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Sex-Dependent Effects of MC4R Genotype on HPA Axis Tone: Implications for Stress-Associated Cardiometabolic Disease

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Abstract

The melanocortin-4 receptor (MC4R) facilitates hypothalamic-pituitary-adrenocortical (HPA) axis responses to acute stress in male rodents and is a well-known to regulator of energy balance. Mutations in the MC4R is the most common monogenic cause of obesity in humans and has been associated with sex-specific effects, but whether stress regulation by the MC4R is sex-dependent, and whether the MC4R facilitates HPA responses to chronic stress, is unknown. We hypothesized that MC4R-signaling contributes to HPA axis dysregulation and metabolic pathophysiology following chronic stress exposure. We measured changes in energy balance, HPA axis tone, and vascular remodeling during chronic variable stress (CVS) in male and female rats with MC4R loss-of-function. Rats were placed into three groups (n= 9-18/ genotype/ sex) and half of each group was subjected to CVS for 30 days or were non-stressed littermate controls. All rats underwent an acute restraint stress challenge on Day 30. Rats were euthanized on Day 31, adrenals collected for weight, and descending aortas fixed for morphological indices of vascular pathophysiology. We observed a marked interaction between *Mc4r* genotype and sex for basal HPA axis tone and acute stress responsivity. MC4R loss-of-function blunted both endpoints in males but exaggerated them in females. Contrary to our hypothesis, *Mc4r* genotype had no effect on either HPA axis responses or metabolic responses to chronic stress. Heightened stress reactivity of females with MC4R mutations suggests a possible mechanism for the sex-dependent effects associated with this mutation in humans and highlights how stress may differentially regulate metabolism in males and females.

LAY SUMMARY:

The hypothalamic melanocortin system is an important regulator of energy balance and stress responses. Here, we report a sex-difference in the stress reactivity of rats with a mutation in this

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COMPETING INTERESTS

The authors declare no competing interests.

system. Our findings highlight how stress may regulate metabolism differently in males and females, and may provide insight into sex-differences associated with this mutation in humans.

Keywords

melanocortin-4 receptor; sex differences; hypothalamic-pituitary-adrenal axis; chronic variable stress; stress responsivity; cardiometabolic disease

1. INTRODUCTION

In response to an acute threat to homeostasis or well-being, the hypothalamic-pituitary-adrenocortical (HPA) axis is engaged. The resulting increase in circulating glucocorticoids acts to mobilize fuels, thereby facilitating appropriate behavioral and/or physiological responses to the stressor (Ulrich-Lai & Herman, 2009). In agreement with this, neuroendocrine circuits underlying stress integration and systemic fuel homeostasis are substantially intertwined (Ulrich-Lai & Ryan, 2014). Pathways involved in the regulation of energy balance also modulate stress responses, and vice versa (Asakawa et al., 2001; J. Chuang et al., 2011; Goodson et al., 2017; Kuo et al., 2007; Karen K. Ryan et al., 2012; K.K. Ryan et al., 2018). For example, the hypothalamic melanocortin system plays a canonical role in the neuroendocrine control of energy balance (Haskell-Luevano & Monck, 2001; Marsh et al., 1999; J. Rossi et al., 2011; Vaisse, Clement, B., & Froguel, 1998; van Dijk, Thiele, Seeley, Woods, & Bernstein, 1997; Wilson, Ollmann, & Barsh, 1999) and it also modulates behavioral and physiological responses to acute stress (Chaki, Ogawa, Toda, Funakoshi, & Okuyama, 2003; J.-C. Chuang et al., 2010; Kheirabad et al., 2015; J. Liu, Garza, Li, & Lu, 2013; J. Liu et al., 2007; Park et al., 2016; Karen K. Ryan et al., 2014; Serova, Laukova, Alaluf, & Sabban, 2013). Activation of the melanocortin-4 receptor (MC4R) by its endogenous agonist α-melanin stimulating hormone (αMSH) as well as its pharmacological agonists reduces caloric intake, increases energy expenditure, and alters peripheral glucose and lipid metabolism (Huszar et al., 1997; Nogueiras et al., 2007; Obici et al., 2001; Karen K. Ryan, Woods, & Seeley, 2012). In addition, MC4R-signaling increases anxiety-like behavior (De Barioglio, Lezcano, & Celis, 1991; Gonzalez, Vaziri, & Wilson, 1996; J. Liu et al., 2013) and increases circulating corticosterone (Dhillo et al., 2002; Lu, Barsh, Akil, & Watson, 2003) in male rats and mice. Conversely, pharmacological blockade or genetic loss of MC4R function increases caloric intake, decreases energy expenditure (Fan, Boston, Kesterson, Hruby, & Cone, 1997; M. Rossi et al., 1998), buffers anxiety-like behaviors (Chaki et al., 2003; Kokare, Dandekar, Singru, Gupta, & Subhedar, 2010; J. Liu et al., 2007; Serova et al., 2013), and blunts HPA axis activation in response to an acute stress challenge, in male rodents (J. Liu et al., 2013; Karen K. Ryan et al., 2014).

Although the HPA axis response to an acutely stressful challenge may be advantageous in the short term, repeated and or persistent stress exposure is associated with increased risk of cardiometabolic disease (McEwen & Stellar, 1993; Steptoe & Kivimäki, 2012). Chronic exposure to elevated glucocorticoids is thought to be an important underlying mechanism (Scholz, Sprague, & Kernohan, 1957; Walker, 2007), since pharmacological glucocorticoid treatment similarly increases risk (M. Handa, Kondo, Suzuki, & Saruta, 1984; Iijima &

Malik, 1988; Krakoff, 1988; Walker, 2007). Therefore, interventions that blunt HPA responses to stress, and/or those that disrupt critical interactions between stress-regulatory and fuel-regulatory pathways, are expected to buffer metabolic pathophysiology associated with chronic stress.

Because activating the hypothalamic melanocortin system is thought to facilitate HPA axis responses to acute stress, we hypothesized that MC4R signaling also contributes to HPA axis dysregulation and metabolic consequences pursuant to chronic stress. Specifically, we predicted that blocking MC4R function during chronic stress would protect against indices of stress-induced HPA axis dysregulation, weight loss, and vascular pathophysiology. We tested this by applying the chronic variable stress paradigm in a well-characterized rat model for genetic MC4R loss-of-function (Almundarij et al., 2016; Mul et al., 2012; Karen K. Ryan et al., 2014). Notably, previous work regarding the role of MC4R on HPA axis regulation has been performed almost exclusively in male subjects. This is especially problematic in light of prominent sex differences in HPA axis responsivity and the strong female bias toward stress-related psychopathologies including metabolic disease (Critchlow, Liebelt, Bar-Sela, Mountcastle, & Lipscomb, 1963; Earls, 1987; R. J. Handa, Burgess, Kerr, & O'Keefe, 1994; Murphy & Loria, 2017; Matia B. Solomon et al., 2015). Understanding sex-specific effects of the MC4R may help inform treatment of both metabolic and stress-related psychological diseases. Therefore, we included both males and females in our experiments.

2. MATERIALS AND METHODS

2.1 Rats

Age-matched male and female rats were generated in-house from breeding pairs heterozygous for a loss-of-function mutation in *Mc4r* (Mul et al., 2012). This mutation introduces a stop codon (K314X) in *Mc4r*, resulting in impaired membrane-binding and subsequent nonfunctionality of the receptor. We have previously demonstrated functional loss of MC4R signaling with this mutation. For example, loss of *in vivo* MC4R function was confirmed by intracerebroventricular administration of agouti-related protein (AgRP), an MC4R inverse agonist, or Melanotan-II, an MC4R agonist, which altered feeding behavior in wild-type rats but did not in homozygous mutant rats (Mul et al., 2012). Genotypes were identified by PCR with forward primers designed against wildtype or mutant *Mc4r* and a common reverse primer (WT-forward: GTCAA GAACT GAGGA AAACC TTCA; mutant-forward: GTCAA GAACT GAGGA AAACC TTCT; reverse: CTTTT TCCCC ACGTC AAAAG TC). Rats were homozygous (HOM, male $n = 13$, female $n = 10$), heterozygous (HET, male $n = 18$, female $n = 18$) or wild-type (WT, male $n = 12$, female $n = 14$) for the mutation. All rats were singly housed in an AAALAC-approved, temperature-(20°C - 22°C) and humidity-controlled facility with a 12-12 light-dark cycle (lights on at 0700 h and off at 1900 h) and allowed *ad libitum* access to standard rat chow (Formulab #5008 LabDiet, MO) and water unless otherwise noted. All animal experiments were approved by the IACUC of the University of California, Davis.

2.2 Chronic variable stress

8-week-old male and female rats were divided into weight-matched (within genotype) chronic variable stress (CVS) and non-stressed control (CON) groups. Rats in the CVS group were subjected to twice daily stressors delivered in randomized order for four weeks. Stressors included crowding (6-8 rats in a standard rat cage for 2h), white noise (~75 dB for 1h), shaker platform (100 rpm for 1h), strobe light (~250 flashes/min for 1h), hypoxia (8% O_2, 92% N_2 for 30 min), no bedding (overnight), and 45° cage tilt (overnight). For the crowding stressor, animals receiving CVS treatment were mixed together within each sex, with particular animals crowded together randomized each time this stressor was used. Sample sizes for each group are detailed in Table 1. The CVS protocol is outlined in Table 2.

2.3 HPA axis testing

On day 17 of CVS treatment, we collected a basal 'unstressed' tail-clip blood sample at 1500h, near the peak of the diurnal rhythm. Briefly, the last 0.5 mm of the tail was removed, and blood was collected into chilled, EDTA-coated tubes. Importantly, samples were collected within 3 minutes of first disturbing the rat's home cage. This timeframe provides a 'snapshot' of unstressed hormones, since it is completed prior to any increases in corticosterone that occur in response to handling the animal (Vahl et al., 2005). On day 30 of CVS treatment, we collected a second, 'unstressed' blood sample at 0900 h, near the nadir of the diurnal rhythm from both CVS and CON rats. Rats were quickly placed into well-ventilated restraint tubes, the tail clipped, and blood collected as above. Rats remained in the restraint tube for 30 minutes and additional samples were collected 15, 30, 60 and 120 minutes after the onset of restraint. In both cases blood was centrifuged at 900 x *g* for 20 min at 4°C. Plasma was stored at −80°C until later analysis. Plasma corticosterone was measured using the Corticosterone Double Antibody RIA Kit (MP Biomedicals, Santa Ana, CA) according to manufacturer's instructions.

2.4 Adrenal weights

Left and right adrenals from each animal were collected at sacrifice (1300 h, on day 31 of CVS treatment), cleaned, and weighed as an indirect index of chronic HPA activation.

2.5 Aortic histology

Descending aortas were collected and cleaned at sacrifice, fixed in 10% neutral buffered formalin and stored at 4°C. A 2-3 mm piece of the descending aorta was later collected 15-18 mm from the diaphragm, embedded in paraffin, sectioned (5 μm) and stained with Masson's trichrome (Research Histology Laboratory, UC Davis). Luminal and medial areas, luminal diameter, and tunica media thickness, were quantified using ImageJ, as in (Goodson et al., 2017).

2.6 Statistical analysis

Data were analyzed by the appropriate mixed-model 2-factor or 3-factor ANOVA, as noted, with Tukey's HSD post-hoc analysis. In all cases, $\alpha = 0.05$.

3. RESULTS

3.1 Basal HPA axis activity

We and others have previously demonstrated that MC4R loss-of-function abrogates acute responses to stress in male rodents (Chaki et al., 2003; J. Liu et al., 2013; Karen K. Ryan et al., 2014; Serova et al., 2013). Therefore, we predicted that MC4R loss-of-function would also protect against increased basal (non-stress) HPA axis activity typically observed during CVS.

Consistent with the known effect of CVS to increase basal HPA axis tone (Ulrich-Lai & Herman, 2009), we observed a main effect of *treatment* ($F(1,61)=7.26$, $p<0.01$), such that CVS-treated rats exhibited greater AM corticosterone compared to CON. Consistent with known sex differences in the HPA axis (R. J. Handa et al., 1994; Kudielka & Kirschbaum, 2004), we observed a main effect of *sex* ($F(1,61)=26.95$, $p<0.001$), such that females exhibited greater AM corticosterone compared to males. However, contrary to expectation, there was no effect of *genotype* and no significant interactions (3-way ANOVA, Figure 1A). For the samples collected in the PM (3-way ANOVA, Figure 1B), near the peak of the diurnal rhythm, we observed a significant *genotype x sex* interaction ($F(2,61)=3.96$, $p<0.05$). Within males, loss of MC4R function decreased plasma corticosterone in a gene dose-dependent manner, though it did not abrogate any increase observed with CVS (i.e., no *genotype x treatment* interaction). Unexpectedly, loss of MC4R function among females *increased* plasma corticosterone in a gene dose-dependent manner. Again, this occurred regardless of treatment. Thus, we conclude that loss of MC4R function has opposing effects on basal HPA axis tone in males vs females, and this is independent of exposure to CVS.

3.2 Adrenal weights

In agreement with the differences in basal HPA axis tone, we observed main effects of *sex* and *treatment* on adrenal weight. For raw, uncorrected values there was a significant main effect of *treatment* ($F(1,73)=38.5$, $p<0.001$) such that CVS induced adrenal hypertrophy relative to CON. As expected (Critchlow et al., 1963), female adrenals were heavier than males' ($F(1,73)=19.67$, $p(sex)< 0.001$). We also observed a main effect of *genotype* ($F(2,73)=3.51$, $p<0.05$) such that adrenals from HOM rats were heavier than those of HET rats (Tukey's post hoc, $p < 0.05$; 3-way ANOVA, Figure 2A). Because sex, treatment, and genotype additionally influence body weight (see Figure 4C), we also analyzed body weight-adjusted values (3-way ANOVA, Figure 2B). We again observed a significant main effect of *treatment* ($F(1,73)=59.62$, $p<0.001$) such that CVS induced adrenal hypertrophy compared to CON. In this analysis, there was also a significant interaction between *genotype* and *sex* ($F(2,73)=4.06$, $p<0.05$). Within each genotype, the corrected adrenal weights from females were heavier than males (Tukey's posthoc, $p<0.001$), and within each sex, there was a gene dose-dependent effect of MC4R loss-of-function to decrease the corrected adrenal weight (Tukey's post hoc, $p<0.001$) — likely as a simple consequence of MC4R-dependent changes in body weight. Taken together, we conclude that CVS induces adrenal hypertrophy, and this occurs independent of *Mc4r* genotype.

3.3 HPA axis response to acute restraint

We challenged both CON and CVS-treated male and female rats with a novel, acute restraint stress on day 30 of CVS. In agreement with our previous work (Karen K. Ryan et al., 2014), the acute restraint-induced increase in circulating corticosterone was blunted with MC4R loss-of-function in CON males (Figure 3A, 2-way RM ANOVA, $F(8,56)=2.88$, p(*genotype x time*)<0.01, Tukey's posthoc). Lower corticosterone levels in the first 30 minutes of HOM males suggest a reduced HPA axis activation. However, contrary to our expectation, this was less apparent among CVS males (Figure 3B, 2-way ANOVA, $F(4,68)=64.27$, p(*time*)<0.001). Conversely, and contrary to our expectation, loss-of MC4R function was associated with an *exaggerated* stress-evoked increase in circulating corticosterone among both CON females (Figure 3C, 2-way RM ANOVA, $F(8,56)=2.73$, p(*genotype x time*) < 0.05, Tukey's post hoc), and CVS females (Figure 3D, 2-way RM ANOVA, $F(8,64)=8.15$, p(*genotype x time*)<0.001, suggesting impaired negative feedback on the HPA axis in females. An integrated analysis of the areas under the curve for all rats (AUC, Figure 3D) revealed an interaction between *genotype* and *sex* (3-way ANOVA, $F(2,61)=9.36$, p<0.001) such that MC4R loss-of-function blunted the corticosterone response among males and exaggerated the corticosterone response among females in a gene dose-dependent manner. Thus, we conclude that MC4R has opposing effects on the restraint-evoked corticosterone response in males vs females, independent of exposure to CVS.

3.4 Energy balance

Because MC4R plays a key role in the regulation of energy balance, and because previous work by ourselves and others suggests MC4R signaling facilitates stress responsiveness, we predicted that MC4R loss of function would abrogate the weight loss and decreased food intake typically observed during CVS.

Consistent with previous findings (Goodson et al., 2017), we observed a main effect of CVS to reduce both chow intake (Figure 4A, 3-way ANOVA, $F(1,73)=7.51$, p(*treatment*)<0.01), weight change (Figure 4B, 3-way ANOVA, $F(1,73)=4.71$, p(*treatment*)<0.05), and absolute body weight (Figure 4C, 3-way ANOVA, $F(1,73)=4.53$, p(*treatment*)<0.05). As expected, there was a significant main effect of *sex* such that males were heavier (3-way ANOVA, $F(1,73)=278.35$, p(*sex*)<0.001) and ate more (3-way ANOVA, $F(1,73)=126.27$, p(*sex*)<0.001) than females. Lastly, as expected, there was also a significant gene dose-dependent effect of *Mc4r genotype* (HOM > HET > WT) on both body weight (3-way ANOVA, $F(2,73)=156.07$, p(*genotype*)<0.001) and chow intake (3-way ANOVA, $F(2,73)=78.20$, p(*genotype*)<0.001). Contrary to our prediction, there was no significant interaction between genotype and stress.

3.5 Vascular pathology

Chronic exposure to either stress or exogenous glucocorticoids increases risk of hypertension and vascular stiffening in both rodents and humans (Goodson et al., 2017; M. Handa et al., 1984; Iijima & Malik, 1988; McEwen & Stellar, 1993; Scholz et al., 1957; Steptoe & Kivimäki, 2012; Walker, 2007). Therefore, we predicted that CVS-treatment would increase related anatomical markers, for example by increasing the intima-medial thickness and/or decreasing the luminal area. Given the findings in *3.1* and *3.3* (above), we

further predicted an interacting effect of sex and genotype on vascular morphology such that MC4R loss-of-function protects against indices of vascular remodeling among males and exaggerates this response among females. Because there are no reports of MC4R expression in the vasculature, and because we likewise observed only very low expression of *Mc4r* mRNA in the aortas (data not shown), we expect any potential effect of genotype occurs indirectly.

As expected, we observed a main effect of CVS-treatment to increase tunica media thickness (Figure 5A, 3-way ANOVA, $F(1,72)=7.98$, $p<0.01$). Moreover, there was a main effect of both *sex* ($F(1,72)=9.16$, $p<0.01$) and *genotype* ($F(2,72)=3.32$, $p<0.05$) such that larger rats, e.g. males and HOMs, exhibited larger tunica media thickness. This is consistent with previous findings that tunica media thickness increases with body weight in both rodents (Ma et al., 2010) and human populations (Woo et al., 2004; Wunsch, de Sousa, Toschke, & Reinehr, 2006). There were no significant interactions. For luminal area (Figure 5B), there was again a main effect of both *sex* (3-way ANOVA, $F(1,72)=60.12$, $p<0.001$) and *genotype* ($F(2,72)=6.81$, $p<0.01$) such that larger rats exhibited a greater luminal area. There was no significant effect of *treatment* and no significant interactions. Finally, when we analyzed media thickness as a ratio to lumen diameter (Pitol et al., 2015; Xiao et al., 2016; Zhu et al., 2018) (Figure 5C), and in agreement with increased risk of cardiovascular morbidity upon exposure to chronic stress (Black & Garbutt, 2002; Chumaeva et al., 2009; Grippo & Johnson, 2009; Lehman, Taylor, Kiefe, & Seeman, 2009; Steptoe & Kivimäki, 2012), we observed a main effect of CVS-treatment to increase this ratio (3-way ANOVA, $F(1,72)=9.82$, $p<0.01$). Contrary to expectation, however, there was no significant difference of *genotype* and no significant interactions. Representative images of aortas are shown in Figure 5D.

4. DISCUSSION

The neural circuitries controlling brain stress integration and systemic metabolism exhibit significant functional overlap (Ulrich-Lai & Ryan, 2014). In agreement with this, metabolic diseases like obesity and diabetes have a high incidence of co-morbidity with stress-associated psychological disorders like anxiety and depression (Anderson, Freedland, Clouse, & Lustman, 2001; Faith, Matz, & Jorge, 2002; Gariepy, Nitka, & Schmitz, 2010; Stunkard, Faith, & Allison, 2003; Ulrich-Lai & Ryan, 2014). Accordingly, interventions targeting these critical neuroanatomical connections may represent a promising strategy for preventing chronic stress-induced metabolic and cardiovascular consequences. However, with regard to the hypothalamic melanocortin system, the present findings do not support this possibility. Consistent with our previous report (Karen K. Ryan et al., 2014), we found that melanocortin 4 receptor (MC4R) loss-of-function significantly diminished the acute stress-induced rise in plasma corticosterone, in naïve male rats (Figure 3). Nevertheless, *Mc4r* genotype did not modulate responses to *chronic* stress. Specifically, MC4R loss-of-function did not protect against chronic stress-associated increases in basal HPA axis tone and adrenal hypertrophy (Figures 1 and 2), nor against chronic stress-induced anorexia and weight loss (Figure 4). Lastly, loss of MC4R function did not reduce chronic stress-induced vascular remodeling (Figure 5).

The MC4R is a G protein-coupled receptor expressed widely in the adult central nervous system (Cone, 2005; Tao, 2010). Its activity is coordinated by opposing actions of its endogenous agonist, αMSH, and its endogenous antagonist, agouti-related protein (AgRP) (Fong et al., 1997; Ollmann et al., 1997; Shutter et al., 1997). In addition, the receptor has intrinsic constitutive activity on which AgRP can act as an inverse agonist (Srinivasan et al., 2004). αMSH producing neurons in the arcuate nucleus of the hypothalamus are activated by restraint stress (J. Liu et al., 2007) and provide melanocortinergic input to MC4R-expressing neurons in key stress and feeding-regulatory brain regions including the paraventricular nucleus of the hypothalamus (PVN), the medial amygdala (MeA), and the nucleus accumbens (NAc) (Balthasar, 2006; Wang et al., 2015). Activation of MC4Rs by αMSH or pharmacological agonists acutely stimulates the HPA axis in male rats and mice (J. Liu et al., 2013) and induces weight loss by reducing caloric intake and increasing energy expenditure in both sexes (Fan et al., 1997; Hamilton & Doods, 2002). Conversely, loss of MC4R function (Karen K. Ryan et al., 2014) oor its pharmacological blockade (Kokare et al., 2010; J. Liu et al., 2007; Serova et al., 2013) blunts acute restraint stress-induced corticosterone elevation in male rats and mice. MC4R loss-of-function also induces weight gain by increasing caloric intake and decreasing energy expenditure in both sexes (Huszar et al., 1997). Our results (Figure 4) are consistent with these reports.

Despite its clear influence on both acute stress responses and energy balance, the present findings indicate that MC4R-signaling does not play a major role in *chronic* stress-induced facilitation of basal HPA-axis tone, anorexia or weight loss. Notably, these outcomes are consistent with previous work identifying MC4Rs in the MeA as a critical mediator for the acute HPA axis response to central melanocortinergic tone in male rats (J. Liu et al., 2013), and supporting that lesions of the MeA blunt neuroendocrine responses to acute, but not chronic, stress in male rats (M. B. Solomon, Jones, Packard, & Herman, 2010). Our findings are inconsistent, however, with another previous study, which concluded that MC4Rs in the NAc are necessary for chronic-stress induced weight loss. Specifically, Lim and colleagues (Lim, Huang, Grueter, Rothwell, & Malenka, 2012) found that male mice treated with AAV-GFP locally in the NAc, and later subjected to chronic restraint stress, lost weight compared to untreated, non-stressed controls, whereas mice treated with AAV-MC4R-shRNA to knockdown *Mc4r* expression in the NAc did not lose weight during chronic restraint stress, again in comparison to the untreated and unstressed wildtype controls. The inconsistent conclusions drawn between this study and ours may result from experimental differences, including differences in model species (rat vs mouse, or whole-body loss-of-function vs virally-directed gene knockdown), differences in the chronic stress model (heterotypic vs homotypic stressors), or from other details of the experimental design (e.g., use of a full-factorial model vs not). Further study will be necessary to resolve the discrepancy.

Our findings are in line with previous reports by ourselves (Goodson et al., 2017) and others (Neves et al., 2009) that chronic stress induces vascular remodeling in male rodents, and with the many epidemiological reports identifying psychological stress as a significant risk factor for cardiovascular disease in human populations (reviewed by (Rozanski, Blumenthal, & Kaplan, 1999; K.K. Ryan, 2014; Steptoe & Kivimäki, 2012)). Specifically, we report that chronic stress increases tunica media thickness, and increases the ratio of tunica media thickness: lumen diameter, in chronically-stressed male and female rats. This is consistent

with known effects of stress to induce hypertension and vascular stiffening (Heagerty, Aalkjaer, Bund, Korsgaard, & Mulvany, 1993), and was independent of *Mc4r* genotype. Although vascular remodeling may be induced in part by chronic glucocorticoid exposure (Fishel et al., 1995) during prolonged or repeated stress exposure, we found no association between basal or stress-induced corticosterone and vascular morphology. That is, although *Mc4r* genotype had opposing effects on HPA axis activity in males and females (discussed below), both sexes exhibited stress-induced vascular remodeling, suggesting glucocorticoid exposure was not the primary mechanism underlying vascular remodeling in this rat chronic variable stress model.

Perhaps the most striking outcome we observed was the marked interaction, between *Mc4r* genotype and sex, for basal HPA axis tone and acute stress responsivity. First, in line with well-documented sex differences in basal and stress-induced HPA axis activity (Goel, Workman, Lee, Innala, & Viau, 2014; R. J. Handa et al., 1994; Matia B Solomon, Jankord, Flak, & Herman, 2011), female rats exhibited greater basal corticosterone when measured at both the nadir and peak of its circadian rhythm, greater restraint-induced increases in corticosterone, and greater chronic-stress induced adrenal hypertrophy, compared to male littermates. Importantly, we (unexpectedly) also observed opposing effects of *Mc4r* genotype to modulate these endpoints depending on sex. In males, MC4R loss-of-function decreased basal corticosterone and blunted the response to acute restraint, in a gene dose-dependent manner. Exactly the opposite effect was observed among female littermates. In female rats, MC4R loss-of-function exhibited greater basal corticosterone and exaggerated responses to acute restraint, also in a dose-dependent manner, resulting in a significant *genotype* x *sex* interaction in the 3-way ANOVA.

The opposing effects of MC4R function on stress reactivity in males and females may provide mechanistic insight regarding the sex-dependent associations between *Mc4r* genotype and eating behavior reported in human populations. In humans, heterozygous *Mc4r* loss-of-function mutations are the most common genetic cause of obesity (Farooqi & O'Rahilly, 2004), and sex has repeatedly been shown to modify the association between *Mc4r* genotype and feeding behavior together with other markers of obesity (Cauchi et al., 2009; G. Liu et al., 2010; Renström et al., 2009). Generally, these studies report a greater effect of the mutation in women compared to men. For example, Horstmann and colleagues (Horstmann et al., 2013) found that only female carriers of a common genetic variant near the *Mc4r* gene exhibited a significant increase in "disinhibited" and "emotional" eating, together with increased gray matter volume in the amygdala. "Emotional eating" has been linked to stress (Adam & Epel, 2007) and our present study found that females with *Mc4r* mutations have heightened stress reactivity compared to males, suggesting a possible mechanism for the sex-dependent effects associated with this mutation in humans. However, in contrast to the human literature but consistent with several rodent studies (Huszar et al., 1997; Ste Marie, Miura, Marsh, Yagaloff, & Palmiter, 2000), we did not find a sex-dependent effect of the *Mc4r* genotype on food intake or body weight in this study. This discrepancy may be due to difficulty in modeling "emotional eating" in rodents. In the current study, for example, rats were not offered the choice to consume palatable foods. It would be interesting, in future work, to investigate if sex-dependent effects of MC4R signaling on energy balance are revealed using a rodent model of binge eating. Furthermore,

the heightened stress reactivity was evident in both CON and CVS females, though more exaggerated in CVS females (Figure 3C–E). Because CVS can alter the female reproductive cycle via disruption of the hypothalamic-pituitary-gonadal axis (Valsamakis, Chrousos, & Mastorakos, 2019), further studies are needed to delineate the role of reproductive hormones to modulate MC4R signaling, specifically with respect to the stress response.

In summary, the present findings support that MC4R-signaling exerts a sex-dependent effect on acute stress-responsivity and basal HPA axis tone. Whereas MC4R loss-of-function facilitated the acute stress-induced rise in plasma corticosterone, in a gene dose-dependent manner in male rats, it had a gene dose-dependent opposing effect in female littermates. Future work will focus on identifying genetic, developmental, and/or hormonal mechanisms contributing to this sex difference, and implications for stress-associated psychopathologies. Importantly, and contrary to our overall hypothesis, *Mc4r* genotype did not modulate responses to *chronic* stress in either sex. Specifically, MC4R loss-of-function did not protect against chronic stress-associated increases in HPA axis tone, adrenal hypertrophy, anorexia, weight loss, or vascular remodeling. Therefore, with regard to these endpoints, we conclude that the hypothalamic melanocortin system is not a critical communication link between brain metabolic and stress systems during exposure to chronic stress.

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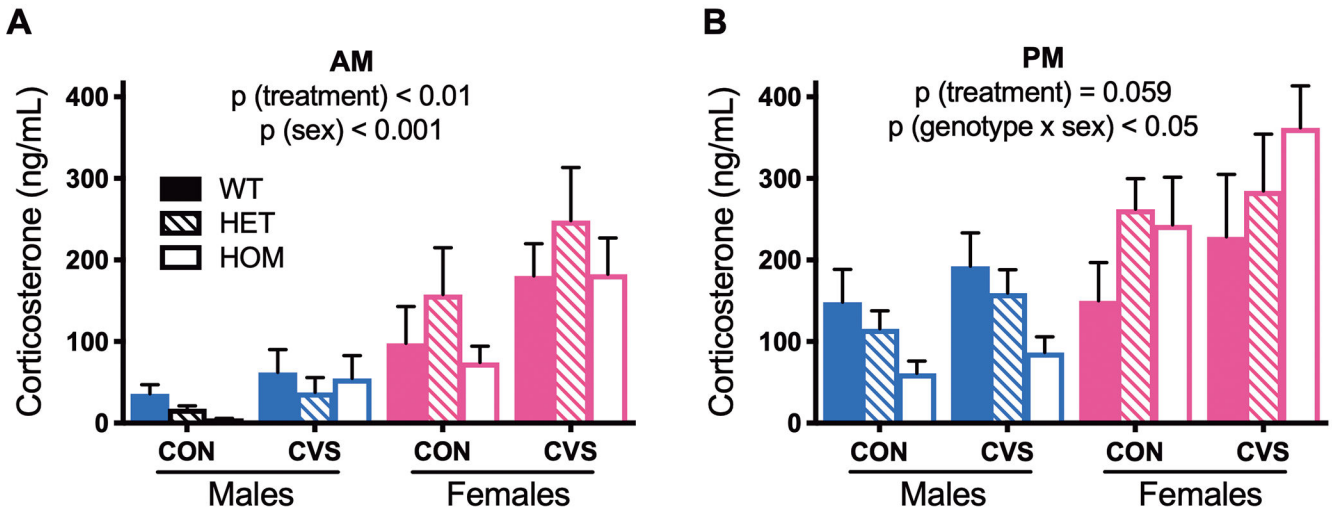

Figure 1. MC4R loss-of-function has sex-dependent effects on basal HPA axis tone.

Rats exposed to chronic variable stress (CVS) exhibited increased basal plasma corticosterone, compared to unstressed littermate controls (CON), when measured near the nadir of its diurnal rhythm (AM) **(A)**. This was independent of genotype. Near the peak of diurnal rhythm (PM) **(B)**, CVS likewise trended towards increased basal plasma corticosterone. Moreover, we observed a significant genotype x sex interaction, such that MC4R loss-of-function dose-dependently decreased and increased corticosterone in males and females, respectively. At both time points, females had significantly higher basal corticosterone than males. WT = wild-type, HET = heterozygous mutant, HOM = homozygous mutant. Data presented as mean ± S.E.M., 3-way ANOVA, n = 3–9/ sex/ genotype/ treatment.

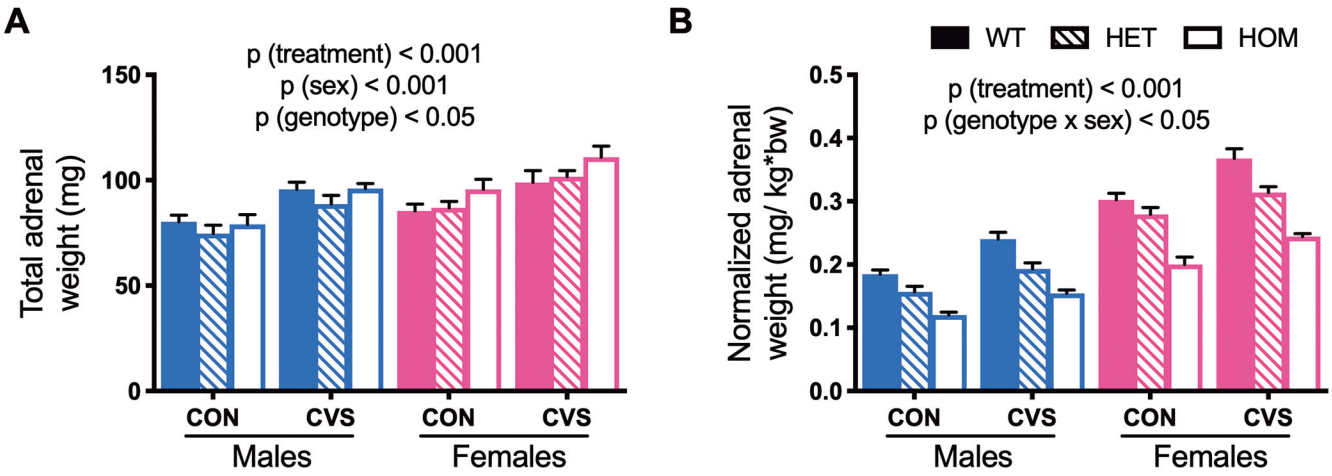

Figure 2. Chronic variable stress induced adrenal hypertrophy independent of genotype.

Adrenals were collected and weighed after 31 days of chronic variable stress (CVS), as an indirect measure of HPA tone. Rats exposed to CVS exhibited increased total adrenal weight compared to unstressed littermate controls (CON) **(A)**, and female adrenals were heavier than males'. When adjusted for body weight **(B)**, MC4R loss-of-function was associated with decreased relative adrenal weight in a dose-dependent manner, likely as a simple consequence of MC4R-dependent changes in body weight (see Fig 4C). WT = wild-type, HET = heterozygous mutant, HOM = homozygous mutant. Data presented as mean ± S.E.M., 3-way ANOVA, n = 4–9/ sex/ genotype/ treatment.

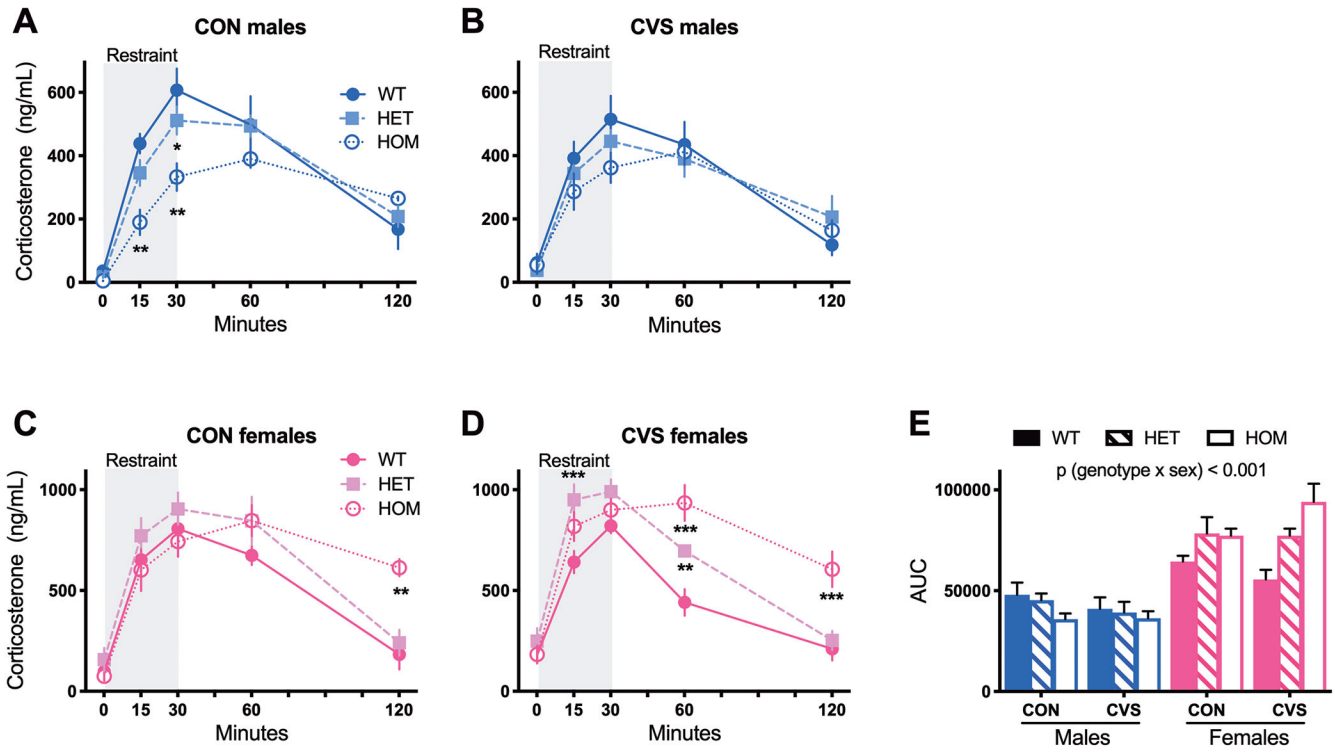

Figure 3. MC4R loss-of-function has sex-dependent effects on the corticosterone response to an acute restraint stress.

Rats were placed in a well-ventilated Plexiglas restrainer for 30 minutes and blood was collected from the tip of the tail vein at indicated times. MC4R loss-of-function blunted the corticosterone response to restraint in unstressed, control (CON) males (**A,** 2-way RM ANOVA, $F(8,56)=2.88$, p (genotype x time) < 0.01) but this effect was less apparent among rats exposed to chronic variable stress (CVS) **(B)**. On the contrary, MC4R loss-of-function heightened the corticosterone response in both CON (**C**, 2-way RM ANOVA, $F(8,56)=2.73$, p (genotype x time) < 0.05) and CVS females (**D**, 2-way RM ANOVA, $F(8,64)=8.15$, p (genotype x time) < 0.001). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Tukey's posthoc. Areas under the curve **(E)** revealed a significant interaction between genotype and sex (3-way ANOVA). WT = wild-type, HET = heterozygous mutant, HOM = homozygous mutant. Data presented as mean ± S.E.M., $n = 3–9$/ sex/ genotype/ treatment.

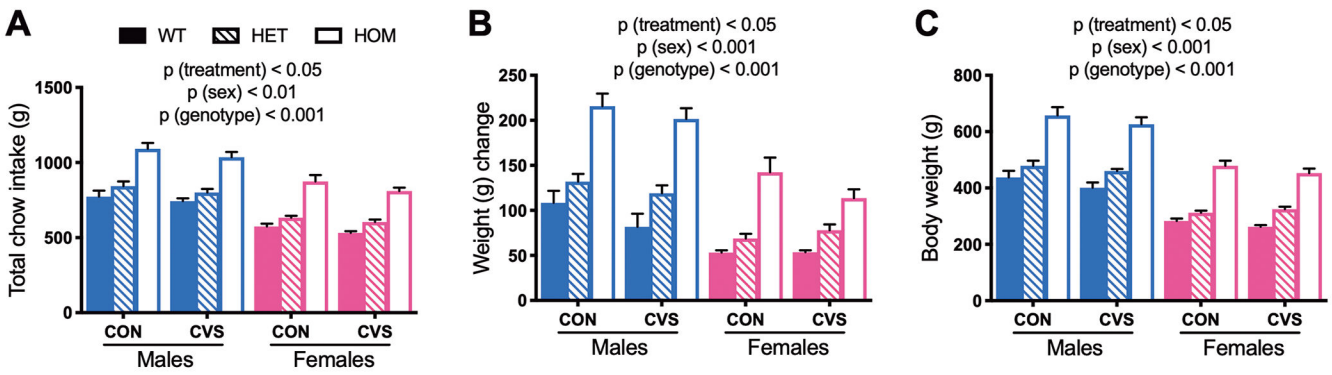

Figure 4. Chronic variable stress decreased food intake and body weight independent of genotype.

Food intake **(A)** and body weight change **(B)** were measured throughout 31 days of chronic variable stress (CVS) treatment. MC4R loss-of-function increased total food intake and body weight change in both sexes in a dose-dependent manner, and males ate more and were heavier than females. This was also reflected in body weight **(C)** at the conclusion of the study. CVS treatment decreased all three measures, compared to unstressed littermate controls (CON), and this was independent of genotype. WT = wild-type, HET = heterozygous mutant, HOM = homozygous mutant. Data presented as mean ± S.E.M., 3-way ANOVA, n = 5–9/ sex/ genotype /treatment.

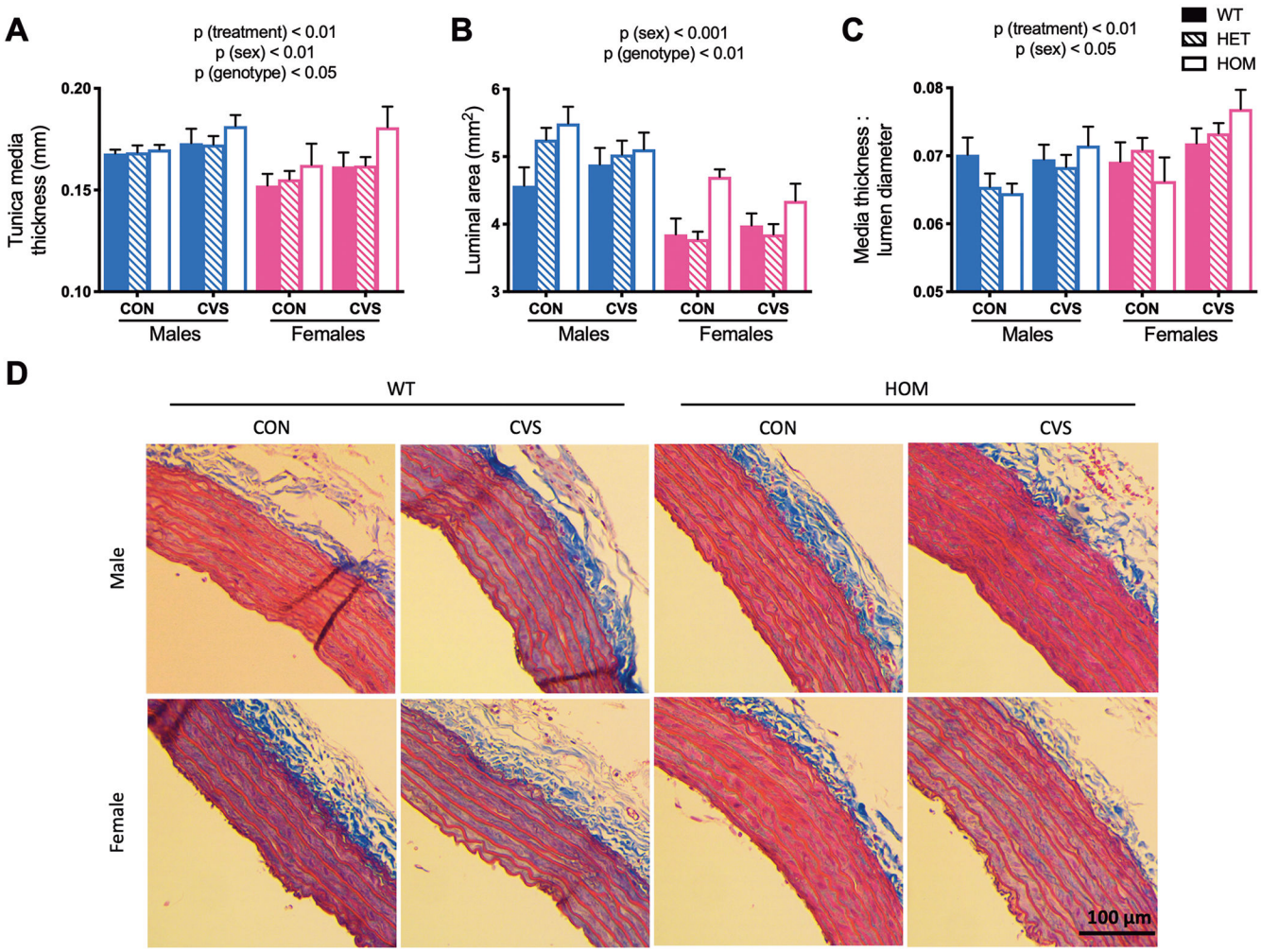

Figure 5. Chronic variable stress induced markers of vascular remodeling independent of genotype.

Descending aortas were collected and fixed in formalin after 31 days of CVS, and a 2-3 mm piece was embedded in paraffin and stained with Masson's trichrome. Exposure to chronic variable stress (CVS) increased tunica media thickness compared to unstressed littermate controls (CON) **(A)**, with additional effects of sex and genotype such that larger rats exhibited larger thicknesses (also see Fig 4C). CVS did not significantly affect luminal area **(B)**, but this also increased with larger rats. Lastly, CVS increased the media thickness : lumen diameter ratio **(C)** independent genotype. Representative images of aortas are shown in **(D)**. WT = wild-type, HET = heterozygous mutant, HOM = homozygous mutant. Data presented as mean ± S.E.M., 3-way ANOVA, n = 3–9/ sex/ genotype /treatment.

Table 1.

Sample sizes of treatment groups.

Sex	Genotype	Treatment	Sample Size	Sex	Genotype	Treatment	Sample Size
Male	WT	CON	6	Female	WT	CON	7
Male	WT	CVS	6	Female	WT	CVS	7
Male	HET	CON	9	Female	HET	CON	9
Male	HET	CVS	9	Female	HET	CVS	9
Male	HOM	CON	6	Female	HOM	CON	4
Male	HOM	CVS	7	Female	HOM	CVS	6

Table 2.

Chronic variable stress protocol.

Experimental Day	AM Stressor	PM Stressor
0	Crowding	Shaker
1	White noise	Crowding
2	Shaker	Tilt
3	Strobe light	No bedding
4	White noise	Hypoxia
5	Shaker	Strobe light
6	Crowding	Tilt
7	Hypoxia	Strobe light
8	Shaker	Crowding
9	White noise	No bedding
10	Shaker	Hypoxia
11	Crowding	Shaker
12	Strobe light	No bedding
13	White noise	Tilt
14	Shaker	Hypoxia
15	Crowding	Shaker
16	Hypoxia	No bedding
17	Shaker	**PM bleed**
18	White noise	Crowding
19	Strobe light	Shaker
20	Crowding	White noise
21	Strobe light	No bedding
22	Crowding	Hypoxia
23	White noise	Shaker
24	Strobe light	Crowding
25	Hypoxia	White noise
26	Crowding	Tilt
27	Shaker	Strobe light
28	Hypoxia	Shaker
29	Crowding	White noise
30	**Restraint bleed**	Strobe light
31	Sacrifice	