UNIVERSITY OF CALIFORNIA, SAN DIEGO

Phenotyping of TRH-DE deficient mice for Energy Balance and Depression- and Anxiety-related Behaviors

A thesis submitted in partial satisfaction of the
requirements for the degree of Master of Science

in

Biology

by

Grace Lee

Committee in charge:

Professor Byungkook Lim, Chair
Professor Brenda Bloodgood
Professor Kathy French
Professor Eric Zorrilla

2016

The Thesis of Grace Lee is approved, and it is acceptable
in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2016

DEDICATION

To Mom and Dad

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ABSTRACT OF THE THESIS

Phenotyping of TRH-DE deficient mice for Energy Balance and Depression- and Anxiety-related Behaviors

by

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Master of Science in Biology

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Professor Byungkook Lim, Chair

Thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE) specifically hydrolyzes released thyrotropin-releasing hormone (TRH), limiting TRH action. Release TRH exerts an anxiolytic-and antidepressant-like effect, and promotes negative energy balance. TRH-DE is highly expressed in brain regions that subserve emotional behavior. Furthermore, central *trhde* overexpression was associated with vulnerability to obesity. The following studies tested the hypothesis that reducing TRH-DE function by targeted gene knockout in mice would cause mice to be leaner, less fatty, with an anti- depressant- or anxiolytic-like phenotype. Adult male and female mice of wild type (WT), hetero-zygous and *trhde* knock-out(KO) genotypes were analyzed for whole-body energy

expenditure, food intake and body composition after chow or high-fat(HF) diet challenge. To assess anxiety- and antidepressant-like phenotypes, adult male and female mice were tested in the elevated plus maze, light-dark box, and forced swim tests. Adult female and male, WT+KO, mice were compared for operant self-administration of food+water and a highly reinforcing sucrose(3%)-saccharin(0.125%) solution. Energy expenditure(EE) analysis revealed that KO mice have an altered circadian profile compared to WT, where KO show higher EE during the later half of the dark cycle. Body composition analysis of chow-reared adult mice(aged 2-11 months) showed that WT mice were heavier (especially in older males), fatter(after 5.3 months) and had more lean mass than KO (p<0.0001). Over a 6-week HF diet challenge, KO ate less across all weeks when compared to HET (p=0.038) and WT(p=0.006) mice. While there were no differences in overall weight gain, KO and HET gained less body fat than WT mice(p<0.05). The time spent in the open arm and light box for EPM and LD, respectively, did not differ by genotype, suggesting similar anxiety-like behavior. In the forced swim test, KO(p=0.01) + HET (p=0.02) mice were less immobile than WT litter- mates, suggesting an antidepressant-like phenotype. Sex x Genotype interactions reflected that male KO mice self-administered more food and water than WT controls following acquisition. In summary, knocking out TRH-DE function had an anti-depressant-like effect on the FST and increased self-administration at FR2 in male KO, but did not show an anxiolytic-like effect, nor impair learning in self-administration. The TRH-DE KO had increased energy expenditure near the circadian nadir, decreased HF diet intake, and less age- and HF-diet-associated body fat accumulation. The results suggest a role for TRH-DE in influencing energy homeostasis and depression, but may not influence anxiety.

CHAPTER 1: INTRODUCTION

Thyrotropin-Releasing Hormone Plays a Major Role in the Central Nervous System, including the HPT axis

Thyrotropin-releasing hormone (TRH) is a tripeptide amide with the structure (pyro)Glu-His-Pro-NH_2 (Boler, Enzmann et al. 1969). As a hypophysiotropic hypothalamic peptide hormone, TRH regulates the hypothalamic-pituitary-thyroid axis (HPT) to cause the release of thyroid stimulating hormone (TSH) from the anterior pituitary after it is released from the paraventricular nucleus (PVN) of the hypothalamus into the median eminence (ME)(Lechan and Segerson 1989, Fekete and Lechan 2014). Released TSH stimulates the release of thyroid steroid hormones, T3 and T4, from the thyroid gland into the circulatory system. Thyroid hormones have many peripheral effects, including modulating energy expenditure through basal and adaptive thermogenesis, fat metabolism, and appetite regulation (Lechan and Fekete 2006).

In-situ hybridization and immunohistochemistry studies showed wide distribution of TRH in the brain; 70% of TRH is expressed outside the hypothalamus, including the raphae nuclei, reticular thalamic nucleus, and olfactory bulb (Heuer, Schafer et al. 1999). Furthermore, nonhypophysiotropic TRH neurons were found to project into the amygdala, the paraventricular thalamic nucleus, and nucleus accumbens (Lechan and Fekete 2006, Wittmann, Fuzesi et al. 2009). This suggests that TRH also facilitates many central actions by working as a neurotransmitter or neuromodulator, including decreasing feeding behavior, increasing locomotion activity, and improving memory.

Released TRH binds to either the type I or type II TRH receptor. Both TRH receptor types have equal binding affinities for TRH and are heterotrimeric G-protein coupled receptors. Yet, the two types are distinct in internalization rates, conformation,

and distribution in the central nervous system and peripheral tissues (Sun, Lu et al. 2003, Joseph-Bravo, Jaimes-Hoy et al. 2015). Additionally, TRH-R2 have not been found in humans (Cao, O'Donnell et al. 1998, Sun, Lu et al. 2003).

Degradation of TRH by Pyroglutamyl Peptidase I and II

TRH is primarily degraded by two types of pyroglutamyl peptidases, I and II. In general, pyroglutamyl peptidases hydrolytically cleave a pyroGlu group from the *N*-terminus of a pGlu peptide and has been found across many species, from mammals to bacteria (Cummins and O'Connor 1998).

Pyroglutamyl peptidase I (PPI) is a soluble cytosolic protein with a low molecular mass compared to type II, ranging from 22 kDa in the human kidney and skeletal muscle to 60 kDa in the rat brain (Cummins and O'Connor 1998). PPI has high substrate specificity for any N-terminus pGlu residue. It is hypothesized that PPI, in conjunction with other cytosolic proteins, is involved in the degradation of damaged pGlu peptides (Cummins and O'Connor 1998).

In contrast, pyroglutamyl peptidase II (PPII, also known as Thyrotropin-Releasing Hormone Degrading Ectoenzyme, henceforth referred as TRH-DE in this report), is a type II integral membrane protein that is primarily expressed in the brain and is highly specific for hydrolyzing released TRH at the pGlu-His bond (Charli, Cruz et al. 1988, Heuer, Schafer et al. 1999, Schomburg, Turwitt et al. 1999, Joseph-Bravo, Jaimes-Hoy et al. 2015). Although PPI is capable of hydrolyzing TRH, TRH is normally stored in secretory vesicles in the cytosol so it is selectively hydrolyzed by TRH-DE in the extracellular space (Joseph-Bravo, Jaimes-Hoy et al. 2015) and not PPI (Charli, Cruz et

al. 1988). Moreover, TRH-DE inhibition specifically causes increased TRH recovery from hypothalamic slices (Charli, Mendez et al. 1989).

TRH-DE Was Overexpressed in Rats at Increased Risk for Obesity

A past microarray analysis in our lab showed that *trhde* was overexpressed in the lateral hypothalamus of two distinct rat models that are at increased risk for obesity (Frihauf 2014). The aim of this study is to explore the role of causal role of TRH-DE in modulating emotional behavior, energy expenditure, and body composition via the use of *trhde* knock out (KO) mice.

CHAPTER 2: THE ROLE OF TRH-DE ON ENERGY BALANCE AND BODY COMPOSITION

With advances in science and public health measures over the past 2 centuries, there has been a decrease in morbidity and mortality rates due to communicable or infectious disease. People began to live longer, eat more, and have a sedentary lifestyle. There was a corresponding change in the cause of morbidity and mortality to chronic, noninfectious diseases like cancer and cardiovascular diseases, which are among the leading causes of death today.

Obesity is established as one of the factors that increases risk of these chronic diseases (Eeg-Olofsson, Cederholm et al. 2009). Furthermore, recent findings have reported that maternal obesity and dietary intake during pregnancy and lactation may affect the offspring's risk of developing cardiovascular and metabolic disease (O'Reilly and Reynolds 2013, Wood-Bradley, Henry et al. 2013). A past study in our lab found that maternal consumption of a Western diet increased vulnerability to obesity and did so even in the offspring of otherwise genetically obesity-resistant rats. While diet and other lifestyle factors contribute to the development of obesity, genetic factors also appear to be involved. Perhaps correspondingly, our lab found that the gene coding for TRH-DE was independently overexpressed in the lateral hypothalamus of rat weanlings that were vulnerable to obesity due to maternal Western diet exposure or, alternatively, due to selective breeding for vulnerability to high-fat diet-induced obesity.

Afferent projections from the nucleus accumbens to the lateral hypothalamus suggest a connection between motivation and appetitive behaviors, including feeding. Indeed, the lateral hypothalamus contains many neuropeptides that modulate feeding or

reward-related behavior, including CART, MCH, and orexin (Berthoud and Munzberg 2011). Relevant to a role for TRH systems in feeding behavior and reward, Pro-TRH neurons also have been observed in the lateral hypothalamus (Wittmann, Fuzesi et al. 2009, Horjales-Araujo, Hellysaz et al. 2014).

Past studies have shown that typical effects of central TRH adminstraion is anorectic: reducing food intake, and increasing energy expenditure (Vijayan and McCann 1977, Schuhler, Warner et al. 2007, Puga, Alcantara-Alonso et al. 2016). Furthermore, serum TRH-DE activity was found to be positively correlated to BMI; euthyroid obese patients were found to have highest TRH-DE activity (Friedman, Yanovski et al. 1995). However, TRH appears to be orexigenic in some circuits (Ao, Go et al. 2006). Recently, TRH neurons were found to be an excitatory input onto agouti-related peptide neurons in the arcuate nucleus of the hypothalamus known to regulate hunger and satiation (Krashes, Shah et al. 2014). Activation of TRH-ergic neurons activates the AgRP neurons, causing increased feeding. While there is some uncertainty to the net effect of TRH on feeding, the involvement of TRH in the HPT axis for energy balance, and the presence of TRH neurons in the nucleus accumbens, amygdala, and lateral hypothalamus, suggests TRH involvement in feeding behavior, memory, and locomotion.

In this chapter, we examine the role of TRH-DE on energy expenditure, and body composition of *trhde* knock out mice due to high fat diet consumption and age. We predicted that a reduction of TRH-DE function by targeted gene knockout would increase energy expenditure and fat metabolism, and decreased food intake.

2.1 METHODS

Subjects

Trhde^{tm1Lex} heterozygote mutant mice on a B6/129s5 background were obtained from UC Davis Mutant Mouse Regional Resource Center (MMRRC) and mated. The resulting male and female *trhde* knock out (KO, Trhde (-/-)), wild-type (WT), and heterozygous (HET) offspring mice were used for this study. The genotypes were determined by PCR. Tails were digested at 55°C overnight in 1x Modified Gitschier Buffer, 1% beta-mercapto ethanol, and 0.5% TritonX-100. DNA was amplified for 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C, 75 seconds at 72°C. The primer sequences (wild type forward: CTC TCG GAC CCG TGG GCT G, wild type reverse: GCA TCA AAT TGT AGT GCA AGC G, knock out forward: GCA GCG CAT CGC CTT CTA TC, knock out reverse: CCA CTG TAA CCT GAG AAG TTC) resulted in PCR products of 494 base pair (KO) and 421 base pair (WT).

The animals were housed in a constant 12 hour light, 12 hour dark cycle at all times (lights on at 06:00 am) with access to food and water *ad-libitum*. All animals were weaned onto *ad-libitum* chow access (LM-485 7012, 17% fat [kcal], 58% carbohydrate, 25% protein; 3.1 kcal/g; 16% saturated, 26% monounsaturated and 58% polyunsaturated fats; 2.7% kcal from saturated fat, Harlan, Indianapolis, IN).

Indirect Calorimetry

Mouse indirect calorimetry was performed with an open-circuit system (Comprehensive Lab Animal Monitoring System; Columbus Instruments, Columbus, OH) over 24 hours (start on 18:00h) on singly housed mice that were acclimated for 3

days. The clear chambers (20 x 10 x 12.5 cm) were equipped with a plastic-mesh floor, water sipper, food tray connected to a balance, and 16 photobeams situated in rows 1.3 cm apart, and 3.3 and 7.3 cm above the floor. Motor activity was measured along the *x*- and *z*- axes, respectively. Chamber exhaust was measured at 10 minute intervals for 60 seconds through oxygen and carbon dioxide sensors to estimate oxygen consumption (VO2) and carbon dioxide production (VCO2). The sensors were calibrated before testing with known concentrations of O_2, CO_2, and N_2 (Praxair, Inc., Danbury, CT). Respiratory quotient and heat formation were calculated as described in (Zorrilla, Sanchez-Alavez et al. 2007), the latter was normalized for lean mass (Wahlig, Bales et al. 2012).

Measurement of body composition

Animal body composition was measured with nuclear magnetic resonance imaging (EchoMRI-1100, software version 2008.01.18M, Echo Medical Systems, Houston, TX).

High fat diet challenge

To determine the effects of TRH-DE deficiency on the response to a high fat diet challenge, male and female *trhde* KO, WT, and HET littermates were individually housed at 58-78 days old and received *ad libitum* water and purified high-fat diet (HF, Research Diets D12451, 20% protein, 35% carbohydrate, 45% fat, 4.73 kcal/g) over six weeks. Food intake and body weights were measured weekly. The high-fat diet food and cages were changed once a week. Baseline (before diet access), week 2, and week 6 body composition was measured by EchoMRI.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using Systat or SPSS data analysis packages. Pairwise comparisons, following significant omnibus tests, involved Fisher's protected LSD tests. Cohort was included as a covariate.

2.2 RESULTS

Indirect calorimetry

At approximately 2 months of age, *trhde* KO and WT mice were analyzed for energy expenditure via mouse indirect calorimetry. A significant Time x Genotype interaction ($F(7,77)=2.606$, $p=0.018$) was observed, reflecting that *trhde* KO mice had increased energy expenditure during the later half (12 AM- 6 AM) of the dark cycle. Neither Genotype x Sex, nor Genotype x Sex x Time interactions were significant. Furthermore, the KO mice presented a blunted amplitude of the circadian rhythm of energy expenditure with a smaller difference between their peak and nadir energy expenditure as compared to WT control littermates. (Figure 1).

Adult body composition measurement: KO mice do not show as much age-associated increase in body fat, but reduced lean mass is evident even at 2 months

All mice were weaned onto *ad-libitum* chow access. To determine differences in body weight, lean mass, and fat composition of adult *trhde* knock-out and wild type (WT) littermates, male and female mice were analyzed via EchoMRI at 3 stages of

development: 1) young adults (2 months), 2) middle aged (5.3 to 6.9 months) and 3) older adults (8.4-11 months).

Young adults did not display differences in Genotype, Sex, or Sex x Genotype interactions for body weight or fat composition. However, an effect of Genotype ($F(2,22)=5.519$; $p=0.01$) and Sex ($F(1,22)=33.163$; $p<0.0001$) was seen as young as two months old for differences in lean mass. At two months, a pairwise analysis showed a significant difference in lean mass between WT and *trhde* KO littermates ($p=0.007$), and between KO and HET littermates ($p=0.01$).

In middle aged adults (5.3 to 6.9 months), there was a significant effect of Genotype ($F(1,24)=11.688$ $p=0.002$), where the KO were lighter than the WT. There was not a significant body weight difference in an analysis of Sex x Genotype interactions. An analysis of body fat composition showed that WT mice were fatter than KO littermates ($F(1,24)=16.567$; $p<0.0001$). There was no difference between male and female adults in fat mass. As seen in the young adults, a similar effect of Genotype ($F(1,24)=5.977$; $p=0.022$) and Sex ($F(1,24)=43.400$; $p<0.0001$) was also seen where the knock-outs and females were less lean than their littermates.

In older adults (8.4-11 months), there was a significant effect of Genotype ($F(1,56)=18.649$; $p<0.0001$) and Sex x Genotype ($F(1,56)=4.617$; $p=0.036$) where knock-outs (specifically females) were lighter than the wild type. Similar to the middle aged adults, WT mice were fatter than KO littermates ($F(1,56)=9.103$; $p=0.004$), where there was no difference between male and female adults in fat mass. In the older adults, there is a similar effect of Genotype ($F(1,56)=21.209$; $p<0.0001$) and Sex ($F(1,56)=115.792$; $p<0.0001$) for lean mass as seen in young adults and middle aged

adults; knock-outs had disproportionally less lean mass than WT littermates, and females had less lean mass than males.

To determine if the effect of Genotype in fat composition is due to differences in body weight or lean mass, we analyzed the body fat composition covarying for lean mass, and the % fat relative to body weight. In middle aged adults, there is a significant Sex difference in per cent fat composition relative to body weight ($F(1,24)=15.616$; $p=0.001$) where males have less % fat than females. There is no effect of Genotype or Sex x Genotype. Starting at 8.5 months in the older adults, the KO mice have less % fat than the WT ($F(1,56)=4.537$; $p<0.05$).

However, in an analysis of fat covarying for lean mass, there was a significant Genotype effect in middle aged adults ($F(1,23)=11.457$; $p=0.003$) where knockouts had less fat. This Genotype difference was not seen in older adults.

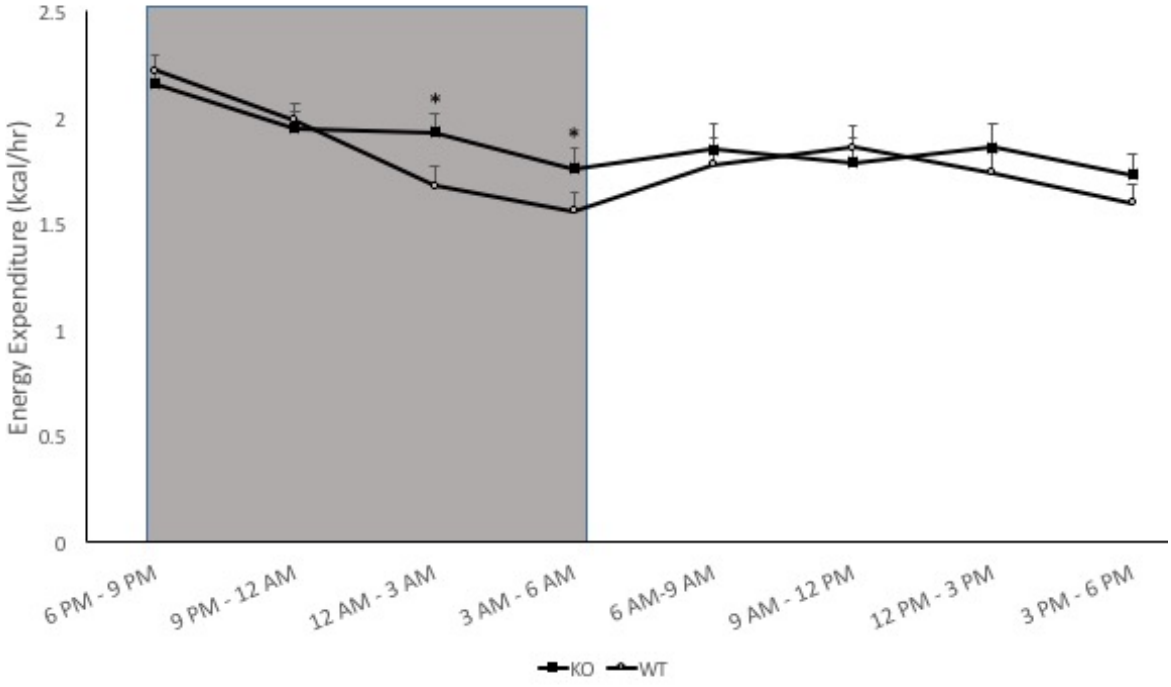
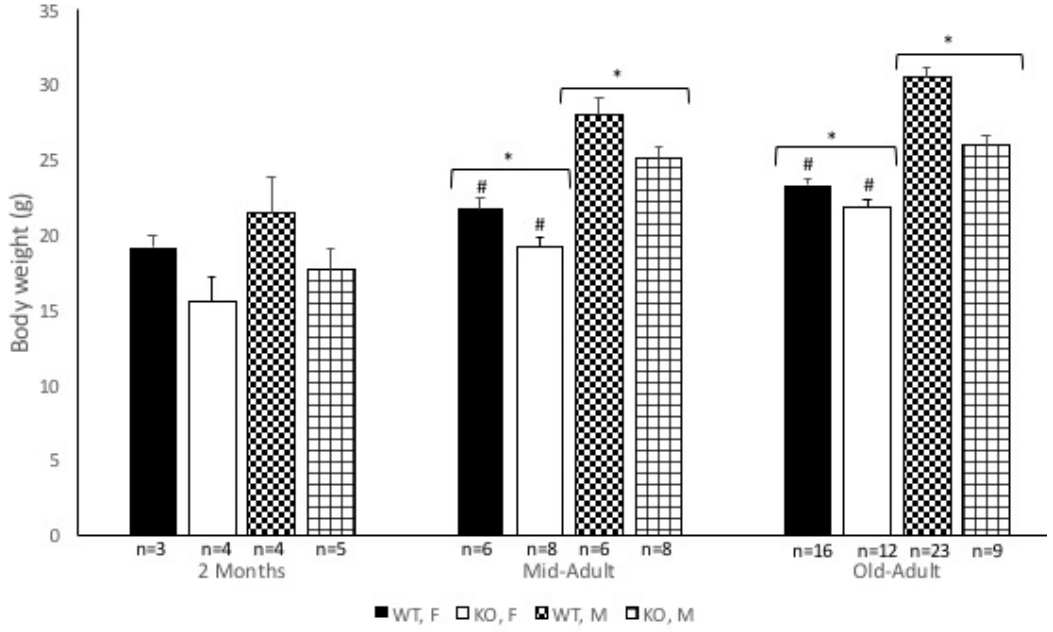
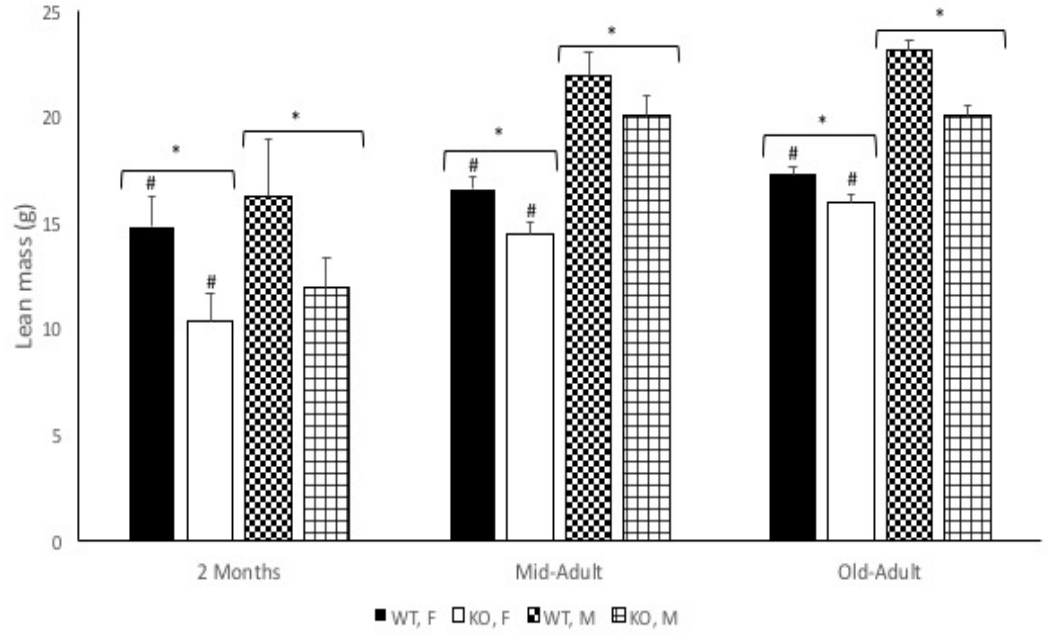


Figure 1. *Trhde* KO mice display increased energy expenditure compared to WT control littermates, with the greatest difference observed towards the end of the dark cycle. Furthermore, the circadian rhythm of the KO does not form a defined nadir and peak as seen in the WT littermates. Data expressed as estimated marginal mean + SEM, covarying for lean mass and accounting for sex. KO (n=8), WT (n=9). (*) Genotype effect, $p<0.05$.

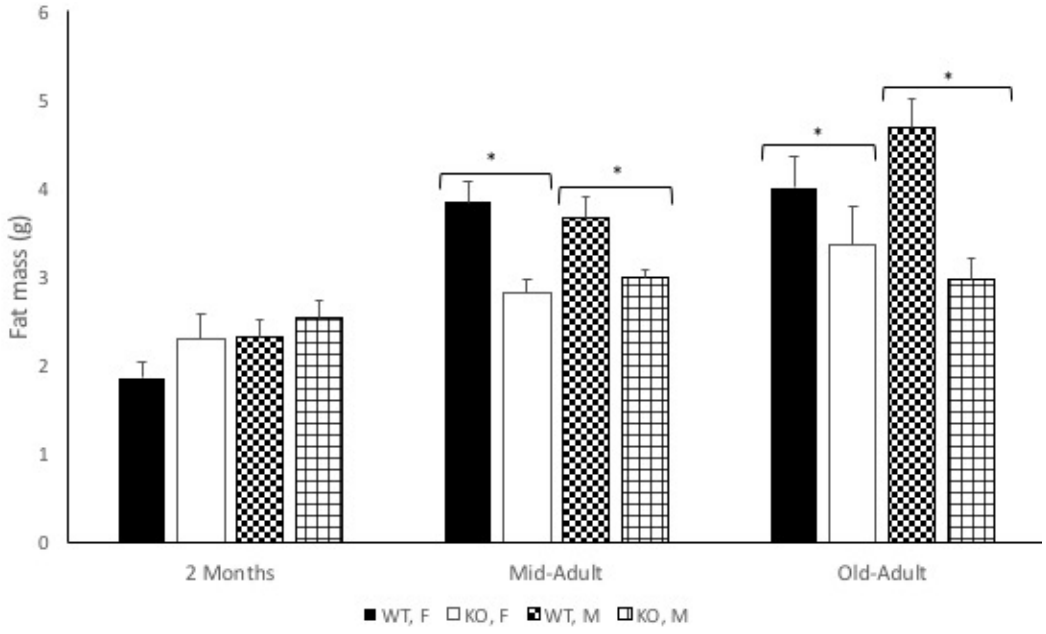
Figure 2. Adult *trhde* KO mice weaned on chow are lighter and have less fat than WT littermates. (a) The Genotype effect becomes greater in males as they age, whereas females don't show this similar pattern in age related weight difference. (b) *trhde* KO mice weaned onto chow have less lean mass than WT littermates (c) Similarly, the Genotype effect is greater in males than females with age; older male KO have much less fat than male WT littermates. This difference is much less pronounced in young adults (2 months). 2 months: female KO (n=4), female WT (n=3), male KO (n=5), male WT (n=4). Middle-aged adults: female KO (n=8), female WT (n=6), male KO (n=8), male WT (n=6). Old adults: female KO (n=12), female WT (16), male KO (n=9), male WT (n=23). Data expressed Means + SEM. (*) Genotype effect, $p<0.05$. (#) Sex effect, $p<0.05$


Body weight (g)
35
30
25
20
15
10
5
0
n=3
n=4
n=4
n=5
2 Months
n=6
n=8
n=6
n=8
Mid-Adult
n=16
n=12
n=23
n=9
Old-Adult
WT, F
KO, F
WT, M
KO, M

A.


Lean mass (g)
25
20
15
10
5
0
2 Months
Mid-Adult
Old-Adult
WT, F
KO, F
WT, M
KO, M

B.



C.

Figure 2 Continued

High fat diet challenge

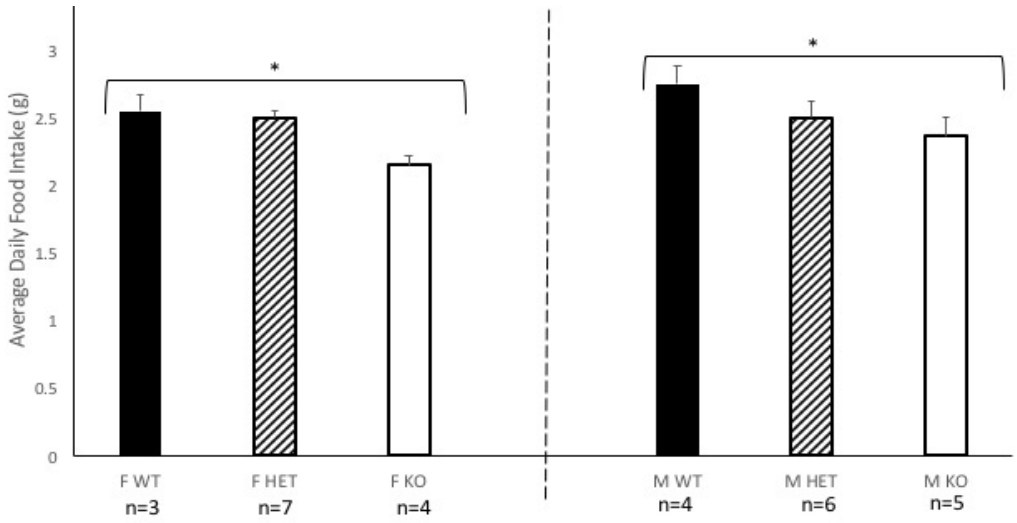
Two month old male and female wild-type, *trhde* KO, and heterozygote mice weaned onto chow were challenged with high fat diet over 6 weeks (Figure 3). There was a significant effect of Genotype for high fat food intake across the 6 weeks ($F(2,22)=4.932$; $p<0.02$). The KO mice significantly ate less high fat diet than their WT ($p=0.006$) and heterozygote ($p<0.05$) littermates. However, there was no difference in food intake between WT and HETs.

While there is a significant Sex effect for the week 6 body weight measurement ($F(1,22)=13.979$; $p=0.001$), there is a strong trend for Genotype ($F(2,22)=3.294$; $p=0.056$) where KO mice are lighter than WT littermates ($p<0.02$) in a pairwise comparison. Surprisingly, there were no significant differences in body weight gain from baseline at 6 weeks among sexes, genotypes, or for Sex x Genotype interactions.

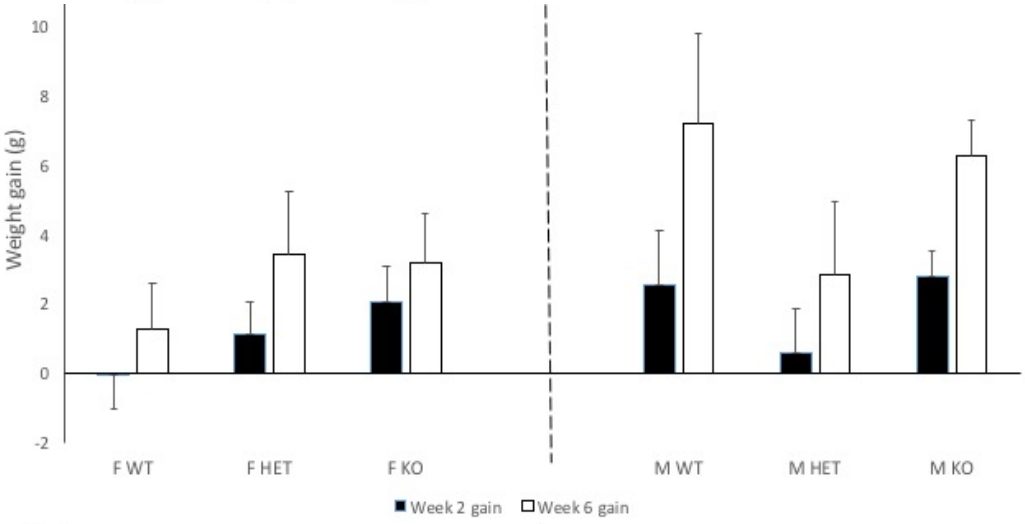
At two weeks into the challenge, there was a strong trend of Genotype ($F(2,22)=2.857$; $p=0.079$) for fat gain, where WT mice gained significantly more fat than KO littermates ($p<0.05$). There was no significant difference in lean mass gain at the two week point. At 6 weeks into the high fat challenge, there was a significant effect of Sex ($F(1,11)=7.092$; $p=0.022$) when male and female WT and *trhde* KO mice were compared for body fat composition. There were no effects of Genotype or Sex x Genotype. However, there was a significant effect of Sex ($F(2,22)=5.491$; $p<0.03$) and Genotype ($F(2,22)=3.496$; $p<0.05$) on fat gained since baseline. Males gained more fat than female mice. With regard to the Genotype effect, WT mice gained more fat (Table 2) compared to *trhde* KO mice ($p<0.02$) and heterozygotes ($p<0.05$). Heterozygotes did not differ in fat gained compared to KO mice.

Furthermore, there was no significant difference in lean mass gain from baseline at the 6 week trial ($F(2,20)=1.542$; $p=0.238$, *ns*). While a comparison of the Means ± SEM seems to suggest a difference in lean mass gain between WT and KO male mice, this analysis did not gain significance.

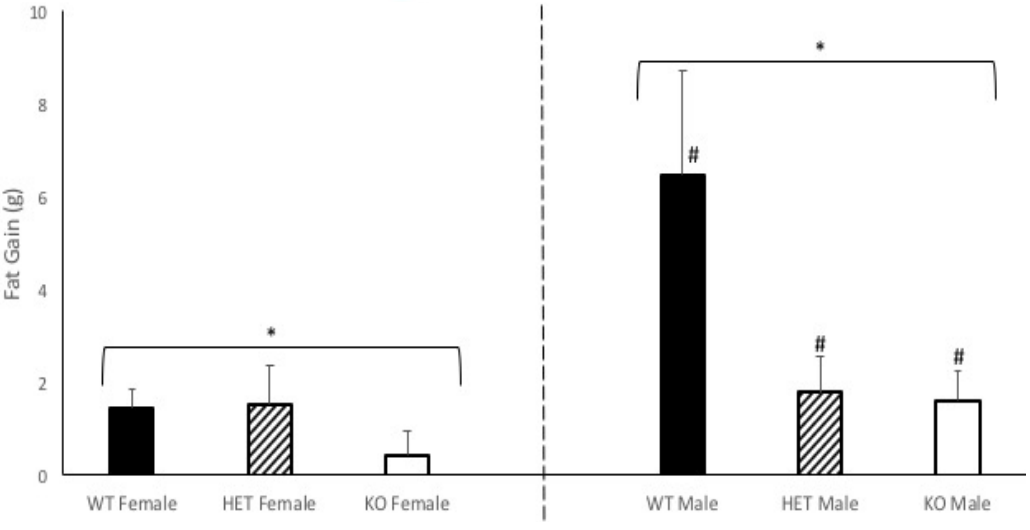
Figure 3. After 6 weeks of *ad-libitum* high fat diet intake, *trhde* KO mice gained less fat from baseline and ate less than the littermates. (a) Across 6 weeks, KO mice, on average, had a lowered daily high fat food intake than heterozyote or WT littermates. (b)No difference in body weight gained from baseline after 2 weeks or 6 weeks of high fat diet intake in analyses of Genotype or Sex x Genotype. At week 6, males were heavier than females. While KO mice were lighter than WT at week 6 ($p<0.02$), there was no Genotype or Sex x Genotype interaction in between subjects. (c) Males gained more fat than females, and WT mice gained more fat than KO or HET littermates. (d) Descriptively, KO mice seemed to gain more lean mass compared to littermates. However, it did not gain significance in the final analysis. Female WT (n=3), Female HET (n=7), Female KO (n=4). Male WT (n=4), Male HET (n=6), Male KO (n=5). Data expressed Means + SEM. (*) Genotype effect, $p<0.05$. (#) Sex effect, $p<0.03$


Average Daily Food Intake (g)
3
2.5
2
1.5
1
0.5
0
*
*
F WT
F HET
F KO
M WT
M HET
M KO
n=3
n=7
n=4
n=4
n=6
n=5

A.


Weight gain (g)
10
8
6
4
2
0
-2
F WT
F HET
F KO
M WT
M HET
M KO
Week 2 gain
Week 6 gain

B.


Fat Gain (g)
10
8
6
4
2
0
*
*
#
#
#
WT Female
HET Female
KO Female
WT Male
HET Male
KO Male

C.

D.

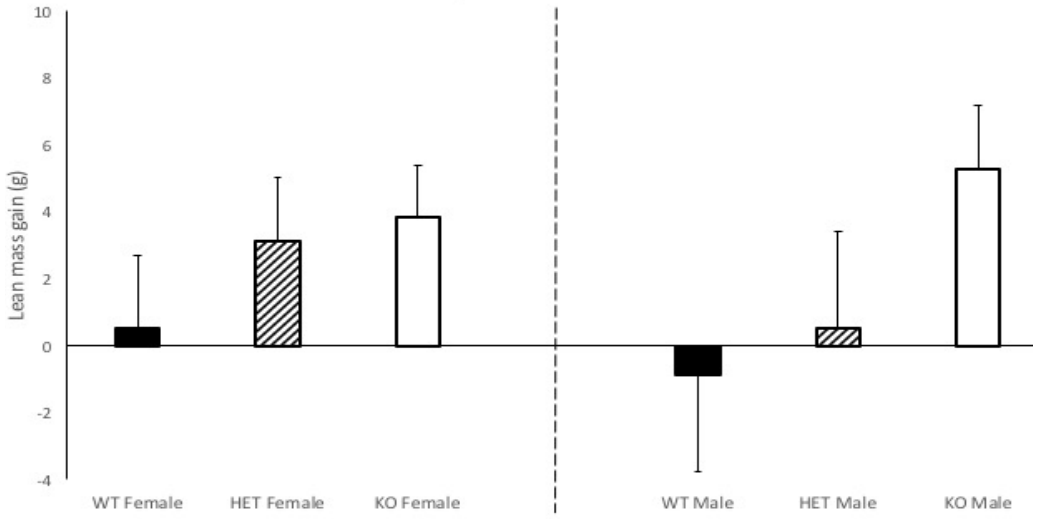


Figure 3 Continued

2.3 DISCUSSION

To determine if *trhde* modulates aspects of energy homeostasis, the current study analyzed differences in energy expenditure, and age-related differences in body weight and body composition of chow-fed *trhde* KO mice across stages of adulthood. In addition, genotypes were compared for their response to a high fat diet challenge.

TRH-DE is needed to maintain a normal circadian rhythm of energy expenditure

Trh has been shown to be mutated in obese patients, along with several other hypothalamic genes associated with the circadian rhythm of food intake (Mariman, Bouwman et al. 2015). Past studies also show a circadian pattern of TRH secretion, TRH-DE activity, and TRH receptor sensitivity (Angel Vargas, Uribe et al. 2002, Szuba, Amsterdam et al. 2005). In male rats, TRH-DE activity was found to be at its nadir in the morning between 9:30 AM and 11:30 AM, which corresponds to the peak energy expenditure of the WT mice in this study. Analysis of energy expenditure showed that male and female *trhde* KO mice had an increased energy expenditure relative to WT controls in the later half of the dark cycle, specifically from 12 AM – 6 AM. Furthermore, *trhde* KO mice presented an altered circadian profile relative to WT littermates. While the WT controls had a defined circadian peak and nadir, the KO mice failed to follow a similar pattern. The period of greatest difference in energy expenditure corresponded to the nadir in WT mice, indicating loss of TRH-DE has taken away the quiet period of energy expenditure. The cause of this blunted effect is not entirely clear. While energy expenditure measures are different from measures of activity levels, one possible explanation may be that the KO mice are more active in general. TRH has been

shown to increase locomotor activity (Lechan and Fekete 2006) in rodents; thus, lack of TRH-DE expression may cause a difference in activity levels, which may correspond to this difference in energy expenditure. Alternatively, Wittmann et.al (Wittmann, Fuzesi et al. 2009) proposed that TRH neurons in the PVN may be activated by cold temperatures to increase heat production via the production of thyroid hormone and activation of the sympathetic nervous system. It is possible that the lack of TRH-DE in the PVN of KO mice may be disinhibiting this TRH signaling pathway, leading to increased energy expenditure.

TRH-DE modulates energy homeostasis: analysis of body composition throughout adulthood and with high-fat diet

Compared to WT, the KO mice were lighter and had less fat and lean mass throughout adulthood. There was no difference in tissue water. In WT females, body weight gained throughout adulthood seemed to asymptote earlier than in males; the genotype effect on body weight continued to increase with age in males.

An analysis of % fat showed genotype differences in only older adults, suggesting an age-dependent role in the fat difference between genotypes. Yet, in an analysis of fat covarying for lean mass, this Genotype effect was seen in younger adults, and not in older adults. When we looked at % fat or grams fat covarying for lean mass while controlling for age in adulthood, the Genotype effect was still significant in both cases. As such, the grand analysis across all groups shows a disproportionate amount of fat in the WT compared to KO, but it is unclear if the cause is age dependent as different analyses led to a different conclusion. Additionally, this analysis shows that this

difference in fat mass cannot be solely on account of the decrease in lean mass between genotypes.

At 6 weeks into the high fat diet challenge, the KO mice were lighter and ate less high fat food across 6 weeks than did littermates. However, there was no difference in Genotype for weight gained over 6 weeks. Analysis of fat gained from baseline showed KO gained less fat compared to WT and HET, so that even a partial loss of *trhde* affected fat gain. A descriptive comparison of Genotype for lean mass suggested that KO gain more lean mass than WT littermates, but this analysis did not gain significance. Nonetheless, the present results show that loss of TRH-DE function also impacts energy balance with high fat diet feeding. The results are consistent with the hypothesis that *trhde* overexpression may be implicated in obesity and, conversely, that TRH-DE inhibitors may oppose high-fat diet-induced weight gain.

CHAPTER 3: THE ROLE OF TRH-DE ON DEPRESSION AND ANXIETY

According to the National Institute of Mental Health, 6.7% of all US adults experienced at least one major depressive episode and 4.1% of all US adults have been diagnosed with severe anxiety disorder. However, new treatment options are needed. Furthermore, the neurobiology of anxiety and depression remain incompletely understood.

T3 thyroid hormone and TRH have long been recognized for their antidepressant effects in humans (Kirkegaard and Faber 1998). For a peripheral effect, T3 hormone and TSH were reported to increase the effectiveness of tricyclic antidepressants (Prange, Wilson et al. 1969, Prange, Wilson et al. 1970). Furthermore, TRH was found to have antidepressant potential in the pargyline/l-dopa potentiation test and was found to have an immediate but brief antidepressant effect after a single injection in a double blind study (Prange, Lara et al. 1972). Yet, there were subsequent studies, some of which were double blind, that did not yield the same result (Stein and Avni 1988).

More recent studies have attempted to elucidate this conflict. Notable among these, one study found decreased TRH mRNA expression in the PVN of patients with major depression when controlled for age, sex, and nonthyroidal illness (Alkemade, Unmehopa et al. 2003). Furthermore, a double blind, placebo controlled study found that nocturnal intravenous TRH administration produced a quick but transient antidepressant effect in some patients with bipolar type 1 and bipolar type 2 major depression (Szuba, Amsterdam et al. 2005). TRH-R1 knockout mice showed increased depressive- and anxiety-like behaviors (Zeng, Schimpf et al. 2007), potentially due to a decreased TRH-ergic response.

In this chapter, we examined the role of TRH-DE on modulating anxiety- or depressive-like behaviors. We predicted that a reduction of TRH-DE function by targeted gene knockout would have an anxiolytic- or antidepressant-like effect.

3.1 METHODS

Subjects

Trhde^{tm1Lex} heterozygote mutant mice on a B6/129s5 background were obtained from the UC Davis Mutant Mouse Regional Resource Center (MMRRC) and mated. The resulting male and female *trhde* knock out (KO, *trhde* (-/-)), wild-type (WT), and heterozygous (HET) offspring mice were used for this study. The genotypes were determined by PCR for 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C, 75 seconds at 72°C. The primer sequences (wild type forward: CTC TCG GAC CCG TGG GCT G, wild type reverse: GCA TCA AAT TGT AGT GCA AGC G, knock out forward: GCA GCG CAT CGC CTT CTA TC, knock out reverse: CCA CTG TAA CCT GAG AAG TTC) resulted in PCR products of 494 base pair (KO) and 421 base pair (WT).

The animals were group housed in a constant 12 hour light, 12 hour dark cycle at all times (lights on at 06:00 am) with access to chow (LM-485 7012, 17% fat [kcal], 58% carbohydrate, 25% protein; 3.1 kcal/g; 16% saturated, 26% monounsaturated and 58% polyunsaturated fats; 2.7% kcal from saturated fat, Harlan, Indianapolis, IN) and water *ad-libitum*. In this study, the subjects were sequentially exposed to the elevated plus maze (EPM), light-dark box (LD), and forced swim test (FST). All behavioral tests were performed during the light cycle. Videotaped behavior was scored naïve to genotype with

custom software designed by Dr. Attila Szücs to score behavioral events and analyzed by ANOVA. A cohort from this study was tested in operant self administration.

Elevated Plus Maze

The EPM apparatus consisted of four arms arranged in a plus shape. Two black open arms (no walls) were aligned across from each other, and two black opaque closed arms, perpendicular to the open arms, extended from a center platform. The open arms were illuminated to approximately 10 *lux*, the closed arms measured at 0 *lux*.

The naïve mice were transported to the experimental room and left to habituate for 1 hour under dim red light before exposure. Mice were placed in the central area facing a closed arm and allowed to explore for 5 minutes. Parameters scored were amount of time spent in the central, open, and closed areas; and number of closed arm and open arm entries. The measures of anxiety in the EPM have been reported to be the per cent time spend in the open arm time, and the per cent open arm entries, where the more time spent or entries into the open arm is thought to be an indicator of less anxiety-like behavior (Rodgers and Johnson 1995). According to the factor analysis of the EPM, the activity measures most correlate to the number of closed arm entries or total arm entries.

Light-dark box

The light-dark box consisted of a larger compartment (29.2 x 27 x 26.6 cm) connected to an adjacent smaller compartment (15.2 x 27 x 26.6 cm) by an entry hole measuring 7.6 x 7.6 cm. The larger “light” compartment was brightly illuminated

approximately 800 *lux* with a light source directly above it. The smaller "dark" compartment was illuminated approximately 20 *lux* due to light shining from the light box thru the entry.

The mice were transported to the same experimental room to habituate under dim red light 30 minutes before exposure. Mice were placed in the smaller compartment facing away from the light box and allowed to explore for 10 minutes. Animals were scored for total time in each compartment, latency to enter the light box, and number of transitions. The measures of anxiety in the LD box include the latency to enter the light box and the total time spent in the light compartment, where a longer latency, or a shorter amount of time spend in the light box each indicates an anxiogenic-like phenotype in rodents (File 2001, Bourin and Hascoet 2003).

Forced Swim Test

Each FST apparatus consisted of a large clear plastic cylinder filled with a foot of water at 25-30°C, the water line was greater than a foot away from the top of the cylinder. The FST is one model of learned helplessness that measures behavioral despair by determining levels of immobility versus active swimming or climbing (Cryan, Valentino et al. 2005). It has been traditionally used to determine potential drugs for clinical antidepressant activity. Furthermore, certain classes of antidepressants have produced different activity behaviors; for example, SSRIs increased swimming behavior while drugs affecting catecholamine activity increased climbing behavior (Cryan, Valentino et al. 2005). The water level was at least a foot below the top of the cylinder.

The mice were tested for 6 minutes, and the last 4 minutes were analyzed for time spent swimming, climbing, or immobile.

Operant Self-Administration

Male and Female TRH-DE null mutant (KO) and WT littermates were compared for operant self-administration. Clear operant testing chambers were equipped with two nose poke sensors, one each responding for liquid and food reinforcement. Each chamber was housed in a larger box equipped with a ventilation fan and a light source programmed to follow the home light-dark cycle. The sensors were located on one side of the chamber wall, separated by a food pellet dispenser, and a well providing liquid reinforcement. A nose poke required a 25 centisecond hold to receive a reward (one food pellet or 20 ul liquid). Mice were trained initially on a continuous reinforcement schedule: FR1 for a 24 hour acquisition phase with water and food pellets (Dustless Precision Pellets, 20 mg, Rodent Grain-Based Diet, BioServ #F0163) where 1 response delivered 1 reinforcer. The FR requirement was increased to FR2 (where 2 responses delivered 1 reward) for the next two days of testing, at 3 hours per session, with water and food pellets. A highly reinforcing 3% sucrose- 0.125% saccharin solution (colloquially called "super-sac") was employed as a FR2 reinforcer during the next 4 test days at 3 hours per session. The super-sac solution was kept at room temperature. Responding at the food lever was not reinforced.

Three hour Progressive Ratio (PR) sessions were employed the next 3 test days with supersac. The progression schedule was determined by the formula:

$$\left[4\left(e^{\#\ of\ reinforcers\ x\ 0.075}\right) - 3\right] * 8$$

The number of water rewards, responses, and breakpoint was recorded. Responding at the food lever was not reinforced.

Operant Self-Administration after Swim Stress

After a month without operant testing for PR with supersac ("deprivation"), adult male and female *trhde* KO and WT littermates were retrained with five 3 hour PR sessions with supersac, as described above. Once the baseline was reestablished, animals were tested daily under the same PR reinforcement schedule over 4 sessions to determine possible differences in response or breakpoint after swim stress.

Swim stress conditions were identical to that described above for the FST test. Animals were housed in a holding cage for 30 minutes to dry before the PR sessions began. All tests were conducted in the morning during the light cycle.

3.2 RESULTS

***Trhde* null mutant mice showed no differences in EPM and Light-dark box tests**

While the EPM and LD tests are exploration based tests used to measure anxiety-like measures, the two tests have different degrees of anxiogenic illumination because the LD test used a greater level of brightness (800 lux) than the EPM (10 lux) (File 2001). Both tests were performed to circumvent the specificity of each test and to ensure that the results can converge to the same conclusion. As shown in Figure 4, *trhde* knock-out mice showed no difference in per cent open arm time, or per cent open arm entries compared to WT or HET littermates in the EPM test. Likewise, in the light-dark box test, *trhde* KO

mice did not significantly differ in latency to first enter the light box, nor in total time spent in the light box, compared to WT or HET littermates.

TRH-DE heterozygote and knock-out mice display greater climbing and swimming behavior than wild-type

To measure the effect of a targeted *trhde* gene knockout in depressive-like behavior, male and female *trhde* null mutant (KO), heterozygote, and WT littermates were exposed to the forced swim test (FST). As shown in Figure 5a, a main effect of Genotype was observed for forced swim immobility ($F(2,60)=4.36, p<0.02$), whereby KO ($p=0.01$) and heterozygote ($p=0.02$) mice were significantly less immobile than WT littermates. A main effect of Sex for climbing reflected that males climbed more than females ($F(1,60)=8.926, p<0.005$). On the other hand, female mice trended to swim more than male mice ($F(1,60)=3.403, p=0.070$). There also was a trend for KO to spend more time swimming than WT littermates ($p=0.054$).

Male TRH-DE knock-out mice showed increased water and food administration at FR2 following acquisition

An effect of Genotype demonstrated that TRH-DE KO mice self-administered more food compared to WT controls at FR2 ($F(1,28)=8.793, p=0.006$). Furthermore, as shown in Figure 6, Sex x Genotype interactions showed that male, but not female, TRH-DE KO mice self-administered more water ($F(1,28)=12.499, p=0.001$) and food ($F(1,28)=5.362, p<0.03$) compared to WT controls at FR2 following the 24 hour acquisition training. However, there were neither sex nor genotype differences seen in

operant self-administration during the initial stage (overnight FR1) of food and water acquisition or for self-administration of the palatable sucrose-saccharin solution.

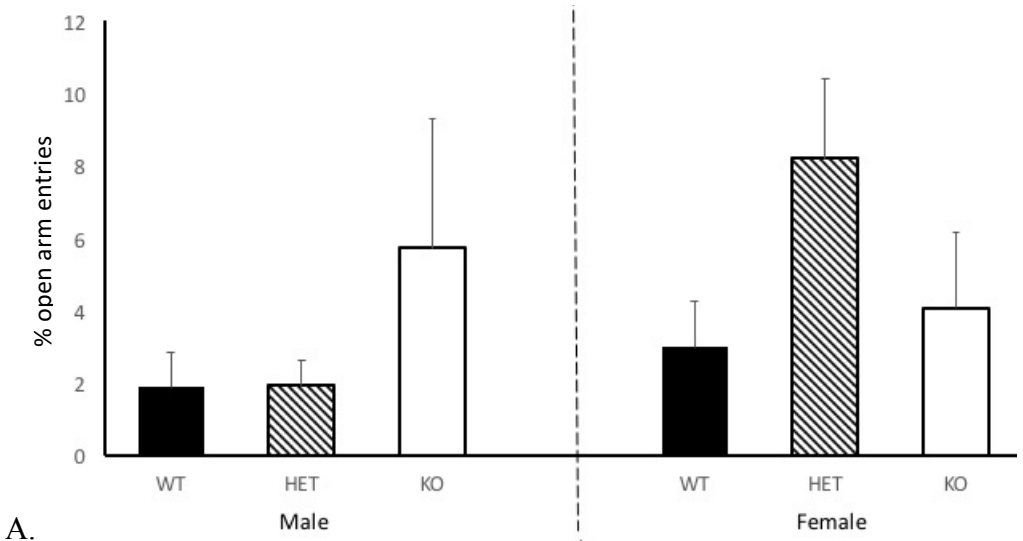
Progressive Ratio response did not change between groups even after swim stress

To measure possible differences in motivation states among groups after a swim stress session, PR schedules with super-sac were performed after the FR2 tests (not pictured).

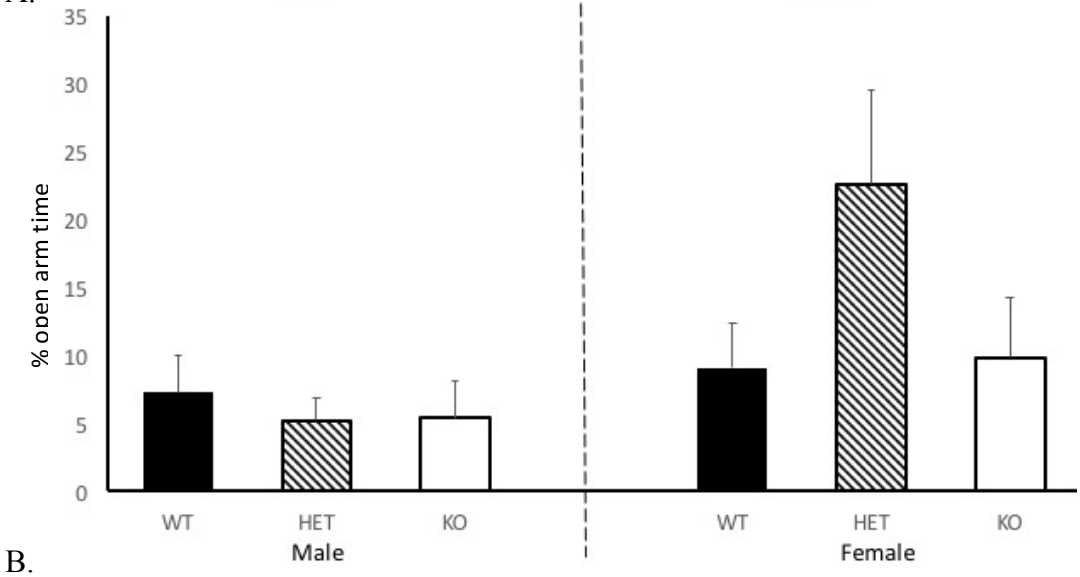
As seen in Figure 7, there was a significant Genotype x Sex interaction ($F(1,15)=5.882$; $p<0.03$) for the re-establish sessions, where the same cohort was challenged with super-sac on a progressive ratio schedule after 1 month had passed since the last PR session with super-sac. Male KO mice tended to respond for a higher number of reinforcers than KO female littermates ($p=0.055$) as did male KO mice over the WT male littermates ($p=0.083$). A paired samples t-test indicated female WT mice responded for a higher number of reinforcers after deprivation ($p=0.002$), as did male KO mice ($p=0.001$) and male WT mice ($p<0.03$). There was no difference in PR response in the session before the month long break (represented as "pre-deprivation"). Additionally, there were no differences of Genotype or Sex x Genotype interactions of Breakpoint, although there was a slight trend towards an effect of Sex ($F(1,23)=3.547$;$p=0.072$).

After one swim stress session, the number of reinforcers rewarded in PR decreased (Figure 8) across all test groups ($F(1,15)=7.381$; $p<0.02$). This effect of Stress was significant throughout all four stress sessions ($F(4,60)=7.116$; $p<0.0001$), including the last stress day compared to Pre-stress ($F(1,15)=10.93$; $p=0.005$). There was no significant effect of Sex, Genotype, or Sex x Genotype observed with stress.

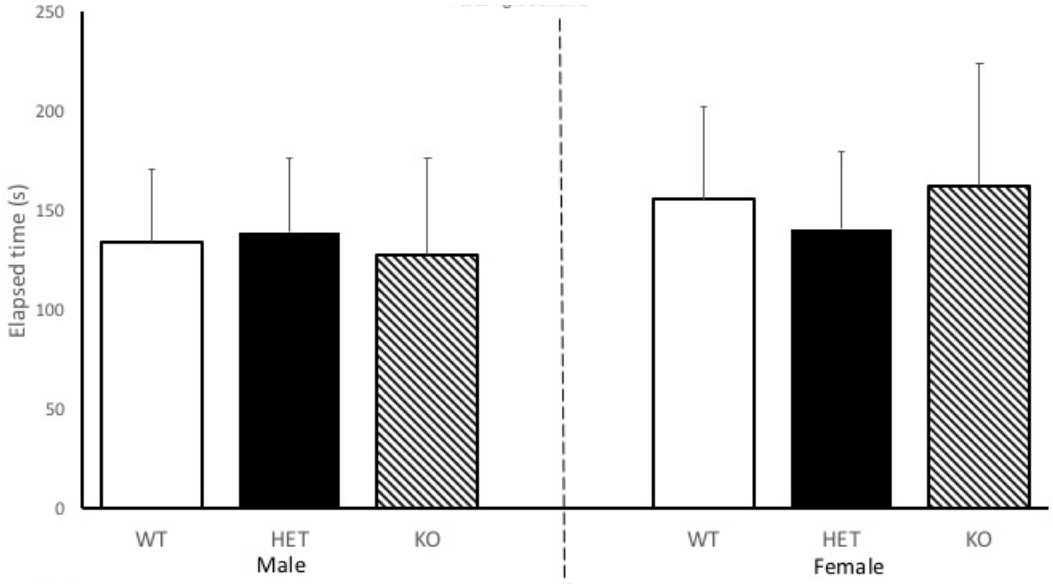
Figure 4. There were no differences between *trhde* KO and WT or HET littermates in tests for anxiety-like behavior. (a-b) Genotype comparisons show no measurable differences in (a) % open arm entries, or (b) % open arm time in Elevated Plus Maze. n=2 in WT group were excluded for leaving EPM apparatus. (c) No differences in total time spent in light box. (d) No differences in elapsed time for latency to enter the light box in the Light-Dark box test. WT (n=25), HET (n=27), KO(n=14). Data expressed Means + SEM.


12
10
8
6
4
2
0
% open arm entries
WT
HET
KO
WT
HET
KO
Male
Female

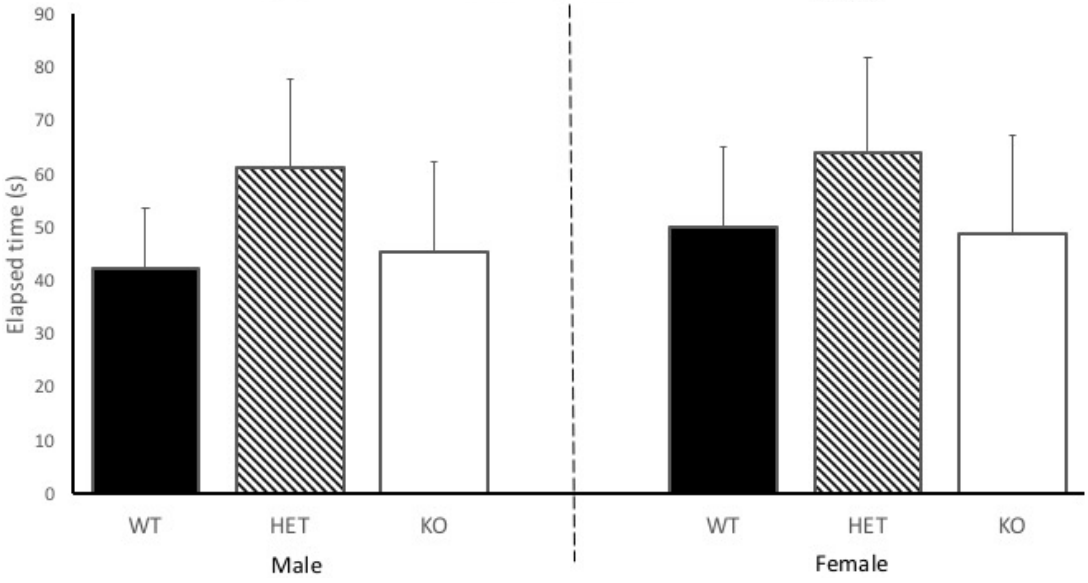
A.


35
30
25
20
15
10
5
0
% open arm time
WT
HET
KO
WT
HET
KO
Male
Female

B.



C.

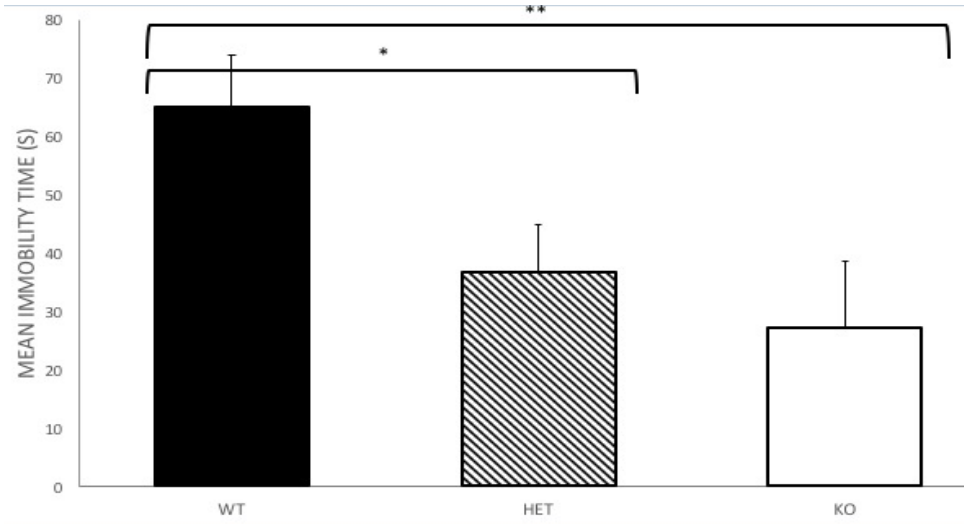


D.

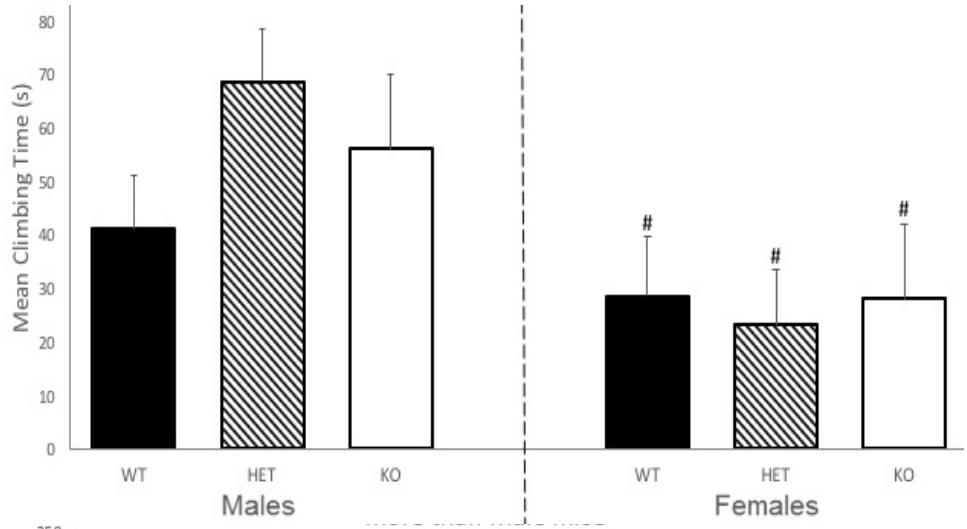
Figure 4 Continued

Figure 5. *Trhde* KO and heterozygote mice display greater climbing or swimming behavior than wild-type littermates. (a) KO and HET mice show decreased immobility in forced swim test compared to WT. Analysis of Genotype accounting for Sex, by 2 x 3 ANOVA. (b) Specifically, male mice showed more climbing behavior ((#) Sex effect, $p<0.005$), (c) whereas female mice trended towards greater swimming behavior ((#) Sex effect, $p=0.07$). HET (n=27), KO (n=14), WT (n=25). Videotaped behavior was independently scored by two trained observers. Data expressed Means + SEM. (*) $p=0.02$, (**) $p=0.01$

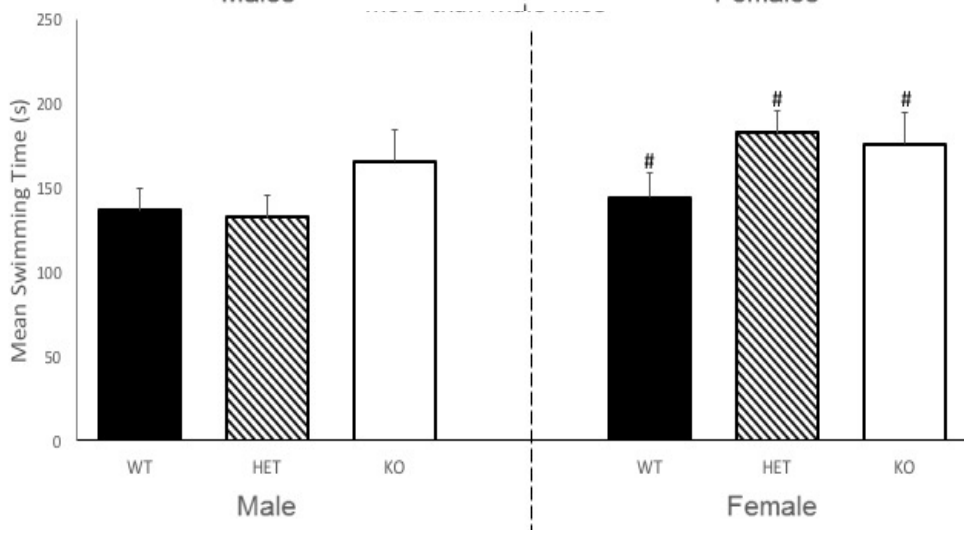
A.

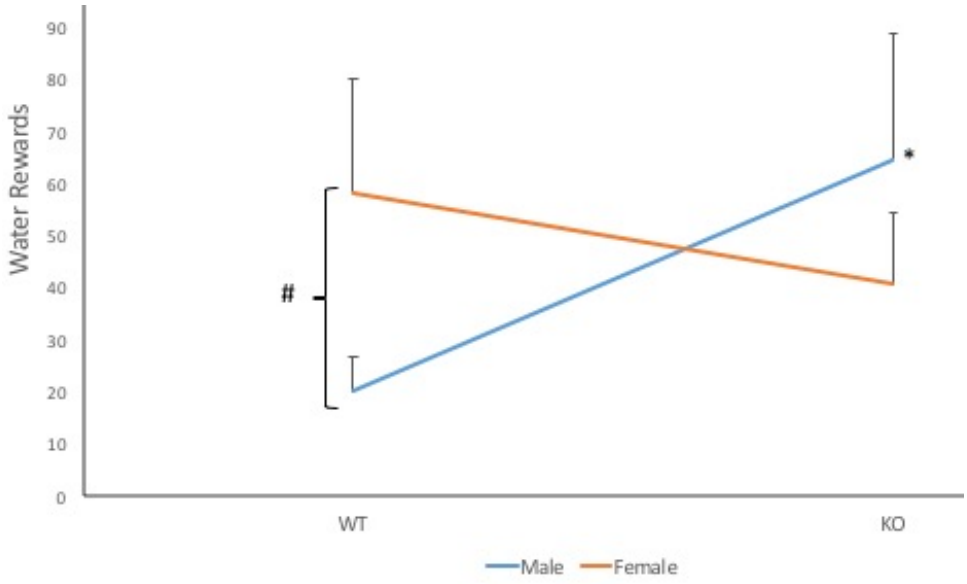

**
*
MEAN IMMOBILITY TIME (S)
80
70
60
50
40
30
20
10
0
WT
HET
KO

B.

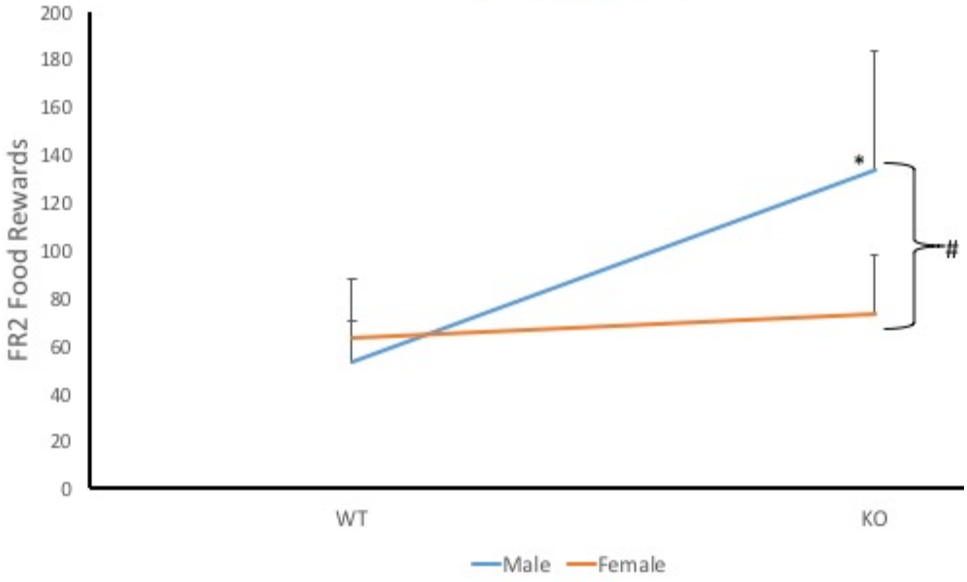

Mean Climbing Time (s)
80
70
60
50
40
30
20
10
0
#
#
#
WT
HET
KO
WT
HET
KO
Males
Females

C.


Mean Swimming Time (s)
250
200
150
100
50
0
#
#
#
WT
HET
KO
WT
HET
KO
Male
Female



A.



B.

Figure 6. Male *trhde* KO mice showed both increased (a) water and (b) food self-administration at FR2 after acquisition compared to WT littermates. (a) Male KO mice had increased water rewards compared to male WT (*) Genotype effect (p=0.001). (#) Sex effect (p=0.005). (b) Male KO mice had increased food rewards compared to male WT (*) Genotype effect (p=0.001). (#) Sex effect (p=0.01). Female WT (n=7), Female KO (n=9), Male WT (n=9), Male KO (n=7). Data expressed Means + SEM.

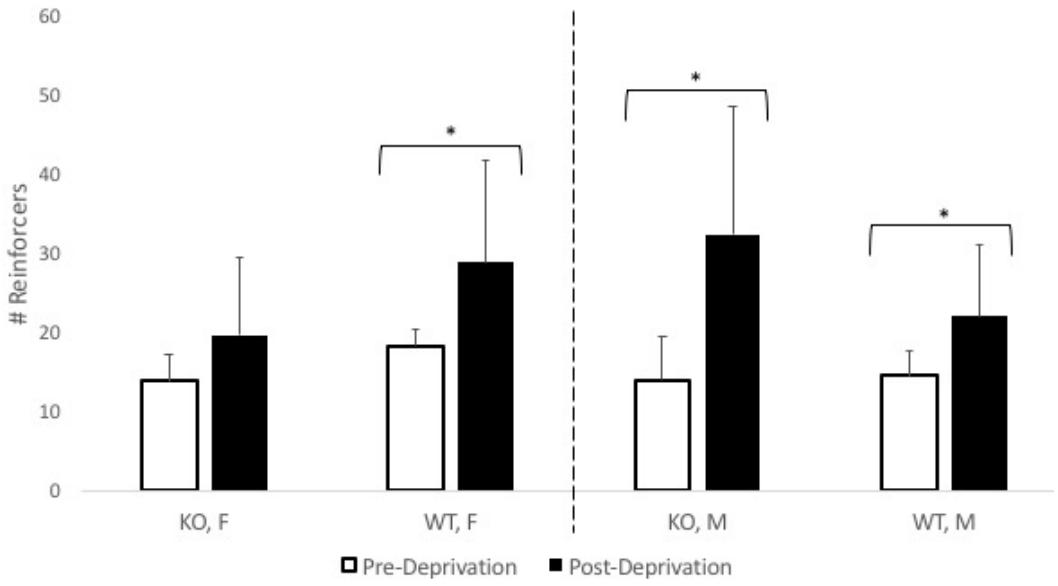
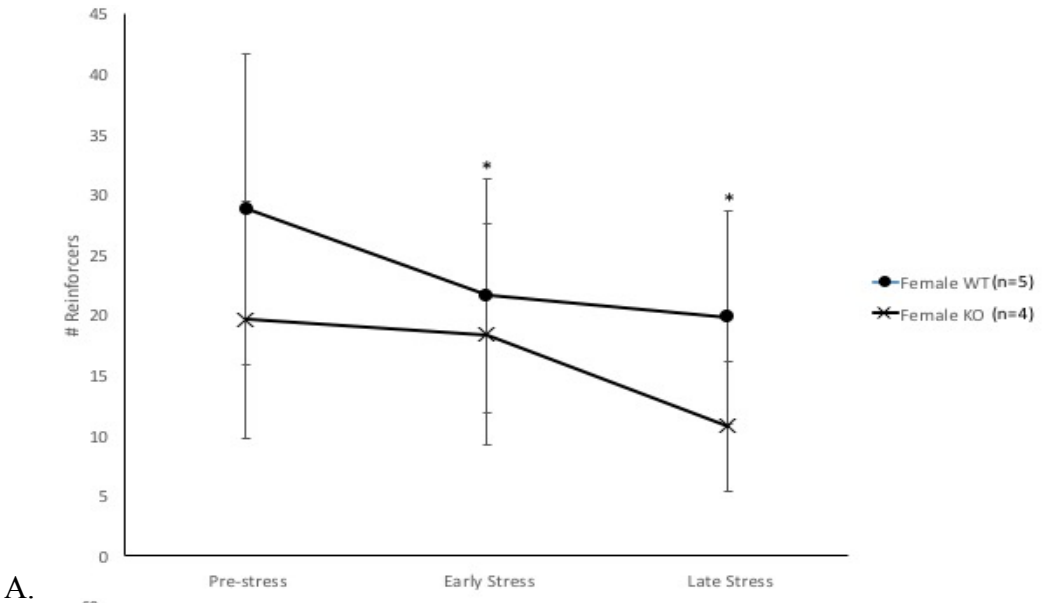
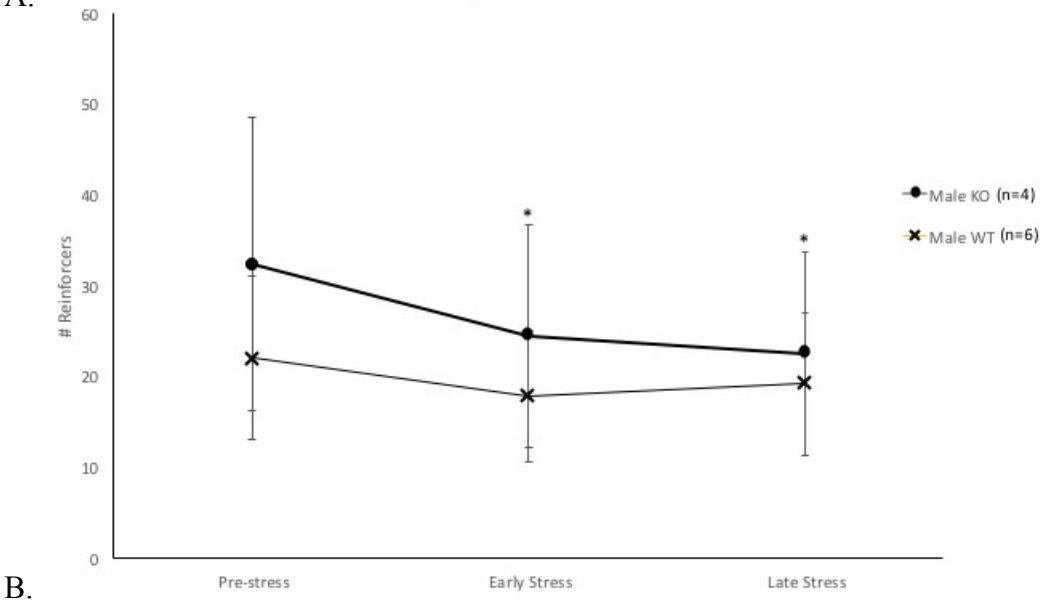


Figure 7. Female *trhde* KO mice respond to super-sac deprivation differently compared to KO or WT littermates. Data expressed Means + SEM. Female KO (n=4), Female WT (n=5), Male KO (n=4), Male WT (n=6). (*) $p<0.05$



A.



B.

Figure 8. Collectively, all groups decreased PR response for reinforcers after swim stress. Early stress values are an average between the first two stress PR sessions. Likewise, late stress values are an average of the last two stress PR sessions. Even after the first stress day, all groups decreased response (p=0.016) for reinforcers. By the last stress day, all groups decreased response for # reinforcers (p=0.005). Data expressed Means ± SEM. Female KO (n=4), Female WT (n=5), Male KO (n=4), Male WT (n=6). (*) p<0.05

3.3 DISCUSSION

FST analysis suggest anti-depressant-like phenotype of trhde knockout mice

As hypothesized, the *trhde* null mutant mice (*Trhde* -/-; KO) showed antidepressant-like behavior in the forced swim test (FST), as defined by decreased immobility. Activity measured was either swimming behavior (defined as horizontal movement), or climbing behavior (defined as vertical movement, typically along the cylinder edges). Surprisingly, the HET mice also displayed greater activity than WT littermates, intermediate to that of the homozygote KO, suggesting even a partial knockout of *trhde* would result in an anti-depressant-like phenotype from the FST. This is consistent with previously described past studies that reported central TRH administration with improving depressive mood in both humans and rodents for a brief period of time (Prange, Lara et al. 1972, Alkemade, Unmehopa et al. 2003, Szuba, Amsterdam et al. 2005). Furthermore, a study with rats showed induction of TRH synthesis after electroconvulsive shock therapy in parts of the brain associated with depression and sleep in humans (Sattin 1998), suggesting this treatment method for depression may work through central TRH action. As TRH-DE is localized to the brain (Heuer, Schafer et al. 1999) and highly specific for degrading released TRH (Charli, Cruz et al. 1988), central TRH action potentially can be modulated by moderating TRH-DE activity or levels.

The circadian pattern of TRH signaling may play a significant role in the anti-depressant potential of TRH. In humans, nocturnal TRH administration presented an anti-depressant response in patients with bipolar type 1 and 2 major depression (Szuba, Amsterdam et al. 2005). Our FST sessions were performed during the light cycle. TRH

signaling has been shown to be most sensitive during the light cycle of rodents (Angel Vargas, Uribe et al. 2002), which aligns with nighttime in humans as the period of decreased activity.

A chief concern that must be addressed with the FST in analyzing antidepressant potential is that of locomotion or activity levels (Cryan, Valentino et al. 2005). As the FST is an activity based test, false positives may occur if a factor, such as a drug, stimulates locomotion. TRH administration has been shown to increase locomotion (Gary, Sevarino et al. 2003) in rats and, although it failed to gain significance, the *trhde* KO mice did trend toward increased activity in the dark cycle but not in the light cycle (Frihauf 2014).

Analysis of total arm entries in the EPM may be used to determine activity (Rodgers and Johnson 1995). Our analysis of total arm entries (not pictured) did not show differences in activity between KO, HET, and WT littermates. As all of our behavioral tests were performed in the light cycle, while it is possible that the *trhde* KO mice may show increased locomotion during the dark cycle, our results on the possible role of TRH-DE in presenting anti-depressant-like effects show potential but warrant further study by measuring anti-depressant potential through a non-activity based assessment like intracranial self-stimulation (ICSS), or by assessing FST results after inducing stress related depression.

Trhde knock out mice did not show an anxiolytic-like response to EPM or LD tests when compared to WT or HET littermates

We predicted that the *trhde* KO mice would demonstrate less anxiety-like behavior than WT or HET littermates; without the degrading enzyme, TRH levels will be increased in those animals and may present a similar anxiolytic-like effect as demonstrated by past studies of TRH and the TRH pathway (Zeng, Schimpf et al. 2007, Gutierrez-Mariscal, de Gortari et al. 2008). Contrary to our hypothesis, neither the EPM nor the LD test demonstrated differences in anxiety response between *trhde* knock out mice and their littermates. While our animals were submitted to the EPM and LD tests during the light cycle, past studies showed that EPM measure of anxiety did not depend on the circadian rhythm of TRH (Gutierrez-Mariscal, de Gortari et al. 2008, Gutierrez-Mariscal, Sanchez et al. 2012). There are few other possibilities, such as a difference in methodology where the apparatus is differently illuminated (File 2001, Walf and Frye 2007). Additionally, these past studies have either measured anxiety levels in rats (as opposed to mice), or used a mouse line raised on a different background (van Bogaert, Groenink et al. 2006, Savignac, Dinan et al. 2011).

As our *trhde* null mutant mice were constitutive knock-outs and have experienced the life-long changes in *trhde* gene expression, our unexpected result may be due to constitutive effects where the neurotransmitter system compensates for the lack of TRH-DE by decreasing ligand production, for example (Groenink, Pattij et al. 2003). When comparing pharmacological studies to those that used constitutive knock-out animals, it is important to consider these compensatory effects on the study. Furthermore, the above cited pharmacological studies are due to acute administration of TRH to which the body may be providing an anxiolytic-like response to the injection. The TRH administration may also be a higher dose compared to endogenous levels. In both cases, the body might

potentially adapt. Future studies may consider differences in anxiety-like response to chronic TRH administration, or a conditional *trhde* KO where *trhde* expression may be turned off acutely.

Zeng, H., et. al (Zeng, Schimpf et al. 2007) observed that TRH-R1 knock-out mice displayed increased anxiety-like behavior, suggesting that the TRH pathway may be involved in providing an anxiety response. A comparison of our KO models would show a difference in modification of neurotransmitter signal transmission. Whereas a receptor KO would change the level of transmission post-synaptically from normal to no signal transmission, a TRH-DE KO would theoretically cause increased transmission due to an increased and sustained TRH level in the synapse. It is possible that differences in the relative level of signal transmission compared to WT littermates may be the determining factor in anxiety response.

It is also possible that previous pharmacological studies with TRH administrations may be activating parts of the brain with little to no *trhde* expression. This possible mismatch may be compared to our knock-out study. While past in-situ hybridization studies found expression of TRH-DE mRNA in parts of the brain, like the amygdala, that modulates anxiety behavior (Heuer, Ehrchen et al. 1998), it is possible that TRH-DE is not expressed on the neurons that modulate the anxiety response circuit.

Operant self-administration reveals Trhde KO mice may learn faster than littermates, with no difference in anhedonia traits even after swim stress

Anhedonia is defined as a certain reduced ability to experience pleasure or interest in rewarding activities. According to the American Psychiatric Association's DSM-V,

anhedonia is one of the primary symptoms of Major Depressive Disorder (MDD). Methods to assess anhedonia in animals are reviewed extensively in (D'Souza and Markou 2010) . In brief, assessment of reward can be measured directly in the brain by the intracranial self-stimulation procedure (ICSS), or by assessing the motivation for rewarding stimuli. One way to assess motivation is by measuring progressive-ratio (PR) response, particularly with a sucrose reward/reinforcer. In a PR schedule, the number of responses an animal has to provide increases after each reward is given; therefore, an animal has to put in more effort each time to receive the next reward until it fails to receive a reward within the allotted time (15 minutes). The amount of effort the animal puts in is thought to be a measure of motivation. The point at which an animal stops engaging in self administration is defined as the breakpoint.

Animals were initially trained on a fixed-ratio (FR) response schedule before they were tested on a PR schedule. As FR schedules of reinforcement are used to train animals to respond in a specific manner for rewards, differences in FR responses may be used to indicate differences in learning and memory. While there were no differences in Genotype, Sex, or Sex x Genotype interactions in the initial acquisition phase (FR1), male *trhde* KO mice self-administered more food and water at FR2 in the following test day. This Sex x Genotype difference was not seen in later sessions of self-administration, including with super-sac, indicating that the male *trhde* KO mice in particular were faster at learning the FR self-administration task as compared to their littermates. This is consistent with previous reports of TRH being implicated for improved learning and memory (Lechan and Fekete 2006).

There were no differences of Genotype, Sex, or Sex x Genotype within each PR session with super-sac. However, to confirm if the differences in depressive-like behavior from the FST were due to genotypic differences in anhedonia, animals were exposed to swim stress before assessed for PR responding. Past studies have suggested that chronic mild stress may cause a decreased sensitivity to reward (Moreau, Scherschlicht et al. 1995). We predicted that the *trhde* KO would be less susceptible to the decreased breakpoint that results from swim stress, in parallel with the anti-depressant-like phenotype in the FST. There were no observed differences in PR among genotypes or sex within each session, however. Rather, all of the groups decreased PR responding with stress, indicating that the reward (in this case, super-sac) diminished in value similarly after stress across genotypes.

Male mice had an exaggerated response to PR conditions after deprivation compared to female mice

Interestingly, all groups increased PR responding with super-sac after a month-long deprivation period, albeit the average response for the female *trhde* KO mice did not obtain significance. This deprivation effect has been previously described in saccharin and in drugs of abuse like ethanol (Zakharova, Malyshkin et al. 2004). Sugar has also been shown to induce similar patterns of craving like those of cocaine, heroin, and methamphetamine where sugar seeking increases after periods of extinction or abstinence (Grimm, Fyall et al. 2005).

Furthermore, male mice had an exaggerated response to deprivation compared to females where male mice responded for more rewards than did females. While the

addictive potential of sugar in rodents has been suggested to be more powerful than the addictive potential of cocaine (Lenoir, Serre et al. 2007), and whereas caloric and non-caloric sweeteners have been shown to be involved in glucose intolerance (Ramasarma and Rafi 2016) and other health problems to the point it has been labeled “toxic” (Lustig 2013), few studies have investigated the possible sex specific differences in sweetened beverage intake or seeking behavior. Here, we report that sugar deprivation may present greater seeking behavior in male mice than in female littermates.

CHAPTER 4: CONLUSIONS

The goal of this project was to characterize how loss of *trhde* expression may functionally affect 1) energy balance throughout adulthood and due to high-fat intake and 2) mood disorders, specifically anxiety and depression. While TRH and thyroid hormone effects on energy and depression or anxiety have been well studied, the effects of TRH-DE are less well-described. Targeting TRH-DE with inhibitors may offer a different, and potentially more beneficial, translational approach to treating obesity and mood disorders. While administration of agonists like TRH may cause off-target effects, targeting TRH-DE's specific location in the CNS may prevent peripheral side effects of TRH administration. Furthermore, drugs that act as agonists may cause possible compensatory effects that result in tolerance; administration of TRH-DE inhibitors may avoid that issue by augmenting the natural physiology, rather than fundamentally altering it.

The studies described in Chapter 2 described the functional role of TRH-DE on energy homeostasis. Loss of TRH-DE function presented an altered circadian profile of energy expenditure. Furthermore, there was a significant difference in fat gain and food intake which was apparent even on a high-fat diet challenge. Presented together, these results show another possible genetic role where *trhde* overexpression may increase obesity risk.

The studies described in Chapter 3 described possible effects of TRH-DE on depression and learning. While initial tests seem to indicate the anti-depressant-like potential of the functional loss of TRH-DE, future studies are needed to ensure the differences are not solely due to activity levels.

Future studies

Future studies may examine the effect of TRH-DE inhibitors in site-specific regions of the limbic brain to determine differences in anxiety or depression levels. These studies can also test the relative effectiveness of different TRH-DE inhibitors to decreasing obesity risk or improving mood.

Future studies may also look at the circuitry of TRH-DE to determine which neurons it is expressed in. Examining the co-expression of TRH-DE to dopaminergic or GABA-ergic neurons, for instance, may elucidate a mechanism of TRH-DE action to support the phenotypes described in this report.

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