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Alterations to Taste Preference in MC4R Haploinsufficiency Manifest Prior to Dietary Induced Obesity and Are Accompanied by Dendritic Spine Alterations to Medium Spiny Neurons of the Nucleus Accumbens in Adulthood

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ALTERATIONS TO TASTE PREFERENCE IN MC4R HAPLOINSUFFICIENCY MANIFEST PRIOR TO DIETARY INDUCED OBESITY AND ARE ACCOMPANIED BY DENDRITIC SPINE ALTERATIONS TO MEDIUM SPINY NEURONS OF THE NUCLEUS ACCUMBENS IN ADULTHOOD

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ABSTRACT

Obesity has reached epidemic proportions in the United States and has become an increasing public health concern for developed nations. Haploinsufficiency of melanocortin receptor 4 has been identified as the single most common monogenetic cause of obesity in humans. Using the MC4R +/- haploinsufficient rat, we sought to determine potential alterations in body weight and morphology, locomotor activity, sucrose concentration preference, and progressive-ratio operant testing in a dietary-induced obesity environment. Rats were placed on four separate diets corresponding to 1.7% saturated fat with 12.2% total kcal/fat, 6% saturated fat with a 40% total kcal/fat, 12% saturated fat with a 40% total kcal/fat, and a 1.7% saturated fat with 12.2% total kcal/fat containing an inflammatory polyunsaturated fat ratio of 20:1 omega-6:omega-3 fatty acids. We found a significant interaction between genetic condition and diet in terms of body weight and waist circumference. Locomotor activity testing showed an increased level of activity for animals on the inflammatory diet, with lower levels of rearing for haploinsufficent animals on the high saturated fat diet. Normal PR schedules failed to produce significant results, but both varying the concentration and the addition of a distracting tone revealed significant effects on motivation for palatable rewards. Finally, we DiOlistically labeled medium spiny neurons in the nucleus accumbens to determine potential alterations to dendritic spine morphologies correlating to impulsive and compulsive behavior. Significant alterations in cumulative frequencies of spine length, spine head diameter, and spine volume are apparent and indicate functional

alterations to the hedonic reward processing center of the brain under high fat, inflammatory, and haploinsufficient conditions. These findings provide insight to potential use of MC4R as a therapeutic target for early-onset dietary-induced obesity, and highlight the effects of inflammatory polyunsaturated fatty acids on hedonic motivational behavior.

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CHAPTER 1: INTRODUCTION

Obesity has currently reached epidemic proportions in the United States, with 1/3 of the adult population classified as having a body mass index (BMI) >30 (Ogden et al., 2014). Over the past 30 years, the rate of adult obesity in the US rose from 15% in 1980 to 35.5% in 2010 (Ogden et al., 2014). A multitude of morbid physiological effects are currently associated with obesity, including high blood pressure, adverse cardiovascular events, type II diabetes (Ogden et al., 2014), and development of various systemic and major organ cancers (Brandon et al., 2009). It is estimated that an additional 1.3 billion USD annually are added to healthcare costs related to obesity (Ogden et al., 2014) , accounting for approximately 0.7-2.8% of an individual country's health care cost worldwide (Fani et al., 2014), highlighting the necessity of preventative therapeutic intervention in obese populations. As the problem of clinical obesity in the United States is expected to continue its trajectory (Ogden et al., 2014), the exploration of functional and psychological mechanisms behind dietary-induced obesity, feeding behavior, and impulsivity/compulsivity for hedonic rewards are paramount to determine the most effective course of therapeutic intervention.

Although obesity results from a complex interplay of peripheral and central nervous system processes, loss-of-function mutations in melanocortin receptor 4 (MC4R) have been identified as the most common monogenetic cause in humans (Calton et al., 2009;Farooqi et al., 2000;Farooqi et al., 2003;Geller et al., 2004;MacKenzie, 2006). To combat the obesity epidemic, it is necessary to consider potential developmental trajectories of hedonic

reinforcement under MC4R haploinsufficient conditions and to determine phenotypic expression points, i.e. pre/post dietary-induced obesity. Adolescent responses to reward are classically considered to be with marked anhedonia and binge behavior (Epstein et al., 2014). Indeed, neurofeedback data shows increased activation of striatal pathways when adolescents viewed images of highly-palatable foods, correlating directly to quantities of circulating leptin, but not insulin, levels (Jastreboff et al., 2014).

The melanocortin receptor family is primarily involved in feeding behaviors, pigmentation, and sexual function in mammals (Adan et al., 2006;Girardet and Butler, 2013) and are concentrated in the hypothalamic nuclei, with populations scattered throughout the cortex, spinal column, and periphery (Tao, 2010). The clinically obese phenotype is only seen in MC4R mutations and not in other members of the subfamily (Calton et al., 2009), causing focus on this particular receptor variant for the understanding and potential treatment of dietary-induced obesity. Previous reports have indicated the presence of a hardwired neuronal pathway projecting from the arcuate nucleus of the hypothalamus (Adan et al., 2006;Boghossian et al., 2010;Tao, 2010) that regulates motivational behavior for feeding through expression of the MC4R inverse-agonists agouti and agouti-related protein (Agrp) expression in the hypothalamus (Ren et al., 2012;Krashes et al., 2014) and amygdala (Boghossian et al., 2010). These brain structures are functionally correlated with resting metabolic weight, energy metabolism, and appetite control in both humans (Hainerova et al., 2011) and mouse models (Girardet and Butler, 2013). Indeed both Agrp and Neuropeptide Y (NPY) have been shown to induce hyperphagy in a dose-dependent manner (Boghossian et al., 2010;Pandit et al., 2014), and Agrp has been found to induce endocytosis of functional MC4R receptors *in vitro* (Breit et al., 2006). Endogenous classic agonists of the receptor,

chiefly the melanocyte-stimulating hormone (MSH) subfamily (α, β, and γ-MSH), are anorexigenic and are metabolically attenuated by central quantities of leptin, glucocorticoids, and various transcriptional neuropeptides (Keen-Rhinehart et al., 2013). Numerous studies investigating local injections of alpha-MSH in the paraventricular nucleus (PVN) have shown reduction in food intake in both rats and mice; see Adan et al., 2006 for review. Further exploration into the molecular function of the receptor and its mutant forms has revealed the obese phenotype results from haploinsufficiency rather than altered receptor function (Ho and MacKenzie, 1999); regardless, meta-analysis of single nucleotide polymorphisms in humans has resulted in identification of a number of missense and frame-shift mutations, each with varying degrees of functionality; see Adan et al., 2006;Farooqi et al., 2000;Farooqi et al., 2003;Hasselbalch et al., 2010;Loos et al., 2008; and Loos, 2011 for thorough review. Most mutant forms of the receptor are inherited in an autosomal dominant pattern, and result in clinical hyperphagy and incomplete suppression of growth hormone (Martinelli et al., 2011) resulting in phenotypic obesity during late adolescence/early adulthood. E-box binding decrease by NHLH2 transcription factor (Wankhade and Good, 2011) and intracellular receptor retention (Lubrano-Berthelier et al., 2003b;Lubrano-Berthelier et al., 2003a) are the molecular results of the misfolded protein; addition of appropriate neurochemical chaperones has resulted in receptor rescue *in vitro* (Rene et al., 2010).

Previously, animal models of obesity have focused primarily on deficiencies of leptin and leptin receptors (Friedman, 2011), resulting in similar clinical phenotypes seen in MC4R mutant animals (Lutz and Woods, 2012). The leptin knockout mouse (ob/ob mouse) clinically manifests hyperphagia, hypothermia, hyperglycemia, hypercorticosteronemia, and

decreases in growth hormone production associated with hypothyroidism (Friedman, 2011). Indeed, centrally-circulating leptin quantities are directly correlated to both weight regulation (Friedman, 2011) and attenuation of the hedonic response from consumption of palatable rewards (Domingos et al., 2014). A number of rat models have been designed to explore leptin dysregulation, specifically the obese Zucker and the Koletsky rats (Chua, Jr. et al., 1996;Lutz and Woods, 2012); both produce nonfunctional extracellular receptor sites of leptin via point mutations, resulting in clinical hyperphagia, insulin resistance, and obesity (Bray, 1977b;Bray, 1977a). Although these models have provided considerable insight into the neurochemical regulatory mechanisms of feeding behavior, loss-of-function mutations in the human leptin gene are exceedingly rare in human populations (Farooqi and O'Rahilly, 2009), limiting the translatability of the model. Created via expansion upon the hypothesis of leptin-induced metabolic regulation, development of the leptin receptor-deficient db/db mouse is characterized by a similar obese phenotype to the ob/ob mouse, yet exhibits a marked increase in rate and severity of hyperglycemia and insulin resistance, appropriating this model toward the study of diabetes mellitus as opposed to dietary-induced obesity (Chua, Jr. et al., 1996). Very few human case studies have addressed this loss-of-function mutation; mice that exhibit the db/db genotype are infertile (Zhang et al., 2012;Donato, Jr. et al., 2011), and there are currently no reports on the reproductive abilities of humans with such a mutation of which the authors are aware.

Branching off these previous models, other constructs have focused on molecular deficits occurring downstream from leptin in this metabolic cascade. Mice lacking proopiomelanocortin (POMC), the precursor of the alpha, beta, and gamma-MSH, has been available for approximately twenty years; these mice exhibit hyperphagy and phenotypic

obesity as well (Challis et al., 2004). Following suit, newer models began exploring loss-of-function mutations at receptor sites downstream of leptin as well; this thinking led to the creation of the MC3R, MC4R, and combined MC3R and MC4R knockout mouse (Rowland et al., 2010;Atalayer et al., 2010), and ultimately the MC4R knockout rat in 2011(Mul et al., 2012). In wild type rats, MC4R mRNA expression begins at postnatal day (PD) 14 in the diencephalon and telencephalon; by PD18 it is expressed throughout the brain, primarily in the amygdala, thalamus, hypothalamus, hippocampus, and dentate gyrus (Tao, 2010). Construction of the knockout MC4R +/- rat (Wistar strain) is the most recent obesity model, created by insertion of a stop codon 54bp upstream of its normal position, causing eight amino acids to be truncated from the C-terminus of the protein chain, resulting in a loss-of-function mutation (Mul et al., 2012). The C-terminus is also crucial for substrate binding in humans (Ho and MacKenzie, 1999), making heterozygotes of this model an accurate and translatable representation of human MC4R haploinsufficiency. The most apparent behavioral phenotype of MC4R haploinsufficiency in rats is hyperphagy and increased longitudinal growth (Srisai et al., 2011;Weide et al., 2003), with differential weight effects becoming statistically significant around PD75 (Mul et al., 2012).

The examination of hedonic reinforcement and incentive behavior seen in physical (i.e. drug) addiction models is thought to mirror behavioral addictions, such as compulsive eating, gambling, and "purging" eating disorders such as bulimia nervosa (Grant and Chamberlain, 2014;Hadad and Knackstedt, 2014). The nucleus accumbens (NAc) has a long history of study as the epicenter for hedonic reward processing, and has been extensively studied in the field of drug abuse; see Motzkin et al. (2014) for review. Ninety-95% of cells in this structure are classified as medium spiny neurons (MSNs), cells rich in

D1 and D2 receptors that receive dopaminergic signals from the ventral tegmental area (Preston et al., 1980). These inhibitory GABAergic neurons are responsible for attenuation of neuronal activity throughout the basal ganglia and frontal cortex; however, continued presence of dopamine releasing drugs (i.e. ethanol, nicotine, and cocaine) creates morphological changes to this brain structure, thought to be the physical result of the addictive process (Bull et al., 2014;Sun and Laviolette, 2014;Pereira et al., 2014) . By assessing synaptodendritic alterations within this system, we can study the effect of gene and diet interactions at the morphological level and determine any changes to the system that mimic those of substance use disorders (Nestler, 2013;Dumitriu et al., 2012a). The main goal of this aspect study is to determine if, under haploinsufficient conditions, dietary-induced obesity alters the morphology of MSN dendritic spines in the NAc, correlating to alterations in hedonically-motivated behavior.

Behavioral aspects of this study will assess potential interactions between MC4R haploinsufficiency and dietary-induced obesity on sucrose concentration preference, locomotor activity, and motivation for sucrose reward (with and without a distracting tone). Current thinking that coordinates compulsive eating with that of drug-seeking behavior begs the question as to whether or not MC4R loss-of-function mutations attenuate a similar drug-seeking response, due to alterations in satiety or neurochemical alterations of dopaminergic signaling in the basal ganglia. These alterations may be inherent due to the loss-of-function mutation and may manifest behaviorally prior to dietary-induced obesity. Administration of selective MC4R agonists have procured clinically relevant weight reduction in dietary-induced obese rats (He et al., 2010a;He et al., 2010b), but have yet to be tested in a MC4R haploinsufficient model of either adolescence or adulthood.

We hypothesize that MC4R haploinsufficiency will result in increased weight gain and early-onset dietary induced obesity reflected in a dose-response according to levels of saturated fat content. We also hypothesize haploinsufficient obese animals will exhibit lower overall locomotor activity, increased preference for high sucrose concentrations, and demonstrate compulsive motivational behavior when presented with a progressive ratio operant task for sucrose reward. It is unknown whether these hypothetical results will occur prior to, or after the onset of, dietary-induced obesity, and whether or not the addition of inflammatory fatty acids exacerbates these behavioral phenotypes. In adulthood, these behavioral changes are expected to be reflected in morphological alterations to dendritic spines on medium spiny neurons of the nucleus accumbens.

CHAPTER 2: MATERIALS AND METHODS

2.1 SUBJECTS

Male Wistar rats (*Rattus norvegicus,* Transposagen, Lexington, KY) (MC4R +/-, *n*=33; control, *n*=33) were weaned at postnatal day 21 and housed in pairs, 1 KO and 1 control animal per cage, and placed on special diets. Prior to weaning, animals were kept with their true dam and litters were caged separately. Originally, female wild-type Wistar P generation rats were bred with MC4R -/- male rats, resulting in the MC4R +/- F1 generation, where litters were culled to males only. This specific breeding step was performed in an effort to control for any potential fetal development confound. After weaning, animals were separated into 4 diet groups, the control group (CON) (n=9 for each group) (1.7% SFA, 12.2 % total kcal from fatty acids), inflammatory (INF) (n=8 for each group) (1.7% SFA, 12.2 % total kcal from fatty acids, 20:1 ratio of omega-6:omega-3 UFA), low-saturated fat (LSF) (n=8 for each group) (6% SFA, 40% total kcal in fatty acids), and high-saturated fat (HSF) (n=8 for each group) (12% SFA, 40% total kcal in fatty acids) (Modified AIN-76 diets, Bio-Serve, Frenchtown, NJ). Rats were weighed, crown-rump length was determined using a metric ruler, and waist circumference was taken with a cloth tape measure 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 calculated using weight (g)/(length(cm)^2. One MC4R +/- KO animal on the INF diet was found deceased on PD 98; missing data for this animal was replaced with column means where appropriate. One MC4R +/- on the CON diet expired previous to our variable PR test, and n(s) were modified accordingly.

Lastly, one animal on the CON-HSF diet expired previous to our distraction task; n(s) were modified accordingly. Animals were maintained in an AAALAC-accredited facility at 21º ± 2 ºC, 50% ± 10% relative humidity and a 12-h light-dark cycle with lights on at 07:00h. All behavioral testing was conducted during the animal's light cycle. The research protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of South Carolina, Columbia, SC; animal assurance number A3049-01.

2.2 Experimental Design

Each group (n=8) was tested monthly for locomotor activity using a 4 (diet) X 2 (genetic condition) with X 10 (litters as a covariate) mixed-factorial design. Seven testing sessions were given, one per month of the study, in addition to one test at weaning (PD 21). Subjects were tested for sucrose preference using a 4 (diet) X 2 (genetic condition) X 5 (sucrose concentration) with X 10 (litters as a covariate) mixed-factorial design. Sucrose preference testing was also performed monthly for a total of seven sessions. For operant testing, subjects were placed on a PR schedule when criterion was reached (see 2.6 Operant Testing Schedule below) using a 4 (diet) X 2 (genetic condition) mixed-factorial design. A total of 6 testing sessions were given. One variable-concentration PR schedule was given over the course of 10 days following the completion of the 6 previous PR testing sessions, using a 4 (diet) X 2 (genetic condition) mixed-factorial design. The distraction task was performed in two consecutive days of operant testing on a 60 minute FR5 schedule with no distraction during the first day and a distracting tone given during minutes 5-25 of the 60 minute test 24 hours later. The distraction task was also performed using a 4 (diet) X 2 (genetic condition) mixed-factorial design.

2.3 Locomotor Activity

Locomotor activity was tested at 21, 30, 60, 90, 120, 150, and 180 postnatal days of age. The testing apparatus consisted of a 40 cm by 40 cm chamber with a circular Plexiglas insert and tracks ambulation and rearing in the X and Y dimensions via infared photocells (Hamilton-Kinder Inc., Ponway, CA). Testing was conducted for one hour under low light conditions to simulate the nocturnal experience when rats are most active. Digipro System Software (v.140, AccuScan Instruments) recorded hits across the photocell grid (32 X 32, spaced 2.5 cm apart) in real time. The dependent measure of basic and fine movements is defined by the monitoring software, Motor Monitor (Hamilton-Kinder Inc, Ponway, CA). In brief, basic movements are defined as clearing of the anchor beam when a new beam is broken, while fine movements (such as head movements) are defined by new beam breakage without clearance of the anchor beam; see Motor Monitor Operations Manual version 3.11 (Hamilton-Kinder Inc, Ponway, CA). Photocell pairs were tuned by the manufacturer to control for extra perspex width due to presence of the circular Plexiglas insert.

2.4 Sucrose Preference Testing

A five-bottle sucrose preference test was administered on days 30, 60, 90, 120, 150, and 180. Habituation to the testing cage was performed on PD 21. Five sucrose solutions (0, 1, 3, 10, and 30% by volume) were available for 20 minute testing sessions. Differences in bottle weight were used for preference analysis. Bottle sequence was block-randomized and continued in a Latin-square procedure over each day of testing to control for any potential bottle preference.

2.5 Operant Testing Apparatus

Operant task chambers (ENV-008; MED Associates, St. Albans, VT) were controlled by Med-PC IV interface software (MED Associates, St. Albans, VT) and were housed in a sound-attenuated cabinet enclosure. The front chamber contained access to a recessed dipper (ENV-202M) through a 5cm X 5cm window with an infrared sensor (ENV-245-CB) to track nose poke time in seconds. Two retractable metal levers (ENV-112BM) on both sides of the opening were located 7.3 cm above a metal grid floor. A dipper with a 0.1 ml cup attached to the end of the arm was raised into the receptacle, which allowed access to the sucrose solution upon completion of the response requirement. A third lever (ENV-112BM) was fixed to the back wall in line to the receptacle and was inactive. All 3 levers were presented in the chamber at the beginning of testing, and rats learned to respond for continuous reinforcement on various ratio schedules (see *operant testing schedule 2.5*) during 82 minute sessions. An operant response to an active lever (left or right) resulted in 4 seconds of access to sucrose solution (5% wt/vol, except when noted), whereas responding on the inactive lever was recorded but not reinforced.

2.6 Operant Testing Schedule

Staring on PD 61, animals were maintained on a fixed-ratio (FR) 1 schedule for a minimum of 3 days; three consecutive days of stable responding resulted in the movement of animals to a FR-3 schedule. Stable responding was operationally defined as greater than 60 rewards over the course of the testing period. Tests ended after subjects obtained 120 rewards. Similarly, after 3 days of satisfactory performance, animals were moved to a FR-5 schedule. After 3 days of satisfactory performance on FR-5 schedule, animals were moved to a progressive ratio (PR). The PR tests were a maximum of two hours in length. The

sequence of progressive-ratio bar-press requirements was 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 179, 603, 737, 901, 1102, 1347, 1646, 2012, according to the methods of Richardson and Roberts (1996). On PD220, animals were placed on the same progressive ratio schedule, this time with 1%, 3%, 5%, 10%, or 30% sucrose concentration. On the days in between progressive ratio testing, a 5% sucrose FR5 schedule was performed, for a total testing time of 11 days (a 0% sucrose concentration progressive-ratio scheduled extinction-prevention day was performed at the end of testing). Concentrations were shifted in a Latin square and starting concentrations were block-randomized.

2.7 Distraction Operant Task

Following varying concentration PR scheduling, we placed rats on an FR5 schedule for 60-minute distraction tasks. During the first task, rats were placed on a normal FR5 schedule for the first 20 and last 20 minutes of a 60 minute test, with the central 20 minutes containing a distracting tone (Mallory Sonalert SC24, Mallory Sonalert Products, Inc., Indianapolis, IN; modified so tone is 5dB above background fan noise per chamber). The day after this test, animals were again placed on an FR5 schedule with no distraction. Finally, on the third day of testing, animals experienced the same distraction tone from minutes 5-25 of the task, with the remaining 35 minutes remaining distraction-free.

2.8 Preparation of Tissue for Dendritic Spine Analysis

Animals were sacrificed on days PD 266-PD 282. On day of sacrifice, animals were deeply anesthetized using sevoflurane (Abbot Laboratories, North Chicago IL) and transcardially perfused with 100 ml of 100 mM PBS wash followed by 200-250 ml of 4% paraformaldehyde buffered in PBS (Sigma-Aldrich, St. Louis, MO). Brains were dissected and post-fixed in 4% paraformaldehyde until tissue processing. After post-fixation, 200 μm

thick coronal slices were cut using a rat brain matrix (ASI Instruments, Warren, MI). Serial coronal slices were then washed in PBS 3 times and placed in tissue cell culture plates (24 well plate; Corning, Tewksbury MA) for DiOlistic labeling.

2.9 Preparation of DiOlistic Cartridges

DiOlistic labeling was performed according to previously described methods (Seabold et al., 2010;Staffend and Meisel, 2011). Approximately 300mg of tungsten beads (Bio-Rad, Hercules, CA) were dissolved in 99.5% pure methylene chloride (Sigma-Aldrich, St. Louis, MO) and sonicated in a water bath (Fisher Scientific FS3, Pittsburgh, PA) for 30-60 minutes. Crystallized DiI (14.5 mg; Invitrogen, Carlsbad, CA) was dissolved in methylene chloride and protected from light until application to the tungsten beads. Following sonication, 100 µl of the bead solution was placed on a glass slide and 150 µl of the DiI solution was titrated on top, and both solutions were slowly mixed using a pipette tip. After allowed to air dry, a razor blade was used to collect the dye/bead mixture onto wax-coated weigh paper and the dye/bead mixture transferred to a 15 ml conical tube (BD Falcon, San Jose, California) with 3 ml ddH2O and subsequently sonicated for 30-60 minutes.

2.10 Preparation of Tefzel Tubing

Tefzel tubing (IDEX Health Sciences, Oak Harbor, WA) was cut into 1.7 M lengths, approximately the same length of the tubing prep station (Bio-Rad, Hercules, CA). Polyvinylpyrrolidone (PVP, 100 mg Sigma-Aldrich, St. Louis, MO) was dissolved in 10 ml ddH2O, vortexed to homogeneity, and then passed through each length of the tubing to aid in bullet adhesion to the tubing. The 3 ml bead/dye solution was then slowly drawn into the tubing and was allowed to spin in the tubing prep station for 5 minutes so beads can

uniformly coat the tube. After slowly draining the water from the tube, the dry tubing was spun in the prep station for approximately 10 minutes with nitrogen gas flow of 0.1 LPM. The nitrogen gas flow through the tubing was adjusted to 0.4-0.5 LPM and the tubing was further spun for 40-60 minutes to ensure the tubing was fully dry. Once dry, tubing was cut into 13 mm segments using the supplied tubing cutter (Bio-Rad, Hercules, CA) and bullets were stored under anhydrous conditions until use.

2.11 DiOlistic Labeling using the Helios Gene Gun

The Helios gene gun (Bio-Rad, Hercules, CA) was loaded with the previously prepared bullets, He gas flow adjusted to 90 PSI, and particles delivered through 3 μm pore filter paper (Millipore, Billerica, MA) directly onto the slice. The barrel was placed approximately 2.5 cm away from the sample, at the top of the well opening on the 24-well plate. After two washes in PBS, sections were stored overnight at 4˚ C to allow ample dye diffusion into the neuronal membrane. Tissue sections were mounted the next day using Pro-Long Gold Antifade (Invitrogen, Carlsbad CA), coverslipped (#1 coverslip; ThermoFisher Scientific, Waltham, MA), and stored in the dark at 4˚ C until confocal microscropy analysis.

2.12 Medium Spiny Neuron Analysis and Spine Quantification

MSNs were analyzed from the NAcc, located approximately 2.20 mm to 0.60 mm anterior to Bregma's landmark, identified by a rat brain matrix (Paxinos and Watson, 2007). 1-2 MSNs per animal were used for analysis. High resolution (1024X1024) Z-stack images were obtained with a Nikon TE-2000E confocal microscope under control of Nikon's EZ-C1 software (version 3.81b). Dendritic spine analysis images were captured using a 60X objective (n.a. = 1.4) with Z plane intervals of 0.15-0.30 μm (pinhole size 30 μm;

backprojected pinhole radius 167 nm) with an internal 1.5X additional magnification. A green helium-neon (HeNe) laser with an emission of 533 nm was used for DiI flurophore excitation. Morphometric analysis of spines was performed using Neurolucida version 11.01.1, utilizing the AutoNeuron and AutoSpine extension modules (MicroBrightField, Williston, VT).

2.13 Dendritic Spine Parameters

Dendritic spine parameters of length, volume, and head diameter were analyzed. Spine lengths were defined as between .01 μm to 5 μm, while longer thin filipodia were not included in analysis (Blanpied and Ehlers, 2004;Ruszczycki et al., 2012). Spine volume parameters were between 0.02 μm^3 and 0.2 μm^3 (Merino-Serrais et al., 2013), and spine head diameters were defined as those measured between 0.3 μm and 1.2 μm (Bae et al., 2012); all parameters were set to remove false positives.

2.14 Statistical Analysis

Body weight, crown-rump length, waist circumference, and BMI were analyzed using multiple regression analysis (Rx64 v3.0.2). Locomotor activity was analyzed using repeated-measures MANCOVA (SPSS v21, IBM), using Roy's Largest Root as a test statistic where appropriate due to the expected high covariation of dependent variables. Sucrose preference, operant testing, and variable concentration operant testing were analyzed using repeated measures ANCOVA with litter as a covariate. The distraction task was analyzed using ANCOVA for each of the 5 minute bins for both FR5 schedules, using Tukey's post-hoc comparisons. Dendritic spine morphological characteristics were analyzed using ANOVA with a Dunnet's post-hoc test for length and head diameter and Tukey's HSD test for volume. All tests were two-sided.

CHAPTER 3: RESULTS

3.1 WEIGHT, LENGTH, WAIST CIRCUMFERENCE, AND BMI

We found that animal mass was ≈654.27g at PND270 with a slope of 241.51g per one unit change in the *log*Day on the control diet and control gene condition. Additionally, the MC4R +/- CON group gained an additional 13.20g compared to their WT counterparts. Surprisingly, this study showed that those on the inflammatory diet actually did not gain weight (≈-11.03g), compared to the WT-CON group, and this effect was slightly reversed in the MC4R +/- INF. Conversely, the effect of the HSF diet alone increased the animal weight by 20.59g (above their standard growth) compared to WT-CON, and this effect was further augmented in the MC4R haploinsufficient animals (12.63g in addition). Finally, this study found that those on the LSF diet (although still receiving 40% kcal/day in fats) gained 7.50g more than their control diet counterparts, and that those with the MC4R-KO gained an additional 7.72g. All r^2 values for weight, length, and waist circumference were greater than 0.95 following a one-phase association curve. Crown-rump length was not significantly different between any groups, but waist circumference specifically highlighted the effects of dietary-induced obesity and genetic condition. Both the LSF groups and HSF groups had a significantly increased waist circumference, weight, and BMI compared to controls, regardless of genetic condition; the effects of diet and gene on BMI become apparent in early adulthood (around PD 90) (see Table B.1).

3.2 Locomotor Activity

A mixed-design MANCOVA revealed a significant main effect of diet on basic movements, fine movements, and rearing [$F(3,769)=174.439$, 17.613, and 7.138, respectively; $p<0.001$)], but no main effect of condition [$F(1, 776)=2.016$, $p=0.110$], indicating MC4R +/- rats were just as active as control rats. There was, however, a significant interaction of condition X diet, [$F(3,772)=7.552$, $\theta=0.029$, $p<0.001$)], as well as an interaction of time, condition, and diet [$F(18,757)=4.721$, $\theta=0.112$, $p<0.001$)], indicating both habituation to the test over time as well as highlighting the main effect of dietary-induced obesity. The interaction of condition and diet over time signifies a cumulative effect of diet and genetic condition affecting locomotor activity over the course of the testing paradigm. Also of note is a main effect of litter, [$F(8,336)=4.721$, $\theta=0.787$, $p<0.001$]. Profile analysis of activity shows a decrease in basic movements and rearing among the MC4R +/- KO LSF and HSF groups across time, as well as a trend in increased activity for WT animals on the inflammatory diet; see fig. 3.2.

3.3 Sucrose Preference 5 Bottle Test

Sucrose preference was analyzed using mixed-factor ANCOVA with litter as a covariate and both diet and genetic condition as between-subject factors. Data was square-root transformed and outliers (indicative of spillage of the testing bottle or deceased animal, noted on master data file) were replaced with the column mean where appropriate. The analysis revealed a significant main effect of condition on the 30% sucrose concentration [$F(3,55)=1.717$, $p<0.05$], as well as an effect of diet [$F(1,55)=3.220$, $p<0.05$]. Diet X condition interactions were not significant for any concentration, nor were covariates of litter. For within-subject effects, the interaction of time and diet was significant for the 0%

[F(15,275)=1.927, $p_{GG}<0.05$] and 3% [F(15,275)=2.268, $p_{GG}<0.05$] concentrations. There were no significant interactions of time and condition, nor were there interactions of time, condition, and diet. It appears as though haploinsufficiency of MC4R does not increase the preference for higher concentrations of sucrose, but rather amount of saturated fat in the diet predicts preference for sucrose. It appears that, regardless of diet or condition, rats prefer the highly palatable 30% sucrose over any other concentration, somewhat contrary to previous reports (Panaro and Cone, 2013).

3.4 Operant Testing for 5% Sucrose Reinforcer

Operant testing was analyzed using repeated measures ANCOVA with litter as a covariate and diet and genetic condition as between-subject factors. Both active lever presses and reinforcers were included as outcome variables. No variables were identified as significant on either outcome variable, indicating motivation for sucrose reward was unaffected by both diet and genetic condition.

3.5 Variable Concentration Progressive Ratio Task

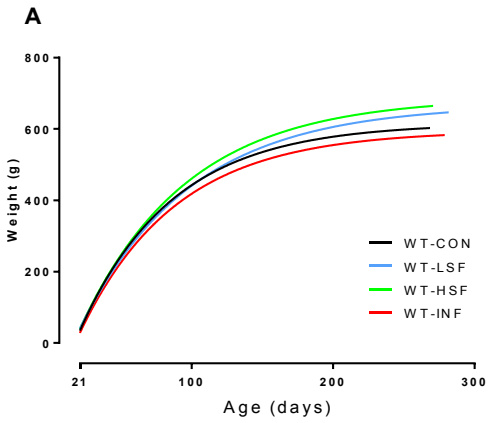
The animals were placed on a 10-day testing cycle during which alternating days of PR-FR5 schedules ran with varying concentrations of sucrose: 1, 3, 5, 10, and 30%, block randomized per group, and shifted in a randomized latin-square throughout testing days. Break-point was used as the outcome variable. ANCOVA analysis revealed a main effect of diet, [F(3,284)=2.794, $p<0.05$], sucrose concentration [F(4,284)=4.808, $p<0.01$], and genetic condition [F(1,284)=4.782, $p<0.05$]. Litter covariate effects were also significant, [F(1,284)=11.402, $p<0.05$], but no interactions of any type were observed. Profile analysis shows decreased responses for both groups on the LSF diet as sucrose concentrations increased, see fig. 3.5.

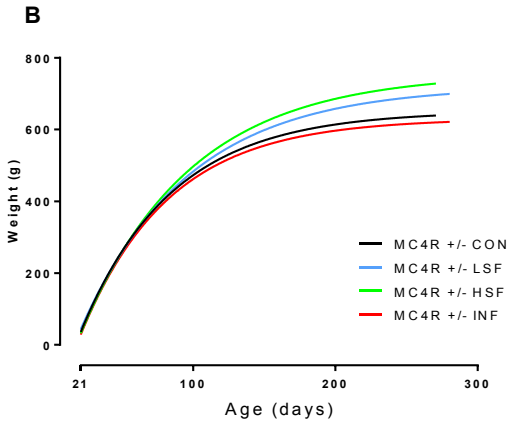
3.6 Distraction Task

The distraction task was analyzed using ANCOVA with 5-minute bins treated as separate variables; 12 bins total. Cumulative number of reinforcers was analyzed as the outcome variable. The test revealed a nearly statistically-significant trend of diet during the first interval, where most of responding occurs (see fig. 6e-h), [$F(3,54)=2.674$, $p=0.056$]. ANCOVA analysis also revealed a robust, yet not significant, trend of genetic condition at interval 5 (when the distracting tone was present): [$F(1,54)=2.674$, $p=0.069$]. These results indicate presence of a distracting tone possibly tends to exhibit less of an inhibitory effect on active lever presses in haploinsufficient animals (see fig. 6e-h.). There was also a significant interaction between condition and diet at interval 11 (minutes 50-55 of the test, [$F(3,54)=2.807$ $p<0.05$]), including an increased responding of MC4R rats on the HSF diet compared to their WT counterparts. During the distraction tone, as well for the last 10 minutes of the test, wild type animals on the inflammatory diet responded significantly more than the other diets, while the MC4R +/- rats on the HSF diet responded significantly higher during minutes 20-30; see fig. 3.6 e-h. These data were compared to a 60-minute FR5 schedule run the day before the distraction task, which yielded significant results when active bar presses were analyzed by ANCOVA [$F(7,88)=5.701$, $p<0.0001$]. Tukey's post-hoc test was performed, showing significant differences between the WT-HSF and WT-INF groups ($p<0.01$), MC4R +/- INF and WT-HSF ($p<0.01$), WT-LSF and MC4R +/- INF ($p<0.0001$), and finally MC4R +/- LSF and MC4R +/- INF ($p<0.0001$); see fig. 3.6 a-d. These data highlight hyperactivity caused by the INF diet (reflected in our locomotor activity results), as well as distraction having less of an effect on active bar presses for MC4R haploinsufficient animals.

3.7 Analysis of Dendritic Spines on Medium Spiny Neurons in the Nucleus Accumbens

Dendritic spine parameters were analyzed using ANOVA with diet and condition as between-subject factors, with Dunnet's post-hoc test using the WT CON group as the control condition. ANOVA revealed a significant effect on spine length [$F(7, 28027)=53.36$, $p<0.0001$], spine head diameter [$F(7, 17837)=11.08$, $p<0.0001$], and volume [$F(7, 9919)=3.819$, $p<0.001$]. In terms of length, post-hoc multiple comparisons showed significant differences between the WT CON group and the WT INF group, as well as significant differences compared to the KO CON, KO LSF, and KO INF groups. Regarding spine head diameter, all groups were significantly different than the WT-CON group. Multiple comparisons of spine volume shows significant differences between WT-LSF and MC4R +/- INF, WT-HSF and MC4R +/- INF, WT-INF and MC4R +/- INF, and MC4R +/- HSF and MC4R +/- INF. All p values <0.01.





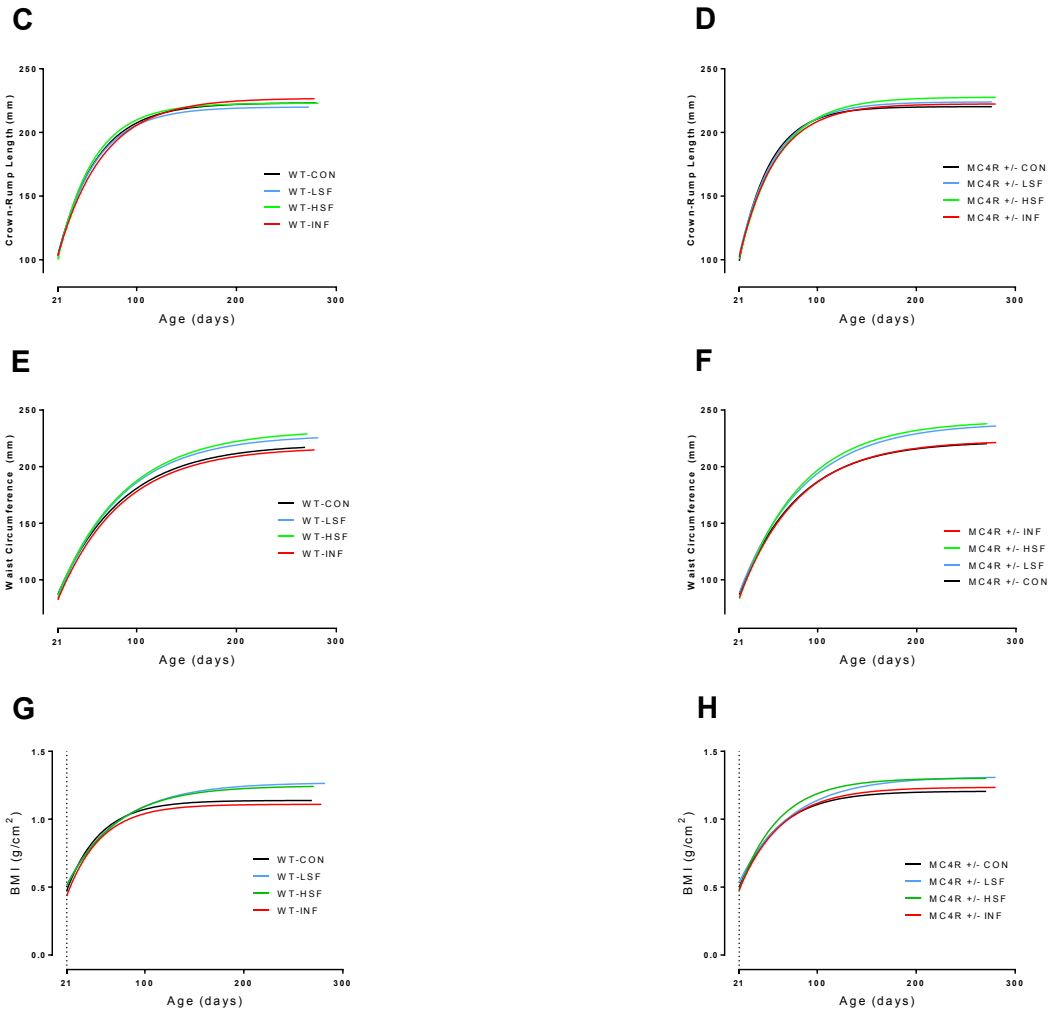


Figure 3.1: Measurements of wild type and MC4R +/- KO Wistar rats following a one-phase association curve. (a-b) Nonlinear curve fits of body weight (g) (mean ± SEM) expressed as a function of day for each group (n=8)*. (c-d) Nonlinear curve fits of crown-rump length (mm) expressed as a function of day (n=8)*. (e-f) Nonlinear curve fits of waist circumference (mm) expressed as a function of day (n=8)*. (g-h) Nonlinear curve fit of Body Mass Index (g/cm^2) as a function of day (n=8)*. *For both wild-type and MC4R +/- animals on the CON diet, n=9.

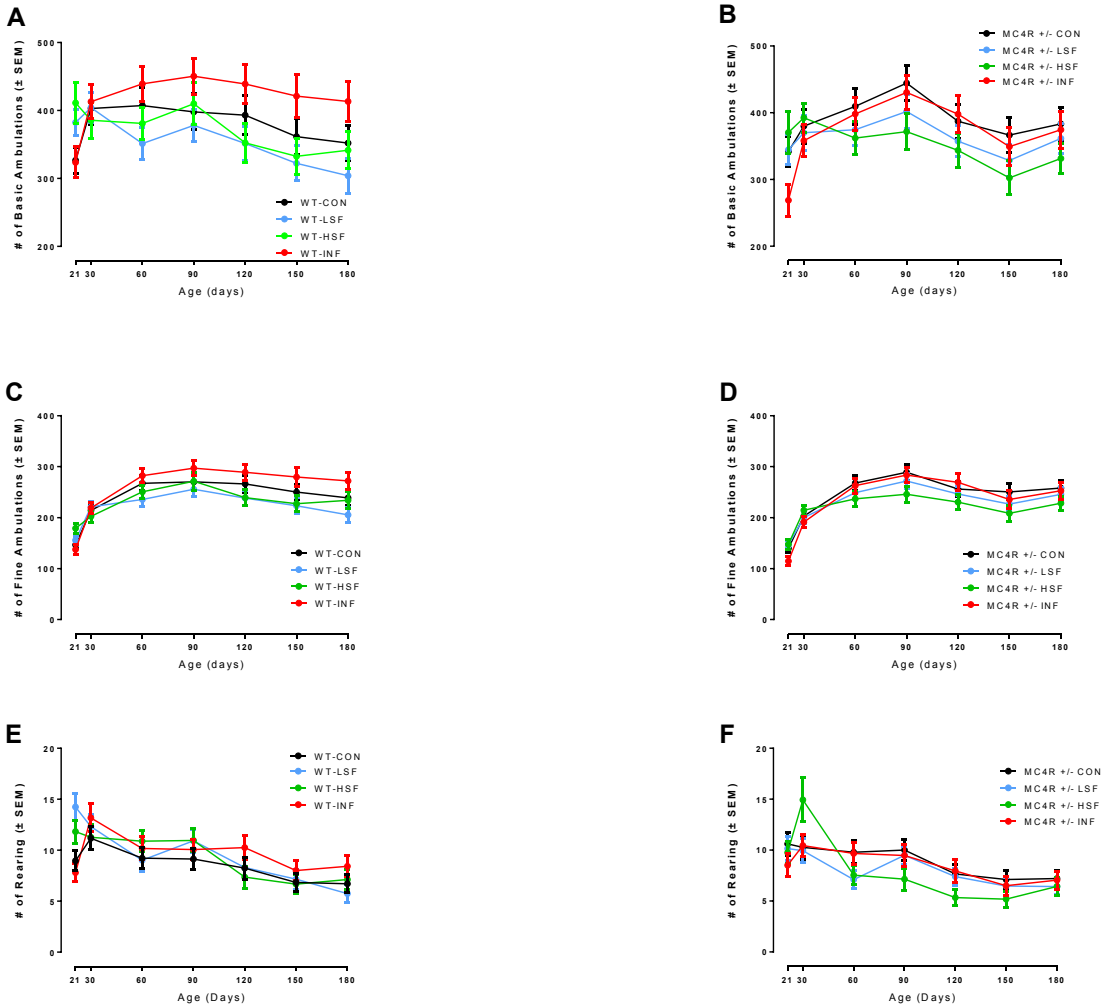
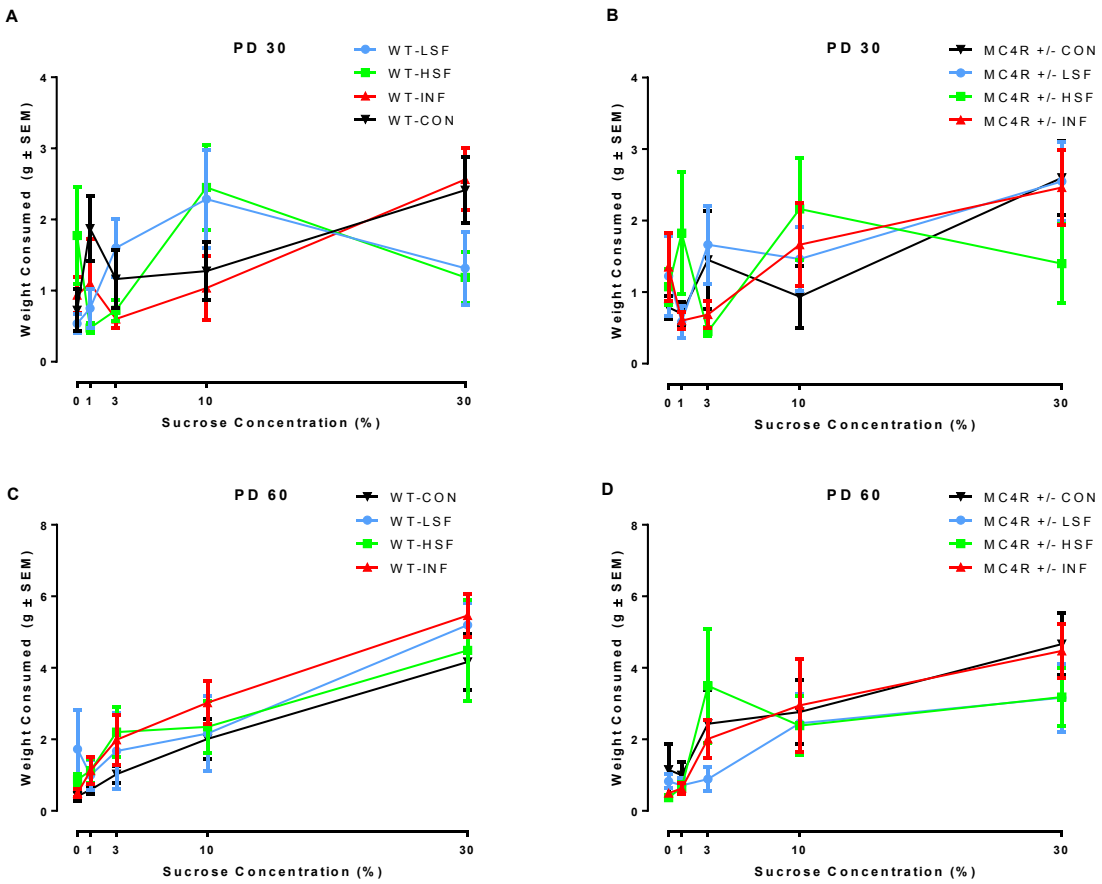
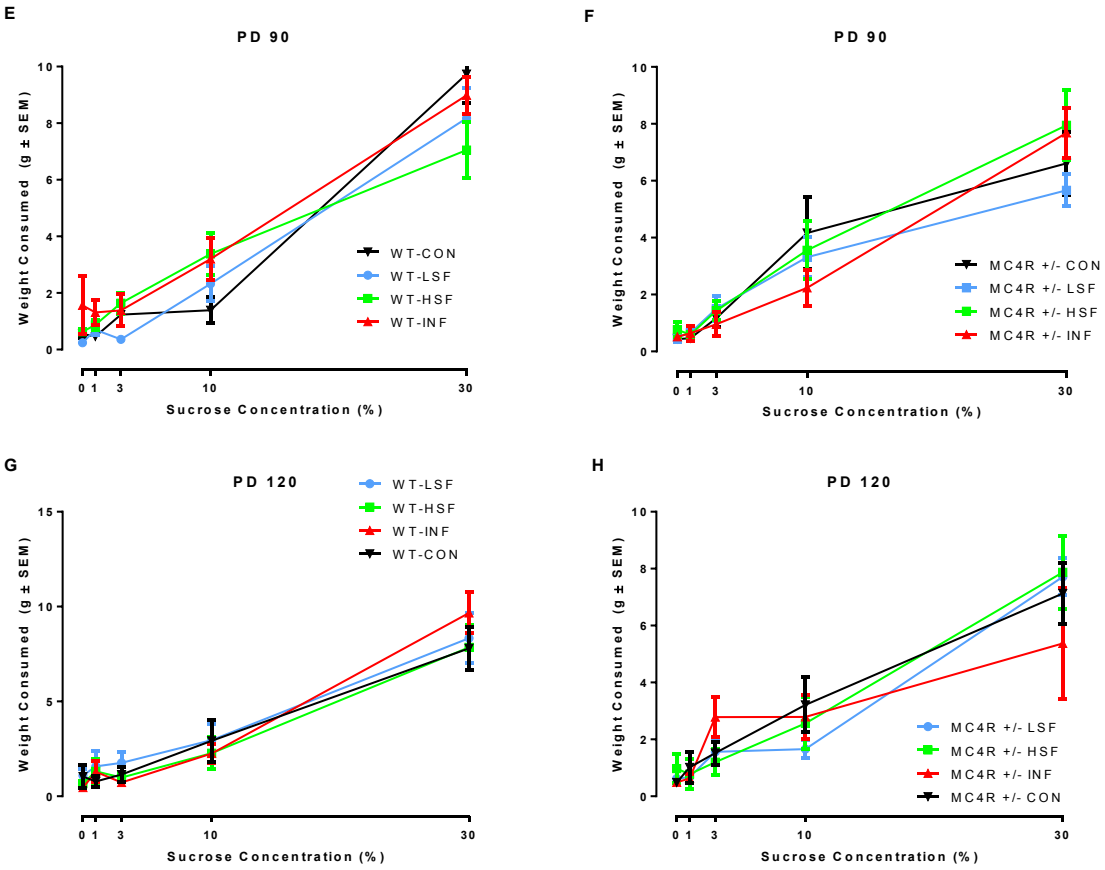


Figure 3.2: Locomotor Activity. Mean (±SEM) of basic ambulations (a-b), fine ambulations (c-d), and rearing (e-f) over 7 separate 60-minute locomotor activity tests conducted on PD 21, 30, 60, 90, 120, 150, and 180. A significant condition X diet interaction ($p<0.001$) indicates effects of dietary induced obesity across all 3 parameters. Beginning in early adulthood, MC4R +/- rats on the HSF diet rear less than any other group. This effect is recovered by PD 150.





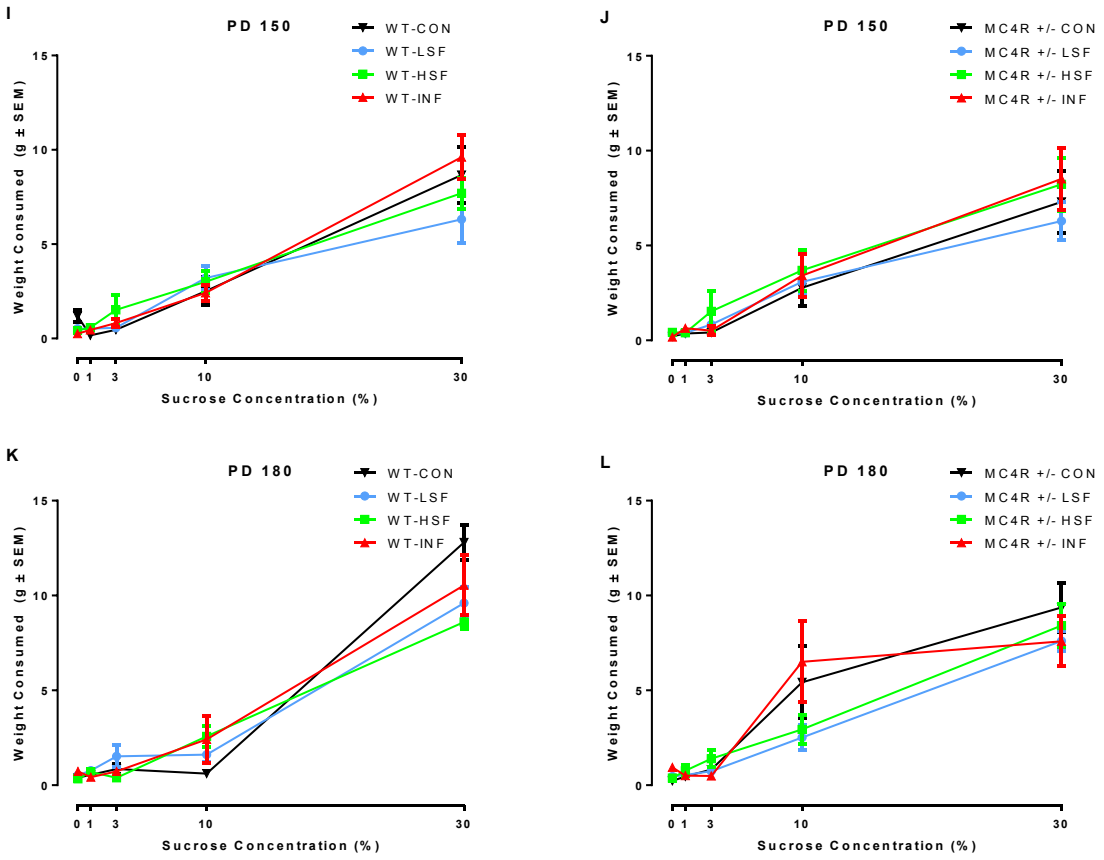


Figure 3.3: Sucrose Preference Test. MC4R +/- KO rats exhibit an altered searching pattern in a 5- choice sucrose preference test. Tests were performed on PD 30 (a-b), 60 (c-d), 90 (e-f), 120 (g-h), 150 (i-j), and 180 (k-l). Alterations to sucrose preference do not seem to be affected unless an animal is fed a high total fat (40% kcal/vol) diet ($p<0.05$). The linear relationships for the MC4R +/- CON and INF groups are altered on the final testing day (PD 180), but are not statistically significant.

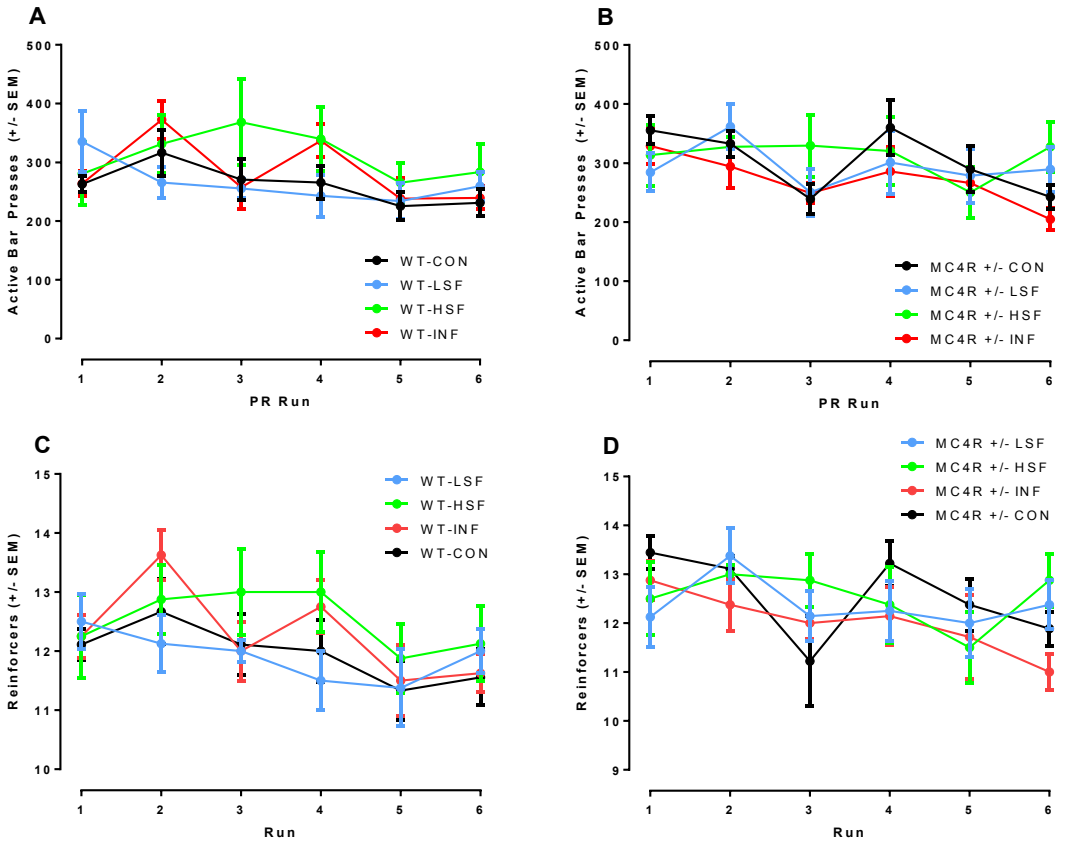


Figure 3.4: Progressive Ratio Operant Testing. On geometric PR schedules, neither diet nor genetic condition yielded a significant main effect on either number of active lever presses or reinforcers obtained. Both active bar presses (a,b) and reinforcers (c,d) failed to reach statistically significant variance among means of all groups.

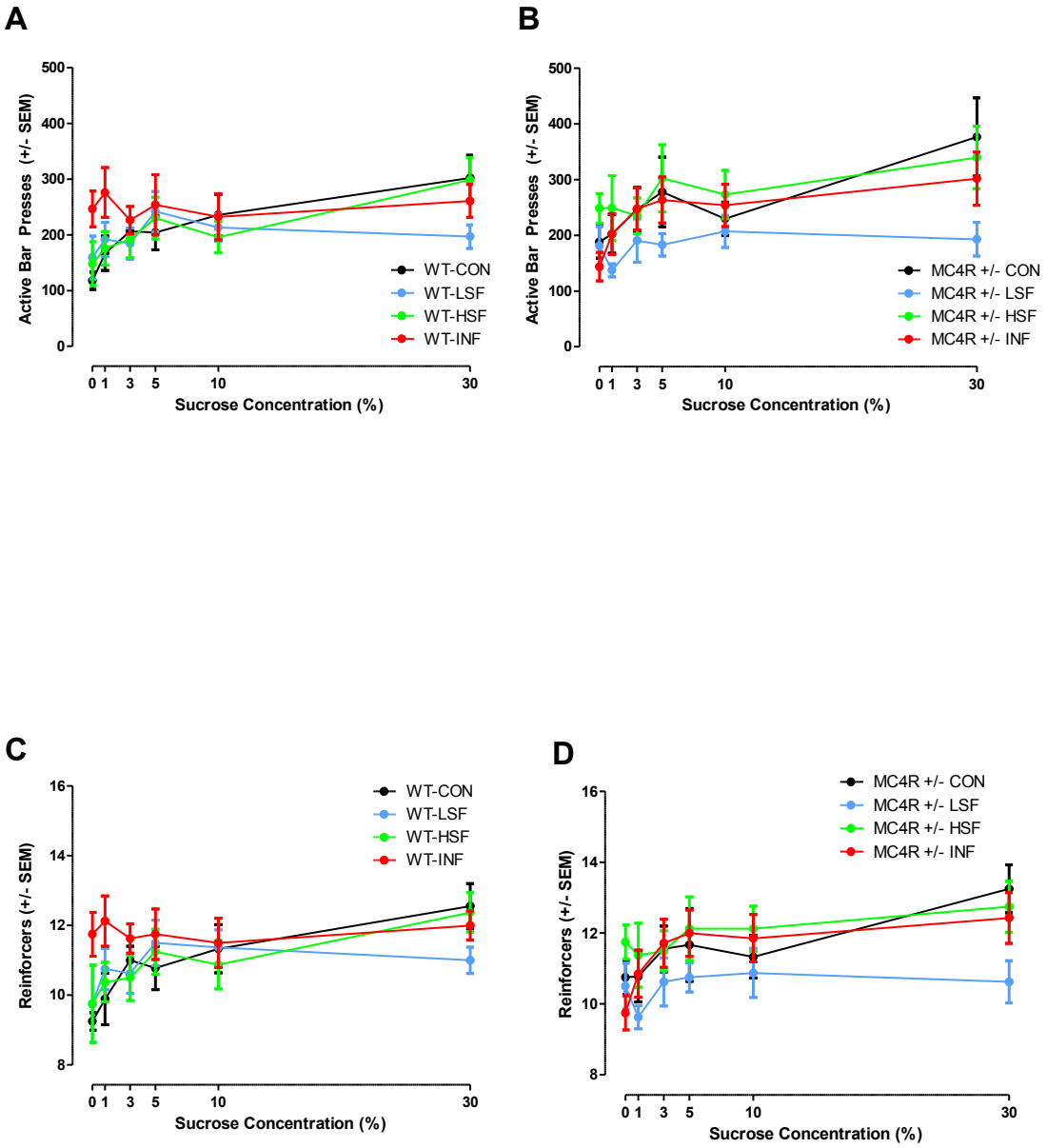
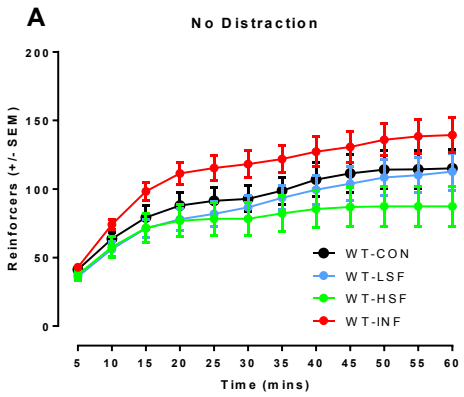
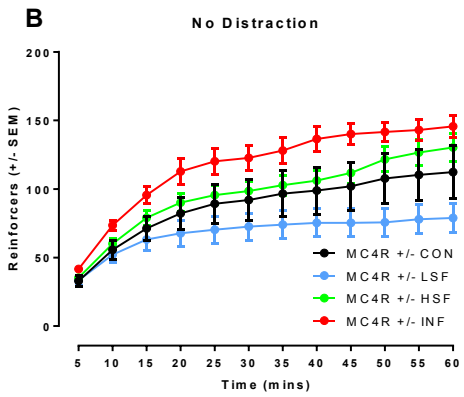
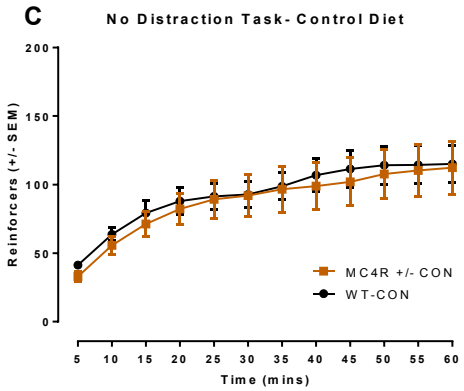
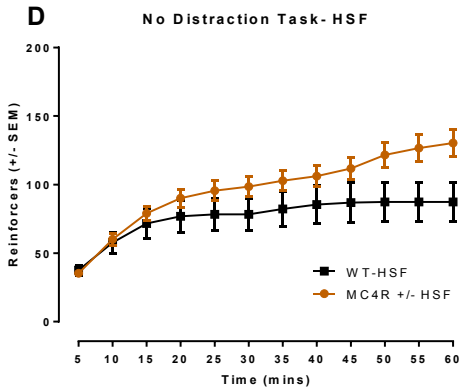


Figure 3.5: Variable Sucrose Concentration PR Testing. On geometric PR schedules using differing concentrations of sucrose, there is a significant effect of diet over time, $p<0.05$. The LSF groups exhibited less motivation for 5% (MC4R +/- only; b,d) and 30% (both groups; c,d) sucrose reinforcers compared to WT groups (a,c); $p<0.05$.









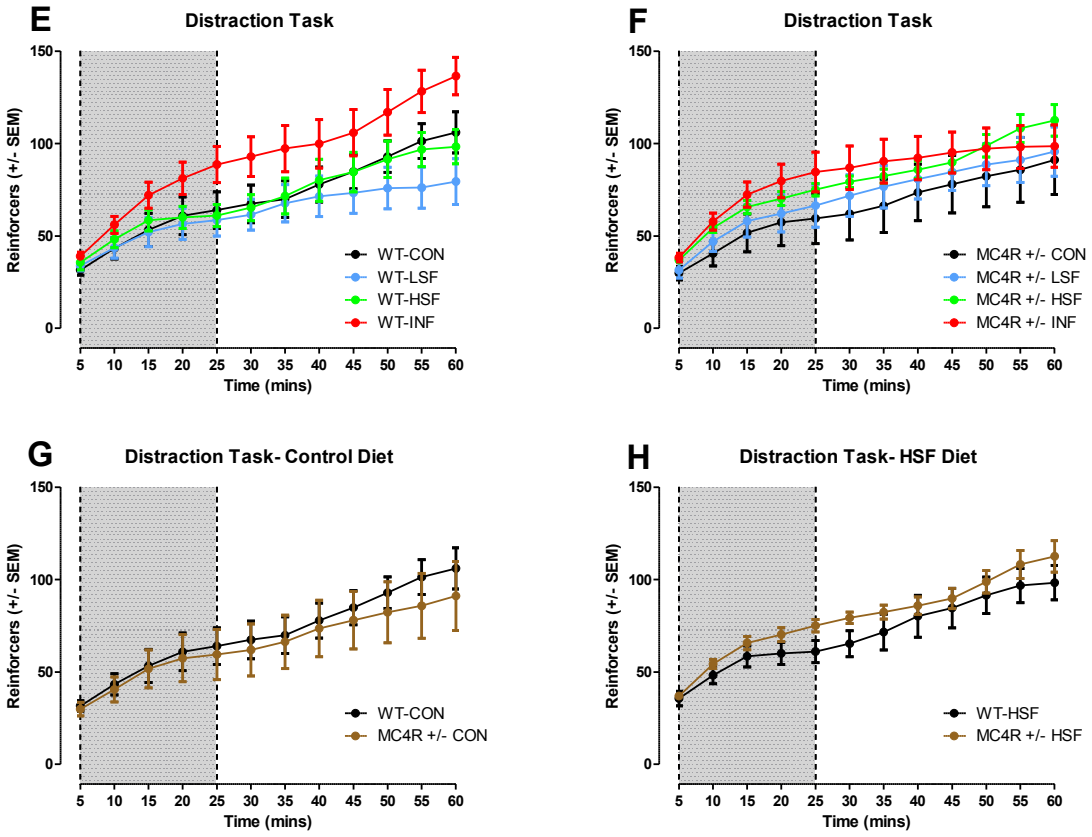
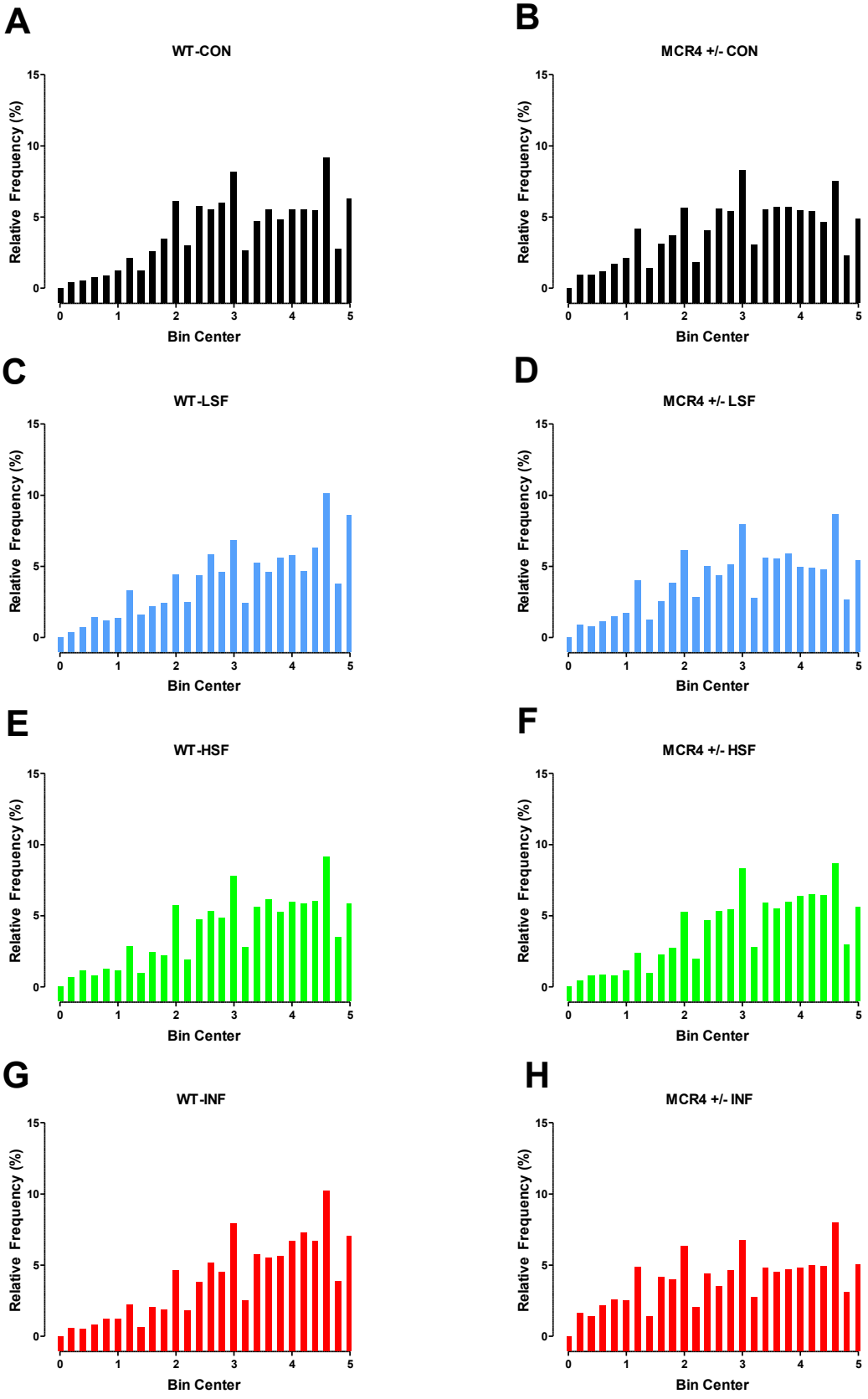
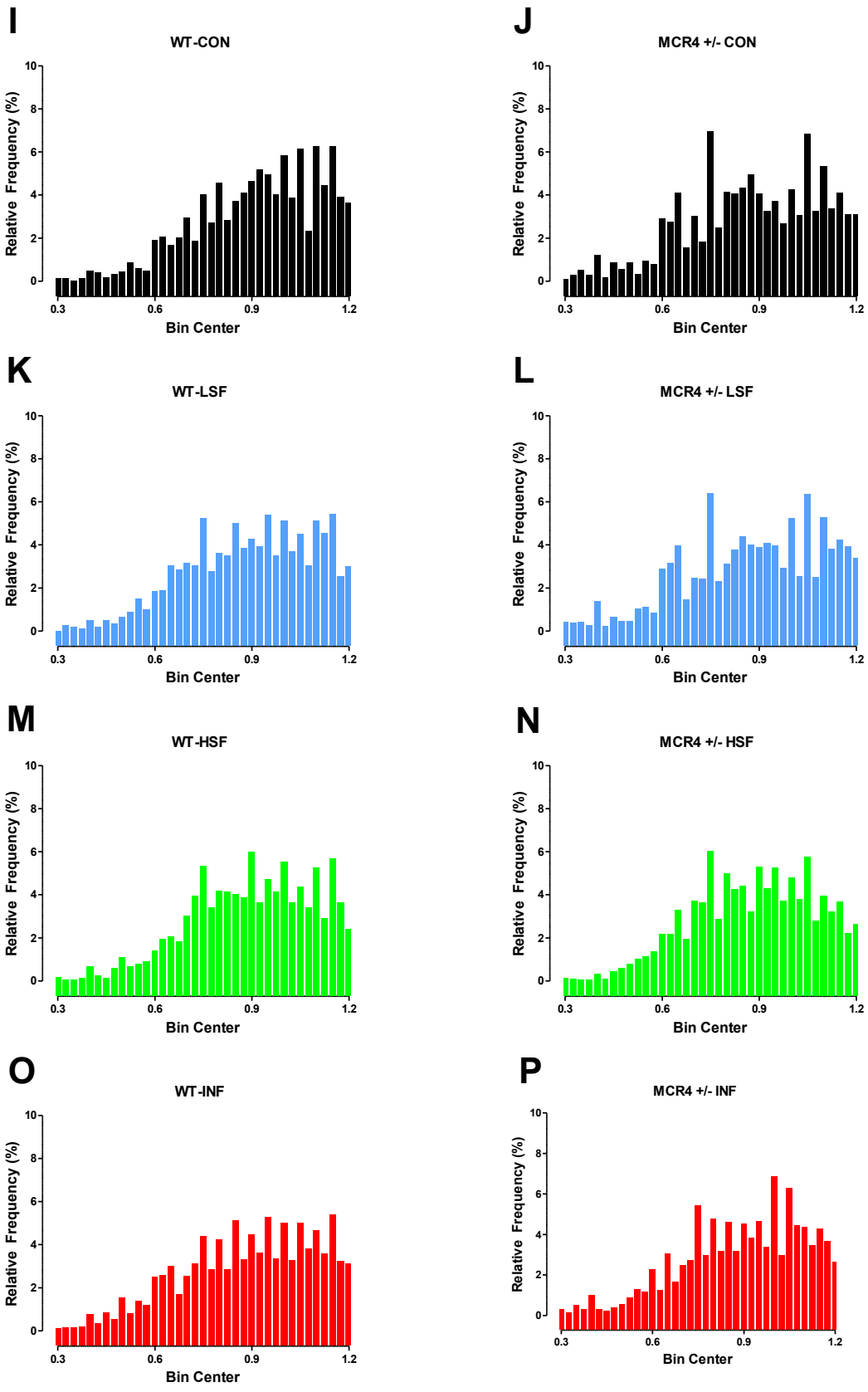


Figure 3.6: Distraction Task. MC4R +/- KO rats exhibit an altered motivational behavior when presented with a distraction tone, engaged during minutes 5-25 of the test (denoted by the shaded area). (a-b) Rats on the INF diet, regardless of condition, exhibit increased responding for 5% sucrose reward on an FR5 schedule. Both groups on the CON diet exhibit similar motivation for reward (c) but MC4R +/- rats on the HSF diet exhibit increased rewards near the end of the 60-minute task (d) ($p<0.0001$). (e-f) the WT-INF group exhibits increased responding during the distraction tone and near the end of the 60-minute testing period, contrary to that seen in the MC4R +/- INF group. (g-h) MC4R +/- HSF rats exhibit compulsive responding behavior for sucrose reinforcement during the presence of a distraction tone (minutes 5-30), opposed to rats in the WT-HSF group ($p=0.075$).





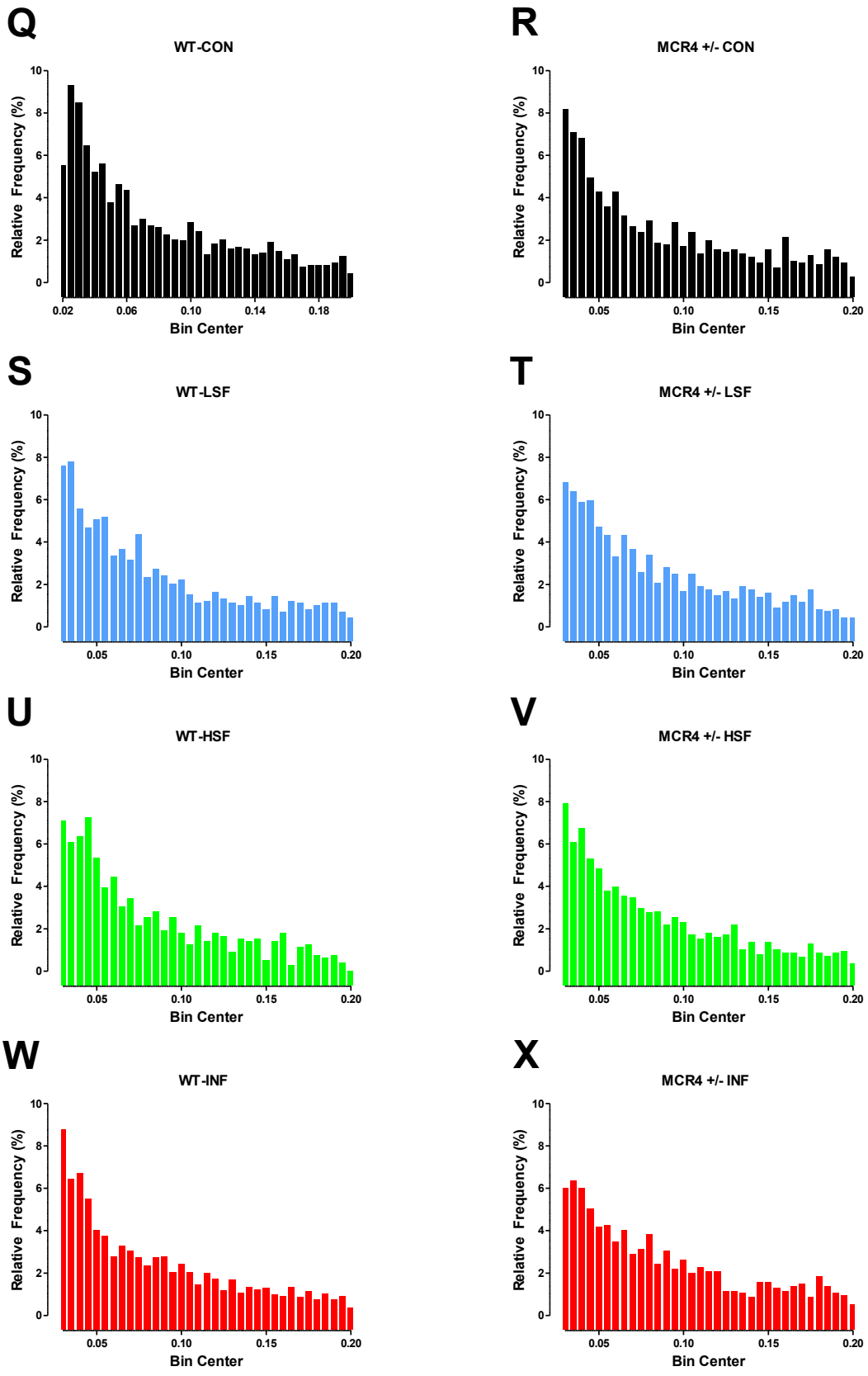


Figure 3.7: Dendritic Spine Analysis of Medium Spiny Neurons in the Nucleus Accumbens. Spine length relative frequencies vary significantly among groups, $p<0.0001$ (a-h). Spine head diameter cumulative frequencies vary significantly when compared to the WT-CON group, $p<0.0001$ (i-p). Spine volume cumulative frequencies were significantly different for animals on the INF diet compared to the WT-CON group, but follow opposite trajectories (larger volume in WT compared to MC4R +/-) $p<0.001$ (q-x).

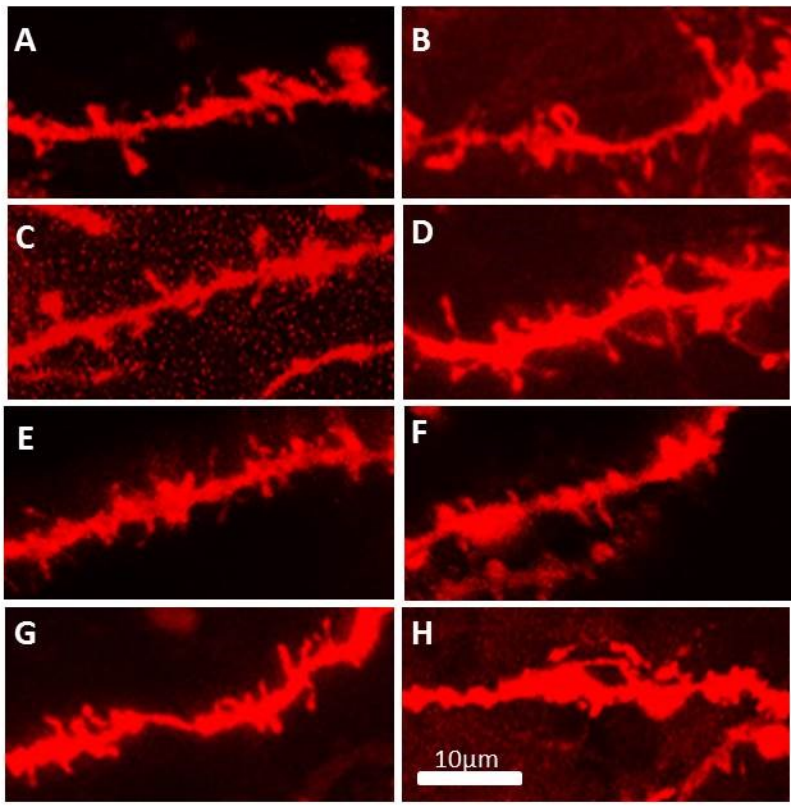


Figure 3.8: DiOlistically labeled medium spiny neurons of the nucleus accumbens (60x). (a) Spines from the WT CON group. Spines have a more pronounced head diameter compared to every other group. (b) MC4R +/- CON group exhibits short, thin spines with a near-equal ratio of neck width to head width. (c) WT LSF group spines are longer than WT-CON but also exhibit a near equal ratio of neck width to head width. (d) MC4R +/- LSF group exhibits a similar phenotype to their CON counterparts. (e) WT HSF group displays numerous long, thin spines at low volume (f) MC4R +/- HSF group spines are the longest out of any group, and head diameters and volume are significantly reduced compared to WT CON spines. (g) WT INF group spines tend to be shorter with less pronounced spine heads. (h) MC4R +/- INF group similarly displays a shorter, stubbier spine phenotype with reduced volume compared to the WT CON group.

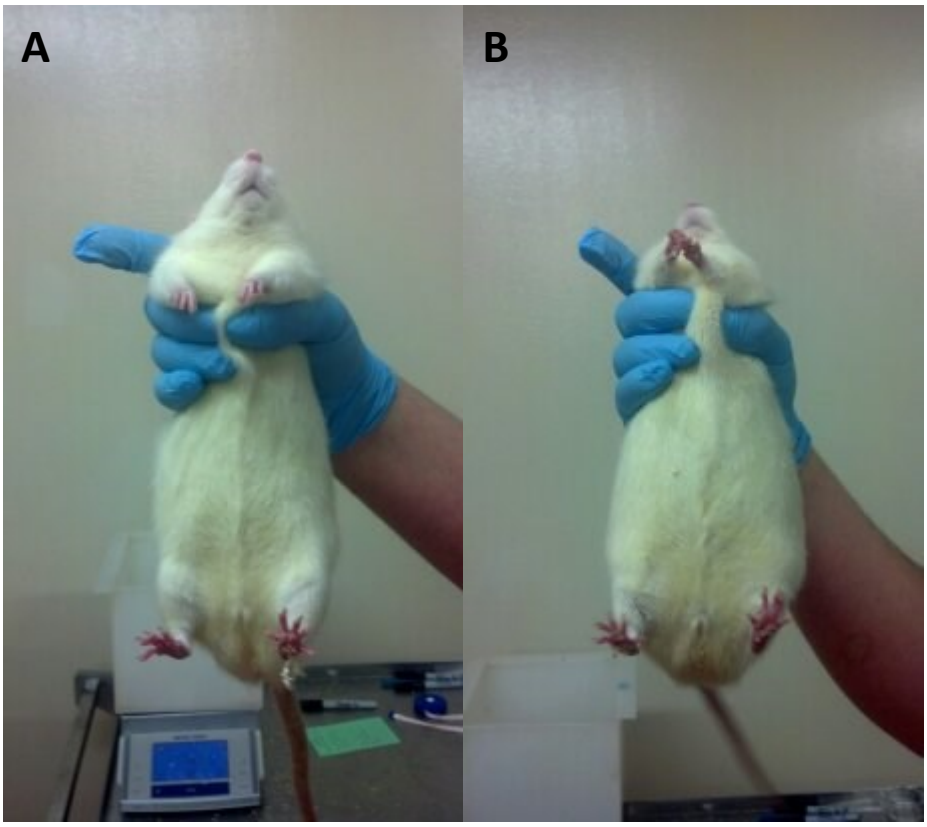


Figure 3.9: MC4R +/- Rats Exhibit Phenotypic Obesity Characterized By an Increased Waist Circumference And Weight. (a) Wild-type Wistar rat on the low saturated fat (6% wt/vol, 40% total kcal from fat) diet. (b) MC4R +/- Wistar rat on the low saturated fat (6% wt/vol, 40% total kcal from fat) diet. Note additional visible mass and waist circumference.

CHAPTER 4: DISCUSSION

The MC4R +/- rat exhibits a similar obese phenotype to that of its human counterpart, and the interaction of diet and genotype result in significant differences in weight observed during adolescence; differences in BMI become statistically significant around late adolescence/early adulthood under HSF conditions. Interestingly, our nonlinear curve fits of weight trajectory show heavier wild type animals in the HSF group than that of MC4R +/- KO rats on the control diet; the opposite is true for the wild type LSF group- these results are important signifiers of dietary-induced obesity working in conjunction with the genetic knockout, and provides promise for MC4R as both a potential therapeutic target and preventative screening tool for early-onset obesity in terms of dietary saturated fat content. Divergence in weights become statistically significant in late adolescence/early adulthood in the rat, correlating to that observed in human MC4R haploinsufficiency (Loos et al., 2008;Lubrano-Berthelier et al., 2003a;Martinelli et al., 2011), highlighting a critical intervention period occurring in conjunction with puberty. As this is a time which is highly focused on the increased hormonal signaling in both the thalamic region and pituitary (Dunkel and Quinton, 2014), it would be expected that the main effect of genetic condition become apparent from this point, continuing throughout adulthood.

MC4R haploinsufficient rats exhibit altered preference for varying concentrations of sucrose prior to the onset of dietary-induced obesity, which has critical implications for therapeutic interventions that may be required for adolescent patients. During our first sucrose preference test, performed at postnatal day 30, haploinsufficient rats appeared to

exhibit markedly increased drinking of the 10% solution bottle while WT rats consumed more of the 30% solution. Under HSF conditions, WT rats exhibit a nearly-perfect linear relationship as a function of sucrose concentration, where haploinsufficient animals exhibit increased consumption of the 10% sucrose solution. Since most soft drinks and fruit juices fall into the range of 10-15% sweet carbohydrate (sucrose or fructose, wt/vol), and are often consumed by children (Malik et al., 2006), altered concentration preference may influence drink choice in adolescence as a function of both MC4R haploinsufficiency and quantity of dietary saturated fat content. Domingos et al. (2013) previously studied the effects of leptin deficiency on sucrose preference using ob^{-}/ob^{-} mice in a Dat-Cre inducible background; concentration preference for sucrose over sucralose, an acaloric artificial sweetener, was reversed using optogenetically stimulated DA neurons in the ventral tegmental area. These results show involvement of the dopaminergic system and support the hedonic aspect of sugars and palatable foods in rats (Benton, 2010;Levine et al., 2003). Subthreshold levels of subcutaneously administered leptin did not alter preference for sucrose nor had any influence on food intake, but threshold levels of injected leptin caused lower responding for sucrose reinforcement (Domingos et al., 2014). Our results are somewhat correlative to this model, especially in the earliest testing day (PD30). Rats in both the LSF and HSF groups preferred 10% sucrose over 30% during this primary session, following previous reports of lower responding for sucrose reinforcement in rat models of dietary-induced obesity (Marco et al., 2012;Davis et al., 2008), which is crucial when considering the overwhelming popularity and consumption of drinks with similar sugar contents. Over time, WT animals exhibit a linear relationship of consumption correlated with sucrose concentration, whereas haploinsufficient animals on the CON and INF diet show a higher preference for 30%

sucrose over 10% sucrose on the final day of testing (PD 180) (see Fig 3). Our results are indicative of two important factors: 1, that fatty diets can change preference for palatable sucrose concentrations in a haploinsufficient environment regardless of saturated fat content, and 2,: that haploinsufficient individuals on fatty diets show preferences similar to their WT counterparts. In a study by Panaro and Cone (2012), MC4R haploinsufficient mice had no alteration for preference of sucrose solution, and homozygous knockouts exhibited *decreased* preference for high fat and high sucrose foods. Panaro and Cone's argument was that mice with these genetic abnormalities seem to be driven by novelty rather than preference for high calorie foods. It is unknown whether altered searching patterns are the result of novelty rather than specific sucrose concentration preference in rats; this is an avenue to explore in future investigations. If this phenomenon *does* manifest itself in rats as well, it would explain our results; dietary-induced obese haploinsufficient rats may present with a reduction in the palatability of high-sucrose concentrations. More research is needed to determine true alterations to sucrose concentration preference, hyperdipsia, and altered approach of novel food sources in a MC4R haploinsufficient background.

Alterations to satiety may also alter the reward processing of individuals who indulge in high-sugar foodstuffs. FMRI analysis of adolescents consuming high-fat milkshakes showed greater activation of the caudate and hippocampus, contrasting to high-sugar milkshakes, which caused greater activation in the bilateral insula and putamen (Stice et al., 2013). Obese individuals tend to exhibit less dopaminergic tone whilst consuming highly-palatable foods, indicating dopamine's attenuating ability food consumption and reward processing (Stice et al., 2013). The gene/environment interaction of MC4R on reward processing in adolescence highlights this as a critical developmental period with

potential long-term influence on food preference and reward value from highly palatable foods. Overconsumption of sucrose during adolescence has been shown to decrease preference and motivation for sucrose rewards later in adulthood (Vendruscolo et al., 2010), which may indicate an adult-onset exhibition of sugar "tolerance".

The effects of dietary-induced obesity on motivational behaviors for sucrose reward manifest in compulsive and clinical ways. Compulsive eating of palatable foods mirror those seen in drugs of abuse, as "abuse" of highly-palatable foods has potential neurochemical correlations between overeating (Vucetic et al., 2011;Levine et al., 2003) and compulsive drug use in addiction (Grant and Chamberlain, 2014;Hadad and Knackstedt, 2014). These challenges are robust, and sweet rewards tend to be more preferable to rats than drugs of abuse (Madsen and Ahmed, 2014). In a study by Velazquez-Sanchez et al. (2014), the investigators used a differential reinforcement task to classify Wistar rats as high-impulsivity trait or low-impulsivity trait before applying a PR operant schedule for highly palatable foods. They found that the high-impulsivity group predicted instances of hyperphagia and addictive-like behavior (Velazquez-Sanchez et al., 2014), albeit through the use of extreme-group designation for "high-impulsive" and "low-impulsive" classification. Our results on repeated performance of a PR operant task for a 5% sucrose reward did not yield statistical significance between groups, but when KO HSF rats were tested in the presence of a distracting tone, the tone had less of a reducing effect on active bar pressing, indicating a possible compulsive mechanism for sucrose reinforcement. Impulsivity/compulsivity has not been studied in the human MC4R haploinsufficient population, and more research into early predictor models of impulsivity in a MC4R haploinsufficient environment is necessary to adopt an appropriate therapeutic angle. Such

data would provide investigators with both a psychological and physiological angle to treat individuals at risk of early-onset dietary-induced obesity.

Our variable-concentration PR task has shown a decrease in active bar presses and reinforcers achieved among the MC4R +/- LSF group compared to all other groups. This dietary-induced attenuation for sucrose reinforcement through concentration of saturated fat content has only been seen in one other study of which the authors are aware. Blaisdell et al. (2014) trained rats on a refined low fat diet (10% total kcals from fat, compared to 13% for the unrefined control diet) and showed decreased active bar pressing on PR3 and PR5 schedules for individuals on refined diet as well as increased weight, suggesting that diet quality is more causative of obesity that concentrations of dietary fat. Exploration of the potential confound by use of refined vs. unrefined sucrose and fatty diets has not been addressed in our study, but represent an interesting clinical observation that must be further investigated regarding studies involving dietary-induced obesity and sucrose reinforcement. In our experiment, the use of a geometric ratio (defined as $n_j=5e^{j/5}-5$) is well suited for examination of satiety, as the requirements for response increase exponentially after each reinforcement; this contrasts with an arithmetic ratio of responding requirements, and correlates to ratios commonly seen in studies of drug abuse (Killeen et al., 2009;Richardson and Roberts, 1996).

Locomotor activity tests yielded somewhat predictable results following our hypothesis; there is slight variation among groups, with wild-type animals in the inflammatory group being the upper-bound (most active) while the MC4R +/- HSF group is the lower-bound. It appears as though weight is correlated to levels of physical activity, as WT INF animals were the most active while weighing the least, and KO HSF animals were

the least active while weighing the most. Interestingly, the inflammatory diet appears to be directly correlated with increased activity compared to other diets, as well as increased motivation for operant rewards and more compulsive activity during a distraction task. Indeed both wild-type and MC4R +/- animals in the inflammatory group weighed less than any other counterpart; there are minor, yet deserving of mention, correlations between dysfunction of the MC4R and clinical attention deficit in humans (Agranat-Meged et al., 2008), especially considering past reports on the decrease in spontaneous activity for MC4R +/- rats (Lutz and Woods, 2012;Mul et al., 2012). This trend of increased basic and fine ambulation whilst on the inflammatory diet is only apparent in the WT condition, indicating that functional MC4R in an inflammatory PUFA environment may be involved in stereotypical behaviors similar to that seen in ADHD models; the receptor has gained attention in this field for that very reason (Agranat-Meged et al., 2008). These results were unexpected and represent an interesting exploratory avenue for future experiments regarding MC4R, inflammatory PUFAs, and detrimental effects on attention and social behavior. Indeed, omega-3 PUFAs comprise a therapeutic avenue for individuals suffering from ADHD (Agranat-Meged et al., 2008;Gillies et al., 2012), and much more investigation into the effects of PUFAs on behavioral disorders is needed.

Perhaps our strongest evidence for neurophysical consequences as a result of both dietary-induced obesity and MC4R haploinsufficiency lies in alterations to spine morphologies in the NAc, the center for hedonic reward and aversive learning (Sun and Laviolette, 2014). The most apparent effects of the interaction between MC4R haploinsufficiency in a dietary-induced obesity environment manifest in alterations to spine head diameter. When compared to the WT CON group, every other group contained a

lesser relative frequency of spines with larger head diameters. Additionally, differences in spine length are apparent when each experimental group is compared to the WT CON group. A high saturated fat diet appears to diminish the haploinsufficiency effects on spine length, indicative of the significant effects of dietary-induced obesity and saturated fat content on the hedonic reward system. Alterations to dendritic spine morphology is considered one of the hallmarks of neuroplasticity (Sala and Segal, 2014) and have been studied in chronic drug abuse models (Quintero, 2013;Gipson et al., 2013;Pal and Das, 2013), but spine morphology is highly motile (Bosch et al., 2014) and recent findings indicate a lack of correlation between spine neck width, neck length, and synaptic potential (Takasaki and Sabatini, 2014). Regardless, cocaine-withdrawn animals present a marked increase in spine head diameter 45 minutes after cessation (Dumitriu et al., 2012b;Toda et al., 2010) which may indicate a reversion in long-term potentiation capability. As substance abuse disorders are thought to physically manifest in reduced neuronal connectivity between the frontal cortex and basal ganglia (Motzkin et al., 2014), neuronal alterations in the NAc seen in this experiment may have both a causative and correlative effect on alterations to reward processing in the rat. Indeed, a high-fat diet in the rat has been shown to attenuate both motivation for sucrose reward as well as amphetamine-induced conditioned place preference (Davis et al., 2008), suggesting a mediating effect of dietary fat on dopaminergic turnover in the mesolimbic system. Additionally, the morphological spine changes seen in rats on the inflammatory diet could also possibly correlate with the incorporation of inflammatory PUFAs into the phospholipid bilayer of the cell; more research into the functional alterations to neural membranes in differing PUFA environments is needed, as

well as exploration into the attenuation effects of dietary saturated fat on motivation for stimulants in a MC4R haploinsufficient background.

As our perspective on obesity and medical intervention shift over time, the results of this study indicate the necessity for both a therapeutic intervention targeting the MC4R receptor as well as an intervention regarding overconsumption of foods rich in saturated fatty acids, particularly for individuals prone to impulsivity. These interventions must occur as early as possible (i.e. pre-obesity) in haploinsufficient patients due to altered substrate preference and reward processing. Indeed MC4R antagonists and reverse-agonists have been investigated in both rat (He et al., 2010a) and human cell culture models; non-selective agonists have been shown to decrease adiposity and hyperphagy *in vivo* as well, but few studies have made it past pre-clinical trials (Fani et al., 2014). Regardless, MC4R has emerged as a key player in the mediation of appetitive behavior prior to clinical manifestation of obesity. These alterations have potentially interactive effects with dietary environments that could cause morphological changes to the hedonic pleasure centers of the brain.

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APPENDIX A: POWER ANALYSIS

Parameter		Partial Eta Squared	Effect Size f	G Power	SPSS Observed Power*
Activity:	BS LITTER	0.041	0.2067	0.26	0.999
	BS DIET	0.038	0.1987	0.42	0.998
	BS GENE	0.024	0.1568	0.26	0.964
	BS CONDITION x DIET	0.035	0.1904	0.42	0.997
	WS TIME	0.182	0.4717	0.99	1
	WS TIME x LITTER	0.084	0.3028	0.83	1
	WS TIME x GENE	0.071	0.2764	0.75	1
	WS TIME x DIET	0.084	0.3028	0.84	1
	WS TIME x GENE x DIET	0.114	0.3587	0.95	1
Sucrose Preference:	BS LITTER	0.056	0.2436	0.61	0.279
	BS GENE	0.098	0.3296	0.89	0.523
	BS DIET	0.231	0.548	0.99	0.745
	BS CONDITION x DIET	0.134	0.3933	0.98	0.604
	WS TIME	0.644	1.345	0.95	1
	WS TIME x LITTER	0.656	1.38	1	0.9
	WS TIME x GENE	0.592	1.204566	1	0.789
	WS TIME x DIET	0.547	1.16	1	1
	WS TIME x DIET x GENE	0.581	1.177	1	0.969
Operant Testing:	BS LITTER	0.038	0.199	0.29	0.229
	BS DIET	0.116	0.3622	0.82	0.58
	BS GENE	0.016	0.1275	0.13	0.119
	BS CONDITION x DIET	0.065	0.2637	0.5	0.328
	WS TIME	0.184	0.4748	0.99	0.48
	WS TIME x LITTER	0.212	0.5187	0.99	0.57

	WS TIME x GENE	0.142	0.4068	0.99	0.352
	WS TIME x DIET	0.265	0.6004	0.99	0.753
	WS TIME x GENE x DIET	0.252	0.5804	0.99	0.717
Variable PR	Litter	0.036	0.1932	0.33	0.831
	Gene	0.015	0.1234	0.16	0.46
	Diet	0.025	0.1601	0.25	0.84
	Gene X Diet	0.015	0.1234	0.16	0.584
Distraction	Litter	0.002	0.0447	0.21	0.23
	Gene	0.001	0.0316	0.13	0.295
	Diet	0.081	0.2968	0.99	0.81
	Gene X Diet	0.095	0.324	0.99	0.396

Parameter	Mean WT	WT SD	Mean MC4R	MC4R SD	Power
Weight	627.84	52	679.46	67.67	0.92
Length	225.9	5.975	228.1613	6.875	0.28
WC	218.9375	8.007	227.677	11.53	0.94
BMI	1.2307204	0.101	1.306103	0.113437	0.79

* SPSS treats the sample effect size as the population effect size for its computation of observed power.

APPENDIX B: BODY MASS INDEX ACROSS TIME

Table B.1
Body Mass Index (g/cm^2) ± SEM at Postnatal Day 21, 60, 90, and 150

	Mean PD 21	SEM	Mean PD 60	SEM	Mean PD 90	SEM	Mean PD 150	SEM
WT-CON	0.496264	±0.020159	0.991490	±0.023467	1.035775	±0.026324	1.089108	±0.024383
WT-LSF	0.506996	±0.009996	1.002901	±0.038325	0.994163	±0.016704	1.151623	±0.022219
WT-HSF	0.553793	±0.027799	0.983502	±0.019304	1.067049	±0.061802	1.163229	±0.023398
WT-INF	0.474004	±0.013341	0.998807	±0.044911	0.955810	±0.024166	1.077883	±0.030939
MC4R +/-CON	0.537939	±0.022369	1.055676	±0.024085	1.067956	±0.021103	1.167590	±0.031672
MC4R +/-LSF	0.534946	±0.025579	1.095936	±0.024041	1.040755	±0.021074	1.217427	±0.013762
MC4R +/-HSF	0.519234	±0.023948	1.197541	±0.168263	1.104197	±0.019672	1.227685	±0.02266
MC4R +/-INF	0.509270	±0.022864	1.014340	±0.019763	1.057411	±0.026653	1.171356	±0.024203