KETOGENIC DIET PARTIALLY ATTENUATES DELETERIOUS EFFECTS OF CHRONIC STRESS

by

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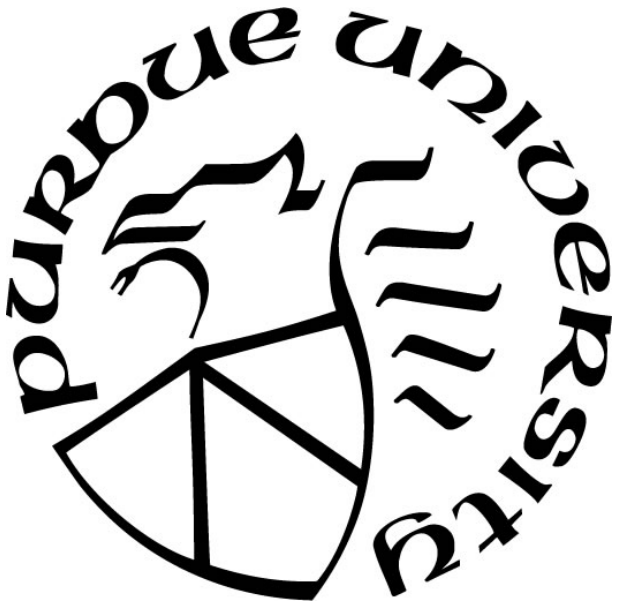

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ABSTRACT

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Ketogenic diets (KDs) are high-fat low-carbohydrate diets that can exert positive effects on physical and neurological health. The more established therapeutic effects of KD are for treating epilepsy and diabetes. However, KD protective effects may apply to other inflammation related disorders associated with Hypothalamic-Pituitary-Adrenal (HPA) axis dysregulation, such as mood disorders. Chronic stress has been shown to elevate cytokine levels, disrupt neuroendocrine homeostasis, and cause anxiety and depressive-like behavior in animal models. In vitro experiments have shown that ketone bodies, a metabolite produced while on KD, can prevent the production of cytokines elevated in response to chronic stress and other pre-clinical experiments have suggested that ketone bodies can prevent anxiety-like behavior. Although this suggests that KDs have anti-inflammatory and mood stabilizing potential, these effects have yet to be explored. In this experiment, we assessed the behavioral and neuroendocrine effects of KD using male and female Long-Evans rats. Animals underwent three weeks of Chronic Mild Stress (CMS) while on KD or control Chow (CH). Body weight and food intake data were recorded daily, and depressive-like behaviors were assayed after the three weeks. Plasma Beta-Hydroxybutyrate (βHB), Corticosterone (CORT) and Interleukin-1 beta (IL-1β) were measured after behavior testing, along with hypothalamic Corticotropin-Releasing Hormone (CRH) and Neuropeptide Y (NPY) mRNA expression. CMS induced weight loss and reduced food intake in the control-diet groups, however the KD-fed male and female rats were resistant to CMS-induced weight loss and reduced food take. Female rats fed KD were protected from CMS-induced reductions in plasma CORT and hypothalamic NPY expression. Collectively, these data suggest anti-depressant potential of KDs against chronic stress, particularly in females.

INTRODUCTION

Lifestyle changes, such as implementing strategies to decrease life stress and adopting healthier eating habits, are known to improve mental health (Walsh, 2011). Hence, these are among the first suggestions that psychologists make when patients report symptoms of affective disorders. Popular stress management strategies include exercise, meditation, and time in nature. Although these have shown to be incredibly beneficial, it is often difficult for those experiencing excessive life stress to have energy and time for such therapies. This makes nutritional modifications appealing since they do not require as much time out of a busy schedule and diet can more quickly improve energy levels. Despite widely accepting that a healthy diet contributes to optimal mental health, there are very few treatment studies investigating the effects of diet-types on mood. Additionally, "healthy eating habits" on the topic are vaguely defined and dietary recommendations to improve mental health are often inconsistent.

This lack of understanding about how dietary changes can impact mood and mental health can result in patients turning to non-empirical sources of information, such as anecdotes from various media sources making indirect untested claims. One common yet unorthodox trend is the adoption of diets that are very high in fat and low in carbohydrates, or ketogenic diets (KDs). Lay reports make widespread claims that drastically reducing carbohydrates in favor of fats can increase energy and mood, thereby making it an appealing dietary regimen for patients with mood disorders as well. These diets are a known effective treatment for epilepsy and diabetes, and are hypothesized to have other positive neuronal benefits (Paoli, Rubini, Volek, & Grimaldi, 2013). Although

there is some evidence that aspects of the diet may increase resilience to stress by protecting from neuroendocrine and immune dysregulation, KD has yet to be tested in a pre-clinical model of affective disorders.

Ketogenic Diets

KDs are high-fat low-carbohydrate diets that have shown to be beneficial to the brain and body. Although the more established neuroprotective effects are in treating epilepsy (Neal et al., 2008), their protective effects may apply to other disorders as well. KD can protect from harmful diet-induced deficits in the brain such as impaired performance on cognitive tasks, decreased brain derived neurotropic factor (BDNF), as well as increased hippocampal inflammation and blood brain barrier permeability (Davidson et al., 2013; Hargrave, Davidson, Lee, & Kinzig, 2015; Hargrave, Davidson, Zheng, & Kinzig, 2016). KDs can also protect from metabolic insult as well as from peripheral immune challenges (Dupuis, Curatolo, Benoist, & Auvin, 2015; Kinzig, Honors, & Hargrave, 2010; Kinzig, Scott, Hyun, Bi, & Moran, 2005; Youm et al., 2015).

Neuroprotective effects of KD seem to rely on the induction of ketosis and elevation of beta-hydroxybutyrate (βHB) (Davidson et al., 2013; Hargrave et al., 2015, 2016). βHB is produced by the liver and elevated in the blood stream when consuming a high-fat low-carb diet, prolonged fasting, or exercising (Mattson, 2014). In vitro experiments have shown that βHB can improve mitochondrial function, decrease spontaneous firing rates in neuronal cells, in addition to the previously described anti-inflammatory potential. (Achanta & Rae, 2016; Youm et al., 2015). Interestingly, injecting βHB subcutaneously decreases food intake in rats, even after the injection clears, suggesting that it can influence behavior (Langhans, Pantel, & Scharrer, 1985).

βHB can enter the brain by active transport via monocarboxylate transporters (Achanta & Rae, 2016), providing a mechanisms by which βHB can enter the central nervous system and influence behavior, such as decreasing anxiety-related behavior as well as influence activity levels (Ari et al., 2016; Murphy, Likhodii, Hatamian, & Burnham, 2005). Many have hypothesized the important role of ketone bodies in homeostatic regulation, however, the mechanisms by which it acts may be more complex than originally thought. The more common theory on the benefits of a KD is that it serves as a better fuel source for neurons compared to glucose (Achanta & Rae, 2016) however, new evidence suggests that βHB can also directly act on an inflammatory pathway stimulated by stress (Youm et al., 2015). These potential therapeutic effects of ketone bodies on the brain are the basic premises underlying speculated anti-depressant effects of KDs.

Depression and Diet

Mood disorders, such as major depressive disorder (MDD) and bipolar depression (BD), constitute neurological changes that result in emotional dysregulation and cognitive deficits. These disorders have a shared diagnostic criterion— having a depressive episode lasting at least two weeks. A depressive episode is categorized by having five or more of the following nine symptoms: depressed mood most of the day, diminished interest or pleasure in activities that are typically pleasurable, significant changes in weight, changes in wakefulness, psychomotor changes, loss of energy, decrease concentration, feeling worthless nearly every day, and suicidal thoughts. These symptoms cause significant distress, and despite the availability of many antidepressant treatments, the World Health Organization (WHO) ranked MDD the highest global cause of "years of life lived with disability" for all age groups (Kessler et al., 2013). Only one

third of patients respond appropriately to treatment, while the other two thirds either don't achieve remission or experience relapses (Rush et al., 2006). Almost all pharmacological treatments act similarly to block monoamine transporters, offering very few distinct alternatives for patients who don't respond appropriately (Leary, Dinan, & Cryan, 2015). The persistently high rates of people living with these disorders, and lack of effective treatments help explain why patients would result to untested therapeutic strategies.

The core of some symptoms can also be the result of energy dysregulation driven by poor diet. Emerging pre-clinical evidence suggests that diets high in fat and sugar alone can cause depression-like symptoms, and epidemiological research revealed depression is often comorbid with metabolic disorders (Luppino, Floriana S.; de Wit, Leonore M.; Bouvy, 2010). There is already evidence suggesting KD can reverse the adverse metabolic effects of high fat high sugar diet, even though the effects on mental health are unclear. Collectively, this link exacerbates the appeal of KDs to subsets of the population suffering from both mental and metabolic problems.

Stress in the Hypothalamus

Chronic stress is known to increase susceptibility to mood disorders. Stressful stimuli activate the sympathetic nervous system, which leads to release of hormones that motivate a "fight or flight." Under normal conditions, this is an acute adaptive response of survival that is mediated in the hypothalamic-pituitary- adrenal (HPA) axis. The HPA axis has several feedback mechanisms to return the brain and body back to homeostasis once a stressful event is over. Chronic activation and constant arousal can lead to dysregulation of these systems and becomes detrimental to homeostatic processes such as

energy regulation (Torres & Nowson, 2007), desensitization of neurotransmitter function in higher up brain regions (Lucassen et al., 2014), and even immune function (Wohleb, Franklin, Iwata, & Duman, 2016).

Three chemical messengers critical in maintaining HPA homeostasis are glucocorticoids, energy regulating hormones, and inflammatory cytokines. Glucocorticoids, such as cortisol (in humans) and corticosterone (CORT, in rodents), are produced in adrenal glands and stimulate a variety of tissues to increase physiological, metabolic, and immune arousal and motivate behavior in case of stressful event. Patients with depression often have abnormal salivary, urinary, and cortisol levels (Nemeroff & Vale, 2005). Corticotropin-releasing hormone (CRH) is produced in the hypothalamus, and plays a critical role in regulating CORT production after acute and chronic exposure to a stressor. It is also found to be abnormal in patients with affective disorders (Claes, 2004).

Energy regulating hormones, such as neuropeptide Y (NPY), are hypothesized to be key integrators of metabolic processes into the HPA stress response. NPY is a 36 amino acid peptide produced in the hypothalamus that stimulates food intake and fat storage after the termination of a stressful event. It also stimulates CORT production, and CRH mRNA expression (Hanson & Dallman, 1995). Patients with depression have abnormal levels of cerebrospinal (Heilig et al., 2004) and plasma NPY (Hashimoto, Onishi, Koide, Kai, & Yamagami, 1996), suggesting that patients have deficits in maintaining neuroendocrine homeostasis. Although the exact role of NPY in HPA feedback loops are not entirely known, it is hypothesized to increase stress resiliency by

balancing out the excitatory effects of CRH and more quickly restore homeostasis (Enman, Sabban, McGonigle, & Van Bockstaele, 2015).

Emerging literature suggests that cytokines are also a major contributor of neuronal damage that characterizes the neurobiology of depression (Najjar, Pearlman, Alper, Najjar, & Devinsky, 2013). Pro-inflammatory markers and immune cells typically have limited access to the brain when the blood brain barrier (BBB) is intact, however, BBB integrity is compromised by chronic stress and a poor diet. Although the precise mechanisms by which this happens is unclear, there is evidence that peripheral inflammation is a major driver of a depressive phenotype (Bhattacharya, Derecki, Lovenberg, & Drevets, 2016). IL-1β correlates with severity of depressive symptoms in humans, and its role in mood regulation is supported by rodent studies that observe changes in depressive behavior after manipulating levels of IL-1β (Goshen et al., 2008). IL-1β is involved in the regulation of several neuronal circuits related to depression such as HPA dysregulation, decrease hippocampal neurogenesis, as well as activity in the amygdala and prefrontal cortex (Inagaki, Muscatell, Irwin, Cole, & Eisenberger, 2012; Koo & Duman, 2008; Miller, Haroon, Raison, & Felger, 2013; Wager-Smith & Markou, 2011). Additionally, IL-1β can reduce the availability of monoamines related to depression—serotonin, dopamine, norepinephrine, and glutamate—by decreasing the production of precursors as well as increasing the expression and function of reuptake transporters (Miller et al., 2013) (Zhu et al., 2010). Recent studies have suggested that beta-hydroxybutyrate (βHB) can inhibit mechanisms of IL-1β synthesis, (Youm et al., 2015), making this a potential mechanism by which βHB can assert anti-depressant effects.

Stress Models

Rodent studies have demonstrated that repeated exposure to stressful psychological or environmental stimuli cause behavioral, neurological, and immune changes analogous to what is observed in humans with affective disorders. The most common pre-clinical models are social defeat stress, restraint stress, and unpredictable stress. These are followed by tests that measure anhedonia, helplessness, anxiety, and/or decrease cognition to assess behaviors that are typical of a depressive-phenotype. Scientists have established a depressive-phenotype characterized by depressive behaviors, elevated corticosterone, disrupted energy regulation, and the newly elucidated increase in cytokine level (Willner, 2016b, 2016a).

Animal models of depression are evaluated by the following criteria: 1) responsiveness to antidepressants—predictive validity, 2) behavioral translation to symptoms of DSM-V—face validity, and 3) their similarity to underlying neurobiological mechanisms—construct validity (Willner, 2016b, 2016a). Of the previously mentioned animal models of depression, chronic mild stress (CMS), scores highest in meeting these criteria. Depressive symptoms modeled by CMS are mainly anhedonia and learned helplessness and can be quantified using behavior tests such as sucrose preference, forced swim, and elevated plus maze (Willner, 2016a) (Willner, Towell, Sampson, Sophokleous, & Muscat, 1987). Effective antidepressant treatments prevent or ameliorate CMS induced symptoms that are highly translatable to human diagnosis. It also models the hypothalamic, behavioral, and immune response analogous to that of humans with depression (Kubera, Obuchowicz, Goehler, Brzeszcz, & Maes, 2011). If effectively

induced, CMS is the model most effective model used to test the mechanisms associated with depression.

Experiment Aims

The goal of the following experiments is to elucidate the effects of KD on the development of depressive-like behavior using a rat model of chronic mild stress (CMS). To do this, we use a KD with macronutrient ratios similar to a strict diets adopted by humans (80% fat, 15% protein, and 5% carbohydrate) (Kinzig et al., 2010). The experimental timeline is illustrated in Figure 1.

We hypothesized that rats consuming KD while undergoing three weeks of CMS will have different behavioral and neuroendocrine patterns than those consuming a regular chow (CH) diet. Further, we hypothesized that the diet-type alone will not disrupt HPA activity or result in negative behavioral outcomes.

Specific Aim 1: Evaluate the effects of KD on the development of a stress-induced depressive-like behavior. Four tests were used to evaluate disordered behavior: daily food intake, anxiety in elevated plus maze (EPM) and open field (OF), locomotor changes in OF, and helplessness in the forced swim test (FST).

Specific Aim 2: Evaluate effects of KD on stress-induced HPA dysregulation. This was quantified by analyzing changes in body weight, terminal plasma corticosterone (CORT) and IL-1β, and hypothalamic CRH and NPY mRNA expression.

Collectively, these experiments will further our understanding of the therapeutic potential of KDs, and the mechanisms by which this diet-type can influence the brain. Sex comparisons will further reveal potential differences in behavioral and neuroendocrine response to both stress and KD.

METHODS

Subjects and Diets

Adult (16-17 week old) male ($N = 33$) and female ($N = 37$) Long-Evans rats were individually housed in suspended wire cages in humidity (55-65%) and temperature (20.5 ± 1 °C) controlled rooms and maintained in a 12:12 hour light/dark cycle unless stated in the stress schedule. Males and females were housed in separate rooms with light/dark cycles staggered by 1 hour to maintain stress and testing time consistent between male and female groups. Animals were weight matched before being assigned to either a KD or control chow (CH) diet. KD (Research Diets D06040601, New Brunswick, NJ) consists of 80% fat, 5% carbohydrates, and 15% protein. Chow diet (Teklad 2018, Envigo, Indianapolis, IN) consists of 18% fat, 58% carbohydrates, and 24% protein. Diet compositions are further described in Table 1. After 4 days of exposure to their assigned diet, animals were randomly assigned to a stress (+) or non-stress (-) subgroup for the remainder of the experiment. There was a total of 4 experimental groups per sex: CH-, CH+, KD-, KD+. Group n for males were 7, 9, 8, 9 respectively, and for females 9, 9, 9, 10 respectively. All animals had *ad libitum* access to food and water in their home cage and in novel cages if stressors lasted 6 or more hours. Animals were weighed daily and food intake was measured by weighting food container daily and subtracting food spillage. All procedures were approved by the Purdue Animal Care and Use Committee (PACUC).

Chronic Mild Stress (CMS)

Animals underwent 21 days of chronic unpredictable mild stress (CMS) while on their corresponding diets. Rats were exposed to the 7 most common stressors (as evaluated in Willner, 2016) in a pseudorandom order manner to prevent habituation and predictability. Stress stimuli are described in Table 2.

Behavior Testing

Food intake was measured daily and final behavior tests were done for 3 consecutive days following the end of CMS. All final testing was done in room adjacent to the vivarium during the first half of the light cycle. Animals were given 10-15 min to habituate to the room before the start of each test. Test order was counterbalanced within each sex to minimize test time bias.

Food Intake

Food intake was recorded to the nearest 0.5g by weighing food daily and subtracting the food weight from the previous day. All food weights were recorded in the second half of the light cycle. If food intake measurements were missed on any given day, linearity was assumed and food weight from day prior and day after were averaged as to not discarded the animal's data. This was done no more than twice per animal out of 25 days.

Open Field Testing (OFT)

The open field test (OFT) quantifies locomotor activity and anxiety-like behavior. The apparatus is 4 feet by 4 feet, with a grid printed on the floor. The rat is placed in the center of the OF apparatus and video recorded for 5 minutes on an aerial view mounted camera. Videos were later scored automatically using Any-Maze Behavior Tracking

Software (Stoelting Co.) for total distance traveled, time spent in the center, and time spent in the corners of the apparatus.

Elevated Plus Maze (EPM)

This behavior assay is used to evaluate anxiety-like behavior. Rats are placed on the EPM apparatus, which is 3 feet off the ground with four arms at 90 degree angles each 6 inches wide and 3 feet long. Two of the opposite side arms are enclosed and the other two are open. Animals were placed in the center of the EPM, and behavior is video recorded from a ceiling mounted camera for 5 minutes. The rat is returned to its cage in immediately after testing. Percent of time spent in open arms was scored manually using a stopwatch.

Forced Swim Test (FST)

This behavior assay is used to measure despair behavior to evaluate depressive-like symptoms in animal models. Animals are placed in a 22x17x25 inch container with 15cm of 25-30 Celsius water or enough so that animals cannot step on the bottom of the container. Animals were monitored during the duration of the test and the test was stopped if animals cannot maintain a swim or float behavior. The test was recorded for 5 minutes using a ceiling mounted camera, then animals were immediately tried off with a towel and returned to their home cage. Videos were later scored manually for time spent immobile, latency to go immobile, and time spent trying to escape using Any-Maze Behavior Tracking Software (Stoelting Co.).

Neuroendocrinology

Animals were sacrificed using pentobarbital (Beauthanasia) approximately 2 hours after FST test for plasma analysis of circulating levels of CORT, βHB, and IL-1β.

Hypothalamic tissue was also dissected for PCR analysis of CRH and NPY. About 3 mL of trunk blood was collected in VACUETTE® K3EDTA blood collection tubes (Greiner Bio-One, Monroe, NC) and centrifuged for 15 min at 4°C, 2500 rpm. Plasma was isolated, aliquoted into three tubes and stored in a -80°C freezer for later analyses. Brains were rinsed with 1x phosphate buffered saline (PBS), hypothalamus were micro-dissected on ice, flash frozen on dry ice, then stored at -80°C.

Beta-Hydroxybutyrate (βHB) Assay

Plasma βHB was measured in duplicate by β-Hydroxybutyrate LiquiColor® assay kit (Stanbio, Laboratory, Boerne, TX) using a microplate reader. First, 1 mM standard solution was diluted with deionized water into five concentrations: 1, 0.75, 0.5, 0.25, and 0.125 mM. 2.5 µL of sample or standard were loaded on a 96 well plate. Reagent 1 was incubated at 25°C for 10 min then 90 µL were dispensed into each well. The absorbance of 505 nm was immediately taken as the baseline reading. After adding 15 µL of reagent 2 per well, the plate was incubated in 25°C for 10 min and a second reading was taken. The difference between the second and the first measurements was used to quantify expression and the concentration of plasma samples were interpolated from the standard curve.

Corticosterone (CORT) Assay

Plasma corticosterone (CORT) concentration was quantified in duplicates using an EIA kit according to the manufactures instructions (Enzo Life sciences, protocol ADI-900-097). Samples were diluted 1:40 using a steroid displacement reagent and standards were serially diluted 1:50. 100 µL of sample or standard were loaded on donkey anti-sheep IgG coated plates, followed by 50 µL of conjugate, then 50 µL of sheep antibody

to rat CORT. Plates were then incubated at room temperature on a plate shaker for 2 hours at 500rpm, then washed 3 times. After wash, 200 μL of pNpp substrate solution was added to every well, then incubated at room temperature for an hour. 50 μL of stop solution was added to every well, then optical density was immediately read at 405mn absorbance using a microplate reader (Multiskan Ascent, Thermo Scientific).

Interleukin- 1 Beta (IL-1β) Assay

Plasma IL-1β was quantified in duplicate with R & D Quantikine ELISA Rat IL-1β/IL-1F2 immunoassay according to the manufacturer's instructions. In summary, Plasma samples were diluted 1:3 using the provided calibrator diluent and the assay standard was serially diluted with a dilution factor of 2 in 8 concentrations. 50 μL of assay diluent was added to rat IL-1β coated well plates, then 50 μL of sample or standard were loaded into each well plate then incubated at room temperature for 2 hours. After the incubation period, wells were washed 5 times, loaded with 100 μL of conjugate, incubated again at room temperature for 2 hours, then washed again five times. After second wash, 100 μL of substrate solution was added to each well, incubated for 30 min at room temperature, protected from light, then 100 μL of stop solution was added. Immediately following the addition of stop solution, 450nm absorbance was measured in a microplate reader (Multiskan Ascent with Ascent software v2.6, ThermoFisher Scientific, Waltham, MA).

RNA Extraction and cDNA Synthesis

Tissues were homogenized in 1 mL TriReagent (RT111, Molecular Research Center, Cincinnati, OH) then 50 μL bromanisole (BCP, Molecular Research Center, Cincinnati, OH) was used to extract RNA. RNA was quantified by spectrophotometer at

260 nm absorbance (Eppendorf BioPhotometer 22331, Hauppauge, NY). First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA) was used to synthesize cDNA from 2 μg total RNA.

Hypothalamic RT-qPCR

Real-time quantitative PCR (qPCR) was performed with PowerUp SYBR Green Master Mix (Applied BioSystems, ThermoFisher Scientific, location?) on an iCycler iQ + MyiQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Primer sequences for β-actin (NM_031144), NPY (NM_012614), and CRH (NM_031019) were designed and/or validated using Primer-BLAST (National Center for Biotechnology Information, NIH), and the thermal profile for each primer was optimized to 90-110% efficiency. Primer sequences are listed in Table 3, along with sequence and thermal conditions. Target genes expression level was normalized to β-actin by ΔΔCt method.

Statistical Analyses

Data were first analyzed by sex via one-way analysis of variance (ANOVA) using GraphPad Prism software (v7.00, GraphPad software Inc., La Jolla, CA). Where appropriate, follow-up Tukey's post hoc multiple comparison test and Bartlett's homogeneity of variance statistic are reported. General treatment effects and sex comparisons were then analyzed using two-way ANOVA (experimental condition by sex). The level of significance for condition and sex were set at $P < 0.05$, and figures are represented as mean with error bars ± standard error of the mean (SEM); n = 7-10 per group.

RESULTS

Behavioral Effects

Food Intake

Daily food intake was lower in all chow-fed animals undergoing CMS (CH+ groups). CMS significantly decreased food intake of CH groups in both sexes and had a stronger effect on females. Multiple comparison tests reveal CH+ males had lower food intake than CH- (*$p = 0.0187$), and KD- (*$p = 0.0136$) (Figure 2[A]). Females also showed effects of diet and stress ($F(2.909, 64) = 9.836$, ****$p < 0.0001$). Multiple comparison test found that CH+ females had lower food intake than CH- females (***$p = 0.0002$), and KD- (**$p = 0.0023$) (Figure 2[B]). KD+ females also had lower food intake than CH- (*$p = 0.0135$), but not its own KD no stress control group ($p = 0.146$). Sex comparison found the decrease of food intake in CH+ groups to be consistent across sexes (*$p = 0.0001$), as well as females having lower food intake overall (*$p < 0.0001$) (Figure 2[C]).

Body Weight

There are significant differences of CMS on body weight change in both sexes (Figure 3). Tukey's multiple comparison tests revealed that males that underwent CMS (CH+) gained less weight than both no-stress groups-- CH- (*$p = 0.0139$), KD- (***$p = 0.0003$). They also gained less weight than KD+ group (**$p = 0.0033$. KD- and KD+ groups were not different from each other ($p = 0.7325$). CH+ females also gained less weight than the CH control group (**$p = 0.0039$), and KD- group (**$p = 0.0048$), but was not different than KD+ (0.1794). The standard deviations between groups was also

significantly different only in females (Bartlett's statistic = 18.92, $p = 0.0003$). When sexes are collapsed for comparison, there is a significant effect of treatment (*$p < 0.0001$), and no differences between sexes ($p = 0.8364$).

Elevated Plus Maze (EPM)

Females that underwent CMS (CH- group) spent more time in open arms of EPM apparatus (Figure 4[B], *$p = 0.339$). This effect was not observed in males (Figure 4[A], $F(3, 29) = 1.885$), $p = 0.1543$). When sexes are collapsed, there was a main effect of treatment ($F(3, 62) = 3.187$, $p = 0.0298$) (Figure 4[C]), presumably driven by females. There are no differences between sexes ($F(1, 62) = 3.163$, $p = 0.0802$) and no interactions between sex and experimental condition (0.6820).

Open Field Test (OFT)

There were no effects of diet or stress on locomotor or anxiety like behavior in the open field (OF) apparatus as quantified by distance traveled (Figure 5[A-C]), time in center of apparatus (Figure 5[D-F]) and time in corners (Figure 5[G-I]). The results for each OF measure are as follows:

Neither males nor females showed significant differences in distance traveled (Male $F(3, 28) = 0.4022$, $p = 0.7525$) (Female $F(3, 32) = 0.7463$ $p = 0.5325$), and two-way ANOVA indicates were no overal experimental effects ($F(3, 60) = 1.098$, $p = 0.3569$) or differences between sexes ($F(1, 60) = 0.5192$, $p = 0.474$). Representative figures are in Figures 5(A-C).

There were no effects of diet or stress on time spent in the center quadrant of the apparatus (Male $F(3, 28) = 0.1266$, $p = 0.9436$)(Female $F(3, 31) = 0.8706$, $p = 0.4668$). Sex comparisons found females overall spent more time in the center than males ($F(1,$

59) = 4.195, *p = 0.045), although diet and stress had no effect (F(3, 59) = 0.1621, p = 0.9215). Representative figures are are in Figures 5(D-F).

There were no effects of diet or stress on time spent in corners of the apparatus (Males F(3, 28) = 0.3444, p = 0.7934) (Females F(3, 31) = 0.4427, p = 0.7241). Two-way comparison indicates were no main treatment effects (F(3, 59) = 0.3898, p = 0.7608) or differences between sexes (F(1, 59) = 1.287, p = 0.2613). Representative figures are in Figures 5(G-I).

Forced Swim Test (FST)

There were no effects of diet or stress on helpessnes behavior in the FST apparatus as quantified by time immobile (Figures 6[A-C]), latency to immobility (Figures 6[D-F]) and time spent attempting to excapt (Figures 6[G-I]). The results for each FST measure are as follows:.

Neither males nor females showed significant differences in time spent immobile (Male F(3, 29) = 0.1022, p = 0.9581) (Female F(3, 32) = 0.6411, p = 0.5942). There were no main treatment effects (F(3, 61) = 0.1607, p = 0.9224) or sexes differences (F(1, 61) = 3.467, p = 0.0674). Representative figures are in Figures 6(A-C).

Neither males nor females showed significant differences in latency to first bout of immobility (Male F(3, 29) = 1.329, p = 0.2841) (Female F(3, 32) = 1.79, p = 0.1689). There were also no main treatment effects (F(3, 61) = 1.749, p = 0.1664) or sexes differences (F(1, 61) = 0.4419, p = 0.5087). Representative figures are in Figures 5(D-F).

There were no effects of diet or stress on time attepting to escape (Male F(3, 29) = 0.2981, p = 0.8264) (Female F(3, 32) = 0.1701, p = 0.9158). Sex comparisons found males overall spent more time attempting to escape than males (F(1, 61) = 5.802, *p =

0.0190), although diet and stress had no effect ($F(3, 61) = 0.1952$, $p = 0.8993$). Representative figures are are in Figures 6(G-I).

Neuroendocrine Effects

Beta-Hydroxybutyrate (βHB) Levels

Terminal βHB was significantly higher in rats consuming KD (Figure 7). Females ($F(3, 33) = 10.76$, $*p < 0.0001$) had slightly lower p values than males ($F(3, 24) = 5.343$, $*p = 0.0058$). However, two-way ANOVA revealed the two are not significant ($F(3, 57) = 2.496$, $p = 0.1197$) and that the effects of KD were consistent across sexes.

Corticosterone (CORT)

Terminal corticosterone (CORT) was analyzed using plasma samples collected approximately 2 hours after forced swim test (FST) (Figure 8). There were no differences between male experimental groups ($F(3, 24) = 0.3009$, $p = 0.8244$). However, females on chow undergoing stress (CH+) had significantly lower CORT than the chow-fed control females (CH-) ($F(3, 32) = 3.15$, $p = 0.0383$). Two-way ANOVA indicates males had overall significantly higher CORT than females ($p = 0.0181$).

Interleukin-1ß (IL-1 ß)

There were no detectable effects of diet type or stress condition on terminal plasma levels of IL-1β in either males ($F(3, 29) = 0.2177$, $p = 0.8833$) or females ($F(3, 32) = 0.4784$, $p = 0.6996$). There were also no overall differences between sexes ($F(1, 61) = 0.04892$, $p = 0.8257$). Notably, all values were toward the lower reliably detectable limit (9 ng), suggesting little to no inflammation in any of the experimental groups. Representative figures are found in Figure 9.

Corticotropin-Releasing Hormone (CRH)

There were no significant effects of diet or stress conditions in hypothalamic CRH mRNA expression in either males ($F(3, 29) = 0.1796$, $p = 0.9094$), or females ($F(3, 30) = 0.5183$; $p = 0.0945$). There were also no differences between sexes ($F(1, 59) = 0.008341$, $p = 0.9275$), of overall treatment effects ($F(3, 59) = 0.3414$, $p = 0.7954$). Results illustrated in Figure 10.

Neuropeptide Y (NPY)

There were no significant effects of diet or stress conditions in males ($F(3, 29) = 0.848$; $p = 0.9094$), however Bartlett's statistic was significant, indicating that the standard deviations of CH- control is less than experimental groups (Bartlett's test = 9.333; $p = 0.0252$). There was no main effect found in female groups either ($F(3, 30) = 2.854$); $p = 0.0538$), however, Tukey's multiple comparison test revealed females CH+ group had lower NPY expression than CH- ($*p = 0.0451$). Females had lower NPY expression than males ($F(1, 59) = 5.55$, $*p = 0.0218$). This seems to be driven by differential effects of treatment since there are no main effects of treatment ($F(3, 59) = 0.5289$, $p = 0.6642$), and CH- groups have roughly the same expression levels. Results are graphed in Figure 11.

GENERAL DISCUSSION

Male and female rats that experienced chronic mild stress (CMS) while on chow diet (CH+) showed some phenotypic characteristics of affective disorders such as decreased body weight and food intake. Differences in time spent in open arms of EPM, plasma CORT, and hypothalamic NPY expression were also observed, but only in female groups. Additionally, our data did not demonstrate any behavioral, neuroendocrine, or inflammatory differences in either KD+ or KD- condition. This suggests that KD animals that underwent stress were protected from stress-induced changes in body weight, food intake, CORT and NPY. Collectively, these data suggest KDs have some anti-depressant potential, particularly in females.

Increased time spent in open arms of EPM and decreased CORT in females is consistent with chronic stress experiments from other groups, and indicative of anxiety-like behavior (McCormick, Smith, & Mathews, 2008). The FST served as an acute stressor, and females that undergo acute stress have higher CORT than those that have undergone chronic stress (Kudielka & Kirschbaum, 2005). The direction of behavior changes is opposite of what is typically observed in males. This dampened response in females is related to differences in adaptive response to chronic stress (Cook & Wellman, 2004). Females exhibit hypo activity in the HPA axis in response to chronic stress, where males exhibit hyper activity. Hypo activity in the HPA axis is also observed in autoimmune disorders, which are more common in women (Bekhbat & Neigh, 2018). This sexual dimorphism has been attributed to the multifaceted role of estrogen. This

phenomenon is still not fully understood, albeit it's critical importance in understanding how and why disorders such as depression develop differently in males and females.

Some data were inconsistent with what has been reported by other experimenters using CMS models. Other experimenters report an increase in time spent floating in the FST, decrease in time spent in the center of OF, changes in plasma CORT and IL-1β, and changes in hypothalamic CRH (Hill, Hellemans, Verma, Gorzalka, & Weinberg, 2012). This could be attributed to a number of issues such as external environmental factors, stressors being too mild, habituation to stress during the light cycle even though timing during the 12 hour period is variable, and even sex of experimenters (Sorge et al., 2014; Willner, 2016b, 2016a). Inconsistencies with changes in CRH and IL-1β could also be due to how plasma and tissue were processed. Animals were sacrificed approximately 2 hours (+/- 15min) after FST, when animals were expected to return to a basal level. The high variability suggests there were some differences in some animals. Perhaps repeated blood collections at different time points post-FST would have revealed differences in stress response. Males that undergo CMS have higher basal levels, but respond differently at 15, 30, and 60 minutes post- acute stress (Bielajew, Konkle, & Merali, 2002). Additionally, NPY was more variable in males under experimental conditions. This greater variability may also be attributed to the timing of tissue collection, given that CORT, CRH, and NPY mechanisms are interrelated.

Overall, these findings provide some empirical support to the psychotherapeutic claims surrounding KDs. This evidence suggest that this diet-type can play a role in preventing stress-induced decreases in body weight and food intake, which are two major symptoms of anxiety and depression identified in the DSM-V. Energy homeostasis is

important in regulating mood, therefore this alone could be driving reports on improved mood and increased energy. One major hypothesis on the metabolic function of KDs is that restricting these types of carbohydrates alone can have a wide array of metabolic and mental health benefits. Adopting a KD limits food choices, and increases awareness of caloric intake. This major lifestyle change that restores energy homeostasis alone may be enough to reverse the negative impacts of "Western Diets" on mental health, such as is reported anecdotally. This major lifestyle change that restores energy homeostasis alone may be enough to reverse the negative impacts of "Western Diets" and improve the mental health such as it is reported anecdotally. Overall, more population data and controlled clinical experiments are needed to determine what aspects of KD are driving reports of improved mood, cognitive function, and elevated energy.

These data also demonstrate that consuming diets very high in fat alone does not increase susceptibility to the development of affective disorders, as is claimed in numerous publications. Other "high fat diet" studies claim that calorie-dense diets high in fat disrupt HPA activity, lead to disordered feeding behavior and induce a depressive-like phenotype (Dutheil, Ota, Wohleb, Rasmussen, & Duman, 2015; Tannenbaum et al., 1997; Zemdegs et al., 2016). In contrast, our experiments demonstrate no adverse effects when animals consumed diets with 80% fat. This suggests that the effects of "high-fat diets" (containing 45-60% fat) are non-linear. These paradoxical findings lead to other questions about the elements of "high-fat diets" that deleterious effects on mental health could be attributed to. One of those being that "high fat diets" or "Western Diets" are typically high in sugars and simple carbohydrates. Further research is warranted not only on the effects of KD on mental health, but also on what other aspects of "Western Diets"

could be causing mood disorders in the first place. More knowledge on the relationship between diet and depression will lead to a broader understanding of what lifestyle changes are best for patients to have optimal mental health.

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APPENDIX A

Table 1

Diet Compositions

Diet Type	Carbs %	Protein %	Fat %	Calorie Density	Product ID
Chow (CH)	58	24	18	3.1 kcal/g	Teklad (2018)
Ketogenic (KD)	5	15	80	6.1 kcal/g	Research Diets (D06040601)

Table 2

Chronic Mild Stress (CMS) Details

Stressor	Description	Duration
Restraint	Restrained in a clear PVC device with a perforated front at an unpredictable time during the light cycle.	30 minutes
Lights	Moved to a neighboring housing room in novel cages, where the lights remained on during the dark cycle.	12 hours*
Flooding	200mL of room temp tap water added to bedding in novel cage during the light cycle.	16 hours*
Cold	Moved to a novel cage and placed in a 4 degrees C chamber at an unpredictable time during the light cycle.	30 min.
Crowding	Temporarily housed with 3-4 unfamiliar conspecifics also undergoing CMS during the light cycle.	6 hours*
Static	Static noise (80dB) played in a neighboring housing room in the middle of the light cycle.	6 hours*
Cage Tilt	Moved to a novel cage during different times of the day tilted 30-45 degrees in home room.	6 hours*

**Note.* Animals had access to food and water in conditions lasting 6 or more hours.

Table 3

Primer Sequences and Thermal Profiles

Target Gene	Primer Sequences	NCBI Sequence	Thermal Profile	Efficiency
CRH	Forward: 5' – CTC TCT GGA TCT CAC CTT CCA C- 3' Reverse: 5' - CTA AAT GCA GAA TCG TTT TGG C - 3'	NM_031019.1	95°C/15s 60°C/15s 72°C/60s	100.4%
NPY	Forward: 5'- TAT CCC TGC TCG TGT GTT TG- 3' Reverse: 5'- TGT CGC AGA GCG GAG TAG TA - 3'	NM_012614	95°C/15s 58°C/15s 72°C/60s	97.6%
ß-actin	Forward: 5' - ATT GGT GGC TCT ATC CTG GC - 3' Reverse: 5'- AAA CGC AGC TCA GTA ACA GTC - 3'	NM_031144.3	95°C/15s 60°C/15s 72°C/60s	90.4%

APPENDIX B

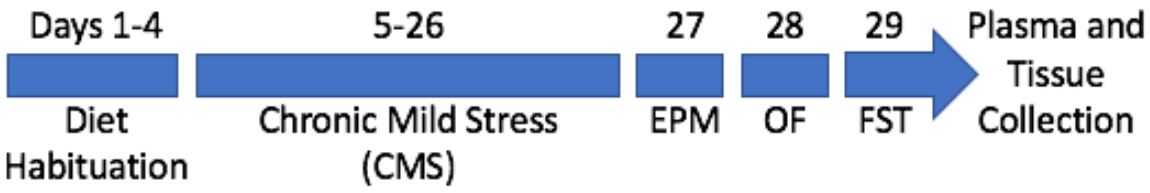

Figure 1. Experimental Timeline. Rats were exposed to either Ketogenic Diet (KD) or Chow (CH) diet for 4 days, then underwent three weeks of Chronic Mild Stress (CMS). After CMS, animals were tested for three consecutive days on Elevated Plus Maze (EPM), Open Field (OFT), and Forced Swim (FST). They were then sacrificed approximately two hours after FST for plasma and tissue analysis.

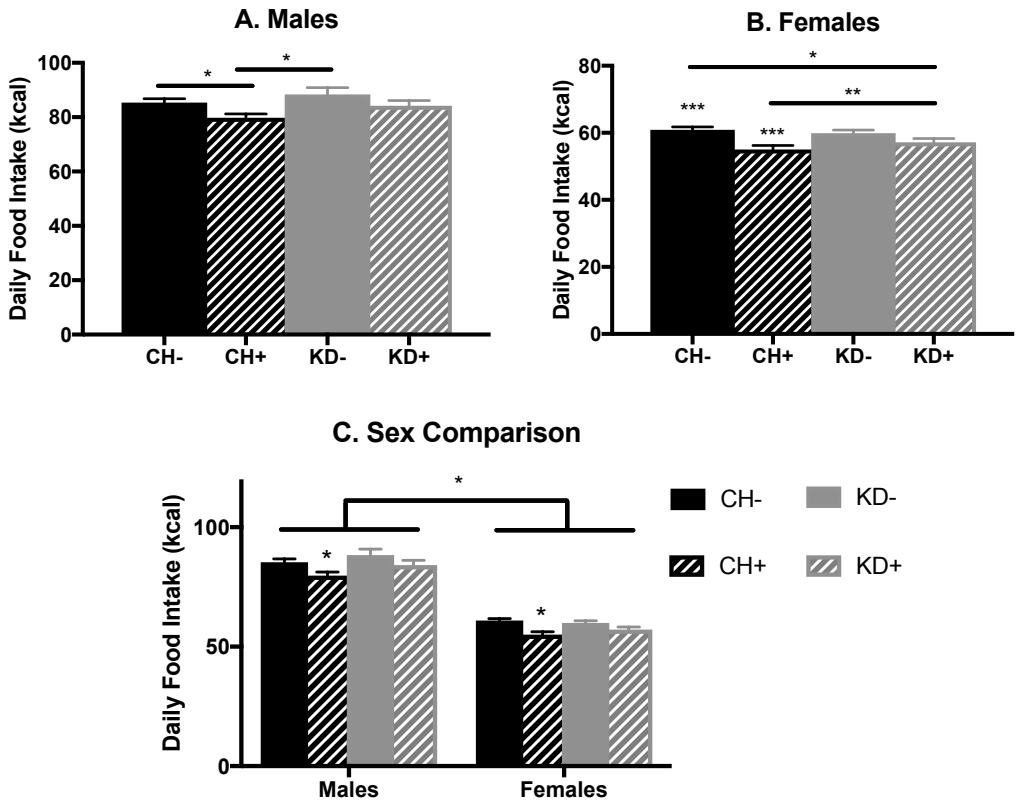

Figure 2. CMS significantly decreased food intake of CH groups in both sexes and had a stronger effect on females. A. There was an overall effect of treatment ($F(2.412, 57.89) = 5.597$, $p = 0.0031$). Multiple comparison tests reveal CH+ males had lower food intake than CH- (*$p = 0.0187$), and KD- (*$p = 0.0136$). B. There was also an overall effect on females ($F(2.909, 64) = 9.836$, ****$p < 0.0001$). Multiple comparison test also found that CH+ had lower food intake than CH- (***$p = 0.0002$), and KD- (**$p = 0.0023$). KD+ females also had lower food intake than CH- (*$p = 0.0135$), but not its own KD no stress control group ($p = 0.146$). C. Sex comparison found the decrease of food intake in CH+ groups to be consistent across sexes (*$p = 0.0001$), as well as females having lower food intake overall (*$p < 0.0001$).

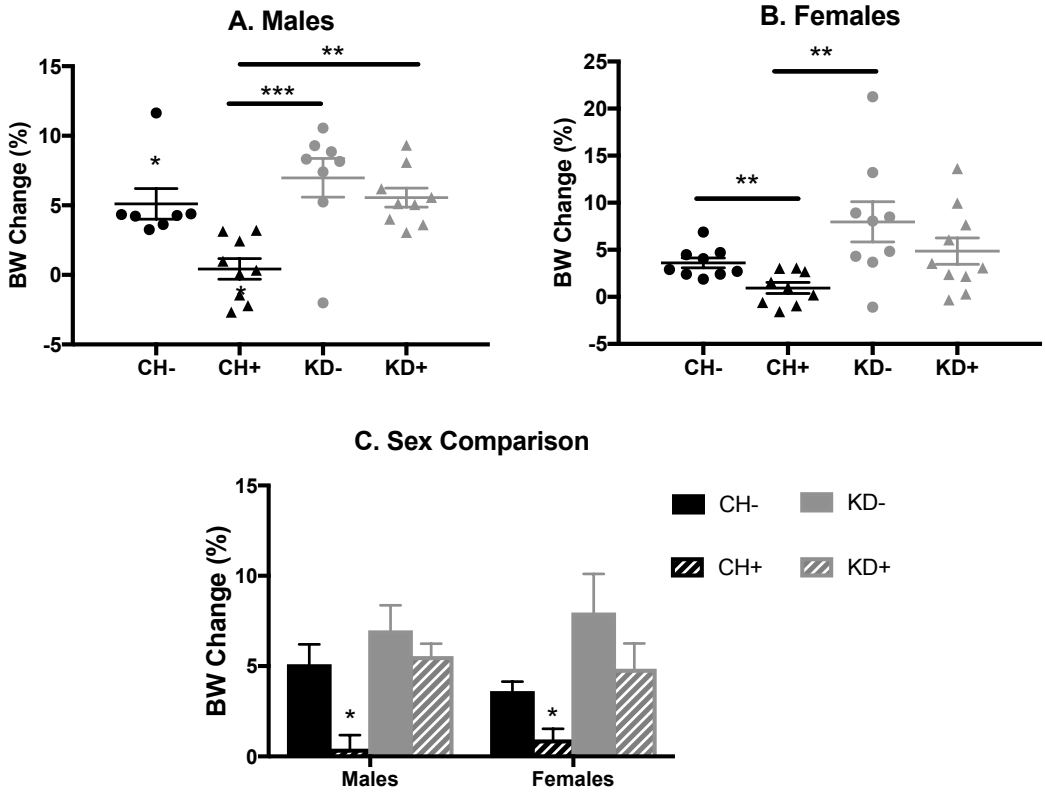

Figure 3. There are significant differences of stress and KD on body weight change in both sexes. A. Tukey's multiple comparison's test suggest CH+ males gained less weight than the CH control group and both KD groups (*p = 0.0003). KD- and KD+ groups were not different from each other (p = 0.7325). B. CH+ females gained less weight than the CH control group (p = 0039), and KD- group (p = 0.0048), but was not different than KD+ (0.1794). KD groups were not significantly different from each other (p = 0.3631). The standard deviations between groups was also significantly different (Bartlett's statistic = 18.92, p = 0.0003). C. When sexes are collapsed, there is a significant effect of treatment (*p < 0.0001), and no differences between sexes (p = 0.8364).

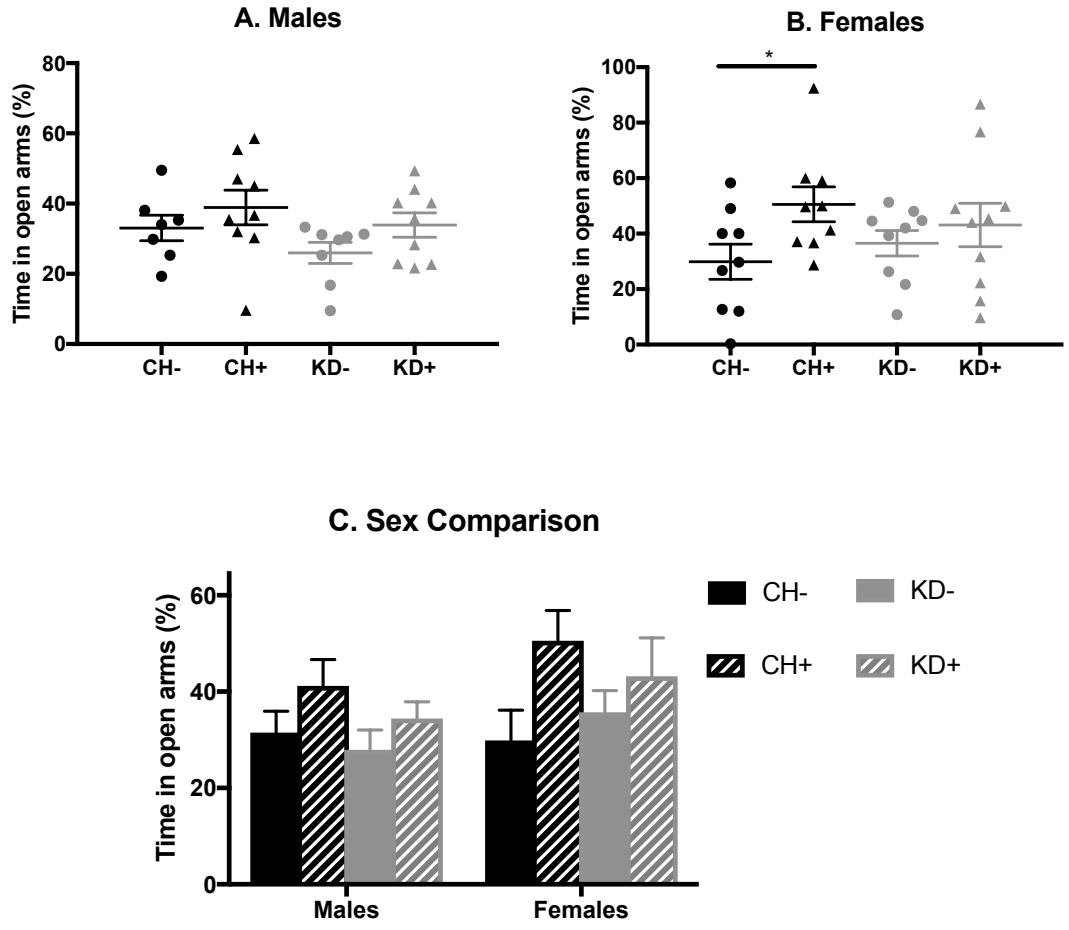

Figure 4. Time spent in open arms of Elevated Plus Maze (EPM). A. There were no significant effects of KD or CMS in males (Fig. A; $p = 0.1543$). B. CH+ females spent more time in open arms than CH- ($p = 0.339$). C. When sexes are collapsed, there is a significant effect of treatment ($*p = 0.0298$). This is likely driven by the effect found in females (Fig. 5B). Sex differences were not significant ($p = 0.0802$) and there were no interactions between sex and experimental condition (0.682).

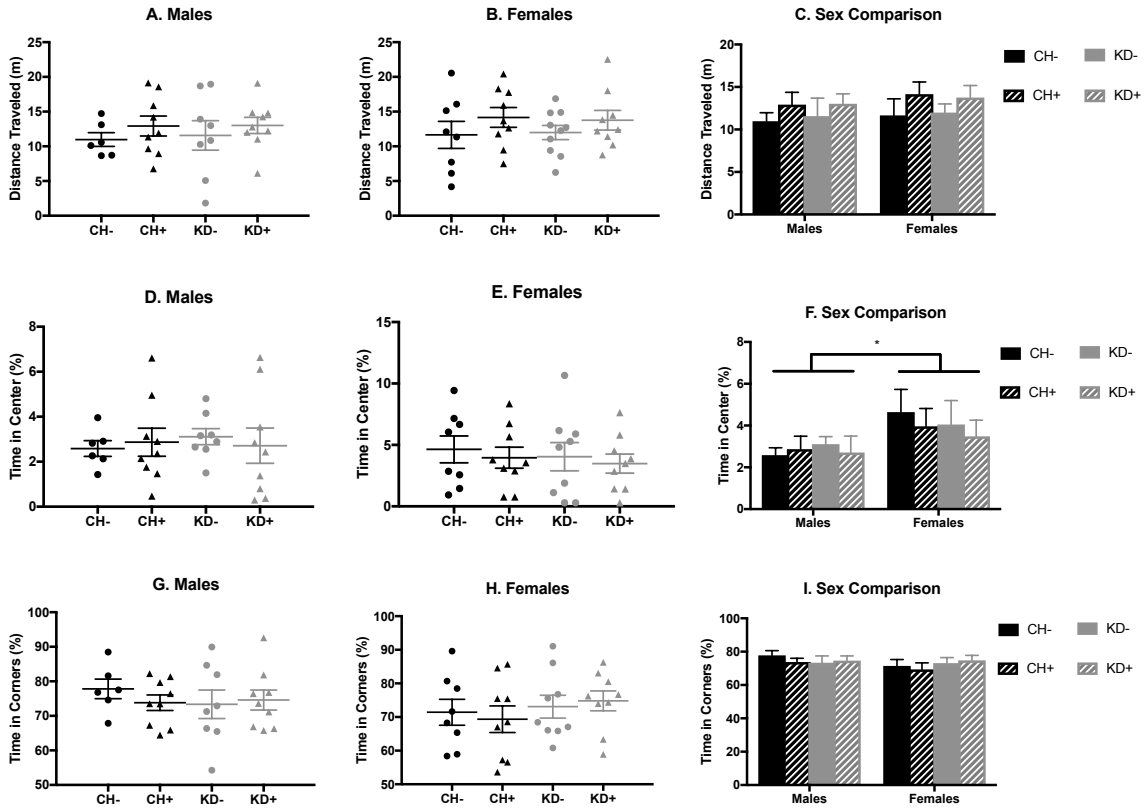

Figure 5. A-C: Distance traveled in Open Field (OF). Neither males (A) nor females (B) showed significant differences ($p = 0.7525$ and $p = 0.5325$ respectively). (C) There were no treatment effects ($p = 0.3569$) or differences between sexes ($p = 0.474$). D-F: Percent of time spent in center quadrants of OF. There were no differences in either sex (D: $p = 0.1512$; E: $p = 0.4668$). F. Two-way analysis found females spent more time in the center than males (*$p = 0.045$), with no effect of treatment ($p = 0.9215$). G-1: Time spent in corners of open field apparatus. Neither males (G) nor females (H) showed significant differences ($p = 0.7934$ and $p = 0.7241$ respectively). Two-way ANOVA (I) indicates were no treatment effects ($p = 0.7608$) or differences between sexes ($p = 0.2613$).

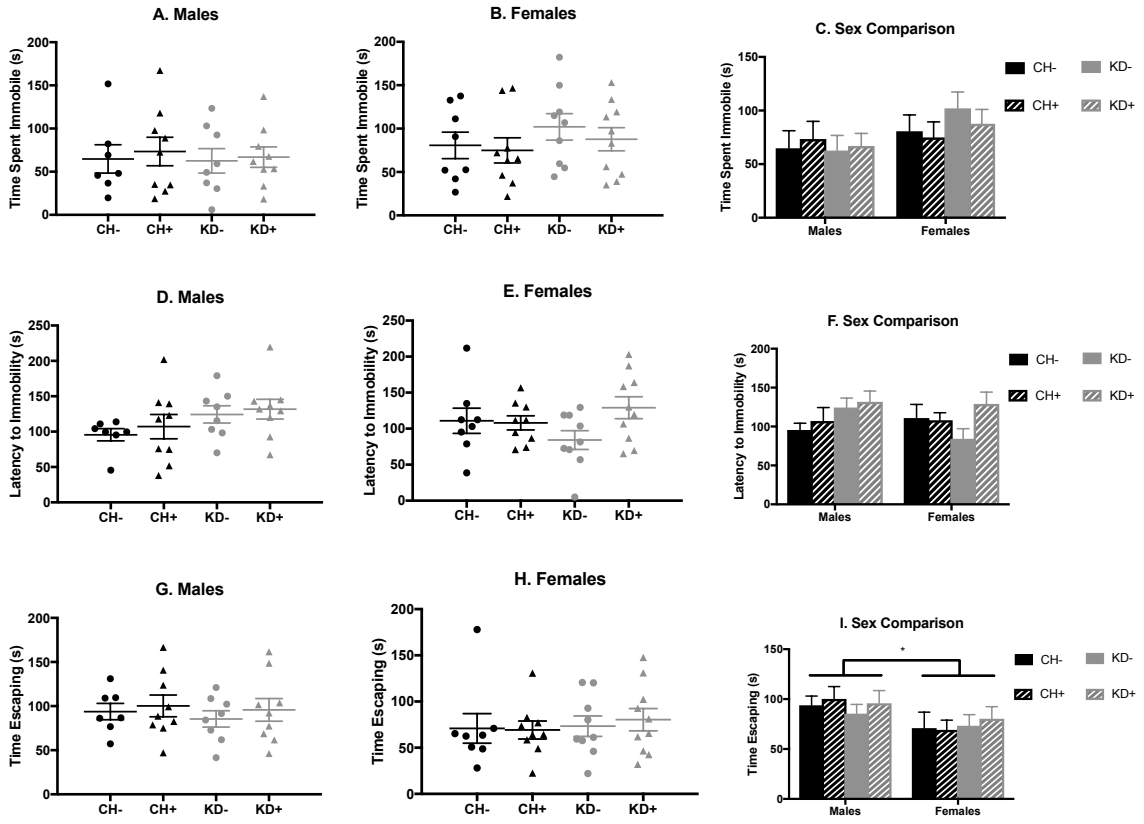

Figure ***6***. A-C: Time spent immobile in FST. Neither males (A) nor females (B) showed significant differences ($p = 0.9581$ and $p = 0.5942$, respectively). Two-way ANOVA (C) show no treatment effects ($p = 0.3569$) or sexes differences ($p = 0.474$). D-F: Time spent immobile in FST. Neither males (D) nor females (E) showed significant differences ($p = 0.2841$ and $p = 0.1689$, respectively). Two-way ANOVA (F) indicates were no treatment effects ($p = 0.5087$) or sex differences ($p = 0.1664$). G-I: Time spent attempting to escape. There were no differences in either sex (G: $p = 0.5554$; H: $p = 0.9158$). I. Two-way analysis found males spent more time attempting to escape than females ($*p = 0.019$), with no effect of treatment ($p = 0.8993$).

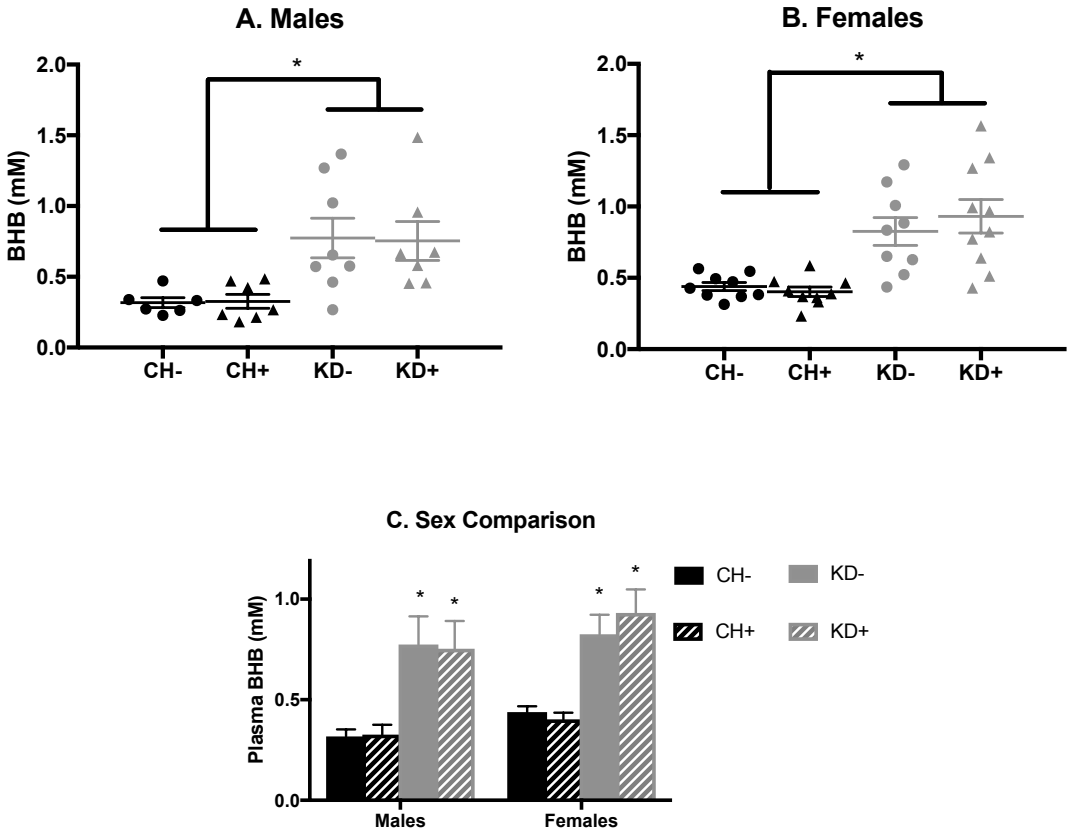

Figure 7. Terminal plasma Beta-Hydroxybutyrate (βHB). Males (A: $p = 0.0058$) and females (B: $p < 0.0001$) consuming KD had elevated βHB. The standard deviations for groups consuming KD were also greater than groups consuming CH (Bartlett's statistic for A: $p = 0.0031$, and B: $p < 0.0001$). When sexes are collapsed (C), there is a main effect of diet ($p < 0.0001$), and no differences between sexes ($p = 0.1197$).

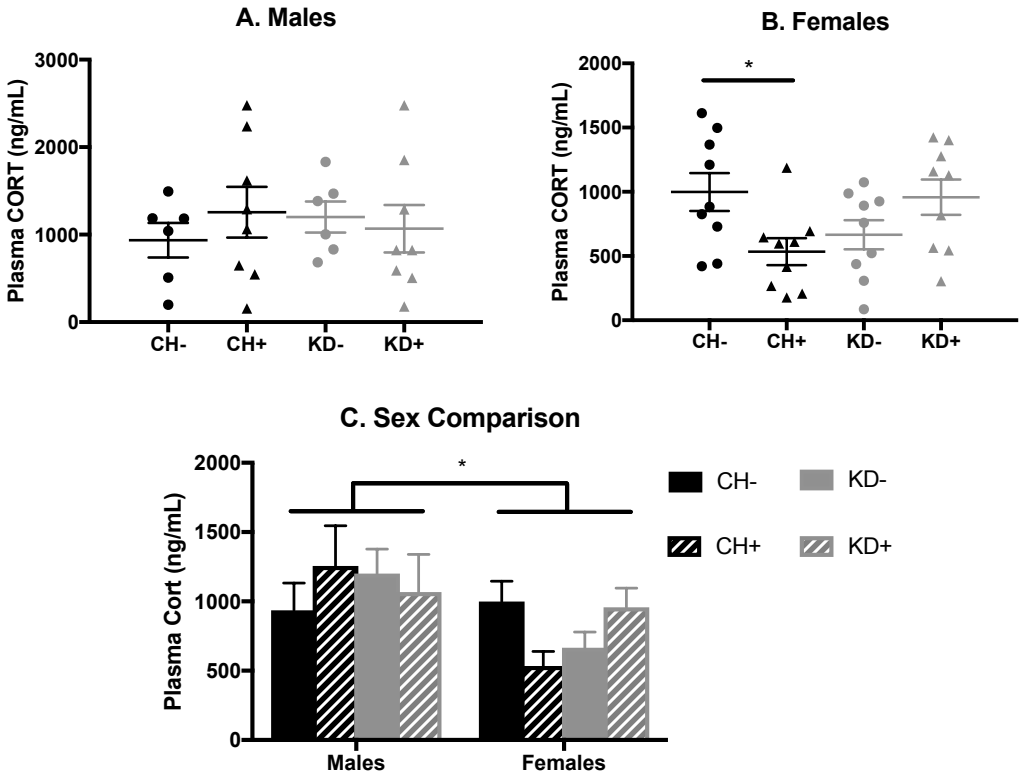

Figure 8. Terminal plasma Corticosterone (CORT). A. There were no differences between male experimental groups ($p = 0.8244$). B. Females on chow undergoing stress (CH+) had significantly lower CORT than the chow control females (CH-) ($p = 0.0383$). C. There were no overall treatment effects when males and females were collapsed ($p = 0.928$), however, males had overall significantly higher CORT than females ($p = 0.0181$).

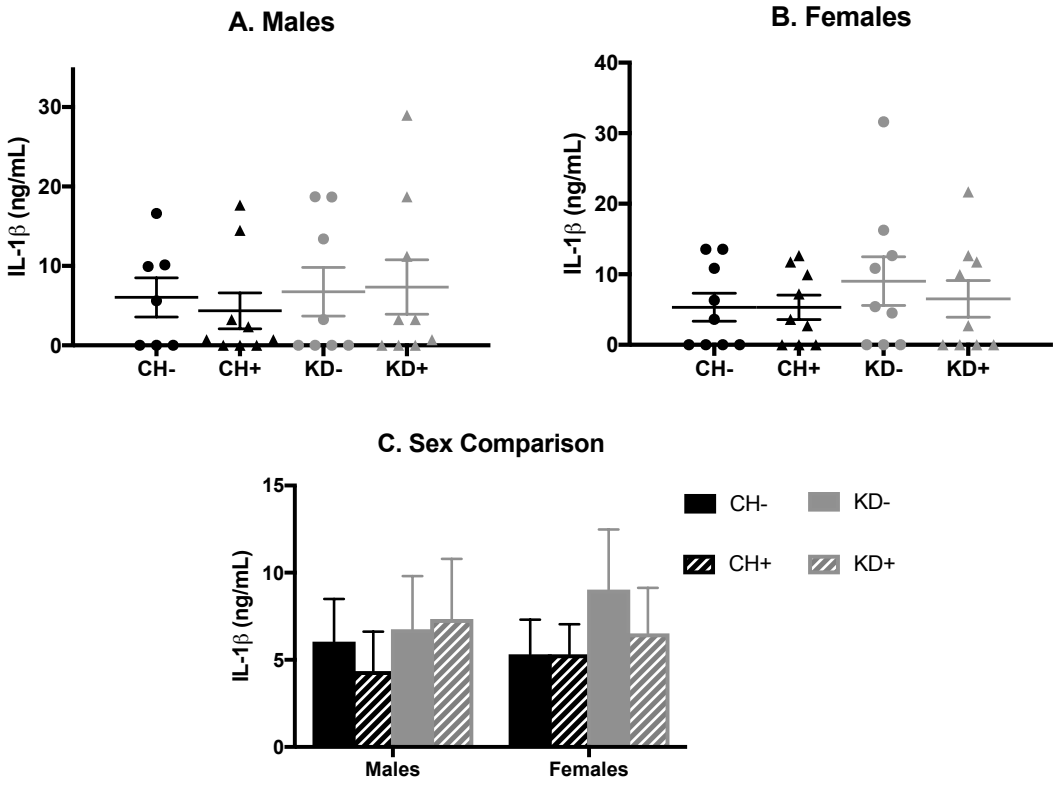

Figure 9. Final plasma IL-1β. Neither males (A) nor females (B) showed significant differences in terminal IL-1β levels ($p = 0.8833$ and $p = 0.6996$, respectively). Two-way ANOVA (C) show no treatment effects ($p = 0.6823$) or sexes differences ($p = 0.8257$).

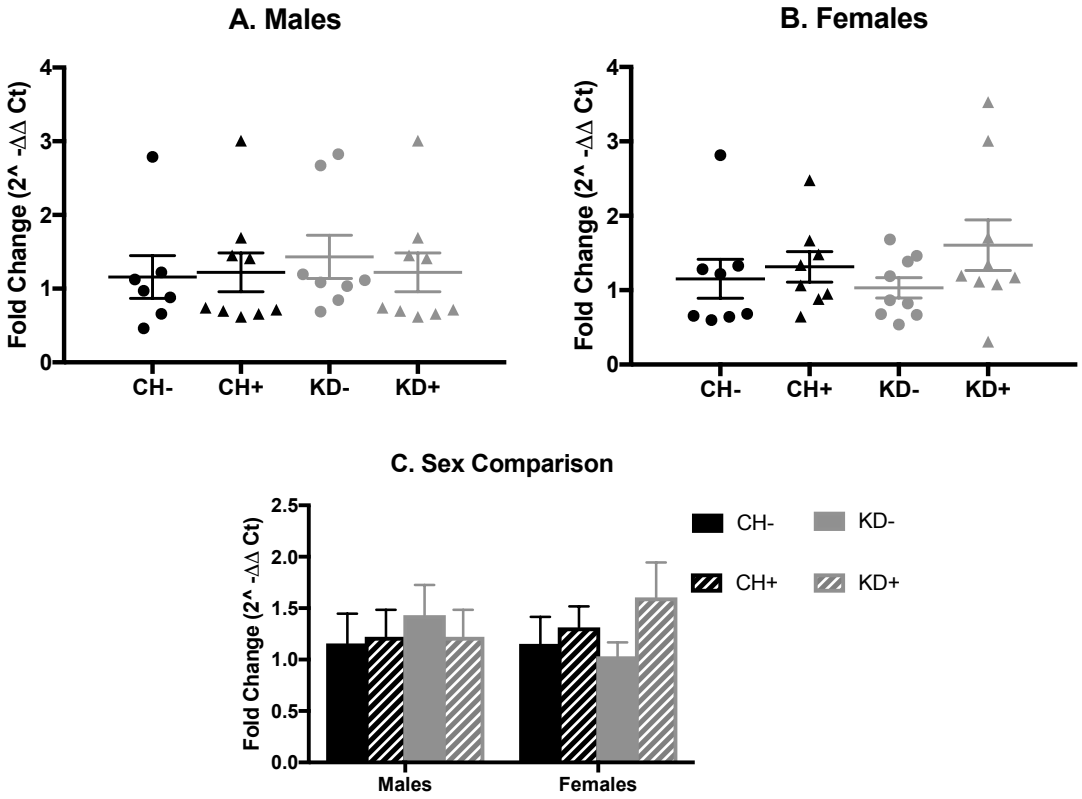

Figure 10. Hypothalamic CRH mRNA expression. Cycle thresholds (Ct) normalized to ß-actin, and ΔΔ Ct was normalized to chow control condition (CH-). There were no significant effects of diet or stress conditions in either males (A; $p = 0.9094$), or females (B; $p = 0.0945$). C. There were no differences between sexes ($p = 0.9275$), of overall treatment effects ($p = 0.7954$).

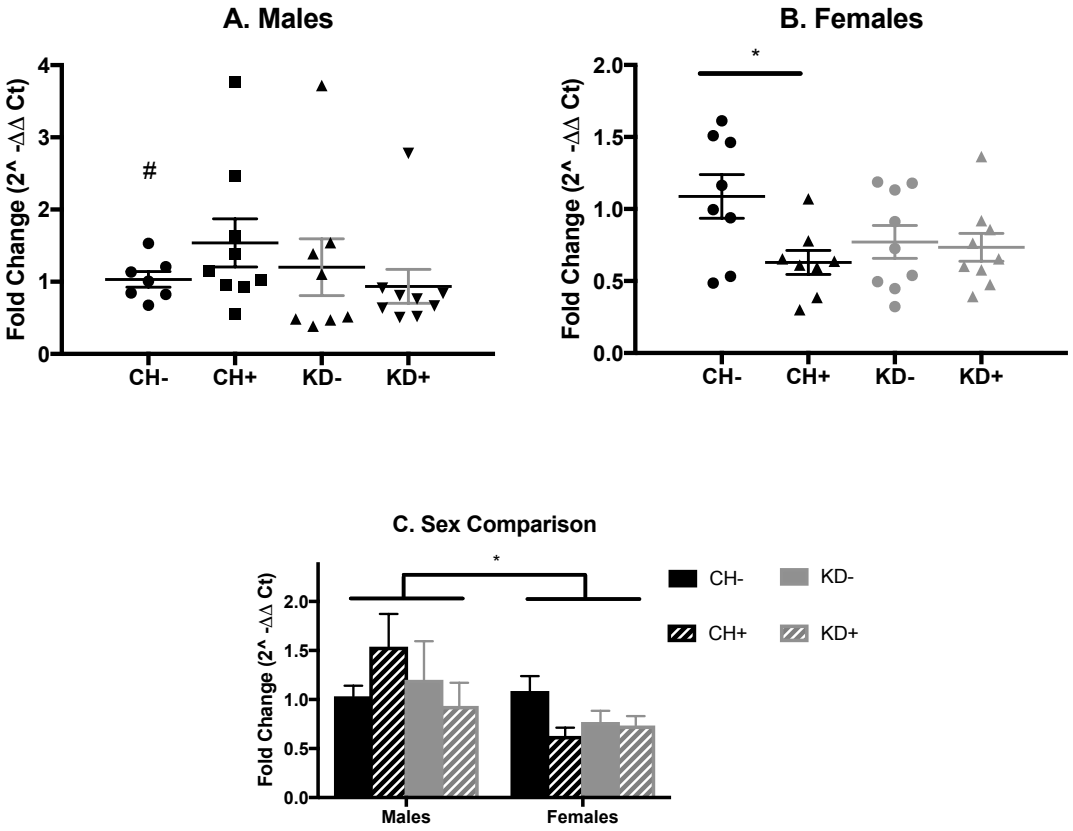

Figure 11. Hypothalamic NPY mRNA expression. Cycle thresholds (Ct) normalized to ß-actin, and ΔΔ Ct was normalized to chow control condition (CH-). A. There were no significant effects of diet or stress conditions in males ($F(3, 29) = 0.848$; $p = 0.9094$), however Bartlett's statistic was significant, indicating that the variation of CH- control is less than experimental groups (# Bartlett's test = 9.333; $p = 0.0252$). B. Although there was no main effect ($F(3, 30) = 2.854$); $p = 0.0538$), Tukey's multiple comparison test revealed females CH+ group had lower NPY expression than CH- (*$p = 0.0451$). C. Females had lower NPY expression than males (*$p = 0.0218$). This seems to be driven by differential effects of treatment since CH- groups have roughly the same expression levels.