

**THE RELATIONSHIP BETWEEN NICOTINE AND BODY WEIGHT: IMPLICATIONS
FOR TOBACCO REGULATORY POLICY FROM RATS & HUMANS**

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Smokers weigh less than non-smokers and former smokers, an observation that has been attributed to nicotine in cigarette smoke. Despite the ability of nicotine to suppress weight gain, body mass index (BMI) is positively associated with smoking intensity. These phenomena suggest a complex relationship between nicotine and body weight: that nicotine impacts body weight, and that body weight may modify nicotine reinforcement. This dissertation tests the hypotheses that self-administered nicotine suppresses body weight and that body weight impacts nicotine reinforcement in rats and human smokers. Experiments tested these hypotheses in a rat model of nicotine self-administration and in human smokers. Experiments in Chapter 2 demonstrate that self-administered nicotine suppressed body weight gain independent of food intake in rats. Acquisition of low dose nicotine self-administration resulted in suppression of weight gain. In contrast, reduction of nicotine dose from a prior higher dose increased weight gain. Experiments in Chapter 3 demonstrated that self-administered nicotine in rats suppressed respiratory exchange ratio, indicating increased fat utilization, prior to a nicotine-induced suppression of weight gain. The experiment in Chapter 4 evaluated weight gain in human smokers randomized to smoke very low nicotine content (VLNC) cigarettes for 6 weeks. These data align with the rat self-administration data in Chapter 2; smokers compliant with VLNC cigarettes gained weight after 6 weeks of use. The current environment is obesogenic. The experiment in Chapter 5 tested the impact of self-administered nicotine on obesity-prone and

obesity-resistant rats and found that nicotine failed to suppress weight gain in obesity-resistant rats. Chapter 6 tested the ability of body weight and/or diet to impact nicotine self-administration. In obese smokers and rats, smoking/nicotine intake was increased, but decreased per body mass, suggesting that nicotine intake is titrated dependent on body mass. In sum, self-administered nicotine acts to suppress weight gain likely via increased fat utilization, and nicotine consumption is titrated dependent on weight. A potential strategy to reduce the health burden of smoking is a reduction in the nicotine content of cigarettes, which is hypothesized to reduce smoking and promote quitting. The data reported in this dissertation has important implications for tobacco regulatory policy.

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PREFACE

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1.0 INTRODUCTION

1.1 THE TOBACCO EPIDEMIC AND REGULATORY SCIENCE

Tobacco use, primarily through cigarette smoking, is the largest cause of preventable death worldwide. Despite the well-publicized health risks associated with smoking, approximately 19% of adults in the United States are smokers, and about half of these smokers are predicted to die prematurely due to tobacco-related illnesses (Centers for Disease et al., 2011). Strategies to reduce the morbidity and mortality caused by tobacco smoke are of immediate need. Nicotine, the primary psychoactive constituent in cigarettes, drives continued use of tobacco products. A potential strategy to reduce cigarette smoking and improve smoking-related public health outcomes is the reduction of nicotine content in cigarettes below a theoretical addictive threshold (Benowitz et al., 1994). Such a reduction would promote quitting in current smokers and prevent initiation in new smokers (Benowitz et al., 1994; Donny et al., 2012). Thus, with the goal of improving public health, The Family Smoking Prevention and Tobacco Control Act, passed in 2009, gives authority to the Food and Drug Administration (FDA) to mandate the content of nicotine in cigarettes to any non-zero level (Donny et al., 2012; Hatsukami et al., 2013; United States. Congress. House. Committee on Energy and Commerce. Subcommittee on Health., 2008). The World Health Organization (WHO) Framework Convention on Tobacco Control calls for established guidelines for the regulation of the content

of cigarettes (World Health Organization Study Group on Tobacco Product, 2012). The most recent report of the WHO study group on tobacco product regulation emphasizes nicotine reduction as a strategy to reduce the immense harm caused by tobacco smoke (World Health Organization Study Group on Tobacco Product, 2015). Recent evidence suggests that reduction of nicotine content in cigarettes reduces smoking in humans (Benowitz et al., 2013; Hatsukami et al., 2013). Such a tobacco control policy could have dramatic implications for the rates of smoking, but may also impact other health-related outcomes.

1.2 CONSIDERATION OF NICOTINE AND WEIGHT REGULATION

In the United States, the past 35 years have been marked by a slow decline in the number of smokers and a dramatic and rapid increase in the rates of obesity. Over 70% of adults and 17% of children and adolescents are considered overweight or obese and deaths due to obesity-related diseases are predicted to surpass mortality caused by tobacco smoke within the decade (Hurt et al., 2010; Mokdad et al., 2005; Stewart et al., 2009). Together, obesity and smoking represent the largest current obstacles in public health. Epidemiological and empirical studies describe an inverse relationship between tobacco smoking or nicotine and body weight (Audrain-McGovern et al., 2011; Jacobs et al., 1981), and desired weight loss or maintenance of reduced body weight is commonly cited as a primary reason for smoking (Fulkerson et al., 2003). Moreover, ex-smokers typically gain an average of 10 lbs within the first year of abstinence (Audrain-McGovern et al., 2011). Oftentimes even the possibility of weight gain after cessation is a strong enough motive to drive continued use (Donny et al., 2011b; Filozof et al., 2004). The decline in smoking rates may in part contribute to the increases in obesity (Chou et al., 2004) and

the motivation to continue to smoke as a method of body weight suppression could be greater in obese smokers (Audrain-McGovern et al., 2011). There is a growing appreciation that body weight and diet may be important environmental factors impacting motivated behaviors (Volkow et al., 2012). Smoking is associated with other maladaptive health behaviors, including increased intake of densely caloric diets (Rupprecht et al., 2015b). It is likely that diet and body weight have an important impact on nicotine-seeking behaviors. These observations imply that body weight may be a critical determinant of smoking and other related health behaviors as a result of a policy regulating the nicotine content in cigarettes. Though obesity and smoking are interrelated, how one might causally affect the other is essentially unstudied.

In the context of nicotine reduction, a regulatory policy mandating low levels of nicotine in cigarettes has two primary implications, as it relates to weight gain. Post-cessation weight gain has been attributed to nicotine withdrawal (Filozof et al., 2004; Gross et al., 1989) (detailed more below), and the possibility exists that reduction of nicotine in cigarettes could result in substantial weight gain in smokers (Rupprecht et al., 2015a). Second, the smoking population is heterogeneous and subpopulations of smokers may continue to smoke despite large reductions in nicotine content. The interrelationship between nicotine and body weight (the impact of nicotine on body weight regulation, and the impact of body weight on nicotine reinforcement) is the focus of the experiments in this dissertation.

1.3 SMOKING FOR WEIGHT LOSS: THE IMPACT OF NICOTINE ON ENERGY BALANCE

1.3.1 Humans.

Smokers weigh less than non-smokers and former smokers (Audrain-McGovern et al., 2011; Rasky et al., 1996). Approximately 10% of smokers report smoking for weight control (French et al., 1995; Klesges et al., 1989). Adolescents, women, and people with obesity may be more likely to report smoking for weight control, though there are also reports describing no effect of these factors (French et al., 1996; Fulkerson et al., 2003; Levine, 2008; Levine et al., 2013). Smoking results in a lower body weight set point (Cabanac et al., 2002). The weight-suppressive effects of smoking have been attributed to nicotine in cigarette smoke. Smokers may use cigarettes as a food replacement, and in this context, smoking may reduce caloric intake (Ogden et al., 1994). However, evidence also suggests that smokers and non-smokers have equal daily caloric intake (Perkins et al., 1992). The impact of cigarette smoke on feeding in human smokers is complex, and seems to be dependent on the caloric or palatable value of the food, as well as satiety-state (Perkins et al., 1995; Perkins et al., 1992). Cigarette smoke can increase basal metabolic rate, which is in part due to nicotine and to the inhalation of smoke (Audrain et al., 1991; Perkins, 1992b; Perkins et al., 1989b).

Cessation from cigarette smoking results in weight gain, which is variable across individuals (Audrain-McGovern et al., 2011). A large magnitude of post-cessation weight gain (i.e., “super-gainers”) is predicted by low weight prior to cessation and heavy levels of daily smoking (Veldheer et al., 2015). Post-cessation weight gain is attenuated by nicotine replacement therapies and cessation medications that act as partial agonists at nicotinic

acetylcholine receptors (nAChR) (Farley et al., 2012; Gross et al., 1989). Individual differences in efficacy for nicotine replacement to attenuate post-cessation weight gain have been reported (Lerman et al., 2004). For example, in post-menopausal women, transdermal nicotine patch can increase weight gain, compared to cessation without replacement therapy (Allen et al., 2005).

The landscape of tobacco product use is changing, with cigarette use at a slow decline, and use of electronic nicotine device systems (ENDS), or e-cigarettes, increasing. Recent evidence suggests that some ENDS users have the expectation of weight control, and that the use of ENDS may regulate weight control (Morean et al., 2017). Given that palatable food consumption is increased following nicotine via nasal spray (Perkins et al., 1992), the combination of nicotine and palatable flavors in ENDS products has important implications for ENDS abuse liability, and its impact on weight regulation. The issues of weight expectations from tobacco use, and the pharmacological actions of nicotine to regulate energy balance in human tobacco users remains relevant.

The impact of smoking and nicotine on weight regulation is complex, and difficult to study in a causal manner in humans. The majority of experiments testing the impact of nicotine or smoking on energy balance occur in current smokers, after the differences in body weight exist. Therefore, it is difficult to attribute differences in caloric intake, energy expenditure, or other parameters to nicotine itself, or long term adaptations due to reduced body weight gain over time. Additionally, weight-concerned smokers are more likely to use smoking as a form of food restriction. Rodent models of nicotine administration represent a tool for the study of the impact of nicotine on energy balance without these confounds.

1.3.2 Rodents.

Nicotinic receptors are expressed almost ubiquitously on central and peripheral cells involved in energy balance (Zoli et al., 2012). Therefore, the regulation of body weight by nicotine is complex, and results of studies focused on nicotine and energy balance are likely dependent on the method, duration, and dose of administration. The regulation of body weight is dependent on energy in (i.e., calories consumed and nutrient absorption) and energy out (i.e., energy expenditure: metabolic rate, adaptive thermogenesis, and physical activity) (Grill et al., 2012). Indeed, previous work has established effects of nicotine on decreased food intake, and increased metabolism, thermogenesis, and physical activity (L. L. Bellinger et al., 2010; Grunberg et al., 1985a; Zoli et al., 2012). An incomplete list of the impact of nicotine on energy balance can be found in Table 1. While nicotine has the ability to regulate many aspects of energy balance, it is commonly accepted that the substantial body weight reduction by nicotine is due to decreased food intake. Of note (Table 1), the vast majority of experiments testing the impact of nicotine on energy balance have used non-contingent experimenter-administered administration. Several experiments have demonstrated that experimenter- and self-administered nicotine differentially impact catecholamine and glucocorticoid release and cardiovascular regulation, factors that influence body weight regulation. The effects of self-administered nicotine on energy balance were experimentally tested, and the results are reported in Chapters 2 – 5 of this dissertation.

Table 1. The impact of nicotine on energy balance in rodents

Publication	Administration route	Dose	Duration	Subject	Weight	Feeding	Other notes
Aceto et al. (1986)	Continuous s.c. infusion	10 mg/kg/day	6 days	Male SD	↓	n/a	↓ water intake
L. Bellinger et al. (2003)	i.p. injection	2 or 4 mg/kg/day	14 days	Male SD	↓	↓ meal size; ↑ meal #	No change in water
L. L. Bellinger et al. (2005)	i.p. injection	1.4 mg/kg/day	12 days	Female SD	↓	↓ meal size; ↑ meal #	
L. L. Bellinger et al. (2010)	i.p. injection	1.4 mg/kg/day	12 days	Male SD	↓	↓	↓ RQ but not EE
Bishop et al. (2004)	Osmotic s.c. minipumps	6 mg/kg/day	12 days	Female SD	↓	↓	↓RQ (acute) but not EE
Blaha et al. (1998)	Osmotic s.c. minipumps	6 mg/kg/day	7 days	Male & Female Fischer	↓	↓ via meal size	No change in estrous cycle
Bowen et al. (1986)	Osmotic s.c. minipumps	4, 8, 12 mg/kg/day	19 days	Female SD	↓	↓	No change in water or activity
H. Chen et al. (2005)	Smoke inhalation	1.2 mg (3x daily)	4 days	Male Balb/C mice	↓	↓	↑ UCP3 in BAT; ↓ UCP1 in WAT
Clarke et al. (1984)	s.c. injection	0.4 mg/kg	1 month	Male SD	↓	↓	↓ water intake
Grebenstein et al. (2013)	Programmed i.v. infusions	60µg/kg/inf (23-h)	9 days	Male SD	↓	↓ in light phase; ↓ meal size	
Grunberg et al. (1985a)	Osmotic s.c. minipumps	4, 8, 12 mg/kg/day	18 days	Male SD	↓	n/a	↑ activity (after ↓ decrease in weight)
Grunberg et al. (1985b)	Osmotic s.c. minipumps	6 or 12 mg/kg/day	17 days	Male SD	↓	↓, but less for sweet foods	
Grunberg et al. (1988)	Osmotic s.c. minipumps	6 or 12 mg/kg/day	17 days	Male & Female SD	↓	↓ of junk food only	
Grunberg et al. (1984)	Osmotic s.c. minipumps	4, 8, 12 mg/kg/day	14 days	Male SD	↓	No change	
Grunberg et al. (1986)	Osmotic s.s. minipumps	4, 8, 12 mg/kg/day	17 days	Female SD	↓	↓ at highest dose	
Mangubat et al. (2012)	i.p. injection	0.5 or 1.4 mg/kg	50 days	Male C57BL/6 mice	↓	↓	↓ fat mass in HED fed only
de Morentin et al. (2012)	s.c. injection	2 mg/kg/12h	17 days	Male SD	↓	↓	↓RQ; ↑ body and BAT temp
Mendez et al. (2016)	Osmotic s.c. minipumps	1 mg/kg/day	14 days	Male SD	↓	↓ meal duration and # (HED)	
Mineur et al. (2011)	i.p. injection	0.1 – 3 mg/kg	30 days	Male mice	↓	↓	
Miyata et al. (1999)	Osmotic s.c. minipumps	1,5, or 9 mg/kg/day	7 days	Male Fischer	↓	↓ meal # & size	Increased DA and 5HT in LHA
Miyata et al. (2001)	Osmotic s.c. minipumps	5 mg/kg/day	5 days	Female Fischer	↓	↓ via meal size; ↑ intermeal interval	Prolonged estrous cycle
Schechter et al. (1976)	i.p. injection	0.8 mg/kg (2 or 3x daily)	5 weeks	Male SD	↓	No change	
Seoane-Collazo et al. (2014)	s.c. injection	2 mg/kg/12h	8 days	Male SD	↓	↓	↓ fat mass; ↑ BAT temp
Wellman et al. (1986)	s.c. injection	0.8 mg/kg (3x daily)	14 days	Male SD	↓	No change	No change in BAT temp
Wellman et al. (2005)	s.c. injection	1.4 mg/kg/day	14 days	Male SD	↓	↓	Rats fed chow or HED
Winders et al. (1993)	Osmotic s.c. minipumps	12 mg/kg/day	14 days	Male SD	↓	↓	

1.3.1 Regulation of body weight by nicotine in obesity.

In the general population, smokers weigh less than non-smokers and smokers who quit gain a substantial amount of weight within the first year of abstinence (Audrain-McGovern et al., 2011); this relationship, however, is not as simple in the obese population. There is a negative correlation between the percentage of smokers and body mass index (BMI) among lean smokers, but this relationship is reversed among overweight, obese, and morbidly obese smokers (Chatkin et al., 2010). Thus, there is a U shape curve associated with percentages of smokers and smoking status as a function of BMI. Furthermore, several other studies report that moderate smokers weigh less than non-smokers, but heavy smokers (i.e., smoking at higher frequencies) are often obese (Chiolero et al., 2007a; Nielsen et al., 2006).

The relationship between heavy smokers and obesity has not been studied longitudinally or causally and may be explained by several factors. First, obesity may enhance nicotine reinforcement, driving cigarette consumption. This idea is explored in more detail below. Alternatively, the relationship may be explained by clustering of other risk behaviors; for example, higher levels of cigarette consumption are linked with low levels of physical activity, low fruit/vegetable intake, and high alcohol consumption (Chiolero et al., 2006). Thus, high rates of obesity among heavy smokers may be due to other independent health risk factors. Thirdly, obese smokers may smoke at higher rates in an effort to suppress body weight, which would indicate that obesity precedes initiation of smoking. A more complete understanding of these pathways is critical in determining how body weight and smoking behaviors may be impacted following the implementation of a nicotine reduction policy.

Despite data suggesting that high BMI is associated with smoking, compared to a rate of obesity of over 35% of obesity in the general population, only 5% of the general population smokes and is obese (Healton et al., 2006). Thus, chronic exposure to nicotine may prevent excess weight gain in individuals who would otherwise be obese. As these data are not available in human smokers, animal models might provide important information to fill this gap. A constant subcutaneous infusion of nicotine suppressed body weight gain in rats that become obese when maintained on a densely caloric diet (i.e., diet-induced obesity) (Seoane-Collazo et al., 2014). In contrast, oral administration of nicotine via drinking water had no effect on body weight in Zucker fatty rats (R. H. Liu et al., 2001; R. H. Liu et al., 2003), a different animal model of human obesity. The total daily dose of nicotine delivered orally to the Zucker fatty rats (R. H. Liu et al., 2003) was substantially lower than the dose delivered by subcutaneous infusion to diet-induced obese rats (Seoane-Collazo et al., 2014), which may explain the difference between the two studies. To our knowledge, there are no other reports of the effects of nicotine or smoking on body weight regulation in an obese population.

The impact of nicotine and smoking on obesity must be evaluated beyond an effect solely on body weight or BMI. Chronic smoking can increase fat accumulation, associated with central obesity and insulin resistance. High BMI is not always correlated with obesity-related illnesses. Waist-to-hip ratio (WHR), a measure of central obesity, is often predictive of the development of Type II diabetes and poor health outcomes. Former smokers who relapse lose weight, approximately 2.5 lbs, but display an increase in WHR (Shimokata et al., 1989). Central obesity increases dose-dependently with cigarette consumption, and this increase in abdominal fat accumulation often occurs independently of changes in BMI or body weight (Barrett-Connor et al., 1989; Shimokata et al., 1989). Smoking also induces insulin resistance (Attvall et al., 1993),

characteristic of Type II diabetes, and it is thought that nicotine in tobacco smoke that contributes to the development of insulin resistance (Eliasson et al., 1996). Indeed, smoking and nicotine consumption is associated with the development of Type II diabetes, which may be mediated by central obesity and insulin resistance (T. Liu et al., 2011; Xie et al., 2009). The contribution of chronic nicotine exposure to the development of insulin resistance is supported in animal models (Wu et al., 2015). Thus, it seems the relationship between nicotine, smoking, and obesity is paradoxical; nicotine may reduce body weight in an obese population and prevent the onset of obesity in an otherwise obese smoker, whereas chronic nicotine exposure may increase central obesity and the development of Type II diabetes in lean smokers, and potentially obese smokers as well.

1.4 THE EFFECTS OF OBESITY ON NICOTINE REINFORCEMENT

Evidence also points to the potential impact of obesity on the degree to which nicotine reinforces behavior. The mechanistic link between the drive for food and psychoactive drugs is clear in humans and rodents, which may underlie the co-occurrence of obesity and substance abuse disorders (Avena et al., 2008; Volkow et al., 2003; Wang et al., 2004). As mentioned above, higher BMI is associated with smoking more cigarettes per day, which may be linked with higher levels of nicotine dependence. In female smokers, childhood-onset obesity is associated with earlier smoking initiation and more severe withdrawal symptoms, but not increases in nicotine dependence or cigarettes consumed per day (Saules et al., 2007). Further, craving for cigarettes significantly increased following two-day abstinence in high-BMI female smokers compared to low BMI counterparts, matched for nicotine dependence and cigarettes per

day (Saules et al., 2004). A longitudinal survey study found that female adolescent obesity is linked to higher levels of nicotine dependence later in life (Hussaini et al., 2011). However, a separate study found a positive correlation between nicotine dependence and BMI in adult men but not women (John et al., 2005b). Results from these studies are not totally consistent, but generally support the notion that obesity might contribute to increased uptake of cigarette use or nicotine dependence.

To our knowledge, there is only one controlled laboratory study investigating the effects of body weight on nicotine reinforcement in human smokers (Blendy et al., 2005). Non-obese and obese non-deprived smokers were asked to take 16 total puffs from 2 cigarettes differing in nicotine content: a normal nicotine content and a very low nicotine content (VLNC) cigarette. Measures of nicotine dependence and cigarettes per day were slightly elevated in the obese smokers. However, nicotine reward, measured by the percentage of total puffs taken from the nicotine cigarette, was lower in the obese subjects. Ratings of liking for the VLNC cigarette, while lower than the ratings of liking for the normal nicotine cigarette, were elevated in the obese compared to non-obese subjects. The data describing a relationship between obesity and smoking behavior are limited and not entirely consistent across studies, but a picture emerges that might support the view that obese smokers may be more nicotine dependent and susceptible to smoking, but derive less reward or liking from the cigarettes. More importantly, perhaps, is that these data highlight the possibility that obese smokers may derive more reward from VLNC cigarettes, indicating the potential for increased acceptance and use of reduced nicotine content cigarettes in obese smokers.

Recent efforts using animal models have focused on the concept that consumption of a densely caloric diet may increase motivated behaviors, such as drug-seeking. Evidence supports

the idea that high fat diet exposure may increase motivation for nicotine reward. In an outbred population of Sprague-Dawley rats that become obese when maintained on a densely caloric diet, only a subset develops insulin resistance. Obese insulin-resistant rats, modeling Type II diabetes, displayed a strong place preference for an environment previously paired with nicotine (Richardson et al., 2014). Interestingly, the obese insulin-sensitive rats do not show a nicotine conditioned place preference. Similarly, in a separate report that did not consider insulin sensitivity, lean mice fed a standard chow diet show conditioned place preference for nicotine, but this is not observed in mice fed a high fat diet (Blendy et al., 2005). Further, rats that become hypoinsulinemic by injection of Streptozotocin, modeling Type I or advanced stage Type II diabetes, show enhanced nicotine self-administration across doses and schedules of reinforcement (O'Dell et al., 2014). These data indicate that perhaps obesity-induced insulin resistance, and not obesity itself, enhances nicotine reinforcement. Limited data from humans are consistent with these claims. Over 40% of adolescents with diabetes reported to be smokers (Reynolds et al., 2011) and quit rates among diabetic smokers are very low (Gill et al., 2005). Further studies investigating specifically whether diet-induced diabetes enhances acquisition and maintenance of nicotine self-administration and smoking behavior is warranted.

Obese smokers represent a unique population and may be especially susceptible to smoking and the weight-suppressive effects of nicotine. Thus, obese smokers should be considered a vulnerable population in a tobacco-reduction policy. It is possible that chronic nicotine exposure may reduce the onset of obesity in a subset of people who are otherwise predisposed to overweightness or obesity. Preliminary work from human and rodent models supports the possibility that reduction of nicotine dose or content will result in substantial weight gain in lean populations (Rupprecht et al., 2016). It is possible that nicotine reduction may result

in the development of obesity and its associated co-morbidities in a subset of smokers. Several lines of research suggest that diet-induced insulin resistance increases susceptibility to smoking and nicotine reinforcement (O'Dell et al.; Richardson et al.), raising the possibility that obese individuals might be more likely to initiate smoking of reduced nicotine content cigarettes, potentially increasing acceptance and use of VLNC cigarettes. On the other hand, insulin-resistant obese smokers may maximally benefit from nicotine reduction, as insulin resistance is a greater determinant of their behavior. Our argument is limited by the number of controlled, experimental studies focused on obese smokers or animal models of obese smokers. This gap in the literature restricts our ability to discern causal from correlative effects and demands future attention to this population, both in human and animal models. A better understanding of how weight and smoking behaviors will be impacted by a potential transition to VLNC cigarettes is critical. Analyses from surveys, such as the National Health and Nutrition Examination Survey, examining the differences in weight at the initiation of and following the cessation of smoking between lean and obese individuals could provide a useful foundation for future work. The initiation of smoking behaviors cannot be experimentally evaluated in humans; thus, animal models are needed to test the self-administration of low doses of nicotine thought to be below the threshold of addiction (Smith et al., 2014) in a model of diet-induced obesity and hypoinsulinemia. The ability to ask these questions in an animal model may lead to a more mechanistic understanding of the link between obesity and smoking. Until we gain a more comprehensive picture of the relationship between nicotine and obesity, careful consideration of obese smokers in nicotine-reduction policy is necessary and future work focusing on this population in human and animal models is of immediate importance.

1.4.1 Modeling lean and obese smokers in rodents.

The bulk of preclinical research on nicotine addiction is modeled by self-administration in male rats that are food restricted on a standard diet low in fat content (Rupprecht et al., 2015c). This paradigm does not accurately model human populations that typically consume more calories than metabolically required (Adebayo et al., 2014; Hill et al., 1998). Sprague-Dawley rats remain lean if maintained on low-fat standard chow (B. E. Levin et al., 2003; B. E. Levin et al., 2005). However, when maintained on a high energy diet (HED), the body weight of a subset of rats (obesity-prone; OP) becomes significantly higher than a separate subset (obesity-resistant; OR) after three weeks of diet exposure (Madsen et al., 2010). The body weight distribution observed in outbred OP and OR rats are among the best animal models of human body weight gain in the United States (Nilsson et al., 2012); the predisposition for obesity is a polygenetic trait and is not expressed until prolonged environmental exposure to a HED (B. E. Levin, 2010). Chapters 5 and 6 utilize OP and OR rats allowed to self-administer nicotine as a rodent model of obese and lean smokers.

1.5 EVALUATING THE RELATIONSHIP BETWEEN NICOTINE AND BODY WEIGHT

The relationship between body weight and nicotine is complex. Nicotine has the ability to suppress body weight, but may also increase central adiposity and contribute to obesity. Preclinical research on nicotine's effects on reduced food intake have most commonly used subcutaneous, non-contingent injections of nicotine, oftentimes using doses within the range that

cause seizures. There is an obvious paucity in the literature of nicotine's effects on energy balance in the most commonly accepted animal model of smoking: intravenous nicotine self-administration in rats. Chapter 2 tests the impact of self-administered nicotine across a range of doses on body weight and food intake. Results suggest that self-administered nicotine, even at very low doses, suppresses body weight independent of food intake. In the context of a regulatory policy mandating very low nicotine levels in cigarettes, reduction of nicotine resulted in substantial weight gain. Chapter 3 uses indirect calorimetry to measure the impact of self-administered nicotine on energy expenditure and results indicate that self-administered nicotine suppresses body weight gain through increased fat utilization, measured by a decrease in respiratory exchange ratio.

Weight gain is a potential consequence of nicotine reduction policy. Chapter 4 examines this possibility through secondary analyses of data from a clinical trial in which smokers were randomized to VLNC cigarettes for six weeks. Men and women with high levels of compliance on investigational VLNC cigarettes had significant weight gain compared to participants in normal nicotine content control groups and participants non-compliant on VLNC cigarettes. Implications of the results for regulatory policy are discussed further in Chapter 4.

The current environment is obesogenic, and study of rodents fed standard, low fat chow is not representative of current populations. In Chapter 5, the impact of self-administered nicotine in OR and OP rats fed HED on body weight and food intake is tested. Self-administered nicotine suppressed weight gain in OP, but not OR rats. These results suggest that individuals resistant to the development of diet-induced obesity may also be resistant to the weight-suppressive effects of nicotine.

Smoking among the obese population is high (Chatkin et al., 2010). This may be due to many factors. The explanation might be as simple as a clustering of unhealthy behaviors, such that, for example, people eating densely caloric foods also smoke (Chiolero et al., 2006). Alternatively, many obese individuals may use smoking as a method of weight reduction (Audrain-McGovern et al., 2011). Finally, obesity has been linked to increased reward-seeking behaviors. This could lead to increased nicotine-seeking or smoking behavior and augment seeking for food reward, causing excess weight gain (Volkow et al., 2012). Nicotine reduces BMI while at the same time increases fat accumulation, central obesity, and insulin resistance (Chiolero et al., 2008), which could contribute to the development of obesity. Moreover, insulin resistance is thought to enhance nicotine reinforcement (O'Dell et al., 2014; Richardson et al., 2014), potentially driving smoking behavior. Together, these factors could create a cycle promoting nicotine consumption in the obese population. Chapter 6 evaluates the impact of body weight on nicotine reinforcement and consumption in human smokers and a rodent model of lean and obese smokers. As noted above, obese smokers represent a potential subpopulation of risk for continued smoking following implementation of product standards regulating low nicotine levels in cigarettes. Nicotine consumption in obesity is tested following large reductions in nicotine. Results from these experiments demonstrate that nicotine consumption is tightly regulated dependent upon body weight.

Work within this dissertation describes the complex relationship between nicotine and body weight. Self-administered nicotine suppresses body weight, likely through increased fat utilization, and the ability of nicotine to suppress body weight is dependent upon obesity status and diet. Very low doses of nicotine suppress body weight, indicating that smokers who initiate cigarette use following a mandated reduction of nicotine content in cigarettes may smoke for the weight suppressant effects of smoking. Large reductions in nicotine dose and content result in increases in weight gain, suggesting that product standards regulating nicotine content to low levels in cigarettes will result in weight gain in smokers. Finally, nicotine consumption is titrated dependent upon body weight, and nicotine reduction may be an effective strategy for reducing

cigarette use in obese and non-obese smokers. The work outlined below extends our understanding of nicotine's actions on energy balance and the impact of body weight on nicotine reinforcement and consumption, and has important implications for regulatory policy.

2.0 SELF-ADMINISTERED NICOTINE SUPPRESSES BODY WEIGHT INDEPENDENT OF FOOD INTAKE IN MALE RATS

2.1 INTRODUCTION

There is an inverse relationship between smoking and body weight, such that smokers weigh less than non-smokers but gain an average of ten pounds in the first year of abstinence (Audrain-McGovern et al., 2011; Williamson et al., 1991). Many smokers cite weight loss as a primary reason for smoking and weight gain for the inability to quit (Pomerleau et al., 2001; Rosenthal et al., 2013; Veldheer et al., 2014).

Nicotine is the primary psychoactive constituent in cigarettes and researchers have suggested that nicotine in cigarettes is most likely responsible for the body weight suppression observed in smokers (Grunberg, 1985; Grunberg et al., 1985a; Grunberg et al., 1984; Grunberg et al., 1986; Winders et al., 1990; Zoli et al., 2012). Studies utilizing rodent models have generally reported that nicotine exposure, primarily via subcutaneous continuous infusion or repeated daily injections, results in a dose-dependent suppression of body weight (Grunberg et al., 1984; Mineur et al., 2011) and decreased food intake (L. L. Bellinger et al., 2010; Mineur et al., 2011; Miyata et al., 2001). Despite reports that nicotine delivery can increase physical activity (Faraday et al., 2003; Faraday et al., 1999) and metabolic rate (de Morentin et al., 2012), the body weight-suppressant effects of nicotine are generally discussed as secondary to a

suppression of caloric intake (Zoli et al., 2012). This conclusion, however, is at odds with data from the clinical literature suggesting that smokers and non-smokers have equal daily caloric intake (Perkins, 1992a; Perkins et al., 1991). The vast majority of experiments examining the effects of nicotine on food intake and body weight have utilized experimenter-administered nicotine, which can produce different effects than self-administered nicotine (Donny et al., 2000). However, few investigators have utilized self-administration procedures to examine the impact of nicotine on food intake or body weight.

The current experiments evaluated the impact of self-administered nicotine, across a range of doses, on body weight and food intake in adult male rats. Results demonstrated that self-administered nicotine suppressed body weight gain independent of food intake and this effect was observed at very low doses. An additional experiment investigated the impact of reducing nicotine dose on body weight; results revealed that reduction of nicotine dose from a large self-administered dose to very low doses resulted in substantial weight gain. These data are important in the context of a reduction of nicotine content in cigarettes, a potential approach to reducing the abuse potential of cigarettes (Hatsukami et al., 2013). The current data provide novel insight into the consequences of nicotine on body weight and offer important implications for the impact of nicotine reduction policy on body weight regulation.

2.2 METHODS

Subjects

Male Sprague-Dawley rats (Harlan Farms, IN, weighing between 200 and 300 g upon arrival) were housed individually in hanging-wire cages on a reverse light-dark 12:12 hr cycle

(lights off at 0700h) in a temperature-controlled facility (between 68 and 70 °F). Rats had free access to standard rodent chow (Purina Rat chow 5001) and water, unless noted otherwise. All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Drugs

Nicotine hydrogen tartrate salt (Sigma, St. Louis, MO) was dissolved in 0.9% saline. Doses of nicotine used for self-administration were 1.875, 3.75, 7.5, 15, and 60 µg/kg/infusion, and for subcutaneous injection, the doses were 0.3 and 1.0 mg/kg (expressed as freebase).

In a subset of experiments (Experiments 3 & 4), a cocktail of cigarette constituents was included in the intravenous nicotine solution. The selected doses of the cocktail of cigarette constituents were based on previous studies (Clemens et al., 2009; Smith et al., 2015), and/or were indexed to a standard dose of nicotine, based on their relative concentrations in cigarette smoke, that supports robust self-administration behavior (30 µg/kg/infusion). The doses used in select self-administration studies were as follows: acetaldehyde (16 µg/kg/infusion), harman (0.1 µg/kg/infusion), norharman (0.3 µg/kg/infusion), anabasine and nornicotine (0.9 µg/kg/infusion), and anatabine, myosmine, and cotinine (0.09 µg/kg/infusion).

The pH of solutions was adjusted to 7.0 (±0.2) using a dilute sodium hydroxide solution. All solutions used in self-administration studies were passed through a 0.22 µm filter to ensure sterility. All intravenous infusions were delivered in approximately 1-s (0.1 ml/kg/infusion). Subcutaneous injections were delivered at 1 ml/kg.

Procedures

Surgery

After at least seven days of habituation post-arrival, rats were anesthetized with isoflurane (2-3% in 100% O₂) and implanted with catheters into the right jugular vein, as described previously (Donny et al., 1995; Donny et al., 1999). Rats were allowed to recover for a minimum of 5 days before self-administration procedures. During the surgical recovery period, catheters were flushed once daily with 0.1 ml sterile saline containing heparin (30 U/ml), timentin (66.67 mg/ml), and streptokinase (9,333 U/ml). Thereafter, catheters were flushed with 0.1 ml heparinized saline (10 U/ml) and heparinized saline (30 U/ml) containing timentin (66.67 mg/ml) prior to and following the self-administration sessions, respectively.

Self-administration

Thirty-eight operant chambers (30.5 cm² x 24.1cm² x 21.0 cm; ENV-008CT; Med-Associates) enclosed inside sound-attenuating chambers, equipped with two nose-poke holes located on the same wall (2.5 cm in diameter and 5 cm above the floor), two white stimulus lights (3.5 cm in diameter, located 6.5 cm above each nose-poke hole), a houselight, and a fan were used in the current studies. An infusion pump was located outside of each chamber, which delivered intravenous infusions during self-administration sessions through tubing connected to each rat's catheter. This tubing was protected in a metal encasing, attached to a swivel system that allowed relatively unrestricted movement.

During daily (7d/wk) 1-h self-administration sessions, fulfilling the required nose-poke responses into the active portal resulted in one infusion of nicotine. Infusions were accompanied

by a 15-sec cue light illuminated above the active nose-poke portal and an unsignaled 1-min timeout, where responses were recorded but had no scheduled consequence. Throughout the 1-h sessions, responses into the inactive nose-poke portal were recorded but had no consequences. In experiments that used food restriction (Experiments 2 & 3) the allotted food amount (20 g/day) was in the home cage when the rat returned from its self-administration session. For self-administration studies, rats in nicotine groups included in analyses passed a patency test, which required displaying physical signs of ataxia within 5-s of intravenous injections of chloral hydrate (up to 60 mg/rat) or methohexital (5 mg/kg). In all experiments, baseline body weights were counterbalanced across drug groups.

Experiment 1. The effect of self-administered nicotine on body weight and food intake

Rats were implanted with intravenous catheters and assigned to self-administer nicotine (60 µg/kg/infusion, n=11) or saline (n=8). Rats weighed 316.6 ± 2.1 g at the start of self-administration. Rats were allowed to respond for drug infusions on a fixed-ratio (FR) 2 schedule of reinforcement for 20 consecutive days. Body weight was measured daily before the self-administration session. Food intake was measured daily over the 23-h period in the home cage, accounting for spillage.

Experiment 2. The effect of subcutaneous nicotine injection on food intake

Given that the results of Experiment 1 are unexpected, the effect of subcutaneous injection of nicotine on food intake was measured in a separate group of rats to replicate previous reports (L. L. Bellinger et al., 2010; Mineur et al., 2011; Miyata et al., 2001). Rats weighing on average 362.8 ± 2.7 g were assigned to a group and injected with nicotine (0, 0.3, or 1.0 mg/kg,

s.c.; n=8 per group) at the onset of the dark cycle. Food intake was measured 1, 3, 6, and 24h post-injection, accounting for spillage.

Experiment 3. The effect of a range of self-administered nicotine doses on body weight under mild food restriction

Given the results of Experiment 1, and that the majority of self-administration studies are performed in rats under mild food restriction (Rupprecht et al., 2015c), the effect of self-administered nicotine on body weight was analyzed from a previously reported experiment (Smith et al., 2014). All rats were food restricted to ~80% of their ad libitum intake (20g/day) at least 5 days before self-administration procedures began. Rats were randomly assigned to self-administer nicotine at one of five doses: 60 µg/kg/infusion (n=65), 15 µg/kg/infusion (n=17), 7.5 µg/kg/infusion (n=15), 3.75 µg/kg/infusion (n=12), or 0 µg/kg/infusion (n=17). In this experiment, the nicotine solution contained a cocktail of constituents found in cigarette smoke. Each drug group differed by nicotine concentration, but the cocktail concentrations remained consistent across different nicotine doses. Rats weighed 268.7 ± 1.5 g on the first day of the experimental period. Rats acquired self-administration of nicotine on a FR1 for one day, FR2 for seven days, and escalated to FR5 for the remainder of the study (Smith et al., 2014). Body weight was measured daily and evaluated for 20 days of self-administration.

To test the possibility that the addition of cigarette constituents could impact body weight regulation, a separate group of rats were food restricted (20g/day) and responded on an FR2 schedule of reinforcement for infusions of nicotine (60 µg/kg/infusion) without (n = 8) or with (n = 11) the cocktail described above, with one minor change. Examination of dosages selected in papers cited by Clemens et al (2009), the paper on which the original cocktail solutions were

based, suggested that the concentrations of the anatabine and anabasine should be reversed (Smith et al.). Thus, the cocktail solution contained 0.9 $\mu\text{g/kg/infusion}$ of anatabine and 0.09 $\mu\text{g/kg/infusion}$ anabasine, along with the other constituents. Average body weight at the start of self-administration was 298.1 ± 4.9 g. Body weight was measured daily and evaluated for 10 days of self-administration.

Experiment 4. The effect of nicotine dose reduction on body weight gain

Body weight regulation following nicotine dose reduction was evaluated post-hoc from a previously published study from our laboratory (Smith et al., 2013). Food restricted (20g/day) rats learned to self-administer infusions of nicotine (60 $\mu\text{g/kg/infusion}$ + cocktail) for 17 days before immediate reduction of nicotine dose, with cocktail doses remaining constant, to one of the following doses: 15 (n=10), 7.5 (n=11), 3.75 (n=11), 1.875 (n=10), or 0 (n=13) $\mu\text{g/kg/infusion}$. Rats responded on an FR5 schedule of reinforcement for the reduction phase of the experiment. Rats weighed 279.6 ± 2.2 g at the start of dose reduction. The control group remained on 60 $\mu\text{g/kg/infusion}$ nicotine with cocktail, which is referred to as “Maintained” (n=11).

Statistics

Data for each experiment were analyzed separately and are expressed as means \pm SEM. All statistical analyses were performed using SPSS. Comparisons between drug group and session (self-administration experiments) or day (feeding experiments) were analyzed by mixed-design and repeated measures ANOVA tests to account for the within-subjects design of the experiments while testing for between-subjects effects of nicotine dose groups. In tests of

repeated measures where Mauchly's Sphericity tests were significant, the data were Greenhouse-Geisser corrected; degrees of freedom reflect this correction where appropriate. Correlations were assessed using a two-tailed Pearson's correlation. The α -level for all tests was set at 0.05. Where appropriate, a Bonferroni adjustment was made to account for the comparison of saline control to many nicotine dose groups. The α -levels were adjusted to 0.0125 and 0.01 for Experiments 3 and 4, respectively, resulting in an overall type I error rate of 0.05.

2.3 RESULTS

Experiment 1. Self-administered nicotine suppressed body weight gain but not food intake.

Rats self-administering 60 $\mu\text{g/kg/infusion}$ nicotine earned significantly more infusions (8.3 ± 1.0 ; $F_{1,18}=26.776$, $p < 0.001$) than the saline group (1.9 ± 0.3), averaged over the final three days of self-administration (Figure 1). Self-administered 60 $\mu\text{g/kg/infusion}$ nicotine suppressed body weight gain compared to intravenous infusions of saline (Figure 2). An ANOVA comparing groups on every fifth day revealed significant differences between groups on Days 10, 15, and 20 ($F_{S1,18} > 12.535$, $ps < 0.003$). There were no significant differences in food intake (expressed as a percentage of body weight) between nicotine and saline groups across days ($p = 0.831$, Figure 2). There were no differences in grams of food consumed between groups ($p = 0.627$; saline = 26.3 ± 0.7 g; nicotine = 24.5 ± 0.7 g on Day 20). Additionally, cumulative food intake during the 20 days of self-administration did not differ between groups when expressed as a percentage of body weight (Figure 2) or in total grams consumed ($p = 0.105$).

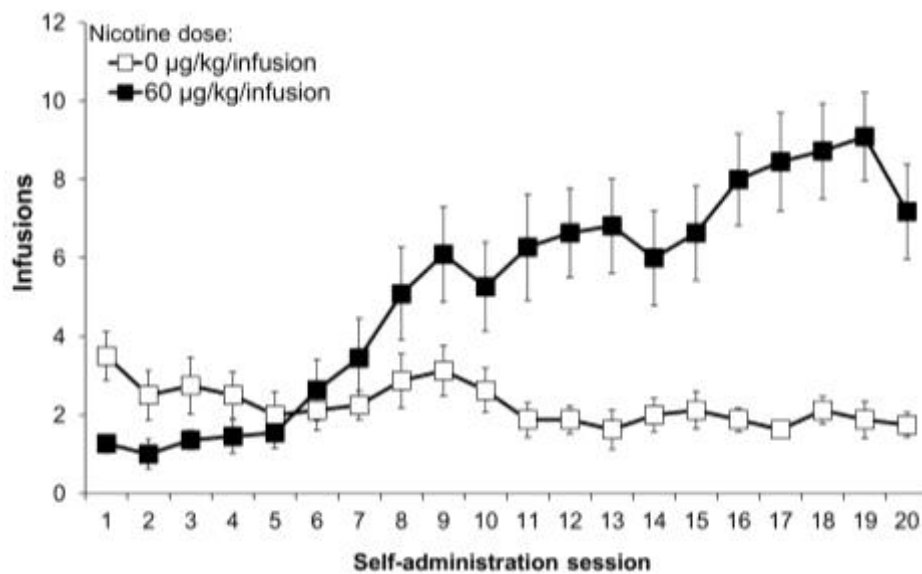


Figure 1. Infusions earned across 1-h daily sessions.

Over 20 daily 1-h daily sessions, rats in the nicotine group earned significantly more infusions than in the saline group.

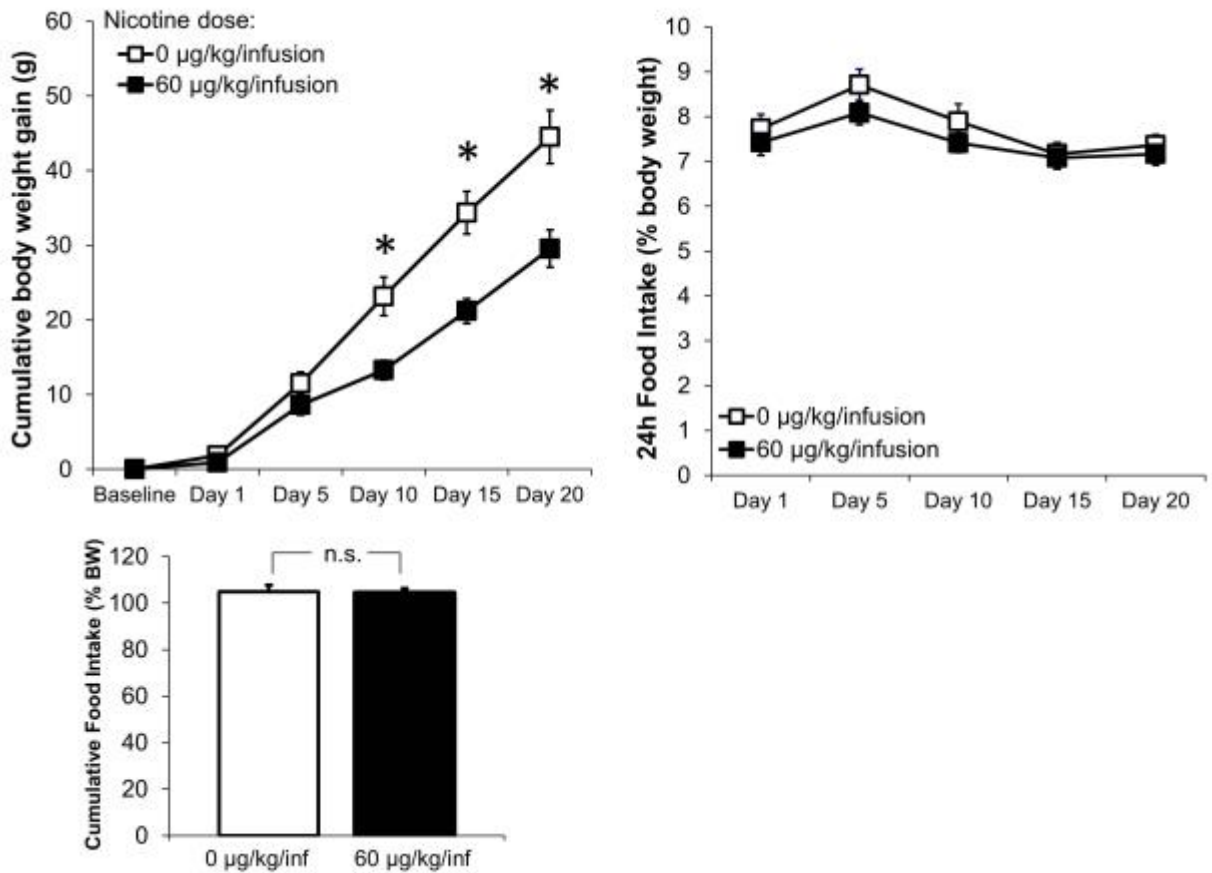


Figure 2. The impact of large dose self-administered nicotine on body weight and food intake

Effects of self-administered nicotine on body weight and food intake. Body weight gain and food intake in rats that self-administered 0 (n=11) or 60 (n=8) µg/kg/infusion nicotine. Self-administered 60 µg/kg/infusion nicotine significantly suppressed cumulative body weight gain, but not 24h food intake, expressed as a percentage of body weight. There was no impact of self-administered nicotine cumulative food intake over the 20-day self-administration period. * indicate $p < 0.05$, between 0 and 60 µg/kg/infusion nicotine.

Experiment 2. Subcutaneously administered nicotine suppressed food intake.

The highest dose of nicotine tested, 1.0 mg/kg, s.c., significantly suppressed food intake by approximately 10% at 3, 6, and 24h post-injection (Figure 3). Repeated measures ANOVA revealed a significant effect of time ($F_{1.833,1} = 908.890, p < 0.001$) with no significant interaction between time and drug group ($p = 0.071$); post-hoc tests revealed significant differences between saline and 1.0 mg/kg nicotine at 3, 6, and 24h ($ps < 0.006$). There was no significant impact of 0.3 mg/kg nicotine on food intake at any time point.

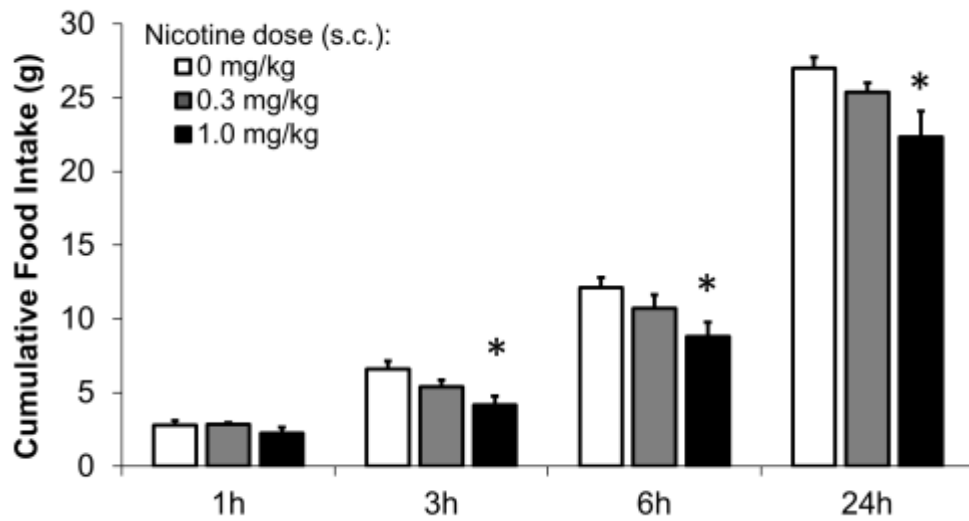


Figure 3. The impact of experimenter-administered subcutaneous injection of nicotine on food intake.

Experimenter-administered nicotine (1.0 mg/kg, s.c.) delivered at the onset of the dark cycle significantly suppressed food intake at 3, 6, and 24 hours. * indicate $p < 0.05$, between 0 and 60 $\mu\text{g/kg}$ /infusion nicotine.

Experiment 3. Self-administered nicotine suppressed body weight gain when food was held constant and restricted.

Self-administered nicotine dose-dependently suppressed body weight gain when food was held constant and restricted (Figure 4). Repeated measures ANOVA revealed a significant main effect of day ($F_{2.78,1} = 430.846$, $p < 0.001$) and significant nicotine group by day interaction ($F_{11.121,4} = 3.702$, $p < 0.001$). Comparisons between groups on every fifth day showed significant effects of nicotine group on Days 5, 10, 15, and 20 ($ps < 0.008$). Analyses further revealed that saline was significantly different from: 60 $\mu\text{g/kg/infusion}$ on Days 5, 10, 15, and 20; 15 $\mu\text{g/kg/infusion}$ on days 10, 15, and 20; 7.5 $\mu\text{g/kg/infusion}$ on Days 10 and 20; and 3.75 $\mu\text{g/kg/infusion}$ on day 10 (all $ps < 0.0125$). Additionally, on Day 20, body weight gain in the 60 $\mu\text{g/kg/infusion}$ group was significantly different from 0, 3.75, and 7.5 $\mu\text{g/kg/infusion}$ groups (all $ps < 0.017$). There was a significant negative correlation between cumulative nicotine intake and cumulative body weight gain (Figure 4; $p < 0.001$). It is noteworthy that total nicotine intake in this experiment is much higher than total nicotine intake reported in Experiment 1, which is likely explained by the differences in feeding status (Donny et al., 1998). Rats under food restriction acquire self-administration behavior more quickly and respond at higher rates (Rupprecht et al., 2015c). In a separate group of rats testing the impact of the addition of constituent chemicals on body weight regulation, there was no significant difference in body weight gain between no cocktail (25.8 ± 8.5 g) and cocktail groups (28.4 ± 5.1 g) after ten days of self-administration ($p = 0.99$).

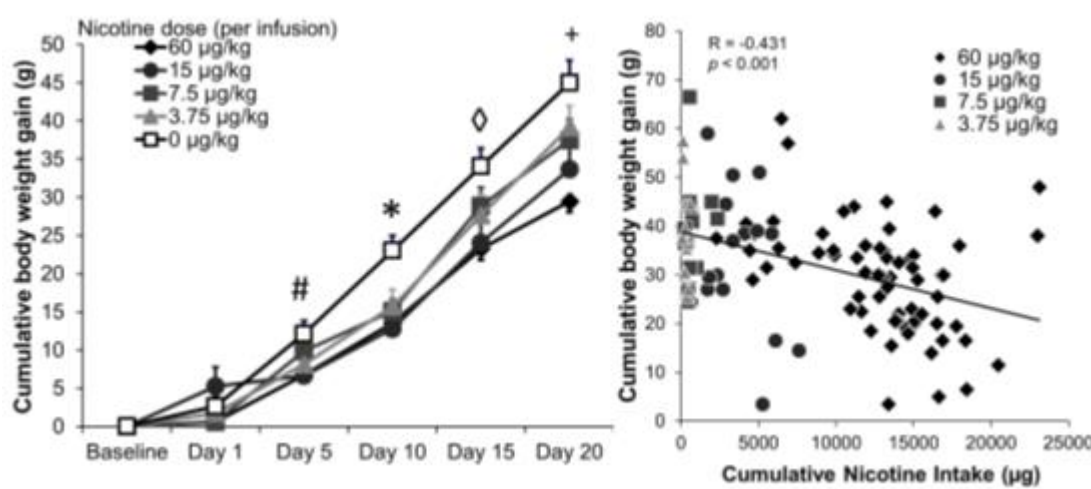


Figure 4. Self-administered nicotine dose-dependently suppresses body weight independent of food intake.

Effects of a range of self-administered nicotine doses on body weight gain. Body weight gain in rats that self-administered 0 (n=17), 3.75 (n=12), 7.5 (n=15), 15 (n=17), or 60 (n=65) µg/kg/infusion nicotine. In rats whose food intake was held restricted and constant, self-administered nicotine dose-dependently suppressed body weight gain. Across all doses, there was a negative correlation between cumulative nicotine intake and cumulative body weight gain on Day 20. # indicates 0 µg/kg/infusion different from 60 µg/kg/infusion, * indicates 0 µg/kg/infusion different from all nicotine doses, ◇ indicates 0 µg/kg/infusion different from 15 and 60 µg/kg/infusion, and + indicates 0 µg/kg/infusion different from 7.5, 15, and 60 µg/kg/infusion; all $ps < 0.0125$.

Experiment 4. Reduction of nicotine dose results in body weight gain independent of food intake.

Reduction of nicotine dose from 60 µg/kg/infusion caused significant weight gain compared to Maintained group (60 µg/kg/infusion) self-administration (Figure 5). Infusions earned in the 7.5 and 15 µg/kg/infusion were similar to the Maintained group following the reduction, but there was a significant reduction of infusions earned in all other groups, such that the 3.75 and 1.875 µg/kg/infusion groups responded similarly to saline (Smith et al., 2013). Repeated measures ANOVA revealed a main effect of day ($F_{2.753,1} = 145.818, p < 0.001$) and a significant interaction between day and dose group ($F_{13.764,5} = 2.802, p = 0.001$). Planned comparisons to identify differences between groups every tenth day revealed significant effects of groups on days 30, 40, and 50 ($ps < 0.002$). Post-hoc analyses showed that the Maintained group was significantly different from 1.875 and 3.75 µg/kg/infusion on Days 30, 40, and 50.

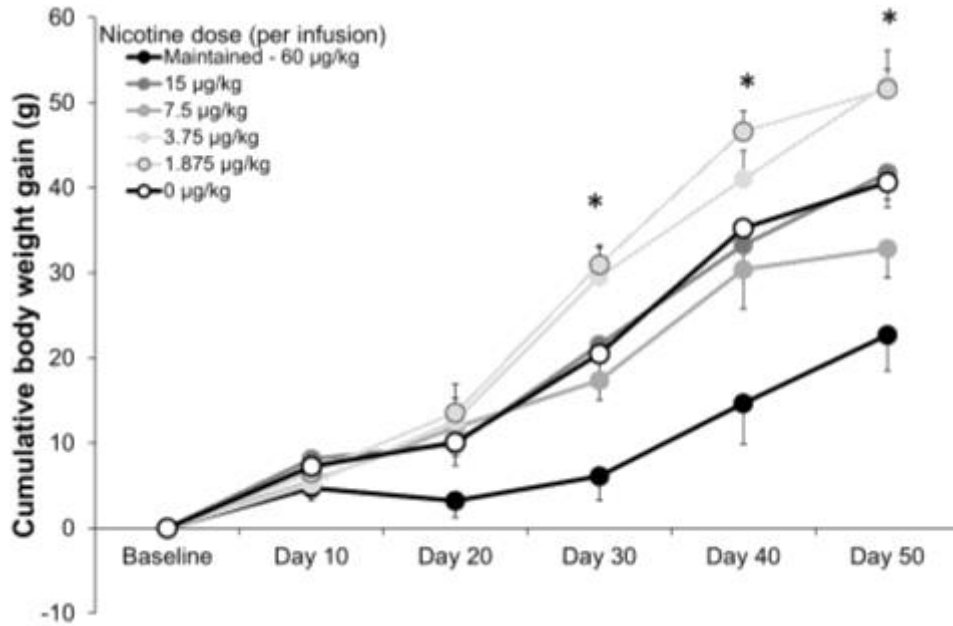


Figure 5. Reduction of nicotine dose results in increased weight gain.

Effects of nicotine dose reduction on body weight gain. Body weight gain in rats where dose was reduced from 60 µg/kg/infusion to: Maintained at 60 (n=11), 15 (n=10), 7.5 (n=11), 3.75 (n=11), 1.875 (n=10), or 0 (n=13) µg/kg/infusion nicotine. Reduction of nicotine dose resulted in significant increases in body weight gain compared to constant self-administration of 60 µg/kg/infusion nicotine. * indicates 60 µg/kg/infusion different from 3.75 and 1.875 µg/kg/infusion. All p s < 0.01.

2.4 DISCUSSION

The present data are the first to demonstrate that self-administered nicotine, across a range of doses, suppresses body weight independent of food intake. These data have important implications for the understanding of the impact of nicotine on body weight and for nicotine regulatory policy. The negative correlation between nicotine intake and body weight gain indicates that total nicotine exposure directly impacts body weight regulation. While it has been reported that smokers and non-smokers have equal daily caloric intake (Perkins, 1992a), this is the first report to our knowledge of nicotine suppressing body weight independent of changes in food intake in a rat self-administration model.

The current data support the view that nicotine, at least when self-administered by adult male rats in daily 1-h sessions, suppresses body weight without simultaneous decreases in food intake. These data differ from a large body of work demonstrating that nicotine suppresses body weight, with the common conclusion made that this is primarily driven by a reduction in food intake (L. L. Bellinger et al., 2010; Mineur et al., 2011; Miyata et al., 2001). Nearly all of these studies have used subcutaneous or intraperitoneal injection or continuous subcutaneous infusion of large doses of nicotine, beyond the range that rats would self-administer (Matta et al., 2007). It is typical that subcutaneous delivery of nicotine at a dose of 1.0 – 1.5 mg/kg suppresses food intake (L. L. Bellinger et al., 2010; Mineur et al., 2011). However, seminal work by Grunberg and colleagues (1984) reported that large doses of nicotine delivered via constant subcutaneous infusion in osmotic minipumps (4 – 12 mg/kg/day) had no impact on food intake, though

resulted in large, dose-dependent suppression of body weight. Furthermore, it is noteworthy that rats develop tolerance to the anorectic effects of daily subcutaneous injections of nicotine (Caggiula et al., 1991). While blood and brain nicotine levels and the time course of absorption differs between intravenous and subcutaneous delivery (Matta et al., 2007), the total amount of nicotine delivered may directly affect feeding behavior. Indeed, it is worth noting that the lowest dose of experimenter-administered nicotine that significantly suppressed food intake (1.0 mg/kg, s.c.) is larger than the total amount of nicotine self-administered in a 1-h session by rats fed *ad libitum* standard rodent chow (ranging from 0.2 – 0.8 mg/kg daily). Additionally, non-contingent nicotine increases corticosterone (CORT) levels compared to self-administered nicotine (Donny et al., 2000). Elevation of CORT results in the suppression of food intake (Calvez et al., 2011; Maniam et al., 2012). Therefore, it is possible that increased CORT levels caused by non-contingent nicotine administration contributes to suppression of food intake, and the absence of this increased CORT during nicotine self-administration allows for the suppression of body weight with no effect on food intake.

To our knowledge, there are few reports on the effects of intravenously infused nicotine on feeding behavior. In contrast with the data presented here, the published studies used 23-h extended access self-administration sessions in which rats were trained to respond for food (45 mg pellets) in the operant chamber. In a report from Grebenstein et al (2013), non-contingent delivery of 60 µg/kg/infusion nicotine during extended access sessions suppressed body weight gain by ~50% and reduced the number of pellets consumed by approximately 20% over 23h. The suppression of food intake by non-contingent nicotine delivery may be directly related to the elevation of CORT following experimenter-administered nicotine, as mentioned above. In a more recent study, however, Bunney (née Grebenstein) et al (2015) extended these results by

demonstrating that self-administration of 60 µg/kg/infusion nicotine in 23-h sessions suppressed chow pellet intake, replicating work by O'Dell and colleagues (2007).

The differences between the effects of self-administered nicotine on food intake in 1-h limited access and 23-h extended access sessions could be due to several reasons. First, total nicotine intake in 23-h sessions is typically greater than in 1-h sessions. In the extended access experiments described above, nicotine intake ranged from ~1.0 – 2.0 mg/kg per day (Bunney et al., 2015; Grebenstein et al., 2013; O'Dell et al., 2007). It is possible that total nicotine exposure ≥ 1.0 mg/kg causes a suppression of food intake, as noted with the current subcutaneous experiment described above. Second, repeated nicotine infusions over 23-h expose rats to many spikes in plasma nicotine levels over a prolonged time course each day. Feeding may be suppressed following each infusion only when plasma nicotine levels are high. Therefore, a suppression of daily food intake by intravenous nicotine is detectable in an extended access procedure, when plasma nicotine levels remain elevated for a longer time period and can contribute to a large cumulative reduction in food intake. However, in these 23-h sessions, rats take the majority of their daily infusions during the active, dark phase (O'Dell et al., 2007). Grebenstein et al (2013) report that the reduction in 23-h food intake by nicotine is largely driven by suppression of food intake during the light cycle, making the possibility that spikes in nicotine plasma levels contribute to food intake suppression unlikely. Regardless, the magnitude of nicotine-induced food intake suppression reported (Bunney et al., 2015; Grebenstein et al., 2013) cannot account for total amount of body weight gain suppression, indicating that intravenous nicotine exposure suppresses body weight gain independent of food intake, at least in part, in an extended access procedure.

There are several advantages of the use of limited access self-administration procedures. First, these procedures allow for the examination of the effects of self-administered nicotine on body weight and food intake in the absence of nicotine dependence and withdrawal. The current data are the first to demonstrate an impact of self-administered nicotine on body weight using a procedure that does not result in nicotine dependence. This is important, as it emphasizes that the effects of nicotine, and potentially nicotine reduction, on body weight will likely be observed in non-dependent smokers. Second, utilization of the 1-h self-administration procedure allows for examining the impact of nicotine on energy expenditure in the absence of changes in food intake. While it is clear a combination of changes in metabolism (de Morentin et al., 2012), physical activity (Faraday et al., 2003; Faraday et al., 1999), and potentially food intake, (L. L. Bellinger et al., 2010; Bunney et al., 2015; Mineur et al., 2011; Miyata et al., 2001; O'Dell et al., 2007) contribute to nicotine-induced suppression of body weight, no other procedure removes the suppression of food intake as a confound; this is critical as clinical literature indicates food intake of human smokers does not differ from non-smokers (Perkins et al., 1991). Third, the use of limited access procedures further demonstrates that the effects of self-administered nicotine are likely dependent upon the daily cumulative effects of nicotine and not singular, isolated spikes in plasma nicotine levels.

When food intake was restricted and held constant, as is standard in most self-administration procedures, self-administered nicotine resulted in a dose-dependent suppression of body weight. These data further emphasize that the body weight-suppressant effects of self-administered nicotine can occur independent of changes in food intake. Across all nicotine doses, there was a negative correlation between nicotine intake and body weight gain, indicating that nicotine exposure directly contributes to the magnitude of body weight suppression. We have

previously reported that 3.75 µg/kg/infusion nicotine is subthreshold for reinforcement (i.e., rats respond at a similar rate for 0 and 3.75 µg/kg/infusion) and that 7.5 µg/kg/infusion nicotine is at threshold, such that only approximately 60% of rats will acquire stable self-administration behavior (Smith et al., 2014). In rats that did not meet standard self-administration criteria at these low doses of nicotine, the nicotine delivered in the few infusions they received suppressed body weight gain. Rats in these groups received very low total daily doses of nicotine (ranging from 8.5 – 15 µg/kg daily), likely as a result of general exploratory behavior and not as a result of primary reinforcement. Therefore, it is likely that the threshold for body weight suppression by nicotine is lower than for reinforcement. These data suggest that doses below the threshold for primary reinforcement may still function to suppress body weight, potentially motivating continued use in weight-concerned smokers following the implementation of a nicotine reduction policy. These results are particularly important regarding the initiation of smoking, as such data from human smokers would become available following the implementation of FDA-mandated nicotine product standards.

Although it is generally accepted that nicotine is the primary constituent in cigarettes responsible for weight loss (Zoli et al., 2012), we conducted an additional experiment to rule out the possibility that a cocktail of select constituents in cigarette smoke may have impacted the results of Experiments 3 & 4. This experiment compared weight gain in rats self-administering nicotine alone and nicotine in the presence of the additional constituents. There was no impact of the addition of the other cigarette constituents on body weight gain, indicating that nicotine, and not the other chemicals, contributes to the body weight suppression reported here.

Body weight can be regulated by changes in energy intake (calorie consumption) and energy output (energy expenditure). In the current studies, self-administered nicotine suppressed

body weight gain independent of food intake, indicating that nicotine likely regulates body weight through increased energy expenditure. This notion is consistent with reports that acute and chronic experimenter-administered injections of nicotine have been shown to increase physical activity (de Morentin et al., 2012; Elliott et al., 2004) and basal metabolism (de Morentin et al., 2012). In rodents, the doses of nicotine used in studies reporting increased energy expenditure are within the range that suppresses food intake, making it difficult to establish an independent role for suppression of food intake or increased energy expenditure in the effect of nicotine on body weight regulation. Nonetheless, it is clear that at moderate experimenter-administered doses, nicotine can increase energy expenditure, which likely contributes to the body weight-suppressant effects reported here. Future experiments monitoring metabolism in a self-administration model are warranted. Data from smokers suggest that nicotine can increase metabolic rate (Perkins, 1992b), further supporting for the idea that self-administered nicotine (via intravenous infusions in rats or via cigarette smoke in humans) may suppress body weight through increased energy expenditure.

The FDA has authority to regulate the nicotine content of cigarettes to a low level (Congress, 2009), which may have unintended consequences on body weight (Rupprecht et al., 2015b). Smoking cessation results in weight gain (Veldheer et al., 2015; Williamson et al., 1991), and rodents gain weight following the cessation of chronic subcutaneous (E. D. Levin et al., 1987; Malin et al., 1992) and intravenous (Greibenstein et al., 2013) nicotine exposures. However, whether reduction of nicotine to a dose below a reinforcing threshold results in body weight gain in rodents was previously unexplored. Reduction of nicotine dose resulted in significant increases in body weight gain in food restricted rats. These data indicate that reduction of nicotine exposure by reducing the dose available in each infusion results in body

weight gain independent of food intake, suggesting that the reduction of nicotine content in cigarettes (thereby reducing total nicotine exposure per cigarette) may result in weight gain in current smokers following a potential mandated reduction of nicotine content in cigarettes.

The results of these experiments provide new insight into the understanding of the body weight suppressant effects of nicotine. In a rodent self-administration model of human smoking, nicotine robustly suppressed body weight gain without concurrent reductions in food intake. These data align with reports from smokers suggesting that the observed body weight differences in smokers and non-smokers are independent of changes in daily caloric intake. The ability of self-administered nicotine to suppress body weight gain independent of food intake is dose-dependent and occurs at very low doses below the threshold for reinforcing behavior. Reduction of nicotine dose results in body weight gain independent of food intake. These data have important implications for nicotine reduction policy, as they suggest that reduction of nicotine in cigarettes to a level that will not maintain smoking will likely cause significant weight gain in current smokers. However, in new smokers low nicotine levels may still reduce body weight, possibly motivating continued use and maintaining exposure to harmful chemicals in cigarette smoke.

3.0 SELF-ADMINISTERED NICOTINE INCREASES FAT METABOLISM AND SUPPRESSES WEIGHT GAIN IN MALE RATS

3.1 INTRODUCTION

Cigarette smoking is the largest cause of preventable deaths worldwide (Centers for Disease et al., 2013; World Health Organization Study Group on Tobacco Product, 2015). Smokers weigh less than non-smokers and former smokers (Audrain-McGovern et al.). Many smokers cite weight loss as a reason for smoking and the fear of weight gain as a reason for relapse or the inability to quit (Pomerleau et al., 2001; Rosenthal et al., 2013; Veldheer et al., 2014). Although the weight-suppressive effects of smoking are clear, the mechanisms underlying this phenomenon are relatively poorly understood.

The weight-suppressive effects of cigarette smoke are often attributed to reductions in food intake, despite evidence that smokers and non-smokers have equal daily caloric intake (Perkins, 1992a; Perkins et al., 1990a). Results of the impact of smoking on energy expenditure in humans are mixed. Studies have shown that basal metabolism is increased, decreased, and unchanged by smoking (Perkins, 1992b; Perkins et al., 1990b; Perkins et al., 1996). Poor cardiovascular or respiratory health in smokers may impact measurements of basal metabolism, which relies on respiration. Therefore, animal models may be useful in the study of the impact of

smoking on body weight and energy expenditure, where the pharmacological effects of drugs can be studied without the confound of the health impact of smoke inhalation.

Nicotine, the primary psychoactive constituent in cigarettes, suppresses weight gain and is likely responsible for the weight-suppressive effects of smoking (Perkins, 1992b; Rupprecht et al., 2016; Zoli et al., 2012). The impact of nicotine on energy balance has been studied extensively in rodents, typically using experimenter, non-contingent administration. Results of studies using experimenter-administered nicotine have shown reduced food intake, increased physical activity, increased thermogenesis, and increased basal metabolism (L. L. Bellinger et al., 2010; de Morentin et al., 2012; Zoli et al., 2012). Typically, doses of nicotine delivered by experimenters to rodents to test parameters related to energy balance are larger than an animal would choose to self-administer, and experimenter- and self-administered nicotine differentially impact processes that contribute to energy balance. Until recently, however, the impact of self-administered nicotine on body weight regulation has been largely ignored.

We have recently demonstrated that self-administered nicotine in male rats during 1-h sessions results in large suppression of body weight independent of food intake (Rupprecht et al., 2016). This suggests that the impact of nicotine on body weight suppression results from increased energy expenditure. The goal of these experiments was to measure of energy expenditure following 1-h nicotine self-administration sessions in male rats, using indirect calorimetry. Understanding the effect of self-administered nicotine on energy balance and weight gain may allow for better health outcomes in smokers as it relates to weight gain, and for the development of pharmacotherapies for the treatment of obesity.

3.2 METHODS

Subjects

Male Sprague-Dawley rats (Harlan Farms/Envigo) weighing between 275 and 300 g upon arrival were housed in a temperature-controlled facility on a reverse light-dark 12:12 hr cycle. Rats were housed paired in tub cages with a plastic divider separating the rats. Rats had free access to powdered Purina Rat chow 5001 and water, unless noted otherwise. All procedures were approved by The Scripps Research Institute Institutional Animal Care and Use Committee.

Drugs

Nicotine hydrogen tartrate salt (Sigma and MP Biomedicals) was dissolved in 0.9% saline. Doses are expressed as free base.

Procedures

Surgery

After at least five days of habituation post-arrival, rats were anesthetized with isoflurane (2-3% in 100% O₂) and implanted with catheters into the right jugular vein, as described previously (Donny et al., 1995; Donny et al., 1999). Rats were allowed to recover for a minimum of 5 days before self-administration procedures. Following surgery, were flushed with 0.1 ml sterile saline containing heparin (30 U/ml), gentamicin (1 mg), and streptokinase (9,333 U/ml). Thereafter, catheters were flushed with 0.1 ml heparinized saline (10 U/ml) and heparinized saline (30 U/ml) containing gentamicin (1 mg) prior to and following the self-administration sessions, respectively.

Self-administration

Operant chambers (Med-Associates) enclosed inside sound-attenuating chambers, equipped with a houselight, a fan, and retractable levers were used in the current experiments. An infusion pump located outside of each chamber delivered intravenous infusions during self-administration sessions through tubing connected to each rat's catheter. The tubing was protected in a metal encasing and allowed for relatively unrestricted movement. One lever was available for the duration of the 1-h self-administration session. A single response on the lever resulted in one infusion of nicotine. Infusions were accompanied by the illumination of a white stimulus light above the lever for 1 sec, followed by a 30 sec timeout period during which the white houselight was extinguished. No infusions were delivered during the 30 sec timeout, but responses were recorded.

All solutions used in self-administration studies were passed through a 0.22 μ m filter to ensure sterility. All intravenous infusions were delivered in approximately 1-s (0.1 ml/kg/infusion). Rats in nicotine groups included in analyses passed a patency test, which required displaying physical signs of ataxia within 5-sec of intravenous injections of methohexital (5 mg/kg).

The effect of self-administered nicotine on energy balance, measured by Comprehensive Laboratory Animal Monitoring System (CLAMS)

Prior to surgery, rats received an Echo MRI to measure lean mass, necessary for accurate measurement of respiratory exchange ratio (RER). Rats were implanted with intravenous catheters and assigned to saline (n=8) or nicotine (60 μ g/kg/infusion, n=8) group based on body

weight. Rats were allowed to respond for drug infusion on a FR1 schedule of reinforcement for 14 consecutive days. Following sessions 1, 2, 7, 8, 13, and 14 of self-administration, rats were placed in CLAMS units for 22.5 hours, and removed for the next self-administration session. While in the CLAMS units, information was collected on RER, heat production, activity (x and z plane counts), drinking, and feeding behavior. Each self-administration session occurred in the final hour of the light cycle, so that the CLAMS sessions could begin at dark onset. Rats were fed ad libitum with the exception of the 1-h self-administration session. After the removal from the CLAMS units on the final day, rats were euthanized with CO₂, catheter pedestals were removed, and a final Echo MRI was conducted.

Eight CLAMS units were available, and so the experiment was conducted as two groups of 8 (n=4 of each drug treatment) staggered by 5 days. Rats were pseudorandomly assigned to drug groups, counterbalanced for body weight and lean mass.

Statistics

Data for each experiment are expressed as means \pm SEM. All statistical analyses were performed using SPSS. Comparisons between drug group and session (body weight and self-administration session) or hour of the CLAMS session were analyzed by mixed-design and repeated measures ANOVA tests to account for the within-subjects design of the experiments while testing for between-subjects effects of nicotine dose groups. Planned post-hoc comparisons were assessed at each day or time point using one-way ANOVA. Data are reported for the second day of each CLAMS exposure (Days 2, 8, and 14), so that the potential impact of the stress of changing housing conditions on energy expenditure data was minimized. The α -level for all tests was set at 0.05.

3.3 RESULTS

Body weight and self-administration

Self-administered nicotine significantly suppressed body weight gain ($p < 0.001$; Figure 6a), beginning on Day 8 of self-administration procedures (absolute body weights at the end of the experiment were saline: 344.37 ± 6.45 g; nicotine: 325.73 ± 8.50 g). Following the final session in the CLAMS units, there was a significant reduction in the percentage of fat mass in the nicotine group ($p = 0.005$; Figure 6b) with no difference between groups in the percentage of lean mass ($p = 0.967$; Figure 6c), although there was a non-significant reduction in lean mass gain in the nicotine group over the course of the experiment. Fat mass gain in the nicotine group was significantly reduced compared to saline ($p =$; Figure 6d). There were no differences in free water ($p = 0.853$) and total water ($p = 0.153$) weight after the final day of CLAMS, as measured by MRI. There were no significant differences in infusions taken between nicotine and saline groups ($p = 0.08$; Figure 7). Average daily nicotine intake was 2.74 ± 0.30 mg/kg/day.

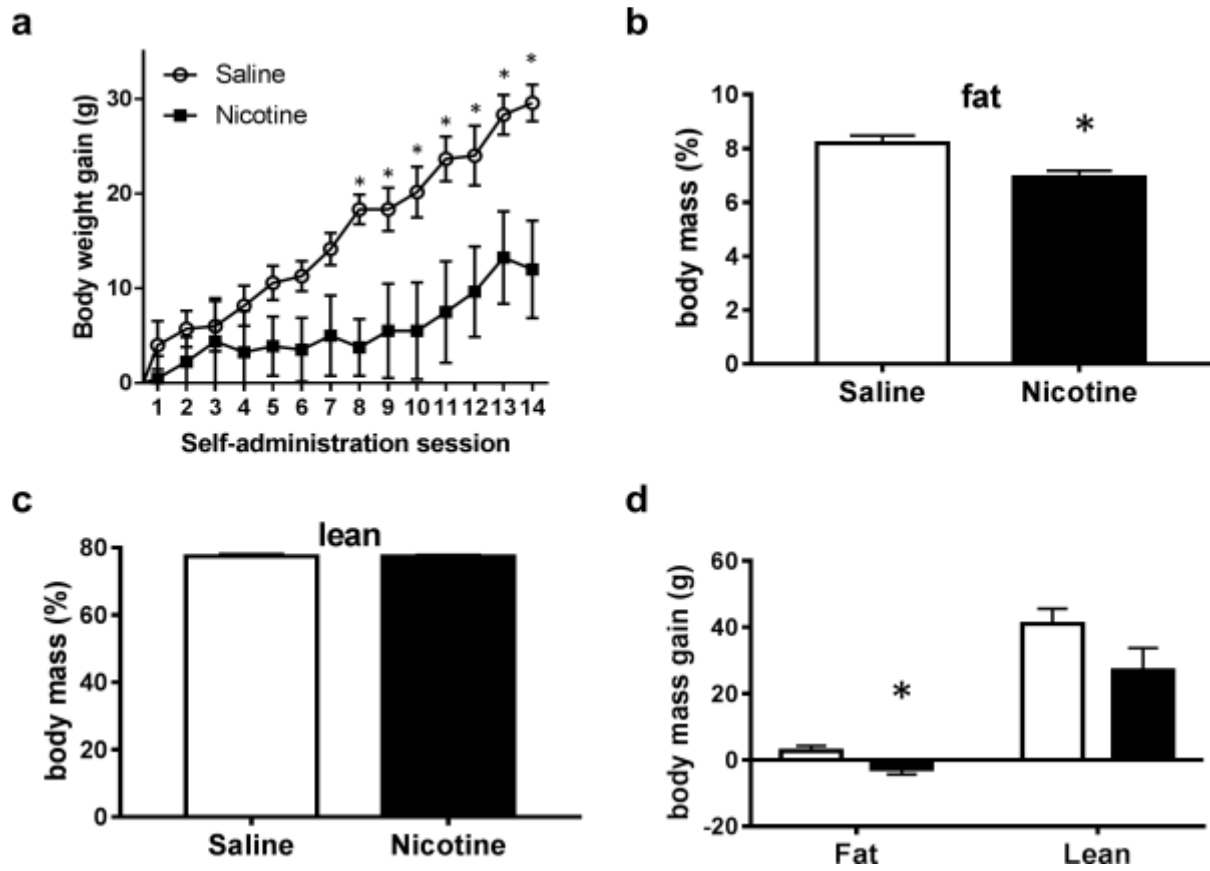


Figure 6. The impact of self-administered nicotine on weight and body mass.

Self-administered nicotine suppressed body weight gain (a) and fat body mass (b), but not lean body mass (c). Fat mass gain was significantly reduced by nicotine, with no change in lean mass gain (d). * indicates $p < 0.05$ between drug groups.

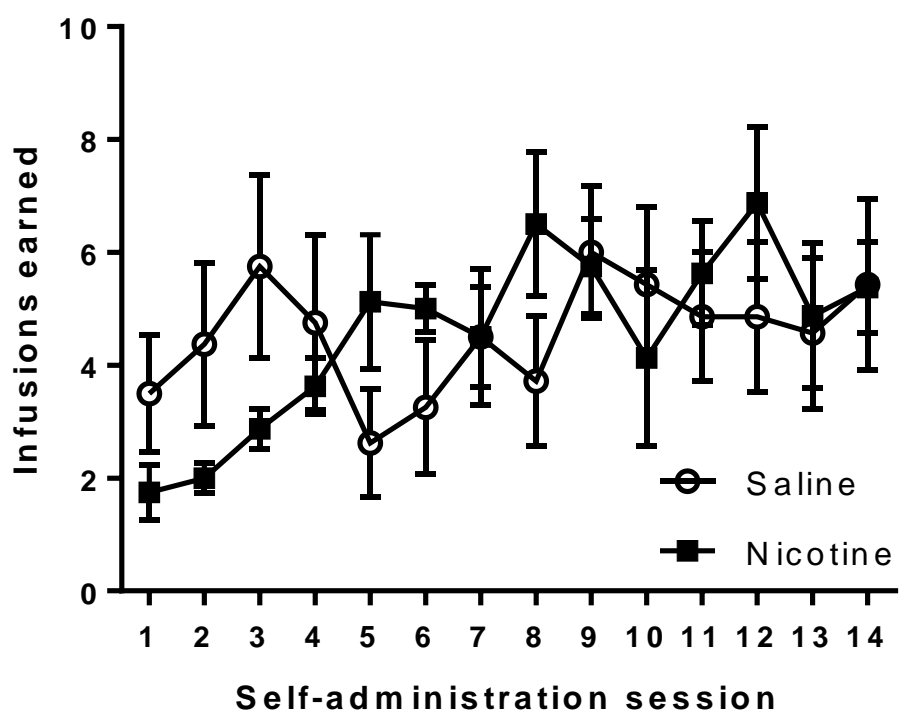


Figure 7. Infusions self-administered in 1-h daily sessions.

Infusions earned during each 1-h session daily. There was no impact of drug group on infusions earned.

Respiratory exchange ratio

Following day 2 of self-administration, there was a significant effect of time ($p < 0.001$; Figure 8a) and group ($p < 0.013$) on RER. Nicotine significantly suppressed RER during the light cycle ($p < 0.003$), but not dark cycle ($p = 0.133$) of the CLAMS session. Following day 8, there was a significant effect of time ($p < 0.001$; Figure 8b), but no effect of group over the 22h session ($p = 0.057$). There was no impact of group during the dark cycle ($p = 0.110$), but nicotine significantly suppressed RER during the light cycle ($p = 0.042$). After the final day of self-administration, there was a significant effect of time ($p < 0.001$; Figure 8c) and group ($p = 0.004$) on RER. Self-administered nicotine suppressed RER during the dark ($p = 0.010$) and light cycle ($p = 0.003$).

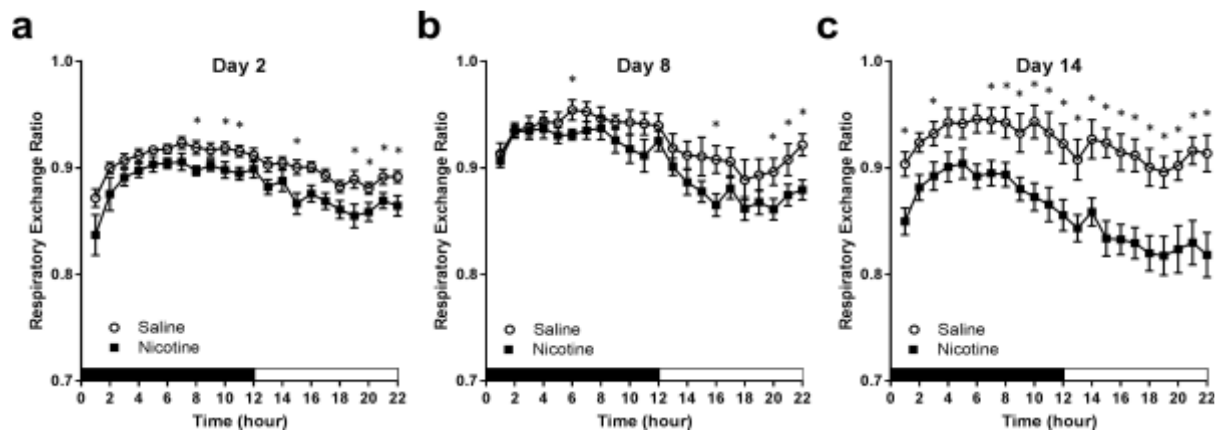


Figure 8. The impact of self-administered nicotine on respiratory exchange ratio.

Self-administered nicotine reduced RER on days 2 (a), 8 (b), and 14 (c). The dark bar indicates dark cycle, and the open bar indicates light cycle during each 22h phase. * indicates $p < 0.05$ between drug groups.

Activity

There was no impact of drug group on X plane total activity counts ($p > 0.164$; Figure 9a, b, c) or Z plane total activity counts ($p > 0.075$) on any day during either phase of the light cycle. There was a significant effect of time ($p < 0.001$), and activity during the dark phase was significantly higher than in the light phase ($p < 0.001$). Of note, on Day 14, activity in the first hour of the CLAMS session was significantly higher than saline ($p = 0.021$; Figure 9c).

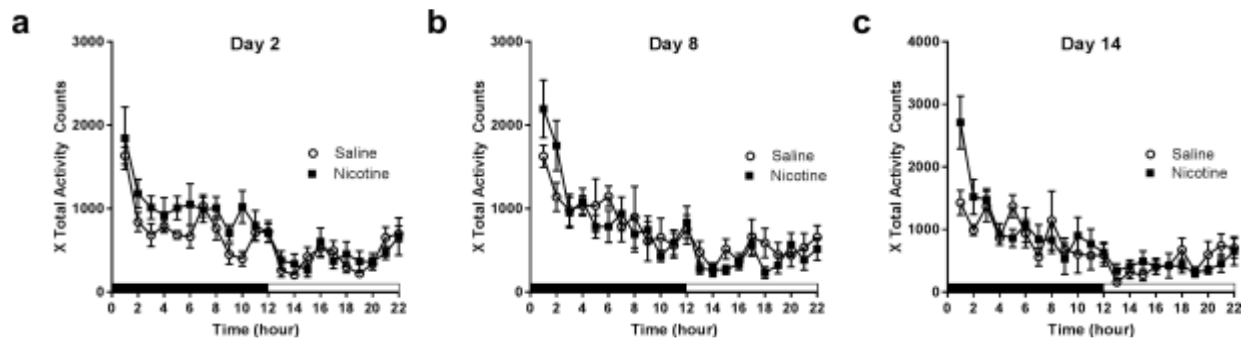


Figure 9. The impact of self-administered nicotine on activity.

Self-administered nicotine did not impact activity in the x plane (a-c) or z plane (d-f). The dark bar indicates dark cycle, and the open bar indicates light cycle during each 22h phase.

Heat

There was no impact of drug group on heat on any day during any phase of the light cycle ($p > 0.230$; Figure 10). There was a significant effect of time ($p < 0.001$) and heat was higher during the dark than the light phase ($p < 0.001$).

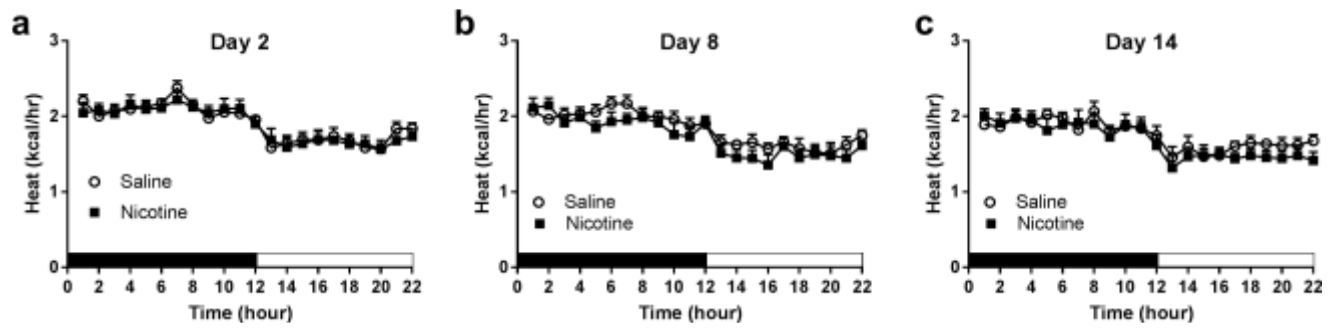


Figure 10. The impact of self-administered nicotine on heat production.

Self-administered nicotine did not impact heat produced on any day. The dark bar indicates dark cycle, and the open bar indicates light cycle during each 22h phase.

Drinking and feeding behavior

Self-administered nicotine transiently suppressed water intake (Figure 11a). On each day, there was a significant effect of time ($ps < 0.002$). Self-administered nicotine significantly suppressed water intake on days 2 ($p = 0.025$) and 8 ($p = 0.019$), but not day 14 ($p = 0.126$). Planned post-hoc comparisons revealed water intake was suppressed after 22h on day 2, and 6 and 22h on day 8. Self-administered nicotine had no impact on food intake, as raw intake ($ps > 0.129$; Figure 11b) and corrected for body weight ($ps > 0.167$; Figure 11c). There were no differences in food intake at any time point ($ps > 0.073$). There was no impact of drug on latency to feed following placement into the CLAMS chambers on any day ($ps > 0.362$; Figure 11d). There was no impact of self-administered nicotine on meal size over the course of 22h ($ps > 0.057$; Figure 11e), though meal size was significantly reduced by nicotine during the light cycle on Day 14 (Table 1). Number of meals consumed over 22h was significantly increased in the nicotine group (Figure 11f) on days 2 ($p = 0.026$) and 14 ($p = 0.012$), which was driven primarily during the light cycle (Table 2). The duration of meals was suppressed on Day 14 ($p = 0.001$; Figure 11g). There was no impact of self-administered nicotine on intermeal interval, with the exception of the light cycle on Day 2 (Table 2).

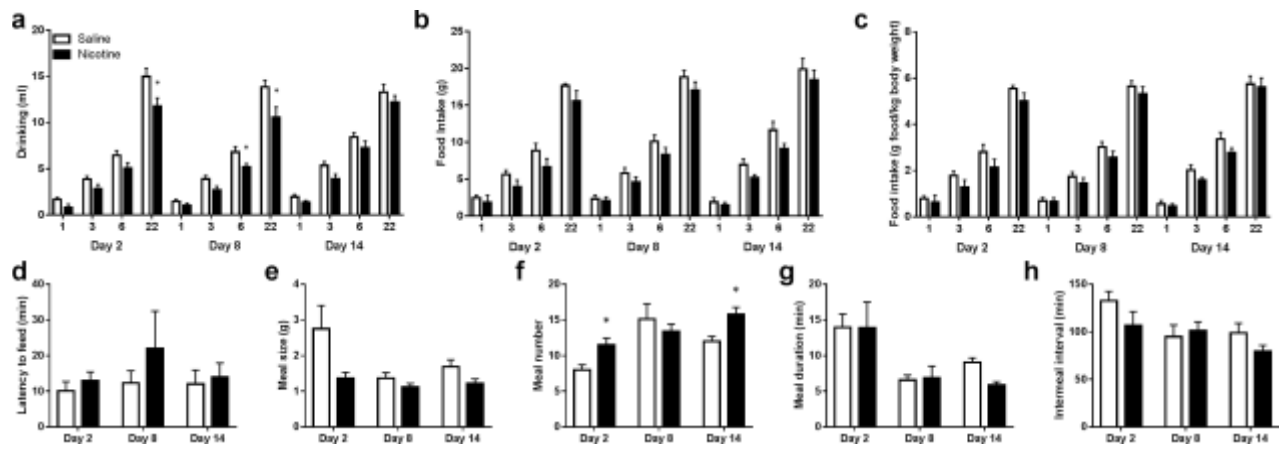


Figure 11. The impact of self-administered nicotine on water and food intake over 22h.

Water intake was reduced by self-administered nicotine on Days 2 and 8 (a), but had no impact on total food intake (b, c). There was no impact of nicotine on latency to feed (d), meal size (e), meal duration (g), or intermeal interval (h). Self-administered nicotine significantly increased meal number on Days 2 and 14 (f). * indicates $p < 0.05$ between drug groups.

Table 2. Meal pattern parameters during dark and light phase of the light cycles. The top line in each box is the average for the dark phase, and the bottom number is average for the light cycle.

* is $p < 0.05$ comparing saline and nicotine within each phase of the light cycle on that day.

	Saline			Nicotine		
	Day 2	Day 8	Day 14	Day 2	Day 8	Day 14
Meal size	2.97±0.82	1.41±0.15	1.75±0.19	1.44±0.18	1.14±0.12	1.26±0.11*
	2.25±0.42	1.30±0.23	1.34±0.26	1.18±0.21	1.12±0.11	1.23±0.33
Meal Number	6.00±0.71	10.29±1.21	9.43±.043	7.75±0.85	10.25±0.77	12.13±0.99*
	2.2.5±0.25	4.85±1.03	2.29±0.29	3.75±0.48*	3.13±0.44	3.66±0.26*
Meal Duration	33.7±18.3	6.83±0.46	9.10±0.75	9.93±1.13	11.6±2.89	6.41±0.64*
	16.0±9.24	6.21±1.25	8.33±1.19	20.1±9.53	6.41±2.00	4.35±0.88*
Intermeal Interval	91.8±13.6	62.1±4.91	60.1±6.62	85.6±11.9	68.1±6.35	54.1±5.23
	313.4±20.8	170.7±38.3	273.7±53.5	151.7±24.7*	235.5±50.3	164.5±14.7

3.4 DISCUSSION

Many smokers cite weight regulation by smoking as a primary reason for smoking and the inability to quit (Pomerleau et al.; Rosenthal et al., 2013; Veldheer et al., 2014). Despite the weight-suppressive effects of smoking and nicotine being studied extensively, the mechanism by which nicotine acts to suppress body weight remains poorly understood. Results of studies in rodents receiving non-contingent nicotine have demonstrated increased energy expenditure and decreased energy consumption through diverse actions of nicotine at its nicotinic cholinergic receptors (nAChR) (Zoli et al., 2012). There is evidence demonstrating that experimenter- and self-administered nicotine may differentially impact physiological responses to nicotine, which could impact weight regulation (Donny et al., 2000; Donny et al., 2011a). Therefore, the use of nicotine self-administration procedures in rodents is particularly useful in evaluating the impact of nicotine on energy balance.

The results of the present experiment demonstrate that self-administered nicotine shifts RER to reflect an increase in fat utilization, without changes in total food intake, activity, or heat production. Changes in RER precede nicotine-induced suppression of weight gain, indicating that increased fat utilization may cause weight reduction following nicotine self-administration. Very low nicotine intake (0.12 mg/kg on Day 2) was sufficient to suppress RER, consistent with recent data demonstrating that very low doses of self-administered nicotine suppress body weight gain independent of food intake (Rupprecht et al., 2016). It has been hypothesized that cumulative nicotine intake over many days may be directly correlated with body weight

suppression. This may explain the magnitude of reduction in RER on Day 14, when total cumulative nicotine intake was highest.

In the current experiment, nicotine intake was low compared to previously published levels of self-administration at this dose over a similar time course. There are several parameters in the self-administration procedure that may explain these low levels of nicotine intake. First, rats were allowed to respond on a lever for infusion of nicotine without previous training for lever responding. Secondly, the 1h/day sessions occurred in the final hour of the light cycle, so that rats could be placed in the CLAMS units at the onset of the dark cycle, when feeding and other behaviors related to energy balance are high. Third, self-administration procedures typically use food restriction to increase levels of behavior (Rupprecht et al., 2015c). In the current experiments, rats were fed *ad libitum*. The self-administration procedure used a compound visual stimulus, which is expected to be mildly reinforcing (Caggiula et al., 2002). This stimulus likely explains the of responding in the saline group, and at a low schedule of reinforcement and high nicotine dose, enhancement of responding for the stimulus by nicotine may not be expected. The similar level of activity at the lever during self-administration between groups offers control of energy expenditure in the operant chamber, indicating that differential activity in the self-administration session likely does not contribute to differences in weight gain between groups.

Nicotine reduced RER most substantially during the light phase, many hours after the nicotine self-administration session. As the half-life of nicotine in a rat is approximately one hour (Kyerematen et al., 1988), this suggests that processes contributing to increased fat utilization occur when nicotine levels in the blood and brain were likely cleared. This result suggests that activation of nAChR is not necessary for increased fat utilization following nicotine

self-administration. There are two likely explanations for the present data. First, that a metabolite of nicotine with a long half-life is responsible for reductions in RER following self-administration. Cotinine is the primary metabolite of nicotine, with a half-life of approximately eight hours in the rat (Kyerematen et al., 1988). Intravenous infusions of cotinine in cigarette smoke to overnight abstinent smokers fails to produce a cardiovascular (Benowitz et al., 1983) or physiologic (Hatsukami et al., 1997) effect. Further, chronic injection of cotinine to mice has been shown to increase weight gain (Riah et al.). Therefore, it is unlikely that cotinine or another metabolite of nicotine acts to decrease RER and weight gain.

Secondly, the possibility exists that nicotine can produce a change that is long lasting, and which remains activated despite a lack of stimulation by nicotine itself. Nicotine administration has been demonstrated to increase lipolysis (Andersson et al., 2001; Friedman et al., 2012; Sztalryd et al., 1996). Evidence supports two parallel pathways by which nicotine could impact lipolysis. First, nicotine has been shown to cause the release of circulating catecholamines, which may in part contribute to increased lipolysis (Cryer et al., 1976). The release of glycerol in subcutaneous fat by intravenous infusion of low dose nicotine to non-smokers is attenuated by local beta-adrenergic and nAChR blockade (Andersson et al., 2001), indicating that nicotine results in lipolysis by catecholamine release and action and local action at adipose tissue. Complicating this idea is evidence demonstrating that while non-contingent intravenous infusion of nicotine causes adrenaline release in rats and humans, there is no impact of self-administered nicotine on adrenaline release in rats (Donny et al., 2000). Therefore, it is unlikely that increased catecholamine release influences results in the present experiment. Current evidence suggests that reduced RER by nicotine, long after nicotine self-administration sessions concluded, is likely driven by lipolysis locally in adipose tissue. The $\alpha 7$ nAChR is

expressed in white adipose tissue and is opened by relatively low levels of nicotine, but is rapidly desensitized (Somm, 2014). An agonist of $\alpha 7$ nAChR has been demonstrated to decrease weight gain in obese, but not normal weight mice (Marrero et al., 2010). Therefore, it is possible that nicotine acts to increase lipolysis at $\alpha 7$ nAChR receptors, and the activation of intracellular processes following nAChR activation act to increase fat utilization after nicotine clearance.

Data from smokers supports the idea that nicotine may increase basal metabolic rate. Administration of nicotine to abstinent smokers via nasal spray results in increases resting metabolic rate (Perkins et al., 1989a), indicating that nicotine increases RER in smokers. The current results from rats compliment and extend what can be learned from a human smoker, demonstrating increased metabolic efficiency in nicotine-naïve rats, before nicotine-induced body weight changes occur. Smoking cigarettes can increase basal metabolic rate (Roth et al., 1944), although increases in energy expenditure without increases in basal metabolic rate have also been reported (Audrain et al., 1991; Perkins et al., 1986). Inhalation of smoke from denicotinized cigarettes can result in small increases in basal metabolic rate, indicating that the non-nicotine constituents in cigarettes or the behavioral action of smoke inhalation impacts metabolism (Perkins, 1992b; Perkins et al., 1989a). However, data from rodent self-administration suggest that the combination of nicotine and non-nicotine constituents in cigarette smoke act to regulate body weight similarly to nicotine alone Rupprecht et al. (2016), indicating that the impact of cigarette smoke on basal metabolism is likely due to behavioral action of inhalation and not additional psychoactive smoke chemicals.

There is a large body of work showing chronic experimenter-administered nicotine suppresses food intake, and some evidence that this occurs via reductions in meal size (L. L. Bellinger et al., 2010; Grebenstein et al., 2013); Wellman et al. (2005). One report of chronic

nicotine injections demonstrated that nicotine acts to suppress food intake without lasting changes in respiratory quotient or energy expenditure (L. L. Bellinger et al., 2010). Therefore, it is possible that in procedures that cause nicotine-induced suppression of food intake, RER shifts back towards carbohydrate utilization in defense of weight set point, and that these specific responses may be dependent upon route, dose, and contingency of administration. In the current data, self-administered nicotine significantly increased meal frequency with non-significant reductions in average meal size. Nicotine dose, route, and duration of administration likely have differential impacts on behaviors related to energy balance (Zoli et al., 2012). These results underscore the importance of examining the impact of nicotine on body weight regulation across many procedures. Regardless, the current data provide further evidence that when nicotine is self-administered in 1-h daily sessions, weight gain suppression occurs independent of changes in food intake.

Nicotine has been previously shown to suppress water intake (Clarke et al., 1984; E. D. Levin et al., 1987). Decreased fluid intake may contribute to rapid weight loss caused by nicotine consumption. However, the current data suggest that over time, tolerance to the hypodipsic effects of nicotine develop, suggesting that negative water balance likely does not contribute to continued weight loss by nicotine across many days. Further, there was no difference in water weight between groups at the end of the experiment. This is in contrast to existing data demonstrating that water intake suppression by nicotine is long lasting, though these effects resulted from high daily nicotine exposure (up to 10 mg/kg/day nicotine) (Clarke et al., 1984). It is possible that tolerance to this develops with nicotine self-administration.

The results of the current experiment demonstrate that self-administered nicotine in male rats suppresses body weight, potentially via increased fat oxidation, without changes in activity,

heat production, or feeding behavior. Together, these results indicate that nicotine-induced body weight suppression relies on decreased RER. Nicotine has been previously reported to increase thermogenesis and slow gastric emptying (de Morentin et al., 2012; Perkins et al., 1996; Scott et al., 1992; Seoane-Collazo et al., 2014). The design of the current experiments cannot rule out the possibility that other parameters not included in our experimental design may contribute to the effect of self-administered nicotine on energy balance. For example, nicotine has been shown to increase thermogenesis. Self-administered nicotine in male rats shifts RER towards fat utilization after nicotine has been cleared and suppresses weight gain.

4.0 REDUCING NICOTINE EXPOSURE RESULTS IN WEIGHT GAIN IN SMOKERS RANDOMIZED TO VERY LOW NICOTINE CONTENT CIGARETTES

4.1 INTRODUCTION

Tobacco use, primarily through cigarette smoking, is the leading cause of preventable mortality, resulting in over 480,000 deaths in the United States annually (Centers for Disease et al., 2011; World Health Organization Study Group on Tobacco Product, 2015). Nicotine is the primary addictive constituent in cigarettes, and in an effort to reduce the public health burden of smoking, the Family Smoking Prevention and Tobacco Control Act gave the Food and Drug Administration (FDA) authority to greatly reduce the nicotine content of cigarettes if doing so would improve public health (Congress, 2009). This policy falls in line with the hypothesis that the reduction of nicotine content in cigarettes to a level below an addictive or reinforcing threshold will suppress nicotine-seeking behaviors in smokers (Benowitz et al., 1994; Donny et al., 2012; Hatsukami et al., 2013). In a recent study, we tested this hypothesis by investigating the effects of cigarettes varying in nicotine content on cigarettes smoked per day and nicotine dependence over a 6-week period (Donny et al., 2015). We found that smokers randomized to smoke very low nicotine content (VLNC) cigarettes containing 2.4 mg of nicotine per gram of tobacco and below for 6 consecutive weeks smoked fewer cigarettes and had lower levels of nicotine dependence compared to those randomized to smoke normal nicotine content cigarettes

(NNC; 15.8 mg of nicotine per gram of tobacco) or their usual brand (Donny et al., 2015). These data support the reduction of nicotine in cigarettes as a strategy for improving smoking-related public health outcomes. However, to fully capture the public health impact of a potential nicotine reduction policy, it is also necessary to identify possible unintended consequences of nicotine reduction, so that policymakers and clinicians may attempt to mitigate them.

The relation between smoking cessation and weight gain is well established. Smokers weigh less than non-smokers and smoking cessation is typically accompanied by weight gain, on average, of 4.5 kg within a year of abstinence (Aubin et al., 2012; Audrain-McGovern et al., 2011; Veldheer et al., 2015). As such, one consequence of a nicotine reduction policy may be weight gain among current smokers (Rupprecht et al., 2015b). Nicotine in cigarettes is likely responsible for the weight-reducing effects of smoking. Use of the transdermal nicotine patch or nicotine gum (Gross et al., 1989) during quit attempts attenuates cessation-induced weight gain, typically in a dose-related manner. Additionally, varenicline, a partial nicotinic agonist FDA-approved for smoking cessation, may offset weight gain among quitters during treatment (Nides et al., 2006). In rats, self-administration of nicotine results in suppression of body weight gain (Bunney et al., 2015; O'Dell et al., 2007; Rupprecht et al., 2016). Moreover, cessation of nicotine self-administration (Bunney et al., 2015) or reduction of nicotine dose to levels below a reinforcing threshold (Rupprecht et al., 2016) results in weight gain. Mice exposed to smoke from NNC cigarettes gained significantly less weight than those exposed to smoke from VLNC cigarettes (Abreu-Villaca et al., 2010). Taken together, evidence points to reductions in nicotine exposure as mediating cessation-induced weight gain, and thus, weight gain is a likely outcome of nicotine reduction (Benowitz et al., 2012).

The aim of this investigation was to examine the effect of an abrupt switch to use of VLNC cigarettes on weight among current smokers. A randomized double-blind, multi-site clinical trial of daily smokers (n=839) not interested in quitting was completed in which participants were assigned to smoke cigarettes varying in nicotine content for six weeks. Here, we evaluated if smoking reduced nicotine content cigarettes in this sample was associated with weight gain. Given the hypothesized primary role of nicotine exposure as the mechanism underlying weight gain and evidence that most participants use other products when randomized to VLNC cigarettes (Benowitz et al., 2015; Nardone et al., In Press), an important analysis focused on the relation between urinary biomarkers of nicotine exposure and weight gain. Furthermore, some evidence suggests that women are more likely to use smoking as a method of weight control and may be more susceptible to post-cessation weight gain (Farley et al., 2012; Levine et al., 2001); therefore, differences in outcomes due to gender were also explored.

4.2 METHODS

Participants

Adult daily smokers were recruited using flyers, direct mailings, television and radio, and other advertisements across 10 sites between 2013 and 2014. Inclusion criteria included: at least 18 years of age, at least five cigarettes smoked per day, expired carbon monoxide (CO) greater than 8 ppm or urinary cotinine greater than 100 ng/ml. Exclusion criteria were: intention to quit smoking in the next 30 days; use of other tobacco products on more than 9 of the past 30 days; serious psychiatric or medical condition; positive toxicological screen for illicit drug use other than cannabis; pregnancy, plans to become pregnant, or breastfeeding; and exclusive use of “roll

your own” cigarettes. All 839 eligible participants provided written informed consent prior to enrollment. The study was approved by accredited Institutional Review Boards at each participating site, and written informed consent was obtained from each study volunteer. All study procedures were conducted in compliance with research ethics outlined in the Declaration of Helsinki.

Study Design

The seven-group, double-blind, randomized trial included a screening visit, a 2-week baseline period during which participants smoked their own usual brand cigarettes, and a 6-week investigational cigarette use period. During the 6-week experimental period, participants were provided with one of seven types of cigarettes varying in nicotine content (mg nicotine per g of tobacco): 0.4 mg/g; 0.4 mg/g high tar (HT); 1.3 mg/g; 2.4 mg/g; 5.2 mg/g; 15.8 mg/g, and usual brand (UB). Average tar yields were 8 to 10 mg; however, for the high tar cigarettes it was 13 mg. The 0.4 HT condition, which contained tobacco filler with the same nicotine content, but differed from 0.4 mg/g cigarettes in filter and ventilation resulting in higher yield (ISO) of tar and nicotine, was added to the design to explore the impact of tar yield on the use and acceptability of VLNC cigarettes. A two-week supply of cigarettes was provided free of charge at each weekly session during the experimental period. During this time, participants were instructed to smoke only the provided investigational cigarettes and received counseling aimed to increase compliance, though there was no penalty for using other nicotine/tobacco products. Study design is described in greater detail in the primary study manuscript (Donny et al.).

Study Assessments & Laboratory Analyses

During each visit to the laboratory, participants were asked to remove shoes and outerwear, and to plant both feet firmly and evenly on the scale surface. Body weight was measured to the nearest 0.1 kg. Biomarkers of nicotine exposure were assessed from urine samples collected at randomization, Week 2, and Week 6. Urinary total nicotine equivalents (TNE), the sum of nicotine and its metabolites and a measure of daily nicotine exposure, were analyzed by liquid chromatography tandem mass spectrometry (Carmella et al., 2013; Murphy et al., 2014; Murphy et al., 2013). Saliva samples for the assessment of nicotine metabolite ratio (NMR), an indicator of CYP2A6 activity and the rate of nicotine metabolism, were collected during the second baseline session (Donny et al., 2015).

Statistical Analyses

Our initial comparison focused on differences in weight gain (defined as each participant's weight at each visit minus his or her baseline weight in kg) by randomized treatment assignment. Baseline weight was the average of three measurements taken at screening and the two, weekly baseline visits. Two participants were found to have a 50kg weight gain at the six-week follow-up period. These records are assumed to have been a data entry error and were removed from all analyses. Differences in weight gain over time were analyzed using a linear mixed model with a random intercept to account for multiple observations from a single individual. Fixed-effects included in the model were treatment group, visit, treatment by visit interaction, baseline weight, age, gender, race, the natural log of salivary NMR, site, time-of-year at enrollment and a site by time-of-year at enrollment interaction. Time-of-year at enrollment

was included in the model by mapping the calendar onto a circle and translating the date into radians and was included as time-of-year impacts weight gain (Le et al., 2003). A Bonferroni-adjusted p-value of 0.01 was used to conclude statistical significance when comparing treatment groups to the 15.8 mg/g control. A secondary comparison was also completed, which compared treatment groups to the Usual Brand control condition.

Within this study population, the average reduction in biomarkers of tobacco use was less than expected given the reduction in nicotine content of the study cigarettes (Donny et al., 2015), indicating likely use of other sources of nicotine (e.g., non-study cigarettes). The use of other nicotine-containing products could potentially mask an effect of the use of VLNC cigarettes on weight gain. Thus, we conducted a subgroup analysis comparing weight gain by compliance status in the combined 0.4 mg/g and 0.4 mg/g HT groups. Compliance status was dichotomized and a participant was considered compliant if their urinary TNE was less than 6.41 nmol/ml at Week 2 and Week 6. This cutoff was established in a prior study in which compliance with 0.4 mg/g cigarettes was enforced (Denlinger et al., 2016). Biochemical confirmation of compliance was not possible in the other cigarette conditions because individual differences in nicotine intake from these cigarettes likely result in greater overlap in the distribution of TNE with smoking NNC cigarettes and no data are available validating such a cutoff. Weight-gain was compared by compliance status over time using a linear mixed-model with a random intercept and fixed-effects for compliance status, visit, compliance status by visit interaction, baseline weight, age, gender, race, baseline cigarettes per day (CPD), natural log of baseline TNE, study site and time-of-year at randomization. Baseline CPD and baseline TNE were previously shown to be associated with biochemical measures of non-compliance and were included in this model to account for potential confounding (Nardone et al., In Press). Gender

was examined as a moderator by adding an interaction for gender and estimating treatments effects within each gender. Finally, the association between weight gain and the natural log of TNE, as the raw TNE data were not normally distributed, at Week 6 in the 0.4 mg/g and 0.4 mg/g HT groups was summarized by Pearson's correlation coefficient.

4.3 RESULTS

Sample characteristics

The overall sample was 41.7 ± 13.2 years old, 57.3% male, smoked 15.6 ± 7.6 CPD, and weighed 85.8 ± 21.8 kg at baseline. Retention exceeded 92% and attrition did not differ by cigarette group. Additional baseline sample characteristics can be accessed in the primary report of these data (Donny et al., 2015).

Cigarette condition failed to significantly impact weight gain

Mean changes in body weight (kg) comparing between each investigational cigarette condition and each of the two control conditions (15.8 mg/g and Usual Brand groups) by week for the entire study sample are shown in Table 2. With the exception of the 0.4 mg/g HT group at Week 4 ($p = 0.009$), there were no significant differences in weight gain when comparing the reduced nicotine conditions with the 15.8 mg/g control group across all treatments groups and week.

Table 3. Effect of smoking reduced nicotine content cigarette on weight gain (kg) over 6 weeks

Treatment Group	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
15.8 mg group as reference group (primary analysis):						
5.2 mg/g	0 (-0.48, 0.48)	0.29 (-0.2, 0.77)	0.01 (-0.47, 0.5)	0.13 (-0.36, 0.62)	0.14 (-0.36, 0.63)	0.01 (-0.48, 0.49)
2.4 mg/g	0.01 (-0.47, 0.5)	0.34 (-0.15, 0.83)	0.08 (-0.41, 0.58)	0.24 (-0.26, 0.73)	0.37 (-0.13, 0.87)	0.22 (-0.27, 0.71)
1.3 mg/g	0.2 (-0.28, 0.68)	0.52 (0.03, 1)	0.25 (-0.24, 0.73)	0.16 (-0.34, 0.65)	0.22 (-0.27, 0.71)	0.18 (-0.3, 0.67)
0.4 mg/g	0.1 (-0.39, 0.59)	0.36 (-0.13, 0.85)	0.11 (-0.39, 0.6)	0.28 (-0.22, 0.77)	0.34 (-0.16, 0.83)	0.18 (-0.32, 0.67)
0.4 mg/g (HT)	0.11 (-0.38, 0.59)	0.51 (0.03, 0.99)	0.23 (-0.25, 0.72)	0.65* (0.16, 1.14)	0.33 (-0.16, 0.82)	0.12 (-0.36, 0.6)
Usual Brand group as reference group (secondary analysis):						
15.8 mg/g	-0.06 (-0.55, 0.42)	-0.04 (-0.53, 0.45)	-0.14 (-0.63, 0.35)	-0.16 (-0.65, 0.33)	-0.43 (-0.92, 0.06)	-0.2 (-0.69, 0.29)
5.2 mg/g	-0.07 (-0.55, 0.42)	0.25 (-0.24, 0.73)	-0.13 (-0.62, 0.36)	-0.03 (-0.52, 0.46)	-0.3 (-0.79, 0.2)	-0.19 (-0.68, 0.29)
2.4 mg/g	-0.05 (-0.53, 0.44)	0.3 (-0.18, 0.79)	-0.06 (-0.55, 0.44)	0.08 (-0.42, 0.57)	-0.06 (-0.56, 0.44)	0.02 (-0.47, 0.51)
1.3 mg/g	0.14 (-0.34, 0.62)	0.48 (0, 0.96)	0.1 (-0.38, 0.59)	0 (-0.49, 0.49)	-0.21 (-0.7, 0.28)	-0.02 (-0.5, 0.47)
0.4 mg/g	0.04 (-0.45, 0.52)	0.32 (-0.17, 0.81)	-0.04 (-0.53, 0.46)	0.12 (-0.38, 0.61)	-0.09 (-0.59, 0.4)	-0.02 (-0.52, 0.47)
0.4 mg/g (HT)	0.04 (-0.44, 0.52)	0.47 (-0.01, 0.95)	0.09 (-0.39, 0.57)	0.49 (0.01, 0.97)	-0.1 (-0.59, 0.38)	-0.08 (-0.56, 0.4)

Reduced nicotine exposure resulted in significant weight gain

Weight gain was significantly negatively correlated with nicotine exposure in the two lowest nicotine content cigarette conditions (0.4 mg/g and 0.4 mg/g HT; $r = -0.21$, $p = 0.001$, 95% CI: -0.34, -0.08; Figure 12). Within the two lowest nicotine content cigarette conditions, smokers compliant with the investigational cigarettes ($n = 45$) gained significantly more weight than non-compliant smokers ($n = 170$), the 15.8 mg/g control group ($n = 119$) and the Usual Brand group ($n = 118$) beginning at Week 3 (Figure 13). Women compliant on study product ($n = 24$) gained significantly more weight than non-compliant women ($n = 76$) and women in the 15.8 mg/g control group ($n = 48$) and Usual Brand group ($n = 46$) (Figure 14a). Likewise, men compliant on study product ($n = 21$) gained significantly more weight than non-compliant men ($n = 94$) and men in the 15.8 mg/g group ($n = 71$) and Usual Brand group ($n = 72$) (Figure 14a). There was no significant interaction between gender and compliance on weight gain.

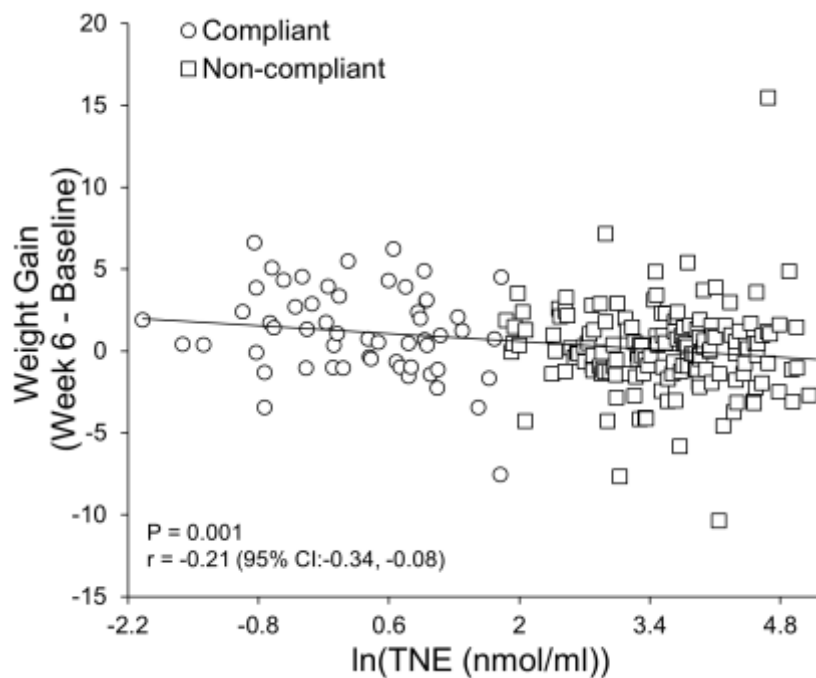


Figure 12. Relationship between nicotine exposure and weight gain.

Within 0.4 mg/g and 0.4 mg/g HT groups, weight gain was negatively correlated with the natural log of TNE.

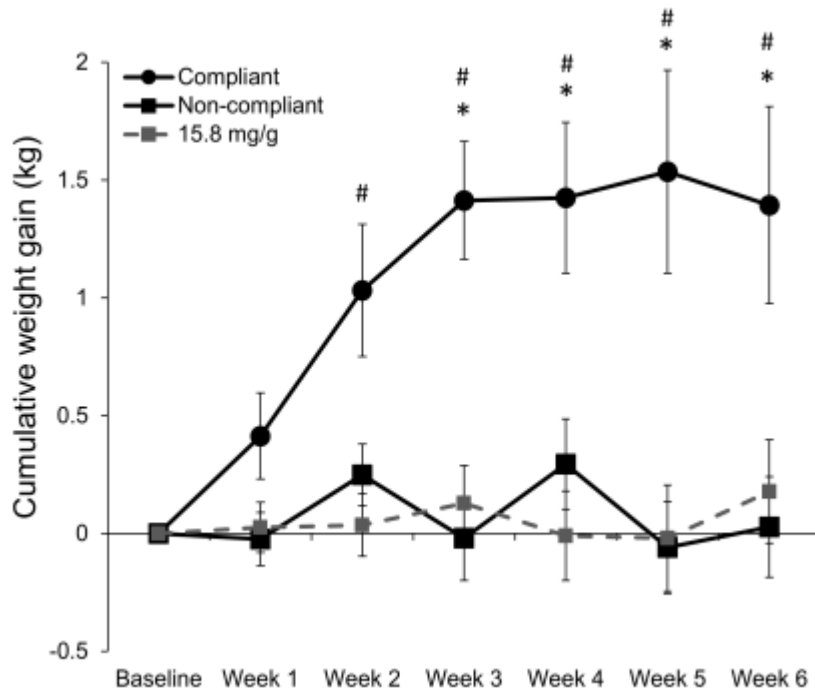


Figure 13. Weight gain over time in compliant and non-compliant individuals

randomized to 0.4 mg/g and 0.4 mg/g HT cigarettes.

Mean cumulative weight gain in individuals compliant (urinary TNE less than 6.41 nmol/ml at Week 2 and Week 6) or non-compliant (urinary TNE greater than 6.4 at Week 2 or Week 6) on 0.4 mg/g and 0.4 mg/g HT cigarettes, and 15.8 mg/g control group. * indicates $P < 0.01$ comparing compliant and non-compliant groups. # indicates $P < 0.01$ comparing compliant and 15.8 mg/g groups. + indicates $P < 0.1$ comparing compliant and usual brand groups.

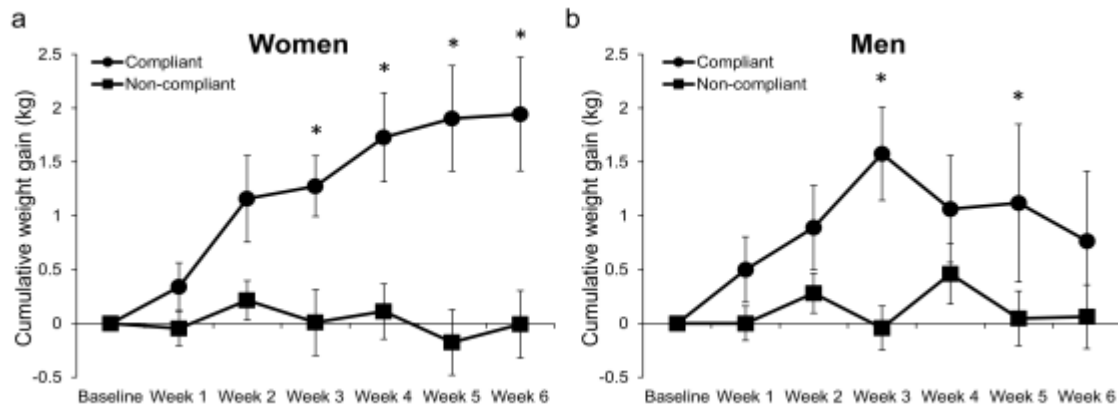


Figure 14. Weight gain over time in compliant and non-compliant men and women randomized to 0.4 mg/g and 0.4 mg/g HT cigarettes.

Mean cumulative weight gain in women (a) and men (b) compliant (urinary TNE less than 6.41 nmol/ml at Week 2 and Week 6) or non-compliant (urinary TNE greater than 6.4 at Week 2 or Week 6) on 0.4 mg/g and 0.4 mg/g HT cigarettes, and 15.8 mg/g control group. * indicates $P < 0.01$ comparing compliant and non-compliant groups.

4.4 DISCUSSION

The implementation of product standards requiring substantial reductions in nicotine content in cigarettes is hypothesized to improve public health by facilitating cessation of smoking. However, the reduction of nicotine content in cigarettes may have other unintended health-related outcomes. The current investigation found that although there was no impact of random assignment to reduced nicotine content investigational cigarettes on weight gain, compliance with investigational cigarettes containing only 2-3% of the nicotine found in NNC cigarettes was associated with resulted in significant weight gain. Furthermore, among individuals smoking cigarettes with the lowest nicotine content, weight gain was negatively correlated with biomarkers of nicotine exposure. These results have important implications for product standards on nicotine and the understanding of nicotine on body weight regulation. The reduction of nicotine content in cigarettes results in an expected amount of weight gain and would likely be observed if product standards requiring low nicotine levels in cigarettes are enacted, assuming people do not substitute other nicotine-containing products.

Compliant participants in the VLNC cigarette condition gained approximately 1.4 kg over 6 weeks of smoking VLNC cigarettes, which is comparable to weight gain reported among abstinent smokers over a similar time period (Emont et al., 1987; Klesges et al., 1989). In the current study, weight gain among compliant smokers occurred primarily within the first three weeks of VLNC use, and then plateaued. This is reassuring, as it suggests that weight gain following reductions in nicotine exposure might be expected to be consistent with long term

changes in weight gain following cessation. Further support for this notion is provided by a study that reported significant body weight gain (approximately 2 kg) in self-reported compliant smokers of cigarettes with nicotine content that was gradually reduced after 26 weeks (Benowitz et al., 2012). Therefore, it is reasonable to expect long term weight gain following nicotine reduction to be similar to that observed in cessation, assuming no use of other nicotine-containing products. A meta-analysis of 62 studies focused on cessation-induced weight gain reported a curvilinear pattern of weight gain over 12 months in untreated abstinent smokers, with weight gain reaching approximately 4.5 kg and plateauing after approximately six months (Aubin et al., 2012). Further, the rate of weight gain in former smokers returns to that of age-matched non-smoker controls following one year of smoking cessation (Audrain-McGovern et al., 2011). Veldheer et al. (2015) recently reported a positive correlation between CPD prior to quitting and ten-year post-cessation weight gain, indicating that a larger change in nicotine exposure results in more robust weight gain. Of note, the sample in the current report was overweight at baseline. Obese and overweight smokers consume more CPD on average than normal weight smokers (Rupprecht et al., 2015b; Veldheer et al., 2015), and therefore may be at risk for larger weight gain following nicotine reduction (Veldheer et al., 2015). Although cessation is associated with an overall increase in weight gain, the impact of quitting on weight gain varies, with approximately 16% of smokers losing weight and 10% gaining over 10 kg in one year (Aubin et al., 2012). The same variability might be expected population-wide following nicotine reduction in cigarettes. Future studies testing the impact of VLNC cigarette use on weight and weight-related health outcomes over longer time periods may confirm this and should more fully capture the impact of nicotine reduction on body weight and health.

Despite the likelihood of weight gain in smokers following nicotine reduction, the overall public health impact of reducing nicotine in cigarettes may be positive if nicotine reduction increases smoking cessation (Benowitz et al., 1994; Donny et al., 2015; Rupperecht et al., 2015b). Indeed, smoking cessation is widely recommended despite the expected gain in weight because the health benefits of quitting far outweigh the negative health consequences of post-cessation weight gain (Health et al., 2004). It is possible that nicotine content could be reduced to a level that would support quitting without resulting in weight gain. However, the lowest nicotine content cigarette tested most reliably decreased multiple measures of dependence and increased quit attempts (Donny et al., 2015), putative predictors of a positive public health impact, even if accompanied by weight gain. There is no indication that the amount of weight gain expected during use of VLNC cigarettes would exceed that of other means of quitting without pharmacotherapy. Research is warranted to determine if NRTs (Filozof et al., 2004; Gross et al., 1989; Schnoll et al., 2012), varenicline (Nides et al., 2006), or bupropion (Farley et al., 2012), which attenuate post-cessation weight gain, would similarly mitigate the weight gain observed in smokers of VLNC cigarettes.

Women more frequently report using smoking as a weight-control method and report fear of weight gain following quitting (Filozof et al., 2004; Levine et al., 2001). Some studies report that post-cessation weight gain is greater among women than men (Filozof et al., 2004; Williamson et al., 1991), but there are also contradictory findings (Aubin et al., 2012). Our study did not reveal significant gender differences, though we did find that women gained more weight on average than men following reductions in nicotine exposure. Additionally, weight gain at Week 3 was equal for women and men, but then plateaued in women and decreased in men. Women were more likely to be compliant on VLNC study product (Nardone et al., In Press), and

within the compliant group, nicotine exposure was lower in women than men (natural log of TNE \pm SEM: 0.08 ± 0.17 in women; and 0.43 ± 0.20 in men). The lower levels of nicotine exposure among women may contribute to the higher average weight gain reported here. Sample size was low and future experiments with sufficient power to address gender differences in weight gain are warranted.

In addition to the results of this study clarifying the effect of VLNC cigarettes on weight gain, it was demonstrated that urinary biomarkers of product compliance can allow for evaluating potential unintended consequences of nicotine reduction where non-compliance could otherwise occlude an effect. Indeed, differences in compliance likely both reduce potential effect size and add substantial variance to measures of unintended consequences related to reduced nicotine exposure per se. An important limitation of focusing on just compliant participants is that they self-selected into compliant and non-compliant groups, which may introduce confounds. Biomarkers of compliance might be utilized to incentivize compliance on study product to better our understanding of the effects of a potential product standard on behavior and health.

These data contribute important information to tobacco regulatory science and provide a greater understanding of the impact of nicotine on body weight regulation. The magnitude of weight gain is negatively related to nicotine exposure, and is similar to what is observed following smoking cessation. Given these results, weight gain is an expected outcome of the implementation of product standards mandating reduced nicotine content in cigarettes. Under the assumption that reductions in nicotine exposure leads to decreased dependence and therefore, increased quitting Donny et al. (2015), the positive public health impact of product standards mandating reductions in nicotine content in cigarettes are likely to outweigh the negative health

consequence of weight gain (Health et al., 2004). Nonetheless, the potential for weight gain must be considered when assessing the public health impact of product standards requiring the reduction of nicotine content in cigarettes. Furthermore, the long-term effect of such strategy must be considered in future research with the goal of mitigating potential weight gain following implementation of product standards reducing nicotine levels in cigarettes.

5.0 SELF-ADMINISTERED NICOTINE DIFFERENTIALLY IMPACTS BODY WEIGHT GAIN IN OBESITY-PRONE AND –RESISTANT RATS

5.1 INTRODUCTION

Tobacco smoke and obesity represent the largest causes of preventable deaths worldwide (Centers for Disease et al., 2013). Over the past 35 years in the United States, rates of cigarette smoking have slowly declined, as the rates of obesity have dramatically increased (Stewart et al., 2009). Abstinence from smoking is typically accompanied by weight gain (Audrain-McGovern et al., 2011; Zoli et al., 2012) and research suggests that smoking cessation is in part responsible for the drastic increase in the rates of overweight in the United States (Flegal et al., 1995). The relationship between smoking and body weight regulation, particularly among the obese population, is poorly understood. Research on the effect of smoking on BMI among obese smokers has resulted in conflicting results (Cooper et al., 2003; Fidler et al., 2007), and is complicated by reliance on self-report data or the challenges of prospective studies. The negative health consequences of smoking are more severe among the obese population (Chiolero et al., 2008; Rupprecht et al., 2015b) and the relationship between smoking and obesity requires more attention.

Although research has consistently demonstrated that higher BMI is associated with higher rates of smoking (Chiolero et al., 2007b), the casual relationship between obesity and

nicotine exposure is unclear. Whether chronic nicotine exposure via cigarette smoke prevents the development of obesity in smokers that would otherwise be obese is unknown (Rupprecht et al., 2015b). It is difficult to assess whether nicotine causes changes in body weight regulation in human smokers; animal models may provide a better opportunity to evaluate this hypothesis. Previous research has demonstrated that large doses of subcutaneous nicotine suppress body weight and food intake in obese rodents (Mangubat et al., 2012; Seoane-Collazo et al., 2014), but the impact of nicotine on body weight and feeding behavior in obesity has not been studied in an animal model of nicotine self-administration.

Outbred rats remain lean when fed chow, but when maintained on a diet modeling the nutritional content of Westernized societies, body weight gain separates into distinct groups: a subset of obesity-prone (OP) and obesity-resistant (DR) rats (B. E. Levin et al., 1989). OP and OR rats are considered among the best animal models of diet-induced obesity and recapitulate many key features of the human condition. The current experiment evaluated the impact of self-administered nicotine on body weight gain and food intake in OP, OR, and chow-fed rats. Results demonstrated that self-administered nicotine suppressed body weight gain in chow-fed and OP rats without suppression of daily food intake. OR rats were insensitive to the weight-suppressive effects of self-administered nicotine.

5.2 METHODS

Subjects

Male Sprague-Dawley rats (n=60; Charles River, Kingston, NY) weighing 275-300 g upon arrival were housed individually in hanging-wire cages on a reverse light-dark 12:12 hr cycle (lights off at 0700h) in a temperature-controlled facility (between 68 and 70 °F). Upon arrival, rats had free access to high energy diet (HED; Research Diets D12266B, New Brunswick, NJ; 31.8% kcal from fat, 25.2% kcal from sucrose, 4.41 kcal/g) and water, unless noted otherwise. All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Drugs

Nicotine hydrogen tartrate salt (Sigma, St. Louis, MO) was dissolved in 0.9% saline; doses are expressed as free base (Rupprecht et al., 2016; Smith et al., 2013). Infusion duration was adjusted daily to account for body weight gain.

Classification of body weight phenotype groups

Body weight was monitored daily while all rats had free access to HED for two weeks. At the end of this two-week period, the twenty rats that gained the most weight were assigned to OP and the twenty rats that gained the least weight were assigned to OR. . The middle tertile gained an intermediate amount of weight and were placed on chow (Purina 5001; 3.36 kcal/g) on Day 15 as a diet control, and will be referred to as the Chow group (n=20). Rats in the Chow group had at least five days of chow exposure before behavioral procedures. Rats assigned to OP

and OR groups remained on HED maintenance. Average body weight for each group on the first day of experimentation was as follows: Chow = 423.3 ± 13.3 g; OP = 445.4 ± 18.2 g; OR = 392.7 ± 21.1 g.

Procedures

Surgery

After two weeks of diet exposure and phenotype group classification, rats were anesthetized with isoflurane (2-3% in 100% O₂) and implanted with catheters into the right jugular vein (Smith et al., 2013; Smith et al., 2014). Rats were allowed to recover for 5-6 days before self-administration procedures, during which catheters were flushed daily with 0.1 ml heparinized saline (30 U/ml) containing timentin (66.67 mg/ml) and streptokinase (8,333 U/ml). Catheters were flushed with 0.1 ml heparinized saline (10 U/ml) and heparinized saline (30 U/ml) containing Timentin (66.67 mg/ml) prior to and following the self-administration sessions, respectively.

Self-administration

Self-administration occurred in 38 operant chambers (Med-Associates) (Rupprecht et al., 2016; Smith et al., 2015). Rats were assigned to drug group (0 or 60 µg/kg/infusion nicotine), counterbalanced by body weight within phenotype group (n=20/phenotype, n=10 receiving saline or nicotine within phenotype). One nose-poke hole was assigned as active, resulting in the delivery of an i.v. infusion after fulfilling the fixed-ratio (FR) 2 response requirement. The other nose-poke hole was inactive; responses at this nose-poke portal were recorded but had no

consequence. Active and inactive nose-poke holes were randomly assigned (left or right). Infusions were accompanied by a 15-sec cue light illuminated above the active nose-poke portal and an unsignaled 1-min timeout, where responses were recorded but had no scheduled consequence. Self-administration occurred in 20 consecutive 1-h daily sessions. Sessions began 1 – 2 h following the onset of the dark cycle, depending on cohort order. Each phenotype group was equally represented in each cohort, and time of day of self-administration session had no impact on dependent measurements.

Rats included in analyses passed a patency test, which required displaying physical signs of ataxia within 5-s of intravenous injections of methohexital (5 mg/kg). Final sample size following patency testing is as follows: Chow saline, n=10; Chow nicotine, n=9; OP saline, n=9; OP nicotine, n=9; OR saline, n=10, OR nicotine, n=7.

Food intake measurements

Food intake measurements, accounting for spillage, were taken every fifth day of experimentation over 24h. Measurements occurred while the rat was out of the home cage, during operant sessions to minimize disruption of food intake. Unlike most self-administration experiments (Rupprecht et al., 2015c), these rats had unrestricted access to food, with the exception of the 1-h self-administration session.

Statistics

Statistical analyses were performed using SPSS. Comparisons between drug group, phenotype, and session (self-administration experiments, every fifth day) or day (feeding experiments) were analyzed by mixed-design and repeated measures ANOVA tests. Planned

comparisons between drug groups within each phenotype were analyzed using one-way ANOVA. The data were Greenhouse-Geisser corrected where Mauchly's Sphericity tests were significant. The α -level for all tests was set at 0.05.

5.3 RESULTS

High energy diet exposure suppressed nicotine self-administration.

Nicotine groups acquired stable behavior, taking more infusions than saline controls ($p < 0.001$). Within the nicotine groups, there was a significant effect of phenotype ($p = 0.042$). Chow rats self-administered more nicotine compared to rats fed HED ($p = 0.011$), though this did not reach significance when separated into OP and OR rats (Figure 15).

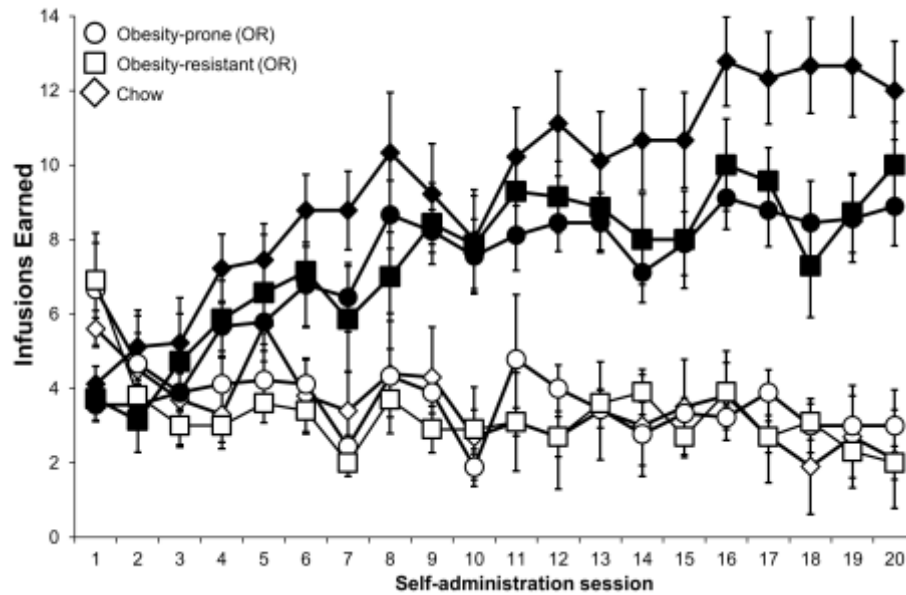


Figure 15. Infusions earned in 1-h daily self-administration sessions.

Infusions earned across 1-h daily self-administration sessions. Open symbols indicate saline, and filled indicate nicotine groups. Across phenotypes, rats earned significantly more nicotine than saline infusions. Within the nicotine groups, Chow rats self-administered significantly more nicotine than HED-maintained groups.

Self-administered nicotine suppressed body weight gain in OP and Chow, but not DR rats.

Self-administered nicotine suppressed BW gain in OP and Chow rats compared to intravenous infusions of saline (Figure 16). There was a significant main effect of day ($p < 0.001$); day*phenotype ($p < 0.001$); day*drug ($p < 0.001$); and three-way interaction ($p = 0.011$). Within phenotype, self-administered nicotine significantly suppressed BW gain in Chow rats on all days tested ($ps < 0.018$; Figure 16 a & b) and in OP rats on Days 5, 10, 15, and 20 ($ps < 0.016$; Figure 16a & c). There was no significant impact of nicotine on weight gain in OR rats (Figure 16a & d).

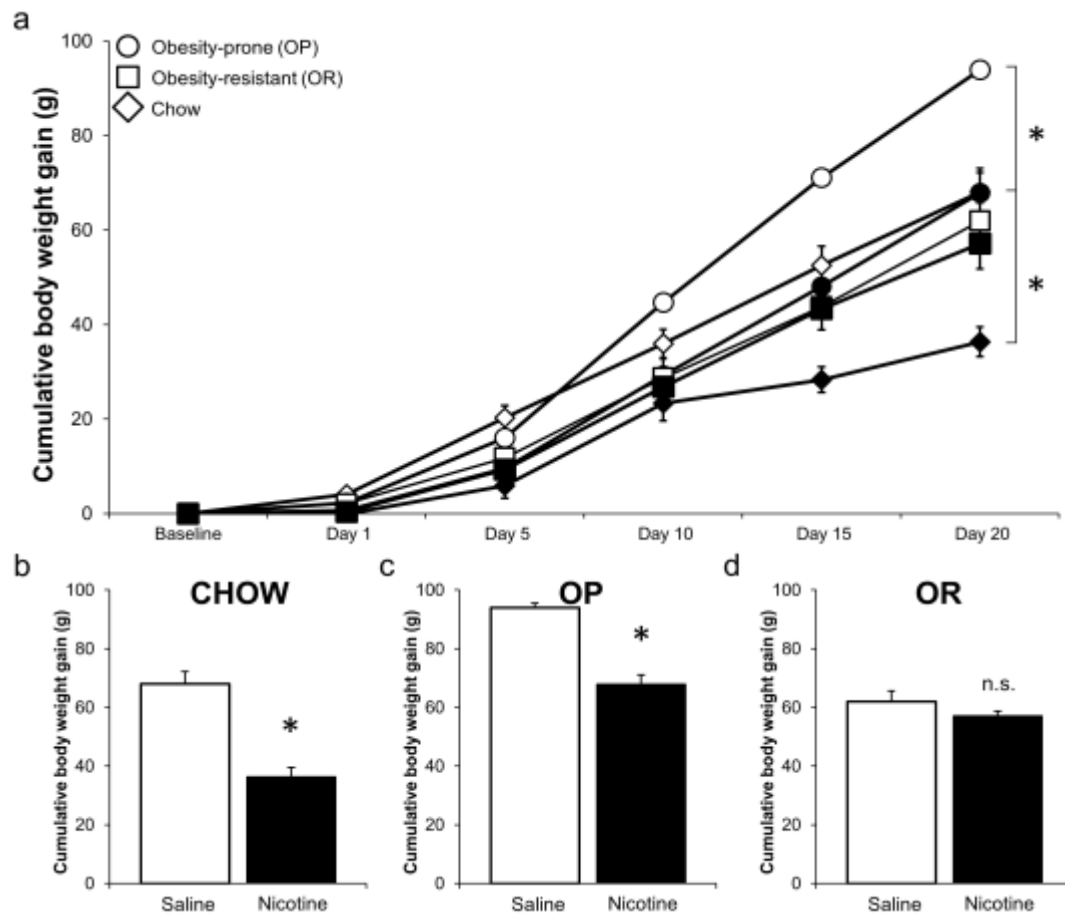


Figure 16. Self-administered nicotine suppressed body weight gain in OP and chow-fed rats.

Self-administered nicotine (60 $\mu\text{g/kg/infusion}$) suppressed body weight gain in OP and Chow rats, but not OR rats (a). Open symbols indicates saline, and filled indicate nicotine groups. For clarity, bar graphs demonstrate suppression of body weight gain in Chow (b), OP (c), and lack of suppression in OR (d) groups after 20 days of self-administration. Data expressed as means \pm SEM. * indicate $p < 0.05$, between 0 and 60 $\mu\text{g/kg/infusion}$ nicotine within phenotype group.

Self-administered nicotine had no impact on food intake.

There was no significant effect of self-administered nicotine on 24h food intake at any measurement, analyzed as kcal as a percentage of BW (Figure 17 shows data from the final 24h of the experiment only, for clarity). There was no effect of day, phenotype, drug, or interaction term. There was no impact of drug on any day when tested within phenotype. Food intake data were transformed to correct for BW as caloric consumption increased significantly in the saline groups over the 20 days of experimentation as BW increased ($p = 0.001$), and to control for potential between subject differences. When food intake was expressed as kcal, there was a significant effect of day ($p = 0.005$), but no significant effect of drug, phenotype, or interaction.

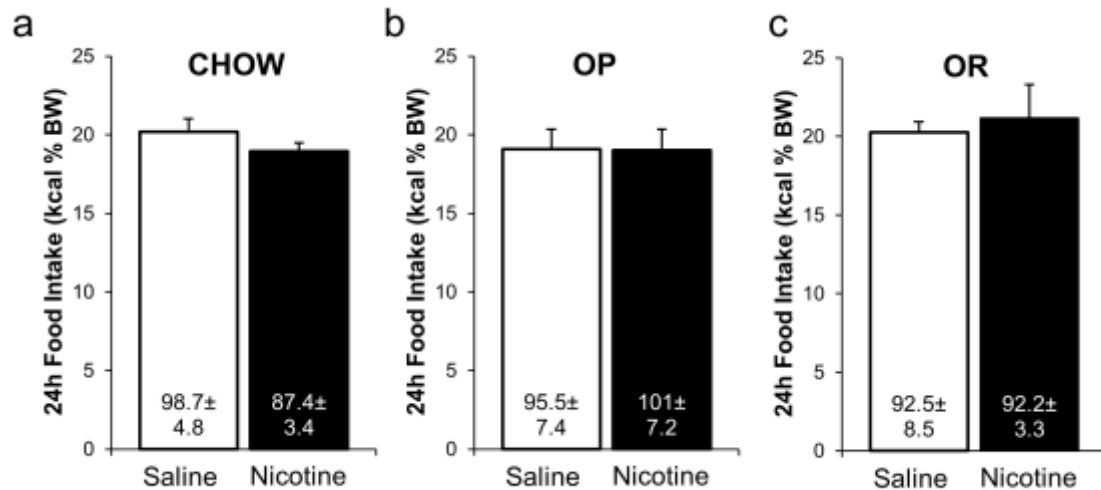


Figure 17. Self-administered nicotine does not impact food intake.

Self-administered nicotine (60 μ g/kg/infusion) did not impact 24h food intake, expressed as kcal as a percentage of BW to account for the between subjects design of the experiment, in Chow (a), OP (b), or OR (c) rats after 20 days of self-administration, when nicotine intake was maximal in all groups. Data expressed as means \pm SEM. Values within each bar are mean 24h kcal consumed on Day 20 of the experiment \pm SEM.

5.4 DISCUSSION

The current data are the first to evaluate the impact of self-administered nicotine on BW in a model of human diet-induced obesity and demonstrate that rats resistant to the development of obesity are resistant to nicotine-induced suppression of BW gain. Many smokers cite weight regulation as a primary reason for smoking initiation and the ability to quit (Audrain-McGovern et al., 2011). However, these data suggest that among smokers consuming a typical Westernized diet, a subset may be resistant to nicotine-induced weight-suppression. Therefore, expectations of weight suppression among many weight-concerned smokers may be unfounded.

In the current data, suppression of BW gain by nicotine in OP and Chow rats occurred independent of food intake, replicating previous results (Rupprecht et al., 2016) and aligning with feeding data from human smokers. We have previously demonstrated that nicotine intake is negatively correlated with BW gain in adult male rats fed standard rodent chow (Rupprecht et al., 2016). The magnitude of weight reduction was greater in Chow than OP rats, possibly due to higher total nicotine consumption in Chow rats. Importantly, the lack of BW suppression seen in OR rats was not accompanied by a compensatory increase in food intake. Weight gain in OP nicotine rats was comparable to both lean (Chow and OR saline groups), suggesting that nicotine exposure may prevent the development of obesity. This aligns with a report in humans demonstrating that obese smokers lose weight compared to normal weight smokers during smoking (Veldheer et al., 2015). It is unclear why rats resistant to the development of obesity are also resistant to the weight-suppressive effects of nicotine. It is possible that nicotine acts to

suppress BW through similar mechanisms as those resulting in the anti-obesity phenotype in OR rats. Therefore, in OR rats, nicotine cannot act to potentiate reduced weight gain. Furthering our understanding of nicotine-induced BW suppression may lead to insights on resistance to diet-induced obesity.

The notion that OP and OR rats have differential responses to psychostimulants is not new. Selectively bred OP rats are more sensitive to the anorectic effects of D-amphetamine compared to selectively bred OR rats when fed chow, but this difference is occluded with HED maintenance (Valenza et al., 2015). Similarly, chow-maintained selectively bred OP rats are more sensitive to cocaine-induced locomotor sensitization than chow-maintained OR rats (Oginsky et al., 2015; Vollbrecht et al., 2015), but this difference is not present in outbred HED-maintained OP and OR rats (Oginsky et al., 2015). It is possible that in the current experiments, OP rats were more sensitive to the locomotor effects of nicotine, potentially contributing to the suppression of BW. However, previous reports suggest that the differential effect of psychostimulants on behaviors related to BW regulation is blocked with HED-maintenance, which is at odds with the current data.

The current experiment was not designed to test whether the impact of self-administered nicotine on BW regulation pre-exists a manipulation of diet, although it provides some clues. It is likely that the extremes of HED-induced BW gain within the Chow group are more OP-like or OR-like. If the impact of nicotine on BW relies solely on the polygenetic predisposition to develop obesity, and not the combination of genetic and environmental factors, then rats that gained the least weight on HED in the Chow group should be resistant to the weight-suppressive effects of nicotine. However, there was no relationship between weight gain during HED maintenance and the impact of nicotine on weight gain in the Chow group (data not shown). This

suggests that resistance to nicotine-induced BW suppression requires consumption of a densely caloric diet. Examining the impact of nicotine on body weight regulation in selectively bred obesity-prone and –resistant rats would remove this confound.

The weight gain in saline and nicotine Chow groups reported here is similar to what is expected in chow-fed rats without previous HED consumption (Rupprecht et al., 2016), though the exclusion of a chow group without HED exposure in the current design limits our interpretation. It is possible that prior maintenance on HED could impact weight gain when returned to chow. Future work may include a group that remains HED-naïve as a control, or utilize selectively bred obesity-prone and –resistant rats, though these rats are no longer commercially available.

Self-administered nicotine in extended access 23-h sessions has been demonstrated to suppress responding for chow pellets (Bunney et al., 2015; O'Dell et al., 2007), but not responding for sucrose (Bunney et al., 2015). Smokers experience intermittent increases in blood and brain nicotine levels, which can be modeled using 1-h or 23-h access protocols. There are several advantages to the use of 1-h nicotine self-administration sessions in the study of body weight regulation (Rupprecht et al., 2016). The robust impact of nicotine on body weight when self-administered over 1-h provides some clues about potential mechanisms of action by which nicotine acts to suppress body weight. As the half-life of nicotine is about one hour in the rat (Adir et al., 1976), a metabolite of nicotine with a half-life lasting several hours, such as cotinine, may act to suppress body weight. Alternatively, nicotine may activate downstream pathways that have long lasting effects on body weight regulation, such as increased brown adipose tissue activity.

Nicotine consumption was higher in Chow rats compared to rats maintained on HED, independent of susceptibility to diet-induced obesity, which may suggest that HED-exposure reduces drug-seeking behaviors. One report supports this idea, demonstrating that HED-exposure impairs cocaine-seeking behaviors (Wellman et al., 2007). In contrast, it is possible that a prior exposure to HED may increase drug-seeking behaviors. Early life (Morganstern et al., 2013) and unpredictable (Puhl et al., 2011) exposure to high-energy diets have been shown to increase drug-seeking when switched to chow. Therefore, it is possible that the behavior observed in the current Chow group is increased in comparison to HED-groups due to the short HED-exposure period. Nevertheless, these self-administration data are at odds with the observation that smokers with obesity smoke more cigarettes per day than normal weight smokers (Veldheer et al., 2015). Future experiments testing the impact of obesity on self-administration across a full dose response curve may provide more insight to this issue.

The results of this experiment provide new insight into the understanding of the interaction between nicotine and obesity and demonstrate that: 1) obesity-resistant rats are also resistant to nicotine-induced suppression of body weight gain; and 2) nicotine may prevent or reduce levels of obesity in a subset of smokers. These data highlight the importance of considering obesity-prone and –resistant rats as separate populations and suggest that expectations of weight regulation by smoking may be unfounded in many weight-concerned smokers.

6.0 NICOTINE CONSUMPTION DEPENDS ON BODY WEIGHT IN RATS AND HUMAN SMOKERS

6.1 INTRODUCTION

Obesity and smoking represent the largest challenges to public health and the negative health impact of the combination of obesity and smoking may synergistically increase the risk for morbidity and mortality (Peeters et al., 2003; Perkins, 1989; Stewart et al., 2009). Obese smokers smoke significantly more cigarettes each day than non-obese smokers (Chiolero et al., 2008; Chiolero et al., 2007a; John et al., 2005a), and obesity may increase the risk for smoking (Chatkin et al., 2010). Some evidence suggests that obese smokers have higher levels of nicotine dependence (Hussaini et al., 2011). Therefore, it is possible that obesity increases susceptibility to smoking.

Large reductions in the nicotine content of cigarettes may improve the public health burden of smoking, a strategy that is being considered worldwide (Benowitz et al., 1994, 2013; Hatsukami et al., 2013). Evidence from human smokers suggests that smoking very low nicotine content (VLNC) cigarettes containing 2.4 mg of nicotine/g of tobacco reduces smoking (Donny et al., 2015). This reduction in cigarettes smoked per day with VLNC cigarettes is accompanied by reductions in nicotine exposure, dependence, withdrawal, and may increase quitting (Donny et al., 2015). Similarly, a threshold for nicotine reinforcement has been shown to

exist in rats; self-administration behavior is not acquired or maintained at 3.75 µg/kg/infusion nicotine and below (Smith et al., 2013; Smith et al., 2014), validating rat self-administration procedures for the study of nicotine reduction. Obese smokers have higher ratings of liking and satisfaction for VLNC cigarettes (Blendy et al., 2005) and may represent a subpopulation of smokers at risk for continued smoking following large reductions of nicotine content in cigarettes (Rupprecht et al., 2015a).

The present experiments examined the impact of obesity on smoking and nicotine consumption. Experiments tested: 1) the impact of body mass index (BMI) on smoking behavior and associated subjective measures before and after randomization to cigarettes of varying nicotine content; and 2) nicotine self-administration in a rat model of obesity, across a range doses. Data provide evidence that daily nicotine consumption is titrated dependent upon body weight, and have important implications for tobacco regulatory policy, as well as treatment strategies for smoking cessation.

6.2 METHODS

Clinical trial

Design

A secondary analysis from a completed clinical trial was performed. A seven-group, double-blind, randomized trial was conducted at 10 sites in the United States. The trial included a screening visit, a 2-week baseline period during which participants smoked their own usual brand cigarettes, and a 6-week investigational cigarette use period. During the 6-week experimental period, participants were provided with one of seven types of cigarettes varying in

nicotine content (mg nicotine per g of tobacco): 0.4 mg/g; 0.4 mg/g high tar (HT); 1.3 mg/g; 2.4 mg/g; 5.2 mg/g; 15.8 mg/g, and usual brand (UB). Average tar yields were 8 to 10 mg; however, for the high tar cigarettes it was 13 mg. The 0.4 HT condition, which contained tobacco filler with the same nicotine content, but differed from 0.4 mg/g cigarettes in filter and ventilation resulting in higher yield (ISO) of tar and nicotine, was added to the design to explore the impact of tar yield on the use and acceptability of VLNC cigarettes. A two-week supply of cigarettes was provided free of charge at each weekly session during the experimental period. During this time, participants were instructed to smoke only the provided investigational cigarettes and received counseling aimed to increase compliance, though there was no penalty for using other nicotine/tobacco products. Study design is described in greater detail in the primary study manuscript (Donny et al., 2015). The study was approved by the Institutional Review Board at each study site and was reviewed by the FDA Center for Tobacco Products.

Participants

Adult daily smokers (n=840) were recruited using flyers, direct mailings, television and radio, and other advertisements between 2013 and 2014. Inclusion criteria included: at least 18 years of age, at least five cigarettes smoked per day, expired carbon monoxide (CO) greater than 8 ppm or urinary cotinine greater than 100 ng/ml. Exclusion criteria were: intention to quit smoking in the next 30 days; use of other tobacco products on more than 9 of the past 30 days; serious psychiatric or medical condition; positive toxicological screen for illicit drug use other than cannabis; pregnancy, plans to become pregnant, or breastfeeding; and exclusive use of “roll your own” cigarettes. All 839 eligible participants provided written informed consent prior to enrollment.

Study Assessments & Laboratory Analyses

Height and weight measurements were collected at the screening visit, which was used to calculate BMI. The average number of cigarettes smoked per day during week 6 was assessed daily using an interactive telephone voice-response system (InterVision Media), which prompted participants to report the number of cigarettes smoked on the previous day. Body weight was measured to the nearest 0.1 kg during each visit to the laboratory, which was used to calculate cigarettes smoked each day per body mass at each time point. The Fagerstrom Test for Nicotine Dependence, Wisconsin Inventory of Smoking Dependence Motives, Minnesota Nicotine Withdrawal Scale, and the 10-item Questionnaire on Smoking Motives were administered during the second baseline and week 6 laboratory visits.

Urinary total nicotine equivalents (TNE), the sum of nicotine and its metabolites and a measure of daily nicotine exposure, were analyzed by liquid chromatography tandem mass spectrometry (Carmella et al., 2013; Murphy et al., 2014; Murphy et al., 2013). Saliva samples for the assessment of nicotine metabolite ratio (NMR), an indicator of CYP2A6 activity and the rate of nicotine metabolism, were collected during the second baseline session (Donny et al., 2015).

Rat experiments:

Subjects

Experiments were conducted using two different rat populations, described in detail below. Upon arrival, rats were housed individually in ventilated tub cages on a reverse light-dark

12:12 hr cycle (lights off at 0700h) in a temperature-controlled facility (between 70 and 74 °F) and had free access to high energy diet (HED; Research Diets D12266B, New Brunswick, NJ; 31.8% kcal from fat, 4.41 kcal/g) or standard rodent chow and water, unless noted otherwise. All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Outbred rat experiments

Male Sprague-Dawley rats (Charles River, Kingston, NY) weighing 275-300 g were fed HED upon arrival. To classify body weight phenotype groups, body weight was monitored daily while all rats had free access to HED for two weeks. At the end of this two-week period, the top third with the highest weight gain was assigned to Obesity-Prone (OP), and the bottom third with the lowest weight gain were assigned to Obesity-Resistant (OR) (B. E. Levin, 1993; Rupprecht et al., 2017). Rats were maintained on HED throughout behavioral procedures unless noted otherwise.

Selectively bred experiments

Male Sprague-Dawley rats selectively bred for obesity-prone (OP) and obesity-resistance (OR) were shipped to the University of Pittsburgh from the University of Michigan (kindly provided by Dr. Carrie Ferrario, this line originated from selectively bred diet-induced obese and diet resistant rats from Dr. Barry Levin. Upon arrival, rats were assigned to HED or chow groups, counterbalanced by body weight within phenotype group. Rats were quarantined for 4.5 weeks before surgical procedures.

Drugs

Nicotine hydrogen tartrate salt (Sigma, St. Louis, MO) was dissolved in 0.9% saline; doses are expressed as free base.

Procedures

Surgery

Rats were anesthetized with isoflurane (2-3% in 100% O₂) and implanted with catheters into the right jugular vein (Smith et al., 2013; Smith et al., 2014). Rats were allowed to recover for 5-6 days before self-administration procedures, during which catheters were flushed daily with 0.1 ml heparinized saline (30 U/ml) containing Timentin (66.67 mg/ml), or gentamicin (1 mg), depending on drug availability at the time of experiments, and streptokinase (8,333 U/ml). Catheters were flushed with 0.1 ml heparinized saline (10 U/ml) and heparinized saline (30 U/ml) containing timentin or gentamicin prior to and following the self-administration sessions, respectively.

Self-administration

Self-administration occurred in operant chambers (Med-Associates) enclosed in sound attenuating chambers (Rupprecht et al., 2016; Smith et al., 2015). One nose-poke hole was assigned as active, resulting in the delivery of an i.v. infusion after fulfilling the response requirement. The other nose-poke hole was inactive; responses at this nose-poke portal were recorded but had no consequence. Active and inactive nose-poke holes were randomly assigned

(left or right). Infusions were delivered after completion of the reinforcement schedule, as detailed below. Infusions were accompanied by a 15-sec cue light illuminated above the active nose-poke portal and an unsignaled 1-min timeout. Self-administration occurred in 1-h daily sessions, seven days per week, 2-3 h following the onset of the dark cycle. Unless noted otherwise, infusion duration was adjusted daily to account for body weight, thereby adjusting nicotine dose to body weight. Rats included in analyses passed a patency test, which required displaying signs of ataxia within 5-s of intravenous injections of methohexital (5 mg/kg).

The impact of obesity on nicotine reinforcement, across a range of doses

Outbred OP and OR maintained on HED (n=20 per group) self-administered 60 $\mu\text{g/kg/infusion}$ nicotine on an FR2 schedule of reinforcement, until rats reached stable behavior (infusions taken within 5% of the previous day for three consecutive days). Thereafter, schedule was increased to FR5, and dose was halved every 7 days to 1.875 $\mu\text{g/kg/infusion}$, and then saline. Final sample size following patency testing is as follows: OP, n=14; OR, n=14. Average body weights on the first day of self-administration were OP: 403.9 ± 11.9 g; OR: 363.8 ± 16.4 g.

A separate group of outbred OP and OR rats (n= 11/group) self-administered 60 $\mu\text{g/kg/infusion}$ nicotine on an FR2 for 14 days before the schedule was changed to a PR (1, 3, 6, 10, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 179, 219, 268, 328, 402, 492). Each dose (60, 15, 7.5, 0 $\mu\text{g/kg/infusion}$ nicotine) was experienced for 4 consecutive days in 4-h sessions. Final sample size following patency testing is as follows: OP, n=11; OR, n=10. Average body weights on the first day of self-administration were OP: 430.5 ± 10.5 g; OR: 388.6 ± 13.2 g.

The impact of diet on nicotine self-administration in OP and OR rats

To test whether increased self-administration of low doses of nicotine relies on pre-existing genetic factors in OR rats or the combination of genetic and environmental factors, nicotine self-administration was tested across three phases of diet exposure. In Phase 1 (Chow), a group of outbred rats fed standard rodent chow ($n = 60$) acquired stable responding for 60 $\mu\text{g/kg}$ /infusion nicotine on an FR2 schedule of reinforcement, before the schedule was increased to FR5. Rats responded for 60, 7.5, and 0 for five consecutive days each. Body weight on day 1 of Phase 1 were: OP: 379.2 ± 25.8 g; OR: 366.4 ± 18.0 g. Following the final day self-administration in Phase 1, all rats were fed HED for 2 weeks, and assigned to OP or OR, as described above. In Phase 2 (HED), the self-administration procedures were repeated. On day 1 of Phase 2, body weights were OP: 550.1 ± 40.1 g; OR: 513.9 ± 49.2 g. Following the final day of Phase 2, all rats were returned to standard rodent chow, and Phase 3 (Chow) tested whether maintenance on HED was required for changes in self-administration. On day 1 of Phase 3, body weights were OP: 640.8 ± 60.3 g; OR: 575 ± 52.2 g. Final sample size following patency testing is as follows: OP, $n=15$; OR, $n=10$.

In a separate experiment, selectively bred OP and OR rats fed chow or HED ($n=8$ per group) self-administered 60 $\mu\text{g/kg}$ /infusion nicotine on an FR2 schedule of reinforcement, until rats reached stable behavior. Thereafter, schedule was increased to FR5, and dose was halved every 7 days to 3.75 $\mu\text{g/kg}$ /infusion, and then saline. Final sample size following patency testing is as follows: OP chow, $n=7$; OP HED, $n=5$; OR chow, $n=6$; OR HED, $n=4$. Average body weights on the first day of self-administration were Prone chow: 493.4 ± 48.4 g; Prone HED: 571.6 ± 49.1 ; Resistant chow: 456.9 ± 38.5 ; Resistant HED: 483.1 ± 27.4 g.

The impact of body weight on unit dose self-administration

In the experiments above, as is typical in drug self-administration experiments, drug dose was adjusted to account daily changes in body weight. In contrast, in human smokers, each cigarette consumed per day is not corrected for body weight, and one cigarette is considered one unit of consumption or reinforcement. Therefore, to test whether body weight impacts self-administration of unit dose nicotine (with one infusion as one unit), without adjusting dose by body weight, outbred OP and OR rats ($n = 11$ per group) self-administered unit dose nicotine, where the infusion duration was held constant based upon a 400 g rat (based on chow-fed rats of the same age). Rats initially responded for 24 $\mu\text{g}/\text{infusion}$, the equivalent of 60 $\mu\text{g}/\text{infusion}$ for a 400 g rat, on an FR2 schedule of reinforcement until stable behavior was reached. Thereafter, dose was halved every 5 days seven times, and then followed by saline. For consistency and easy comparison to other experiments, doses in this experiment will be reported as the comparable $\mu\text{g}/\text{kg}/\text{infusion}$ based on a 400 g rat, referred to as “Unit Dose”. Final sample size following patency testing is as follows: OP, $n=8$; OR, $n=10$. Body weights on the first day of self-administration were OP: 508.8 ± 18.0 g; OR: 448.0 ± 10.0 g.

Locomotor activity and non-drug reinforced responding

To test whether there are existing differences in locomotor behavior that may impact self-administration behavior, outbred OP and OR rats ($n = 13/\text{group}$) were placed in a novel open field apparatus for 30 minutes and locomotor activity was measured.

A separate group of OP and OR rats ($n = 12/\text{group}$) learned to respond for mildly reinforcing visual stimulus (VS; 1-sec stimulus light cue and 60-sec offset of the houselight) presentations on an FR2 schedule of reinforcement.

Statistics

Statistical analyses were performed using SPSS. For clinical trial analyses, the two normal nicotine conditions (15.8 mg/g and usual brand) were combined and compared to four VLNC conditions (2.4 – 0.4 mg/g), as these conditions had similar effects on cigarettes smoked per day and nicotine exposure in the overall sample (Donny et al., 2015; Tidey et al., 2017). The 5.2 mg/g condition was excluded from analyses because its effects on cigarettes smoked per day and nicotine exposure in the overall sample were mixed. Effects of BMI on outcome measures at baseline and interaction between BMI and cigarette nicotine content on outcome measures at post-randomization week 6 were analyzed by general linear model regression. Analyses from baseline adjusted for age, race, and gender. Post-randomization analyses adjusted for age, race, gender, and the variable of interest.

In rat experiments, comparisons between phenotype, dose, and diet were analyzed by mixed-design and repeated measures ANOVA tests. In each experiment, an average of the infusions taken on last 2 or 3 self-administration sessions at each dose was used for statistical tests. Planned comparisons between drug groups within each phenotype were analyzed using one-way ANOVA. The data were Greenhouse-Geisser corrected where Mauchly's Sphericity tests were significant. The α -level for all tests was set at 0.05.

6.3 RESULTS

Human experiments

Characteristics of participants by BMI are listed in Table 4.

Table 4. Characteristics of participants by BMI.

	Under & Normal Weight (BMI < 25)	Overweight (BMI: 25 – 29.9)	Obese (BMI > 30)
Sample size (%)	252 (30.0)	243 (29.3)	335 (39.9)
BMI	22.17 ± 1.93 (16.86 - 24.84)	27.23 ± 1.37 (25.06 -29.84)	36.46 ± 6.80 (30.00 - 81.73)
Weight (kg)	65.99 ± 9.27 (40.6 - 88.9)	81.15 ± 8.77 (59.4 - 111.0)	104.16 ± 20.20 (65.9 - 177.3)
Age	38.85 ± 14.86 (18-71)	42.53 ± 12.7 (18 - 68)	43.12 ± 11.77 (19 -74)
Gender (%)			
Male	159 (63.1)	164 (67.5)	152 (45.4)
Female	93 (36.9)	79 (32.5)	183 (54.6)
Race (%)			
White	157 (62.3)	151 (62.1)	156 (46.6)
Black/African American	85 (33.7)	89 (36.6)	166 (49.6)
American Indian/Alaskan Native	10 (4.0)	7 (2.9)	20 (6.0)
Asian	9 (3.6)	3 (1.2)	5 (1.5)
Native Hawaiian/Pacific Islander	2 (0.8)	4 (1.6)	3 (0.9)
Other	7 (2.8)	7 (2.9)	15 (4.5)

Baseline: obese smokers smoke more cigarettes per day, but have lower nicotine exposure

At baseline, obese smokers smoked significantly more cigarettes per day than normal weight smokers ($p = 0.002$; Figure 18a). As we hypothesize that body mass is a determinant of smoking behavior, cigarettes smoked per day as a function of body weight (kg) was analyzed. Cigarettes smoked per day as a function of body weight, normal weight smokers consumed significantly more than overweight and normal weight smokers ($p < 0.001$; Figure 18b). This pattern was reflected in total nicotine equivalents (Figure 18c). There was a significant effect of BMI on total nicotine equivalents ($p = 0.027$). There was no significant impact of BMI status on nicotine metabolite ratio ($p = 0.466$; Figure 18d) or expired carbon monoxide ($p = 0.713$; Figure 18e).

There were no differences in nicotine dependence by BMI status, as measured using the WISDM ($p = 0.546$; Figure 19b) and the FTND ($p = 0.570$; Figure 19c) scales. There was no impact of BMI on ratings of craving, using the Questionnaire on Smoking Motives ($p = 0.532$; Figure 20a) or withdrawal, using the Minnesota Nicotine Withdrawal Scale ($p = 0.760$; Figure 20b).

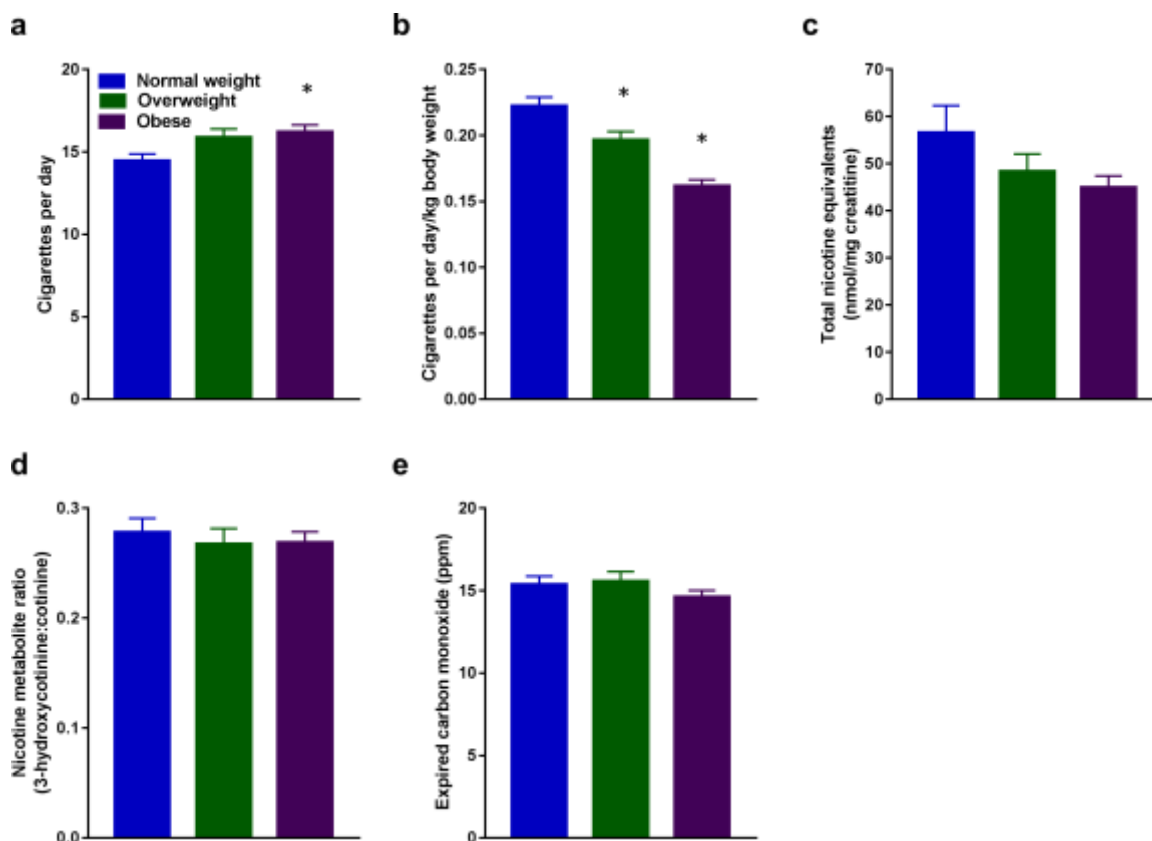


Figure 18. Smoking related behaviors by BMI at baseline, while smoking usual brand cigarettes.

Cigarettes smoked per day is significantly higher in obese smokers compared to normal weight smokers (a). As body weight may be a determinant of smoking behavior, cigarettes per day as a function of body weight was evaluated (b). Obese and overweight smokers consumed significantly fewer cigarettes per day as a function of body weight compared to normal weight smokers. This pattern was reflected in total nicotine equivalents, a urinary measure of nicotine exposure (c). There was a significant impact of BMI on total nicotine equivalents. There was no impact of BMI on nicotine metabolite ratio (d) or expired carbon monoxide (e). * indicates $p < 0.05$, compared to normal weight smokers.

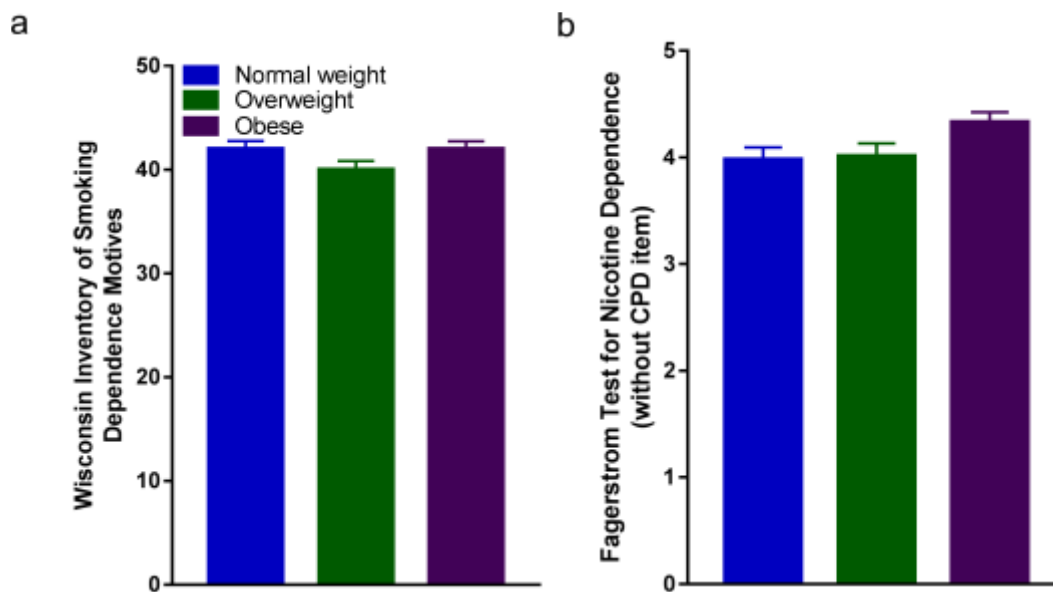


Figure 19. Measures of dependence by BMI at baseline, when smoking usual brand cigarettes.

There was no impact of BMI on nicotine dependence, as measured by the Wisconsin Inventory of Smoking Dependence Motives (a; 37-item questionnaire, 11-77 scale) or the Fagerstrom Test for Nicotine Dependence, with the cigarettes per day item removed (b).

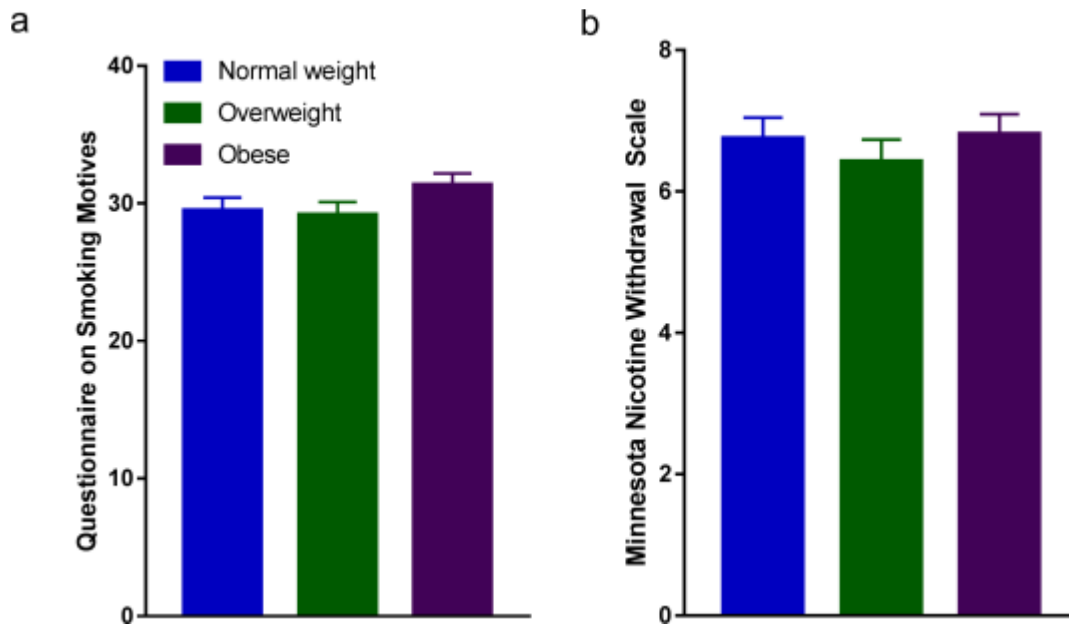


Figure 20. Measures of craving and withdrawal by BMI at baseline, when smoking usual brand cigarettes.

There was no impact of BMI on nicotine craving, measured by the Questionnaire on Smoking Motives (a) or withdrawal, measured by the Minnesota Nicotine Withdrawal Scale (b).

VLNC cigarettes reduced cigarette consumption, exposure, and subjective measures of dependence and craving

At week 6, cigarettes smoked per day was significantly reduced by VLNC use ($p < 0.001$). There was no effect of BMI ($p = 0.263$), and a significant interaction ($p = 0.032$) on cigarettes smoked per day (Figure 21a). Within the VLNC cigarette group, obese smokers smoked significantly more cigarettes per day compared to normal weight smokers ($p = 0.012$). VLNC use significantly suppressed cigarettes smoked per day as a function of body weight ($p < 0.001$). There was no significant effect of BMI ($p = 0.210$) and a significant interaction ($p = 0.030$; Figure 21b). There was a significant reduction in TNE in the VLNC group ($p < 0.001$), but no significant effect of BMI ($p = 0.333$) or interaction ($p = 0.271$). There was a significant effect of cigarette nicotine content ($ps < 0.001$), but not BMI and no significant interaction on Measures of dependence, withdrawal, or craving.

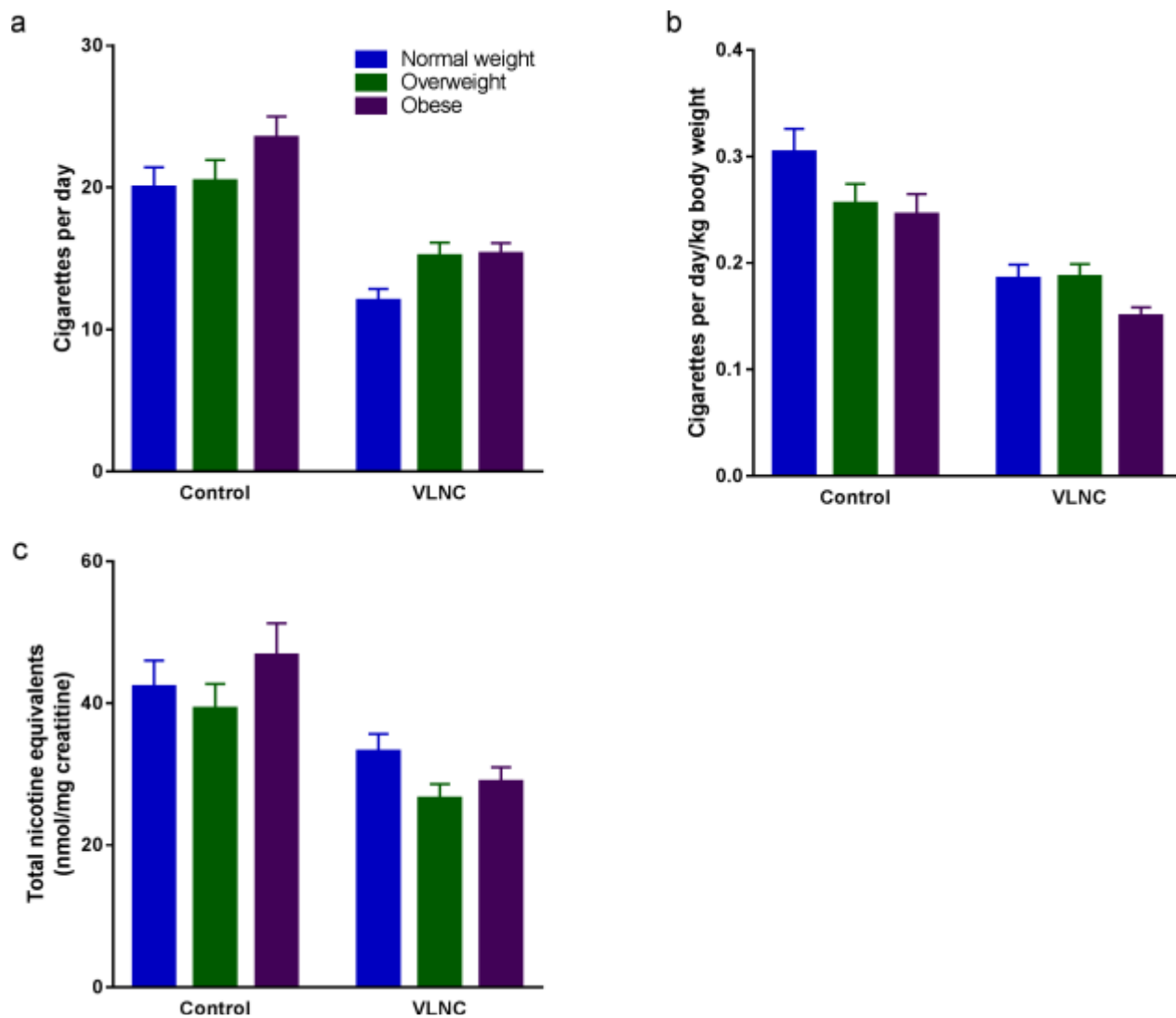


Figure 21. Smoking and nicotine exposure at week 6.

VLNC cigarettes reduced cigarettes smoked per day (a), cigarettes smoked per day as a function of body weight (b), and nicotine exposure (c) across BMI groups.

Rat experiments

Resistance to diet-induced obesity increases low dose nicotine self-administration

Outbred OR rats took significantly more infusions compared to OP across a range of doses on an FR5 schedule of reinforcement (Figure 22a). There was a significant effect of dose ($p < 0.001$), phenotype ($p = 0.041$), and interaction ($p = 0.022$). OR rats took more infusions at all doses of nicotine compared to saline, within subject ($ps < 0.001$). In OP rats, the number of infusions taken at 1.875 $\mu\text{g/kg/infusion}$ was not significantly higher than saline ($p = 0.230$). All other doses were self-administered at a higher rate compared to saline in OP rats ($ps < 0.005$). Active responses were significantly higher than inactive responses at all doses above saline for the OR group and above 3.75 $\mu\text{g/kg/infusion}$ nicotine for the OP group. There were no differences between groups in inactive responding (ranging on average between 3 and 15 responses, across doses). There were no differences between in nicotine consumption (mg) across doses.

In a separate group of rats, OP rats took more infusions of nicotine on a PR schedule of reinforcement (Figure 22b). There was a significant effect of dose ($p < 0.001$), phenotype ($p = 0.047$), but no interaction ($p = 0.356$). Post-hoc analyses demonstrated that OR rats responded significantly more for 15 $\mu\text{g/kg/infusion}$ nicotine for the OP group.

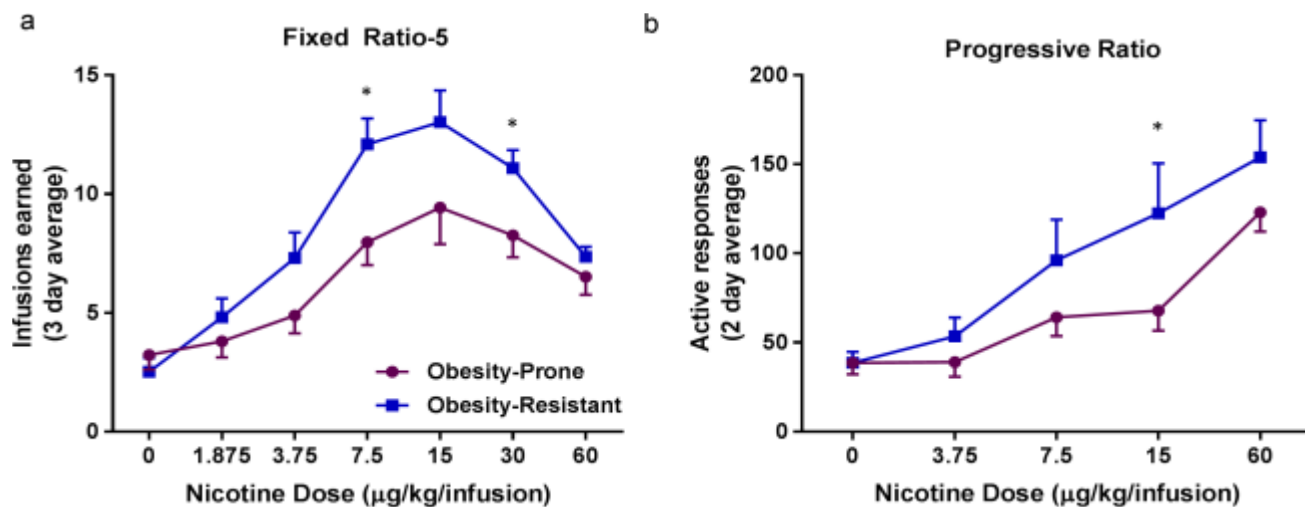


Figure 22. Nicotine self-administration in obesity-prone and obesity-resistant rats fed HED.

Obesity-resistant rats self-administered higher numbers of infusions on a fixed-ratio schedule of reinforcement (a). In a separate group of rats, obesity-resistant rats responded more for nicotine infusions on a progressive ratio schedule of reinforcement (b). * is $p < 0.05$ between phenotype groups at a specific nicotine dose.

Increased low dose nicotine self-administration in OR rats requires HED exposure

HED consumption increased nicotine self-administration in OR, but not OP rats (Figure 23). In outbred OP and OR rats naive to HED (Phase 1), there was no impact of phenotype on nicotine self-administration ($p = 0.415$; Figure 23a). Consumption of HED (Phase 2) increased self-administration in OR rats at 7.5 and 60 $\mu\text{g/kg/infusion}$ nicotine, compared to OP rats (Figure 23b), and within-subject to Phase 1 ($p < 0.007$). Self-administration in OR rats remained high following the removal of HED and replacement with chow ($p = 0.027$; Figure 23c).

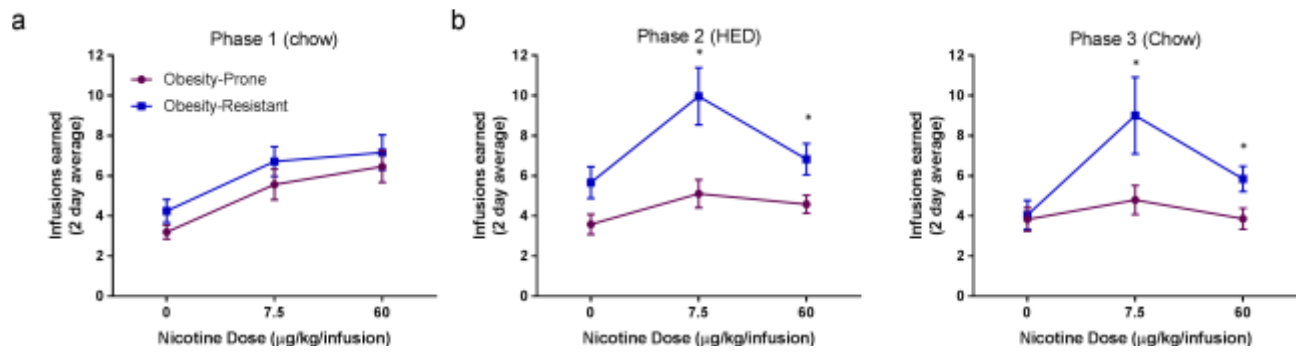


Figure 23. Enhanced nicotine self-administration in obesity-resistant rats requires HED exposure.

There were no differences in nicotine self-administration between obesity-prone and obesity-resistant groups when fed chow, before exposure to HED (a). Maintenance on HED significantly increased nicotine self-administration in obesity-resistant rats only (b). Obesity-resistant rats had increased self-administration following removal of HED and maintenance on chow (c). *

indicates $p < 0.05$ between groups at a specific nicotine dose.

The sample size in experiments using selectively bred rats is insufficient for statistical tests due to Power analyses, but the data are informative. Therefore, a descriptive analysis of data follows. Maintenance on HED increased self-administration in selectively-bred OR rats (Figure 24). At 7.5 $\mu\text{g/kg/infusion}$ nicotine, OR HED rats self-administered more infusions than OR chow rats at trend levels, as revealed by independent samples t-test ($p = 0.051$). In selectively-bred OR rats fed chow, self-administration was similar to OP groups. There was no impact of diet on nicotine self-administration in selectively bred OP rats.

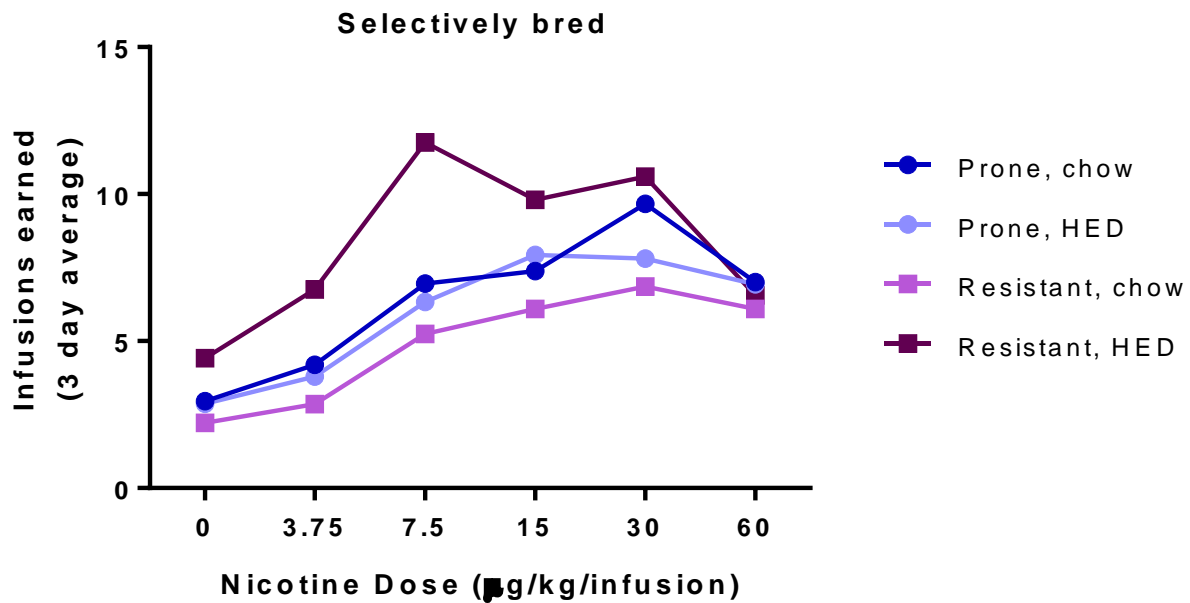


Figure 24. Nicotine self-administration across a range of doses in selectively bred obesity-prone and obesity-resistant rats.

Low dose self-administration was enhanced in obesity-resistant rats fed HED, compared to all other groups. There was no impact of diet on obesity-prone rats, and there was no impact of phenotype on self-administration in chow fed rats.

Obesity increases nicotine self-administration as unit dose

When nicotine infusions were not corrected for body weight, outbred OP rats self-administered significantly more infusions than OR rats, across a range of doses. (Figure 25). There was a significant effect of dose ($p < 0.001$), but not phenotype ($p = 0.132$) or interaction ($p = 0.213$). Post-hoc analyses revealed that OP rats self-administered significantly more infusions at 60, 30, and 15 unit dose/infusion. There were no differences in nicotine consumption (mg)/kg body weight between groups.

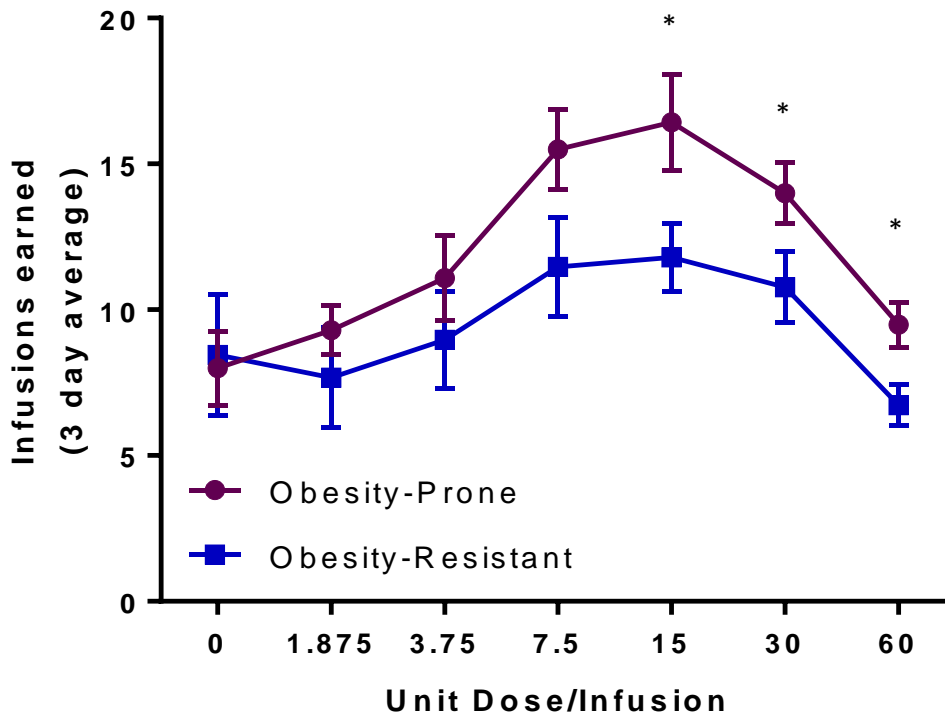


Figure 25. Nicotine self-administration in obesity-prone and –resistant rats as unit dose/infusion.

Obesity-prone and –resistant rats fed HED responded for nicotine infusions, when infusion duration was based upon a 400 g rat, and was stable for the entire experiment. Obesity-prone self-administered more infusions than obesity-resistant. * indicates $p < 0.05$ between phenotype groups at a specific nicotine dose.

Obesity does not impact locomotor activity or VS responding.

There was no impact of phenotype on locomotor activity ($p = 0.368$; Figure 26a).

There was no impact of phenotype on VS presentations earned ($p = 0.536$; Figure 26b).

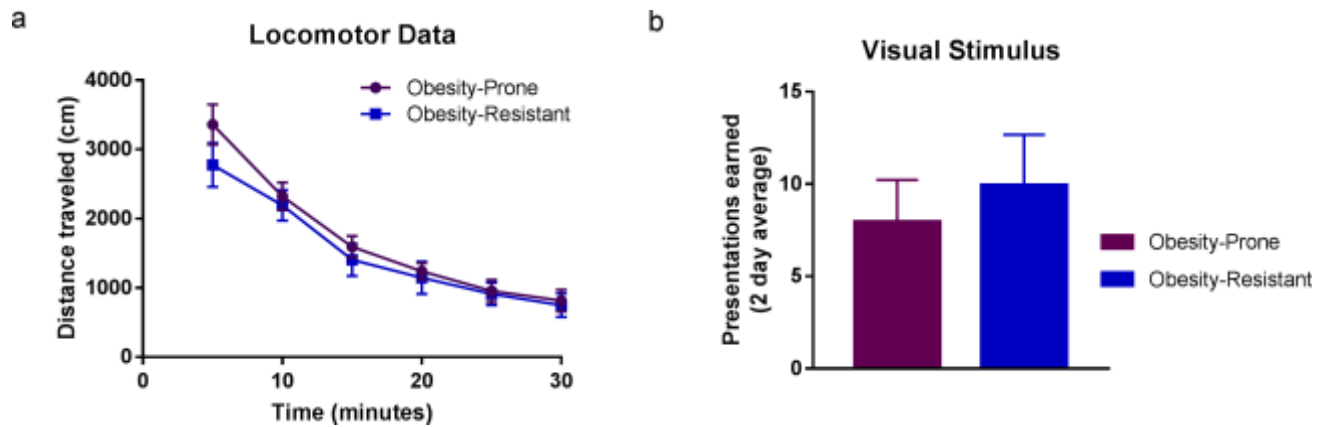


Figure 26. Locomotor activity and non-drug reinforced responding in obesity-prone and -resistant rats

There was no impact of phenotype on locomotor behavior in a novel open field (a) or on responding for mildly reinforcing visual stimulus presentations (b).

6.4 DISCUSSION

The implementation of product standards requiring substantial reductions of nicotine content in cigarettes is hypothesized to improve public health by reducing smoking and facilitating quitting (Benowitz et al., 2013; Donny et al., 2012; Hatsukami et al., 2013). Evidence suggests that smokers with obesity may be at risk for continued smoking following mandated reductions in nicotine content in cigarettes (Rupprecht et al., 2015a). The current investigation found that smokers with obesity smoke more cigarettes per day, but consume fewer cigarettes per day as a function of body mass. Nicotine exposure was low in obese smokers compared to normal weight smokers. Likewise, obesity-prone rats had higher levels of nicotine intake, but less nicotine intake when infusions were corrected as a function of body mass. Together, these data indicate that the consumption of nicotine, via cigarettes in human smokers or infusions in self-administering rats, is titrated dependent upon body weight. Reduction of nicotine content in cigarettes and nicotine dose resulted in reductions in nicotine consumption across obese and lean groups, suggesting that the reduction of nicotine content in cigarettes may be an effective strategy for reducing smoking across BMI groups.

Obesity impacts drug distribution and pharmacokinetics. Nicotine is distributed primarily in lean mass and has very low affinity for adipose tissue (Hukkanen et al., 2005; Urakawa et al., 1994). Total lean body mass is increased in obesity, although the percentage of lean mass per total body mass is reduced (Cheymol, 2000). The percentage of fat mass in obesity is increased. Therefore, obese individuals may require higher levels of nicotine consumption per lean body

mass, reflected as increased CPD (Figure 18a) and increased unit dose self-administration (Figure 25) in the present data, but lower nicotine consumption as a percentage of lean body mass, reflected as fewer CPD/kg (Figure 18b) and fewer nicotine infusions/kg (Figures 22-24). Large reductions in nicotine content or dose, containing ~ 15% of the nicotine content of control cigarettes or ~12% of nicotine dose to maintain high levels of self-administration behavior, resulted in reduced nicotine consumption independent of obesity status. It is likely that distribution of nicotine in lean mass at these reduced nicotine contents and doses is very low, and no longer acts to reinforce behavior. These data support nicotine reduction policy as an effective strategy for improving public health outcomes related to smoking.

Data from nicotine replacement therapy use in obese and non-obese subjects may provide additional evidence for titration of nicotine consumption by body weight. Transdermal nicotine patch is less effective in maintaining smoking quit rates in obesity (Lerman et al., 2004; Swan et al., 1997), primarily among overweight and obese women (Strong et al., 2015). Efficacy of transdermal nicotine patch as a cessation tool in obesity may be due to differences in nicotine pharmacokinetics. Peak nicotine concentrations were reduced in obese men vs. non-obese men following application of a transdermal nicotine patch (Prather et al., 1993). One study compared the efficacy of transdermal nicotine patch and nicotine nasal spray in promoting quitting in obese and non-obese smokers (Lerman et al., 2004). Nicotine nasal spray was more effective in promoting quitting in obese compared to non-obese smokers. Obese subjects self-administered significantly more nicotine nasal spray than non-obese subjects, indicating that titration of nicotine dose in obesity is more effective for nicotine replacement in obesity than transdermal nicotine patch (Lerman et al.).

Although smokers with obesity smoke significantly more cigarettes per day, there was no impact of BMI on measures of nicotine dependence, withdrawal, or craving. These results are further support for the notion that increased cigarette and nicotine consumption in obese subjects is due to titration of nicotine levels in the blood and brain, and not subjective factors.

In the current experiments, it is difficult to distinguish between nicotine consumption and nicotine reward, as consumption was dependent upon body mass. One previous report has evaluated nicotine reward in obese humans and rats, using procedures that control for subject's nicotine consumption (Blendy et al., 2005). Mice that became obese when fed a high fat diet did not exhibit a conditioned place preference, but mice fed a normal fat diet showed a nicotine conditioned place preference, which may indicate reduced nicotine reward in obesity or by high fat diet. Using a choice procedure in which obese and non-obese smokers were allowed to take 16 puffs from a normal nicotine content or VLNC cigarette, obese smokers took significantly fewer puffs from the normal nicotine cigarette compared to non-obese smokers. If proportion of normal nicotine content choice is an effective measure of nicotine reward, it is likely that obese smokers have reduced nicotine reward. However, choice of normal nicotine content cigarettes was 48% by obese smokers, which may suggest that obese smokers were unable to discriminate between the two cigarettes. It is possible that, in obese smokers, 8 total puffs of a normal nicotine content cigarette insufficient to reach blood and brain nicotine levels to detect nicotine. Likewise, a nicotine conditioned place preference may require a higher dose in obese mice, which was not tested. Regardless, data suggest that nicotine reward may be reduced in obesity (Blendy et al., 2005), but total body weight may have influenced outcomes.

Although VLNC cigarettes reduced smoking across BMI group, the average number of VLNC cigarettes smoked per day at week 6 in smokers with obesity was higher than normal weight smokers. This may indicate that obese smokers are more sensitive to non-nicotine aspects of smoking. This could include non-nicotine constituents in cigarettes that may modify use, such as chemicals that inhibit monoamine oxidase (Smith et al., 2016). Additionally, evidence suggests that conditioned reinforcement may be higher in obesity (Robinson et al., 2015; Vollbrecht et al., 2015), and suggests the possibility that obese smokers may be more sensitive to smoking-related cues. However, increased nicotine-related cue-seeking in obesity may be unlikely given that obesity-prone and obesity-resistant rats responded at similar levels for infusions of saline control delivered with previously nicotine-paired cues, whether infusions were delivered as a unit dose or dose/kg. Evaluation of the number of VLNC cigarettes smoked per day over a longer period to better evaluate extinction of smoking-related cues is warranted.

An additional important observation in these data is that obesity-resistant rats fed chow self-administer similar levels as obesity-prone rats fed chow and HED. Therefore, there is likely some physiological or neurobiological change induced by HED specifically in rats resistant to obesity to increase nicotine self-administration, particularly at doses at the peak and descending limbs of the dose response curve, when infusion duration accounts for body weight. There are many published records of differences in physiological and neurobiological properties between obesity-prone and –resistant rats, though data suggests that most changes are exclusive to the obesity-prone individuals (Clegg et al., 2005; Irani et al., 2007; Irani et al., 2009; B. E. Levin, 1990a, 1990b; Madsen et al., 2010; Robinson et al., 2015; Vollbrecht et al., 2015). An exception to this is that orexin receptor expression is elevated in selectively-bred obesity-resistant chow fed rats (Teske et al., 2013), although the impact of HED consumption on orexin receptor expression

in OR rats is unexplored. Orexin neurotransmission has been related to nicotine reinforcement and self-administration (Kenny, 2011). It is possible that differences in orexin neurotransmission at least in part explain the behavioral phenomenon in the present results. Future experiments should explore a mechanism underlying this behavioral phenomenon.

In summary, nicotine consumption is titrated by an individual based upon body weight in human smokers and rats. Individuals with obesity consume significantly more units of nicotine (cigarettes in smokers and infusions in rats), but fewer units per kg of body weight. Large reductions of nicotine content or dose result in reductions in nicotine consumption independent of obesity. These results have important implications for understanding drug use in obesity, and for nicotine reduction policy.

7.0 GENERAL DISCUSSION

Tobacco use, primarily through cigarette smoking, is the largest cause of preventable death worldwide. Despite the well-publicized health risks associated with smoking, approximately 19 percent of adults in the United States are smokers, and about half of these smokers are predicted to die prematurely due to tobacco-related illnesses (Centers for Disease et al., 2013). Epidemiological and empirical studies describe an inverse relationship between tobacco smoking or nicotine use and body weight (Audrain-McGovern et al., 2011; Jacobs et al., 1981), and desired weight loss or maintenance of reduced body weight is commonly cited as a primary reason for smoking (Fulkerson et al., 2003). There is a negative correlation between the percentage of smokers and body mass index (BMI) among lean smokers, but this relationship is reversed among overweight, obese, and morbidly obese smokers (Chatkin et al., 2010). Thus, there is a U-shape curve associated with percentages of smokers and smoking status as a function of BMI. Furthermore, several other studies report that moderate smokers weigh less than non-smokers, but heavy smokers (i.e., smoking at higher frequencies) are often obese (Chiolero et al., 2007a). Therefore, the relationship between nicotine and body weight requires attention, and was the focus of this dissertation. This general discussion reviews the results of the experiments in Chapters 2-6, and provides a more in depth discussion of potential mechanisms to explain the impact of self-administered nicotine on energy balance, as well as increased nicotine consumption in obesity.

Self-administered nicotine, even at doses that do not support high levels of nicotine-taking behaviors, suppressed body weight gain independent of food intake. These data provide evidence that the dose response curve for body weight reduction by nicotine is shifted to the left compared to nicotine reinforcement. Therefore, it is possible that reductions of nicotine content in cigarettes may not reinforce smoking behavior, but could be effective for weight suppression, which may encourage weight-concerned smokers to continue to smoke. Low levels of daily nicotine intake suppressed body weight gain and decreased respiratory exchange ratio (RER), suggesting that self-administered nicotine shifts macronutrient utilization towards increased fat metabolism. These changes were not accompanied by changes in physical activity, food intake, or heat. These data offer support for increased fat utilization as the primary source of increased energy expenditure by self-administered nicotine, acting to suppress weight gain.

A potential strategy to improve smoking-related public health outcomes posits that reducing the nicotine content in cigarettes below an addictive threshold would promote quitting in current smokers and prevent initiation of smoking (Benowitz et al., 1994; Donny et al., 2012). Evidence from rat self-administration experiments suggests that reduction of daily nicotine consumption results in weight gain independent of changes in food intake. Human smokers randomized to smoke very low nicotine content (VLNC) cigarettes and were compliant in smoking their investigational produce gained a significant amount of weight. This weight gain was the approximate level expected in completely abstinent smokers over a similar amount of time. It has been proposed that the health benefits of quitting smoking outweigh the post-cessation weight gain. If VLNC cigarette use promotes quitting, the reduction of nicotine content in cigarettes should have an overall positive impact on public health, despite associated weight gain.

Most societies consume a “Westernized diet,” which is high in fat and sugar content. Some individuals consuming a Westernized diet are prone to diet-induced obesity, and others are resistant to the development of diet-induced obesity. Two separate experiments evaluated obese and lean individuals as it relates to nicotine-related behaviors. First, self-administered nicotine suppressed body weight gain in obesity-prone (OP) rats fed HED, but not obesity-resistant rats fed HED, suggesting that rats resistant to diet-induced obesity are also resistant to the effects of nicotine on weight suppression. Second, nicotine consumption per body mass is reduced in obesity, but nicotine unit (cigarettes per day or stable infusion duration) is increased in obese rats and humans. Potential mechanisms of action are explored below.

7.1 METHODOLOGICAL CONSIDERATIONS AND OTHER FACTORS UNADDRESSED IN OUR MODEL

Intravenous self-administration is often considered the gold standard test for abuse liability because of its clear face validity and because responding for the drug is a function of drug reinforcement (Henningfield et al., 2016). A nicotine reduction policy targets the reinforcing effects of nicotine, making self-administration an ideal model for understanding how nicotine reduction is likely to impact behavior and other health-related behaviors. As discussed in Chapter 2, there are clear advantages to the use of limited access self-administration procedures in the study of the impact of nicotine on energy balance. However, utilization of limited access nicotine self-administration in male rats has some limitations. A few of these limitations are discussed below.

Smokers experience increases in blood and brain nicotine levels throughout the day with each cigarette smoked. Limited access self-administration procedures cannot capture the cyclical rise and fall in nicotine blood and brain levels experienced in smokers. It is possible that this pattern of nicotine exposure impacts factors that influence energy balance. Experiments using 23-h self-administration in male rats have demonstrated suppression in total 45mg pellets consumed (Bunney et al., 2015), though it is unclear whether this is due to the pharmacological action of nicotine on food intake or a consequence of reduced body weight. A separate experiment showed that tolerance develops to the anorectic effects of self-administered nicotine (O'Dell et al., 2007).

7.2 NICOTINE-INDUCED SUPPRESSION OF WEIGHT GAIN: POTENTIAL MECHANISMS AND SITES OF ACTION

Identification of a specific mechanism by which nicotine acts to suppress weight gain is made difficult by the complexity of nAChR expression and the impact of nicotine on nAChR function and expression. nAChRs are expressed on nearly every cell in the body, and the subunits that comprise nAChRs have diverse expression and functional properties in their interaction with nicotine (Changeux et al., 1984; Dani, 2015), and with varying affinity for nicotine (Dani & Heinemann 1996). Generally, chronic nicotine binding at nAChR results in activation and desensitization of the receptor. There is evidence for the expression of nonfunctional nAChR (Margiotta et al., 1987), and that low dose nicotine can cause desensitization without activation of nAChR (Dani et al., 1996). The interaction between nicotine and nAChR is complex and relies on many factors, which include the route, dose, and

chronicity of administration. These factors likely influence the impact of nicotine on energy balance (Zoli et al., 2012).

The complexity of the interaction of nAChR with nicotine and the fact that contingent vs. non-contingent nicotine may differentially impact energy balance (Rupprecht et al., 2016) outcomes make it difficult to draw upon previously published work to inform the current findings. Given the available data, likely sites of action include brain, pituitary, sympathetic nervous system, and adipose tissue. This discussion of the action of nicotine in the body as it relates to energy balance is not comprehensive, but meant to supplement discussions in previous chapters. Although these potential sites of action are discussed separately, the likely possibility exists that activation is occurring in series or in parallel.

7.2.1 Nicotine action at nAChR to suppress weight gain.

It is assumed that the action of nicotine to suppress weight gain is due to activation of the nAChR. However, several experiments have shown that blockade of the nAChR using mecamylamine, a nAChR antagonist, fails to attenuate the impact of nicotine on weight gain (Aceto et al., 1986; Schechter et al., 1976). However, this form of pharmacological blockade is complicated by the fact that the half-life of nicotine outlasts the half-life of mecamylamine. Specific actions of nicotine, such as lipolysis, have shown to be blocked by mecamylamine when infused to a more local target (Andersson et al., 2001). This may suggest evidence for nicotine acting at nAChR at many levels to suppress weight gain. However, the possibility that nicotine acts independently of cholinergic signaling to suppress weight gain cannot be ruled out based on the currently available data.

The most widely expressed nAChR subtypes in the brain and body are $\alpha 4\beta 2$ and $\alpha 7$ (Gotti et al., 2007). Administration of sazetidine-A, a partial to full agonist with high affinity for $\alpha 4\beta 2$ nAChR, suppresses weight gain (Hussmann et al., 2012). Cytisine is a partial agonist at $\alpha 4\beta 2$ nAChR, and partial to full agonist at $\beta 4$ -containing and $\alpha 7$ nAChR. Non-contingent delivery of cytisine can act to suppress weight gain and food intake (Grebenstein et al., 2013; Mineur et al., 2011). A selective agonist at the $\alpha 7$ nAChR reduces food intake and weight gain in obesity (Marrero et al., 2010; McFadden et al., 2014). Although limited, these results suggest that nicotine does indeed act at nAChR to mediate energy balance.

The time course of nicotine to act on systems that are likely in part responsible for the suppression of weight gain is discussed in Chapter 3. However, throughout the following subsections, it is important to keep in mind that the effects of nicotine on systems involved in weight regulation remain activated, or possibly become activated, after nicotine is cleared, and evidence does not support a role for nicotine metabolites acting to suppress weight gain (Riah et al., 1999).

7.2.2 Brain nAChR in energy balance.

Experimental studying the action of nicotine in the brain to modulate energy balance has focused on cell types within the hypothalamus and the brainstem. Nicotine may act at nAChR expressed on diverse cell types in the hypothalamus, including proopiomelanocortin (POMC), neuropeptide Y (NPY), Agouti-related peptide (AgRP), and orexin, to suppress food intake (Frankish et al., 1995; Li et al., 2000; Mineur et al., 2011). Reports have demonstrated both increased and decreased mRNA levels of these hypothalamic neuropeptides following nicotine exposure, it is difficult to attribute activation of one or more of these neuropeptides on the impact

of nicotine on energy balance, particularly when considering the cumulative effect of activation of all hypothalamic peptides. Nicotinic receptors are expressed in the brainstem, primarily localized to the nucleus tractus solitarius (NTS) and the A2 and C2/3 regions (Wada et al., 1989). Nicotine may act on these receptors to suppress meal size (Guan et al., 2004), which may be mediated by prolactin-releasing peptide (B. Sun et al., 2005), a hindbrain neuropeptide which suppresses meal size (Maniscalco et al., 2012).

Mice chronically treated with nicotine have reduced body weight gain, where mice with knockout of cannabinoid receptor type 1 (CB1) fail to show this effect (Bura et al., 2010). Rimonabant, a CB1 inverse agonist, may mitigate post-cessation weight gain in smokers (Rigotti et al., 2009), despite serious psychological side effects of the drug. The interaction between nicotine and the endocannabinoid system to control weight regulation may occur at the neural level, although CB1 receptors are expressed throughout the body.

7.2.3 Nicotine increasing pituitary hormone release

Cigarette smoke and nicotine have been shown to have effects on the endocrine system. Pituitary hormone release, including adrenocorticotropin hormone (ACTH), growth hormone, and vasopressin, is stimulated by smoking normal nicotine content, but not low nicotine content cigarettes (Seyler et al., 1986), indicating that pituitary hormone release is due to nicotine. Growth hormone stimulates lipolysis in adipose tissue (Vijayakumar et al., 2010), and it is possible that increased lipolysis by nicotine is caused by the release of growth hormone.

7.2.4 Sympathetic activation by nicotine to regulate energy balance.

Nicotinic receptors are the primary mediator of neurotransmission in sympathetic ganglia, and it makes sense that nicotine may act to increase sympathetic tone to target tissue to suppress weight gain. Experimenter- and self-administered nicotine differentially impact sympathetic output, as measured by catecholamines in plasma (Donny), indicating that self-administered nicotine may not have an effect of sympathetic drive. However, it is conceivable that self-administered nicotine acts to increase sympathetic drive, increasing adrenergic signaling to more local regions. Smokers have increased norepinephrine in urine, which may be due to nicotine or smoke itself. Nicotine can increase sympathetic tone (Yoshia 1990; Haass & Kubler 1997), but whether this contributes to weight regulation is unclear. Global pharmacologic blockade of sympathetic ganglia failed to suppress nicotine-induced weight gain suppression (unpublished results from our lab). These results are complicated by the half-life of drugs used for ganglionic blockade and potential side effects of chronic ganglionic blockade.

7.2.5 The impact of nicotine on adipose tissue to regulate energy balance.

As discussed in Chapter 3, nicotine has been shown to increase lipolysis in adipocyte culture, in explanted adipose tissue from nicotine-treated rats, and increase respiratory exchange ratio. These data indicate that nicotine increases fat utilization, which may lead to decreased weight gain. It is clear that nicotine impacts functions related to lipolysis, but there are no data casually linking increased lipolysis to suppression of weight gain by nicotine. The following paragraphs discuss previously published reports on the effect of nicotine on adipose tissue, and offer potential roles for those findings in the current data.

It has been previously reported that large doses of subcutaneous nicotine increase brown adipose tissue (BAT) temperature and the mitochondrial uncoupling protein 1 (UCP1), indicating increased thermogenesis (de Morentin et al., 2012; Seoane-Collazo et al., 2014; Yoshida et al., 1999). Although increased thermogenesis in BAT by nicotine has been reported many times, there is no link between nicotine increasing thermogenesis and weight regulation. One study reported increased BAT thermogenesis in rats treated chronically with nicotine, but no change in weight (Lupien et al., 1988). While a role for nAChR in sympathetic ganglia in thermoregulation is clear (M. Sun et al., 2007), evidence supporting increased BAT thermogenesis by nicotine to suppress weight gain remains unconvincing. In fact, unpublished data from our lab consistently revealed that self-administered nicotine significantly suppressed BAT temperature immediately following the session, and after many days of nicotine self-administration, there was no difference in BAT temperature. Previously published work investigating the effect of nicotine on BAT temperature and UCP1 used experimenter-administered nicotine. It is possible that contingency of nicotine administration impacts its effects on BAT, which could be directly related to the inability of self-administered nicotine to increase catecholamine (Donny et al., 2000).

Until recently, it was thought that only BAT was responsible for adaptive thermogenesis, but it has been established that there are depots of brown-like fat in white adipose tissue (WAT) with thermogenic properties (Dempersmier et al., 2015; Harms et al., 2013). Importantly, these brown-like cells or depots exist in rodents and humans (Ishibashi et al., 2010). White fat cells can be induced to become beige cells (i.e., brown-like fat cells in white fat depots), also called brite cells (brown in white), by certain stimuli, a process known as “browning.” Beige cells can be detected in WAT depots by measuring specific molecular signatures or by histological

confirmation (de Jong et al., 2015; Dodd et al., 2015). Repeated subcutaneous injection of nicotine induces increased UCP1 in WAT and produces a brown-like multilocular phenotype (Yoshida et al., 1999). It is rare that a pharmacological treatment reduces body weight in the absence of an effect on food intake or activity. Further, browning of WAT increases energy expenditure (Dodd et al., 2015).

7.3 THE INTERACTION BETWEEN NICOTINE AND BODY WEIGHT

There is a bidirectional relationship between nicotine and body weight: nicotine impacts weight regulation, and body weight influences nicotine consumption. The interaction between weight and nicotine is particularly interesting in obesity-resistant rats fed HED. Rats resistant to obesity fed HED are resistant to nicotine-induced suppression of weight gain and have elevated levels of low dose nicotine self-administration. Together, these data suggest that obesity-resistant rats are less sensitive to nicotine. Evidence presented in this dissertation (Chapters 5 and 6) suggests that this altered sensitivity to nicotine requires both the genetic predisposition for obesity-resistance and exposure to HED. Behavioral, neurobiological, and physiological differences between these phenotypes have been previously studied. However, the obesity-prone rats, both before and after HED exposure, have changed behavioral, physiological, and neurobiological changes compared to a chow-fed control. Consistently, the obesity-resistant rat functions similar to an outbred Sprague-Dawley chow-fed rat. Therefore, the majority of previous literature on this topic does not inform the data reported here. Several differences between obesity-prone and obesity-resistant rats that may inform the current data are discussed

below. This discussion is not meant to be comprehensive, but to highlight a few possibilities of differences between obesity-prone and –resistant rats that could account for the altered sensitivity to nicotine.

Genome wide association (GWA) studies have demonstrated that genetic variation at *CHRNA5-CHRNA3-CHRNA*, which encodes nAChR, correlates with intensity of smoking. Studies have reported that increased T-allele copies, which is associated with higher levels of smoking, is inversely correlated with BMI (Freathy et al., 2011; Varga et al., 2013). There was no association between allelic copy and BMI in non-smokers. This suggests the possibility that a gene x environment interaction may contribute to weight suppression by nicotine, or that lower BMI results in increased intensity of smoking. The interaction between genetic variability in *CHRNA5-CHRNA3-CHRNA* with diet has not been explored. It is possible that HED has a differential impact on nAChR function and expression in obesity-prone and –resistant rats. This could lend support for decreased sensitivity to nicotine in obesity-resistant rats. Experimental investigation of this hypothesis is challenging, for the reasons listed above describing the complexity of nAChR and its interaction with nicotine.

Alterations in WAT function have been shown to induce obesity-resistance in mice that would otherwise become obese when fed HED. For example, mice lacking acyl CoA:diacylglycerol acyltransferase 1 (DGAT1), an enzyme involved in triglyceride synthesis, are resistant to diet-induced obesity (H. C. Chen et al., 2003). Likewise, mice deficit in acylation-stimulating protein (ASP), which stimulates triglyceride synthesis in adipose tissue, are resistant to diet-induced obesity (Xia et al., 2002). Additionally, beigeing of WAT can block diet-induced obesity (Dodd et al., 2015). These changes in WAT are associated with increased secretion of adipocyte-derived hormones, such as adiponectin. Cultured adipocytes incubated with nicotine

released adiponectin dose-dependently (R. H. Liu et al., 2004). Evidence suggests that adiponectin can act centrally to regulate behaviors, such as locomotor activity (Miyatake et al., 2015). Concentrations of plasma adiponectin may correlate with increased ratings of hedonic odorants (Trellakis et al., 2011). The link between adiponectin, weight regulation, and behavior in obesity-resistant rats is unclear, though it provides a potential mechanism through which these rats may have decreased sensitivity to nicotine.

Changes in diet produce rapid changes in the gut microbiome (David et al., 2014; Walker et al., 2011), and gut microbes are thought to influence behavior (Dinan et al., 2017). HED suppresses total microbiome bacterial count, and increases the proportion of bacteroidales and clostridiales, independent of propensity for obesity development (de La Serre et al., 2010), though enterobacteriales were specifically increased in obesity-prone rats, suggesting that obesity-resistant rats fed HED have different gut microbiome profiles from obesity-prone HED and chow-fed. Although less studied, evidence suggests that smoking impacts the gut microbiome. Smokers have increased bacteroidal bacteria count (Benjamin et al., 2012). It is possible that changes in gut microbiome influence body weight regulation and nicotine reinforcement in obesity-resistant rats.

There were no differences in nicotine metabolite ratio in obese and normal weight smokers, indicating that there are no differences in CYP2A6, the primary enzyme for nicotine metabolism. However, it is possible that differences in nicotine metabolism exist in obesity-prone and -resistant rats. Increased levels of hepatic CYP enzymes were reported in obese mice induced by injection of monosodium glutamate (Tomankova et al., 2015) and the genetically obese Zucker rat (Irizar et al., 1995). Altered nicotine metabolism may account for changes in

obesity-prone and resistant rats. However, it is expected that levels of CYP enzymes would be increased in obesity-resistant rats to account for the reduced sensitivity.

7.4 IMPLICATIONS FOR TOBACCO REGULATORY POLICY

Large reductions in nicotine content in cigarettes may reduce smoking and promote quitting, thereby improving public health outcomes. The data presented here have important implications for tobacco regulatory policy. First, weight gain is an expected outcome of reduced nicotine exposure. Should nicotine reduction indeed promote quitting, the weight gain associated with nicotine reduction will likely not offset the positive health gains from quitting or reducing smoking. Nicotine replacement therapies shown to mitigate post-cessation weight gain may prevent weight gain during or following nicotine reduction. An ongoing clinical trial is assessing smoking and other health outcomes in smokers randomized to smoke normal nicotine content or VLNC cigarettes, with or without patch, and weight gain in this sample should be evaluated. Post-cessation weight gain in the first month of abstinence predicts continued abstinence rather than relapse (Hall et al., 1986). Secondly, very low doses of nicotine in rats naïve to nicotine were effective to suppress weight gain. It is possible that weight-concerned naïve smokers may initiate VLNC smoking as a weight control method. However, given the rise of other tobacco product availability, such as electronic nicotine delivery systems, which are effective for weight suppression, this possibility seems unlikely. Finally, nicotine reduction will likely be an effective strategy to reduce smoking behavior in lean and obese smokers.

7.5 FUTURE DIRECTIONS

The results of the experiments described within this dissertation improve our understanding of the complex relationship between nicotine and body weight. The work described here presents the opportunity for many more studies to more closely investigate the relationship between nicotine and body weight. Listed below is a non-comprehensive list of future research directions.

1. Chapters 2 and 3 demonstrate that self-administered nicotine suppresses weight gain independent of food intake and suppresses RER, indicating increased fat utilization. As discussed above, the use of pharmacological blockade in the study of the impact of self-administered nicotine on energy balance poses many problems. Therefore, a primary future direction in this area will be the use of mouse models. Self-administration in mice with specific deficits in nAChR in brain, fat, or other target tissue may be important moving forward. For example, mice with specific deficits of $\alpha 7$ nAChR in white adipose tissue may fail to show decreased RER and weight gain.
2. The focus on male rats in the study of the impact of nicotine on body weight regulation ignores two important populations: females and adolescents. Female smokers are more likely to be weight-concerned smokers and to use smoking as a weight regulation strategy (Fulkerson et al., 2003; Levine et al., 2001). Separating subjective motives for smoking and weight loss from the pharmacological actions of nicotine in human smokers is difficult. Some evidence in rats suggests that nicotine may potentiate weight loss in females compared to males, but other reports suggest that there is no interaction with sex (L. L. Bellinger et al., 2005; Bishop et al., 2004; Blaha et al., 1998). A comparison of the impact of

nicotine self-administration on weight regulation in males and females is unexplored and is warranted. Adolescents represent another population that may be more likely to be weight-concerned smokers. A perception of overweightness in adolescents increases risk for smoking (Yoon et al., 2016). Rodent self-administration data suggest that there is no impact of nicotine on body weight in adolescence (Natividad et al., 2013). This experimental design allowed rats to self-administer nicotine for four consecutive days at a time, and it is possible that more chronic daily self-administration of nicotine would produce suppression of weight gain in adolescent rats, particularly as suppression of weight gain by nicotine in the current experiments was seen after at least 5 days of self-administration. Unpublished data from our lab testing nicotine self-administration in adolescents for 16 consecutive days support the data from Natividad et al, suggesting that adolescent rats may be resistant to nicotine suppression of weight gain.

3. Chapter 5 demonstrated resistance to nicotine-induced weight suppression in obesity-resistant rats. Changes in energy expenditure to explain these results are unknown. Measuring RER, activity, and heat production in these rats following self-administration would give important insights into these results.
4. Exploring differences in nAChR in obesity-prone and –resistant rats is an important future direction. Characterizing nAChR expression density with autoradiography, as well as electrophysiological properties in response to specific nAChR agonists in slice may provide important insight into differences in nAChR function and expression in obesity-prone and –resistant rats. For example, $\alpha 5$ -containing nAChR in the medial habenula regulate nicotine consumption (Fowler et al., 2011), and $\beta 2$ -containing nAChR in the ventral tegmental area mediate nicotine reinforcement (Picciotto et al., 1998), specific

changes in these receptors in these regions may give insight into the behavioral actions of nicotine in obesity-prone and resistant-rats.

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