Fall 2019

Motivational and Physiological Dysregulation Due to Development and Onset of Obesity via Melanocortin 4 Receptor +/- Haploinsufficiency

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Bachelor of Arts
University of South Carolina, 2016

Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Arts in

Experimental Psychology

College of Arts and Sciences

University of South Carolina

2019

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Abstract

Obesity is one of the leading most health risks around the world, being especially problematic in the United States. A combination of high-fat diets and genetic abnormalities are to blame for the ever-growing number of obese individuals. Melanocortin 4 receptors are vital for regulating energy expenditure and feeding behaviors; mutations of the receptors have been found to be the leading monogenetic cause of obesity. Using MC4R +/- haploinsufficient rats being fed a range of dietary fat, we investigated the physiological and motivational differences using a locomotor task, an operant task with fixed and progressive ratios, as well as a distraction operant task. Percentage of lipid deposits in the liver of each rat was also analyzed using the Area Fraction Fractionator probe for stereological measurements. MC4R +/- haploinsufficiency resulted in a phenotypic resemblance for adult-onset obesity that is worsened by poor dietary fat consumption. Results from the operant tasks indicate that motivational deficits due to MC4R +/- haploinsufficiency can be seen prior to the onset of obesity. Post-obesity motivational deficits may be dependent on dietary fat consumption. Given the full results, the MC4R circuit ties closely with the motivational dopamine circuit providing a possible target in the prevention of adult-onset obesity before developme

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Chapter 1. Introduction

Obesity in the United States is a major health risk for both children and adults. The epidemic has caused serious consequences that affect the individual, communities, as well as the economy. In 2014, reports estimated that the national total cost of overweight and obese individuals was $149.4 billion yearly, with an average cost of the individual being $1901 yearly (Kim & Basu, 2016). Over the past few decades, trends in obesity have continued to increase. Specifically, childhood obesity has seen a large increase in the past 30 years. An increase from 7% to 18% prevalence of obesity in children ages 2 to 19 has been seen since 1988. Extreme obesity has also seen an increase reaching 5.8% in the same age children (Ogden et al., 2016). It is clear there is a drastic increase in the obese population seen over the past 30 years.

Obesity is a multifactorial issue. The lack of exercise and a high-fat diet lifestyle that many Americans have adopted is the most common issue. Besides societal negligence, many other genetic and central nervous system dysregulations contribute to obesity. The leading monogenetic cause of obesity in the United States is the deletion or mutation of the melanocortin-4 receptor (MC4R). The mutation has been estimated to affect approximately 6% of the clinically obese population (Farooqi et al., 2003). The melanocortin system as a whole controls regulatory behaviors that include body weight, energy intake, energy expenditure, as well as sexual function (Ho and Mackenzie 1999).

The arcuate nucleus of the hypothalamus is a relay area that regulates and reflects metabolic status. The arcuate nucleus is part of the melanocortin system that is regulated by leptin (Adan et al., 2006). The arcuate nucleus is compromised of two main pathways; the anorexigenic and the orexigenic. The two pathways have been studied for decades beginning with the early lesion studies in the arcuate nucleus (Hetherington and Ranson., 1940, Anand and Brobeck., 1951).

Melanocortins are products of the proopiomelanocortin (POMC) prohormone (Gantz & Fong, 2003). Neurons that contain melanocortins can be found in many areas of the brain but are primarily found inside the arcuate nucleus. POMC neurons, for example, have feeding behavior pathways that project to the dorsal vagal complex of the brainstem and the intermediolateral cell column of the spinal cord (Mercer et al., 2013). The activity of the melanocortin system is regulated by endogenous melanocortin receptor agonists, α-melanocyte-stimulating hormone (α-MSH), β-MSH, γ-MSH (which are all derivative of POMC), as well as agouti-related protein (AgRP). POMC neurons release α-MSH, which serves as an agonist to melanocortin receptors, thus causing a suppression in appetite. AgRP neurons activate the opposite pathway, which inhibits appetite suppression activated by a negative energy balance that is regulated by leptin. AgRP is as an inverse agonist for two of the major receptors of the melanocortin system, melanocortin 3 and melanocortin 4 (Adan et al., 2006). While the melanocortin system has five different receptors, MC4R is the most relevant to obesity because of its relationship with the POMC and AgRP pathways. The MC4Rs are G protein-coupled receptors that are located downstream of POMC neurons. Because of their location and

function, the MC4Rs are the most influential of the MC receptors in regards to feeding behavior and energy expenditure, as well as general energy homeostasis (Smith et al., 2007: Krashes et al., 2016).

The most unique aspect of MC4Rs is that deletion or mutation of the receptors reacts in a functional gene dose-response manner. Briefly, the heterozygous mutation shows a lesser loss of function compared to that of the homozygous mutation (Tao & Ya-Xiong, 2010). The current knowledge of the MC4R functioning has allowed the creation of both mice and rats with MC4R dysregulations that have been used to study the role of MC4Rs in as obesity.

Control wild type rats have Mc4R mRNA expression that begins around postnatal day 14. By postnatal day 18, it is expressed throughout the brain, primarily in the hypothalamus, amygdala, thalamus, and the hippocampus (Tao & Ya-Xiong, 2010). The MC4R haploinsufficient rats are created using Wistar strain rats that have a mutation that produces a stop codon located further upstream than its usual position, which results in the truncation of 18-amino acids in helix 8 of the receptor. The mutation results in the phenotypic change in progressive obesity, hyperphagia, decreased grooming behavior, as well as reduced ambulatory activity in the rat (Mul et al., 2012). The resulting progressive obesity mimics human obesity in normal populations; thus suggesting that the MC4R haploinsufficient is a valid model to its human counterpart. Other phenotypical changes in the MC4R haploinsufficient rat are lesser known. Changes in taste preference as well as motivation regarding food acquisition and consumption require further research.

The consequences of obesity, specifically that of a high-fat diet, are well known. One such consequence is a range of non-alcoholic fatty liver diseases. Steatosis, which is a milder form of steatohepatitis, is the accumulation of lipid deposits in the liver (Lieber et al.,2004). The disease was previously thought to only occur with heavy alcohol use but has since been directly linked to high fat dietary food (Ludwig et al., 1980). Interestingly, melanocortin activity in the amygdala has been shown to regulate dietary fat appetite control (Boghossian et al., 2009). Steatosis and the effects of dietary fat have been studied using rats previously (Ahmed et al., 2009); however, no study has shown the possible effects of MC4R mutation.

The present study investigates the possible physiological and motivational changes due to MC4R haploinsufficiency, dietary fat, or the relationship between the two. Given the known dose-dependent effects of mutation of MC4Rs, the haploinsufficient +/- rat was chosen to model the development of adult-onset obesity. Selection of diets (0% -12% saturated fat) was specifically chosen to be clinically relevant to a range of modern diets. The addition of the inflammatory group allows for a unique control compared to the other dietary groups. Consumption of high-fat diets should also enhance the progression of obesity. Our guiding hypothesis is that the trajectory to obesity is preceded by alterations in motivational systems, including neuroadaptations in the central nervous system; these alterations in motivational systems will have persistent functional consequences for vulnerability to excessive caloric intake in an obesogenic environment, and the extent of central nervous system neuroadaptations will be exacerbated in an obesogenic environment.

Chapter 2. Methods

2.1 Subjects

Male Wistar Rats (MC4R +/-, n=33; control, n=33) taken from 10 different litters, were weaned at postnatal day 21. After weaning, animals were housed in pairs with one haploinsufficient rat and one control rat per cage and randomly assigned to a diet. Physical characteristics such as weight, crown-rump length, and waist circumference were measured on postnatal days 21-23, 27-29, 34-36, 41-43, 48-50, 62-64, 76-78, 90-92, 120-122, 152-154, and day of sacrifice. BMI was also calculated using weight (g)/(length(cm)2. Animals were kept in an AAALAC accredited (D16-00028) facility at 21 ±2 degrees Celsius, 50% ± 10% relative humidity on a 12-hour light/dark cycle with lights on at 07:00h. All behavioral testing was conducted during the light cycle.

2.2 Dietary Group

Dietary groups were randomly assigned to each cage. The diets include a control diet (n=9 per group) (1.7% Saturated Fatty Acids SFA, with 12.2% total kcal from fatty acids), an inflammatory diet (n=8 per group) (1.7% SFA, with 12.2% total kcal from fatty acids, 20:1 ratio of omega-6:omega-3 unsaturated fatty acids), a low-saturated-fat diet (n=8 per group) (6% SFA, with 40% total kcal from fatty acids), and a high-saturated fat diet (n=8 per group) (12% SFA, with 40% total kcal from fatty acids). While in home cages, animals have ab libitum access to food and water. Diets were chosen to replicate a range of possible diets related to human counterparts

2.3 Timeline and Experimental Design

Animals began activity tasks post weening starting at day 30. All animals repeated locomotor activity and sucrose preference tasks throughout a significant portion of their lifespan. Prior to the development of obesity, animals were assessed using a fixed ratio and progressive ratio operant tasks to assess motivation. Following the onset of obesity, motivation was assessed similarly, using variable progressive ratio and distraction operant tasks. Post sacrifice, steatosis analysis occurred. The overall study design can be seen in table 1.

Table 2.1: Overall study design

Activity Tasks. Postnatal days 30, 60, 90, 120, 150, and 180		
Sucrose Preference Task	Locomotor Activity	
Pre-Obesity Motivational Tasks. Postnatal days 61-120		
Fixed Ratio 1, 3, 5	Progressive Ratio	
Post-Obesity Motivational Tasks. Postnatal day 120-Sacrifice		
Variable Progressive Ratio	No Distraction Task	Distraction Task
Post Sacrifice		
Steatosis Analysis		

2.4 Locomotor Activity

The testing apparatus for the locomotor activity task was a 40 cm by 40 cm square chamber with a circular Plexiglas insert to promote movement. The chamber tracks ambulation and rearing using infrared photocells on an X and Y dimension (Hamilton-Kinder Inc., Ponway, CA). Photocells were tuned by the manufacturer to control for the Plexiglas insert. The test was administered at postnatal days of age 21, 30, 60, 90, 120, 150, and 180 under low light conditions to simulate the nocturnal experience when rats are active. The hits across the photocell grid (32 X 32, spaced 2.5

cm apart) were recorded using Digipro System Software (v. 140, AccuScan Instruments) in real time. Motor Monitor software (Hamilton-Kinder Inc, Ponway, CA) was used to record and monitor movements inside the chamber. Basic movements were defined as a clearing of a beam when a new beam is broken, while rearing was defined as a breaking of an overhead beam.

2.5 Sucrose Preference Test

Sucrose preference testing was administered on days 30, 60, 90, 120, 150, and 180. The animals were habituated to the testing cage on postnatal day 21. For a 20-minute testing session, five sucrose solutions (0, 1, 3, 10, and 30% by volume) were available to the animal. Bottle weight differences were used for the preference analysis. Potential position preference was controlled for by using block randomization and Latin-Square procedure on the bottle sequence.

2.6 Operant Testing Apparatus

The operant task chambers (ENV-008; MED Associates, St. Albans, VT) were housed in a sound attenuated cabinet. The front of the chamber had access to a recessed dipper through a 5cm by 5cm window with infrared sensors to track nose poke time in seconds. The dipper has a 0.1ml cup attached, which is raised into the chamber to allow access to the cup. The cup contains a sucrose solution upon the completion of the required responses. On each side of the opening, 7.3cm above the metal grid floor are two retractable metal levers. On the back wall of the apparatus is a third inactive lever that is located in line with the receptacle. At the beginning of testing, all three levers were presented. Animals underwent various ratio schedules to learn to respond

for continuous reinforcement during 82-minute sessions. After correct operant responses to the active lever, the sucrose solution is presented for 4 seconds, whereas responding on the inactive lever is recorded but not reinforced.

2.7 Fixed and Progressive Ratio Task

On Postnatal day 61, animals underwent a fixed-ratio (FR) 1 schedule for at least 3 days. After three consecutive days of stable responding, defined by greater than 60 rewards during the test period, the animals would be moved to an FR-3 schedule. Similarly, after 3 consecutive days of stable responding, now defined by 120 rewards on the FR-3 schedule, animals were moved to an FR-5 schedule. Upon 3 consecutive days of stable responding on the FR-5 schedule, animals underwent a progressive ratio test. The sequence of lever pressing requirements were 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 179, 603, 737, 901, 1102, 1347, 1646, and 2012, for a maximum of two hours in length for each test.

2.8 Variable Progressive Ratio task

On postnatal day 220, animals underwent the same progressive ratio schedule, with varying concentrations of sucrose reward (1%, 3%, 5%, 10%, or 30%). Each animal received a test for each concentration with a 5% sucrose FR-5 schedule on days in between tests. The total testing took 10 days with a 0% sucrose concentration PR schedule on the last day for extinction prevention. Starting concentrations were block randomized with concentrations shifted using a Latin square design

2.9 Distraction Task

Upon completion of the progressive ratio task around postnatal day 230, animals performed an FR-5 schedule distraction task for 60 minutes. The first test is an FR-5 schedule with a distracting tone (5dB above background fan noise of the chamber) during the middle 20 minutes of the 60-minute test period. The next day, animals were placed on an FR-5 schedule again, with no distraction. Lastly, on the third day of testing, animals were tested on the same FR-5 schedule with the distracting tone played from minutes 5-25, with no tone being played during the remaining of the testing period.

2.10 Steatosis Analysis

Livers of all animals were extracted and stored in -80°C freezer until processed. 32 livers (n=4 per group) were randomly selected to undergo stereology procedures. Each liver was sectioned to 20 micron slices using a cryostat (Shandon Cryotome). Every 18th slice was mounted, then, underwent a histological staining process using Oil Red O to stain for lipid deposits. The following was the histological staining procedure:

- Slices were mounted and placed in a 10% PFA solution for 8-10 minutes
- Washed with distilled water
- Placed in 100% propylene glycol for 3-5 minutes
- Placed in Oil Red O heated to 60 Degrees C for 8-10 minutes
- Placed in an 85% propylene glycol and distilled water solution
- Finally washed once more with distilled water.

To estimate percent volume of fat in each liver a Nikon Eclipse E800 (Nikon, Melville, NY) equip with a motorized LEP MAC 5000 XYZ stage (Ludl Electronic Products, NY) and

Stereoinvestigator (MicroBrightfield Williston, VT, Version 11.09) were used. The Area Fraction Fractionator probe allows randomly selected sampling sites to be determined and used to estimate volume with a sampling grid. For each slice, four sampling sites were determined with a 200x200 um square with markers 8um apart (total of 625 markers) laid over each sampling site. From the stereological count, an accurate estimation of percent volume of fat was calculated by taking points counted divided by total points.

2.11 Statistical analysis

All Statistical analyses were done using IBM SPSS v 24 (IBM Corp., Somers, NY). Graphs and curve fits were made using GraphPad Prism 5.02 (GraphPad Software, Inc. La Jolla, CA). On postnatal day 98, one MC4R +/- haploinsufficient animal on the inflammatory diet was found deceased. Missing data for the animal was replaced with means where appropriate. To detect if there was an effect of litter, we conducted a repeated-measures ANOVA on the bodyweight data using litter and genetic condition as variables. The data was used for its theoretically truer distribution of variance compared to other data sets. Following text from "Some statistical and experimental considerations in the use of the analysis-of-variance procedure" by Denenberg VH (1984) regarding nested designs, a more stringent alpha of .05 was used for our criteria. Litter was found non-significant at this alpha level $F(8,56)=1.783$, $P=.100$. Given that litter was found not significant, statistical analysis proceeded without regard of litter.

Bodyweight was analyzed using a mixed model ANOVA with genetic condition and diet as between-subjects factors and time (day) as a within-subject factor. Two

separate mixed model ANOVAs were run for basic movement and rearing during the locomotor activity tasks. Similarly, condition and diet were between-subject factors while time was a within-subject factor. Sucrose preference task was also analyzed using mixed models ANOVA. The same factors as the previous analyses were used, as well as the addition of the within-subject factor of sucrose concentration.

The progressive ratio task was analyzed using a simple between-subjects ANOVA using genetic condition and diet. The variable progressive ratio task was analyzed using mixed models ANOVA using the same factors as the progressive ratio in addition to sucrose concentration as a within-subjects factor. The distraction was analyzed using mixed models ANOVA as well. The within-subjects factor for the analysis was the 5-minute bins that were recorded throughout the task.

Lastly, steatosis was analyzed using a between-subjects ANOVA. All behavioral measures were tested with the percentage of lipids in the liver using Pearson's Correlation analysis. Measures that were found significantly correlated were then put into a linear or multiple regression model.

Chapter 3. Results

3.1 Both MC4R +/- haploinsufficiency and consumption of high-fat diet cause obesity.

Bodyweight data was used to assess the effect of MC4R +/- haploinsufficiency. Both genetic condition, $F(1,26)=25.499$, $P\leq.001$, as well as diet, $F(3,26)=3.837$, $P\leq.05$ were found significant, however, there was no interaction between the two variables. Figure 1 depicts the differences between both the MC4R +/- haploinsufficient and dietary group. Most interestingly, there are notable differences in weight starting as early as day 120, confirming the idea that haploinsufficiency can be a model for adult-onset obesity.

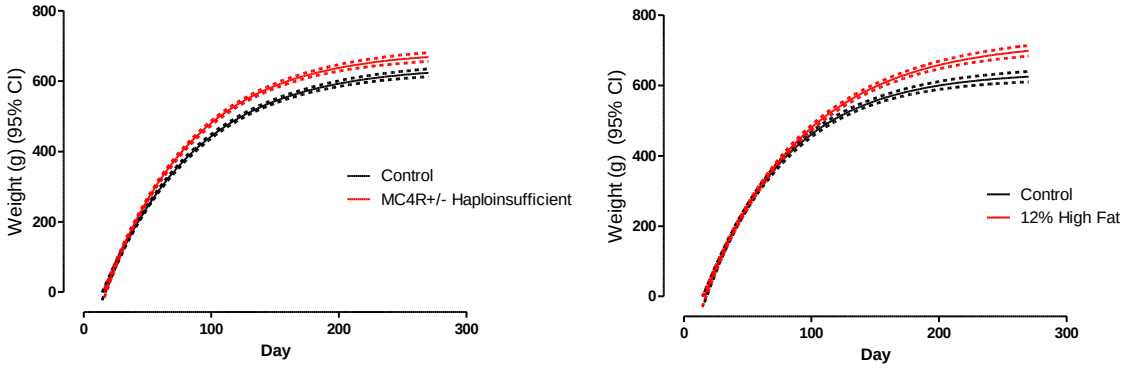

Figure 3.1: Bodyweight of both control and MC4R +/- Haploinsufficient animals. Haploinsufficient animals show a greater peak than the control counterparts do with an exaggerated difference between the high-fat diets and the control diets. Divergence of body weight can be seen around day 120 for both groups.

3.2 MC4R +/- haploinsufficiency combined with poor diet produces deficits only found in rearing.

Locomotor activity was analyzed by creating an area under the curve for each genetic group and dietary condition across all trials, as depicted in figure 2. Basic movement measures show no difference between genetic group, however, indicate a slight decline with an increase in dietary fat. For rearing, control and MC4R +/- haploinsufficient animals yield divergent outcomes. As dietary fat increases, control animals yield an increase in rearing. Conversely, the MC4R +/- haploinsufficient animals yield a decrease in rearing as dietary fat increases. Control and MC4R +/- haploinsufficient animal curve fits for the rearing are 0.92 and 0.98 respectively, with a significant difference between the two lines at $F(2,2)=62.17$, $P \leq .05$.

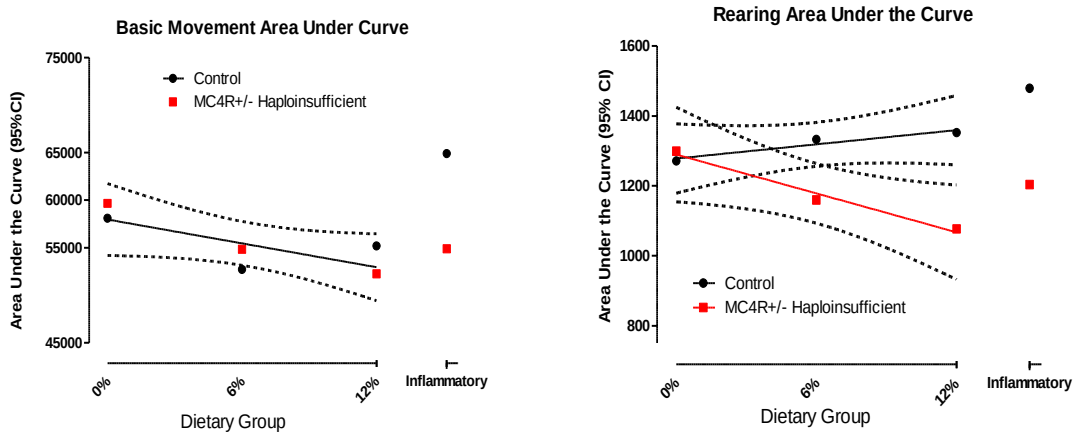

Figure 3.2: Area under the curve measures for both basic movement and rearing were fit to curves. Basic movement yielded no difference between genetic group but indicated a slight decline as dietary fat increased. Rearing yielded divergent results for control and MC4R +/- haploinsufficient animals.

3.3 Motivational deficits due to MC4R +/- Haploinsufficiency are seen prior to obesity onset.

The fixed ratio and progressive ratio operant tasks were used to analyze motivational differences. The tasks were assessed prior to the onset of obesity. None of the fixed ratio operant tasks revealed a significant effect of either genetic condition or diet.

The progressive ratio operant task was assessed specifically at postnatal day 105. At this time point, the effect of the haploinsufficiency was of peculiar interest. Because of that, only the effect of the haploinsufficiency of the animals on the control diet was tested. The results found a significant effect $F(1,16)= 11.645$, $P\leq.05$. The increase responding in the control MC4R +/- haploinsufficient group can be seen in figure 3.

3.4 Post-obesity MC4R +/- haploinsufficiency motivational deficits are dependent on consumption of dietary fat.

The variable progressive ratio operant task was assessed at postnatal day 220 to investigate motivational differences with varied reward concentrations post obesity onset. Diet was found significant, $F(3,283)=4.269$, $P\leq.05$. An interaction of condition and diet was also found significant, $F(3,283)=2.632$, $.P\leq.05$. Animals fed the control diet showed a similar linear increase in responding with an increase in sucrose concentration, regardless of their genetic condition.

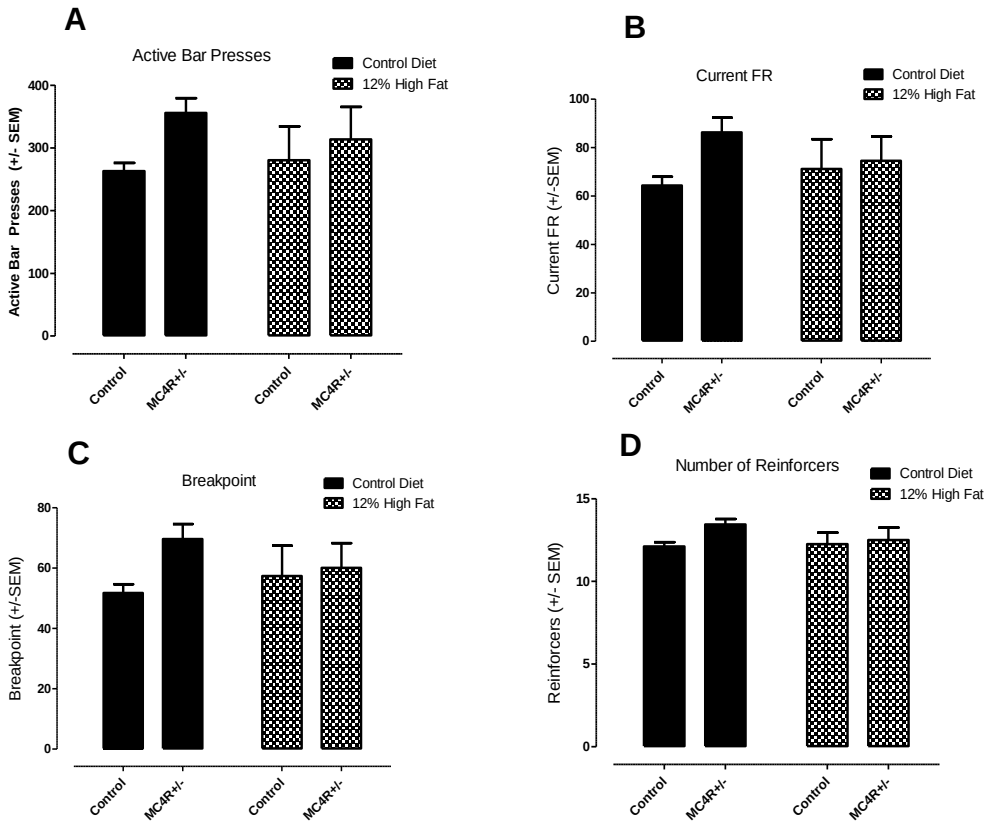

Figure 3.3: Results from the progressive ratio task started around postnatal day 105. MC4R+/- animals fed the control diet show a clear increase in active lever pressing, indicating increased motivation for food compared to their control counterparts. The same task with animals fed the high saturated fat diet did not yield the same results. The effect of the high-fat diet lessens the difference between the control and MC4R+/- groups. A: Active lever presses. B: Current fixed ratio schedule. C: Breakpoint. D: Number of Reinforcers received

MC4R+/- animals fed the high saturated fat diet show increased responding regardless of the sucrose concentration reward. The control counterparts only reach similar responding levels with the highly rewarding 30% sucrose concentration. The effect of diet and genetic condition on the variable ratio task can be seen in figure 4.

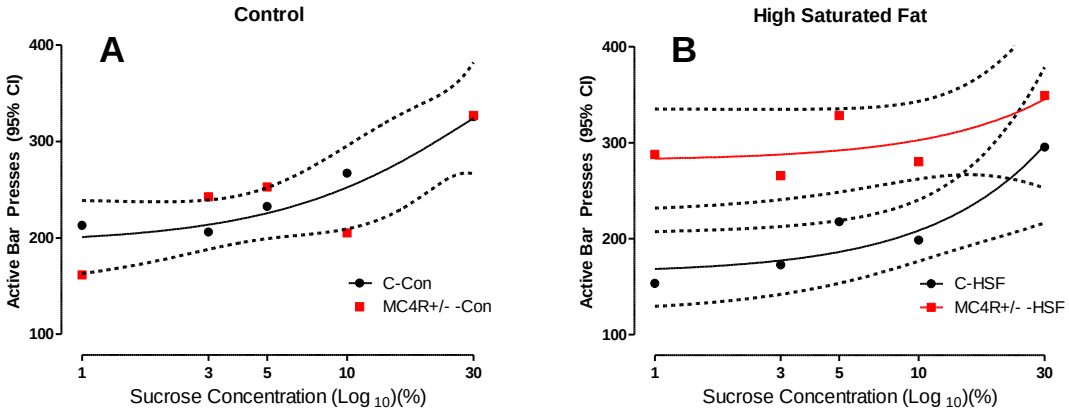

Figure 3.4: Results from the variable progressive ratio task starting at postnatal day 220, separated by dietary group. A: Animals fed the control diet do not show any difference in consumption regardless of genetic group or sucrose concentration. B: MC4R+/- haploinsufficient animals fed the high saturated fat diet show an increase in responding for a lower sucrose concentration than the control counterparts. However, at a high sucrose concentration responding rates for both groups indicate no difference.

For the distraction task analysis, the third day of testing was used, with the distracting tone being played from minute 5 to 25. Only the intervals during the distraction period were used for the analysis. The distraction task did not reveal any significant differences between the groups.

The FR5 schedule used between the distraction tasks on postnatal day 230, labeled the no distraction task, was also analyzed using an ANOVA. The analysis revealed a significant effect of condition, $F(7,88)=5.701$, $P \leq 0.05$. Performing a Tukey's post hoc analysis revealed a significant difference between the control animals fed the high saturated fat diet and their MC4R +/- haploinsufficiency counterparts. Figure 5 depicts the differential responding in the MC4R +/- haploinsufficient group during the FR5 schedule that is not seen during the distraction task. Both the variable ratio task and the late age FR5 task show MC4R +/- haploinsufficiency motivational deficits are seen only in groups consuming high dietary fat.

3.5 Development of Steatosis is linearly dependent on dietary fat consumption with no relationship to behavior

The steatosis analysis found a significant effect of diet $F(1,3)=5.40$, $p \leq .05$. Following an ANOVA, a Bonferroni post-hoc test determined the difference was attributed to the control group and the 12% high fat group. Not surprisingly, it seems that the accumulation of fat in the liver has a direct linear relationship with the percentage of fat in the diet.

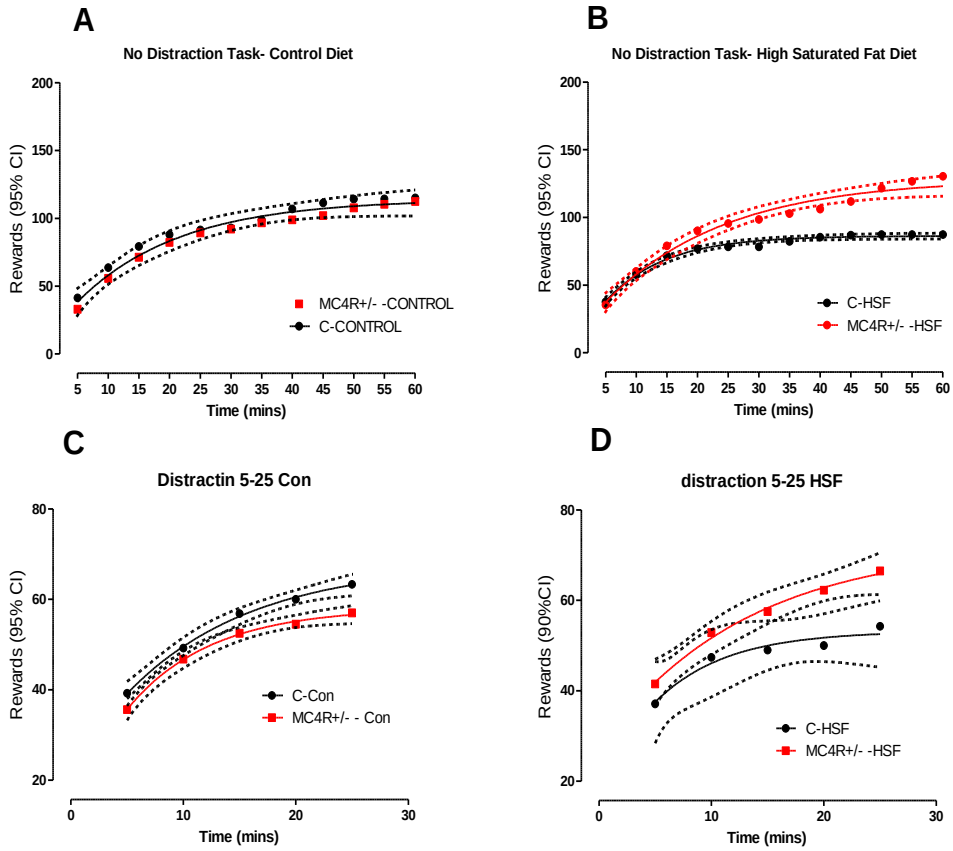

Figure 3.5: Distraction and no distraction FR5 tasks starting on postnatal day 230. A: During the no distraction task, animals fed the control diet did not differ in rates of responding. B: MC4R +/- KO animals fed the high saturated fat diet respond at higher rates than their control counterparts. C and D: The presence of a distracting tone alters the number of rewards earned by the MC4R +/- haploinsufficient animals fed the high saturated fat diet down to a non-significant level regardless of dietary fat being fed.

All behavioral measures were analyzed to investigate if steatosis can be predicted by behavior. To test the accuracy of steatosis measures, a regression analysis using bodyweight data to predict lipid deposits were used. The analysis resulted in an R2 of .681. The robust result allows for more confident analysis of other behavioral measures.

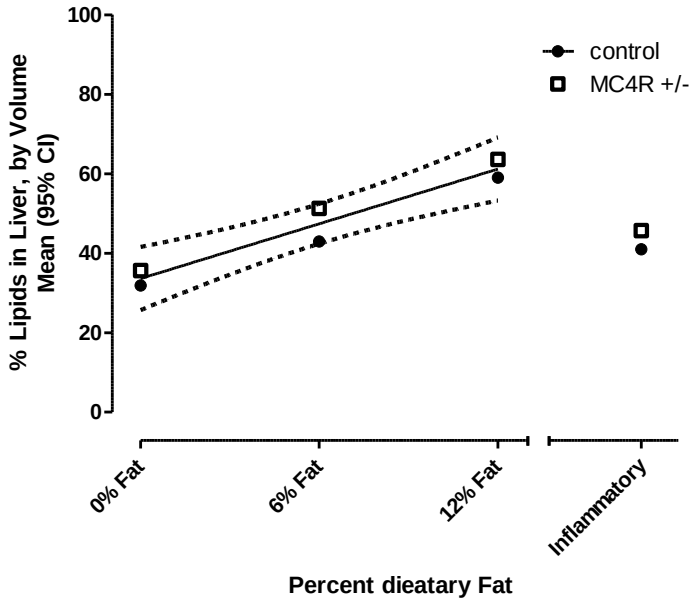

Figure 3.6: A clear linear relationship is observed between the percentage of dietary fat and the percentage of lipids in the liver. The inflammation group drops down to almost control level indicating that this effect is not due to inflammation. No effect of MC4R +/- haploinsufficiency is indicated.

Sucrose preference measures (consumption of water, 1%, 3%, 10%, and 30% sucrose concentrations) on day 90 resulted in an R2 of .426 using a linear regression model. Both the water and the 10% sucrose bottle measures were significant coefficients, with an overall significant ANOVA $F(5,31)=3.855$, $P \leq .05$. No other measure had an R2 greater than .2.

Chapter 4. Discussion

It is clear that the MC4R haploinsufficient rat shows a resemblance in phenotypic expression to its human counterpart. Motivational changes in the MC4R+/- haploinsufficient rat, however, seem to be intricately entangled with two other major factors; age and diet. The results from the progressive ratio task, the variable progressive ratio task, and the distraction task were all performed at different ages that help create a timeline of the development of obesity and motivational changes. During the initial stages of obesity around postnatal day 100, MC4R +/- haploinsufficient animals fed the control diet showed increased motivation towards food-related rewards. After control and MC4R +/- haploinsufficient animals exhibited differential weights on postnatal day 220, animals fed the high saturated fat diet began to express divergent motivational outcomes during the tasks. The time course could indicate a shift in motivational changes from the development of obesity to the maintenance of obesity. Pre-obesity, MC4R +/- animals fed the control diet yielded an increase in motion towards reward, while the high saturated fat diet mitigated the effect of the MC4R +/- haploinsufficiency. Conversely, during later stages of obesity, the animals fed the high saturated fat diet showed increased motivation for reward, regardless of the value of the reward (shown by the variable progressive ratio task), to help maintain their already rewarding dietary consumption habits.

Analysis of weight shows a significant difference in late adolescence or early adulthood period between the control and MC4R haploinsufficient groups. The weight differences are seen regardless of the diet being fed, indicating that the MC4Rs are the underlying cause of the weight differences that could be exacerbated by the effect of high-fat diets. Observed differences in weight are shown around the adolescent developmental period in humans as well (Loos et al., 2008, Lubrano and Berthelier et al., 2003). It is no surprise that we see similar results in animals as we do humans. The underlying mechanisms of weight gain are the current focus of many researchers.

The results from the behavioral tasks indicate an exceptional role of motivation in the MC4R+/- haploinsufficient rat. Age and diet seem to influence motivational differences in the animals as well. During the early stages of the development of obesity, MC4R +/- animals that are not receiving already rewarding high-fat diet display an increase in motivation towards food related rewards. In the adult animals that have fully developed obesity, it seems that the maintenance of their obesity becomes the source of the motivational differences, causing the animals already fed the high saturated fat diet to display increased responding to food related rewards. The signs of motivational differences during the early stages of obesity could indicate a dysregulation in reward pathways in the brain even prior to the development of obesity. While animals on different diets displayed motivational deficits at different time points during the development of obesity, an underlying dysregulation of the reward pathway could be the source. The idea of a reward circuity malfunction is not farfetched. There

are known connections between the MC4R and dopamine circuits that are related to the motivation in receiving and consuming palatable food.

Reward related areas of the brain are highly connected with the melanocortin system. POMC and AGRP neurons from the arcuate nucleus of the hypothalamus have projections to areas such as the ventral tegmental area, the nucleus accumbens, as well as the lateral hypothalamus (King and Hentges., et al 2011: Bagnol et al., 1999: Cui., et al 2012). Similarities in brain areas cause cross interaction between the two systems that affect feeding behaviors, motivation, and the relationship between the two. He, Zhi-Gang et al compiled a large body of literature to show the abundant collaboration between the two systems (He, Zhi-Gang et al., 2015). D1 and D2 receptors have also been found to be co-localized with MC4R in the striatum and Nucleus Accumbens. Cui et al found that procedural memory activity shown from the D1 receptors are interconnected through the MC4Rs, specifically in the striatum (Cui et al.,2012). Yoon and Baik 2015 found that both MC4R and D2 receptors work cohesively inside the bed nucleus of the stria terminalis. Inside the ventral tegmental area, it has been shown that injections of melanocortins have decreased consumption of palatable rewarding sucrose solutions during a two bottle sucrose preference task (Yen, Haw-Han et al., 2013). Along with the connection to dopamine, previous studies have linked motivational differences between control and MC4R haploinsufficient groups (Vaughan et al., 2006: Cui., et al 2013). The studies used both a progressive ratio and a fixed ratio respectively with motivational differences uncovered; however, age and dietary differences were not observed. A key difference between the previously cited studies and ours was the

accessibility to food. Both studies use a form of food restriction while our animals had ad libitum access to their food. Availability to food, specifically with differing levels of saturated fat, further emphasizes the complex connection between the MC4R and dopamine reward systems. The complex relationship generalizes more uniquely to human individuals with MC4R deficits that have an abundant availability of easily accessible food.

Stereology revealed intriguing results in reference to the accumulation of lipid deposits in the liver. The control group and the high saturated fat group showed statistical significance when analyzed. More accurately, there is a linear relationship between the percentage of dietary fat with the percentage of lipid deposits in the liver. It appears that even when fed a control diet there is still fat that can be found in the liver of both the control and MC4R haploinsufficient animals. It is normal to have fat in the liver. The results conclude that both groups of animals have around 30% volume of fat in their livers. Control animals seem to have an above average volume of lipid deposits; however, even the animals on the control diet had access to food ab libitum. The constant access to food might have increased their base level of fat in the liver especially compared to humans when food is not necessarily available at all times (i.e. while working, school, or simply following a normal three-meal diet). The 6% saturated fat diet shows a slight increase of fat in the liver, raising it to about 40-50%. The 12% saturated fat group jumps the volume of lipids to around 55-60% in the liver. Looking at these results it appears that every 6% additional saturated fat increased the percentage of lipids in the liver by around 10-15%. These results coincide with previous findings of

the effect of dietary fat creating a similar representation of steatosis in our animals as they did with theirs. (Ahmed et al 2009).

To further develop the relationship between the high fat dietary consequences and MC4R haploinsufficiency, we investigated if any of the behavioral measures were predictive of the amount of fat accumulated in the liver. After running multiple regression models, it is unclear if there is a relationship. Building models accounting for pre and/or post obesity, no model reached statistical significance. While modeling a behavioral predictor for steatosis could have great clinical relevance, it is not surprising that no effect was found.

We hypothesized that the MC4R +/- haploinsufficient animals would display increased obesity early in age due to an increased motivation to food related rewards indicated by behavioral differences. We also hypothesized that the percentage of lipid deposits in the liver would be indicative of the behavioral tasks. Steatosis does act in a dose-dependent relationship with dietary fat, however, using Pearson's correlation and multiple regression analyses; it does not seem that any behavioral measures are predictive of the development of steatosis. Our results conclude an elaborate connection between motivation, the MC4R circuit, and dietary fat. Motivational deficits seem to be influenced by the stage of obesity as well as dietary fat being consumed. The results emphasize the importance of a healthy low saturated fat diet. The knowledge of motivational differences caused by MC4R deficits reveals a potential new clinical target for the treatment of obesity in the underlying mechanisms of the dopamine reward circuitry connected to MC4R receptors.

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