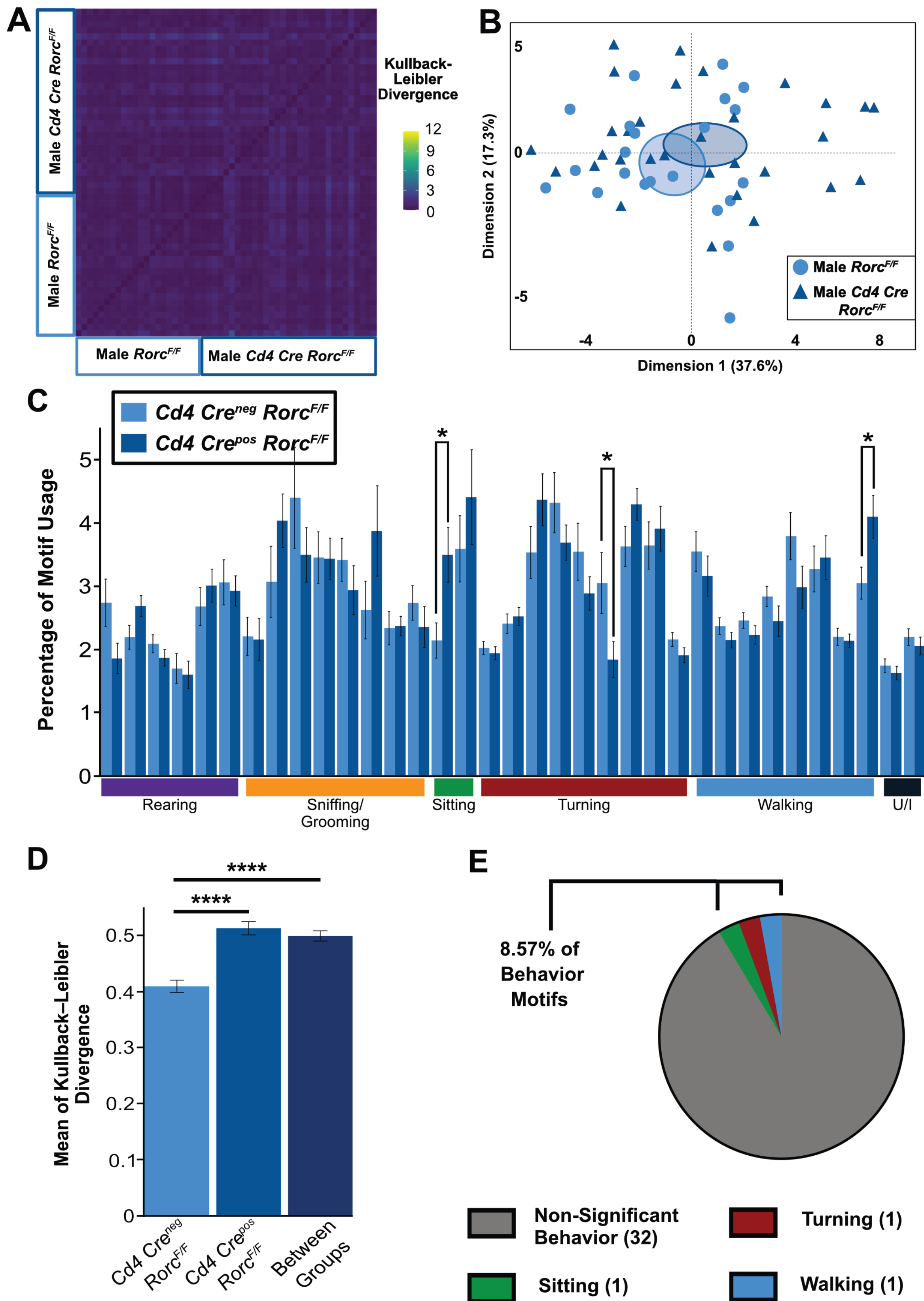

Figure 9: *Rorc* Depletion does not Impact Subtle Behaviors in Male Mice at Baseline: (A) Kullback-Leibler divergence heatmap and (B) PCA plot of DeepLabCut analyzed behaviors between groups (n=22 or 32/group). Combined N=2, male mice. (C) Percentage and grouping of motif usage by *Rorc* KO vs littermate controls in DeepLabCut analyzed videos (n=22 or 32/group). (D) Means of Kullback-Leibler Divergence scores in littermate controls, *Rorc* KO mice, and between groups (n=22 or 32/group). Multiple T tests (p= <0.0001). T tests (Supplemental Table 1). (E) Quantification and grouping of significantly changed motifs (by % usage) in *Rorc* KO vs littermate controls. Male mice.

UCRS Induced Anxiety- or Depressive-like Behaviors are not Affecte by the Lack of Rorc in Th17 cells:

While no behavioral differences in male mice were observed between groups at baseline, females are known to experience depression at a higher rate than males[247]. Thus, we aimed to examine the impact of *Rorc* in Th17 cells in female mice. However, at baseline no differences in escape behavior, anhedonia, or anxiety-like behaviors were observed between female *Rorc* KO mice and controls at baseline (Fig. 10A-B). Similarly, our machine learning approach was not able to detect differences in behaviors as shown through the Kullback-Leibler Divergence plot (Fig. 11 C and D), PCA plot (Fig. 11A), and motif usage breakdown plots (Fig. 11 B and E). To examine the impacts of *Rorc* KO in a stressful environment, female mice were exposed to 3 weeks of UCRS, a stronger model of stress know to induce anxiety- and depressive-like behaviors and changes in

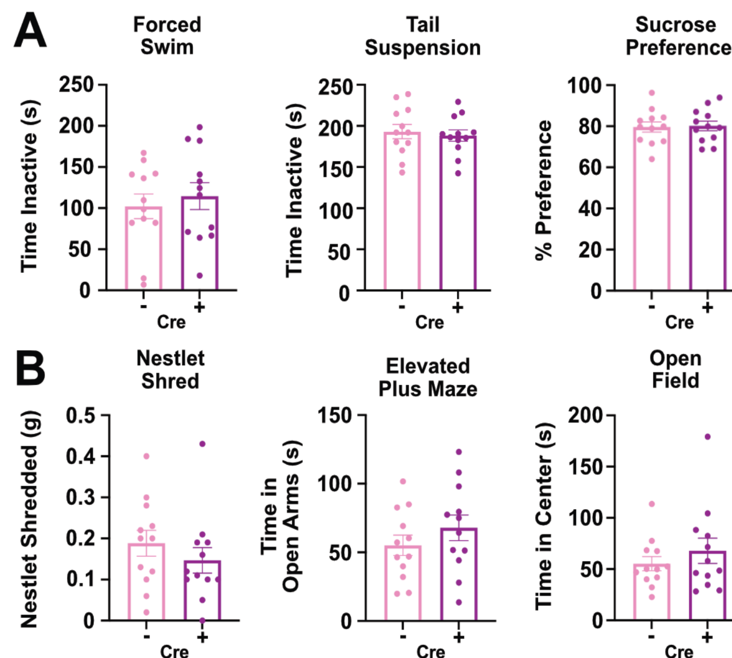

Figure 10: Loss of *Rorc* in T cells does not Impact Depressive- or Anxiety-like Behaviors in Female Mice. No differences in the (A) forced swim, tail suspension, or sucrose preference tests between female KO and littermate controls (n= 12/group). No differences in (B) the nestlet shred test, elevated plus maze, or open field test between female *Rorc* KO and littermate controls at baseline (n=12/group).

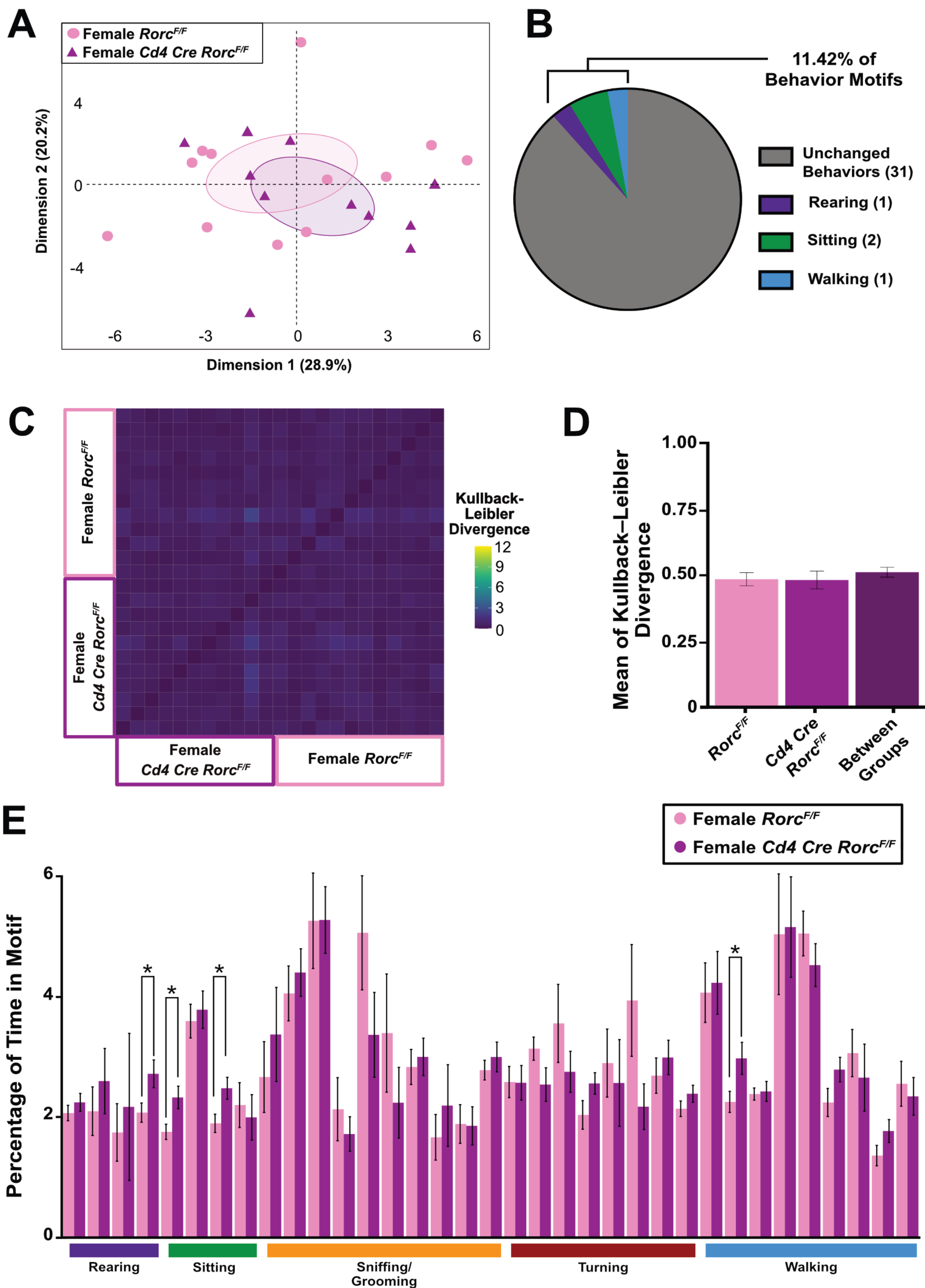

Figure 11: ***Rorc* Depletion does not Impact Subtle Behaviors in Female Mice at Baseline.** No differences in the (A) PCA plot of DeepLabCut analyzed baseline behaviors between groups (n=12/group). (B) Quantification and grouping of significantly changed motifs (by % usage) at baseline in female *Rorc* KO vs littermate controls. No changes in the (C) Kullback-Leibler divergence heatmap or (D) means of baseline Kullback-Leibler Divergence scores in female littermate controls, *Rorc* KO mice, and between groups (n=12/group). Multiple T tests. (E) Percentage and grouping of baseline motif usage by female *Rorc* KO vs littermate controls in DeepLabCut analyzed videos (n=12/group). T tests.

spine density of the basolateral amygdala[248]. After exposure to stress, the nestlet shred test revealed that *Rorc* KO mice demonstrate a significant increase in anxiety-like nesting behaviors, whereas the elevated plus maze showed a significant decrease in anxiety-like behaviors, and the open field test showed no difference between groups (Fig. 12A). In addition, no differences between groups were observed in escape behavior (Fig. 12B). Ultimately, our unbiased computational approach also did not detect any genotype driven differences as seen in the Kullback-Leibler Divergence plot (Fig. 13C and D) and the PCA plot (Fig. 13A). No differences in groups were detected in any of the 35 behavior motifs identified between groups (Fig. 13 B and E). In summary, although we saw a significant increase in anxiety-like behavior in the nestlet

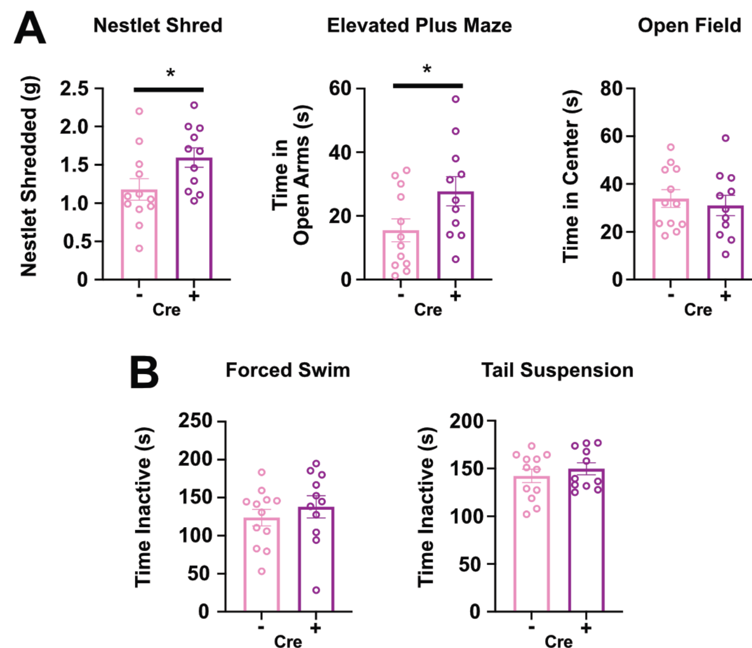

Figure 12: UCRS does not Induce *Rorc* Driven Changes in Female Mice: (A) Nestlet shred, elevated plus maze and open field behaviors after 3 weeks of UCRS between female *Rorc* KO and littermate controls (Nestlet Shred: p= 0.0392, Elevated Plus Maze: p= 0.0453). (B) Forced swim and tail suspension tests after 3 weeks of UCRS between female *Rorc* KO and littermate controls. n= 12/group.

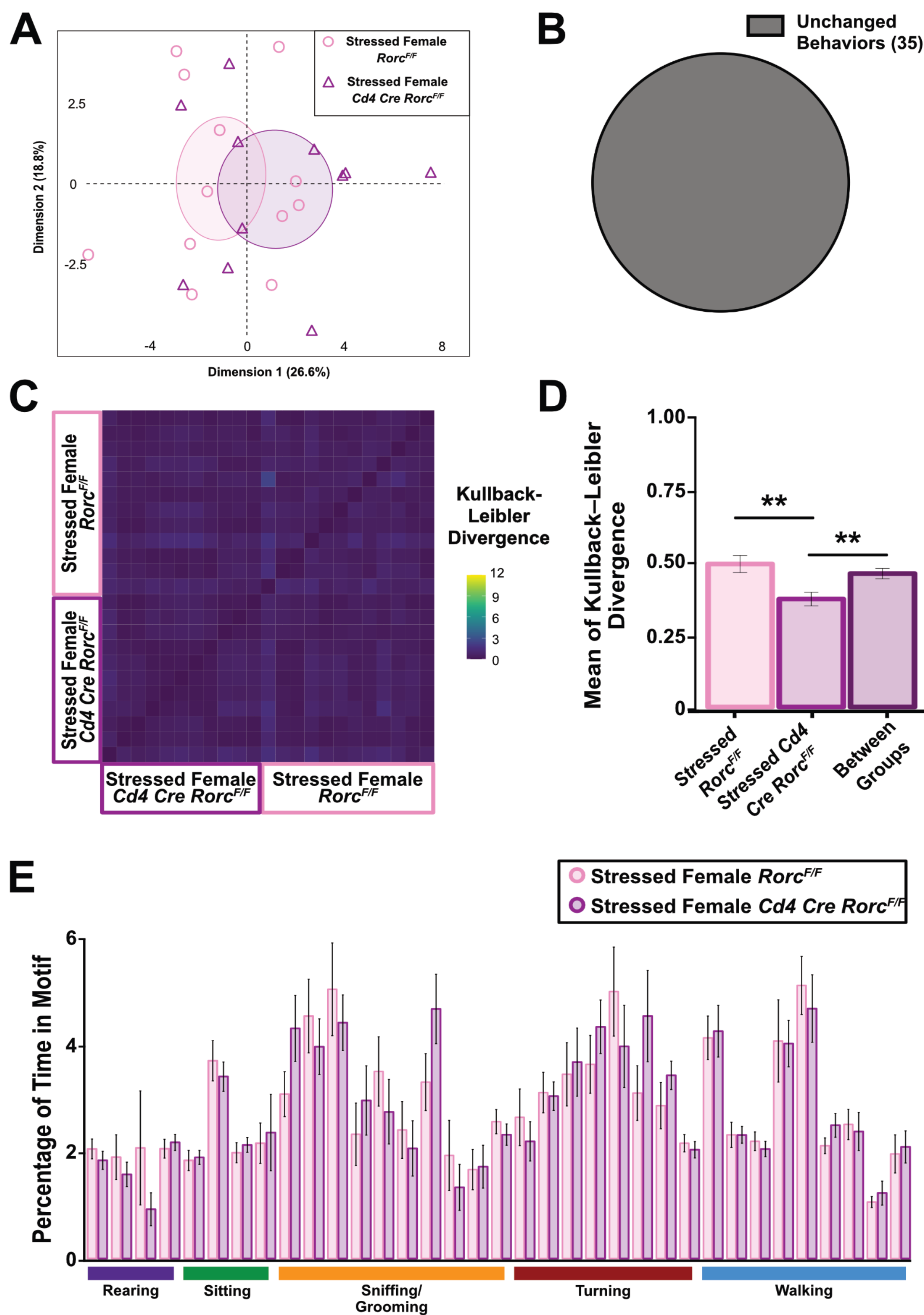

Figure 13: Stress Exposure does not Induce Subtle Behavior Differences in Rorc Knockout mice: (A) PCA plot of DeepLabCut analyzed behaviors after 3 weeks of UCRS between groups. (B) Quantification and grouping of significantly changed motifs (by % usage) after 3 weeks of UCRS in female *Rorc* KO vs littermate controls. (C) Kullback-Leibler divergence heatmap and (D) means of Kullback-Leibler Divergence scores after 3 weeks of UCRS in female littermate controls, *Rorc* KO mice, and between groups. (E) Percentage and grouping of motif usage by stressed female *Rorc* KO vs stressed female littermate controls after 3 weeks of UCRS in DeepLabCut analyzed videos. n=12/group. Multiple T tests used in D (p= 0.0015, p= 0.0032).

shredding test between *Rorc* KO and control animals, our other behavioral tests did not support this trend and our unbiased behavioral analysis showed no changes between groups. Based on these data, *Rorc* KO in T cells does not significantly impact depressive-like behaviors in female mice before or after UCRS.

Discussion:

Depression presents a major social, health, and economic concern across the world, representing the number one cause of disability[249]. The root causes of this debilitating disorder have yet to be elucidated. With treatments remaining inconsistent and new options lacking, new research is needed to further our understanding of this disorder. T lymphocytes have been gaining support as a potential mediator of MDD pathology. Here, we examine the role of T cell specific RORγT in a mouse model of depression. We demonstrate that deletion of *Rorc* from CD4+

T cells, induces no changes in anxiety or depressive-like behaviors at baseline or after stress exposure. This is supported by previous work showing that increasing Th17s is not sufficient to change the susceptibility of mice to social defeat stress[250]. Others have suggested that increased Th17s are responsible for stress-induced depressive-like behaviors and have supported this claim by transferring Th17s into mice and finding increases in depressive-like behaviors[235]. However, this only suggests that an increase in Th17s may be sufficient to drive these behaviors[235], not that it is necessary. Additionally, the origin of IL-17 is not precisely defined. IL-17 can be produced from many cell types and be induced in alternate ways that may contribute to depression outside of Th17s[251, 252]. Supporting this concept, it has been found that IL-17 from γδT cells regulates

anxiety-like behaviors in mice[253]. Additionally, multiple transcription factors (STAT3, NF-κB, KLF4, etc) and microRNA can act on IL-17 producing cells to induce IL-17 production[254]. This regulation can act in synergy with RORγT or independently of it, as with KLF4[254], suggesting that IL-17 could be produced without *Rorc* and outside of Th17 cells. These data and our own work suggest that if there is a role of IL-17 in the onset of depression, this cytokine does not solely originate from CD4+ cells or is produced by alternative cell types (ILC3s, γδT cells, or others) altogether.

The notion that Th17 cells play a critical role in the onset of depression has been growing in popularity but remains controversial. Several human studies have demonstrated that changes in IL-17 are correlated with depression[223, 236, 237, 255] while others have not[238, 239]. While further work is needed to fully understand the role of IL-17 in the onset of depressive symptoms, our work demonstrates that these cells are not necessary for the induction of stress-induced depression. Instead, we suggest there are alternative means or sites of IL-17 production in stress-induced depression. If no such mechanisms can be identified, a need to develop novel approaches to identify other potential causes of depression symptoms, such as the microbiome or other inflammatory signals is required.

Materials and Methods:

All methods were performed in accordance with guidelines and regulations of the University of Virginia and approved by the University of Virginia Animal Care and Use Committee.

Mice:

B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ (CD4Cre)[256] (#022071), B6(Cg)-*Rorc*^{tm3Litt}/J (Roryt^{fl}) [244] (#008771), and *AHR*$^{tm3.1Bra}$/J (Ahr^{fx}) (#006203) [257] mice were purchased from Jackson Laboratories. Mice were bred in house. Mice were kept on a 12-hour light/dark schedule. All behavioral interventions were performed between 8am and 3pm and animals were sacrificed between 7am and 1pm. All mice were housed in cages of up to 5 animals from birth until initiation of the stress protocol. All mice exposed to stress were at least 8 weeks of age and age matched to control animals. Stressed mice were housed individually without enrichment to enhance stress[258]. Naïve animals were housed in standard cages in groups of 2-5 mice of the same sex. All procedures were approved by the University of Virginia ACUC (protocol #3918).

Experimental Autoimmune Encephalomyelitis:

EAE was induced in 6-8-week old female *Cd4 Cre Ahr*$^{F/F}$ and *Ahr*$^{F/F}$ mice as previously described[259]. Briefly, mice are subcutaneously injected with MOG 35-55 and Complete Freund's Adjuvant. Two intraperitoneal injections of pertussis toxin are administered on days 0 and 1. Thirty-minute videos of mice were taken at day 25 post immunization and used for analysis. Healthy controls were age matched females.

Stress Experiments:

In our model of Unpredictable Chronic Stress (UCS), mice were exposed to a 2 hour period of a daily stressor (restraint, strobe light, or white noise). After the daily stressor, mice were placed in an overnight stress (cage tilt, 24 hour light exposure, wet bedding, or 2x cage change) until the

next induction of 2 hour stress (Supplemental table 2). UCS protocols were maintained for 3 weeks. For Unpredictable Chronic Restraint Stress (UCRS) experiments, mice were exposed to chronic restraint (ventilated 50mL conical vials) for a period of 2 hours daily for a period of three weeks. Once removed from restraint, an overnight stressor of either cage tilt, wet bedding, or 2x cage change was used (Supplemental table 3). All daily stressors were carried out between 8am and 5pm. Overnight stressors were started upon removal from the daily stressor and remained in place until the next day's daily stressor.

Behavioral Tests:

The forced swim, tail suspension, sucrose preference, open field, elevated plus maze, novel object recognition, marble burying, and three chamber social preference tests we performed as previously described[260-267]. All testing was recorded on a Hero Session 5 GoPro and analyzed with Noldus behavioral analysis software.

DeepLabCut:

Animal pose estimation: Animal pose estimation was performed by using a deep-learning package, DeepLabCut[246] (https://github.com/DeepLabCut/DeepLabCut). We generated a DeepLabCut convolutional neural network to analyze open field test videos, which is trained in a supervised manner: 16 manually labeled points were selected as references of transfer learning. 15 randomly selected videos were used for building a training dataset. Finally, the performance of the neural network is evaluated by researchers.

Unsupervised behavior classification: Estimated mouse poses from DeepLabCut were further analyzed by Variational Animal Motion Embedding (VAME)[268], which classifies animal behavior in an unsupervised manner (https://github.com/LINCellularNeuroscience/VAME). We trained a unique VAME recursive neural network for each experiment, which classifies each frame of the open field test video into 1 of the 35 behavioral motifs. Then, all behavior motifs were annotated and evaluated by blinded researchers. With annotated frames, we were able to calculate the percentage of time usage of each motif, which is then used for principal component analysis and Kullback-Leibler divergence analysis.

RNA Extraction and Quantitative PCR:

For RNA extraction, cultured cells were pelleted, frozen, lysed, and RNA extracted using the Bioline Isolate II RNA mini kit as per manufacture's protocol (BIO-52073). RNA was quantified with a Biotek Epoch Microplate Spectrophotometer. Normalized RNA was reverse transcribed to cDNA with either the Bioline SensiFast cDNA Synthesis Kit (BIO-65054) or Applied Sciences High-Capacity cDNA Reverse Transcriptase Kit (43-688-13). cDNA was amplified using the Bioline SensiFast NO-ROX kit (BIO-86020), according to manufacturer's instructions. The TaqMan GAPDH probe (Mm99999915_g1) was measured as a normalizer for each sample. The TaqMan probes Cyp1a1 (Mm00487218_m1), Ahr (Mm00478932_m1), Rorc (Mm01261019_g1), and Il17a (Mm00439618_m1) were used to measure transcript levels from the samples. Results were analyzed with the relative quantity ($\Delta\Delta$Cq) method.

CD4 T cells isolation and differentiation:

Naïve CD4 T cells were harvested and skewed as previously described[269]. After skewing, cells were washed and frozen for qPCR analysis or treated with an Ahr antagonist (I_3S-250250 µM, Sigma-Aldrich I3875), agonist (CH223191-10 µM, Tocris Bioscience 301326-22-7), or vehicle control (DMSO-Fisher Scientific D128-1) for 24 hours prior to freezing.

Tissue Harvest and Digestion:

After experimental manipulation, mice were perfused with 0.9% saline plus 5units/mL heparin (Medefil; MIH-3333) and tissues of interest were harvested and processed for flow cytometry as described below.

Small Intestine:

Whole small intestine was collected from the animals, flayed open and rinsed with ice cold HBSS (Gibco, 14175-095). Tissue was cut into ~2cm pieces and stored in 30mL of 5% FBS (R&D systems, S12450H) in HBSS until processing. Small intestine was shaken at 37°C for 20 minutes to remove mucus and debris. Gut pieces were filtered over mosquito net, placed in fresh 30mL of 5% FBS in HBSS, and shaken at 37°C for another 20 minutes. Samples were again filtered over mosquito net. Pieces were cut using a razor blade until fine slurry was created. Slurry was incubated in gut digestion buffer: Collagenase 8 (Sigma, C2139-5G), DNAse (Worthington, LS002139) in 5% FBS in HBSS-/- for 40 min, shaken at 150rpm at 37°C. Once digested, the solution was filtered through a 70µm filter and washed three times with 5% FBS in HBSS.

Lymph nodes and Peyer's Patches:

Single cell suspension in RPMI was prepared from Peyer's Patches and lymph nodes after fat removal by mechanical dissociation and subsequent filtration using sterile 70μm filters.

Meninges:

Meninges were dissected from skull caps in ice cold RPMI and digested in the digestion buffer: Collagenase 2 (Gibco, 17101-015), collagenase D (Sigma, 11088882001) and DNAse (Worthington, LS002139) for 20 minutes at 37°C. Once digested, meninges were physically dissociated with a 1mL pipette and filtered through a sterile 70μm filter.

Flow cytometry

Single cell suspensions were incubated with CD16/32 Fc Block and then stained with a 1:200 antibody dilution (1:100 for transcription factors). For intranuclear staining, the eBioscience FoxP3/Transcription Factor Staining Kit (00-5523-00) was used per manufacturer's instructions. Antibodies used are as follows: 488-conjugated CD8 (53-008182), APCe780-conjugated TCRβ (47-5961-82), e450-conjugated CD4 (48-0042-820, PE-conjugated RORγT (12-6981-82), and PE-Cy7-conjugated FoxP3 (25-5773-82), all purchased from Invitrogen. APC-conjugated CD45.2 (109813) was purchased from BioLegend. A Live/Dead discrimination dye Ghost Dye Violet 510 (Tonbo Biosciences; 13-0870) was used on all samples. OneComp eBeads (Thermo Fisher Scientific, 01-111-42) were used for all color controls except for the viability dye in which cells were used. Flow cytometry was performed using a Beckman Coulter Gallios flow cytometer and data were analyzed with FlowJo software v10.7.1.

Statistical Analysis:

All statistical analyses-except those associated with DeepLabCut-were performed in GraphPad Prism 9. Analyses involving two groups were performed using a two-tailed T test. If the variances between groups were significantly different, a Welch's correction was applied. Outliers were excluded if they fell more than two standard deviations from the mean. For all analyses, the threshold for significance was at $p<0.05$. Repeats for each experiment are specified in the figure legend corresponding to the respective panel. All p values and statistical tests are reported in Supplemental table 1.

Chapter 4: The Gut Microbiome and Depression

In recent years, work has begun to associate the onset of depression with changes in the gut microbiome[270]. The gut microbiome is defined as the dynamic population of microorganisms that live in the gastrointestinal (GI) tract, and is estimated to be composed of over 100 trillion organisms[271]. The composition of the microbiome is driven by many different factors, ranging from method of birth to environment and diet, and is known to change with age[272-274]. In general, there is a symbiotic relationship between the gut microbes living in the GI tract and the host[275]. For example, while larger organisms provide a home and nutrients to microbes, microbes can shape the immune system, protect against pathogens, change gut permeability, and regulate energy metabolism[276-279]. As such, it has been well established that dysregulation of the microbiome can potentiate disease states. Much of the original work establishing the

microbiome's role in disease has been in the context of intestinal ailments, such as irritable bowel syndrome and colitis [280]. However, as research has progressed, it has been shown that the microbiome can play a role in a wider variety of disorders than previously expected.

Evidence for Microbiome Changes in Depression:

Both animal models of depression and human patients with depression have exhibited changes in their microbiomes[281]. While many changes in the composition of the microbiome have been reported in depression or depressive-like models, a few trends have consistently surfaced. It is generally believed that increases in some groups of bacteria, like Bacteroidetes, have an overall negative impact on depressive outcomes, while increases in others, like Lactobacillus or Bifidobacterium, have a positive outcome on mood disorders[281-283]. However, the exact mechanisms for how specific bacteria impact mood are not fully understood. While human works have demonstrated causation between specific bacterial changes and depression, mouse models have been able to provide stronger links between bacteria and changes in physiology. In 2017, Marin et. al demonstrated that supplementation of *lactobacillus Ruteri* into the diet of mice experiencing depressive-like symptoms reversed behavioral changes[284]. These data suggest that *lactobacillus* plays an important role in either the initiation or maintenance of depressive-like symptoms in mice. Further highlighting the critical role for the microbiome in the onset of depression, data has shown that depressive-like behaviors can be transferred to animals through fecal microbiome transfers, suggesting that the microbiome is sufficient to drive depressive-like behaviors[285, 286]. This has also been demonstrated in our own lab by Andrea

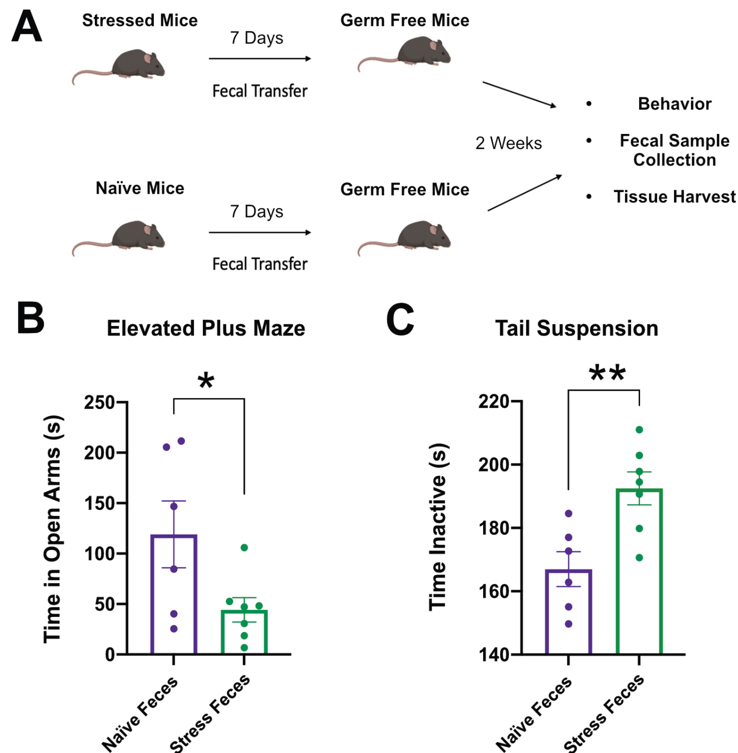

Figure 14: Microbiota from Stressed Mice Drives Anxiety- and Depressive-like Behaviors: (A) Schematic representing microbiome transfer experimental design. (B) Elevated plus maze demonstrating anxiety-like behaviors in ex-germ free mice that have received a stressed microbiome. (C) Tail suspension test demonstrating depressive-like behaviors in mice that received a stressed microbiome. Male mice, n=6-7/group, t-tests. Data courtesy of Andrea Merchak.

Merchak who gave germ free mice fecal pellets from either stressed or naïve mice and found that depressive- and anxiety-like behaviors were transferred into the ex-germ-free cohort (Fig. 14A-C). Together, these examples provide strong evidence that the microbiome contributes to the onset of depressive symptoms and may be a therapeutic target for depression treatments.

While there is clear evidence for a role for the microbiome in depression, understanding the factors that induce dysbiosis is necessary to understanding the true causes of this disorder. Depression is multifactorial; however, stress has been shown to be a major depression risk factor in both humans and in mice[71, 72, 128, 217, 250, 287-296]. Both stressful events and

direct exposure to stress hormones have been shown to bring the onset of depressive-like symptoms in animal models [118, 262, 284, 290, 297-303]. Importantly, stress and changes in the microbiome have also been extensively linked. In 1974 it was shown that mice exposed to environmental and dietary stress exhibited changes in their microbiome[304]. This work has been expounded upon in countless studies demonstrating that stress exposure is sufficient to change the composition of the microbiome in animal models ranging from mice to primates[217, 250, 284, 288-290, 302, 305-309]. While not as tested in humans, stress exposure is known to change the human microbiome and bacterial supplementation can help mitigate those effects[310-312]. For example, medical students exposed to academic stress were given fermented milk supplemented with or without *Lactobacillus casei,* a probiotic. Students that received the *Lactobacillus* experienced preserved gut diversity and reduced abdominal dysfunction compared to those who did not[312]. While more work around the human microbiome and stress is needed, these data validate that stress impacts the human microbiota and preventing these changes can have beneficial impacts on health.

Literature clearly supports the role of the microbiome in depression. As such, the microbiome has become a viable therapeutic target. In mice, this approach has shown success with microbe-targeting treatments reversing depressive-like behaviors[284, 286, 289, 306, 313, 314]. However, probiotics have had mixed results in humans[315, 316]. This is largely due to the complexity and heterogeneity of the human microbiome[317-319]. To bypass these limitations, more work is needed to identify the upstream mediators of microbiome dysbiosis in stress-induced depression. One possible mediator is the mucosal layer.

The Mucosal Layer and the Microbiome

The intestinal mucosal layer is a critical component of gut health. Dogma suggests the main function of the mucus layer is to provide protection for intestinal epithelial cells against the harsh digestive environment and microbiome[320]. However, recent works have demonstrated the complexity of this layer and the integral role it plays in maintaining gut homeostasis.

Mucus is a gel-like layer made of highly glycosylated proteins called mucins. Coating many epitheliums within the body, mucins come in two distinct types: soluble mucins and membrane bound mucins. Soluble mucins compose the gel-like structure that coats an organ, while membrane bound mucins remain attached to the cell epithelium and form the glycocalyx[321]. One of the main functions of mucus is to act as a protective barrier for an organ or tissue, preventing unwanted pathogen interactions[322]. This is especially critical in the gut, where trillions of microbes have the potential to trigger the host immune system and cause disease[323]. In addition to this protective function, the gut mucus layer also serves as a nutrient reservoir and anchor point for commensal bacteria[321].

The mucosal layer is uniquely positioned to shape the microbiome. As the natural food source and anchor point for the microbes that live in the gut, the mucosal layer serves as a connection point for the microbiome[321]. Although the microbiome can influence the mucus layer, mucins can also impact the content of the microbiome and therefore alter communication between the gut and the brain. Indeed, the mucosal layer can select for specific bacteria based on patterns of mucin glycosylation in the gastrointestinal (GI) tract[323]. It has also been demonstrated that stress, a major risk factor for depression, can shift the O-glycosylation patterns of the mucosal layer, supporting the idea that stress may be able to change the mucosal

layer in a way that induces dysbiosis. Additionally, glycocalyx mucins have been shown to influence the shape of microvilli, critical structures of the intestines involved in nutrient absorption and epithelial and microbiome interactions[324, 325]. Increased oxidative stress and inflammation, also associated with chronic stress, have been shown to change microvilli structures, further supporting the idea that stress could tune the microbiome composition[326]. Strikingly, genome wide association studies have implicated mucins in depression[56, 327]. For example, in a study examining genetic mutations in treatment refractory depression, pathway analysis revealed changes in the O-linked glycosylation of mucins[327]. Additionally, mucin 13 has been specifically implicated in a genome-wide association study of depressive symptoms in a Hispanic community[56]. Taken together, this suggests that stress induced mucus changes may be responsible for microbiota dysbiosis. This induction of dysbiosis could then continue to feedback on depression through previously described contributors to depression, such as microbial metabolites or the immune system.

Depression remains a significant public health concern. Much work has been dedicated to the discover and production of depression treatments. However, depression treatments are effective in only a subset of patients and often do not correct all symptoms. In light of this, more work is needed to investigate the root causes of depression in order to provide alternative management options for the disease.

Because stress represents a major risk factor for depression, areas of the body that are impacted by stress should be looked at to identify potential sources of initiating factors. The microbiome has been shown to be heavily impacted by stress and changes to its composition are associated with depressive outcomes. However, therapeutic targeting of the microbiome

remains difficult given the complexity of bacterial interactions and the variability between patients. Additionally, the way in which stress changes the microbiome is not understood. This gap in knowledge may be the missing link capable of connecting many of the factors that propagate depressive states and providing a targetable option for future therapeutics. Logically, it follows that closer examination of what areas stress can act on near the microbial niche may provide clues to how it can cause dysbiosis. One such candidate lies in the mucosal layer of the small intestine.

Chapter 5: The Role of Mucin 13 in Stress-Induced Depression

Given the importance of microbiome dysbiosis in depression and the role of the mucosal layer in microbiome homeostasis, I wanted to examine the relationship between mucins and depression. I utilized a newly developed mouse line that lacked mucin 13 in combination with our robust model of unpredictable chronic mild restraint stress to test the hypothesis that the mucosal layer is a mediator of stress-induced dysbiosis. The following chapter is from my first author publication entitled "Mucin 13 Regulates Microbiome Dysbiosis and Depressive-like Behaviors in a Model of Chronic Mild Restraint Stress".

Abstract

Depression is a common mental health condition with a large impact on the economy and society. While depression etiology is multifactorial; chronic stress is a well-accepted contributor

to the disease. Depression is associated with altered gut microbial signatures that can be replicated in animal models. While targeted restoration of the microbiome has been shown to reduce depressive-like behaviors in mice, the complexity and diversity of the human microbiome has complicated therapeutic intervention in patients. To circumvent the current microbiome therapeutic limitations, there is a critical need for identifying pathways responsible for microbiome dysbiosis. Here, we identify that that the mucosal layer, and specifically, expression of the transmembrane protein mucin 13, can regulate microbiome composition in a model of stress-induced depression. To demonstrate this, we use a model of unpredictable chronic mild stress to induce anxiety- and depressive-like behaviors in mice and alter the microbiome. After stress exposure, we a significant reduction in mucin 13 expression across the intestines. We further present gene expression data correlating *Muc13* expression reductions and stress-induced alterations in circadian rhythms. Furthermore, we show that deleting *Muc13* leads to gut dysbiosis, and baseline behavioral changes normally observed after stress exposure. Lastly, we demonstrate that *Muc13* deletion increases susceptibility to stress-induced behavioral changes. Together, these results demonstrate that mucosal layer disruption is an initiating event in stress-induced dysbiosis and offers mucin 13 as a potential new therapeutic target for microbiome dysbiosis in stress-induced depression.

Introduction:

Depression and anxiety impact millions of people worldwide[328]. While many treatments exist for these disorders, high rates of treatment-resistant cases remain[329]. While many factors can influence the onset of depression and anxiety, stress is thought to be a large contributing

factor[330, 331]. Depression has also recently become associated with changes in the gut microbiome[313, 332]. Gut microbiome dysbiosis has been observed in both humans and animal models of depression and has been targeted as a potential treatment for mental health disorders[284, 317, 333]. In fact, targeted restoration of the microbiome has been shown to reduce depression symptoms in humans and depressive-like behaviors in mice[284, 334]. While promising, efforts to therapeutically manipulate the host microbiome remain inconsistent[317]. These inconsistencies are thought to be due to the complexity of the microbiome, unknown microbe-microbe interactions, failure of therapeutic microbes to colonize, availability of resources for microbes, and heterogeneity between hosts[317-319]. Unfortunately, these challenges limit the potential for broadly applicable microbiome therapeutics to treat disease states, including depression. To circumvent the current microbiome therapeutic limitations, there is a critical need for identifying conserved regulators of the microbiome that can be targeted with new therapeutics. Here, we address this gap in knowledge by investigating how stress initiates a change in the microbiome.

Our results demonstrate that stress disrupts the mucosal layer by driving reductions in the transmembrane protein, mucin 13. We further show that reductions in mucin 13 correlate with stress-induced alterations in circadian rhythms, suggesting a connection between stress, circadian rhythm changes, and mucosal layer disruptions. Finally, we demonstrate that deletion of *Muc13* drives both microbiome dysbiosis and depressive-like behaviors at baseline and renders animals more susceptible to behavioral changes after stress exposure.

Results:

Unpredictable Chronic Mild Restraint Stress Drives Anxiety- and Depressive-like Behaviors and Modifies the Gut Microbiome Composition:

To investigate the biological underpinnings of stress-induced microbiome dysbiosis we exposed mice to unpredictable chronic mild restraint stress (UCMRS), a model known to induce anxiety- and depressive-like behaviors and alter the gut microbiome (Fig. 15A)[284]. After three weeks, mice exposed to UCMRS, but not naïve controls, showed a significant increase in anxiety-like behaviors characterized by an increase in nestlet shredding and decrease in time spend in the open arms of the elevated plus maze were observed (Fig. 15B). Depressive-like behaviors presented with increases in the time spent inactive in the tail suspension and forced swim tests, in combination with a decrease in sucrose preference (Fig. 15B). In addition to behavioral readouts, levels of murine stress-associated markers were measured in serum by mass spectrometry (Fig 15C and Fig. 16A-G). An increase in murine cortisol levels and a decrease in both serotonin and glutamate in the serum of stressed mice were observed, complementing our behavioral data showing that UCMRS induces robust anxiety- and depressive-like phenotypes in mice (Fig. 15C)[335, 336].

We next evaluated the microbiome composition changes in mice exposed to UCMRS by performing 16S sequencing on isolated fecal DNA prepared from stressed animals and naïve controls. No changes in alpha diversity (Fig. 15D) or evenness (Fig. 17A) were observed between groups. However, Non-metric Multidimensional Scaling (NMDS) of sample beta diversity revealed significant separation between naïve and UCMRS exposed animals by PERMANOVA analysis (Fig. 15E)[337]. As expected, stress drove significant changes in the percent abundance of several

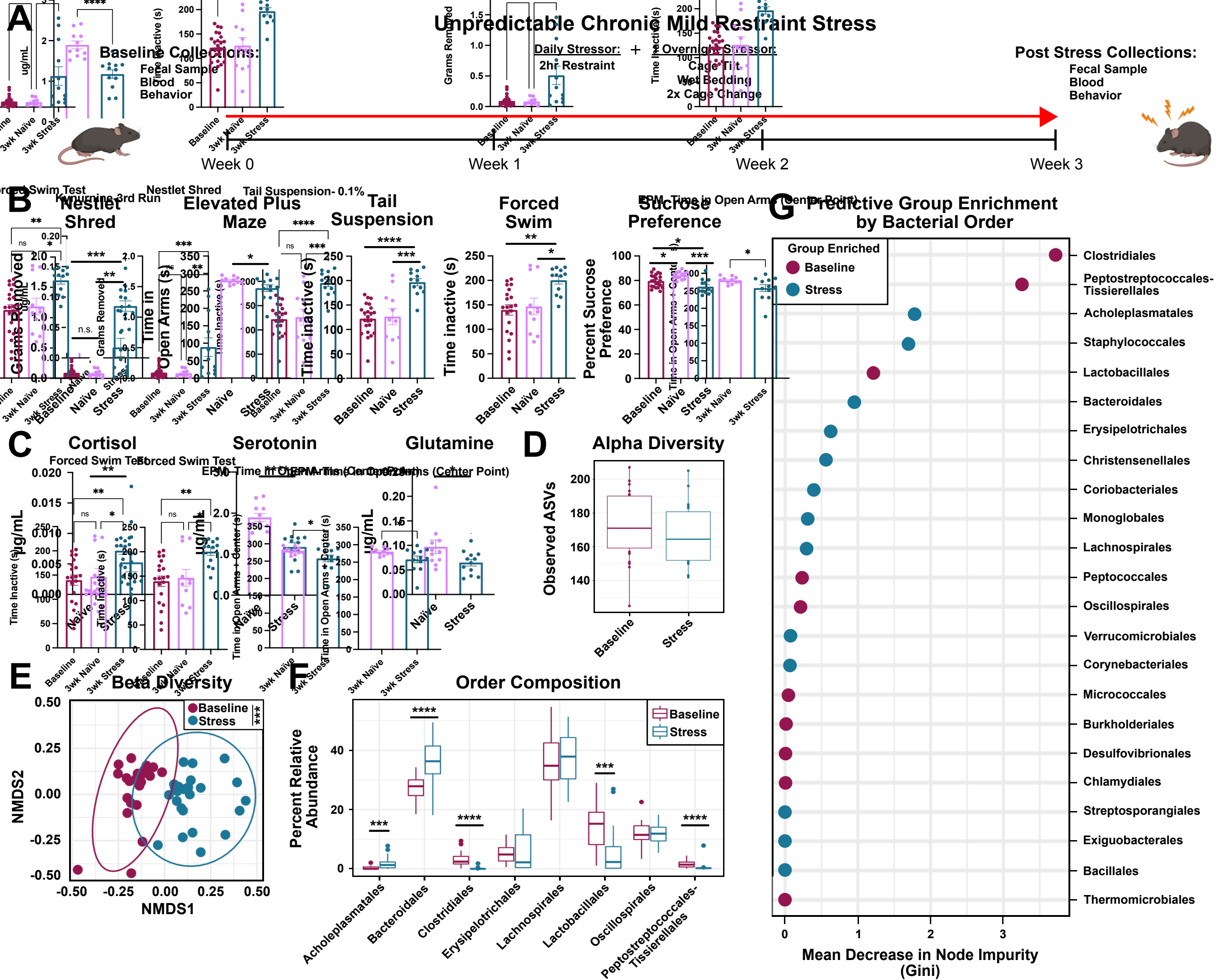

Figure 15: Unpredictable Chronic Mild Restraint Stress Induces Anxiety and Depressive-like Behaviors and Microbiome Dysbiosis: (A) Schematic of Unpredictable Chronic Mild Restraint Stress (UCMRS). (B) Nestlet shred (1WANOVA), elevated plus maze (t-test), tail suspension (1WANOVA), forced swim (1WANOVA), and sucrose preference (1WANOVA) tests between baseline, naïve controls, and UCMRS exposed animals. Male mice, n=11/12 per group. Representative graphs of 2 experiments. (C) Mass spectrometry analysis of cortisol, serotonin, and glutamine in the serum of naïve or UCMRS animals (t-tests). (D) Alpha diversity plot showing observed ASVs between baseline and UCMRS exposed mice (t-test). (E) Beta-diversity Non-metric Multi-dimensional Scaling (NMDS) plot comparing baseline and stress fecal microbiome samples (PERMANOVA). (F) Relative abundances of bacterial orders >1% (Wilcoxon Rank Sum test with Bonferroni correction for multiple comparisons). (G) Random forest model predicting bacterial orders that best discriminate between baseline and UCMRS groups. Importance is based on the mean decrease in node impurity, with larger values being more important to the model. Male mice, n=24/group, N=1.

bacterial orders including reductions in Clostridiales, Lactobacillales, and an expansion of Bacteroidales (Fig. 15F and Fig. 17B). Lastly, a random forest model accurately predicted the bacterial orders associated with either baseline or stressed groups, strengthening the link between stress and microbiome dysbiosis (Fig. 15G). Taken together, these results demonstrate that unpredictable chronic mild restraint stress (UCMRS) induces anxiety- and depressive-like behaviors in mice, alters murine stress hormone levels, and changes microbial composition at the bacterial order level.

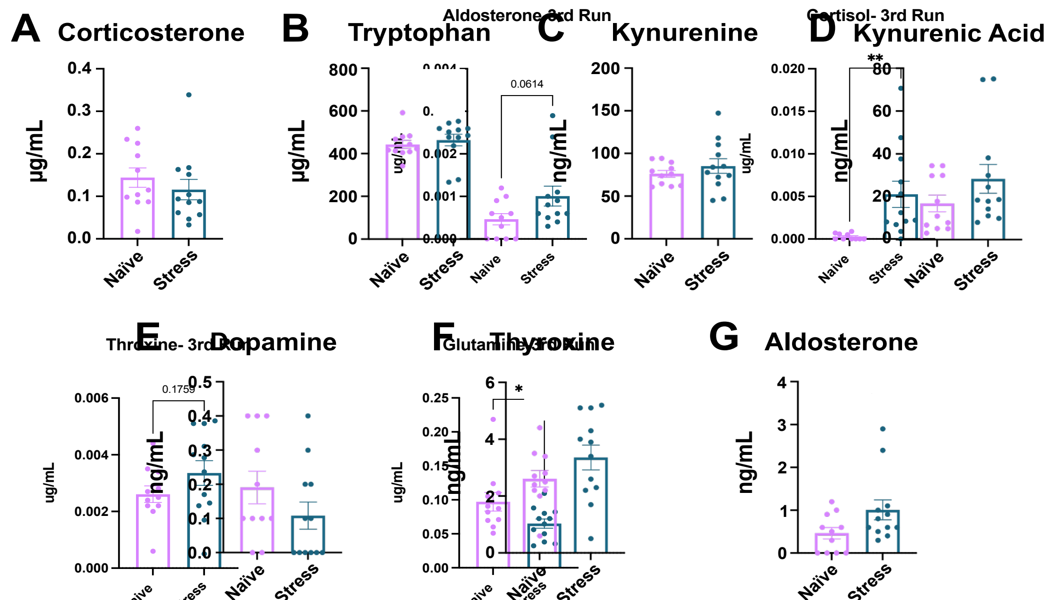

Figure 16: Mass Spectrometry of Serum Molecules Associated with Stress: Mass spectrometry analysis of serum from naïve or stress mice. (A) corticosterone, (B) tryptophan, (C) kynurenine, (D) kynurenic acid, (E) dopamine, (F) thyroxine, (G) aldosterone. Male mice, n=11/12 per group, N=1.

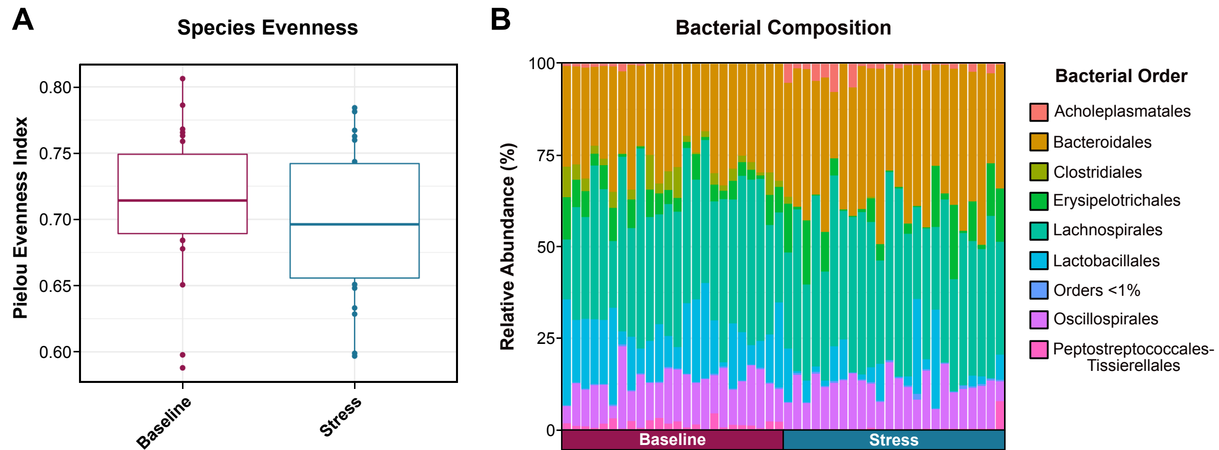

Figure 17: Bacterial Evenness and Composition from Baseline or Stress Mice: (A) Pielou evenness index for baseline or stress mice. (B) Individual order relative abundances for baseline or stress mice. Male mice, n=24/group, N=1.

Unpredictable Chronic Mild Restraint Stress Reduces Mucin 13 Expression *In Vivo*:

While it is well accepted that stress can change the microbiome in humans and mice (Fig. 15), the mechanism leading to a change in bacterial composition remains unknown[281, 289, 309, 311, 317]. Thus, we sought to examine how stress exposure induces microbial dysbiosis by identifying host specific mediators of bacterial changes. As the mucosal layer, composed of proteins called mucins, provides both an anchor point and nutrient reservoir for bacteria [338-340], we hypothesized that a stress-induced change in mucus composition could induce microbiome dysbiosis by changing the microbial niche. To test this, we examined mucin RNA expression from individual sections of the intestines in mice exposed to UCMRS and naïve controls (Fig. 18). Interestingly, we found that of all the mucins expressed in the gut (muc1, 2, 3/17, 4, and 13) only mucin 13 was significantly reduced across the small and large intestine (Fig. 18B-C and Fig. 19A-C). To confirm these results, we examined mucin expression in another strain of mice that had been exposed to stress. We found that in BALB/cJ mice exposed to stress, mucin 13 was also

reduce in the small intestine, while mucin 2 remained unchanged in the small intestine (Fig. 19D-E). Mucin 2 is highly expressed in the colon and could not be quantified by qPCR because the transcript was more abundant than all housekeeping genes tested. Taken together, these results suggest that stress induces changes in the mucosal layer in a protein specific manner, and that these changes are conserved across different mouse strains.

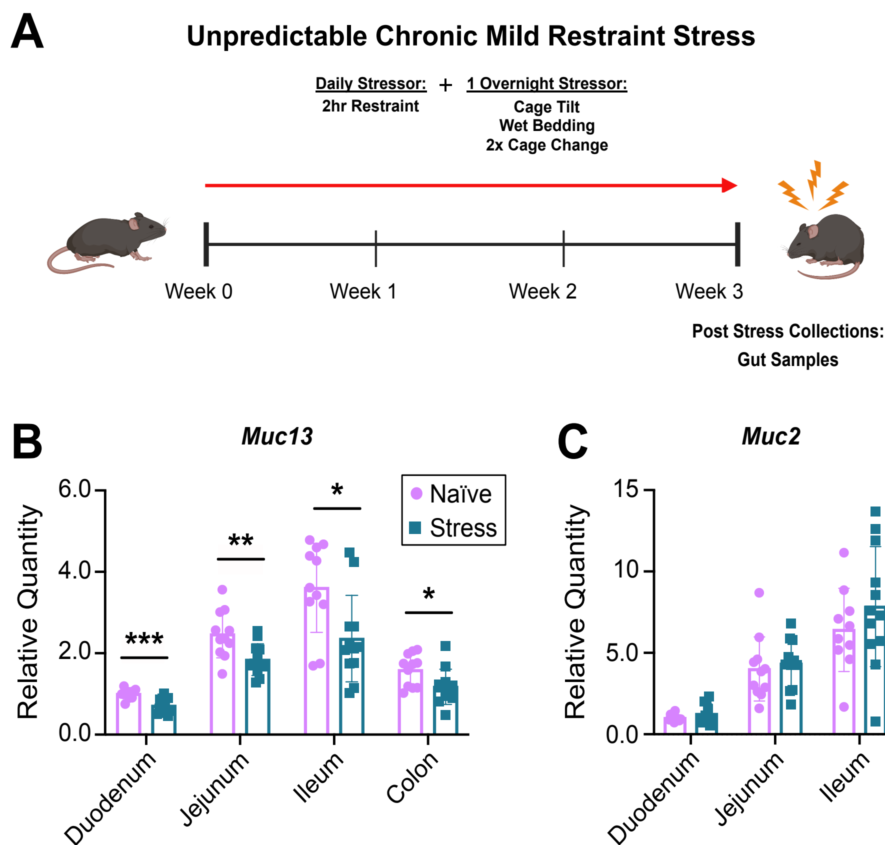

Figure 18: Mucin 13 Expression is Reduced after UCMRS Exposure: (A) Schematic representing Unpredictable Chronic Mild Restraint Stress (UCMRS). (B) Relative quantity of *Muc13* transcripts by qPCR in individual sections of the intestine. (C) Relative quantity of *Muc2* transcripts by qPCR in individual sections of the intestine. 2WANOVA with Multiple Comparisons. Male mice, n=11/12 per group, representative graph of 2 experiments.

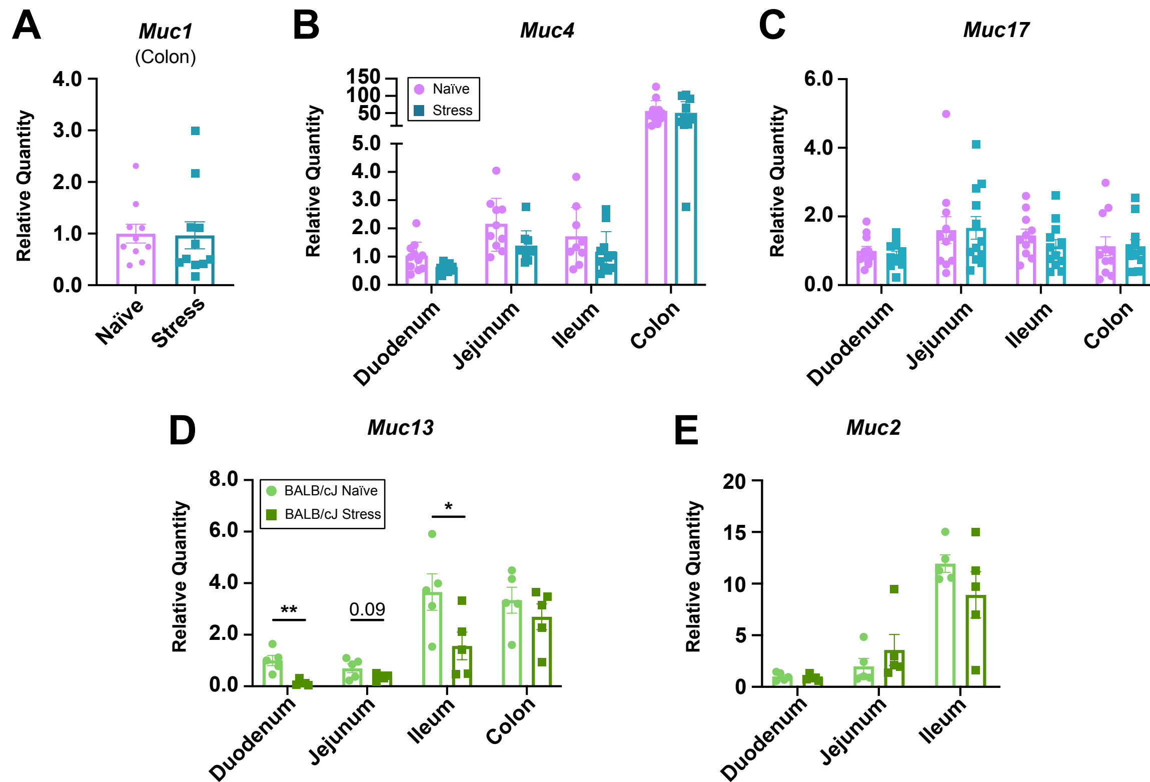

Figure 19: Mucin Expression Changes in Stress and BALB/cJ Mice: (A) Relative quantity of *Muc1* expression in colon of naïve or stress mice by qPCR. *Muc*1 was not detected in other sections (t-test). Relative quantities of (B) *Muc4* and (C) *Muc3/17* expression in the intestines of naïve or stress mice by qPCR. Multiple t-tests. Male mice. Experiments A-C, n=11/12 per group. Experiments D-E, n=5 per group. N=1.

Stress Hormones, Cytokines, Vagal Tone, and the Microbiome do not Reduce Mucin 13 Expression

To understand the mechanism behind these stress-induced reductions in mucin 13, we began by exploring pathways and systems documented to be impacted or driven by stress. First, we tested the direct impact of cortisol, a major stress hormone, on intestinal tissues.

Organoids derived from both human and mouse intestinal tissues were treated with hydrocortisone, a precursor to cortisol (Fig. 20A) [341, 342]. Interestingly, no reductions in mucin 13 expression were observed in either human (Fig. 20B) or mouse (Fig. 20D) organoids, suggesting

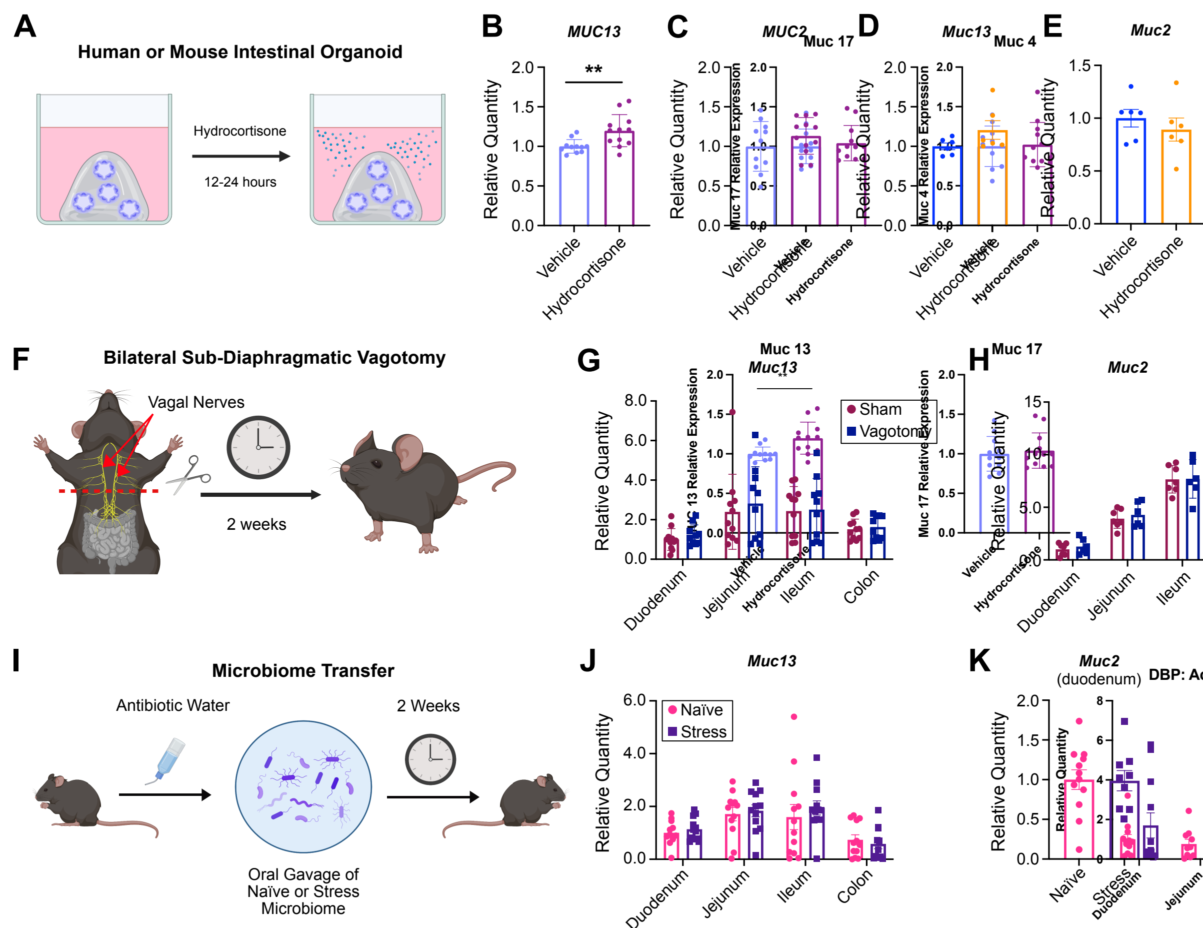

Figure 20: Stress Hormones, the Vagal Nerve, and the Microbiome in Mucin Expression Changes: (A) Schematic representing treatment of organoids with hydrocortisone. Relative quantity of (B) *MUC13* and (C) *MUC2* in human duodenal organoids treated with 200µM hydrocortisone for 12 hours. N=1, n=12 per group. T-tests. Relative quantity of (D) *Muc13* and (E) *Muc2* in mouse jejunal organoids treated for 24 hours with 5ng of hydrocortisone. N=1, n= 6 per group. T-tests. (F) Schematic representing bi-lateral sub-diaphragmatic vagotomy experimental design. Relative quantities of (G) *Muc13* and (H) *Muc2* transcripts in the intestines of sham or vagotomized mice. N=2, n=10 per group. Male Mice. 2WANOVA with multiple comparisons. (I) Schematic representing microbiome transfer experimental design. Relative quantities of (J) *Muc13* and (K) *Muc2* transcripts in the intestines of animals receiving a naïve or stress fecal microbiome. Male mice, n=12 per group. *Muc2* only detected in the duodenum. 2WANOVA with multiple comparisons (J) or t-test (K).

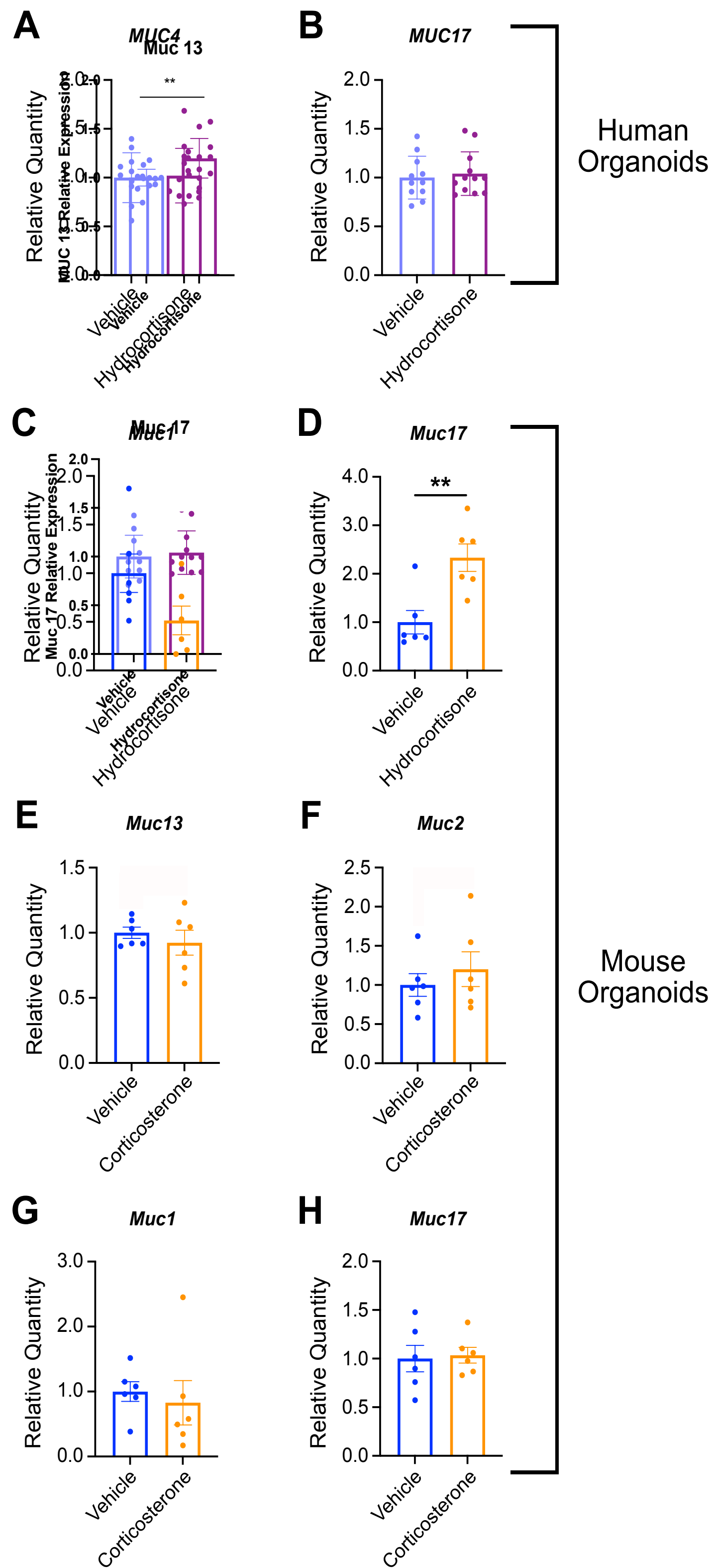

Figure 21: Mucin Changes in Human and Mouse Organoids: Relative quantities of (A) *MUC4* and (B) *MUC17* in human duodenal organoids treated with 200µM hydrocortisone for 12 hours. N=1, n=12 per group. T-tests. Relative quantities of (C) *Muc1* (D) *Muc17* of mouse jejunal organoids treated for 24 hours with 5ng of hydrocortisone. N=1, n= 6 per group. T-tests. Relative quantities of (E) *Muc13*, (F) *Muc2*, (G) *Muc1*, and (H) *Muc17* in mouse jejunal organoids treated for 24 hours with 150 µg of corticosterone. N=1, n= 6 per group. T-tests.

that hydrocortisone does not directly act on intestinal cells to reduce mucin 13 expression *in vitro*. To gain a full picture of cortisol's direct impact on intestinal cells, we also examined expression of other mucins expressed in organoids. We found that none of the mucins tested (Muc1, Muc2, Muc3/17, MUC2, MUC4, and MUC17) had reductions in expression after treatment (Fig. 20C and E and Fig. 21A-H). These results suggest that cortisol does not directly impact the intestinal production of mucin 13 by enterocytes.

The immune system has long been understood to be influenced by changes in stress hormones[343]. In addition, the immune system been known to be impacted by the microbiome and can also impact the mucosal layer [344]. Thus, we hypothesized that stress may be inducing changes in the immune system that would indirectly reduce mucin 13. To test this, we exposed two human gut cell lines known to express mucins (HT-29 and Caco-2) to several cytokines known to be associated with depression, stress, or changes in the intestines (IFNγ, TNFα, IL-1β, IL-17a, IL-6, IL-33, and IL-4) (Fig. 22A) [234, 345, 346]. We then extracted RNA and looked for changes in mucin expression. However, none of the cytokines tested demonstrated a reduction in *muc13* expression *in vitro* (Fig. 22B-L). Interestingly, IL-1β, IFNγ, IL-4 and IL-33 were found to increase muc13 expression *in vitro* in at least one of the cell types tested (Fig. 22B-C, G-H, K-L). These results suggested that while increasing exposure to cytokines *in vitro* did not decrease mucin 13 expression, it was possible that a reduction in cytokines *in vivo* may correlate with the observed reduction in mucin 13. Thus, we sought to determine if IL-1β, IFNγ, IL-4 and IL-33 were reduced in the intestines after stress exposure *in vivo*. We again examined intestinal tissue from naïve or UCMRS exposed mice but found no changes in any of the cytokines tested (Fig. 22M-P). These

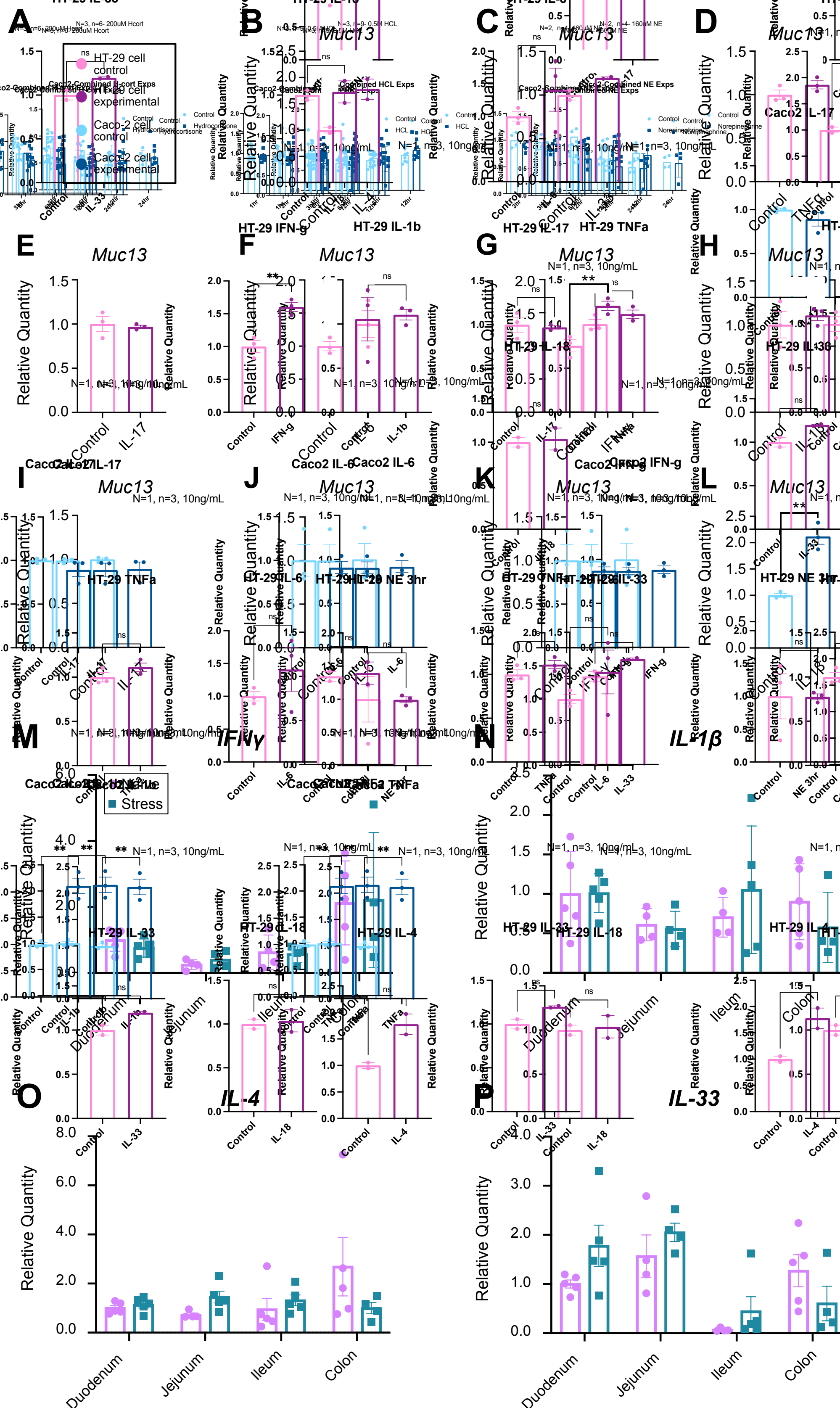

Figure 22: Impacts of Cytokines on Mucin 13 Expression: (A) Key explaining the color coding of graphs B-L. Relative quantities of *Muc13* after exposure to 10ng of (B) IL-4, (C) IL-33, (D) TNF-α, (E and I) IL-17, (F and J) IL-6, (G and K) IFN-γ, or (H and L) IL-1β in Caco-2 or HT-29 cells. T-tests, N=1. Relative quantities of *in vivo* expression of (M) IFN-γ, (N) IL-1β, (O) IL-4, or (P) IL-33 in the intestines of naïve or stress mice. 2WANOVA, Male mice, n=5 per group, N=1.

results suggest that stress-induced changes in cytokines are unlikely to be driving the observed reductions in mucin 13 after UCMRS exposure (Fig. 22).

In addition to the immune system and stress hormones, vagal tone is known to be strongly impacted by stress and altered in depression[347]. We therefore sought to examine the impacts of vagal tone in mucin 13 expression changes by manipulating the Vagal nerve *in vivo*. To do this, we performed both vagal nerve stimulation (VNS) (Fig. 23C) or bilateral subdiaphragmatic vagotomy (Fig. 20F). In the vagotomy paradigm, mice were allowed to recover for 2 weeks before having RNA extracted from each section of the intestines (Fig. 20F). Samples were collected from the VNS animals 30 hours after stimulation. Mucin expression was examined in both groups. Surprisingly, no changes in mucin 13 expression were observed in either experimental model (Fig. 20G and Fig. 23D). Changes in mucin 2 were also not detected in either group, nor were changes observed in muc4 or 3/17 in the vagotomy experiment (Fig. 20H and Fig. 23A-E). Together, these data demonstrate that neither bilateral sub-diaphragmatic vagotomy nor VNS drive reductions in mucin 13 expression, suggesting that stress may not act acting through the Vagal nerve to induce mucosal changes.

Finally, while our central question aimed to understand how stress changes the microbiome, it is possible that changes in the microbiome could be inducing further changes to the gastrointestinal tract. In fact, the microbiome is well known to influence the mucus layer [340, 348]. To ensure that the changes in mucin 13 expression observed in stress were not induced by changes in the microbiome, we transferred fecal microbiomes from naïve or UCMRS exposed mice into mice treated with antibiotics for 2 weeks (Fig. 20I). Two weeks post reconstitution, intestinal samples were collected and processed for RNA extraction. Expression of mucin and 13

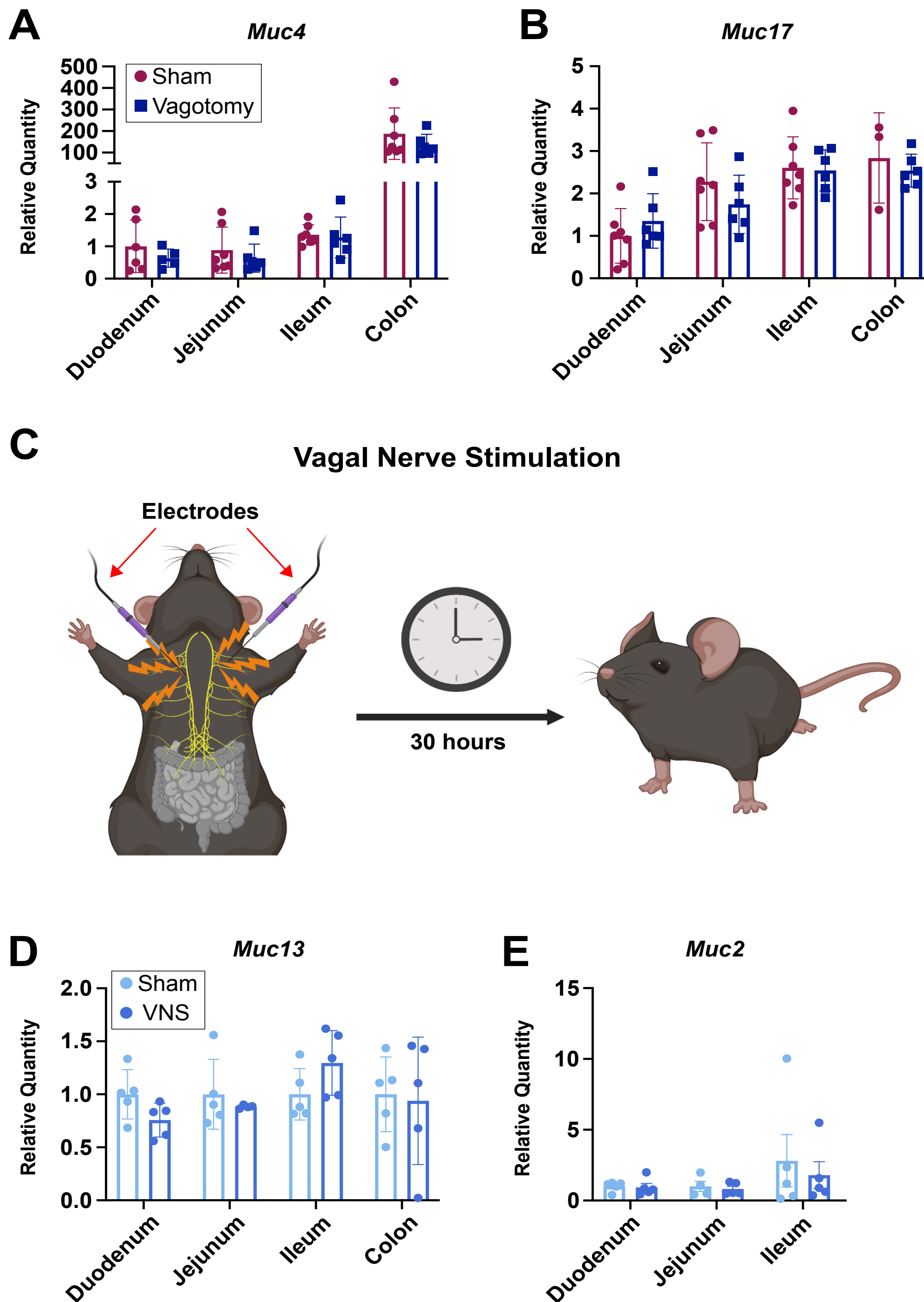

Figure 23: Mucin Changes in Bilateral Sub-diaphragmatic Vagotomy and Vagal Nerve Stimulation: Relative quantities of (A) *Muc4* and (B) *Muc17* in the intestines of vagotomized mice or sham mice. Male mice, n=6 per group, N=2, 2WANOVA. (C) Schematic of vagal nerve a experimental design. Relative quantities of (D) *Muc13* and (E) *Muc2* in the intestines of stimulated or sham mice. Male mice, n=6 per group, N=1, 2WANOVA.

was examined by qPCR. No change in mucin 2 was detected in the duodenum between groups (Fig. 20K). Mucin 2 in the remaining sections was unquantifiable due to amplification before housekeeping probes. This was expected as introduction of a microbiome induces strong mucin 2 production[349]. Importantly, no change in mucin 13 was detected between groups (Fig. 20J), suggesting that mucin 13 changes precede microbiome dysbiosis.

Circadian Genes are Significantly Changed in Stress Exposure

After examining several of the most likely mediators of stress-induced mucin 13 changes, we performed bulk RNA sequencing on the duodenal tissue of stressed and naïve mice to identify genes that were significantly changed after stress exposure (Fig. 24A). Surprisingly, genes associated with circadian rhythms were significantly changed (Fig. 24B). We then confirmed our sequencing results via qPCR. RNA from individual sections of the intestines were quantified with qPCR. As with RNA sequencing, we found circadian genes were disrupted in our model of UCMRS (Fig. 24C-E). Both *Dbp* and *Ciart* were found to be upregulated after stress exposure, while *Bmal* expression was significantly down-regulated (Fig. 24C-E). We then investigated if additional circadian genes (*per1*, *per2*, *per3*, and *rev-erb*α and β) were disrupted in stress by qPCR (Fig. 25A-E). Surprisingly, we found no changes between stress exposed and naïve groups, suggesting that only a subset of circadian genes are impacted by stress exposure (Fig. 25A-E). These results also suggest that circadian rhythms are disrupted, rather than simply shifted as there is not a significant change in all circadian genes in the feedback loop (Fig. 25A-E). Taken together, these data suggest that stress significantly impacts circadian gene expression. Further work is needed

to determine how the changes in circadian rhythms relate to the downregulation of mucin 13 expression.

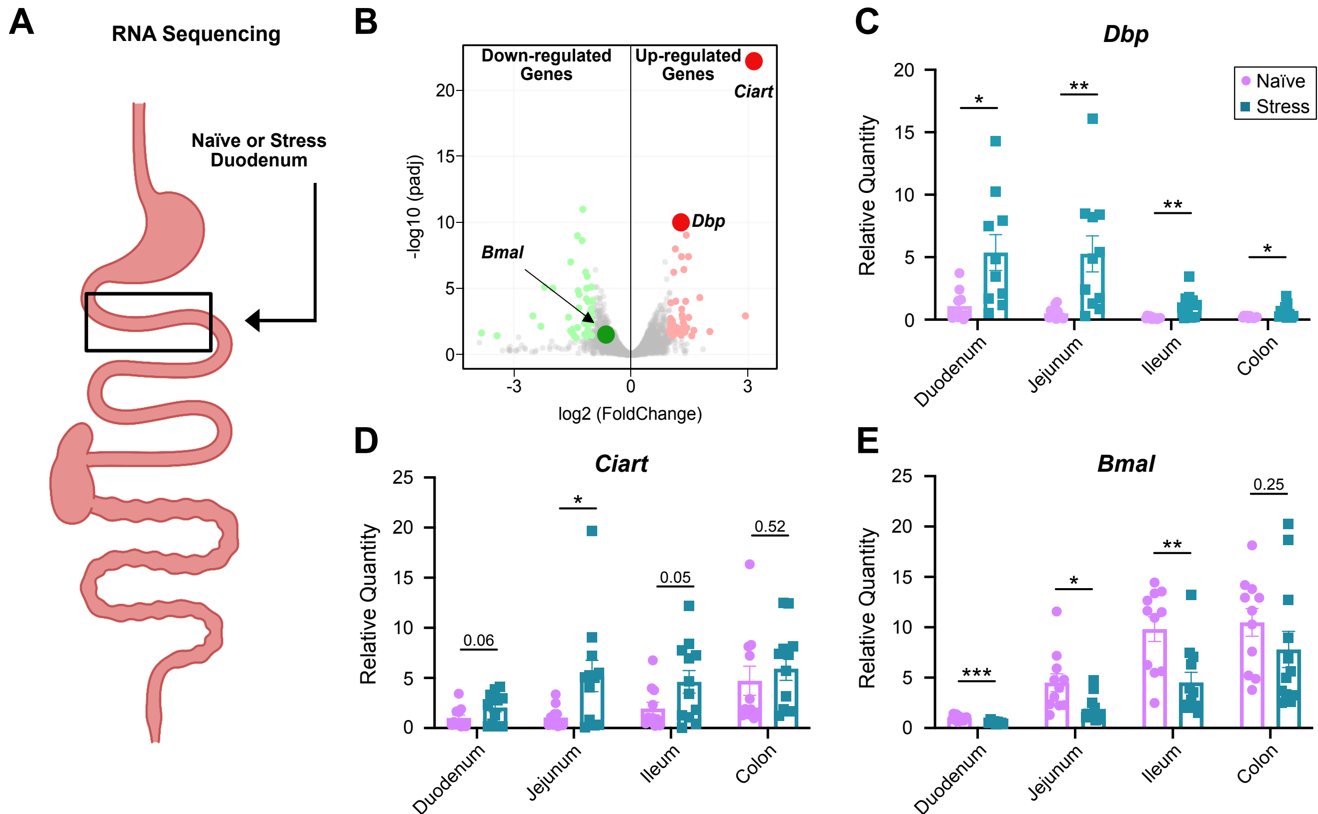

Figure 24: Clock Gene Expression Changes in the Intestines after Stress Exposure: (A) Schematic representing RNA sequencing experimental design. (B) Volcano plot of mouse duodenal genes altered by stress. Each dot is representative of 1 gene. Up-regulated genes with an adjusted p-value of less than 0.05 and log2 fold change greater than 1 are indicated by red dots. Down-regulated genes with an adjusted p-value of less than 0.05 and log2 fold change less than -1 are indicated by green dots. Relative quantities of (C) *Dbp*, (D) *Ciart*, or (E) *Bmal* in the intestines of naïve or stress mice. Male mice, n=11-12 per group. Multiple T-tests, representative of 2 experiments.

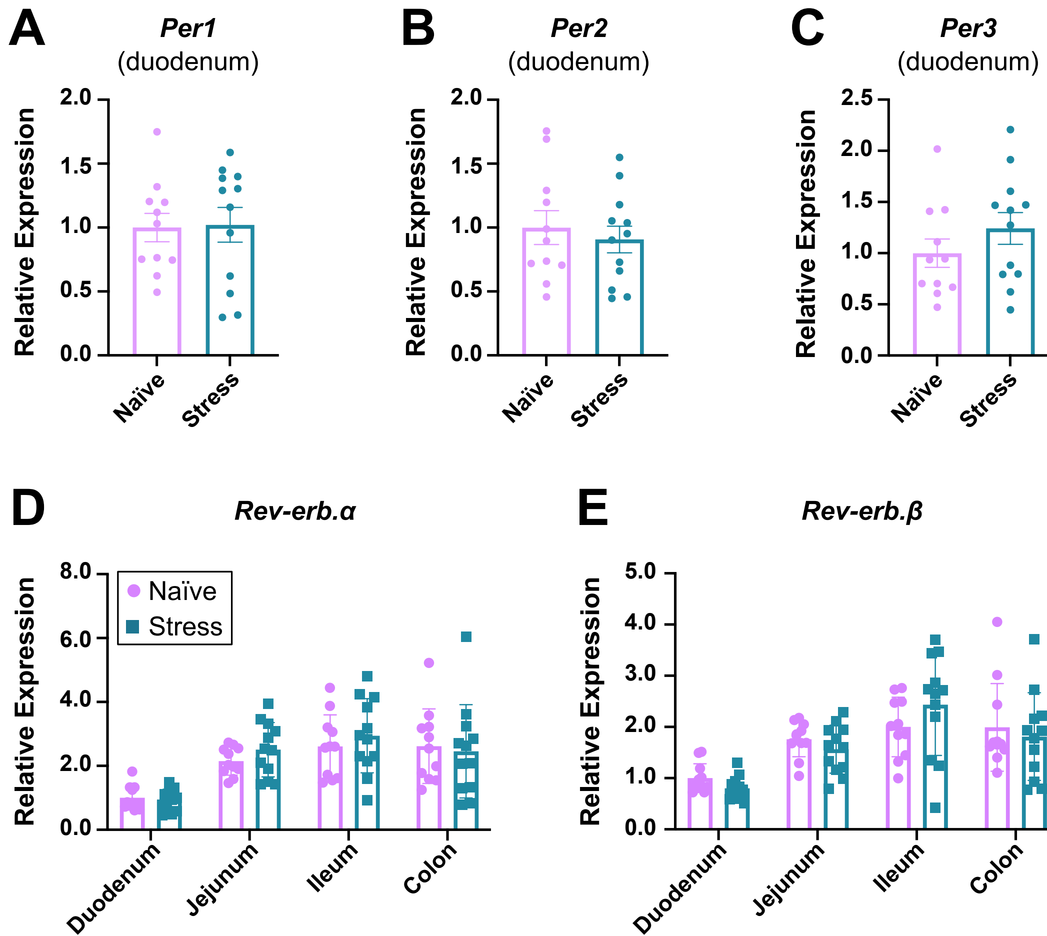

Figure 25: Additional Clock Gene Expression Changes after Stress Exposure: Relative quantities of (A) *Per1*, (B) *Per2*, and (C) *Per*3 in the duodenum of naïve or stress mice. T-tests. Relative quantities of (D) *Rev-erb*α and (E) *Rev-erb*β in the intestines of naïve or stress mice. 2WANOVA. Male mice, n=11-12 per group, representative of 2 experiments.

Mucin 13 Deletion Induces Baseline Behavioral and Microbiome Changes and Lends Mice More Susceptible to UCMRS:

Given our data demonstrating that mucin 13 is specifically downregulated in stressed animals, we sought to examine if deleting mucin 13 impacted both the microbiome and depressive- and anxiety-like behaviors in mice. Using the *i*-GONAD system, we created a mucin 13 knockout (*muc13*$^{-/-}$) line by deleting a 475bp region of the gene that contained the start codon

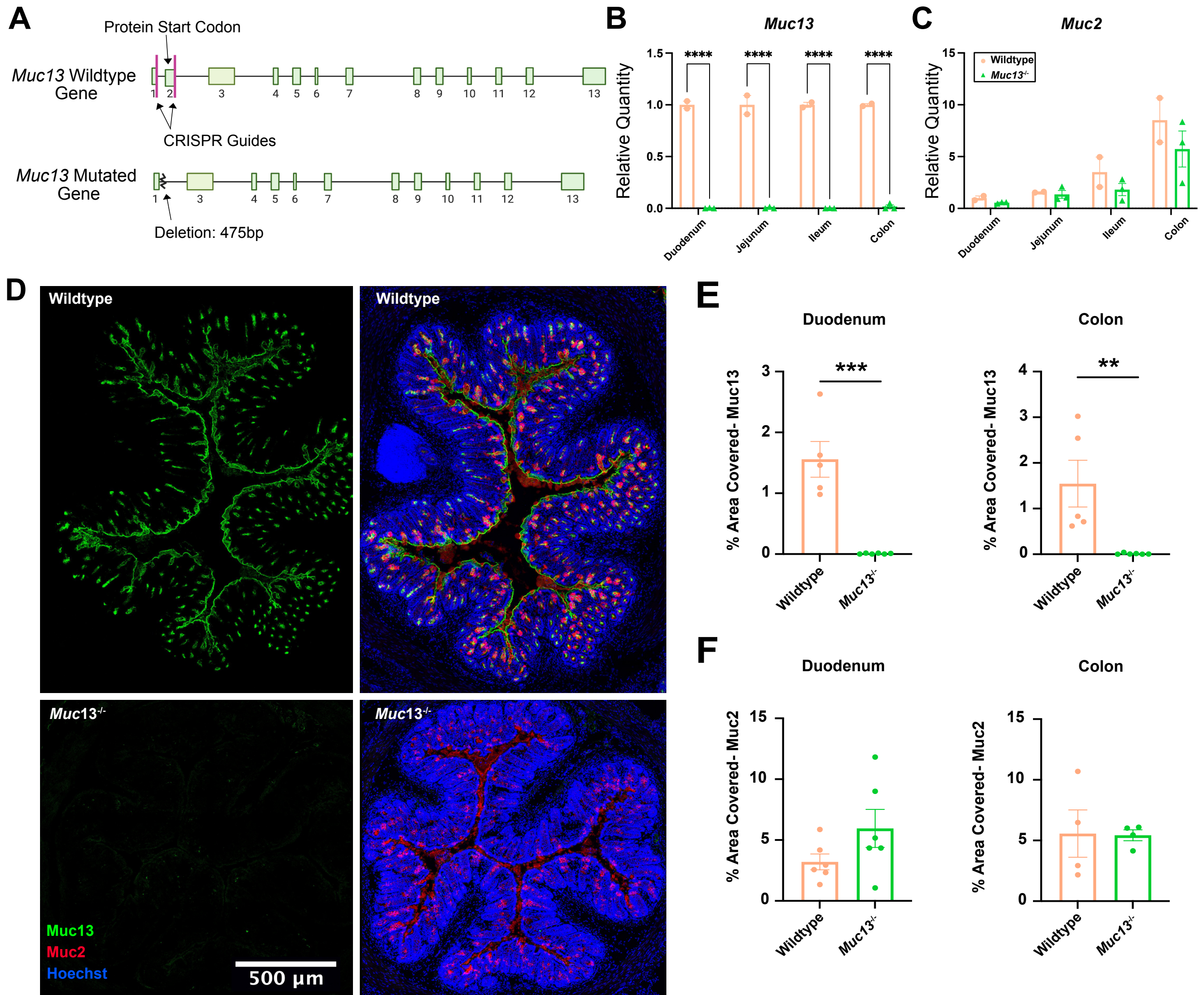

Figure 26: Mucin 13 Deletion Validation: (A) Schematic of mucin 13 exon 2 deletion. Relative quantities of (B) *Muc13* and (C) *Muc2* in the intestines of wildtype or *Muc13*$^{-/-}$ mice. Male mice, n=2-3 per group, N=1. 2WANOVA. (D) Representative images of immunofluorescence staining of Muc13, Muc2 and Hoechst in the colon of wildtype or *Muc13*$^{-/-}$ animals. Quantification of immunofluorescence of (E) Muc13 and (F) Muc2 in the duodenum and colon of wildtype or *Muc13*$^{-/-}$ mice. Male mice. T-tests, n=4-6 per group. Representative of 2 experiments.

(Fig. 26A) [350, 351]. Validation of the knockout was performed using both RNA transcripts (Fig. 26B) from individual sections of the intestine and immunofluorescence (Fig. 26D-E). Mucin 2 was also examined to check for knockout specificity and compensation by other mucins. No changes were observed in Muc2 at the transcript or protein levels (Fig. 26C and F). After model validation, we sought to understand the impacts of mucin 13 deletion on microbiome composition. We

compared 16S sequencing results from fecal samples collected from wildtype and *muc13*$^{-/-}$ animals, separated by genotype at weaning, at baseline and after UCMRS exposure (Fig. 27A). Interestingly, we found that *muc13*$^{-/-}$ animals clustered distinctly from their wildtype controls, suggesting that mucin 13 deletion significantly impacts the composition of the microbiome at baseline (Fig. 27B). In addition, while wildtype controls saw a significant shift in their microbial composition after 1 week of UCMRS, muc13 KO samples remained clustered together (Fig. 27B), suggesting that baseline *muc13*$^{-/-}$ microbial signatures are not as impacted by stress as baseline signatures in wildtype mice. Importantly, samples from wildtype animals exposed to 1 week of UCMRS microbial shifted towards both groups of the *muc13*$^{-/-}$ animals, further supporting the idea the *muc13*$^{-/-}$ animals have microbial signatures that mimics stress induced microbiome

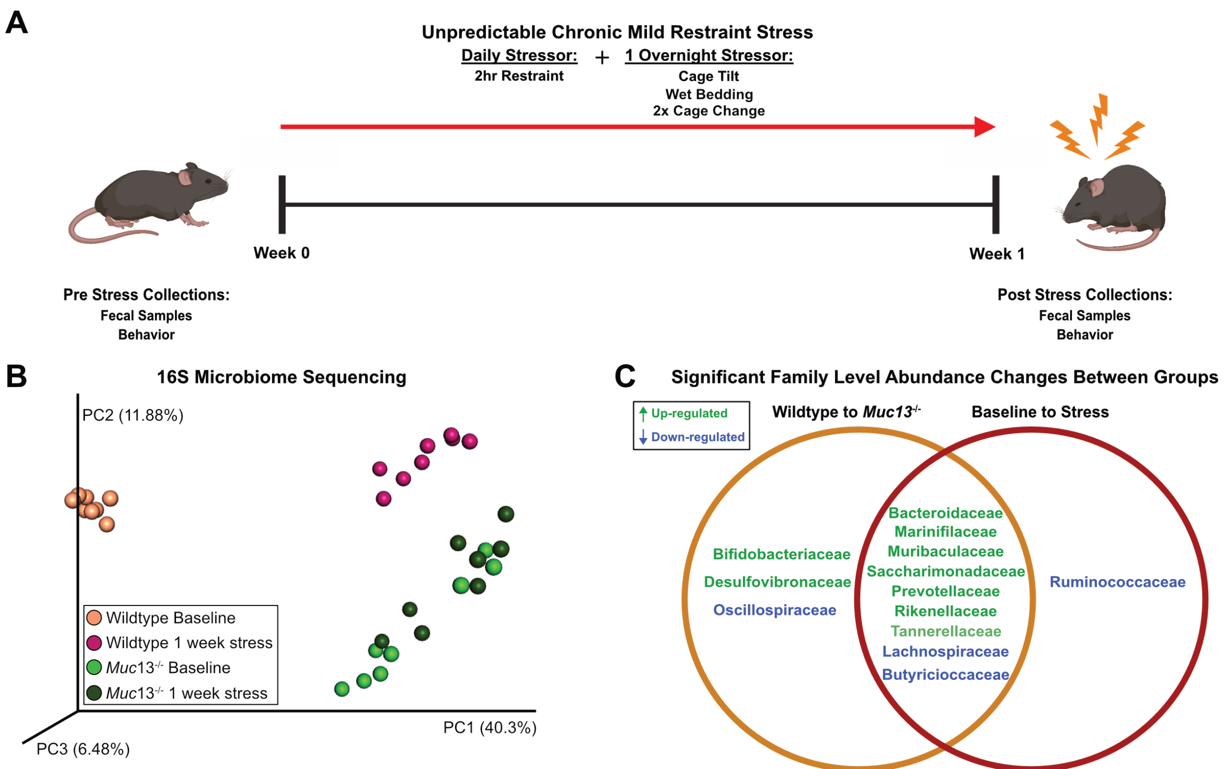

Figure 27: Microbiome Changes in *Muc13*$^{-/-}$ Mice at Baseline and after Stress Exposure: (A) Schematic representing experimental design of 1 week Unpredictable Chronic Mild Stress (UCMRS) exposure. (B) PCA plot of 16S fecal microbial sequencing in wildtype and *Muc13*$^{-/-}$ animals at baseline and after 1 week of stress exposure. Male mice, n=8 per group, N=1. (C) Venn diagram comparing significantly changes families from 16S fecal microbiome sequencing between wildtype and *Muc13*$^{-/-}$ animals to families changes between wildtype baseline and stress exposed animals. Male mice, 2WANOVA, n=13 per group, N=1.

dysbiosis (Fig. 27B). To further understand the similarities between the *muc13*$^{-/-}$ microbiome and a UCMRS exposed microbiome, we compared the significantly changes bacterial families between wildtype and *muc13*$^{-/-}$ animals to the significantly changed bacterial families between baseline and animals exposed to 1 week of UCMRS. Of the bacterial families that had significant changes in either group, 69% of those changes overlapped (Fig. 27C). This again suggests that mucin 13 deletion induces a change in the microbiome that closely mimics changes in the microbiome observed after UCMRS exposure.

Given the connection between microbiome changes and mental health, we sought to examine if mucin 13 deletion impacted anxiety- and depressive-like behaviors[284]. Both *muc13*$^{-/-}$ animals and wildtype controls were subjected to the open field, nestlet shred, tail suspension and forced swim tests (Fig. 28A-D). Behavior was collected at baseline for all animals (Fig. 28A-D, left columns). Interestingly, at baseline, we observed no differences in anxiety-like behaviors in the open field or nestlet shredding tests between *muc13*$^{-/-}$ and wildtype controls (Fig. 28A-B, left columns), but did see strong depressive-like behaviors by the tail suspension and forced swim tests (Fig. 28C-D, left columns). In addition, to gain a full picture of the impacts of mucin 13 deletion on susceptibility to stress induced-anxiety- and depressive-like behaviors, we subjected mice to 1 week of UCMRS. 1 week UCMRS is generally considered a subclinical model of stress that does not induce behavioral changes in wildtype animals, thus we hypothesized that if *muc13*$^{-/-}$ deletion rendered animals more susceptible to stress, they would exhibit anxiety- and depressive- like behaviors before wildtype animals [352]. Our results demonstrated that after 1 week of UCMRS, *muc13*$^{-/-}$ animals had significant reductions in the amount of time spent in center in the open field test and significant increases in the amount of nestlet removed in the

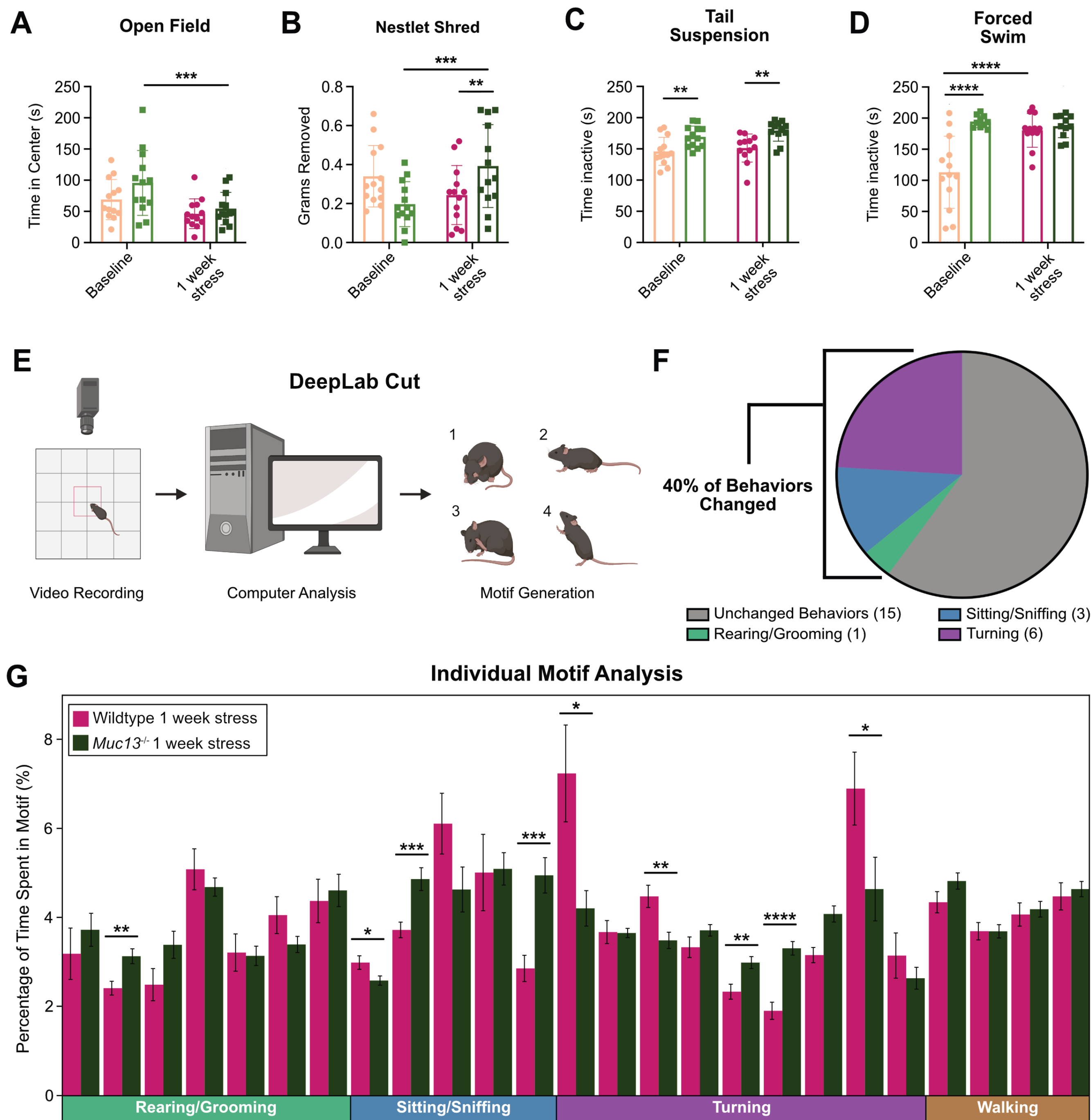

Figure 28: Behavioral Changes in *Muc13*$^{-/-}$ Mice at Baseline and after Stress Exposure: (A) Open field and (B) nestlet shredding tests comparing anxiety-like behaviors between wildtype and *Muc13*$^{-/-}$ animals at baseline and after 1 week of stress exposure. (C) Tail suspension and (D) forced swim tests comparing escape behaviors between wildtype and *Muc13*$^{-/-}$ animals at baseline and after 1 week of stress exposure. Male mice, 2WANOVA, n=13 per group, N=1. (E) Schematic representing DeepLabCut experimental design. (F) Pie chart representing quantified behavioral motifs changed between wildtype and *Muc13*$^{-/-}$ animals. (G) Individual motif analysis comparing behaviors after 1 week of stress exposure in wildtype and *Muc13*$^{-/-}$ animals. T tests, male and female mice, n=13-24 per group, representative of 3 experiments.

nestlet shred test compared to baseline. These results indicate that 1 week of UCMRS is sufficient to induce anxiety-like phenotypes in *muc13*$^{-/-}$ animals, but not their wildtype counterparts (Fig. 28A-B, green columns). Unsurprisingly, in the tail suspension and forced swim tests, we saw no increase in time spent inactive as the *muc13*$^{-/-}$ already demonstrated such behaviors at baseline and hit a ceiling effect (Fig. 28C-D, green columns). To complement these classic behavioral assays, we also examined more subtle behavior changes through the unbiased computational modeling system known as DeepLabCut (Fig. 28E-G and Fig. 29) [246, 287]. 10-minute videos of mice exploring an open field box were obtained and each frame was broken down into 25 individual motifs (Fig. 28E). Each motif was characterized and grouped into a behavioral classification and changes in behavioral groups were quantified between *muc13*$^{-/-}$ and wildtype animals (Fig. 28E-G). Baseline analysis revealed that of the 25 distinct motifs, 16% were significantly changed between groups (Fig. 29A-B). Suggesting that, like in classical behavioral assays, distinctions between *muc13*$^{-/-}$ and wildtype animals could be detected at baseline. Similarly, the DeepLabCut software was able to detect differences between groups after 1 week of UCMRS exposure. After

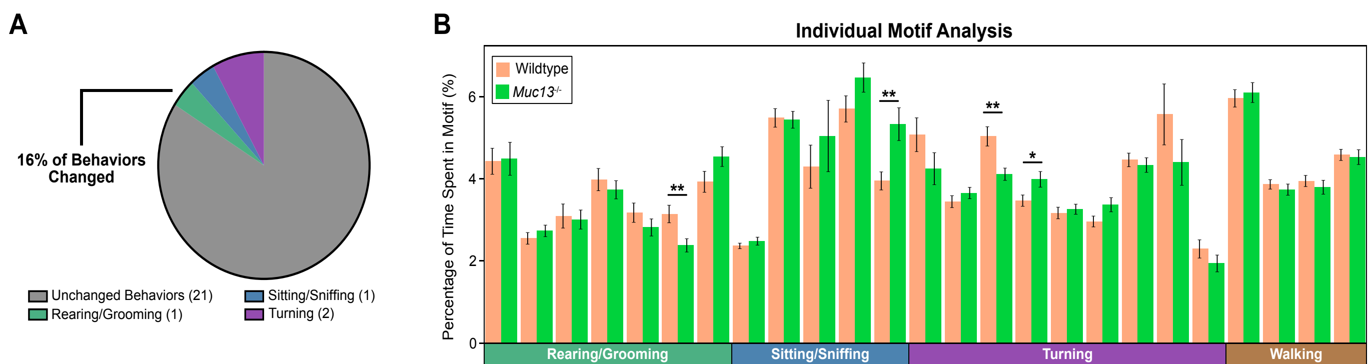

Figure 29: DeepLabCut Analysis of Baseline Behaviors: (A) Pie chart representing quantified behavioral motifs changed between wildtype and *Muc13*$^{-/-}$ animals. (B) Individual motif analysis comparing baseline behaviors in wildtype and *Muc13*$^{-/-}$ animals. T tests, male and female mice, n=13-24 per group, representative of 3 experiments.

stress exposure, behavioral changes were more pronounced between groups with 40% of behavioral motifs being changed between *muc13*$^{-/-}$ and wildtype controls (Fig. 28F-G). These results support our classical behavioral findings and demonstrate that mucin 13 deletion is sufficient to induce behavioral changes, including depressive-like behaviors, at baseline and render animals more susceptible to behavioral changes after stress exposure. As a whole, our results show that mucin 13 deletion is able to drive both microbiome dysbiosis that mirrors stressed microbiomes and depressive-like behaviors at baseline. In addition, deletion of mucin13 allows animals to be more susceptible to stress induced microbiome changes.

Discussion:

Our work demonstrates that in addition to inducing microbiome changes and anxiety- and depressive-like behaviors in mice, stress also reduces expression of a key component of the mucosal layer, mucin 13. While the mechanisms behind stress-induced mucin 13 reductions are still being investigated, it likely does not directly involve stress hormones, the immune system, the Vagal nerve, or the microbiome. Interestingly, we present correlative evidence to suggest that stress also disrupts circadian rhythms, suggesting there may be a connection between changes in circadian genes and reductions in mucin 13 expression. However, more work is needed to elucidate the connection between circadian rhythms and mucin 13 gene expression. Finally, we demonstrate that deletion of mucin 13 is sufficient to induce microbiome and behavioral changes that mimic stress-induced depressive phenotypes at baseline. In addition to these baseline changes, mice lacking mucin 13 also are more susceptible to behavioral changes after sub-clinical exposure to unpredictable chronic mild restraint stress.

While spanning several areas of the body and connecting disparate fields, our results are supported by previously published literature. For example, it is well known that there is a reciprocal relationship between the mucus layer and microbes [340]. In fact, disruption of this critical layer results in sweeping microbiome changes, heightened inflammation, and disease onset [340, 353-360]. Additionally, stress is known to alter the glycosylation patterns of mucins[361]. As changes in glycosylation are also known to alter the microbiome, the connection between stress and mucin induced microbiome changes is well supported [362, 363]. Furthermore, the connection between mucins and depression has also been suggested in the literature. Specifically, single nucleotide polymorphisms (SNPs) in mucin 13 have been identified in GWAS studies of depressed populations[56, 364, 365]. In addition, SNPs in o-glycosylation have also been identified in populations with treatment resistant depression[327, 365]. Together, these data support our results suggesting that mucin 13 is an important driver of microbiome dysbiosis and depressive-like behaviors in mice.

Mechanistically, more work is needed to determine how stress induces reductions in mucin 13. However, our results suggest a connection between mucin changes and circadian rhythms. This connection is also supported by current literature. First, it is has become clear known that stress and sleep are connected, as stress induced significant changes in circadian rhythms [366-368]. In addition, changes in circadian rhythms also have known impacts on the intestine by regulating the microbiome, intestinal regeneration, and the intestinal immune system[369-371]. More strikingly, however, is demonstrated the connection between alterations in circadian rhythms and depression. Multiple studies have connected SNPs in *BMAL* and other clock genes to depression in humans, suggesting that alterations in circadian rhythms are

detrimental in depressed states[372-374]. In addition to these links between stress, circadian rhythms, and depression, research has also connected changes in mucin 13 to changes in clock genes. In fact, in a study looking at an animal model lacking *Bmal*, researchers noted a significant downregulation of mucin 13 expression[375]. Further supporting this idea, the UCSC genome browser demonstrates that Bmal is capable of binding to an enhancer of mucin 13. Additionally, this enhancer region is conserved across humans and mice, demonstrating the potential therapeutic potential of findings related to Bmal induced mucin 13 changes. Taken together, these data support the premise that stress induced changes in circadian rhythms that downregulate the expression of mucin 13. This reduction then alters the microbial niche in a way that induces microbiome changes that mirror stress induced dysbiosis and induces depressive-like behaviors in mice. However, more work is needed to solidify the hypothesized connection between stress induced-circadian disruptions and stress induced-mucin 13 reductions.

Our results have brought to light a key aspect in stress-induced depression. We have demonstrated that a transmembrane mucin is indirectly regulated by stress in such a way that interfering with its homeostatic expression patterns induces microbiome dysbiosis and depressive-like behaviors in mice. We believe that this change is a critical step in initiating microbiome dysbiosis in stress-induced depression and that this change may be a targetable upstream mediator of dysbiosis that could be broadly applicable to patients with stress-induced depression as mucin 13 is conserved across the mucosal layer of humans. In addition, while directly related to stress-induced depression, our results provide the basis for further research targeting transmembrane mucins as a broadly applicable intervention point for any disease that

presents with or is driven by pathogenic microbiome dysbiosis, such as colitis or Parkinson's Disease[[280, 310, 376-378]].

Materials and Methods:

Mice:

All C57BL/6j and BALB/cJ mice were purchased from Jackson Laboratories (strain #000664). Mice were bred in-house. Mice were kept on a 12-h light/dark schedule. All behavioral interventions were performed between 8 am and 3 pm and animals were sacrificed between 7 am and 1 pm. Animals were housed as previously described[287]. All procedures were approved by the University of Virginia ACUC (protocol #3918). All experiments were conducted and reported according to ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines)

Stress Experiments. Unpredictable Chronic Mild Restraint Stress (UCMRS) experiments were performed as previously described[287].

Behavioral Tests. The forced swim, tail suspension, sucrose preference, open field, elevated plus maze, and nestlet shred tests performed as previously described[260-262, 265, 287]. All testing was recorded on a Hero Session 5 GoPro and analyzed with Noldus behavioral analysis software.

Mass Spectrometry: Metabolomics was performed with targeted mass spectrometry as previously described[379].

Fecal DNA Extraction: DNA was isolated from fecal pellets using the phenol/chloroform method. Briefly, a solution of 200mM TrisHCL (pH 8), 200mM NaCl, 20mM EDTA, and 20% SDS was added to fecal pellets. Pellets were transferred in solution to a tube containing 0.1mm zirconia/silica beads (Biospec #11079101z) and a 3.2mm steel ball (Biospec #11079132ss). Pellets were subjected to beadbeating for 4min on high at room temp. Samples were centrifuged and the aqueous layer transferred to a new tube. Samples were then further processed with QIAquick PCR purification kit (Qiagen #28106) as per manufacturer's instructions.

16S rRNA Gene Sequencing

The V4 region of the 16S rRNA gene was amplified from each sample using a dual indexing sequencing strategy[380]. Samples were sequenced on the MiSeq platform (Illumina) using the MiSeq Reagent Kit v2 (500 cycles, Illumina #MS102-2003) according to the manufacturer's protocol with modifications found in the Schloss Wet Lab SOP (https://github.com/SchlossLab/MiSeq_WetLab_SOP).

16S Sequence Analysis

All processing and analysis of 16S rRNA sequencing data was performed in R (version 4.1.2) [381]. Raw sequencing reads were processed for downstream analysis using DADA2 (version 1.22.0)[382]. Processing included inspection of raw reads for quality, filtering of low-quality reads, merging of paired reads, and removal of chimeric sequences. Length distribution of non-chimeric sequences was plotted to ensure lengths matched the expected V4 amplicon size. Taxonomy was assigned to amplicon sequence variants (ASVs) by aligning reads with the Silva reference database (version 138.1)[383].

Microbiota diversity and community composition were analyzed using the packages phyloseq (version 1.38.0), microbiome (version 1.16.0), and vegan (version 2.5.7)[384-386]. The packages tidyverse (version 1.3.0), and ggplot2 (version 3.3.5) were used for data organization and visualization[387, 388]. Random forest analysis was performed using the and randomForest (version 4.6.14), vegan (version 6.0.90), and pROC (version 1.18.0) packages [389-391].

Data Sharing

Raw sequencing reads will be deposited in the sequence read archive (SRA) and bulk RNA sequencing will be submitted to the Gene Expression Omnibus (GEO) database at the time of publication.

Random Forest

Samples were first divided into training (70% of samples, divided equally between Baseline and Stressed samples) and test (30% of samples) sets. The training set was used to tune the "mtry" parameter of the model, while the test set was used to validate model performance. Feature importance was determined using the Gini index, which measures the total decrease in node impurity averaged across all trees.

DeepLabCut. *Animal pose estimation:* Animal pose estimation was performed by using a deep-learning package, DeepLabCut (https://github.com/DeepLabCut/DeepLabCut) [246]. We generated a DeepLabCut convolutional neural network to analyze open field test videos, which is trained in a supervised manner: 16 manually labeled points were selected as references of

transfer learning. 15 randomly selected videos were used for building a training dataset. Finally, the performance of the neural network is evaluated by researchers.

Unsupervised Behavior Classification: Estimated mouse poses from DeepLabCut were further analyzed by Variational Animal Motion Embedding (VAME)[268], which classifies animal behavior in an unsupervised manner (https://github.com/LINCellularNeuroscience/VAME). We trained a unique VAME recursive neural network for each experiment, which classifies each frame of the open field test video into 1 of the 25 behavioral motifs. Then, all behavior motifs were annotated and evaluated by blinded researchers.

RNA Extraction and Quantitative PCR. For RNA extraction, cultured cells were pelleted, frozen, and lysed. RNA was extracted using the Bioline Isolate II RNA mini kit as per manufacture's protocol (BIO- 52073). RNA was quantified with a Biotek Epoch Microplate Spectrophotometer. Normalized RNA was reverse transcribed to cDNA with either the Bioline SensiFast cDNA Synthesis Kit (BIO-65054) or Applied Sciences High- Capacity cDNA Reverse Transcriptase Kit (43-688-13). cDNA was amplified using the Bioline SensiFast NO- ROX kit (BIO-86020), according to manufacturer's instructions. Probes are listed in Sup. Table 1.

Cell culture:

HT-29 and Caco-2 cells were obtained from ATCC and cultured as per ATCC website instructions with McCoy's 5A media (Thermofisher #16-600-082) or EMEM (ATCC #30-2003), respectively. Cells were treated with either human IL-4 (Peprotech #200-04), IL-33 (Peprotech #200-33), TNFα (Peprotech #300-01A), IL-17 (Peprotech #200-17), IL-6 (Peprotech #200-06), IFN-γ (Peprotech #300-02), or IL-1β (Peprotech #200-01B), for 3hours at 10ng/mL.

Organoid Culture: Mouse duodenal organoids were collected and cultured as previously described[392]. Crypts were plated in 50µL matrigel (Corning # 356237) and grown in intesticult media (Stemcell Technologies # 06005**).** Organoids were allowed to mature for at least 1 week to reach maturity before treatment. Mouse organoids were treated with either 5ng/mL of hydrocortisone (Sigma # H4001) or 150µg/mL of corticosterone (Cayman Chemical # 16063) for 24 hours. Concentrations were determined from mass spectrometry of hormones from serum (Fig. 15 and 16). Human organoids were generously gifted from the Moore lab at the University of Virginia. Crypts were plated in 50µL matrigel (Corning # 356237) and grown in intesticult media (Stemcell Technologies # 06010). Human organoids were treated with 200µM of hydrocortisone (Sigma # H4001) for 12 hours. After treatment cells were frozen for RNA extraction.

Vagal Nerve Stimulation and Sub-Diaphragmatic Vagotomy: Surgeries were performed as previously described[393]. Animals receiving vagal nerve stimulation or sham surgery were allowed to recover for 30 hours before sample collection. Animals receiving sub-diaphragmatic surgery or sham controls were allowed to recover for 2 weeks before sample collection.

Mucin-13 Knockout Generation:

Mucin-13 knockout line generation was carried out in compliance with policies of the Association for Assessment of Laboratory Animal Care and approved by the University of Virginia Animal Care and Use Committee (protocol #3795). For timed pregnancies, animals were mated

overnight at 5pm and separated at 7am. Jackson C57BL/6j mice were housed on a 12-hour light/dark cycle with food and water ad libitum. Males were singly-housed and females group-housed (5 females per cage). Females were checked for estrus daily and females in estrus were transferred to a male cage from 5pm to 7am. At 7am, females were checked for vaginal plugs; those with plugs were set aside for surgery to be performed at 4pm, ~16 hours post-assumed copulation.

Mutant lines were generated using the iGONAD technique as described using a BTX ECM 830 Electroporation System (Harvard Apparatus). [350, 351]. Briefly, the muc13 sequence was taken from the UCSC genome browser, mouse assembly Dec. 2011 (GRCm38/mm10), Genomic Sequence (chr16:33,794,037-33,819,927)[394]. Exons 1 and 2 including the intervening intron where analyzed with CRISPOR (http://crispor.tefor.net/)[395]. Exon 2, containing the protein start sequence, was targeted for excision at these two target sequences + PAM sites: Exon2_protein_start_sequence GCAAGAGCAGCTACCATGAA (AGG) and Exon2_end_of_exon AGTCTCCTTTTGGTGACCGT (GGG). Alt-R S.p. HiFi Cas9 Nuclease, tracrRNA, and crRNA XT for the two target sequences were purchased (IDT). Prior to surgery, the Alt-R CRISPR/Cas9 reagents were prepared according to IDT guidelines: crRNA-XT and tracrRNA were annealed to form the gRNA, then complexed to the S.p HiFi Cas9 nuclease, and then diluted with sterile Opti-MEM with Fast Green FCF to aid visualization.

Statistical analysis. All statistical analyses-except those associated with DeepLabCut-were performed in GraphPad Prism 9. Analyses involving two groups were performed using a two-tailed T test. If the variances between groups were significantly different, a Welch's correction was applied. Outliers were excluded if they fell more than two standard deviations from the

mean. For all analyses, the threshold for significance was at $p < 0.05$. Repeats for each experiment are specified in the figure legend corresponding to the respective panel.

Chapter 6: Future Directions and Conclusions

While depression has been continuously and rigorously studied for nearly a century, more work is needed to fully understand its etiology. Evidence is clear that there is a role for neurotransmitters and brain circuitry in depression. However, current therapeutics that target these disturbances do not adequately reverse depression symptoms universally. Thus, there is a need to further understand the root causes and sequential steps leading to depression. One such are of work has been in the microbiome. Like disruptions in neurotransmitters, research has shown a clear link between depression and microbiome alterations. Again, therapeutics designed to address the observed dysbiosis, while promising, cannot be broadly applied to the general population. Instead, research must turn towards identifying therapeutic targets that are conserved across the human population and able to be manipulated without off-target health consequences. Through my doctoral work, I have examined two routes through which stress-induced depression may be acting: Cytokines and alteration of the mucosal layer composition. While both works have addressed the hypotheses asked of them, more work must be accomplished to fully understand their roles in depression.

Future Directions for Cytokines in Stress-Induced Depression:

My doctoral work addressed the role of Th17 produced IL-17 played in stress-induced depression. While my data disproved the hypothesis that IL-17 produced from Th17s cells is necessary for the onset of depression in mice, more work can be done to identify the route through which stress causes depression. This can be addressed by answering the following lines of investigation:

Investigate the role of IL-17 from other cells in stress-induced depression

While my results demonstrated the IL-17 from Th17s did not induce depressive-like behaviors in mice (chapter 3), they do not rule out the role of IL-17 completely. As discussed, IL-17 has long been connected to depression[238]. Canonically, IL-17 is thought to be produced by RORγT+ Th17s. However, IL-17 can also be produced by other cells such as γδ T cells or ILC3s[251]. Thus, to understand the full picture of IL-17 in stress-induced depression, more work can be done to identify which cell type is critical to depression onset.

This question can mainly be addressed by using antibodies depleting IL-17 producing cell types or knockout line in combination with adoptive transfer models. This has been partially addressed in works looking at γδ T cells and anxiety-like behaviors in mice[253]. In this work, the authors demonstrate that anxiety-like behaviors are likely regulated by IL-17 produced from γδ T cells. To complement this work, a similar study examining the role of IL-17 produced from ILC3 is needed to demonstrate if these cells play a similar role in regulating anxiety- or depressive-like behaviors.

In addition, as our knockout model has a constitutively expressed Cre recombinase, there could be compensation from other cytokines making an effect from a single cytokine (IL-17) hard to distinguish. In order to address this, the cytokine profiles of our knockout mice can be evaluated at baseline and under stressful conditions. This could be accomplished through both *in vitro* and *in vivo* assays. First, T cells and other immune cells can be isolated from the knockout mice and stimulated *In vitro*. ELISAs, flow cytometry and qPCR could all be used to determine if other cytokines are more highly expressed in this model. In addition, the cytokine and immune profile from the knockout mice could be profiled both at baseline and after stress exposure to see if in physiological conditions, there are changes in the expression of other cytokines.

Identifying other immune mediators of anxiety- or depressive-like behaviors:

To date, literature has correlated increases in IL-17 with depressive behaviors in mice in humans. Additionally, work has been done to suggest that anxiety-like behaviors are regulated by $\gamma\delta$ T cells[253]. While important, these studies leave room for further investigation into the causes of depressive-like symptoms in mice.

This question remains largely open ended and will likely require a variety of techniques and lines of investigation to uncover. As many aspects of the immune system are tied to depression (IL-1β, TNF-α, IFN-γ, etc.), there are many directions research can take[131, 201, 396, 397]. One technique to avoid costly and lengthy experiments is to examine changes in the blood, intestines, and brain in mice with multi-omics approaches. Classic bulk RNA sequencing or newer

technologies, like MERSCOPE, will be able to give overviews of transcriptional changes in depressive-like states. Once viable targets are identified, similar experiments to the ones I have conducted in my doctoral work can be applied to understand the target's impact on propagating stress-induced depression.

While the goals mentioned include direct steps to take to identify the pathways mediating stress-induced depression, longer term plans can also be considered. Once a route (or multiple routes) of stress-induced depression have been identified, druggable targets need to be established. These targets should be well conserved in patients and have limited off-target impacts when perturbed by drugs.

Future Directions for Mucins in Stress-Induced Depression:

My work has demonstrated that mucin 13 regulates microbiome dysbiosis and depressive-like behaviors in stress-induced depression. I have shown that in stress, mucin 13 is significantly reduced. Furthermore, after mucin 13 deletion, there is a significant shift in the microbial composition that mimics changes observed after stress exposure. In addition to this dysbiosis, *Muc13* deletion induces depressive-like behaviors at baseline and renders animals more susceptible to stress-induced behavioral changes in a sub-clinical model of stress. While these findings contribute much to the mucin and depression fields, more questions remain. Here I will discuss some open lines of investigation in mucin 13 regulation of stress- induced impacts:

Developing Techniques to Quantify Mucin 13 Protein

Glycoproteins are notoriously hard to quantify. The highly o-glycosylated branches interfere with numerous technical approaches aimed at determining the number of mucins present in samples. In addition, lack of specific antibodies renders even more techniques useless for quantification. To ensure that our results are representing true and biologically relevant phenomena, we need to address this limitation. Collaborative work to quantify mucin 13 by mass spectrometry is underway by collaborators at Yale[398]. Specifically, the Malaker lab has developed a "mucinase" that is specifically able to digest the O-glycosylated chains on mucins in a way that allows for protein identification and quantification by mass spectrometry. These results would allow for specific quantification of mucus without the noise associated with non-specific glycan digests.

Identifying the Mechanisms behind Stress Induced Mucin 13 Changes

While still in progress, my work has identified a likely candidate for inducing the reduction of mucin 13 expression after stress exposure: circadian rhythms. RNA sequencing between naïve and stressed duodenal tissues suggested that multiple circadian genes were significantly altered. I was able to confirm these results with qPCR, however more work is needed to determine the role circadian rhythms play in mucin expression changes. This question can be addressed with several experiments.

First, we aim to confirm that changes in circadian rhythms reduces mucin 13 expression. In addition to our RNA sequencing data, literature supports a connection between circadian rhythms and mucin 13 expression. First mucin 13 is known to cycle in the distal colon[399]. In addition, the UCSC genome browser demonstrates that Bmal is capable of binding to an enhancer of mucin 13. Additionally, this enhancer region is conserved across humans and mice, demonstrating the therapeutic potential of findings related to Bmal induced mucin 13 changes. To test this hypothesis experimentally, we will utilize both mouse organoids and mouse models. First, using mice that lack *Bmal* expression, we will determine if *muc13* is reduced at baseline by examining RNA expression of the transcript across the intestines. In addition, to determine if this expression change is driven by whole body circadian rhythms or by cell specific expression of *Bmal* we will culture organoids from the jejunum of these mice and again examine mucin 13 expression by qPCR. Results will determine if mucin 13 expression is driven by *Bmal* expression and if this change is driven by cell-intrinsic or extrinsic factors. However, because these experiments are derived from mice with constitutively excised mucin 13, there is room for adaptations from the host to mask these changes. To address this potential limitation, we are currently breeding *Villin* Cre, *Bmal*$^{fl/fl}$ mice that will have *Bmal* excised only in *Villin* expressing cells (intestinal epithelial cells). We are generating lines that contain both a constitutive deletion and an inducible deletion. These lines will allow us to specifically ask if *Bmal* expression in the intestinal epithelium of mice regulates mucin 13 expression. We will also examine behavioral readouts and the microbial composition of these mice to determine if they have similar phenotypes to the *Muc13*-/- mice.

The above experiments address only the role of *Bmal* in mucin 13 expression changes. However, our sequencing results also suggested that both *Ciart* and *Dbp* were significantly upregulated after stress exposure. These hypotheses are more difficult to test given the lack of *Ciart* and *Dbp* knockout lines and the fact that they are upregulated after stress exposure. To gain a clearer picture of the roles of these circadian genes in mucin expression, we will derive organoids from the intestines of wildtype mice and use both an siRNA against *Ciart* or *Dbp* and a transfection model where *Ciart* or *Dbp* are over expressed. In these models, we will look for either increases or decreases in mucin 13, respectively. If these results show promise, we will use the *i-GONAD* system to create a knockout line and examine if deletion of either *Ciart* or *Dbp* is protective against stress-induced microbiome dysbiosis and depressive-like behaviors.

If circadian genes do not prove to regulate mucin 13 expression, I will move to investigate the role of microRNA in mucin 13 regulation. MicroRNA has been shown to target and regulate mucin 13 and represents another viable candidate for stress-induced mucin changes[400].

Understand how Mucin 13 Alters the Microbiome

While stress can alter mucin 13 expression and mucin 13 perturbations can alter the microbiome, how mucin 13 changes regulate the microbiome remains unknown. Mucin 13 is a highly conserved transmembrane mucin that is expressed primarily in the intestines[338]. Of all the transmembrane mucins, mucin 13 is the shortest and is unlikely to provide a binding site for bacteria[320]. Thus, reductions in mucin 13 are unlikely to directly impact the microbial niche. However, mucin 13 has been demonstrated to be protective against inflammation by preventing cellular apoptosis [401]. It is possible that a stress-induced reduction in mucin 13 can manipulate

microbial composition by altering general epithelial turnover and thus availability of other mucin glycans to bacteria. Additionally, the thickness of the glycocalyx is critical to regulating microvilli shape and budding[325]. Given that microvilli budding and shape also regulate antimicrobial peptide (AMP) release, it is feasible that a reduction in mucin 13 could sufficiently alter the shape of microvilli to alter AMP secretion and change the bacterial environment[402].

To test these open questions, a combination of *in vivo* and *in vitro* techniques can be used. To test the rate of intestinal epithelial death, TUNNEL or propidium iodide staining can be used both in intestinal tissue harvested from mice with or without mucin 13 expression and in organoids derived from mucin 13 knockout animals as previously described[403, 404]. In addition to these methods, a similar method for investigating small intestine mucin turnover could be applied to epithelial turnover. As a measure of mucin turnover, researchers injected mice with a GalNAz antibody that labeled transmembrane mucins and sacrificed animals at staggered points over 12 hours and quantified the amount of staining left in the intestine[405]. A similar approach could be taken with an antibody against Villin, a marker for intestinal epithelial cells. Together, these results would determine if mucin 13 deletion impacted the amount of cellular apoptosis in the intestines.

In addition to influencing cell death, mucin 13 deletion can plausibly influence the shape of microvilli by altering the thickness of the glycocalyx and antimicrobial peptide release[325]. Testing this hypothesis is technically challenging but can be addressed through electron microscopy and tomography as previously described[406]. Through this technique, the thickness of the glycocalyx and number of microvesicles being released can clearly be quantified. In

addition to these results, immunofluorescence in combination with fluorescence *in situ* hybridization (FISH) or RNAscope can be used to determine the distance between bacteria and the epithelial layer. This distance can be used as a proxy for antimicrobial peptide release in the small intestine as there is no inner mucus layer and AMPs are used to control the distance of microbes to the epithelium[320]. Finally, a method for determining the amount of AMPs present in the lumen must be used to bring together all results and determine if mucin 13 reductions alter the glycocalyx thickness in a way that alters AMP release. This can accomplished with mass spectrometry as previously described[407].

Taken together, these experiments would determine if mucin 13 reduction induces changes in cellular apoptosis or antimicrobial peptide release and allow for greater insights into the ways that mucin 13 reductions can influence the microbiome.

***Develop Methods for Mucin 13 Delivery* in vivo**

I have established foundational evidence for the role of mucin 13 in regulating both the microbiome and depressive-like behaviors in stress-induced depression. While I have demonstrated that mucin 13 deletion is sufficient to microbiome homeostasis and depressive-like behaviors in mice, I have not demonstrated that restoration of mucin 13 is sufficient to restore the changes back to baseline.

Altering mucin 13 expression *in vivo* is currently technically challenging. However, methods for success exist. One option is through viral overexpression of mucin 13. In this scenario, a mucin 13/GFP virus targeting the stem cell niche in the intestines would need to be

created and delivered in a way that could effectively access the cells it targets. Technically, this is possible through superior mesenteric artery injection as previously described[408]. However, limitations to this method still exist as the viral infection rate remains low and it is unclear how successful overexpression will be.

An alternative plan to viral overexpression of mucin 13 is to deliver mucin 13 protein to the intestines. This can be done in several ways with varying degrees of technical difficulty. First, it is possible to deliver mucin proteins through the diet of mice. I have tested the delivery of porcine stomach mucus by mixing the powder and food with success. Similarly, it is possible to gavage a known quantity of mucins directly into the stomach of mice. A third theoretical option for mucin delivery could be through hydrogels that protect the mucin proteins and are only degraded in different sections of the intestines. As both pH and the microbiota composition change throughout the intestine, it is possible to coat mucin proteins in different "layers" of glycan containing gels that could be degraded along different parts of the intestine to ensure even delivery[409].

However, with all these techniques there is currently no way to identify how much exogenous mucin protein reaches the intestines. For this to be a successful option for mucin 13 delivery, we must first purify a very large amount of mucin 13 (something that is currently extremely cost prohibitive) and tag those proteins with a fluorescent marker that can be used to identify the exogenous protein from host mucins. In addition, it is currently unknown if exogenous mucins can integrate into the host epithelium. This can be determined with fluorescently tagged mucin 13 proteins. However, if exogenous mucin is not able to integrate

into the epithelium as is, modifications to the protein will have to be made. Theoretically, conjugating mucin 13 to an antibody that targets the intestinal epithelium would allow for the delivered mucin 13 to be as close to the membrane as possible. This process would not be conducive for mucin 13 signaling in the intestines but would allow insights into if the presence of the mucin 13 protein influences host dynamics.

Admittedly, these approaches are technically difficult and costly. However, these experiments remain critical to understanding if mucin 13 overexpression in the context of stress induced depression can reverse microbiome changes and depressive-like behaviors.

Investigate the Translational Relevance of Mucin 13 Changes in Stress-Induced Depression

My work has clearly shown that mucin 13 can regulate the microbiome and depressive-like behaviors in mice. However, the translational relevance of these findings is yet to be defined. Mucin 13 is the most conserved transmembrane mucin and as such is likely to have important evolutionary consequences[410]. Thus, it is reasonable to hypothesize that mucin 13 reductions in mice may have similar consequences in humans. In addition, depression and circadian changes are strongly linked in humans, suggesting our hypothesized mechanisms could also be conserved in humans[411, 412].

To examine this line of inquiry, we must utilize intestinal samples from human patients experiencing depression symptoms. Biopsies collected from both the duodenum and colon of

control patients and patients diagnosed with depression that are not taking medication for depression and do not show obvious signs of other clinical pathologies are ideal. RNA from these biopsies can be collected and examined for differences in mucin 13 expression and circadian genes. Additionally, fecal samples from these patients should be collected to examine if microbiome changes are also found. These results would determine if our findings in the mouse in stress-induced depression are conserved in human depression. If mucin 13 changes are conserved in humans, therapeutics targeting mucin 13 will need to be developed.

Conclusions

Stress-induced depression remains a worldwide problem. While decades of research have been dedicated to understanding the etiology of depression and therapeutics have been developed, high rates of treatment-resistant cases remain. Given the need for further understanding, I have dedicated my doctoral work to the biological underpinnings of depression in hopes of identifying novel therapeutic targets.

In the course of my work, I first investigated how the stress-induced changes could be influencing behavior. Based on evidence that IL-17 and Th17s are changed in depression in humans and mice, and work from our own lab demonstrating an expansion of Th17s in the small intestine of mice, I investigated the role of CD4+ cell produced IL-17. This was accomplished by deleting the master transcription factor for IL-17, *Ror*γt, in CD4+ t cells. Functionally, this deletion is believed to eliminate the Th17 cell population. After the deletion of Th17s, I examined knockout and control mice for depressive- and an anxiety-like behaviors. To my surprise, I found

no effect of Th17 deletion on either depressive- or anxiety-like phenotypes at baseline or after stress exposure. Based on this evidence, I concluded that the *Rorγt* driven production of IL-17 is not necessary to drive changes in depressive- or anxiety-like behaviors.

In addition to my work investigating the regulation of stress-induced behavioral changes, I dedicated the majority of my thesis work to understanding how stress initiates microbiome dysbiosis. Based on data generated from a previous graduate student, Ioana Marin, showing that laxatives were sufficient to induce microbiome dysbiosis and depressive-like behaviors, I hypothesized that stress was inducing dysbiosis by disrupting the mucosal layer. To test this hypothesis, I examined changes in gene expression of the main mucus proteins, mucins. Surprisingly, I found that of the mucins tested, only mucin 13 was significantly changed. After demonstrating a significant reduction in mucin 13 expression I sought to identify the mechanism by which stress-induced changes in mucin 13. After much work, I identified that stress-induced mucin changes also correlated with stress-induced changes in circadian rhythms, suggesting that mucin 13 may be regulated by alterations in circadian gene expression. While I am still working on providing stronger evidence for clock genes regulating mucin 13, I believe this is a viable mechanistic route. Finally, I sought to test the role of mucin 13 in microbiome dysbiosis and depressive-like behaviors. To accomplish this, I created a novel mouse line in which mucin 13 was deleted. After confirming successful deletion of mucin 13, I tested knockout and control animals for alterations in the microbiome and behavioral changes. Results demonstrated the mucin 13 deletion is sufficient to drive microbiome dysbiosis that mimics the dysbiosis observed in stress-induced depression and depressive-like behaviors in mice. Together, these data demonstrate that stress induces a significant reduction in mucin 13 in the intestines of mice that is possibly

driven by alterations in circadian rhythms. In addition, my Ph.D. work identifies mucin 13 as a key regulator of the microbiome in stress-induced depression.

My work has both disproven a popular hypothesis for the onset of depression behaviors and highlighted a novel function for an understudied protein in the intestines. While the implications of these discoveries for depression treatments are yet to be investigated, I believe they hold promise and if well utilized, could have a widespread and positive impact in the field.

Chapter 7: References

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