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Consumption of dietary emulsifiers increases sensitivity to social stress in mice: a potential role for the COX molecular pathway

by

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ABSTRACT

Chronic low-grade inflammation and exposure to stress are key contributing factors in the etiology and progression of many neuropsychiatric disorders. Dietary emulsifiers are commonly added to processed foods and are classified by the Food and Drug Administration (FDA) as generally recognized as safe (GRAS). Recently, however, it has been revealed that these additives at translationally relevant doses can cause low-grade inflammation, gut dysbiosis, and may even increase baseline anxiety-like behavior. The latter finding suggests that dietary emulsifiers impact brain areas that modulate stress responses. We used RNA-Seq to examine whether chronic consumption of either polysorbate 80 (P80) or carboxymethylcellulose (CMC) is associated with changes in gene expression in the amygdala and PVN and used Ingenuity Pathway Analysis to identify enriched molecular pathways that may underlie an anxiety-like phenotype. Emulsifier consumption resulted in alterations in gene expression of various immediate early, stress-related, and immune-related genes in brain regions that are known to be important in the generation of behavioral and neuroendocrine responses to stress-provoking stimuli. We also hypothesized that emulsifier-treated mice exhibit sensitized behavioral, hormonal, and neuronal activity responses to stress. To test this hypothesis, C57Bl6/J mice were subjected to acute defeat conditions after 12 weeks of emulsifier or water consumption. When subjected to social defeat, emulsifier-treated mice showed increases in social avoidance and circulating corticosterone as well as alterations in neuronal activity as measured by c-Fos immunofluorescence. Subsequently, given the observed increased expression of PTGS2 (COX-2) in the amygdala, we tested the hypothesis that increased inflammation through the COX pathway is a mechanism driving emulsifier-induced increases is stress sensitivity. Groups were as described above, but mice were also divided into aspirin (25mg/kg/day) and placebo

intervention groups. We found that aspirin, a COX pathway inhibitor, appears to block the increase in social avoidance observed in emulsifier-treated mice. These data demonstrate that ingestion of dietary emulsifiers at concentrations analogous to those ingested by humans increases sensitivity to social stress in mice. Further, it appears that the COX pathway may be a prime mechanistic candidate by which emulsifier-induced increases in sensitivity to social stress occurs.

INDEX WORDS: Food additive, Dietary emulsifier, Inflammation, Social stress, Social defeat, Cyclooxygenase, Amygdala, Paraventricular nucleus, Corticosterone, Carboxymethylcellulose, Polysorbate-80

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DEDICATION

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1 INTRODUCTION

1.1 Overview

Various studies provide evidence that immunological stress, often in the form of low-grade inflammation, may be a mechanism underlying mood disorders. Chronic physical stressors like inflammation and chronic psychological stressors such as social stress can disrupt the hypothalamic-pituitary-adrenal (HPA) axis, which is the main modulator of the body's neuroendocrine response to stress [1-6]. It has been proposed that a chronic inflammatory state ultimately leads to a hyperactive HPA-axis, resulting in increased long-term release of glucocorticoids, which could then result in anxiety- and depression-like phenotypes [7-9]. Further, chronic psychological stress can amplify the HPA response to an immune challenge and, reciprocally, inflammatory stress can amplify the HPA response to a psychological stressor, suggesting that there could be feedback or feedforward loops that change HPA axis responsivity [10, 11]. The immune system interacts with stress-regulatory brain areas to affect the brain and behavior by altering neurochemical signaling, neuroplasticity, and neuroendocrine processes [3, 12]. Reciprocally, stress exposure can activate inflammatory immune responses [13].

A potential environmental source of immunological activation or stress for humans is chronic consumption of a pro-inflammatory diet. Certain dietary components, such as trans fatty acids and saturated fatty acids, have been shown to stimulate chronic, low-grade inflammation and thereby could increase susceptibility to developing a neuropsychiatric disorder [6, 14-16]. Food additives, such as emulsifiers that are used as food stabilizers, are commonly added to processed foods. These additives are generally viewed as innocuous and are classified by the Food and Drug Administration (FDA) as "generally recognized as safe"

(GRAS). Recently, however, it has been revealed that these additives at translationally relevant doses can cause systemic inflammation [17, 18]. It has also been suggested that mice consuming emulsifiers may display an anxiety-like behavioral phenotype [18]. It is not known, however, whether emulsifier-treated animals exhibit normal behavioral and neuroimmune responses to stress. Arguably, the most common stressor experienced by most animals is social stress. Social stress is of particular interest because it is a salient, naturally occurring stressor experienced by all social animals, including rodents and humans [19]. Social stress in humans has been shown to contribute to the etiology and progression of mood and anxiety disorders, and, similarly, non-human animals that experience social stress exhibit anxiety- and depression-like phenotypes [20-24]. Altogether, it is conceivable that a chronic, low-grade inflammatory state induced by emulsifier consumption may increase physiological and behavioral responses to social stress. **Thus, the purpose of this project is to test whether dietary emulsifiers increase sensitivity to social stress and, if so, whether they do so, at least in part, by stimulating a pro-inflammatory state.**

1.2 Emulsifiers

Dietary emulsifiers are the most common food additive found in processed foods[25]. In fact, processed foods and beverages usually contain one or more emulsifiers that are used to stabilize mixtures, extend shelf life, and to enhance texture, uniformity, and volume [26]. Although these additives are recognized as safe for consumption by the FDA, it has been recently reported that these additives at translationally relevant doses can cause systemic inflammation and gut dysbiosis [14, 17, 18]. Specifically, the consumption of emulsifiers reduces microbial diversity and increases inflammation-promoting proteobacteria. Further, emulsifiers may disrupt the protective mucosal barrier in the gut,

allowing bacteria to translocate to the intestine's epithelial lining and resulting in an innate immune response and a pro-inflammatory cascade [17, 27]. Animals fed emulsifiers may be more susceptible to inflammation-driven disorders such as colitis, metabolic disorders, and colorectal cancer [17, 28]. Further, these phenotypes are transferrable via microbiota transplant from emulsifier-treated animals, suggesting that microbiota composition is a mechanism by which these disordered phenotypes arise [17, 28]. Emerging data indicate that emulsifier-treated animals may also display a baseline anxiety-like behavioral phenotype [18], suggesting that emulsifier-induced inflammation may also impact the brain. The mechanism whereby emulsifiers might contribute to the development of an anxiety-like behavioral phenotype, however, has yet to be determined.

It is possible that altered gene expression in areas of the brain that play a major role in modulating anxiety and stress responding, such as the amygdala and paraventricular nucleus of the hypothalamus (PVN), may be contributing to the anxiety-like behavioral phenotype following ingestion of emulsifiers. It is also possible that emulsifiers alter immune-related gene expression in the brain and that this contributes to an altered behavioral and neuroendocrine stress response, as many studies have demonstrated that alterations in neuro-immune signaling pathways can affect stress responding[29, 30]. Thus**, the purpose of Aim 1 was to test whether the consumption of emulsifiers, at doses that are used by the food industry, is sufficient to alter gene expression in the brain.** Such a result would suggest that dietary emulsifiers have a much more profound impact on the brain than has been previously known. The discovery of what gene pathways are impacted by emulsifiers could also give us an idea of potential mechanisms whereby these compounds might alter responses to stress.

1.3 Social Defeat Stress

Social stress is a commonly encountered stressor for most animals and is a major contributing factor in the etiology of a variety of stress-related diseases in humans [19]. The most common form of social stress experienced by animals is social defeat stress. Social defeat models, wherein an animal loses, or is defeated, in a confrontation with a conspecific, are widely used and are thought to have strong ethological and face validity [31-33]. Social defeat stress reliably induces anxiety- and depression-like phenotypes and is a naturally occurring stressor present in the environment of social animals [19, 32]. Humans and many other animals show increased social avoidance after social defeat [22, 31, 33, 34], and social avoidance is a prominent symptom of a variety of neuropsychiatric disorders including mood and anxiety disorders, post-traumatic stress disorder, and schizophrenia [21, 35, 36]. For these reasons, social defeat is often used in studies that investigate the relationship between stress and neuropsychiatric disease.

In the context of studying how social stress may interact with inflammation to produce or exacerbate neuropsychiatric disorders, it has recently been demonstrated that social defeat-induced anxiety in mice is correlated with various peripheral pro-inflammatory cytokines [37]. Thus, the use of social defeat as a stressor to test stress sensitivity in emulsifier-treated animals is ideal because emulsifiers stimulate peripheral inflammation, which we predict will make them more susceptible to social stress. **Thus, the purpose of Aim 2 is to test whether emulsifier consumption increases sensitivity to social stress.**

1.4 Inflammation, Social Stress, and Neuropsychiatric Disorders

Inflammation is an immune response mediated by immune cells and various molecular signals, such as cytokines and prostaglandins, working in concert to eliminate

pathogens, irritants, and unhealthy or damaged cells. Acute inflammation lasts hours to days and ceases immediately after the removal of the immune threat. In conditions where the threat is sustained or becomes dysregulated and anti-inflammatory molecular mediators are unable to inhibit the pro-inflammatory immune reaction, a chronic inflammatory state may result [38]. This chronic activation can result in increased tissue damage and can increase susceptibility to neuropsychiatric disease by disrupting neuroendocrine homeostasis [14, 39].

Elevated pro-inflammatory markers such as c-reactive protein (CRP), interleukin-6 (IL-6), interleukin-17 (IL-17), tumor necrosis factor alpha (TNFa), and interleukin 1 receptor antagonist (Il-1Ra) are associated with neuropsychiatric disorders including anxiety, depression, post-traumatic stress disorder (PTSD), and schizophrenia [6, 40-42]. Further, various autoimmune disorders including rheumatoid arthritis, diabetes, hepatitis, sepsis, Crohn's disease, and psoriasis are associated with a greater risk of developing a mood disorder [43-45]. Studies in both humans and mice that have experimentally induced a proinflammatory state through endotoxin exposure or exogenous administration of cytokines support the hypothesis that inflammation is a driving factor in the development of mood disorder, as subjects in these studies demonstrated various neuropsychiatric symptoms such as anhedonia, social avoidance, anxiety, and cognitive impairment [46-48]. Collectively, these findings demonstrate a positive association between inflammatory mechanisms and neuropsychiatric symptoms.

Stressors, particularly social stressors, can increase inflammatory activity in a manner that may promote neuropathology as discussed earlier. Furthermore, the relationship between social stress and inflammation appears to be bidirectional, as inflammation can increase neural and behavioral sensitivity to social stress and vice versa [49]. Under acute

conditions this bidirectional relationship is highly adaptive, in that stress-induced immune activation prepares the body for potential physical harm from an environmental threat and reciprocally, immune-induced sensitization of the stress response increases vigilance when the body is vulnerable [50, 51]. Under chronic conditions, however, social stress and inflammation can result in an anxiety and depression-like phenotype. Social stressors may interact with pro-inflammatory conditions to produce or exacerbate neuropsychiatric disorders, as both dietary inflammation and social stress are major predisposing risk factors in the development of stress-related disease in humans [14, 23, 24, 52]. Further investigation of inflammatory mechanisms that underlie increased susceptibility to stress-related pathology are essential to inform novel, improved therapeutic strategies to treat these disorders.

1.4.1 Prostaglandins

Peripheral inflammation from the gut can signal to the central nervous system (CNS) by multiple routes such as systemic circulation of cytokines which can disrupt the blood brain barrier, migration of gut-derived immune cells that translocate to the CNS, and afferent vagus nerve fibers [53-56]. In response to afferent inflammatory signals, a neuroinflammatory response is mounted in the CNS that consists of the release of inflammatory cytokines. A primary mediator of this cytokine cascade are prostaglandins, which are synthesized by cyclooxygenase (COX) enzymes in endothelial and perivascular cells as well as by microglia, the brain's resident immune cells [57-59]. Under inflammatory conditions, prostaglandins regulate HPA activity [60, 61]. Reciprocally, chronic activation of the HPA-axis through repeated restraint stress elevates constitutive COX1 and inducible COX2 cyclooxygenase in the PFC, hippocampus and hypothalamus [62]. Interestingly, basal expression of COX2 is restricted to areas of the brain that regulate stress responding, which

includes the basolateral amygdala where it is upregulated after stress exposure [63-65]. Other studies show that increased COX2 expression in the brain promotes susceptibility to chronic mild unpredictable stress in rats, and COX2 inhibition decreases anxiety-like behavior in mice and decreases glutamatergic activity in the amygdala [66, 67]. Further, pharmacological inhibition of the COX pathway has shown promise in clinical trials investigating its therapeutic potential to reduce symptoms of major depression [68, 69]. Taken together, the COX molecular pathway may be a prime mechanistic candidate driving emulsifier-induced increases in sensitivity to stress and a promising alternative or adjunctive treatment for stress-related neuropsychiatric disorders. **Thus, the purpose of AIM 3 is to test the hypothesis that inflammation, specifically through the COX pathway, is a mechanism driving emulsifier-induced stress sensitivity.**

1.5 Specific Aims Overview

1.5.1 Aim 1: Does consumption of dietary emulsifiers alter gene expression in brain regions that modulate the stress response?

Emulsifier-treated animals show increased peripheral inflammation and perhaps baseline anxiety-like behavior [18]. It is not yet known if emulsifiers induce inflammation centrally. It is possible that emulsifiers induce immune-related gene expression in the brain and that this contributes to altered stress behavior, as many studies have demonstrated that alterations in neuro-immune signaling pathways can affect stress responding[29, 30]. Altered gene expression in areas of the brain, such as the amygdala and PVN, that modulate behavioral and physiological responses to stress could be responsible for the anxiety-like behavioral phenotype. Further, identifying immune and stress-related genes may elucidate candidate mechanisms by which emulsifier consumption might alter stress-related behavior.

The purpose of this aim was to determine whether a supposedly innocuous dietary manipulation, namely the addition of low concentrations of emulsifiers, could elicit changes in gene expression in the brain. This is examined in two brain areas, the amygdala and the PVN, that are known to be critical for regulating behavioral and neuroendocrine responses to stress and known to be dysregulated in stress-induced neuropsychiatric disorders [32]. Using RNA-Seq, differential gene expression within the amygdala and PVN is compared between animals that consume emulsifiers in their drinking water versus controls drinking plain water.

1.5.2 Aim 2: Does emulsifier consumption increase susceptibility to social stress?

Initial data suggest that emulsifier-treated animals may display a baseline anxiety-like behavioral phenotype on a subset of behavioral tests thought to be reflective of anxiety-like responses [18]. It is not yet known whether animals that consume emulsifiers also show an enhanced stress response when subjected to stress. **We hypothesize that emulsifier-treated animals show increased sensitivity to social stress.** Experiment 2a will test whether emulsifier consumption increases social avoidance in response to social defeat, which is a highly salient social stressor. Experiment 2b will test whether emulsifier consumption increases serum corticosterone following social defeat. Experiment 2c will examine c-Fos protein expression, a marker of neuronal activity, in brain regions that are critical for behavioral and hormonal responses to stress to determine whether emulsifiers increase activation of stress-related brain regions following exposure to social defeat stress.

1.5.3 Aim 3: Does an anti-inflammatory intervention, specifically Cox pathway inhibition, ameliorate emulsifier-induced increases in vulnerability to social stress?

Increased COX2 expression in the brain enhances susceptibility to chronic mild unpredictable stress in rats, and Cox pathway inhibition decreases anxiety-like behavior in

mice [66, 67]. Thus, the Cox pathway may be a prime mechanistic candidate driving emulsifier-induced stress sensitivity. **We hypothesize that aspirin, a Cox pathway inhibitor, can ameliorate the increased vulnerability to social stress induced by emulsifiers.** Experiment 3a will test whether treatment with low-dose aspirin (25mg/kg/day) decreases social avoidance in emulsifier-treated animals. Experiment 3b will test whether treatment with low-dose aspirin decreases the corticosterone response to a social stressor.

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2 DIETARY EMULSIFIER CONSUMPTION ALTERS GENE EXPRESSION IN THE AMYGDALA AND PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS IN MICE

2.1 Introduction

Chronic low-grade inflammation has recently been identified as a key contributing factor in the etiology and progression of neuropsychiatric disorders [1, 2]. The immune system interacts with stress regulatory brain areas to affect brain and behavior by altering neurochemical signaling, neuroplasticity, and neuroendocrine processes [3, 4]. One common source of low-grade inflammation originates from the gastrointestinal tract when the host/microbiota interaction is altered through agents such as dietary factors. Indeed, select dietary components can lead to chronic, low-grade inflammation and may thereby increase susceptibility to developing a neuropsychiatric disorder [2, 5]. Food and drink additives, such as emulsifiers, are commonly added to processed foods and many beverages to improve texture, consistency, and to extend shelf-life. Although these additives are generally viewed as innocuous and are classified by the Food and Drug Administration as Generally Recognized As Safe, it has been recently reported that these additives at translationally relevant doses can cause systemic inflammation [5-7]. Specifically, consumption of emulsifiers, such as carboxymethylcellulose (CMC) and polysorbate-80 (P80), appear to reduce microbial diversity and to increase inflammation-promoting proteobacteria. CMC and P80 appear to act through different mechanisms on the intestinal microbiota in a way that promotes chronic intestinal inflammation that manifests as colitis in genetically susceptible mice and metabolic deregulations in mice that are not genetically susceptible [6, 8]. Importantly, these phenotypes are abolished in germ-free mice and are transferrable via microbiota transplant from emulsifier-treated mice to germ-free mice,

suggesting that the intestinal microbiota is both required and sufficient to drive emulsifier-induced detrimental effects [6, 8]. Interestingly, previous data suggest that emulsifier-treated animals may also display an anxiety-like behavioral phenotype [7], suggesting that emulsifier-induced shifts in the microbiome may also impact the brain. The mechanism whereby emulsifiers contribute to the development of an anxiety-like behavioral phenotype, however, has yet to be determined.

Stress-related disorders are often characterized by dysregulation of the hypothalamic-pituitary-adrenocortical (HPA) axis [9], the neuroendocrine cascade that is a key modulator of the body's response to stress[9]. Furthermore, the HPA axis has a bi-directional relationship with the innate immune system as well as with the gut microbiota[10, 11]. Sustained activation of the HPA axis can affect gut microbial composition and gastrointestinal permeability[12]. Conversely, antibiotic-induced gut dysbiosis can sensitize the HPA axis, resulting in excessive stress hormone release after acute stress exposure[13]. It is possible that altered gene expression in areas of the brain that play a major role in modulating anxiety and stress responding, such as the amygdala and paraventricular nucleus of the hypothalamus (PVN), may be responsible for the anxiety-like behavioral phenotype following ingestion of emulsifiers. It is also possible that emulsifiers induce inflammation in the brain and that this contributes to an altered behavioral and neuroendocrine stress response, as many studies have demonstrated that alterations in neuro-immune signaling pathways can affect stress responding[14, 15]. Hence, the purpose of this present study was to test whether consumption of emulsifiers at doses used by the food industry are sufficient to alter gene expression in the brain in a way that might illuminate how these compounds alter stress responding and anxiety-like states. Using RNA-sequencing, we investigated whether emulsifier consumption alters gene expression in two critical stress-

modulatory brain regions, the amygdala and PVN. We hypothesized that ingestion of emulsifiers alters immune and stress-related gene expression in both the amygdala and PVN. Overall, we present a comprehensive genome-wide analysis of differentially expressed genes after emulsifier consumption in two brain regions that modulate the stress response.

2.2 Results

2.2.1 Verification of emulsifier-induced metabolic syndrome and low-grade inflammation

To verify that the animals used in the RNA-Seq and qRT-PCR validation experiment display the previously described low-grade inflammation and altered metabolic phenotype, the body weight of each subject was measured throughout the duration of the study and organs were weighed at the time of sacrifice. Animals that consumed emulsifiers showed a significant increase in weight over time (**Figure 2.1.a**; p=.0279) and increased adiposity, measured by fat pad weight (**Figure 2.1.b**; CMC vs Water, p=.0002 and P80 vs Water, p=<.0001). Mice that consumed emulsifiers also had significantly shorter colons (**Figure 2.1.c**; CMC vs Water, p=<.0001 and P80 vs Water, p=.008) and increased spleen weight **(Figure 2.1.d**; CMC vs Water, p=<.0001 and P80 vs Water, p=.0015), a phenotype that results from low-grade inflammation. Altogether, these data verify that the animals used in the RNA-Seq and qRT-PCR validation study display a phenotype consistent with metabolic syndrome and low-grade inflammation.

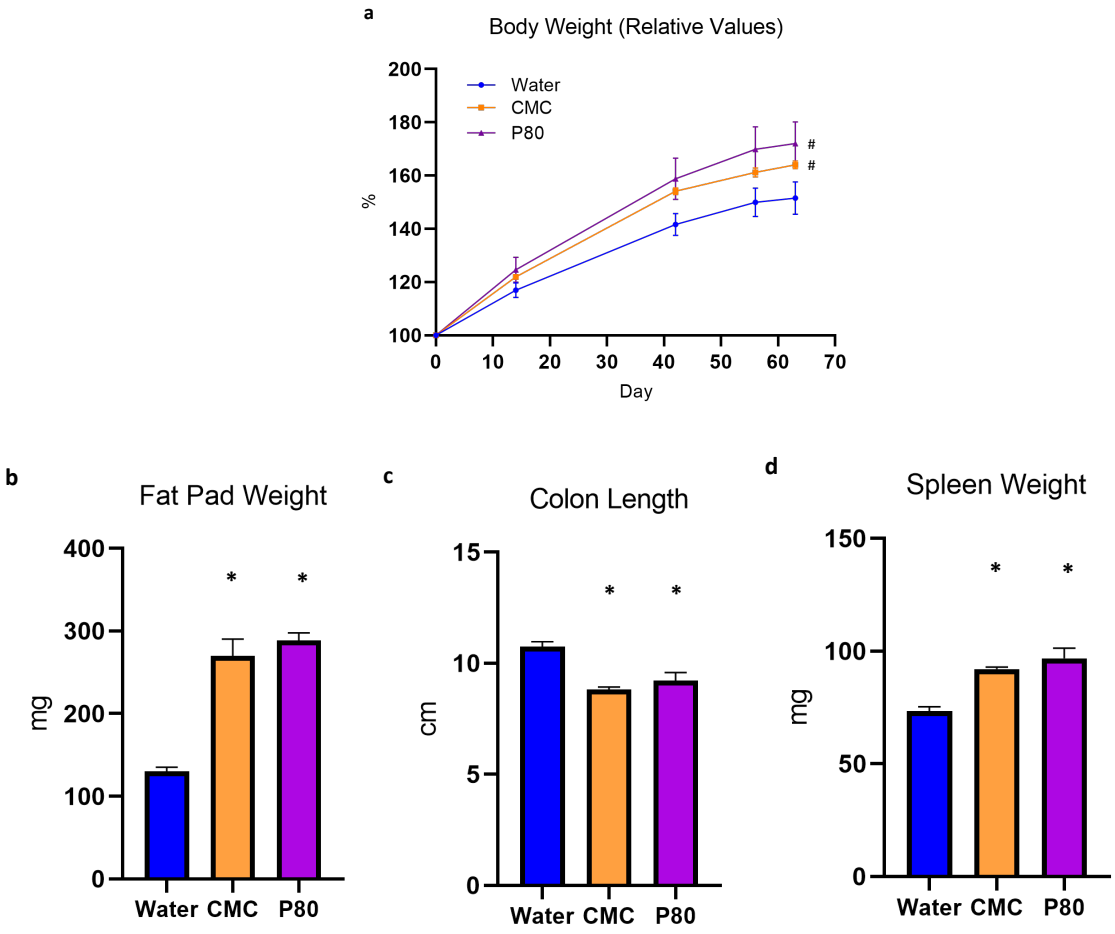

Figure 2.1 Emulsifier consumption results in metabolic syndrome and low-grade inflammation

*Note. (***a**) Relative body weight over time. Body weight is expressed as a percentage compared to the initial body weight (Day 0) defined as 100%. Ingestion of emulsifiers significantly increased fat-pad mass (**b**), decreased colon length (**c**), and increased spleen weight (**d**) compared to controls. Significance was determined using a repeated measure one-way ANOVA with a Geisser-Greenhouse correction (#$p < 0.05$) or unpaired t-tests (*$p < 0.05$). N=5 in each group.

2.2.2 Consumption of dietary emulsifiers alter hypothalamic and amygdala gene expression

To identify potential mechanisms by which emulsifier exposure could alter anxiety-like behavior, we applied an unbiased approach and performed total mRNA sequencing of the amygdala and PVN regions, known to play a central role in stress-responding and anxiety-like behaviors. Importantly, we observed that emulsifier consumption is associated with the modulation of gene expression in the amygdala and the PVN. Volcano plots and Venn diagrams

were generated to broadly show the number of genes that were changed in the amygdala and PVN after each emulsifier treatment compared to water-only controls. **Figure 2.2** demonstrates that a relatively low number of genes were found to be significantly differentially expressed (padj < .05, Log2 FC > 1 and <-1) in the amygdala (**Figures 2.2.a and 2.2.b**) and PVN (**Figures 2.2.c and 2.2.c**). Only genes that fit differential expressed gene (DEG) criteria were used for subsequent analyses (Figures 3-5 and Tables 1-3). As presented in **Figure 2.3**, the number of shared and different DEGs between emulsifier conditions in each brain region is relatively low compared to the total number of variables studied (18,778). In total, 243 genes were differentially expressed in the amygdala and PVN of emulsifier-treated animals compared to control animals. Counts include 56 unique DEGs in the amygdala after CMC treatment and 59 with P80 treatment. In addition, 52 unique DEGs were counted in the PVN after CMC treatment and 76 with P80 treatment. 9 DEGs were shared between CMC- and P80-treated mice in the amygdala and 9 DEGs were shared between CMC- and P80-treated mice in the PVN. Importantly, of the differentially expressed genes shared in common between CMC and P80, several were immediate early genes (IEGs), which are widely used as a molecular marker of neuronal activity. Emulsifier treatment increased expression of IEGs such as NR4A3, EGR2, FOSB, and FOSL2 in the amygdala and PVN, suggesting that both CMC and P80 may increase neuronal activity in these brain regions. A hierarchical heat map (**Figure 2.4**), grouping genes with similar transcriptional profiles into clusters, was included to show the direction of differential expression of each DEG and global DEG patterns occurring within (comparing individuals within each group) and between (water versus emulsifier) conditions.

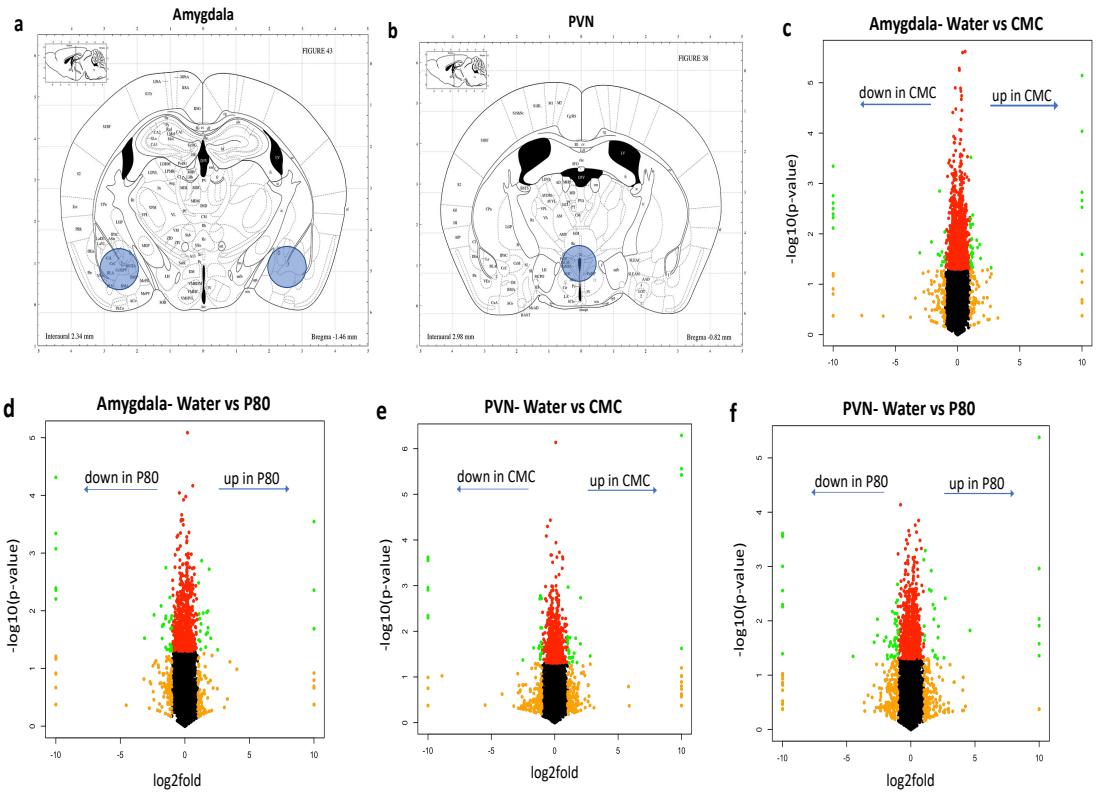

Figure 2.2 Dietary emulsifier consumption alters gene expression in the amygdala and paraventricular nuclei of the hypothalamus

Note. Wild-type (WT) mice were exposed to plain drinking water or water containing CMC or P80 (1.0%) for 12 weeks and brain tissue was harvested. The extent of tissue punch in the amygdala (**a**) and PVN (**b**). Sections adapted from Paxinos, George, and Franklin 2001.Total RNAs were extracted, mRNAs purified, and subjected to library preparation and sequencing. Genes were filtered to keep only genes expressed in at least one condition (average FPKM (Fragments Per Kilobase Million) > 1 in at least one group) and were visualized on volcano plots. (**c**) Water-treated versus CMC-treated, amygdala. (**d**) Water-treated versus P80-treated, amygdala. (**e**) Water-treated versus CMC-treated, paraventricular nucleus. (**f**) Water-treated versus P80-treated, paraventricular nucleus. For each gene, the difference in abundance between the two groups is indicated in log2 fold change on the x-axis (with positive values corresponding to an increase in the emulsifier-treated group compared with the water-treated group, and negative values corresponding to a decrease in the emulsifier-treated group compared with the water-treated group). Log2 fold values that exceeded 10 or -10 are recorded as 10 or -10. Significance between the two groups is indicated by −log10 p-value on the y-axis. Red dots correspond to genes with adj. $p<0.05$ between emulsifier-treated and water-treated groups. Orange dots correspond to genes with at least a one-fold decreased or increased expression in the emulsifier-treated group compared with the water-treated group. Green dots represent differentially expressed genes (adj. $p<0.05$, log2 FC >1 and <-1) between control and emulsifier groups. Only genes in green were used for subsequent analysis.

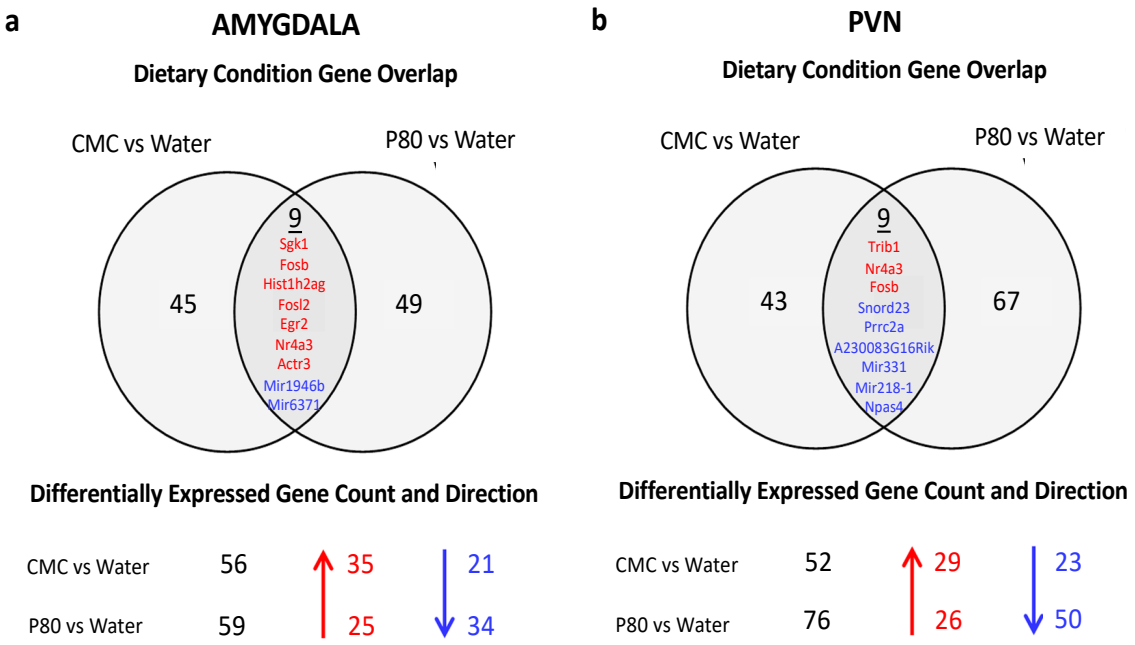

Figure 2.3 Quantified DEGs in the amygdala and PVN after treatment with P80 or CMC

Note. Quantified genes were differentially expressed and met analysis criteria with an adjusted p-value of less than 0.05 and exceeded log2fold change of 1 and -1. Venn-diagram shows the overlap of significant differentially expressed genes between CMC and P80 treatments. Upregulated DEGs are in red and downregulated DEGs are in blue.

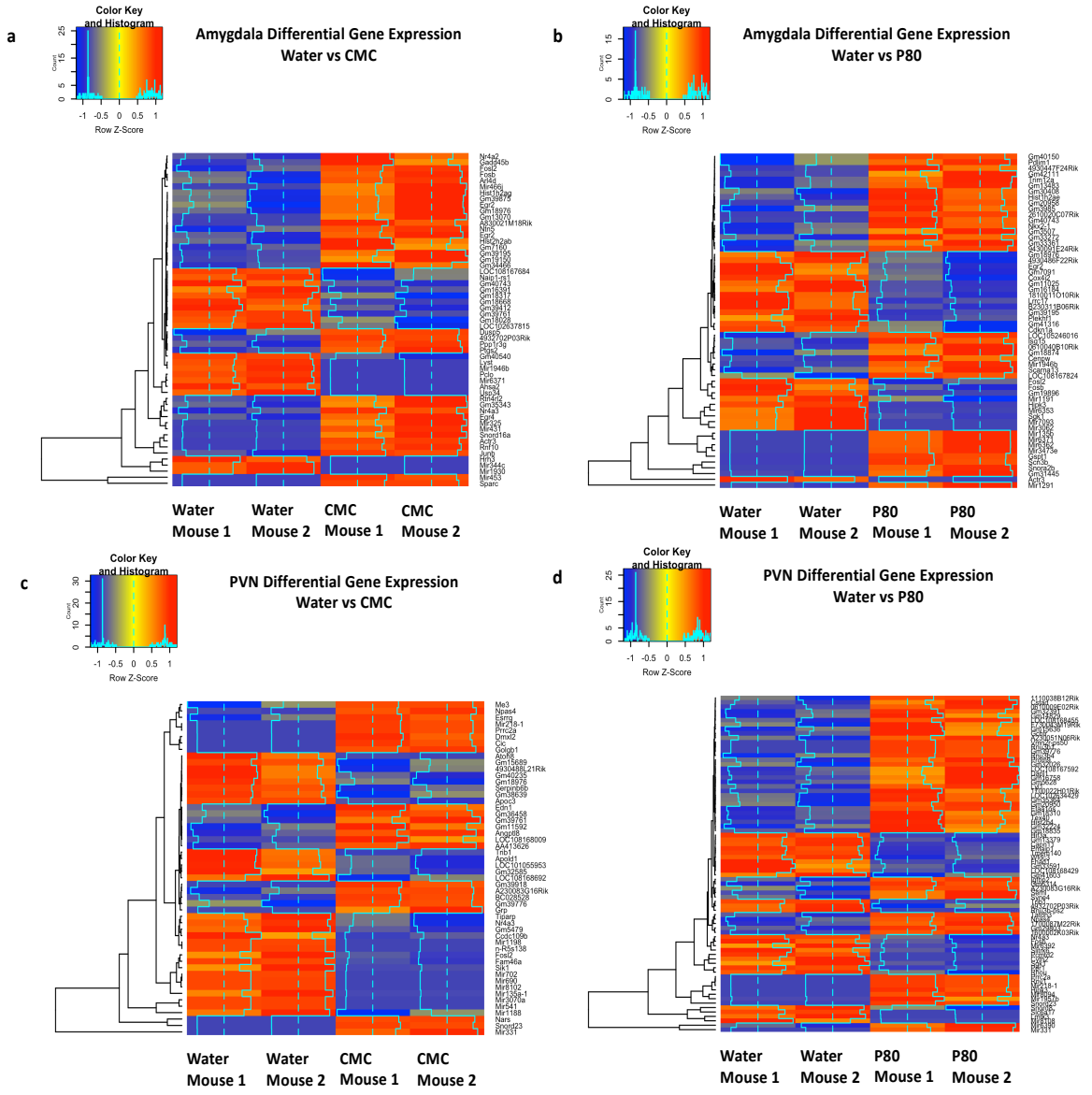

Figure 2.4 Heat maps show z-score normalized relative expression of differentially expressed genes that met criteria maps show z-score normalized relative expression of differentially expressed genes that met criteria

Note. Heatmaps show z-score normalized relative expression of differentially expressed genes that met criteria (FC >1, adj.p-value <0.05). Hierarchical clustering based on Euclidian distance of 56 differentially expressed genes in the amygdala after CMC treatment and 59 with P80 treatment. 52 differentially expressed genes in the PVN after CMC treatment and 76 with P80 treatment are listed. Genes are indicated in rows and animals are in columns. Blue indicates downregulation and red upregulation of the differentially expressed genes. Heatmaps were generated using the gplots and RColorBrewer libraries in R

2.2.3 Gene Ontology

To explore the potential biological meaning of the identified differentially expressed genes, we next used EnrichR to perform gene ontology to identify biological processes or molecular functions (**Table 2.1 and Table 2.2**) involved[16-18]. Analysis of gene ontology yielded statistically significant (p-value < .05) enriched terms. An important stress and immune function term, "glucocorticoid receptor binding" was enriched in both CMC and P80 conditions, which may suggest possible HPA-axis dysfunction under both emulsifier conditions. DEGs known to code for protein that regulate glucocorticoid receptor (GR) binding include Nuclear Receptor Subfamily 4 Group A Member 3 (NR4A3, ^), Nuclear Receptor Subfamily 4 Group A Member 2 (NR4A2, ^), and Serum- and Glucocorticoid-inducible Kinase 1 (SGK1, ^). Specifically, NR4A3 and NR4A2 transcription factors inhibit GR-dependent repression of the POMC gene, a gene that encodes the precursor protein needed to synthesize ACTH. Further, both NR4A3 and NR4A2 have been shown to regulate HPA-axis neuroendocrine activity within the PVN, pituitary, and adrenals[19]. SGK1 potentiates and prolongs GR activation following cortisol stimulation by increasing GR phosphorylation and nuclear translocation [20]. SGK1 is both a downstream target of GR signaling and also affects GR activation by positively regulating the long-lasting effects of glucocorticoids[21]. The increase in differential expression of the above-mentioned genes in our dataset may suggest alterations in HPA-axis activity in animals that consume emulsifiers. Interestingly, several metabolism-related pathways were also enriched in the PVN as a result of emulsifier consumption. For example, for the CMC-induced deregulated DEGs, these pathways included "regulation of triglyceride metabolic process," "regulation of lipoprotein lipase activity," "regulation of lipid biosynthetic process," "acylglycerol homeostasis," and "triglyceride homeostasis," whereas the P80-induced

deregulated DEGs included metabolic-related terms such as “insulin-like growth factor” I and II binding. These data suggest possible neural mechanisms that may contribute to the dysfunctional metabolic phenotype previously described in emulsifier-treated mice[6].

Table 2.1 Gene Ontology of differentially expressed genes in the amygdala of emulsifier treated mice

Gene Ontology Functional Enrichment Analysis			
Amygdala- CMC vs Water			
GO ID	***Biological Process Description***	***p-value***	***DEGs***
GO:0031643	positive regulation of myelination	4.02E-04	RNF10;EGR2
GO:0045444	fat cell differentiation	5.46E-04	NR4A2;EGR2;NR4A3
GO:0031646	positive regulation of nervous system process	1.10E-03	RNF10;EGR2
GO:0031641	regulation of myelination	2.31E-03	RNF10;EGR2
GO:0099643	signal release from synapse	6.24E-03	PCLO;HRH3
GO:0007269	neurotransmitter secretion	6.52E-03	PCLO;HRH3
GO:0031620	regulation of fever generation	1.37E-02	PTGS2
GO:0031394	positive regulation of prostaglandin biosynthetic	1.37E-02	PTGS2
GO:1903208	negative regulation of hydrogen peroxide-induced	1.37E-02	NR4A3
GO:2000253	positive regulation of feeding behavior	1.37E-02	NR4A3
GO ID	***Molecular Function Description***	***p-value***	***DEGs***
GO:0035259	glucocorticoid receptor binding	2.64E-04	NR4A2;NR4A3
GO:0001228	DNA-binding transcription activator activity, RNA	2.19E-03	NR4A2;EGR2;NR4A3;EGR4;FOSL2
GO:1990837	sequence-specific double-stranded DNA binding	1.31E-02	NR4A2;RNF10;EGR2;EGR4;FOSB;JUNB
GO:0016907	G protein-coupled acetylcholine receptor activity	1.91E-02	HRH3
GO:0099528	G protein-coupled neurotransmitter receptor activity	1.91E-02	HRH3
GO:2001069	glycogen binding	2.18E-02	PPP1R3G
GO:0017017	MAP kinase tyrosine/serine/threonine phosphatase	2.72E-02	DUSP5
GO:0008330	protein tyrosine/threonine phosphatase activity	2.72E-02	DUSP5
GO:0035497	cAMP response element binding	2.72E-02	NR4A3
GO:0061665	SUMO ligase activity	2.72E-02	EGR2
Amygdala- P80 vs Water			
GO ID	***Biological Process Description***	***p-value***	***DEGs***
GO:0048539	bone marrow development	1.49E-02	LRRC17
GO:0086018	SA node cell to atrial cardiac muscle cell signaling	1.49E-02	SCN3B
GO:0014037	Schwann cell differentiation	1.49E-02	EGR2
GO:0044843	cell cycle G1/S phase transition	1.54E-02	CDKN1A;GSPT1
GO:0060441	epithelial tube branching involved in lung	1.79E-02	NKX2-1

GO:0032020	ISG15-protein conjugation	1.79E-02	ISG15
GO:0060373	regulation of ventricular cardiac muscle cell membrane	1.79E-02	SCN3B
GO:0044058	regulation of digestive system process	1.79E-02	SGK1
GO:0060371	regulation of atrial cardiac muscle cell membrane	2.08E-02	SCN3B
GO:0086015	SA node cell action potential	2.08E-02	SCN3B
GO ID	***Molecular Function Description***	***p-value***	***DEGs***
GO:0017080	sodium channel regulator activity	5.51E-03	SCN3B;SGK1
GO:0086006	voltage-gated sodium channel activity involved in	1.49E-02	SCN3B
GO:0019871	sodium channel inhibitor activity	2.38E-02	SCN3B
GO:0004861	cyclin-dependent protein serine/threonine kinase	2.96E-02	CDKN1A
GO:0061665	SUMO ligase activity	2.96E-02	EGR2
GO:0017081	chloride channel regulator activity	4.41E-02	SGK1
GO:0004129	cytochrome-c oxidase activity	4.41E-02	COX4I2
GO:0051371	muscle alpha-actinin binding	4.41E-02	PDLIM1
GO:0031625	ubiquitin protein ligase binding	4.53E-02	CDKN1A;EGR2;ISG15
GO:0010314	phosphatidylinositol-5-phosphate binding	4.70E-02	PLEKHF1

Note. Using EnrichR, differentially regulated gene lists were evaluated for significant enrichment against the following gene set libraries: GO Biological Process, GO Molecular Function (all from http://www.geneontology.org). The top 10 enriched terms were selected and ranked based upon the combined score that was calculated by the EnrichR platform following Z-score permutation background correction on the Fisher exact test p-value. Results were filtered for $p < 0.05$. Upregulated genes in red and downregulated genes in blue.

Table 2.2 Gene Ontology of differentially expressed genes in the PVN of emulsifier treated mice

PVN- CMC vs Water			
GO ID	***Biological Process Description***	***p-value***	***DEGs***
GO:0090209	negative regulation of triglyceride metabolic process	9.88E-05	APOC3;SIK1
GO:0045601	regulation of endothelial cell differentiation	2.36E-04	ATOH8;APOLD1
GO:0048660	regulation of smooth muscle cell proliferation	2.81E-04	EDN1;NR4A3;TRIB1
GO:0010613	positive regulation of cardiac muscle hypertrophy	9.88E-04	EDN1;NR4A3
GO:0046887	positive regulation of hormone secretion	1.22E-03	EDN1;GRP
GO:0051004	regulation of lipoprotein lipase activity	1.35E-03	ANGPTL8;APOC3
GO:0051055	negative regulation of lipid biosynthetic process	1.48E-03	APOC3;SIK1
GO:0010611	regulation of cardiac muscle hypertrophy	1.76E-03	EDN1;NR4A3
GO:0055090	acylglycerol homeostasis	1.91E-03	ANGPTL8;APOC3
GO:0070328	triglyceride homeostasis	2.94E-03	ANGPTL8;APOC3
GO ID	***Molecular Function Description***	***p-value***	***DEGs***
GO:0016615	malate dehydrogenase activity	1.55E-02	ME3
GO:0005179	hormone activity	1.76E-02	EDN1;GRP
GO:0031434	mitogen-activated protein kinase kinase binding	2.06E-02	TRIB1
GO:0035259	glucocorticoid receptor binding	2.32E-02	NR4A3
GO:0008140	cAMP response element binding protein binding	2.32E-02	SIK1
GO:0035497	cAMP response element binding	2.57E-02	NR4A3
GO:0055102	lipase inhibitor activity	2.57E-02	APOC3
PVN- P80 vs Water			
GO ID	***Biological Process Description***	***p-value***	***DEGs***
GO:0007616	long-term memory	2.34E-03	NPAS4;SGK1
GO:0046329	negative regulation of JNK cascade	6.97E-03	PER1;HIPK3
GO:0048660	regulation of smooth muscle cell proliferation	1.49E-02	NR4A3;TRIB1
GO:0007614	short-term memory	1.89E-02	NPAS4
GO:1903208	negative regulation of hydrogen peroxide-induced neuron death	1.89E-02	NR4A3
GO:2000253	positive regulation of feeding behavior	1.89E-02	NR4A3
GO:1903749	positive regulation of establishment of protein localization to	1.92E-02	PMAIP1;RHOU
GO:0015820	leucine transport	2.26E-02	SLC6A17
GO:0061469	regulation of type B pancreatic cell proliferation	2.26E-02	NR4A3

GO:2000323	negative regulation of glucocorticoid receptor signaling pathway	2.26E-02	PER1
GO ID	***Molecular Function Description***	***p-value***	***DEGs***
GO:0051998	protein carboxyl O-methyltransferase activity	2.26E-02	PCMTD2
GO:0031995	insulin-like growth factor II binding	2.63E-02	IGFBP2
GO:0031434	mitogen-activated protein kinase kinase binding	3.00E-02	TRIB1
GO:0035259	glucocorticoid receptor binding	3.37E-02	NR4A3
GO:0035497	cAMP response element binding	3.74E-02	NR4A3
GO:0046975	histone methyltransferase activity (H3-K36 specific)	4.10E-02	PRDM9
GO:0070679	inositol 1,4,5 trisphosphate binding	4.10E-02	CYTH2
GO:0070513	death domain binding	4.10E-02	DAPL1
GO:0031994	insulin-like growth factor I binding	4.83E-02	IGFBP2

Note. Using EnrichR, differentially regulated gene lists were evaluated for significant enrichment against the following gene set libraries: GO Biological Process, GO Molecular Function (all from http://www.geneontology.org). The top 10 enriched terms were selected and ranked based upon the combined score that was calculated by the EnrichR platform following Z-score permutation background correction on the Fisher exact test p-value. Results were filtered for $p < 0.05$. Upregulated genes in red and downregulated genes in blue.

2.2.4 Enriched Pathways

To gain a mechanistic understanding of the altered gene expression observed during emulsifier consumption, we next performed bioinformatic analysis using INGENUITY® Pathway Analysis (IPA) to examine the biological relevance of altered gene pathways using a systems biology approach. Genes imported into IPA had a stringency filter set for statistically significant genes (fold-change >1, adj *p*-value < 0.05) within the amygdala and PVN of emulsifier vs water controls. The top 5 canonical pathways (CPs) are presented in **Table 2.3**. These analyses revealed sets of genes within the amygdala and PVN tissue that were highly enriched in primarily immune-related canonical pathways. Interestingly, though both CMC and P80 conditions resulted in enriched immune-related pathways within the amygdala and PVN, neither condition had a common pathway shared. Among CMC-treated animals, the top dysregulated canonical pathways in the amygdala were MIF regulation of innate immunity (p = 9.47E-05), corticotropin-releasing hormone (CRH) signaling (p = 2.45E-04), Coronavirus pathogenesis pathway (p = 2.79E-04), CD40 signaling (p = 3.48E-04), and ILK signaling (p = 6.81E-04). Within the PVN, the top canonical pathways included opioid signaling pathway (p = 4.14E-06), G-protein coupled receptor signaling (p = 8.10E-05), CRH signaling (p =9.13E-05), CREB signaling in neurons (p = 1.95E-04), and CDK5 signaling (p = 2.19E-04). **Figure 2.5** depicts the IPA predicted molecular interactions of DEGs within the CRH pathway. Functionally, glucocorticoid synthesis may be Nur77/Nr4a1 dependent and pro-inflammatory prostaglandin synthesis may be COX2 dependent within the canonical CRH pathway. Among P80-treated animals, the top dysregulated canonical pathways in the amygdala were IL-17A signaling in gastric cells (p = 1.08E-03), T-cell receptor signaling (p = 1.39E-03), CD28 signaling in T-helper cells (p =1.67E-03), TNFR2 signaling (p =1.84E-03), and IL-17A signaling

in fibroblasts (p = 2.22E-03). Within the PVN, the top canonical pathways included role of JAK2 in hormone-like cytokine signaling (p = 6.22E-04), opioid signaling pathway (p = 1.52E-03), role of Wnt/GSK-3 signaling in the pathogenesis of Influenza (p =4.63E-03), human embryonic stem cell pluripotency (p =5.02E-03), and role of JAK1 and JAK3 in cytokine signaling (p =5.49E-03). In addition, analysis of significant disease pathways in IPA showed considerable overlap between emulsifier conditions but also showed regional differences that likely reflect the region-specific response to emulsifier treatment (**Figure 2.6**). Altogether, these results demonstrate that CMC and P80 consumption induces limited but physiologically relevant alterations in gene expression in brain regions controlling physiological and behavioral concomitants of stress and anxiety.

Table 2.3 Top five IPA enriched canonical pathways of differentially expressed genes in the amygdala and PVN of emulsifier treated mice.

Top Canonical Pathways		
Amygdala- CMC vs Water		
Name	***p-value***	***Overlap***
MIF Regulation of Innate Immunity	9.47E-05	7.1% 3/42
Corticotropin Releasing Hormone Signaling	2.45E-04	2.8% 4/145
Coronavirus Pathogenesis Pathway	2.79E-04	2.7% 4/150
CD40 Signaling	3.48E-04	4.6% 3/65
ILK Signaling	6.81E-04	2.1% 4/190
Amygdala- P80 vs Water		
Name	***p-value***	***Overlap***
IL-17A Signaling in Gastric Cells	1.08E-03	8.7% 2/23
T Cell Receptor Signaling	1.39E-03	2.9% 3/104
CD28 Signaling in T Helper Cells	1.67E-03	2.7% 3/111
TNFR2 Signaling	1.84E-03	6.7% 2/30
IL-17A Signaling in Fibroblasts	2.22E-03	6.1% 2/33

PVN- CMC vs Water		
Name	***p-value***	***Overlap***
Opioid Signaling Pathway	4.14E-06	3.8% 9/236
G-Protein Coupled Receptor Signaling	8.10E-05	3.0% 8/267
Corticotropin Releasing Hormone Signaling	9.13E-05	4.3% 6/139
CREB Signaling in Neurons	1.95E-04	1.9%
CDK5 Signaling	2.19E-04	4.8% 5/104
PVN- P80 vs Water		
Name	***p-value***	***Overlap***
Role of JAK2 in Hormone-like Cytokine	6.22E-04	9.4% 3/32
Opioid Signaling Pathway	1.52E-03	2.5% 6/236
Role of Wnt/GSK-3 Signaling in the	4.63E-03	4.7% 3/64
Human Embryonic Stem Cell Pluripotency	5.02E-03	3.1% 4/131
Role of JAK1 and JAK3 in c Cytokine Signaling	5.49E-03	4.4% 3/68

Note. Emulsifier-treatment is primarily associated with inflammation and other immune-related canonical pathways. The top categories were ranked in accordance with their P-value of overlap. Overlap refers to the number of molecules from the dataset that map to the pathway listed divided by the total number of molecules that define the canonical pathway from within the IPA knowledgebase.

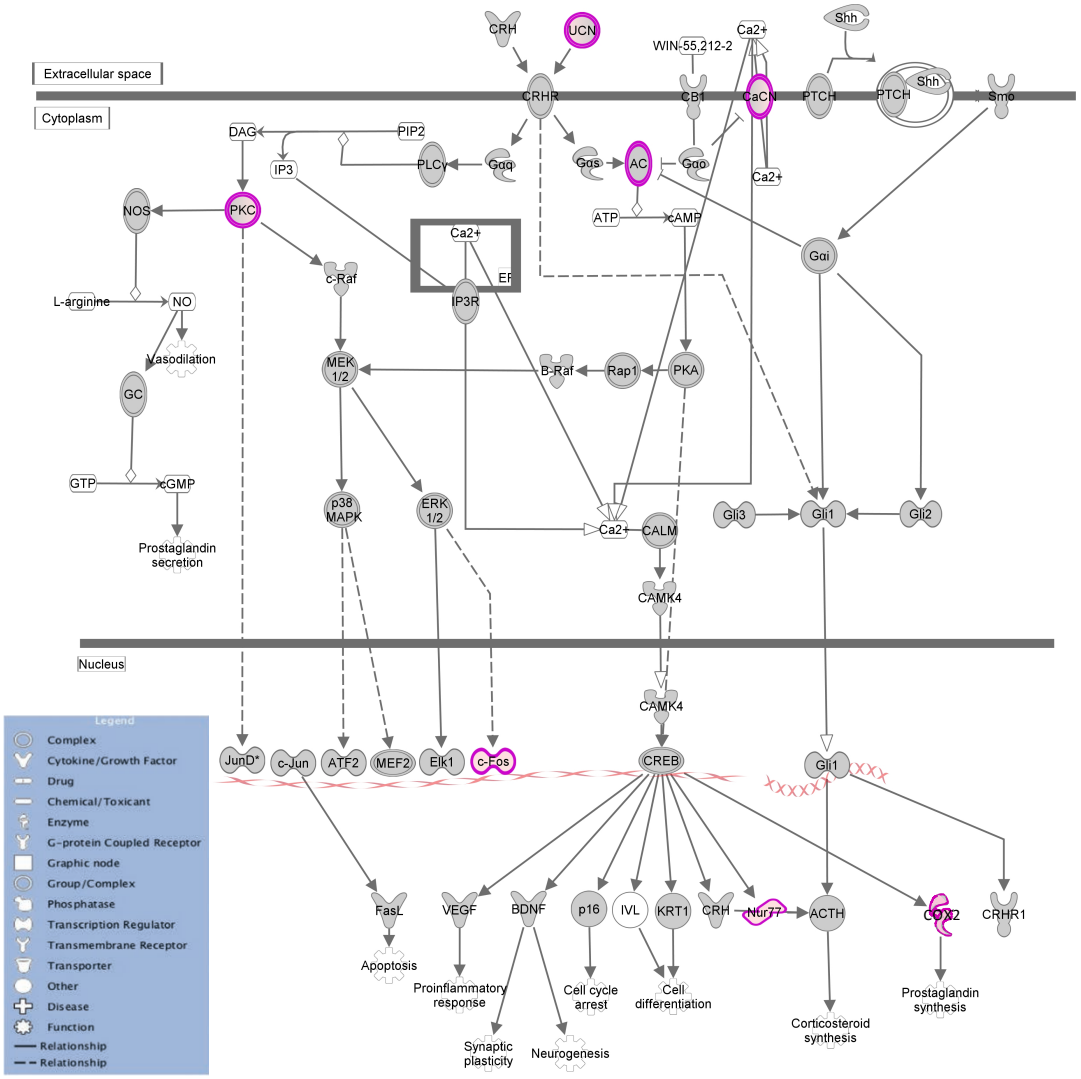

Figure 2.5 Corticotropin-Releasing Hormone Pathway Identified by Ingenuity Pathway Analysis

Note. The CRH pathway is identified as one of the significant pathways by IPA within the PVN of CMC-treated animals (p= 9.13E-05). All up-regulated DEGs in our dataset are shown here in pink and overlaid onto the CRH molecular pathway.

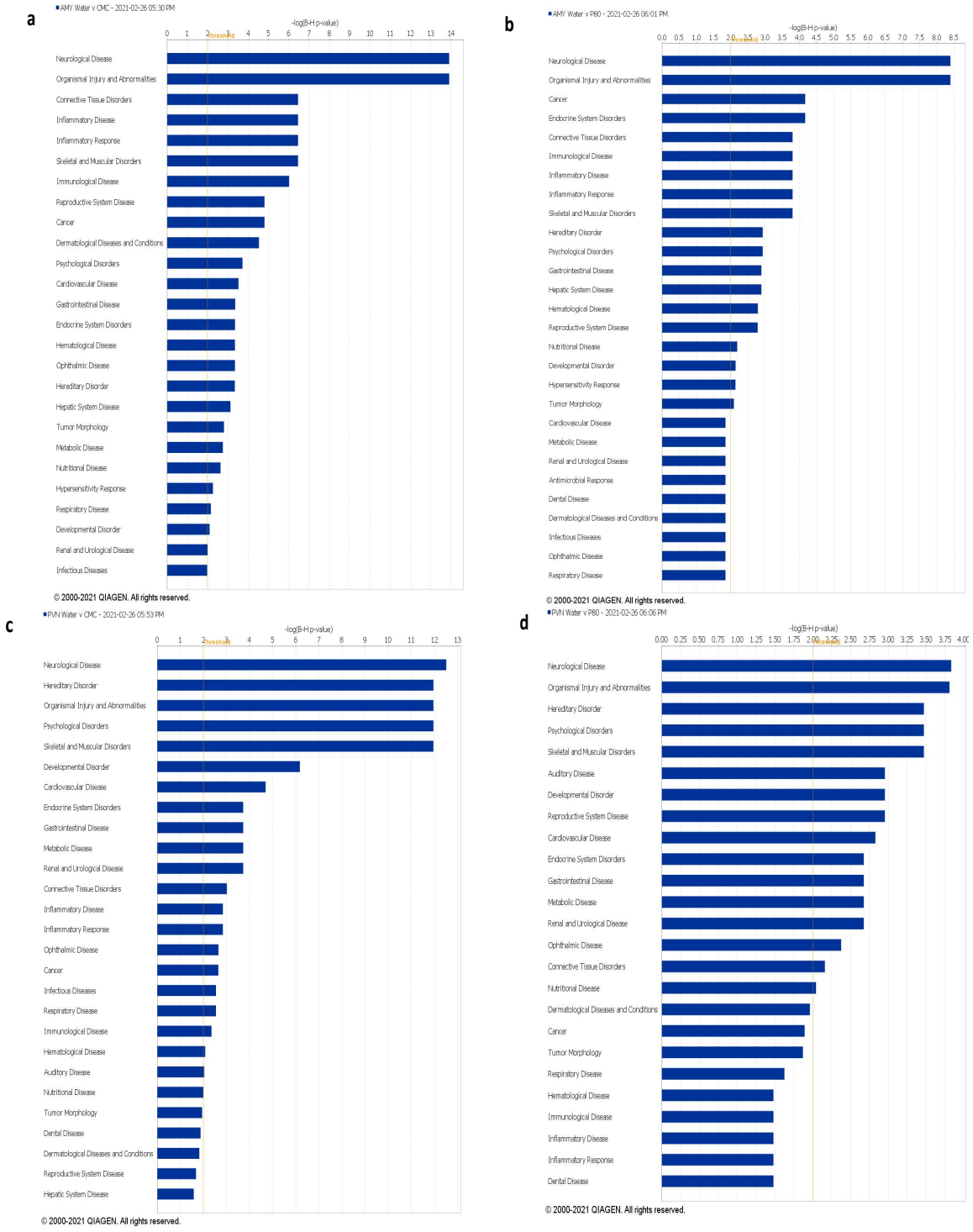

Figure 2.6 Top enriched IPA disease pathways

Note. Top diseases in the amygdala after CMC treatment (**a**) or P80 treatment (**b**) and PVN after CMC treatment (**c**) or P80 treatment (**d**). Threshold indicates minimum significance level [–log (p-value) from Fisher's exact test].

2.2.5 Validation of RNA-Seq results by quantitative real-time PCR (qRT-PCR)

Six DEGs were selected due to their involvement in stress responding and immune function according to previous studies. SGK1, NR4A3, PRRC2A, FOSB, PTGS2, and EGR2 were selected to validate RNA-Seq results by qRT-PCR (**Table 2.4**). Expression levels calculated via RNA-Seq were significantly positively correlated to expression levels determined via qRT-PCR (**Figure 2.7**; R2=0.8815, p<0.0001). For all genes tested, qRT-PCR data strongly correlate with RNA-Seq data except for PTGS2 expression levels within the PVN. Expression levels of PTGS2 measured by qRT-PCR were higher than indicated by RNA-Seq expression. Overall, the correlation observed between qRT-PCR and RNA-seq further strengthens the emulsifier-induced alteration in gene expression described above, validating the RNA-seq approach on punched brain regions to investigate the impact of altered host/microbiota relationship on the central nervous system.

Table 2.4 List of primer sequences used for qRT-PCR.

qRT-PCR Primers		
Gene	**Forward-sequence**	**Reverse-sequence**
Sgk1	TCCTGAGGTCCTCCATAAGCA	GTGCCTTGCCGAGTTTGTAAT
Nr4a3	TTCTGACGGCCTCCATTGAC	CTCCCCAAATCCTCGAAGGC
Prrc2a	GGACTCTGCCGGGGTTAAT	AGAAGACCTCAGGGTACGGA
Fosb	GCCAGGAACCAGCTACTCAA	CTTGTTCCGCTCTCTGCGAA
Ptgs2	CTGACCCCCAAGGCTCAAAT	TCTGCTCTGGTCAATGGAGG
Egr2	GCCGTAGACAAAATCCCAGT	AGCTACTCGGATACGGGAGA
Ywhaz	GATCCCCAATGCTTCGCAAC	CCAGTCTGATGGGGTGTGTC

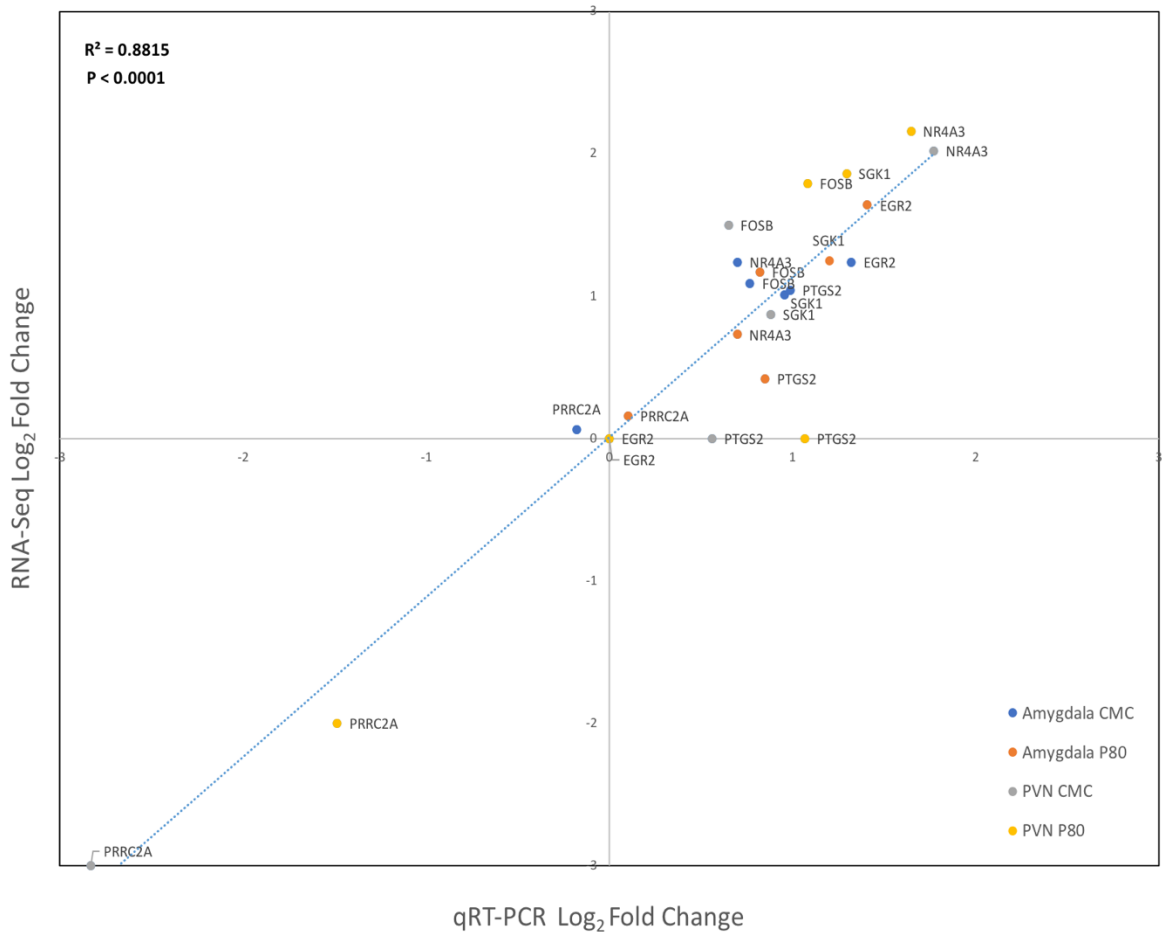

Figure 2.7 Validation of RNA-Sequencing results by quantitative RT-PCR.

Note. Log2 fold change correlation between RNA-Seq and quantitative real-time PCR (qRT-PCR) for six differentially expressed genes (SGK1, NR4A3, PRRC2A, FOSB, PTGS2, EGR2; n=5) within Amygdala CMC, Amygdala P80, PVN CMC, and PVN P80 conditions. Correlation between RNA-Seq and qRT-PCR gene expression (R2=0.8815, p<0.0001).

2.2.6 Consumption of dietary emulsifiers does not result in dehydration

One potential confounding factor that could drive gene expression changes in the current study is changes in hydration[22] among groups if the emulsifiers altered water intake or absorption. To eliminate the possibility that gene changes were secondary to changes in hydration rather than to the emulsifier treatment, itself, various hydration measures were compared between emulsifier-treated animals and water controls. Measures included liquid

intake, plasma osmolarity, and a hydration ratio obtained by echo magnetic resonance imaging (eMRI). There were no significant differences in liquid intake between groups (CMC vs water, p=.86 and P80 vs water, p=.19) over 3 days at the end of the treatment phase of the study (**Figure 2.8.a**). Additionally, neither the hydration ratio of CMC (p=.47) nor P80 (p=.12) differed significantly from water-treated controls (**Figure 2.8.b**). There were no significant differences in plasma osmolarity, a measure of hydration status, between groups (CMC vs water, p=.78 and P80 vs water, p=.52) (**Figure 2.8.c**). Altogether, all three hydration measures indicate that emulsifier treatment does not result in changes in liquid intake, dehydration, or changes in body composition, thus this is unlikely to be a confounding factor driving differential gene expression seen in emulsifier conditions versus control.

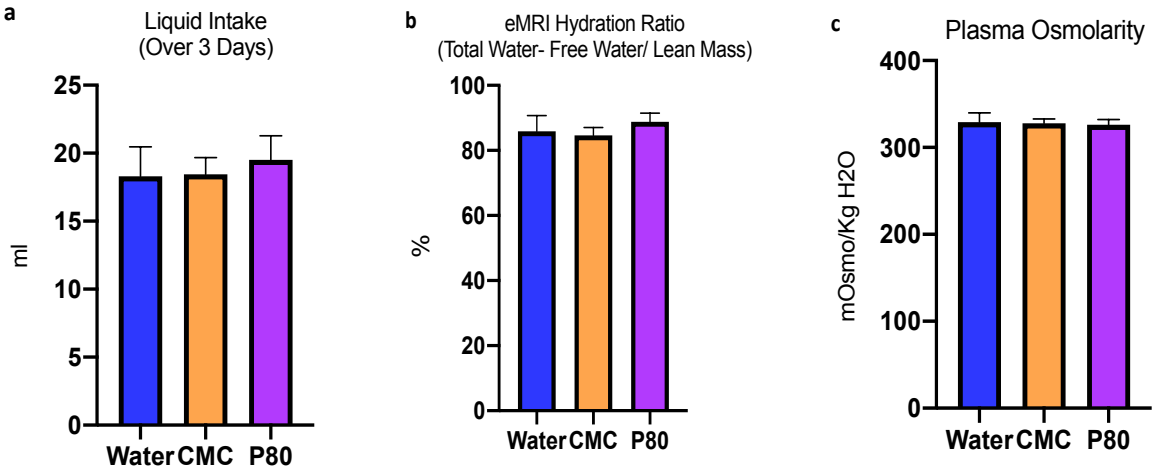

Figure 2.8 Hydration measures

Note. There were no differences among groups in liquid intake (**a**), hydration ratio as measured by eMRI (**b**), or plasma osmolarity (**c**). N=10 in each group.

2.3 Discussion

The present study tested the hypothesis that emulsifier intake, at doses that are directly relevant to those ingested by humans, can influence gene expression in brain regions that are known to be important in the generation of behavioral and neuroendocrine responses to stress-

provoking stimuli. The current findings illustrate the novel finding that emulsifier consumption induces genetic alternations within the amygdala and PVN that could be associated with the previously reported anxiety-like phenotype[7]. It appears that emulsifier treatment increased expression of IEGs such as NR4A2, NR4A3, EGR2, JUNB, FOSB, and FOSL2 in the amygdala and PVN, suggesting that both CMC and P80 may increase neuronal activity in brain regions that modulate stress responding. Because no additional procedures were experienced by the animals before euthanasia, the increase in IEGs suggests that the emulsifiers may have increased sensitivity to the mild stress of transport and handling that occurred just before tissue collection.

Increased neural activity in the amygdala and PVN is associated with an anxiety-like phenotype in rodents and may thus be brain regions within which emulsifiers act to increase anxiety-like behavior[23]. Further, differential expression of multiple HPA axis regulatory and responsive genes in emulsifier-treated animals indicates possible dysregulation of the HPA axis. Analysis of gene ontology shows emulsifier-induced alterations in the function of "glucocorticoid receptor binding" in both CMC and P80 conditions. NR4A2, NR4A3, and SGK1 are known to code for proteins that regulate glucocorticoid receptor function, and all three of these showed increased differential expression in emulsifier-treated animals. NR4A2 (NURR1) and NR4A3 (NOR1) are immediate early genes induced by growth factors, inflammatory signals, and glucocorticoids and are known to regulate the HPA axis at the hypothalamic, pituitary, and adrenal level [24, 25]. In addition, we found emulsifier-treated animals had decreased expression of PCLO and increased expression of SGK1, which are known biomarkers of clinical depression, a disorder often characterized by HPA axis dysregulation. More specifically, decreased expression of PLCO is associated with increased activity in the amygdala of depressed patients[26]. Increased expression of SGK1, a glucocorticoid receptor (GR)-inducible gene, is

found in the hypothalamus and the serum of patients with depression[20]. SGK1 is known to prolong GR activation[27], which makes this gene a prime, potential molecular mechanism by which emulsifiers may sensitize the stress response.

Neuroinflammation is another possible mechanism by which emulsifier consumption may alter behavior. It is previously established that emulsifier consumption causes low-grade inflammation in the periphery[6, 28]. The present study is the first to provide evidence that emulsifiers may cause immune activity centrally, as well. The top enriched canonical pathways in emulsifier-treated animals were largely comprised of altered immune pathways in both the amygdala and PVN. These potential changes in central immune activation are highly relevant, as increased neuroinflammation is proposed to be a driving factor in the expression of anxiety and depression-like behavior[29]. Indeed, recent studies have demonstrated that decreasing innate immune system activity ameliorates social defeat-induced social anxiety and depression in mice [29]. One immune DEG of high interest that increased in the amygdala as a result of emulsifier consumption was PTGS2 (COX2), a gene responsible for coding the enzyme that converts arachidonic acid to pro-inflammatory prostaglandins. Other studies show that increased COX2 expression in the brain increases susceptibility to chronic mild unpredictable stress in rats, and COX2 inhibition decreases anxiety-like behavior in mice and decreases glutamatergic activity in the amygdala[30, 31]. Taken together, Cox-2 may be another prime mechanistic candidate driving emulsifier-induced stress sensitivity. Further connecting neuro- inflammation to stress sensitivity, the top canonical pathway identified in the amygdala of CMC-treated mice was “MIF Regulation of Innate Immunity”. Macrophage migration inhibitory factor (MIF) is released from immune cells in response to infectious stimuli and is also considered an endocrine hormone

capable of participating in HPA axis regulation[32]. Moreover, MIF is highly expressed in the brain and plays a key role in anxiety- and depression-like behaviors[33, 34].

It is important to note that both blood and vasculature were likely present in our brain samples. Several genes expressed in vasculature endothelial cells were found to be altered in the PVN and amygdala of emulsifier-treated animals and are known to play a role in blood-brain barrier function. Decreased expression of PCDHGA5, the gene that codes for a cadherin-like adhesion protein, was found in the PVN of CMC-treated mice. This gene plays a critical role in the barrier-stabilization properties of the blood-brain barrier (BBB)[35]. Decreased expression of this gene may indicate compromised vasculature within the brain. In addition, increased expression of ARNO (cytohesion-2) was found in the PVN of P80-treated mice. This gene plays a critical role in the permeability of the BBB. Increased expression of ARNO is associated with increased vessel permeability[36]. Lastly, increased expression of SPARC was found in the amygdala of CMC-treated mice. Pro-inflammatory molecules are known to increase SPARC expression in endothelial cells. SPARC alters BBB properties through increased paracellular permeability and decreased transendothelial electrical resistance (TEER)[37]. The presence of these genes is of particular interest as both neuroinflammation and BBB dysfunction are implicated in the neurobiology of stress-related disorders, namely, major depressive disorder[38].

A limitation to this study is the exclusion of females from our dataset. Due to the high per sample cost of RNA-seq, only males were included in this study. Diet-induced inflammation may have sex-specific effects on gene expression in areas of the brain critical for stress responding, such as the amygdala and PVN [39]. The exclusion of females from this study is a clear limitation, as sex differences in the prevalence of both inflammatory disease and stress-

related disorders exist in the clinical population[40, 41]. A critical future direction will be to investigate sex differences in emulsifier-induced gene expression in brain regions critical for the modulation of the stress response [7, 42]. Another consideration is the fact that the 1 mm tissue punches of PVN and amygdala clearly captured several distinct subregions. Therefore, the DEGs captured here are summative of these heterogeneous regions. Future studies could use single-cell RNA-Seq to examine DEGs in identified cell groups.

In conclusion, these data are the first to show that chronic emulsifier ingestion, known to be associated with microbiota alterations and low-grade inflammation, induced altered gene expression in key areas of the brain that control stress-related behavior. The transcripts and pathways highlighted here provide a more concrete understanding of the molecular mechanisms by which emulsifier consumption may affect HPA-axis functioning and stress-associated behaviors. Future experiments will test the extent to which emulsifiers sensitize behavioral and neuroendocrine stress responding, specifically assessing the role of the discussed DEGs in driving these phenotypes. Ultimately, the data obtained from this project highlight potential molecular neural mechanisms by which food additives, like emulsifiers, may affect inflammatory processes and behavior.

2.4 Methods

2.4.1 Animals

Adult male C57BL/6J mice, 8 weeks of age (The Jackson Laboratory, Bar Harbor, ME), were group-housed (5 mice per cage) in ventilated OptiMouse plastic cages with AlphaDri bedding. Mice were maintained on a 12:12 hour light/dark cycle, and food and water were supplied *ad libitum*. All procedures were approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Georgia State

University and with national regulations and policies. This study was performed in accordance with Animals in Research: Reporting In Vivo Experiments (ARRIVE) guidelines.

2.4.2 Procedure

Mice (n=5/group) received drinking water containing either 1% sodium carboxymethylcellulose (CMC; Sigma, St. Louis, MO), 1% polysorbate-80 (P80; Sigma), or water without an emulsifier, a control group. Our previous work has shown that similar inflammatory and metabolic changes occur following emulsifiers administered in either food or water [6]. Body weights were measured throughout the duration of the study. After 12 weeks of respective emulsifier or control treatment, animals were removed from their home cage, immediately anesthetized with isoflurane, and then decapitated. Brains were rapidly extracted, flash-frozen in ice-cold isopentane, and then stored at -80˚C until sectioning. Fat-pads, colons, and spleens were also collected for analysis of weight or length (colon).

2.4.3 RNA extraction

Brains were sectioned coronally at 300 μm on a cryostat and bilateral 1mm tissue punches were taken of the central/basolateral amygdala and the PVN. For RNA-seq and real-time, quantitative polymerase chain reaction (RT-qPCR), total RNA was extracted from tissue punches using a Trizol extraction method as described earlier [6]. In brief, brain tissue was homogenized in Trizol, followed by the addition of chloroform, and then centrifuged to separate the RNA aqueous layer from other cell contents. RNA was then alcohol-precipitated and resuspended in 30 μl nuclease-free water. RNA concentration and purity were measured using a nanodrop spectrophotometer, and the two samples from each group with the highest concentration and purity were sent for Illumina sequencing as described below.

2.4.4 mRNA sequencing library preparation

Total RNA extracted from the bilateral amygdala and PVN punches of animals treated with either CMC, P80, or emulsifier-free drinking water (N=2/group) were used to create mRNA sequencing libraries. Our initial study used a small number of samples taken from individual animals to maximize the number of group comparisons, while still allowing for the statistical identification of possible differentially expressed genes that could be subsequently validated with quantitative PCR using a larger group of animals [43, 44]. The mRNA sequencing library was constructed using the TruSeq RNA Sample Preparation kit (Illumina, San Diego, CA) following the manufacturer's instructions. In brief, PolyA-containing mRNAs were purified using oligo-dT attached magnetic beads followed by RNA fragmentation. cDNA was synthesized from RNA primed with random hexamers using reverse transcriptase. 3' overhangs and 5' overhangs were repaired using End Repair Mix. After adenylation of the 3' end, indexing adaptors are added to cDNA in preparation to be hybridized onto a flow cell. PCR was performed with a PCR Primer Cocktail that selectively amplifies DNA fragments with adaptors.

2.4.5 Sequencing

Each sample was uniquely indexed (barcoded) to allow for the pooling of all samples in a single sequencing run. A 75-cycle sequencing run was performed on the Illumina NextSeq 500 at the Genomics Core at Cornell University (Ithaca, NY). Data were processed using the standard Illumina processing pipeline to segregate each multiplexed samples' reads. The resulting data files were in fastq format and included at least 20 million reads per sample.

2.4.6 Analysis Pipeline

Various programs were used to assess sequence quality, determine differential gene expression, and perform ontological and pathway analysis. The FastQC program

(https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess the quality of reads and to provide various statistics such as total reads, total sequences, GC content, and sequence duplication levels. GC content for each sample fit the theoretical distribution, thus indicating no contamination or systemic bias within the samples. FASTQ quality filter (http://hannonlab.cshl.edu/fastx_toolkit/commandline.html#fastq_quality_filter_usage) was used to filter low-quality sequences (base quality score less than 30) leaving only sequences with high-quality scores. High-quality reads were then aligned to mm10 mus musculus reference genome using Bowtie2 and Tophat2 to identify gene sequences[45]. Gene expression levels were measured using Cufflinks and differential expression was measured using Cuffdiff [46]. Fragments Per Kilobase of transcript per Million mapped reads (FPKM) expression scores were obtained for each gene. Log2fold differential expression scores for each gene are calculated by dividing the average FPKM score for each experimental group by the average FPKM score for the water control group and converting the fold change to a logarithmic 2 scale after which a p-value was calculated[47]. P-score values were FDR adjusted (q-value) using the Benjamini-Hochberg correction [48]. Only genes that had an adjusted p-score value (q-value) < 0.05 and at least a log2fold score of 1 and -1, which are widely accepted cutoffs, were used for subsequent analysis[49]. Lastly, EnrichR and Ingenuity Pathway Analysis (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA) were used to perform gene set enrichment and to analyze DEGs to predict significant canonical pathways and diseases. Overlap with canonical pathways were based on well-established metabolic and signaling pathways that are within the INGENUITY knowledgebase, and enrichment for diseases were based on the annotations for diseases and disorders in the IPA curated knowledgebase. The Fisher's Exact Test was used to calculate the statistical significance of the overlap of DEG. R was used to generate expression

heat maps using the heatmap.2 function from the ggplot2 library and volcano plots were generated using in-house scripts.

2.4.7 RT-qPCR validation

Six genes of interest that were observed to be differentially expressed in the RNA-Seq dataset were selected for further RT-qPCR validation. Animals (n=5/group) were treated as described in Experiment 1 (RNA-Seq) and then gene expression was measured in the amygdala and PVN. In brief, RNA from tissue punches of the amygdala and PVN was synthesized into cDNA using a SuperScript IV First Stand Synthesis System to manufacture's specifications. Primers for genes of interest were designed using the NCBI primer design tool and only sequences that were exclusively specific to the gene of interest were used. Primer efficiency and specificity were verified; primer sequences are listed in Supplementary Table 1. cDNA was amplified using primers and Quantifast SYBR green PCR reaction mix via real-time PCR to allow for quantification of total gene expression. PCR cycle conditions were 95˚C for five minutes then 40 amplification cycles of 95˚C for 10 seconds to denature and 60˚C for 30 seconds to anneal and elongate. A melting curve was run for each primer to verify the formation of only one product. Data were normalized to the well-validated housekeeping gene, YWHAZ using the ΔΔct method because previous research has shown that YWHAZ expression remains stable under inflammatory conditions[50], and we verified that it did not vary by condition in this study. RT-qPCR relative gene expression within each brain region was compared between emulsifier and water control groups.

2.4.8 Hydration measures

2.4.8.1 Liquid intake. To verify that the emulsifier treatment did not alter the volume of liquid ingested, liquid intake was measured in an additional cohort of animals (N=10/group) that

were also treated as in Experiment 1 (RNA-Seq). Thus, after 12-weeks of emulsifier or control treatment, animals were singly housed to determine the volume ingested by each individual over a 72 hr period. Mice were given a 50ml sipper tube filled with respective emulsifier or water. Volume within the sipper bottle was logged daily for 3 consecutive days to determine the total ml of fluid ingested.

2.4.8.2 Echo Magnetic Resonance Imaging (eMRI). In addition to the liquid intake measures, the body composition of each subject was measured using an EchoMRI 1100 (EchoMRI LLC, Houston, TX, USA) to determine whether emulsifier intake altered body composition or hydration compared to that observed in mice drinking only water with no emulsifier added. Briefly, animals were weighed and inserted into the eMRI restrainer tube. The tube was then inserted into the eMRI machine and body composition was measured. This procedure is non-invasive and does not require the use of anesthesia. Tubes were disinfected with 70% ethanol between testing each mouse. Parameters were measured in triplicate with each measure lasting approximately 30 seconds. Measures collected included body mass, total body fat, lean body mass, free water, and total body water content. A hydration ratio was obtained by using the following equation: (total water – free water/ lean mass).

2.4.8.3 Plasma Osmolarity. Immediately after the completion of eMRI measures, animals were sacrificed by decapitation and trunk blood was collected in heparinized tubes. Blood was spun down immediately in a refrigerated microcentrifuge at 1,500 x G for 10 minutes. The plasma layer was isolated and plasma osmolality was quantitatively determined using the Advanced Instruments Model 3300 Micro-Osmometer (Advanced Instruments, Norwood, MA, USA) according to the manufacturer's instructions.

2.5 References

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3 COX INHIBITION AMELIORATES SENSITIVITY TO SOCIAL STRESS IN MICE THAT CONSUME DIETARY EMULSIFIERS

3.1 Introduction

Stress-related disorders are often characterized by enhanced immune activation and dysregulation of the hypothalamic-pituitary-adrenocortical (HPA) axis, the main modulator of the body's neuroendocrine response to stress [1-4]. Chronic low-grade inflammation has been identified as a factor that can potentiate stress responsivity and fuel the progression of neuropsychiatric disorders [5, 6]. In addition, dietary components that disrupt the gastrointestinal microbiome have recently been identified as an environmental factor that can underlie chronic inflammatory conditions in clinical populations, and these components might thus increase susceptibility to stress-related disorders [7, 8]. Emulsifying agents such as carboxymethylcellulose (CMC) and polysorbate-80 (P80) are added to processed foods and beverages to improve consistency and extend shelf-life. While these dietary components have been deemed safe for consumption at low doses by the Food and Drug Administration, it has recently been demonstrated that these additives can cause gut dysbiosis, promote systemic inflammation, and alter immune and stress-related genes in the brain [8-11]. Recently, it was also reported that animals fed emulsifiers at percent doses that are approved for human consumption may show increased basal, anxiety-like behavior. However, it is not known whether emulsifier consumption potentiates stress responding [10].

We have recently demonstrated via RNA-Seq that chronic emulsifier consumption results in alterations in gene expression of various immediate early, stress-related, and immune-related genes in brain regions that are known to be important in the generation of behavioral and neuroendocrine responses to stress-provoking stimuli [11]. Increased neural activity, as indicated

by increased expression of immediate early genes (IEGs) in stress-modulatory brain regions such as the paraventricular nucleus and amygdala, is known to be associated with an anxiety-like phenotype in rodents [12]. Further, differential expression of HPA axis regulatory genes and altered expression of immune-related genes in the brain can contribute to an altered behavioral and neuroendocrine stress response [13, 14]. Altogether, the neurogenetic profile of animals that consume emulsifiers suggests that these animals might be hypersensitive to stressful stimuli. Thus, the purpose of this project was to test the hypothesis that the consumption of emulsifiers exacerbates stress responding and that it does so at least in part via increased neuro-immune signaling. First, we examined whether the consumption of emulsifiers at doses the FDA considers safe for humans enhances behavioral, neural activational, and hormonal responses to an acute social defeat stressor.

Next, we evaluated the role of the COX inflammatory pathway as a mechanistic candidate driving social stress sensitivity in mice that chronically consume dietary emulsifiers. We previously reported that cyclo-oxygenase 2 (PTGS2/COX2), a gene responsible for coding the enzyme that converts arachidonic acid to pro-inflammatory prostaglandins, increased in the amygdala following emulsifier consumption [11]. Clinically, COX-2 activity may contribute to the pathophysiology of stress-related psychiatric disorders given that peripheral COX-2 expression is increased in subpopulations of patients with major depression, and pharmacological inhibition of COX-2 has been shown in clinical trials to decrease depressive symptoms of patients with major depression [15-18]. Rodent studies indicate that increased COX2 expression in the brain susceptibility to chronic mild unpredictable stress in rats and that COX2 inhibition decreases anxiety-like behavior in mice and decreases glutamatergic activity in the amygdala [19, 20]. To determine whether emulsifier-induced differences in stress responsivity were related

to differences in inflammatory processes, we tested whether concomitant aspirin treatment (COX inhibitor) would ameliorate emulsifier-induced increases in responsivity to social stress.

3.2 Methods

3.2.1 Animals

Adult male, C57BL/6J mice were obtained from the breeder at 8 weeks of age (The Jackson Laboratory, Bar Harbor, ME). Upon arrival, they were group-housed 5 per cage in ventilated, Allentown, NexGen plastic cages with corncob bedding until behavioral manipulations began. Male CD-1, retired breeder mice (Charles River Laboratories) were singly housed in the cages described above and were used as resident aggressors during social defeat. After social defeat training or control manipulations, all C57BL/6J mice were then singly housed. All mice were maintained on a 12:12 hour light/dark cycle, and food and water were supplied ad libitum. All manipulations were performed during the first three hours of the dark, or active, phase of the daily light-dark cycle. All procedures were approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Georgia State University and with national regulations and policies. This study was performed in accordance with Animals in Research: Reporting In Vivo Experiments (ARRIVE) guidelines.

3.2.2 Procedure

Dietary manipulations: As per our earlier studies [9-11], to test the effects of emulsifiers on stress-induced endpoints, C57BL/6J mice (n=7-8/group) were supplied with water bottles containing either water with 1% sodium carboxymethylcellulose (CMC; Sigma, St. Louis, MO), water with 1% polysorbate-80 (P80; Sigma), or a water-only control as in our previous study [9, 11]. We have previously shown that similar results were obtained following emulsifier ingestion in either food or drinking water [9]. After 12 weeks of emulsifier or control treatment, C57BL/6J

mice were subjected to a 3 x 5-minute social defeat as detailed below. In experiments testing an anti-inflammatory intervention, the groups described above were further divided into aspirin (25mg/kg/day) or no aspirin groups. Aspirin was added to the drinking water or respective emulsifier treatment from weeks 6 to 12 (n=7-8/per group) as per Roher et. al [21].

3.2.3 Social Defeat

Screening of aggressor CD-1 mice used in defeat training: CD-1 mice with consistent levels of aggressive behaviors, as determined from a 3-day prescreening process, were used in social defeats of C57BL/6J subjects. Briefly, each day a C57BL/6 mouse was placed in the home cage of a CD-1 aggressor for 5 minutes. Only CD-1 mice whose latency to attack was consistently less than 30 seconds were used for subsequent social defeat training.

Social Defeat Training and No Defeat Controls: Subjects were subjected to three, 5-min defeat sessions on a single day with a 5 min inter-trial interval between each session during which mice were returned to their home cages. The 5 min defeat sessions were timed from the onset of the first attack by the RA. All animals were monitored during the defeat experience to ensure that no physical injuries occurred. During training, no defeat controls were placed into an empty RA/CD1 cage for the same duration of time as defeated animals to control for handling and exposure to a novel cage with social odors. All social defeats and control manipulations occurred during the first three hours of the dark phase of the daily light-dark cycle. All subjects were singly-housed following defeat/novel cage exposure for the duration of the experiment.

3.2.4 Testing of Anxiety-like Behavior

Social Avoidance Testing: 24 hours after social defeat, the social avoidance of experimental animals was tested as in Golden et. al [22]. Briefly, the time animals spent investigating an empty box placed on one side of a neutral arena over 2.5 minutes was compared

to the time spent investigating the box containing a novel CD-1 stimulus over the subsequent 2.5 minutes. Investigation of the empty box provided a baseline measure of non-social investigation of a novel object, while also serving to habituate the animal to the box. A preference index was calculated as [(interaction time, stimulus mouse-present)/(interaction time, stimulus mouse-present + interaction time, empty)] to indicate the overall level of social avoidance while controlling for the possible confounding variable of novel object avoidance (or neophobia)[23, 24]. The social preference index varies from 0 to 1, where a preference index > 0.5 indicates a preference for social interaction and an index < 0.5 indicates social avoidance.

3.2.5 ELISA

Serum Corticosterone and Prostaglandin E2 (PGE2)- Animals were subjected to social defeat as described above and trunk blood was collected 20 minutes after the final social defeat. After collection of the whole blood, it was allowed to clot at room temperature for 30 minutes. Blood was centrifuged and serum was isolated for use in an enzyme-linked immunosorbent assay (ELISA). Corticosterone and PGE2 were measured with commercially available ELISA kits according to the manufacturer's instructions (ENZO Life Sciences, Ann Arbor, MI, USA). Standards and samples were added in duplicate to the wells of the microtiter plate. Optical density was measured using an MD SPECTRAmax plate reader. The serum concentration of corticosterone and PGE2 was calculated according to the standard curves.

Fecal Lipocalin 2 (Lcn-2) – To collect feces, mice were placed in a clean cage 20 minutes before euthanasia and 3-5 fresh fecal pellets were collected from the cage and flash frozen. Fecal pellets were reconstituted in PBS containing 0.1% Tween 20 (100 mg/ml) and vortexed for 20 min to get a homogenous fecal suspension. Samples were then centrifuged for 10 min at 12,000 rpm and 4°C. Clear supernatants were collected and stored at −20°C until analysis

[25]. Lcn-2 levels were estimated in the supernatants using an Lcn-2 ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis MN, USA). Standards and samples were added in duplicate to the wells of the microtiter plate. The concentration of Lcn-2 was calculated according to the standard curve.

3.2.6 Immunofluorescent Labeling

90 minutes after social defeat, animals were deeply anesthetized with isoflurane and then perfused with 0.05 M PBS followed by 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde overnight at 4°C and then placed into a 30% sucrose solution. Brains were coronally sectioned at 40μm on a cryostat. A series of coronal sections were obtained from the infralimbic (IL) and prelimbic (PL) areas of the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), paraventricular nucleus (PVN), and basolateral (BLA). Sections were incubated with an anti-c-Fos antibody (1:1000, Abcam, Cat# ab222699) overnight at 4 °C, followed by incubation with an Alexa Fluor 488 conjugated goat anti-rabbit antibody (1:1000, Abcam, Cat# ab150077) for 1 h at room temperature. Sections were cover-slipped and nuclei counterstained with 4′,6-diamidino-2-phenylindole (DAPI) antifade mounting media. 10x images were captured with a Keyence microscope with a digital camera. Positive c-Fos cell counts were quantified using CellProfiler and were normalized to total cell counts in each respective region analyzed.

3.2.7 Physiological Parameters: Hydration, Locomotion, and Body Composition

Liquid intake: To verify that the emulsifier and aspirin treatments did not alter the volume of liquid ingested, liquid intake was measured over 7 days (N=7-8/group). Upon being singly housed, mice were given a 50ml sipper tube filled with one of the emulsifiers in water or

plain water. Volume within the sipper bottle was logged daily for 7 consecutive days to determine the total ml of fluid ingested.

Open Field Testing: 48 hours after social defeat, exploratory behavior was measured. Individual mice were placed in the center of a transparent plastic (50 cm × 50 cm) chamber, and their behavior was monitored for 10 min with an overhead video-tracking system (Med Associates Inc., Fairfax, VT). Total distance traveled was used as a measure of general activity, to control for the potential confound of differences in overall health driving effects.

Echo Magnetic Resonance Imaging (eMRI): In addition to the liquid intake measures, the body composition of each subject within the aspirin experiment was measured using an EchoMRI 1100 (EchoMRI LLC, Houston, TX, USA) to determine whether emulsifier or aspirin intake altered body composition or hydration compared to that observed in mice drinking only water. Briefly, animals were weighed and inserted into the eMRI restrainer tube. The tube was then inserted into the eMRI machine and body composition was measured. This procedure is non-invasive and does not require the use of anesthesia. Tubes were disinfected with 70% ethanol between testing each mouse. Parameters were measured in triplicate with each of the three measurements lasting approximately 30 seconds. Measures collected included body mass, total body fat, lean body mass, free water, and total body water content. A hydration ratio was obtained by using the following equation: (total water – free water/ lean mass).

3.2.8 Statistics

Datasets were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA) and SPSS (SPSS Inc., Chicago, IL). Assumptions of ANOVA were tested and met. Statistical significance was assessed using analysis of variance and significant main effects and interactions relevant to the hypotheses were probed with Fisher's LSD or Tukey's *post hoc* tests. Sample

sizes and statistical tests are indicated in the figure legends and were considered statistically significant when <0.05. All plots show mean ± SEM. *p*-values and effect sizes are indicated in the results section. The effect size value was evaluated using partial eta-squared (η_p^2).

3.3 Results

3.3.1 Emulsifier consumption increases defeat-induced social avoidance but not novel object avoidance

In Experiment 1, we examined social avoidance behavior 24 hours after exposure to defeat (D) or no defeat (ND; novel cage exposure) to determine whether emulsifier consumption behaviorally sensitizes animals to social stress (experimental timeline shown in **Figure 3.1a**). No defeat controls demonstrated a preference for social interaction regardless of dietary treatment compared to defeated mice, which displayed a significant reduction in social preference index ($F(1,54)$ 43.739; $p<.001$; $\eta_p^2 = .448$; **Figure. 3.1b**). Interestingly, pairwise comparisons revealed that defeated animals that consumed emulsifiers showed a significant reduction in social preference index compared to defeated animals that consumed water (D CMC v D Water; =.026) and (D P80 v D Water; p=.03), suggesting that dietary emulsifier consumption increases social avoidance after defeat. In addition, social defeat significantly reduced time spent investing a novel object (empty box) ($F(1,54)$ 31.061; $p<.001$; η_p^2 .365**; Figure. 3.1c**), however, no significant differences were found between dietary groups. Together, these data suggest that emulsifier consumption increases social anxiety but not general anxiety-like behavior in response to defeat.

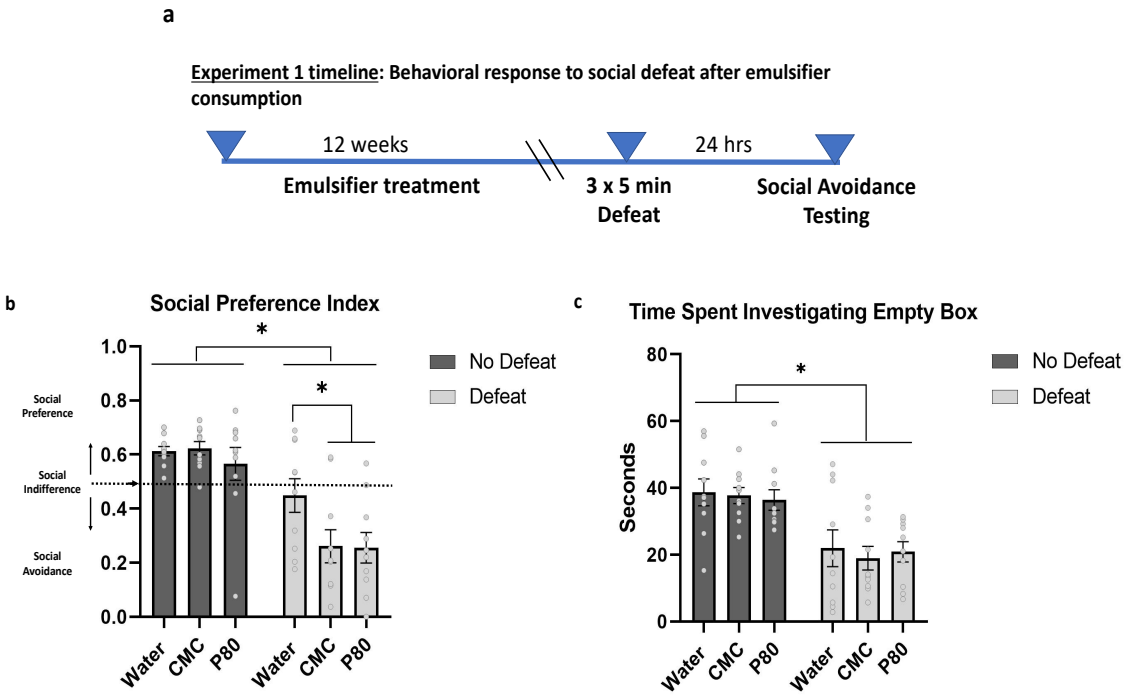

Figure 3.1 Exp 1 Dietary emulsifiers increase social avoidance after social defeat but do not affect social preference in non-defeated mice and do not alter defeat-induced avoidance of a novel object (empty box trial).

Note: Mice were given drinking water, alone, or water containing 1% of either CMC or P80. Experiment timeline (**a**) social preference index (**b**) and time spent investigating empty box (**c**) across defeated and no defeat dietary groups (N= 10 per group, data presented as means ±SEM; individual data points represent 1 or more animals). 2x3 ANOVA analysis was conducted and multiple comparisons were made using Tukey's *post hoc* tests. *p <.05

3.3.2 Emulsifier consumption increases neural activity in stress-modulatory brain regions

Activation of neurons induces the expression of immediate early genes, such as c-fos and subsequently c-fos protein, which are reliable markers of neuronal activity [26, 27]. Increased neural activity in areas of the brain that modulate the stress response is associated with increased behavioral sensitivity to various psychological stressors [12, 28, 29]. Our previously published RNA-Seq data indicate that animals that chronically consumed emulsifiers showed increased immediate early gene expression in the amygdala and PVN compared to controls that consumed water [11]. In Experiment 2, we evaluated whether protein expression of the immediate early

gene c-fos is increased in various stress-modulatory brain regions under control or social stress conditions as a result of chronic emulsifier consumption (**Figure 3.2a**). Representative examples of c-Fos and DAPI staining and how they were quantified are shown in **Figure 3.2b**.

The medial prefrontal cortex (mPFC) is a critical structure that regulates the HPA axis response to psychological stressors [27, 30]. The mPFC represses HPA-axis activity largely through functionally distinct subregions such as the prelimbic cortex (PL) and infralimbic cortex (IL) that are heavily interconnected with stress modulatory regions, such as the basolateral amygdala (BLA) and project directly to PVN [27, 31]. Defeat significantly increased c-Fos expression compared to the ND group in the PL ($F= 4.775$; $p=.039$; $\eta_p^2 =.166$) and IL ($F= 7.591$; $p=.011$; $\eta_p^2 =.24$***;*** **Figure 3.2c**). Pairwise comparisons revealed that animals that consumed P80, specifically, showed significantly greater c-Fos expression in the defeat condition compared to P80-treated animals in the ND group ($p=.008$). This effect was only present in the IL and not in the PL subregion of the mPFC.

The nucleus accumbens (NAc) is a stress-responsive brain region that mediates goal-directed behavior and salience [30]. The NAc receives input from both the BLA and PVN, which are stress-sensitive brain regions that modulate HPA activity [31]. Defeat significantly increased c-Fos expression compared to the ND group in the NAc ($F= 8.487$; $p=.008$; $\eta_p^2 =.261$; **Figure 3.2d**). Pairwise comparisons revealed that animals that consumed CMC, specifically, showed significantly greater c-Fos expression in the defeat condition compared to CMC-treated animals in the ND group ($p=.046$).

Within the PVN, defeat significantly increased neuronal activity compared to the ND group ($F=118.08$; $p<.01$; $\eta_p^2 = .831$; **Figure 3.2e**). A significant dietary effect was present only between CMC-treated mice and controls within the ND condition ($p=.005$). Within the BLA,

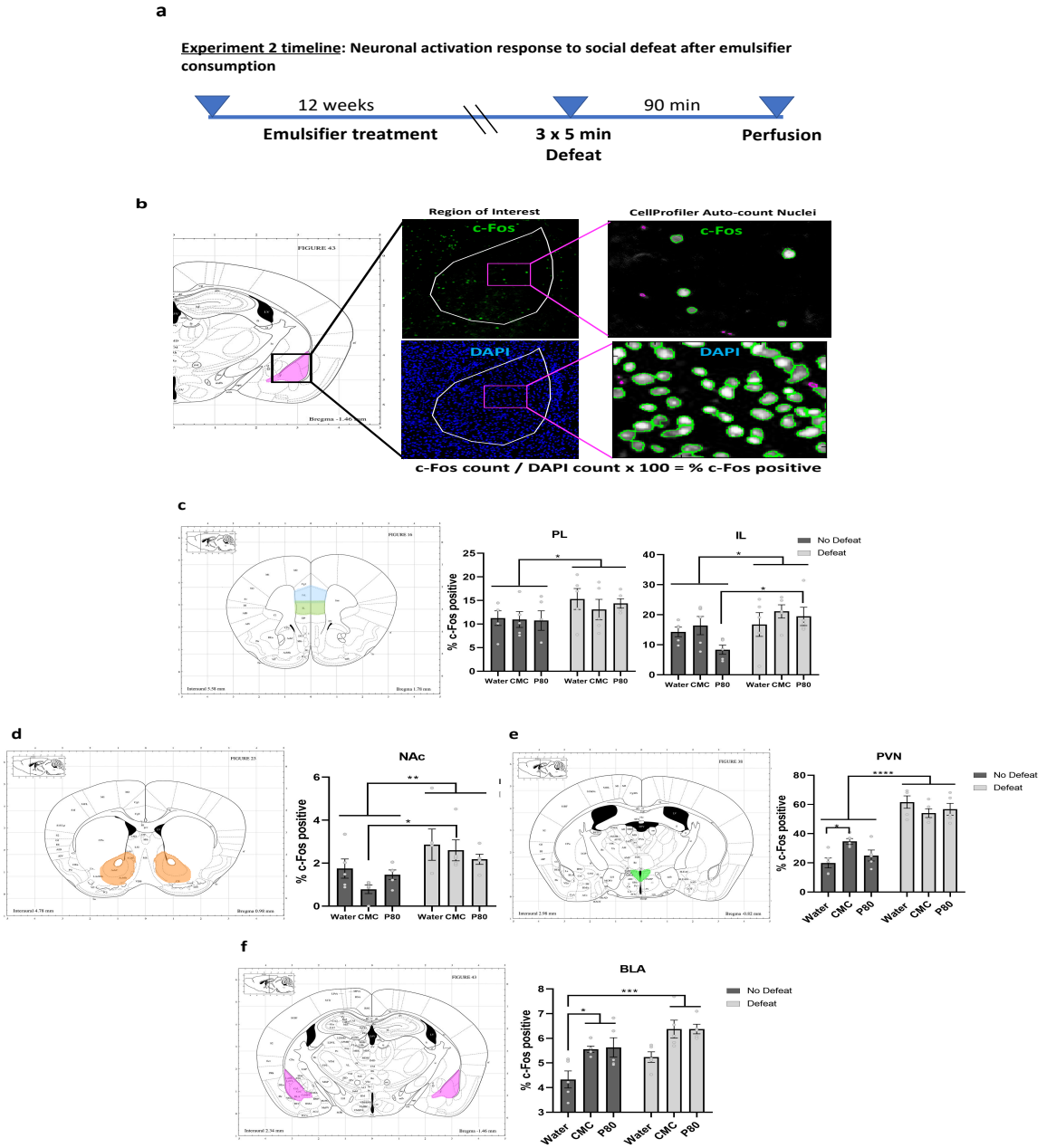

Figure 3.2 Emulsifier consumption elicits distinct c-Fos expression patterns across various stress-modulatory brain regions under basal and stress conditions

Note: Experiment 2 timeline (a). Immunofluorescent c-Fos+ cells and DAPI-labeled nuclei within brain regions of interest were counted automatically with CellProfiler. As an example, within the BLA region of interest, c-Fos+ and DAPI+ nuclei recognized and auto-counted by CellProfiler are outlined in green. Immunofluorescent artifacts or partial nuclei/cells outlined in magenta are not counted. % c-Fos positive cells were computed using the following equation [c-FOS counts/ DAPI counts x100]. Counts were obtained within the corresponding coordinates from Paxinos and Franklin's mouse brain atlas as adapted in sections shown on left of each panel (b-f). Quantification of % c-Fos positive cells within each brain region under no defeat and social defeat conditions across dietary conditions were calculated and data are presented as means ± SEM. N=5 per group and individual data points represent 1 or more animals. 2x3 ANOVA analysis was conducted and multiple comparisons were made using Tukey's post hoc tests. * p <.05, ** p<.01, *** p<.005, **** p<.001 (c-f).

there was a significant main effect of defeat ($F=12.098$; $p=.002$; $\eta_p^2 = .335$ Figure. 2f) and a significant main effect of diet ($F=12.098$; $p=.002$; $\eta_p^2 = .335$). CMC and P80-treated groups within the ND condition showed increased BLA neural activity compared to control animals that consumed water (CMC $p=.02$, P80 $p=.04$). CMC and P80-treated groups within the defeat condition also showed significantly increased neural activity compared to their water-treated controls (CMC $p=.006$, P80 $p=.005$). Increased c-Fos expression within the emulsifier conditions is of particular concern, as BLA hyperactivity is associated with anxiety-like behavior and HPA-axis dysfunction [32].

3.3.3 Aspirin treatment ameliorates the heightened sensitivity to social defeat in emulsifier-treated animals

We recently reported that animals consuming CMC or P80 show increased expression of *COX2 (PTG2)* in the amygdala [11]. Other studies suggest that increased COX2 expression in the amygdala is associated with increased susceptibility to chronic, mild, unpredictable stress in rats, and COX inhibition decreases anxiety-like behavior in mice [19, 20]. Thus, we identified the COX pathway as a possible mechanistic candidate driving the increased sensitivity to social stress in mice that consumed emulsifiers. In Experiments 3 and 4 (timelines in **Figure 3.3a**), animals were treated as in Experiment 1 but further divided into aspirin or no aspirin groups. Aspirin (25mg/kg/day) was added to the drinking water or respective emulsifier treatment from weeks 6 to 12. In Exp. 3, we measured social avoidance behavior 24 hours after exposure to defeat or ND to determine whether COX inhibition ameliorates the increased sensitivity to social stress that was observed in animals that consume dietary emulsifiers. No defeat controls demonstrated a preference for social interaction regardless of dietary or aspirin treatment compared to defeated animals, which displayed a significant reduction in social preference index

($F(1, 78) = 27.470$; $p < .001$; $\eta_p^2 = .260$; Figure. 3b). Within the no aspirin groups, pairwise comparisons showed that defeated animals that consumed water did not significantly differ from no defeat animals that consumed water, however, defeated, CMC-treated animals and defeated P80-treated animals showed a significant reduction in social preference index compared to their respective no defeat counterparts (D CMC vs ND CMC; p=.003) (D P80 vs ND P80; p=.008). Within the aspirin group, a significant difference was found between defeat and ND in the water-treated animals (p=.022), however no significant differences were found between defeated animals and ND in emulsifier-treated animals.

A significant interaction between dietary condition and aspirin treatment was found when evaluating time spent investigating the empty box ($F = (2, 78)$ 5.103 $p = .008$ $h_p^2 = .116$; **Figure 3.3c**). Pairwise comparisons revealed a significant decrease in time spent investigating the empty box in defeat animals compared to no defeat animals in every dietary condition across aspirin treatments except in aspirin-treated animals that consumed CMC (No Aspirin; D water vs ND water, p=.002, D CMC vs ND CMC, p=.003, D P80 vs ND P80, p=.007) (Aspirin; D water vs ND water, p=.003, D P80 vs ND P80, p=.01)

To evaluate whether emulsifier treatment increases hormonal sensitivity to social stress and if aspirin ameliorates emulsifier-induced hormonal sensitivity, a separate group of animals (Experiment 4) received emulsifier and aspirin treatment as described above and were sacrificed 20 minutes post-defeat. A factorial ANOVA was conducted to analyze the interaction of diet, defeat, and aspirin on serum corticosterone. The interaction of the three variables was significant ($F (2, 78) = 3.994$, $p = .022$; $\eta_p^2 = .093$; Figure. 3d). The interaction of diet and aspirin was significant ($F (1, 78) = 23.502$, $p < .001$; $\eta_p^2 = .232$) and the interaction of defeat and diet was

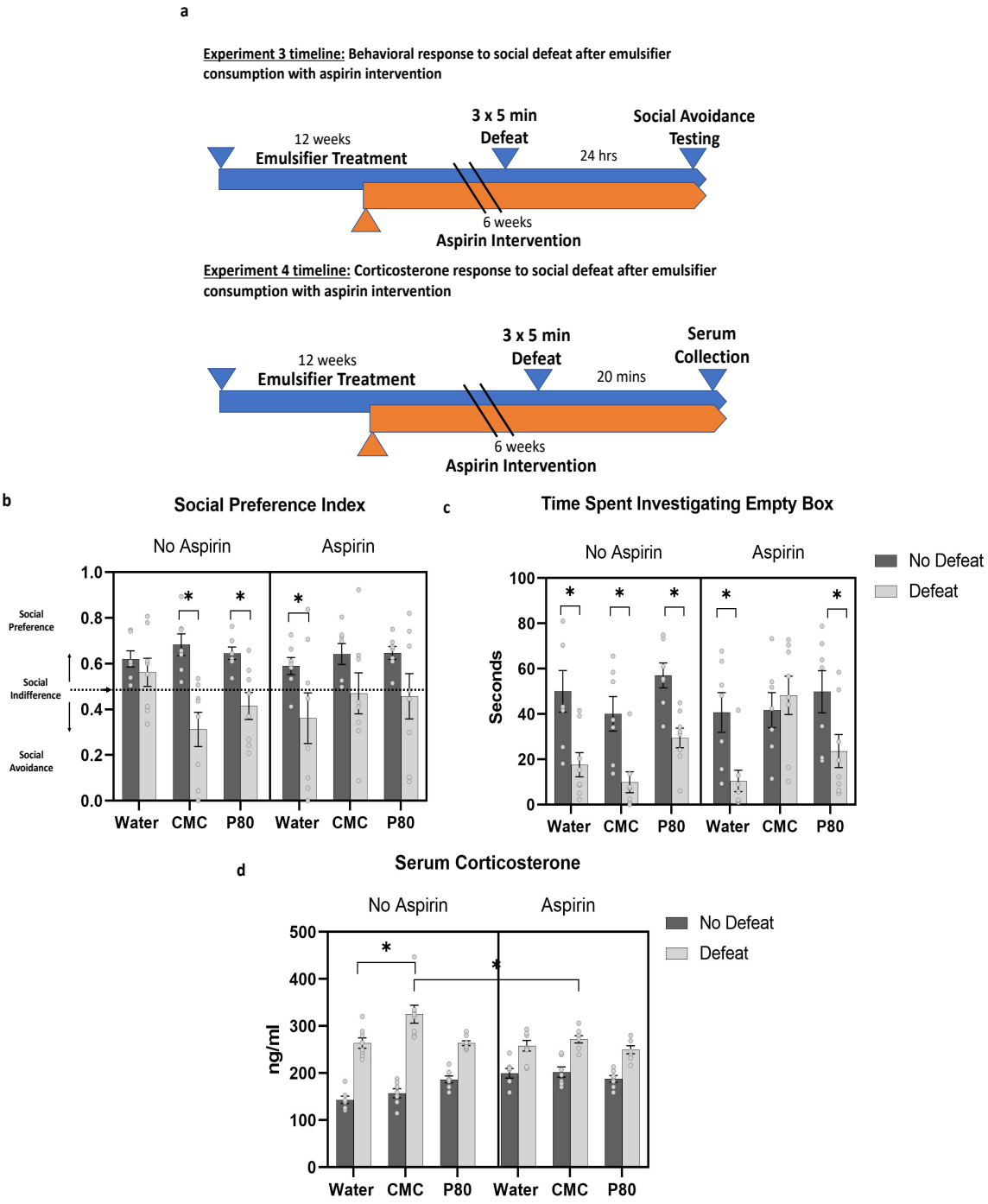

Figure 3.3 Aspirin treatment reverses the emulsifier-induced enhancement of defeat-induced social avoidance and corticosterone release.

Note: Experimental timelines for Experiments 3 and 4 (a). Social preference index across defeated and not defeated dietary groups that were given aspirin or not (b). Time spent investigating empty box across defeated and not defeated dietary groups that were given aspirin or not (c). Serum corticosterone concentration 20 minutes post-defeat (d). N= 7-8 per group and individual data points represent 1 or more subjects; data presented as means ± SEM. 2x2x3 ANOVA analysis was conducted and pairwise comparisons were made using Fisher's LSD post hoc tests. *significantly lower than no defeat group p <.05 (b-c).

significant (F (2, 78) = 5.631, p = .005; η_p^2 = .126). Results also indicated main effects for defeat (F (2, 78) = 23.854, p < 001 η_p^2 = .750) and diet (F (2, 78) = 5.222, p = .007; η_p^2 = .118). Defeated animals showed significantly higher serum corticosterone levels (p <. 001). Within the defeated group, pairwise comparisons indicated that animals given CMC that also received aspirin showed significantly lower serum corticosterone (p < .001). When looking at differences based on diet, animals on a P80 diet showed significantly higher serum corticosterone within the non-defeated, non-aspirin group compared to water (p = .006) and CMC (p = .059). For defeated animals that did not receive aspirin, those given CMC had higher serum corticosterone than those given water alone (p <.001) and P80 (p < .001). There were no significant differences in serum corticosterone across diets within the aspirin-treated animals regardless of defeat status. Pairwise comparisons across aspirin-treated, non-defeated mice indicated that serum corticosterone was significantly higher for the water (p < .001) and CMC (p = .004) animals compared to animals that did not receive aspirin, suggesting that 6-week treatment with low-dose aspirin may increase basal circulating corticosterone.

3.3.4 Emulsifier or aspirin treatment does not result in dehydration or general malaise

Potential confounding factors that could drive behavioral, hormonal, and brain activity changes found in the current study are changes in fluid ingestion/hydration and physiological symptoms associated with general malaise (**Figure 3.4a**). To assess the possibility that effects seen in this study were secondary to changes in hydration or sickness rather than to the emulsifier or aspirin treatments, themselves, various physiological and behavioral parameters were compared across aspirin and dietary treatments after the social avoidance testing (full timeline for Exp. 3 is shown in **Figure 3.4a**). Hydration measures included liquid intake and a hydration ratio obtained by echo magnetic resonance imaging (eMRI). Parameters used to assess

overall health also included body mass, fat mass, and locomotor activity. No significant differences between groups were found in any measure across aspirin or dietary groups except for liquid intake (**Figure 3.4b**), where animals that consumed P80 showed significantly more liquid intake over 7 days than did animals that consumed water ($p<.001$). These data suggest that animals that consume P80 may display polydipsia, a condition often associated with an anxiety-like or abnormal metabolic phenotype [33]. However, alternative interpretations such as increased palatability of P80 cannot be ruled out without doing preference testing. This polydipsia did not, however result in changes in hydration (**Figure 3.4c**), body mass (**Figure 3.4d**), or fat mass (**Figure 3.4d**).

3.3.5 Verification of emulsifier-induced intestinal inflammation and aspirin suppression of PGE2

Lipocalin-2 (LCN2), also known as neutrophil gelatinase-associated lipocalin (NGAL), is a highly validated biomarker of gastrointestinal inflammation [34]. LCN2 is strongly expressed in conditions where gastrointestinal damage, bacterial infection, or intestinal inflammation is present [35, 36]. It has been previously reported that animals that consume emulsifiers show elevations in fecal LCN2 [9], so we included at the end of Exp. 4 this measure to assess emulsifier-induced intestinal state (experimental timeline is shown in **Figure 3.5a**). We found that emulsifier treatment significantly increased fecal LCN2 levels ($F(2, 84) = 5.534$, $p = .006$, $\eta_p^2 = .116$; **Figure. 3.5b**), further supporting the contention that emulsifier treatment induces a pro-inflammatory state. *Post hoc* comparisons revealed a significant increase in fecal LCN2 in CMC-treated mice ($p = .048$) and P80-treated ($p=.006$) mice compared to water-treated controls. Aspirin treatment had no significant effect on LNC2 levels, suggesting that aspirin did not amplify or ameliorate emulsifier-induced intestinal inflammation.

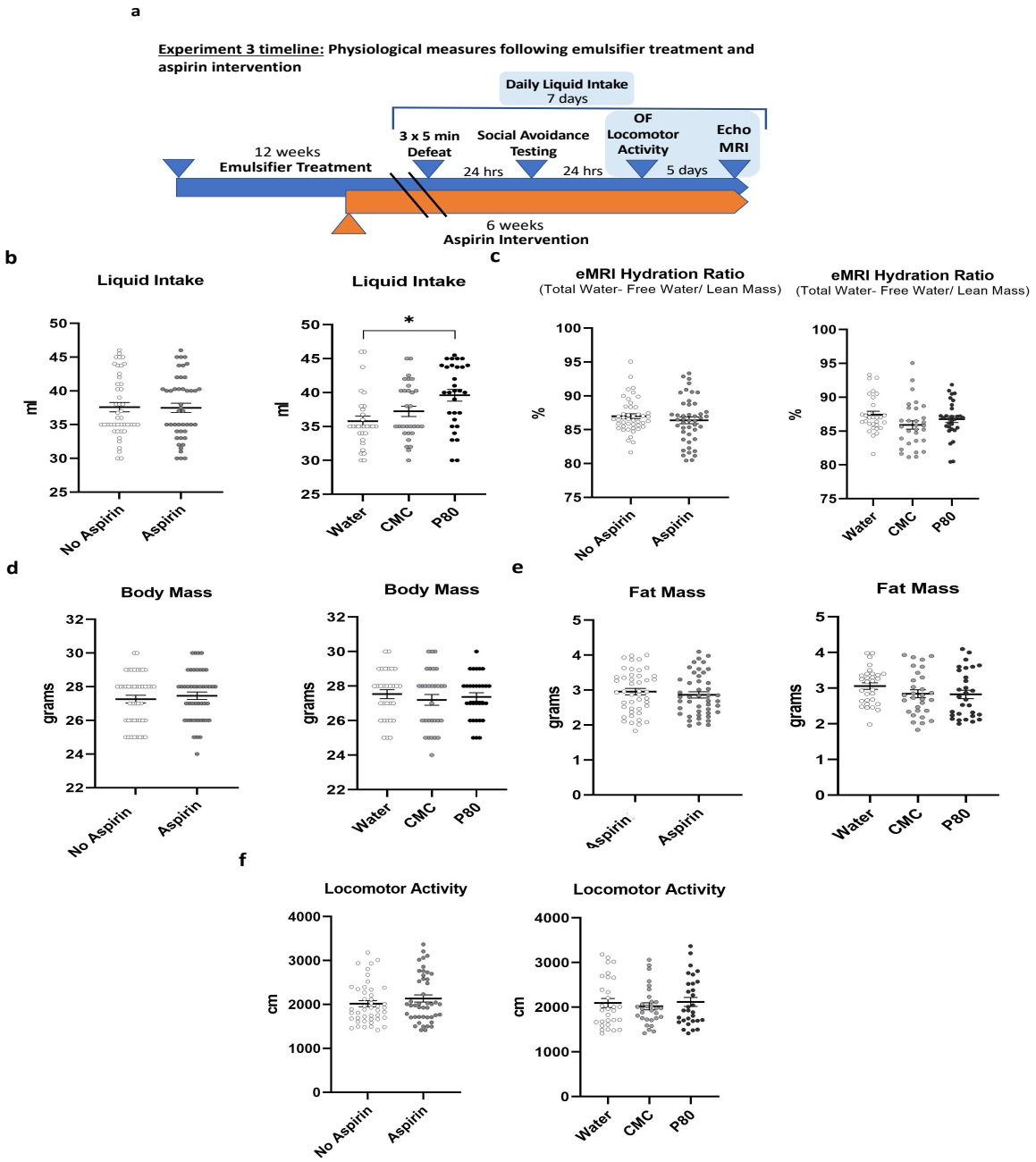

Figure 3.4 Emulsifier or aspirin treatment does not result in physiological or behavioral changes that would be expected if the treatments were associated with dehydration or general malaise.

Note: An expanded experimental timeline for Experiment 3 is shown (a), liquid intake over 7 days between no aspirin and aspirin groups shows the addition of aspirin did not significantly affect liquid intake but that P-80 resulted in increased water intake ($p < .05$) (b), hydration ratio as measured by eMRI between no aspirin and aspirin groups and hydration ratio between dietary groups indicated no effect of treatment on hydration, (c), no effect of treatment on body weight between no aspirin and aspirin groups or between dietary groups (d), no effect of treatment on fat mass between no aspirin and aspirin groups or between dietary groups (e), no effect of treatment on distance traveled over 10 minutes in the open field between no aspirin and aspirin groups or between dietary groups (f). N=45 per experimental group for

aspirin vs no aspirin comparisons and N=30 per experimental group for dietary comparisons, as data displayed in each panel are collapsed across defeat and either aspirin treatment or dietary treatment because acute defeat did not have a significant effect on physiological parameters. Data are presented as means ± SEM. ANOVA analysis was conducted, and multiple comparisons were made using Tukey's post hoc tests. *p <.05 (b-f)

Prostaglandin E2 (PGE2) is elevated peripherally and centrally under inflammatory conditions. PGE2 is synthesized from arachidonic acid by COX1 and COX2 and modulates the production of pro-inflammatory cytokines [37]. NSAIDs inhibit COX production of PGE2, therefore PGE2 measurement is often used to indicate the effectiveness of NSAIDs such as aspirin [38, 39]. We found a significant main effect of aspirin on serum PGE2 ($F(1, 78) = 17.037$, $p < .001$, $\eta_p^2 = .179$; **Figure 3.5c**).

3.4 Discussion

Herein we provide the first evidence that dietary emulsifiers at percent doses regularly consumed by humans can potentiate the neural activational, behavioral, and hormonal responses to a relatively mild psychogenic stressor, namely acute social defeat stress. Emulsifier consumption, alone, does not seem to illicit robust behavioral or hormonal responses until paired with an additional salient stressor, suggesting that these food additives may drive susceptibility to stressors. An exception to this, however, is that under basal conditions without social stress, we do see increased neural activity within stress-modulatory regions of the brain such as the PVN of CMC-treated mice and increased neural activity within the BLA, in both P80 and CMC-treated mice. The BLA is critical for the expression of anxiety and for the assignment of salience to threating cues [40-43] Thus, it is possible that emulsifier consumption may "prime" fear circuits to illicit a stronger anxiety-like behavioral response to social stress. The present data are also consistent with previous research indicating that stress-modulatory brain regions show increased neural activity under inflammatory conditions [44-47].

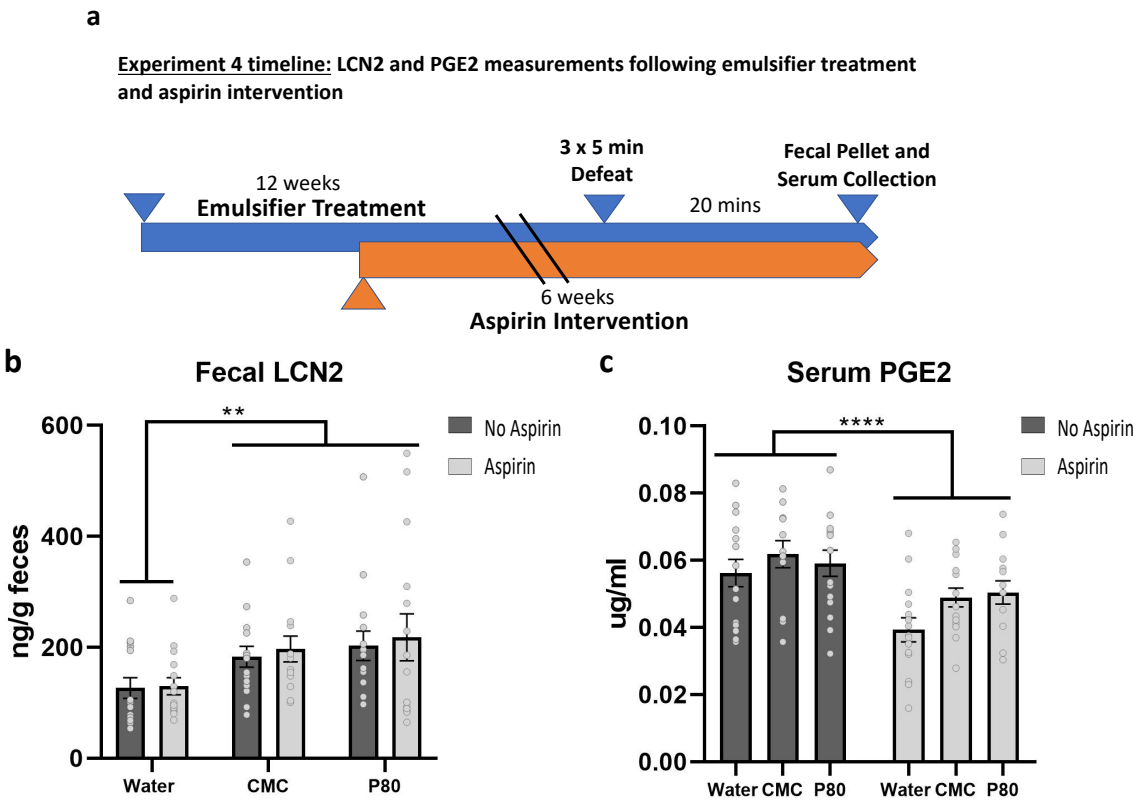

Figure 3.5 Inflammatory marker LCN2 increases after dietary emulsifier consumption and PGE2 decreases with aspirin treatment.

Note: An expanded timeline for Experiment 4 is shown in (a). Fecal LCN2 concentrations were significantly increased after emulsifier consumption (b), and serum PGE2 was reduced after aspirin intervention (c). Data are collapsed across defeat status for aspirin or dietary treatment because acute defeat did not have a significant effect on inflammatory markers. Data are presented as means ± SEM, and data points represent 1 individual. N= 15 per group. ANOVA was conducted, and multiple comparisons were made using Tukey's post hoc tests. ** p<.01, **** p<.001 (b,c)

Mechanistically, it is reasonable to postulate that the enhanced responsivity to social stress following emulsifier consumption may be due, at least in part, to the inflammatory COX pathway, as inhibition of this pathway via aspirin administration reduced the emulsifier-induced enhanced expression of social avoidance and corticosterone release. In principle, COX inhibition may elicit anxiolytic effects via one or more mechanisms of action such as preventing prostaglandin synthesis and the elevation of pro-inflammatory cytokines, increasing endocannabinoid (eCB) levels, and/or reducing thromboxane TXA2. Many studies provide strong evidence that COX inhibition of prostaglandin-driven inflammation decreases anxiety-like

behavior and HPA-axis hyperactivity[48-50]. Alternatively, there is evidence that suggests COX inhibition may have anxiolytic effects via eCB signaling, as substrate-selective inhibition of COX-2, which increases brain eCB levels without decreasing prostaglandin levels, causes cannabinoid receptor 1-dependent anxiolytic actions in mice [51]. Finally, it is also possible that reductions in thromboxane TXA2, which is a platelet agonist and vasoconstrictor, contribute to the anxiolytic-like actions of COX inhibitors. Metabolites of thromboxane TXA2 are highly correlated with circulating cortisol levels in patients with major depressive disorder, however, very little is currently known regarding its role in the regulation of stress responses and behavior [52].

Aspirin is an irreversible COX1 and COX2 inhibitor. Both COX1 and COX2 are known to induce PGE2, which engages the HPA axis during an immune challenge [53-55]. Given that our recent studies suggest that emulsifier ingestion stimulates the release of immune markers and that PTGS2 cyclooxygenase gene expression is enhanced in the brains of animals that consume emulsifiers [9, 11]. future studies should go beyond a non-selective COX manipulation to selectively inhibit COX1 or COX2. In addition, aspirin was administered systemically and its metabolite, sodium salicylate, can readily pass the blood-brain barrier to inhibit COX action centrally. Therefore, we cannot conclude from the present study whether aspirin affected the HPA axis and behavior through a peripheral or central mechanism [56, 57]. However, various studies suggest that the COX pathway affects HPA axis function centrally [58]. Additional experiments in which COX inhibitors are administered selectively to specific stress-modulatory brain regions will elucidate whether emulsifier-induced increases in sensitivity to social stress occur via central COX action in a regionally specific manner.

A potential confound we must highlight is that long-term aspirin use can be associated with an increased likelihood of gastrointestinal damage, which can be of particular concern given that emulsifier consumption may disrupt the mucosal barrier integrity in the gut [59]. Histology of the gastrointestinal tract will need to be conducted to determine whether the damage is present. Importantly, however, we measured body mass, liquid intake, and locomotor activity, none of which were significantly impacted by emulsifier or aspirin treatment. Furthermore, LCN2, a biomarker of intestinal damage and inflammation, did not increase following aspirin administration, indicating that aspirin did not amplify emulsifier-induced intestinal inflammation. Additionally, there are other studies indicating that low-dose aspirin does not cause intestinal damage in mice [21, 60, 61]. The studies that do report aspirin-induced intestinal damage used high doses of aspirin, upwards of 200mg/kg/day, over multiple days [62, 63]. Altogether, these data suggest that the aspirin effects reported herein are not likely due to aspirin-induced intestinal damage.

Taken together, the present data demonstrate that ingestion of dietary emulsifiers, at concentrations commonly ingested by humans, increases sensitivity to social stress and that emulsifier consumption may impact social behavior and HPA-axis function at least in part via the COX-molecular pathway. Thus, it is possible that consumption of dietary emulsifiers poses a safety concern in humans wherein inflammation and dysregulation in stress responding can ultimately lead to or exacerbate neuropsychiatric disease.

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4 FINAL CONCLUSIONS

4.1 Summary of current findings

It is only relatively recently that scientists have begun to recognize that the immune system can impact brain and behavior and vice versa. For instance, we now know that chronic inflammation can disrupt the HPA axis, increase susceptibility to social stress, and serve as an underlying mechanism driving mood disorders [1-6]. Therefore, identifying and evaluating environmental factors that may cause inflammation and immunological stress is important so that we can learn how the increased risk resulting from this exposure can be mitigated. Dietary emulsifiers are the most common food additives; they are generally viewed as innocuous and are classified by the Food and Drug Administration (FDA) as generally recognized as safe (GRAS). It has recently been demonstrated, however, that these additives, at translationally relevant doses, can cause systemic inflammation and gut dysbiosis [7, 8]. Because recent studies have suggested that emulsifier intake may increase basal anxiety-like behavior, we sought to determine whether emulsifiers increase sensitivity to salient stressors, such as social defeat, and to identify mechanisms that may underlie an anxiety-like phenotype. This project aimed to test the overarching hypothesis that a dietary emulsifier-induced, proinflammatory state increases sensitivity to social stress. First, we sought to determine whether the consumption of emulsifiers, at doses that are used by the food industry, is sufficient to alter gene expression in the brain thus offering a potential mechanism whereby these compounds may alter responses to stress (Aim 1). Next, we tested whether emulsifier consumption increases sensitivity to social stress. We investigated this aim by observing behavioral, hormonal, and neural activational responses to social defeat stress in mice that consumed emulsifiers (Aim 2). Lastly, we tested the

hypothesis that inflammation, specifically through the COX pathway, is a potential mechanism driving emulsifier-induced stress sensitivity (Aim 3).

In Aim 1, we used RNA-Seq to measure gene expression in brain regions that are known to be important in the generation of behavioral and neuroendocrine responses to stress-provoking stimuli. This was measured in groups consuming either water containing 1% carboxymethylcellulose or polysorbate 80 or plain water for 12-weeks. A total of 243 genes were differentially expressed in the amygdala and PVN of emulsifier-treated mice compared to controls. Interestingly, there was minimal overlap of differentially expressed genes in CMC- and P80-treated animals, suggesting that each emulsifier may act via distinct molecular mechanisms to produce an anxiety-like phenotype. The neurogenetic profile of emulsifier-treated animals, which included differential expression many stress- and inflammation-related genes, suggested that they would exhibit an increased sensitivity to stressors compared to controls, which ultimately drove the investigation of this phenotype in Aim 2. In particular, we found that emulsifier treatment increased expression of IEGs in the amygdala and PVN, suggesting that both CMC and P80 increase neuronal activity in brain regions that modulate stress responding. Further, differential expression of multiple HPA axis regulatory and responsive genes in emulsifier-treated animals indicated possible dysregulation of the HPA axis. Lastly, the top enriched canonical pathways in emulsifier-treated animals were largely comprised of altered immune pathways in both the amygdala and PVN. These potential changes in central immune activation are highly relevant, as increased neuroinflammation is proposed to be a driving factor in the expression of anxiety and depression-like behavior [9]. One immune-related gene of high interest that was differentially expressed (enhanced) in the amygdala of emulsifier-treated mice as a result of emulsifier consumption was PTGS2 (COX2), a gene responsible for coding the

enzyme that converts arachidonic acid to pro-inflammatory prostaglandins and regulating the HPA axis [10, 11]. Thus, this pathway was identified as a prime mechanistic candidate that may drive an anxiety-like phenotype.

Given the evidence provided in Aim 1, which highlighted several genetic markers of HPA axis dysregulation in animals that consumed emulsifiers, Aim 2 investigated whether emulsifier consumption, at percent doses regularly consumed by humans, increased sensitivity to acute stress. We found that the consumption of these dietary emulsifiers potentiated the behavioral, hormonal, and neuroactivational responses to a mild psychogenic stressor, namely acute social defeat stress (ASDS). We did not find that emulsifier treatment, alone, elicited robust behavioral or hormone responses until paired with ASDS. This finding suggests that these emulsifiers may not be stimulating anxiety-like processes at baseline but may, instead, promote susceptibility to stress. By contrast, however, we found that emulsifier treatment, alone, was sufficient to illicit greater, basal neural activity, particularly within the BLA, even in the absence of a stressor. Given that the BLA is a critical node in the brain controlling the signs and symptoms of fear and anxiety and is thought influence the salience of social threat [10-13], it is possible that emulsifier consumption "primes" fear circuits such that stronger anxiety-like behavioral responses are then produced in response to stressors such as social stress.

In Aim 3, we sought to test a mechanism by which emulsifier consumption might sensitize mice to social stress. We had identified the COX inflammatory pathway as a mechanistic candidate based on our finding in Aim 1 that mice consuming dietary emulsifiers show increased amygdala expression of COX2. We found that systemic inhibition of the COX pathway via low-dose aspirin administration ameliorated the enhanced social avoidance and circulating corticosterone seen in emulsifier-treated animals, leading us to conclude that the

observed enhancement in stress responsivity observed in Aim 2 may be due, at least in part, to the inflammatory COX pathway. Paradoxically, we found that controls consuming aspirin, alone, without the emulsifier additive also showed increased social avoidance after social defeat when compared to no aspirin control animals. This suggests that our aspirin regimen may only be beneficial under inflammatory conditions and, under control conditions, may in fact exacerbate social avoidance when inflammation is not present.

In sum, the present study tested the hypothesis that emulsifier intake, at doses that are directly relevant to those ingested by humans, can influence gene expression in brain regions that are known to be important in the generation of behavioral and neuroendocrine responses to stress-provoking stimuli. The current findings illustrate the novel finding that dietary emulsifier consumption induces genetic alternations within the brain, in particular the amygdala and PVN, that could be associated with the previously reported anxiety-like phenotype [8]. Increased neural activity in the amygdala and PVN is often associated with an anxiety- or depressive-like phenotype in rodents and these may thus be brain regions within which emulsifiers act to increase anxiety-like behavior [12]. Further, differential expression in emulsifier-treated animals of multiple genes known to regulate the HPA axis and its response to stress indicates possible emulsifier-induced dysregulation of the HPA axis.

4.2 Limitations and Future Directions

The results of our studies lay a strong foundation for others to further explore the impact of and mechanisms whereby emulsifier consumption impacts stress responsivity. To our knowledge, this is the first demonstration that these dietary additives may impact responses to even a relatively mild social stressor. We wish to highlight some important limitations to the studies presented here, however, so that future studies can address these points.

Most obviously, the exclusion of females from this study is a clear limitation, as sex differences in the prevalence of both inflammatory disease and stress-related neuropsychiatric disorders exist in the clinical population [13, 14]. Previous studies [8] revealed potential sex differences in anxiety-like and social behaviors in emulsifier-treated mice. Thus, it is entirely possible that there are sex differences in the sensitivity to stress following emulsifier intake. Though the social defeat stress model used in our study is a highly salient and translationally relevant stressor [15, 16], it is not useful in female mice because CD1 aggressors will only defeat males. Future studies should examine the effect of emulsifiers in other species, such as Syrian hamsters, which are more conducive to testing sex differences in sensitivity to social defeat because both sexes can be readily and similarly defeated by same-sex conspecifics. Future studies could also use other types of stressors that can be equally applied to both males and females to determine if sex differences in stress sensitivity are present in animals that consume emulsifiers. An additional benefit of using a variety of stressors is that the data would reveal whether the observed increase in stress sensitivity in emulsifier-treated animals is generalized or is specific only to social stress.

Another limitation is that our current test of the proposed inflammatory mechanism of action was very non-selective COX inhibition with aspirin treatment may affect HPA axis functioning via several, downstream effector pathways. The use of a more specific manipulation such as a selective COX inhibitor or the use of a conditional COX1 or COX2 knockout mouse would delineate more specifically whether the effects seen in our study were due to inhibition of the COX1 or COX2 pathway. Further, the route of aspirin administration used limits the conclusions we can make regarding how and where aspirin is acting. In Aim 3, aspirin was administered systemically. Though its metabolite, sodium salicylate, can readily pass the blood-

brain barrier to inhibit COX action centrally, we cannot conclude whether the effects on HPA axis function behavior occurred through a peripheral or central mechanism. Experiments in which selective COX inhibitors are administered selectively to specific stress-modulatory brain regions could elucidate whether emulsifier-induced social stress sensitivity acts via central COX action, and if so, whether it does so in a brain region-specific manner.

The use of a selective COX2 inhibitor or central administration may also help mitigate the potential confound that long-term, systemic aspirin use may result in gastrointestinal damage, as the prostaglandins generated via the COX1 pathway are largely responsible for the integrity of the mucosal barrier in the gut [17]. As discussed in the previous chapter, we did include some measures to assess whether aspirin, itself, had deleterious effects on our subjects. Encouragingly, we found that animals receiving aspirin did not show obvious physiological signs associated with sickness nor did they exhibit higher fecal LCN2, a marker of intestinal damage. Thus, we concluded that aspirin-induced intestinal damage or general malaise is not likely driving the effects observed in our study, however, histological examination of the gastrointestinal tract was not done in our studies. Future work could examine whether there is evidence of damage following chronic administration of systemic COX inhibitors.

4.3 Final Summary and Impact

In conclusion, these data are the first to show that chronic emulsifier ingestion, which is associated with alterations in gut microbiota and with low-grade inflammation [7, 8, 18], alters gene expression in key brain areas that control stress-related behavior. The transcripts and pathways highlighted here provide a more concrete understanding of the molecular mechanisms by which emulsifier consumption may alter both HPA-axis functioning and stress-associated behaviors. Further, these data demonstrate that ingestion of dietary emulsifiers, at concentrations

commonly ingested by humans, increases sensitivity to social stress and that emulsifier consumption may impact social behavior and HPA-axis function via the COX-molecular pathway. From a translational perspective, it is possible that the long-term consumption of emulsifiers may sensitize an individual to social stressors, which could further enhance overall vulnerability to stress and increase the likelihood of developing stress-related neuropsychiatric disorders. For this reason, consuming dietary emulsifiers may pose a safety concern to consumers' health as dysregulation in stress responsiveness can ultimately lead to neuropsychiatric disease. More evidence clearly needs to be obtained so that this possibility can be further tested. In the meantime, we maintain that the FDA should consider that certain food additives, such as pro-inflammatory emulsifiers, could act as susceptibility factors to highly detrimental neuropsychiatric disorders and should continue to evaluate whether such additives should continue to be regarded as "generally recognized as safe."

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