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Murine Genetic Variance in Sugar and Fat-Conditioned Flavor Preferences: Roles of Dopamine, Opioid and NMDA Receptors and Nutritive Sensing

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Murine Genetic Variance in Sugar and Fat-Conditioned Flavor Preferences: Roles of Dopamine, Opioid and NMDA Receptors and Nutritive Sensing

By

Tamar T. Kraft

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2019
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By,

Tamar T. Kraft

This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Murine Genetic Variance in Sugar and Fat-Conditioned Flavor Preferences: Roles of Dopamine, Opioid and NMDA Receptors and Nutritive Sensing

By

Tamar T. Kraft

Advisor: Professor Richard J. Bodnar, PhD.

Abstract

As obesity and diabetes have emerged as a severe public health crisis, understanding the mechanisms underlying the consumption of sugars and fats has become a topic of vigorous study. From a biological standpoint, genetic dispositions, neurochemical and hormonal influences, and predetermined orosensory and postingestive signals that modulate the hunger and satiety process may govern physiological aspects of the obesity puzzle. In addition to an innate appetite and attraction for simple carbohydrates and fats, learning plays an important role in modulating preferences for sugar- and fat-rich foods in rodents, including inbred mouse strains. Marked genetic variance has been observed among murine strains in sugar and fat appetite as well as the development and persistence of sugar preferences. In particular, SWR and BALB/c inbred mouse strains differ in their sweet taste sensitivity, exhibit robust intakes of sugars and fats, and develop strong and persistent sucrose-conditioned flavor preferences (CFP). These two strains also display strong and divergent sensitivity to dopamine (DA) D1 and opioid receptor antagonists in reducing spontaneous intake of sucrose and fat as well as in the acquisition (learning) and expression (maintenance) of sucrose-CFP. Murine strain differences have also been observed in the ability of sucrose and glucose, but not fructose, to elicit CFP following intra-gastric sugar infusions, and in the differential responsiveness of strains to post-oral actions of fructose. Six approaches were employed to further examine the role of genetic variance in responsiveness of and preferences for sugars and fats in inbred SWR and BALB/c mice. A
first study examined the relative preference for fructose and sucralose and sucralose + saccharin (S+S) solutions in SWR and BALB/c mice and found that ad-libitum-fed SWR, but not BALB/c mice reversed their initial preference for S+S over fructose after experience. This study also compared initial and subsequent preferences following experience for 8% fructose and 8% glucose solutions in the two strains as an index of the post-oral reinforcing actions of the two sugars. Both ad-libitum-fed and food-restricted SWR mice strongly preferred glucose to fructose in direct choice tests, whereas food-restricted, but not ad-libitum-fed BALB/c mice displayed this preference. A second study examined whether systemic administration of opioid (naltrexone: NTX) and dopamine D1 (SCH23390: SCH) receptor antagonists reduced intakes of non-nutritive 0.2% saccharin and nutritive 8% fructose solutions in BALB/c and SWR mice. Although saccharin intake was reduced similarly by SCH and NTX in BALB/c and SWR mice, SWR mice exhibited greater potencies of opioid (1.9-fold) and DA D1 (4-fold) receptor antagonism of fructose intake relative BALB/c mice. A third study examined whether BALB/c and SWR mice exhibited differential sensitivity to NTX and SCH in altering the expression (maintenance) and acquisition (learning) of fructose-CFP. SCH was more effective than NTX in reducing the expression of fructose-CFP in both strains. Whereas BALB/c mice displayed hastened extinction of acquisition of fructose-CFP following SCH, but not NTX, SCH eliminated fructose-CFP acquisition and NTX hastened extinction of fructose-CFP in SWR mice. A fourth study examined whether BALB/c and SWR mice exhibited differential sensitivity to the NMDA receptor antagonist, MK-801 in altering acquisition and expression of both sucrose- and fructose-CFP. Although acquisition of fructose- and sucrose-CFP was eliminated by MK-801, NMDA antagonism was more potent in BALB/c relative to SWR mice. MK-801 mildly reduced the magnitude of the expression of sucrose- and fructose-CFP in BALB/c mice, but blocked the expression of fructose-, but not sucrose-CFP in SWR mice. A fifth study examined whether BALB/c and SWR mice exhibit differential sensitivity to NTX and SCH in altering the acquisition and
expression of fat-CFP. BALB/c and SWR mice exhibited similar fat-CFP in preferring the CS+ flavor paired with a 5% Intralipid solution over a CS- flavor paired with a 0.5% Intralipid solution. Whereas SCH blocked the expression of fat-CFP in both BALB and SWR mice, NTX reduced this response in BALB/c, but not SWR mice. In contrast, acquisition of fat-CFP was eliminated by SCH in SWR, but not BALB/c mice. Acquisition of fat-CFP was marginally impaired by NTX in BALB/c, but not SWR mice. A sixth study examined whether BALB/c and SWR mice exhibit differential sensitivity to MK-801 in altering acquisition and expression of fat-CFP. MK-801 eliminated acquisition of fat-CFP in both BALB/c and SWR mice with the latter’s response appearing to turn into an avoidance response. Expression of fat-CFP was more effectively eliminated by MK-801 in BALB/c relative to SWR mice. The myriad behavioral differences observed in BALB/c and SWR strains indicate a crucial role for genetic background in mediating the neurochemical and behavioral substrates of sweet and fat intake as well as the development and persistence of sweet and fat preferences.
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ABBREVIATIONS

CFP, Conditioned Flavor Preferences

CS+, Conditioned Stimulus Preferred

CS-, Conditioned Stimulus Less Preferred

DA, Dopamine

MK-801, non competitive antagonist of NMDA

NMDA, N-methyl-D-aspartate

NTX, Naltrexone; opioid antagonist

QTL, Quantitative Trait Locus

SCH23390, Dopamine D1 antagonist

S+S, sucralose + saccharin

Veh, Vehicle
Significance and Specific Aims:

As obesity and diabetes have emerged as a severe public health crisis, understanding the mechanisms underlying the consumption of sugars and fats has become a topic of vigorous study. From a biological standpoint, genetic dispositions, neurochemical and hormonal influences, and predetermined orosensory and postingestive signals that modulate the hunger and satiety process may govern physiological aspects of the obesity puzzle (Smith & Dockray, 2006). Correspondingly, the availability and accessibility of cheap, calorically dense food provides environmental explanations in the obesity equation (Dragone, 2009). In addition to an innate appetite and attraction for simple carbohydrates and fats, learning plays an important role in modulating preferences for sugar- and fat-rich foods in rodents, including inbred mouse strains (Pinhas et al., 2012). Marked genetic variance has been observed among murine strains in sugar and fat appetite as well as the development and persistence of sugar preferences. In particular, SWR and BALB/c inbred mouse strains differ in their sweet taste sensitivity, exhibit robust intakes of sugars and fats, and develop strong and persistent sucrose--conditioned flavor preferences (CFP). The first specific aim examined the relative preference for fructose and sucralse + saccharin (S+S) solutions in SWR and BALB/c mice to determine their sensitivity to post-oral fructose appetition after experience. The first specific aim also compared initial and subsequent preferences following experience for 8% fructose and 8% glucose solutions in the two strains as an index of the post-oral reinforcing actions of the two sugars.

The differential sensitivity of BALB/c and SWR mice to DA D1 and opioid receptor antagonism of sucrose and fat intake has been observed as well as their ability to affect the acquisition and expression of sucrose-CFP (Dym et al., 2007, 2009, 2010, 2012). Sugar-CFP involves the systematic comparison of more-preferred calorically dense sugars (e.g., sucrose, fructose) relative to less-preferred non-nutritive sweet (e.g., saccharin) solutions. Given that the effects of DA D1
(SCH23390) and opioid (naltrexone) receptor antagonism upon fructose and saccharin intake has not
been evaluated in inbred mouse strains, the second specific aim examined the dose-dependent abilities
of systemic naltrexone and SCH23390 to reduce intakes of 0.2% saccharin or 8% fructose solutions
in BALB/c and SWR mice.

DA D1, but not opioid, receptor antagonism blocks the acquisition and expression of sucrose-CFP in sham-feeding rats (Yu et al., 1999, 2000a, 2000b) and of fructose-CFP in real-feeding rats
(Baker et al., 2003, 2004). Both DA D1 and opioid receptor antagonism reduced the expression
(maintenance) of an already-learned sucrose-CFP in BALB/c and SWR mice (Dym et al., 2012).
However, the acquisition of sucrose-CFP is blocked by DA D1, but not opioid receptor antagonism in
SWR mice, and by opioid, but not DA D1 receptor antagonism in BALB/c mice, indicating a double
dissociation between receptor antagonist effectiveness and inbred mouse strain (Dym et al., 2012). A
major neurochemical candidate mediating many forms of learning is glutamate, especially acting
through its NMDA receptor, which has been shown to play a crucial role in learning, memory and
synaptic plasticity (see review: Rezvani, 2006). Specifically, blockade of the NMDA receptor with
the non-competitive antagonist, MK-801, selectively blocks the acquisition, but not the expression of
fructose-CFP (Golden and Houpt, 2007). To examine whether these differential patterns of
acquisition and expression CFP effects persist for sugars, the third and fourth specific aims examined
whether BALB/c and SWR mice exhibit differential sensitivity to naltrexone and SCH23390 (Specific
Aim 3) and the NMDA receptor antagonist, MK-801 (Specific Aim 4) in altering the acquisition and
expression of fructose-CFP.

Acquisition and expression of fat-CFP in real-feeding rats is markedly affected by DA D1, but
not opioid receptor antagonists (Dela Cruz et al., 2012a, 2012b). NMDA receptor antagonism
eliminates the acquisition, but not expression of fat-CFP in rats (Dela Cruz et al., 2012b). The fifth
and sixth specific aims examined whether BALB/c and SWR mice exhibit differential sensitivity to
naltrexone and SCH23390 (Specific Aim five) and the NMDA receptor antagonist, MK-801 (Specific Aim 6) in altering the acquisition and expression of fat-CFP.
Chapter 2

Background.

One third of the total number of deaths in the United States results from the “new normal” of an overweight country and sedentary lifestyle (Mokdad et al., 2004). According the most recent statistics delineated by the Campaign to Obesity, the following statistics are alarmingly concerning: a) Two-thirds of adults and nearly one-third of children struggle with overweight and obesity today; b) if obesity rates stay consistent, 51 percent of the population will be obese by 2030; c) whereas twenty years ago, no state had an obesity rate above 15 percent, today there are 41 states with obesity rates over 25 percent, according to the Trust for American's Health; and d) Since 1980, the rate of obesity in children and adolescents has almost tripled.

It is proposed that a complex interaction between biological and environmental factors governs the development of obesity. Therefore, this proposal examined the dynamic interplay of nature and nurture as well as the potential pharmacological substrates that govern palatable intake of sugars and fats in inbred mice strains. These variables have been typically studied in isolated fashion with many genetic and pharmacological manuscripts. In order to assess relationships can be made among these factors, this background section examined: 1) genetic influences on palatability by reviewing the use of inbred mice strains, 2) genetic variance in sweet intake and preferences, 3) the pharmacology of conditioned flavor preferences, 4) the concepts of appetition and satiation in post-oral control of intake, 5) genetic variance in the pharmacology of sugar and fat intake and preferences, and 6) rationales for the six specific aims.

Section 1. Rodent Models of Obesity: Overview of Rodent Models

Obese Rodent Models: Historically, the first rodent models of obesity include the hypothalamic obese rat (Hetherington et al., 1940; Brobeck et al., 1943) and the dietary obese rat (Ingle, 1949) as well as the genetic mutants of inbred and outbred rodent strains. Additionally, there was the discovery of Ob/Ob mice, (Ingalls et al., 1950), typical C57Bl/6J mice whose ob gene
became mutated with the homozygotic offspring. Phenotypically, these offspring displayed hyperphagia, reduced energy expenditure, insulin resistance associated with hyperglycemia and hyperinsulinemia, became profoundly obese, and showed marked increases in food intake. Nearly a half a century later, the basic mechanism underlying these biological and behavioral changes were elucidated as being a result of a functional leptin deficiency which was directly was related to the development of obesity (Halaas et al. 1995; Friedman and Halaas, 1998). This leptin deficiency occurs as a single-base spontaneous mutation of the ob gene, which in turns prematurely terminates leptin synthesis, and consequently its secretion in the body (Lutz & Woods, 2012). Additionally, Ob/ob mice were used as an animal model for type II diabetes and have led researchers to understand the role leptin plays in food intake, body weight (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995; Stephens et al. 1995) and metabolic rate (Pelleymounter et al. 1995).

Another mouse model, the db/db mouse, is profoundly diabetic, an effect attributed to leptin resistance, and not to alterations in leptin levels as found in the ob/ob mice. (e.g., Chen et al., 1996). These db/db mice are also characterized by hyperphagia and reduced energy expenditure leading to marked early-onset obesity. (Lutz & Woods, 2012).

Analogous to the db/db mouse are the obese Zucker (fa/fa) and Koletsky rats which are both characterized as leptin-resistant obese rodents due to mutations in the leptin receptor, more specifically the extracellular domain of the leptin receptor. A similar phenotype of hyperphagia and reduced energy expenditure are developed in these strains, leading to morbid obesity (de Jonge, L., & Bray, G. A., 1977; Bray and York, 1979) as well as an impaired glucose tolerance, and a growth deficit possibly related to a lower activity of the GH/IGF-1 axis and hypothyroidism. Undetectable levels of mRNA expression has been observed as the key mutation (Chua et al., 1996; Crouse et al., 1998; da Silva et al., 1998; Friedman, 1997; Takaya et al., 1996; Wu-Peng et al., 1997) in Koletsky rates, whereas the fa/fa mutation of Zucker fatty rats is associated with a processing defect of the
leptin receptor. For the Zucker fa/fa rodents, the leptin receptor is produced but retained intracellularly. This structural conformation leads to reduced numbers of leptin receptors on the cell surface, which causes decreased leptin binding and signal transduction. Koletsky rats are more hypertensive and have a more severe phenotype of insulin resistance than Zucker fatty rats. Zucker rats were bred to be a genetic model for research on obesity and hypertension. There are two types of Zucker rat: a heterozygous lean Zucker rat, denoted as the dominant trait (Fa/Fa) or (Fa/fa); and the characteristically homozygous obese (or fatty) Zucker rat, represented as expression of its a recessive trait (fa/fa) (Bray and York, 1971; Kurtz et al. 1989; Takaya et al. 1997; Zucker and Antoniades, 1972; Zucker and Zucker, 1961). Obese Zucker rats have high levels of lipids and cholesterol in their blood, are resistant to insulin without being hyperglycemic, and gain weight from an increase in both the size and number of fat cells (Kava et al. 1990, Ikeda et al., 1986; Stern et al. 1972; Terrettaz et al. 1986,). Obesity in Zucker rats is primarily linked to their hyperphagic nature that is accompanied by excessive hunger. However, food intake does not fully explain the hyperlipidemia or overall body composition (Kava et al. 1990; Kurtz et al. 1989).

Inbred Mouse Strains: Comparisons of genetically altered inbred mouse strains have also produced incredibly meaningful data in the quest to examine traits related to energy balance and obesity. The differential phenotypes observed in these strains display continuous variations in traits and are exceptionally complex, undoubtedly due to dynamic interplay of the varying contributions of genetic susceptibility and interacting environmental factors (Pomp et al., 2008). A mouse strain can be considered ‘inbred’ if it is maintained by sibling (sister x brother) mating’s for 20 or more generations. Being homozygous at virtually all of their genetic loci, these inbred mice strains are as genetically identical as possible. Each inbred strain possesses a unique genotype, and comparisons among strains can further isolate genotype differences as a function of behavioral differences (e.g., www.jax.org/jaxmice). Identifying divergent behavioral responses between strains can ultimately
lead to chromosomal localization of that given behavior through quantitative trait loci (QTL) analyses. QTL analyses are used to localize chromosomal regions, and ultimately genes, critically involved in such differences. Furthermore, genetic models using inbred mice can be potentially more instructive than using transgenic mouse models because they are not subject to possible confounding variables associated with mutations, such as developmental, compensatory, and multiple behavioral effects of the mutated gene (see review: Mogil and Grisel, 1998). In addition to inbred mice, a commonly used outbred wild-type strain (e.g., CD-1) is also used in strain surveys, which allows for direct comparisons of ingestive responses to inbred strains. In contrast to inbred strains, outbred mice are genetically heterogeneous with respect to one another, and therefore any variance in their behavioral responses cannot be attributed to genetic factors. Thus, the use of an outbred strain introduces an additional control when investigating the role of the genetic contribution to ingestive responses. (Dym et al., 2007). Inbred mice have been used to examine the genetic variance involved in many aspects of food intake (see reviews: Bodnar et al., 2013; Reed et al., 1997; West and York, 1998).

Section 2. The Importance of Distinguishing among Sucrose, Glucose, and Fructose intake

In the most simplistic explanation, sugars such as fructose, glucose and sucrose are simple carbohydrates that are classified as either monosaccharides or disaccharides. Monosaccharides such as fructose and glucose are the simplest, most basic units of carbohydrates and are made up of only one sugar unit. They also form the essential building blocks of sucrose, a disaccharide. Thus, disaccharides are just a pair of linked sugar molecules. They are formed when two monosaccharides are joined together and a molecule of water is removed - a dehydration reaction. The following will describe the differences and similarities of these three sugars.

Glucose is considered to the most important monosaccharide in that it is the body’s preferred energy source. As glucose circulates in the blood, it is referred to as ‘blood sugar’, relying on
glucokinase or hexokinase to initiate metabolism. The large majority of ingested carbohydrates is converted into glucose and is used in the body for either immediate energy if necessary, or converted by the liver as glycogen for later use. Insulin secretion occurs primarily in response to elevated glucose concentrations in the blood, which in turns facilitates the entry of glucose into cells (Schaefer et al., 2009). Thus, this is one marked difference between glucose and fructose.

While fructose is a monosaccharide sugar found naturally in many fruits and vegetables, it is very different from other sugars. This is due to a differential metabolic pathway that is not the preferred energy source for animal muscles or the brain. Fructose is only metabolized by the liver after intestinal absorption in the bloodstream, and does not initiate insulin release, another key difference between it and glucose. This marked difference causes fructose to behave more like fat in the body than like other simple carbohydrates in that it is more lipogenic, or fat producing (White, 2013).

Sucrose is commonly known as table sugar, and most fruits and vegetables naturally contain the disaccharide sucrose. Upon sucrose consumption, the beta-fructosidase enzyme separates sucrose into its constituents of glucose and fructose, and their specific metabolic and transport mechanism then occur. The glucose response then occurs in its usual manner causing insulin secretion, whereas fructose metabolism by the liver is initiated. The body will use glucose as its main energy source and the excess energy from fructose, if not needed, will be metabolized as part of fat synthesis, which is stimulated by the insulin released in response to glucose (Schaefer et al., 2009). The discernible differences among these three sugars only serve to underscore the complexity of their metabolic characteristics. Although fructose has long been thought to be the “enemy” of dieters and diabetics alike due to findings related to high fructose corn syrup, divergent results from research done on the effects of non-nutritive sweeteners such as dextrose and sucralose paints a much more esoteric picture.
that only opens up a Pandora’s box of uncertainty in our understanding of the complex nature of the behavioral and metabolic characteristic of sugars and non-nutritive sweeteners.

**Non-nutritive sweeteners:** Developed at the end of the 19th century, non-nutritive sweeteners (NNS) have been introduced as a non-caloric sugar replacement recognized as General Recommended as Safe by the FDA. Until recently, NNS were considered to be a godsend, delivering the sweet taste of sugars without the calories and glycemic effect. However, there is a growing literature suggesting that the use of NNS might dysregulate energy balance, and thereby contribute to obesity and other negative health outcomes (Fowler et al., 2008, Swithers et al., 2009, 2010, Pepino et al., 2015). Despite being perceived as 10-20 times sweeter than sugars by humans, NNS offer little to no energy when ingested, an important distinction when compared with fructose, sucrose and glucose. NNS do not undergo the complex metabolism observed for sugars, and thus may differ in its ability to condition learning and maintenance of its intake. Given that the only alluring characteristic of NNS is its artificial sweet taste in the absence of nutritional value, sugars possess an evolutionarily advantage mechanism of survival and so this proposal will examine whether animals may display differential preferences for nutritive and non-nutritive sweeteners.

**Orosensory and Post-Ingestive Characteristics of Conditioned Flavor Preferences (CFP)**

**for Sugars and Fats:** In addition to the important role that innate, genetic components play in development of food preferences, environmental factors play a strong role as well. The CFP paradigm has been used to demonstrate that learning is involved in food selection, specifically in the preference of food (see reviews: Capaldi, 1992; Sclafani, 1990, 1995, 1997). The CFP paradigm is typically viewed as a form of Pavlovian or classical conditioning. In one version of this paradigm, the novel cue flavor (e.g., dilute cherry Kool Aid) acting as the conditioned stimulus (CS+) is systematically paired with an unconditioned stimulus (US) solution, which has a nutritive source (e.g. sucrose, glucose, fructose). A second novel cue flavor (CS-, e.g., dilute grape Kool Aid) is paired
with a nonnutritive (e.g. saccharin) solution. The CS+ and CS- are offered to the animals in single-bottle exposures on alternate days with the nutritive and nonnutritive pairings administered respectively via the mouth (flavor-flavor conditioning) or pairing two flavored saccharin solutions with intragastric, intraduodenal or intravenous infusion of the sugar as compared to water (flavor-nutrient conditioning). In this one-bottle acquisition phase, the animals learn to associate the cue flavor with the post-ingestive consequence of the nutrient and overall acceptance of the CS+ and CS-flavors is assessed by measuring the absolute intake. During the expression phase, preference is evaluated by having the animal undergo a two-bottle test with both cue flavors mixed in a saccharin solution (flavor-flavor conditioning) or with no infusions (flavor-nutrient conditioning). Due to the post-ingestive consequences of the nutrient in flavor-nutrient conditioning, animals display strong preferences (70-90%) for the CS+ over the CS- cues in rats, and is very resistant to extinction (Drucker, et al., 1994; see review, Sclafani, 1997).

Flavor-flavor conditioning, occurs when the CS+ does not typically result in a post-ingestive consequence, and learning occurs mainly from associating the cues with the hedonic orosensory properties of the food stimuli. Flavor-flavor conditioning can be established in two ways. One way is through the sham-feeding preparation in which animals are fitted with a gastric fistula (Van Vort and Smith, 1983, Weingarten and Watson, 1982). When the fistula is closed while the animal is consuming the solution, the food is digested normally (real-feeding), and when the fistula is opened, this allows the stomach contents to empty (sham-feeding) and minimize post-ingestive learning (Young et al., 1974). However, one drawback to using the sham feeding preparation is that it may not block all neural and hormonal feedback from the gut because sham-feeding does not completely prevent food digestion and absorption (Sclafani and Nissenbaum, 1985a, 1985b). A second way to establish a flavor-flavor conditioned preference is by using a nutrient that is relatively ineffective in producing a flavor-nutrient conditioned response, but possesses highly palatable orosensory qualities.
Ingestion of a previously-novel flavor immediately followed by a non-nutritive saccharin solution results in learned preference for that flavor (Holman, 1975). The monosaccharide, fructose, is ineffective in producing CFP through intragastric infusions relative to glucose or sucrose (Sclafani and Ackroff, 1993; Sclafani et al., 1999). However, the combination of a previously neutral flavor with a fructose solution conditions a preference (Sclafani and Ackroff, 1994). Thus, in flavor-flavor conditioning, combining a previously neutral flavor with solutions that are inherently reinforcing due to their sweet taste or viscous consistency, preferences can be conditioned for stimuli that lack post-ingestive consequences (Ackroff and Sclafani, 1999, Sclafani and Ackroff, 1994). However, glucose and sucrose elicit CFP both through flavor-flavor and flavor-nutrient processes (Sclafani & Ackroff, 1994; Sclafani et al; 1993, 1999).

From an early age, rodents are also attracted to the flavor of fat (e.g., corn oil) and nonnutritive fat substitutes (e.g., mineral oil, sucrose), which may be mediated in part by taste receptors for fatty acids (Ackroff & Sclafani, 2009; Passilly-Degrace, Gaillard, & Besnard, 2009). In addition, both the postingestive actions and orosensory properties of fat are rewarding and condition a CS+ flavor preference (Ackroff & Sclafani, 2009; Sclafani, 1999).

Section 3. Pharmacology of Sugar and Fat Conditioned Flavor Preferences (CFP) in Rats

Role of Dopamine (DA) Receptors in Rat CFP: One neurochemical candidate for the mediation of CFP was brain dopamine based on its involvement in food reward and motivation (see reviews: Berridge and Robinson, 1998; Smith, 2006; Wise, 1989). Initial evidence of DA involvement in CFP involved the study of rats trained to drink a mildly bitter tastant paired with intragastric polycose (CS+) and a mildly sour tastant paired with intragastric infusions of water (CS-). After training, exposure to the CS+ and CS- without intragastric pairings resulted in significantly increased extracellular DA levels in the NAC following voluntary intake of the CS+, but not CS-.
solutions (Mark et al., 1994). Administration of the DA D2 receptor antagonist, raclopride, reduced preference for a flavored 10% sucrose solution compared to a second flavor paired with saline (Hsiao and Smith, 1995). Systemic treatment with a DA D1 receptor antagonist (SCH23390), but not a DA D2 receptor antagonist (raclopride), blocked flavor-nutrient conditioning by intragastric sucrose infusions (Azzara et al., 2001). DA D1 and D2 receptor antagonists both interfered with preference expression during flavor-flavor CFP paradigms. Rats treated systemically with SCH23390 or raclopride during sham-feeding training sessions failed to display preferences for the sucrose-conditioned CS+ flavor comparable to control animals (Yu et al., 2000a, 2000b). Moreover, both antagonists dose-dependently reduced an already-trained preference for the CS+ flavor, indicating that DA D1 and D2 signaling are involved in the acquisition (learning) and expression (maintenance) of a conditioned preference (Yu et al., 2000a, 2000b). One possible limitation of the sham-feeding study was that the animals consumed substantially more of the flavored sucrose solution than the flavored saccharin solution during training, and therefore were more familiar with the CS+ flavor. Therefore, in a subsequent study (Baker et al., 2003), a rationed portion (16 ml) of a distinctly flavored fructose and saccharin solution (CS+) and another flavored saccharin solution (CS-) was employed in real-feeding rats. Systemic treatment with SCH23390 and, to a lesser degree, raclopride blocked acquisition of fructose-flavor conditioning, whereas both DA D1 and D2 receptor antagonism significantly reduced the expression of a CS+ fructose-CFP (Baker et al., 2003). In examining the neural substrates of these responses, intracerebral injections of SCH23390, and to a lesser degree, raclopride, significantly reduced the expression of a CS+ fructose-conditioned preference following administration into the amygdala (Bernal et al., 2008b) and shell of the NAC (Bernal et al., 2008) during a fructose-CFP. The acquisition of this preference was unaffected by NAC or amygdalar DA antagonists, but the DA D1 antagonist hastened extinction of the learned response. DA D2 receptor antagonism in the medial prefrontal cortex was more effective than DA D1 antagonism in dose-
dependently reducing the expression of fructose-CFP (Malkusz et al., 2012). SCH23390 administration into the shell and core of the NAC, amygdala and medial prefrontal cortex interfered with acquisition, but not expression of a flavor-nutrient CFP in rats (Touzani et al., 2008, 2009, 2010). Systemic DA D1 and D2 receptor antagonism in rats also reduces acquisition and expression of fat (corn oil)-CFP (Dela Cruz et al., 2012a) though not to the same degree as observed for fructose-CFP (Baker et al., 2003). Whereas fructose-CFP engages only flavor-flavor conditioning, fat-CFP engages both flavor-flavor and flavor-nutrient conditioning. Interestingly, DA D1 and D2 antagonists reduce the acquisition and expression of glucose-CFP to a similar degree as well (Dela Cruz et al., 2014), by putatively engaging both flavor-flavor and flavor-nutrient conditioning, is, like fat-CFP,

**Role of Opioid Receptors in Rat CFP:** Opioid receptors have been extensively implicated in sweet and fat intake per se through the ability of general (naltrexone (NTX)) and specific opioid receptor antagonists to inhibit sugar, NNS or fat intake (see reviews: Bodnar, 2004, 2015). Surprisingly however, systemic NTX treatment failed to block flavor-nutrient conditioning by intragastric sucrose infusions (Azzara et al., 2000). NTX failed to interfere with preference expression during flavor-flavor CFP paradigms despite systematically reducing overall intake of the CS+ and CS- solutions. Systemic NTX during sham-feeding training sessions failed to affect preferences for the sucrose-conditioned CS+ flavor comparable to control animals (Yu et al., 1999). Moreover, NTX failed to affect an already-trained preference for the CS+ flavor, indicating that opioid receptor signaling is not involved in the acquisition (learning) and expression (maintenance) of a conditioned preference (Yu et al., 1999). Further, Baker and co-workers (2003) found that NTX failed to block acquisition or expression of fructose-flavor conditioning. Intracerebral NTX injections into the nucleus accumbens, amygdala or medial prefrontal cortex failed to affect the expression of fructose-CFP (Bernal et al., 2010; Malkusz et al., 2014). Finally, systemic opioid receptor antagonism in rats failed to affect acquisition and expression of fat-CFP (Dela Cruz et al., 2012b). Thus, opioid receptor
antagonism, unlike DA receptor antagonism, plays little or no role in sugar- or fat-CFP in rats despite its clear ability to inhibit sweet and fat intake.

**Role of NMDA receptors in Rat CFP:** Learning, memory and Synaptic plasticity have been shown to be mediated by glutamate and specifically by NMDA receptor transmission. (Li & Tsien, 2009). This is largely assumed to be due to vital interactions between dopamine and NMDA transmission in crucial brain areas including the VTA, NaC, AMY and mPFC, all areas considered to be influential in food related incentive learning, (Kelley, 2004; Ranaldi et al., 2011). Golden & Houpt (2007) found that systemic NMDA receptor blockade with the non-competitive antagonist, MK-801, significantly reduced the acquisition but not the expression of fructose CFP. Fat-CFP acquisition, but not expression was also blocked by MK-801 treatment (Dela Cruz et al., 2012b). Further, the acquisition, but not the expression of flavor-nutrient CFP elicited by intragastric glucose was significantly reduced by administration of the competitive NMDA receptor antagonist, AP5 into the amygdala. Moreover, and interaction between dopamine and NMDA systems was observed by the elimination of the acquisition of intragastric glucose-CFP by AP5 administration into one amygdala, and by SCH23390 administration into the other amygdala (Touzani et al., 2013). Thus multiple neurochemical systems working in a distributed brain network mediate the actions of flavor-flavor and flavor-nutrient CFP. The next section will now focus on the abilities of sugars to mediate post-ingestive signals leading to the initiation and ending of intake.

**Section 4: Roles of Appetition and Satiation in post-oral control of intake**

Meal size and post-meal eating can be suppressed by multiple hormonal and neural signals generated by ingested nutrients. These signals are governed by the availability of sugar and fat-rich foods, which, under certain circumstances, can override these satiety signals leading to overeating and obesity. The palatability of sugar- and fat-rich foods may induce overeating but the additional post-oral actions of these nutrients can increase overall food consumption. (Sclafani, 2013). In a seminal
study conducted by Holman (1975), post-oral nutrient actions were demonstrated to enhance food preferences using a conditioned flavor paradigm. The findings illustrated that rats showed a significant (~66%) CS+ preference (lemon) paired with intragastric infusions of an eggnog diet (US) versus another flavored solution such as anise (CS-) paired with water solutions. Over the ensuing three decades, intragastric (IG) nutrient infusions (i.e., milk, glucose, casein) were able to condition flavor preferences in food-restricted rats (Puerto et. al., 1976; Deutsch & Wang, 1977; Sherman et.al., 1983; Balker et al., 1987). The findings of these experiments suggested the notion that the effective “reinforcer” for the conditioned flavor preference was due to the inherent characteristic of the caloric or nutrient properties of the solutions. (Sherman et al., 1983; Baker et al., 1987). However in subsequent studies, non-food restricted rats acquired strong preferences for CS+ solutions paired with IG self-infusions of sugars, fat and milk. (Lucas & Sclafani, 1989; Sclafani et al., 1993; Azzara & Sclafani, 1998; Sclafani & Glendinning, 2005; Ackroff & Sclafani 1994). Based on these findings alone, perhaps animals do not need to be in an energy-depleted state to acquire nutrient-based flavor preferences, or even to take it one step further perhaps energy depletion may not independently be sufficient enough to induce or reinforce a flavor preference.

Using the breadth of work done by the Sclafani laboratory, a remarkable notion is suggested; is it possible that the palatability of sugar and fat-rich foods may not only stimulate appetite, but also produce post-oral actions that continue to enhance intake? Although a food’s satiating effects is often dictated by the type and amount of post-oral food reward, could there be a separate and distinct system that mediates appetite. This will be revisited in the rationale for Specific Aim 1.

**Section 5: Murine genetic variance in the pharmacology of sugar and fat intake and preferences**

Strong genetic variance has been observed in inbred mouse strains intake of sweet solutions such as sucrose and saccharin. (See review: Bodnar, Lewis-Levy & Kest, 2013). Our laboratory examined strain differences in sucrose intake among inbred and outbred mouse strains across sucrose
concentrations (0.0001–20%: Lewis et al., 2005). A, C57BL/6, C57BL/10, CD-1, SJL and SWR strains consumed the greatest amount and percentage of kilocalories of sucrose, whereas AKR, CBA, C3H/He and DBA/2 strains consumed the least sucrose. BALB/c, DBA/2 and 129P3 strains consumed intermediate amounts of sucrose on these measures. A parallel study (Lewis et al., 2007) demonstrated genetic variance for intake of an emulsified fat solution, Intralipid, with the greatest sensitivity observed for BALB/c, AKR, C57BL/6, DBA/2 and SWR mice, intermediate sensitivity for CD-1, C57BL/10 and SJL mice, and less sensitivity for A, CBA, C3H/He and 129P3 mice. In addition to its high caloric density, dietary fat has a hyperphagic effect, in part as a result of its high palatability. Fat taste receptors have been found to be implicated in governing fat intake. The recent identification by Laugerette et al. 2015 indicates CD36 involvement in orosensory detection of dietary lipids, spontaneous fat preference, and digestive secretions. GPR40 and GPR120 has been shown to mediate the taste of fatty acids (Cartoni et al., 2010). Marked genetic variance was observed in naltrexone’s inhibition of sucrose intake with the greatest sensitivity observed in the C57BL/10 and C57BL/6 strains, intermediate sensitivity in BALB/c, C3H/He, CD-1 and DBA/2 mice, and the least sensitivity in 129P3, SWR and SJL strains (Dym et al., 2007). Marked genetic variance was also observed in the ability of DA D1, but not D2 receptor antagonism to inhibit sucrose intake with strong (129P3, SJL, C57BL/6, BALB/c), intermediate (DBA/2, SWR, C3H/He, C57BL/10) and weak (CD-1) effects observed following SCH23390 (Dym et al., 2009). Finally, SCH23390 reduced Intralipid intake in inbred mice with the greatest sensitivity observed in DBA, CD-1, SWR, SJL, C57BL/6, lower sensitivity in 129P3 and C57BL/10 mice, but not in BALB/c mice. Correspondingly, naltrexone reduced Intralipid intake in inbred mice with the greatest sensitivity observed in DBA, SWR and SJL mice, moderate sensitivity in CD-1, C57BL/10, C57BL/6 and 129P3 mice, but not in BALB/c mice (Dym et al., 2010).
Murine genetic variance was also observed for development of sugar-CFP (Pinhas et al., 2012) in inbred strains possessing “sensitive” (SWR, SJL, C57BL/10, C57BL/6) or “sub-sensitive” (DBA/2, BALB/c, C3H/He, 129P3) forms of sweet taste receptors (T1R2/T1R3: Reed et al., 2004). Robust sucrose-CFP was observed in SWR, DBA/2, BALB/c, SJL, C3H and 129P3 mice, whereas more modest effects were observed in C57/BL/6, C57BL/10, and outbred CD-1 mice. However, the magnitude of fructose-CFP ranged from being robust (BALB/c, SWR, C3H) to modest (DBA/2, C57BL/10, CD-1, 129P3), and absent (C57BL/6, SJL). The magnitude of sucrose-CFP was invariably stronger than fructose-CFP, an effect attributed to differences in the orosensory and postingestive actions of the two sugars. As stated previously, fructose acts via orosensory factors or flavor-flavor conditioning whereas sucrose acts via both orosensory and post-ingestive factors (both flavor-flavor and flavor-nutrient conditioning.). Post-oral actions that enhance the intake and preference for sugar and fat rich foods are known as appetitive processes (Sclafani, 2013; Sclafani & Ackroff, 2012). Sweet-sensitive C57BL/6 and sub-sensitive 129 mice displayed similar post-oral appetition responses to IG sucrose infusions (Sclafani & Glendinning, 2003). However, differences in post-oral sugar appetition were observed in C57BL/6 and FVB sweet-sensitive strains tested with glucose and fructose (Sclafani, et al., 2014, 2015). Whereas IG glucose conditioned preferences in both strains, IG fructose conditioned preferences only in FVB mice. Further, whereas naïve FVB and C57BL/6 mice strongly preferred a non-nutritive 0.1% sucralose +0.1% saccharin (S+S) solution to 8% fructose, FVB, but not C57BL/6 mice switched their preference to fructose over S + S, indicating genetic variance in fructose’s post-oral reinforcing action.

This dissertation proposal examined two of the specific murine inbred mouse strains, BALB/c and SWR mice. This is due to their robust sensitivity to sugars and fats (Lewis et al., 2005, 2007), the differential ability of opioid and DA D1 antagonism to alter sugar and fat intake (Dym et al., 2007, 2009, 2010), and their ability to acquire and maintain both sugar (sucrose and fructose)-t-CFP (Pinhas
et al., 2012). These mice were also previously examined for their pharmacological effects upon sucrose- and fructose-CFP. Dym et al., (2012) observed a double-dissociation in the ability of DA D1 and opioid receptor antagonism to affect sucrose-CFP. Unlike rats, both DA D1 and opioid receptor antagonism significantly reduced the expression of sucrose-CFP in both strains. However more interestingly a double dissociation was observed in that, DA D1 receptor antagonism eliminated the acquisition of sucrose-CFP in SWR but not BALB/c mice, whereas opioid receptor antagonism eliminated the acquisition of sucrose CFP in BALB/c but not SWR mice.

Section 6. Rationales for the six specific aims

Specific Aim 1: The first specific aim examined the relative preference for fructose and sacralose + saccharin (S+S) solutions in SWR and BALB/c mice to determine their sensitivity to post-oral fructose appetition after experience. The first specific aim also compared initial and subsequent preferences following experience for 8% fructose and 8% glucose solutions in the two strains as an index of the post-oral reinforcing actions of the two sugars. These data was published in Physiology & Behavior 153 (2016) 64-69.

Recent studies indicate that C57BL/6J (B6) and FVB mouse strains differ in post-oral fructose conditioning. This was demonstrated by their differential flavor conditioning response to intragastric fructose and their preference for fructose versus a non-nutritive sweetener (Sclafani et al., 2014; Sclafani et al., 2015). The present study extended our analysis of post-oral fructose appetition to SWR and BALB/c inbred mice, which are sweet sensitive and sub-sensitive respectively. These strains were of interest because they both show robust flavor conditioning responses to fructose.

Specific Aim 2: The second specific aim examined the dose-dependent abilities of systemic naltrexone and SCH23390 to reduce intake of 0.2% saccharin or 8% fructose solutions in BALB/c and SWR mice. These data were published in Pharmacology, Biochemistry and Behavior 131 (2015) 13-18.
Sugar and fat intake in rodents are mediated in part by brain dopamine (DA) and opioid neurotransmitter systems although important strain differences exist. Thus, whereas as sucrose intake of BALB/c and SWR mice were reduced by DA D1 (SCH23390: SCH) receptor antagonism, opioid (naltrexone: NTX) receptor antagonism reduced intake only in BALB/c mice (Dym et al., 2007, 2009). Both SCH and NTX reduce fat intake in SWR, but not BALB/c mice (Dym et al., 2010). The present study extended this pharmacological analysis to caloric and non-caloric sweeteners by examining whether fructose (8%) or saccharin (0.2%) intakes were differentially suppressed in BALB/c and SWR mice by SCH or NTX over a 2 h time course.

**Specific Aim 3:** The third specific aim examined whether BALB/c and SWR mice exhibit differential sensitivity to naltrexone and SCH23390 in altering the acquisition and expression of fructose-CFP. These data were published in *Physiology and Behavior* 151 (2015) 213-220

Sugar appetite is influenced by unlearned and learned preferences in rodents. In the present study, we investigated the effects of DA D1 and opioid receptor antagonists on the expression and acquisition of fructose-CFPs in BALB/c and SWR mice. This was of interest because prior studies indicated that sucrose and fructose differ in their conditioning actions. Real-fed sucrose involves a both flavor-flavor learning and flavor-nutrient learning (Sclafani et al., 1995; Sclafani et al., 2004). In contrast, a fructose-CFP is assumed to be reinforced only by sweet taste (flavor-taste learning) because IG fructose solutions failed to provide a CFP in rats as well as C57BL/6 mice (Sclafani et al., 2012a; Zukerman et al.,2013a). Thus, the disruptive effects of DA and opioid antagonists on fructose-CFPs may differ from those observed with sucrose-CFPs.

**Specific Aim 4:** The fourth specific aim examined whether BALB/c and SWR mice exhibit differential sensitivity to MK-801 in altering the acquisition and expression of sucrose- and fructose-CFP. These data were published in *Pharmacology, Biochemistry and Behavior* 148 (2016) 76-83.
Conditioned flavor preferences (CFP) are elicited by sucrose and fructose relative to saccharin intake in rats and inbred mice (Pinhas et al., 2012). Whereas, dopamine, but not opioid receptor antagonists differentially interfere with the acquisition (learning) and expression (maintenance) of sugar-CFP in rats (Malkusz et al., 2015), these antagonists differentially affect acquisition and expression of sucrose- and fructose- CFP in BALB/c and SWR inbred mice (Dym et al., 2012; present dissertation). Given that NMDA receptor antagonism with MK-801 blocks acquisition, but not expression of fructose-CFP in rats (Golden and Houpt, 2007), the present study examined whether MK-801 altered the expression and acquisition of sucrose and fructose-CFP in BALB/c and SWR mice.

**Specific Aim 5:** The fifth specific aim examined whether BALB/c and SWR mice exhibit differential sensitivity to naltrexone and SCH23390 in altering the acquisition and expression of fat-CFP. These data were published in *Pharmacology, Biochemistry and Behavior* 110 (2013) 127-136.

Sugar and fat appetites are influenced by unlearned and learned responses to orosensory and post-ingestive properties, which are mediated by dopamine (DA) and opioid transmitter systems. In BALB and SWR mice, acquisition and expression of sucrose conditioned flavor preferences (CFP) are differentially affected by systemic DA D1 and opioid receptor antagonism. The present study examined whether fat-CFP occurred in these strains using preferred (5%) and less preferred (0.5%) Intralipid solutions, and how SCH and NTX altered its acquisition and expression.

**Specific Aim 6:** The sixth specific aim examined whether BALB/c and SWR mice exhibit differential sensitivity to the NMDA receptor antagonist, MK-801 in altering the acquisition and expression of fat-CFP. These data were published in *European Journal of Pharmacology* 799 (2017) 26-32.

Conditioned flavor preferences are elicited by fat (Intralipid) in inbred mouse strains with BALB/c and SWR mice displaying among the most robust preferences. DA D1 and opioid receptor
antagonism differentially reduces the acquisition (learning) and expression (maintenance) of fat-conditioned flavor preferences in these two strains. Because noncompetitive NMDA receptor antagonism with MK-801 differentially altered sugar-conditioned flavor preferences in these strains, and because NMDA receptors are involved in fat intake, the present study examined whether MK-801 differentially altered expression and acquisition of fat-conditioned preferred in BALB/c and SWR mice.
Chapter 3

BALB/c AND SWR INBRED MICE DIFFER IN POST-ORAL FRUCTOSE APPETITION AS REVEALED BY SUGAR VERSUS NON-NUTRITIVE SWEETENER TESTS

Introduction

Sugar appetite in rodents depends on both stimulation of oral sweet taste receptors (Bachmanov and Beauchamp, 2007) and post-oral sugar sensors (Sclafani and Ackroff, 2012). Inbred mouse strains vary in their taste response to sugars and non-nutritive sweeteners, which is attributed, in part, to genetic differences in the T1r3 component of the T1r2/T1r3 sweet taste receptor (Reed et al., 2004). Some strains have a "sensitive" form of the receptor which results in increased preferences and intakes of a variety of nutritive and non-nutritive sweet solutions, while other strains have a "sub-sensitive" form of the receptor which produces reduced preferences and intakes of these sweetener solutions, particularly at low concentrations (Bachmanov and Beauchamp, 2007). Sugar intake and preference are also influenced by post-oral nutritive effects via a process referred to as appetition to distinguish it from the satiation process that inhibits sugar intake (Sclafani, 2006; Sclafani and Ackroff, 2012). Post-oral appetition is most clearly demonstrated by the intake and preference-stimulating effects produced by intragastric (IG) sugar infusions in mice and rats (Sclafani and Ackroff, 2012). Conceivably, inbred strain variations in sugar preferences may be influenced by strain differences in post-oral appetition as well as by differences in sweet taste sensitivity. Sclafani and Glendinning, investigated this possibility in sweet-sensitive C57BL/6J (B6) mice and sub-sensitive 129 mice, which differ substantially in their oral intakes of sucrose. Both strains, however, displayed similar post-oral appetition responses to IG sucrose infusions. This and other findings indicate that post-oral sugar appetition is not mediated by gut T1r3 receptors (Sclafani et al., 2010).

More recently, Sclafani and co-workers observed a difference in post-oral sugar appetition in B6 and FVB mice, which are both sweet-sensitive strains with high oral intakes of sugar. In this case,
the mice were tested with glucose and fructose. Whereas IG glucose infusions stimulated intake of, and preference for, a flavored (CS+) saccharin solution in both strains, IG fructose failed to condition preferences in B6 mice but conditioned significant CS+ preferences in FVB mice. The differential post-oral actions of fructose were also revealed in sugar vs. non-nutritive sweetener choice tests (Sclafani et al, 2014; Sclafani et al., 2015). Like B6 mice, naïve FVB mice strongly preferred a 0.1% sucralose + 0.1% saccharin (S+S) solution to 8% fructose in an initial 2-day two-bottle test. However, after the mice had separate 2-day choice tests with S+S and fructose versus water, the FVB mice preferred fructose to S+S, whereas the B6 mice continued to prefer S+S to fructose. Taken together, these data indicate that fructose has a post-oral reinforcing action in FVB mice which conditions a preference for the initially less-preferred 8% fructose over 0.1% S+S after separate experience with both sweeteners.

The present experiment extended our analysis of post-oral fructose appetition to SWR and BALB/c inbred mice, which are sweet-sensitive and sub-sensitive strains, respectively (Reed et al., 2004). These strains were of interest because in a survey of inbred mouse strains, they both acquired strong preferences for a CS+ flavor added to an 8% fructose + 0.2% saccharin solution over a CS-flavored 0.2% saccharin-only solution (Pinhas et al., 2012). In contrast, B6 mice failed to prefer the fructose-paired CS+ flavor. At the time, the fructose-conditioned preference in the SWR and BALB/c mice was attributed to flavor-taste learning reinforced by the sugar’s sweet taste since fructose was known to have little or no post-oral reinforcing actions in B6 mice or Sprague-Dawley rats (Sclafani and Ackroff, 2012; Sclafani et al., 1993; Sclafani et al., 1999). However, in view of the post-oral fructose appetition recently discovered in FVB mice (Sclafani et al., 2014), it is possible that the fructose-conditioned flavor preferences observed in SWR and BALB/c mice were due in part to post-oral conditioning in these strains. To evaluate this possibility, Experiment 1 determined the relative preference for fructose and S+S solutions in SWR and BALB/c mice before and after they had
separate experience with the two sweeteners. As noted above, unlike B6 mice, FVB mice switch their preference from S+S to fructose after experience with the sweeteners which is indicative of post-oral fructose appetite. In a second experiment we compared the preference for 8% fructose and 8% glucose in the two strains, which provides an index of the differential post-oral reinforcing actions of the two sugars.

**Experiment 1: Fructose vs. Sucralose + Saccharin Preferences**

**Materials and Methods**

*Subjects:* Adult male SWR and BALB/c mice obtained from the Jackson Laboratories (Bar Harbor, ME) were adapted to the laboratory for 1 week. The starting body weights of the SWR (25.6 g) and BALB/c mice (25.7 g) were similar. The animals were singly housed in plastic tub cages in a room maintained at 22°C with a 12:12-h light-dark cycle and given ad libitum access to chow (LabDiet Standard Laboratory Rodent Diet #5001, PMI Nutrition International, Brentwood, MO) and water except where noted. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Queens College and were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

*Test Solutions:* Solutions of 8% fructose (Sigma Aldrich Laboratories, St. Louis, MO) and a mixture of 0.1% sucralose (Tate & Lyle, Dayton, OH) and 0.1% saccharin (Sigma Aldrich Laboratories) (S+S) were prepared with tap water on a w/w basis because intakes were measured by weight. The S+S solution was selected based on the finding that B6 mice strongly preferred it to 8% fructose or 8% glucose in 1-min two bottle tests, suggesting that it was “sweeter” than the sugar solutions. The solutions were available through stainless steel sipper spouts attached to 50-ml plastic tubes that were placed on the grid top of the cage and fixed in place with springs. Fluid intakes were measured to the nearest 0.1 g by weighing the drinking bottles on an electronic balance. Spillage in
this study was minimal as demonstrated by recording the change in weight of two tubes that were placed on an empty cage.

Procedure: SWR mice (n=8) and BALB/c mice (n=10) were given *ad-libitum* access to chow and two bottles of water for 4 days. They were then given a series of 2-day two-bottle tests as in our prior study [18]: Test 1 (days 1-2): fructose vs. S+S; Test 2 (days 3-4): fructose vs. water; Test 3 (days 5-6): S+S vs. water; Test 4 (days 8-9): fructose vs. S+S. The mice were given water vs. water for one day (day 7) between Tests 3 and 4. The left-right position of the sweetener and water bottles were switched from the first to second day of each test to control for potential position effects.

Because daily fructose and S+S intakes of the BALB/c mice were rather low, which is characteristic of this strain (Lewis et al., 2005; Ramirez and Fuller, 1976) a second group of nine BALB/c mice was tested which had restricted access to food to stimulate their sweetener intakes. These mice were given daily chow rations that maintained their body weights at 85-90% of their *ad libitum* level for two weeks prior to testing, and throughout the four 2-bottle preference test series.

Statistics: Daily solution intakes were averaged over the 2 days of each test, and sweetener preferences were expressed as percent solution intakes (e.g., fructose intake/total intake x 100).

Intakes were analyzed using a mixed model analysis of variance (ANOVA) with test and solution as repeated factors. One ANOVA included results from Tests 1 (naïve mice) and 4 (experienced mice), and evaluated whether relative intakes of fructose and S+S changed across the two tests within groups. A second ANOVA included results from Tests 2 and 3, and compared the intakes of each sweetener vs. water within groups. Percent sweetener intakes within groups were analyzed with t-tests. Additional between groups ANOVAs were performed as described below.

Results

SWR mice. The SWR mice consumed more S+S than fructose in Test 1, but more fructose than S+S in Test 4, although only the Test 4 difference was significant (*Sweetener x Test* interaction,
(F(1,7)= 94.4, p<0.0001; Figure 1A). Seven of the eight mice drank more S+S than fructose in the first test, whereas all 8 mice consumed more fructose than S+S in Test 4. The percent fructose intake increased from 39% in Test 1 to 66% in Test 4 (t(7)= 7.15, p<0.0001). In Tests 2 and 3, SWR mice consumed more fructose and S+S than water (F(1,7)= 36.0, p<0.0001) and their sweetener intakes and percent intakes did not differ.

**BALB/c mice.** The ad-libitum fed BALB/c mice consumed significantly more S+S than fructose in both Tests 1 and 4 (F(1,9)= 112.7, p<0.0001), and their percent fructose intakes remained low in both tests (20% and 27%, Figure 1B). Overall intakes increased from Test 1 to 4 (F(1,9)= 5.8, p<0.05). In Tests 2 and 3, the mice consumed more fructose and S+S than water (F (1,7)= 36.0, p<0.0001), and they also consumed more (p<0.05) S+S than fructose (Sweetener x Test interaction, (F (1,7)= 12.1, p<0.01). Their percent S+S intake also exceeded that of their percent fructose intake (76% vs. 65%, t (9)= 3.0, p<0.05).

Similar to the ad-libitum fed mice, the food-restricted BALB/c mouse consumed significantly more S+S than fructose in Tests 1 and 4 (F (1,8)= 33.9, p<0.0001), and their percent fructose intakes were low in both tests (18% and 13%, Figure 1C). In Tests 2 and 3, the mice consumed more fructose and S+S than water (F (1,8)= 31.3, p<0.001) and they also consumed much more (p<0.001) S+S than fructose (Sweetener x Test interaction, (F (1,8)= 45.1, p<0.001). However, their percent S+S and fructose intakes relative to water did not to differ. The food-restricted BALB/c mice consumed significantly more fluid than did the ad-libitum fed BALB/c mice in Tests 1 and 4 (F (1,17) = 32.3, p<0.0001) as well as in Tests 2 and 3 (F(1,17) = 22.2, p<0.001). In both cases, this was due primarily to the elevated S+S intakes of the food-restricted mice (Sweetener x Group interaction (F(1,17) = 15.4 and 16.1, p<0.01). The percent sweetener intakes in Tests 2 and 3 of the food-restricted BALB/c mice also exceeded those of the ad-libitum fed BALB/c mice (F(1,17) = 5.5, p<0.05; Figure 1). Overall, the ad-libitum fed SWR mice consumed more sweetener than did the ad-libitum fed BALB/c
Figure 1. (Chapter 3). Fructose and Sucralose+Saccharin preference tests in BALB/c and SWR Mice. Intake (mean, +SEM) in 2-day, two-bottle tests with 0.1% sucralose + 0.1% saccharin (S+S) vs. 8% fructose (Test 1), fructose vs. water (Test 2), S+S vs. water (Test 3), and S+S vs. fructose (Test 4). A. Mean intakes of ad-libitum fed SWR mice in Tests 1-4. B. Mean intakes of ad-libitum fed BALB/c mice in Tests 1-4. C. Mean intakes of food-restricted BALB/c mice in Tests 1-4. Significant differences (p < 0.05) within each test are denoted by asterisks (*). Numbers atop bars represent mean percent preference for that solution.
mice in Tests 1 and 4 (F(1,16) = 124.7, p<0.001) as well as in Tests 2 and 3 (F(1,16) = 31.1, p<0.001).

The food-restricted BALB/c mice consumed significantly more fluid than did the *ad-libitum* fed BALB/c mice in Tests 1 and 4 (F (1,17) = 32.3, p<0.0001) as well as in Tests 2 and 3 (F(1,17) = 22.2, p<0.001). In both cases, this was due primarily to the elevated S+S intakes of the food-restricted mice (Sweetener x Group interaction (F(1,17) = 15.4 and 16.1, p<0.01). The percent sweetener intakes in Tests 2 and 3 of the food-restricted BALB/c mice also exceeded those of the *ad-libitum* fed BALB/c mice (F(1,17) = 5.5, p<0.05; Figure 1). Overall, the *ad-libitum* fed SWR mice consumed more sweetener than did the *ad-libitum* fed BALB/c mice in Tests 1 and 4 (F(1,16) = 124.7, p<0.001) as well as in Tests 2 and 3 (F(1,16) = 31.1, p<0.001). In contrast, the *ad-libitum* fed SWR and food-restricted BALB/c mice did not differ in their overall intakes in Tests 1 to 4, but they did differ in their sweetener intakes. Specifically in Test 4, the *ad-libitum* fed SWR mice consumed more fructose and less S+S than did the food-restricted BALB/c mice (Group x Test x Sweetener interaction, (F(1,16) = 58.3, p < 0.0001).

**Experiment 2: Fructose vs. Glucose Preferences**

Our prior studies of B6 and FVB mice indicated that, while the two strains differed substantially in their post-oral conditioning response to fructose, both strains displayed stronger IG conditioning responses to glucose than to fructose (Sclafani and Ackroff, 2012; Sclafani et al., 2014). Consistent with this finding, both strains also consumed significantly more glucose than fructose in separate sugar vs. water tests, and strongly preferred glucose to fructose in a 2-day, two-bottle test (Sclafani et al., 2014). In view of these findings, Experiment 2 compared the relative preferences of SWR and BALB/c mice for isocaloric 8% fructose and 8% glucose. The BALB/c and SWR mice were tested under both *ad-libitum* and food-restricted conditions given the significant effect of food restriction on the sugar intakes and preferences of BALB/c mice observed in the first experiment.
Materials and Methods

The *ad-libitum* fed SWR (n=7) and BALB/c (n=9) mice from Experiment 1 were used except for one mouse from each strain that died after the first experiment. The mice were maintained on *ad-libitum* food and given a series of 2-day, two-bottle tests as follows: Test 1 (days 1-2) 8% fructose vs. water, Test 2 (days 3-4) 8% glucose vs. water, Test 3 (days 6-7) 8% fructose vs. glucose. The mice were given two-bottle access to water only on day 5 between tests 2 and 3. The mice were then food-restricted as in the first experiment and given the same series of preferences tests described above. The left-right position of the sugars was switched from the first to second day of each test to control for potential position effects.

Results

**SWR mice.** The SWR mice consumed much more sugar than water in Tests 1 and 2, and therefore an ANOVA was performed only on the sugar intake data (Figure 2). When tested under *ad-libitum* food and food-restriction conditions, the SWR mice consumed significantly (*p* < 0.01) more glucose than fructose (*F*(1,6) = 45.6, *p*<0.001) in the sugar vs. water tests (Tests 1 and 2). Percent glucose intakes were also higher than percent fructose intakes in both food availability conditions (*F*(1,6) = 5.7, *p*<0.055). In addition, SWR mice consumed more glucose under food restriction than with *ad-libitum* food, while fructose intakes did not vary with deprivation state (State x Sugar interaction, *F*(1,6) = 5.6, *P* < 0.056). In Test 3, SWR mice in *ad-libitum* and food-restriction states consumed substantially more glucose than fructose (*F*(1,6) = 37.6, *p*<0.001), but they did not differ in their absolute or percent sugar intakes (Figure 2).

**BALB/c mice.** Overall, BALB/c mice consumed more sugar under food restriction than *ad-libitum* feeding states in Tests 1 and 2 (*F*(1,8) = 9.5, *p*<0.01), and they tended to consume more glucose than fructose, but this difference was not significant (Figure 3). However, percent glucose intakes
Figure 2. (Chapter 3) Fructose and Glucose preference tests in SWR Mice. Intake (mean, +SEM) in 2-day, two-bottle tests with 8% fructose vs. water (Test 1), 8% glucose vs. water (Test 2), and 8% glucose vs. 8% fructose (Test 3) in SWR mice. A. Mean intakes of SWR mice fed *ad-libitum* in Tests 1-3. B. Mean intakes of food-restricted SWR mice in Tests 1-3. Significant differences (p< 0.05) within each test are denoted by asterisks (*). Numbers atop bars represent mean percent preference for that solution.
exceeded percent fructose intakes in both feeding states (F(1,8) = 5.6, p<0.05). In Test 3, BALB mice drank significantly more glucose than fructose under food restriction, while they drank similar amounts of the two sugars with *ad-libitum* feeding (State x Sugar interaction, (F(1,8) = 8.1, p<0.05). In addition, more glucose was consumed in the food-restricted state than in the *ad-libitum* state (p<0.01), whereas fructose intakes did not differ as a function of food availability. Consequently, the percent glucose intakes were higher in the food-restricted relative to the *ad-libitum* state (75% vs. 51%, t(8) = 3.56, p<0.01).

Overall, SWR mice consumed much more sugar than did BALB/c mice under *ad-libitum* and food-restricted conditions in Tests 1 to 3 (F(1,14) = 216.6, p <0.0001). The *ad-libitum* fed SWR mice also displayed greater preferences for fructose and glucose over water than did the *ad-libitum* fed BALB/c mice (fructose: 88% vs. 68%; glucose: 93% vs. 75%, F(1,14) = 6.0, p<0.05). In addition, the *ad-libitum* fed SWR mice displayed a greater preference for glucose over fructose in Test 3 than did the *ad-libitum* fed BALB/c mice (87% vs. 51%, t(14) = 3.50, p < 0.01). In contrast, the food-restricted SWR and BALB/c mice did not significantly differ in their preferences for fructose and glucose over water in Tests 1 and 2 (fructose: 88% vs. 71%, glucose 98% vs. 82%) or their preference for glucose over fructose in Test 3 (78% vs. 75%).

**Discussion**

Experiment 1 investigated whether fructose has post-oral reinforcing actions in SWR and BALB/c mice as it does in FVB mice, but not B6 mice (Sclafani et al., 2014). This outcome seemed plausible given the finding that SWR and BALB/c, but not B6 mice acquired significant preferences for a flavor mixed into a fructose+saccharin solution over a flavor mixed into a less preferred saccharin-only solution (Pinhas et al., 2012). The finding that SWR mice reversed their initial preference for non-nutritive S+S over fructose in Test 1 to a fructose preference over S+S in Test 4
Figure 3. (Chapter 3) Fructose and Glucose preference tests in BALB/c Mice. Intake (mean, +SEM) of 2-day, two-bottle tests with 8% fructose vs. water (Test 1), 8% glucose vs. water (Test 2), and 8% glucose vs. 8% fructose (Test 3) in BALB/c mice. A. Mean intakes of BALB/c mice fed *ad-libitum* in Tests 1-3. B. Mean intakes of food-restricted BALB/c mice in Tests 1-3. Significant differences (p<0.05) within each test are denoted by asterisks (*). Numbers atop bars represent mean percent preference for that solution.
after separate experience with the two sweeteners in Tests 2 and 3 strongly indicates that fructose exerts post-oral reinforcing actions in this inbred strain. A similar observation found S+S to fructose preference shift in FVB mice exposed to the same test experience (Sclafani et al., 2014). In addition, IG fructose infusions were found to condition flavor preferences in FVB mice. In contrast, B6 mice failed to develop a preference for fructose over S+S, although they strongly preferred glucose to S+S consistent with the post-oral reinforcing effect of glucose, but not fructose, observed in this strain (Sclafani et al., 2015).

In contrast to ad-libitum fed SWR mice, ad-libitum fed BALB/c mice did not reverse their preference for S+S over fructose, but rather displayed strong S+S preferences in both Tests 1 and 4. Because the ad-libitum fed BALB/c mice consumed less than half as much sweetener as did the SWR mice in the 2-day tests, it was possible that their low intakes provided insufficient post-oral feedback to enhance their fructose preference. We therefore tested a separate group of food-restricted BALB/c mice, which consumed significantly more of the sweeteners than did the ad-libitum fed BALB/c mice. Yet they also did not reverse their preference for S+S over fructose. Their failure to develop a fructose preference is particularly noteworthy given their need for the energy provided by the sugar but not the S+S. We previously observed that food-restricted B6 mice also failed to develop a preference for nutritive fructose over non-nutritive S+S (Sclafani et al., 2015). These findings challenge the notion that the energy value of sugars accounts for their preference over non-nutritive sweeteners (Beeler et al. 2012; see also Sclafani et al., 2015).

In earlier inbred mouse survey of sugar conditioning (Pinhas et al., 2012), it was assumed that the fructose-conditioned flavor preference was due to the sweet taste of the sugar rather than a post-oral reinforcing action given the inability of IG fructose infusions to induce a flavor preference in B6 mice. The finding of fructose-conditioned preferences in sweet-sub-sensitive BALB/c mice (and other strains), but not sweet-sensitive B6 mice seemed inconsistent with this interpretation (Pinhas et al.,
The B6 mice, unlike BALB/c mice, consumed more flavored saccharin than flavored fructose+saccharin solution during training, which suggested that the enhanced saccharin preference of B6 mice, relative to BALB/c mice, may have contributed to their failure to acquire a fructose-based flavor preference. However, this interpretation was questioned by the finding that SWR mice also consumed considerably more saccharin than fructose+saccharin during training yet acquired a preference for the CS+/fructose solution. That fructose has post-oral reinforcing actions in SWR, but not B6 mice appear to explain why SWR, but not B6 mice developed a fructose-based flavor preference in the earlier study (Pinhas et al., 2012). The present findings, however, fail to explain why BALB/c, but not B6, mice acquired a fructose preference. The overall significance of these findings are discussed further in the General Discussion.
Chapter 4

DOPAMINE D1 AND OPIOID RECEPTOR ANTAGONIST-INDUCED REDUCTIONS OF FRUCTOSE AND SACCHARIN INTAKE IN BALB/c AND SWR INBRED MICE

Introduction

Sugar appetite in rodents is mediated in part by brain dopamine (DA) and opioid transmitter systems. Thus, sugars can release brain DA (e.g., Avena et al., 2006; Hajnal and Norgren, 2001; Hajnal et al., 2004; Hernandez and Hoebel, 1988, 1990; Rada et al., 2005) and opioids (e.g., Castro and Berridge, 2014; Colantuoni et al., 2001; Papaleo et al., 2007; Pomonis et al., 2000; Yamamoto et al., 2000). Opioid receptor antagonism with naloxone or naltrexone (NTX) significantly reduces intakes of sucrose (Glass et al., 1996; Kirkham and Cooper, 1988a, 1988b; Levine et al., 1982, 1995; Rockwood and Reid, 1982; Sclafani et al., 1982) and saccharin (Cooper, 1983; Lynch, 1986; Lynch and Libby, 1983) as well as fat (Cooper et al., 1985; Weldon et al., 1996) in rats. DA receptor antagonism also significantly reduces intake of sucrose (Bello and Hajnal, 2006; Geary and Smith, 1985; Muscat and Willner, 1989; Schneider et al., 1986, 1990; Sclafani et al., 1982; Tyrka et al., 1992) and fats (Baker et al., 2001; Imaizumi et al., 2000; Rao et al., 2008; Weatherford et al., 1988, 1990; Yoneda et al., 2007) in rats. Whereas DA D1 receptor antagonists consistently reduce sugar and/or fat intake in rats, DA D2 receptor antagonists alternatively reduce, increase or fail to affect these forms of intake in rats (Corwin and Wojnicki, 2009; Muscat et al., 1991; Phillips et al., 1991; Pritchett and Hajnal, 2011; Tyrka and Smith, 1993; Wong et al., 2009).

The comparison of inbred mouse strains offers the additional ability to study the role of genetic factors governing DA and opioid receptor modulation of sugar intake. Our laboratory found that marked strain differences occurred in the reduction of sucrose intake following NTX over a wide dose range (0.01-5 mg/kg) in eleven inbred strains (Dym et al., 2007), and following the DA D1 antagonist, SCH23390 (SCH), but not the DA D2 antagonist, raclopride over a wide dose range (50-
1600 nmol/kg) in eight inbred strains (Dym et al., 2009). Correspondingly, marked strain differences occurred in the reduction of fat (Intralipid) following NTX and SCH in 8 inbred strains (Dym et al., 2010). In addition to the intrinsic palatability of sugars, sucrose and fructose illicit conditioned flavor preferences (CFP) that are also subject to marked differences across eight inbred strains (Pinhas et al., 2012).

Two of these inbred strains, SWR and BALB/c mice, displayed the most robust and durable sucrose-CFP (Pinhas et al., 2012), and thus were evaluated for DA D1 and opioid antagonist mediation of the expression and acquisition of sucrose-CFP (Dym et al., 2012). Whereas sucrose-CFP expression in both BALB/c and SWR mice was significantly reduced by SCH and NTX, sucrose-CFP acquisition was significantly reduced by NTX, but not SCH in BALB/c mice, and by SCH, but not NTX in SWR mice (Dym et al., 2012). A subsequent study (Kraft et al., 2013) demonstrated that these strains displayed fat (Intralipid)-CFP, and that its acquisition and expression were significantly reduced by SCH and NTX in BALB/c mice, but only by SCH in SWR mice. SWR and BALB/c mice, which possess different variants of the Tas1r3 taste receptor gene, thereby making the SWR strain more sweet-sensitive than the BALB/c strain (Reed et al., 2004), also display interesting associations and dissociations in their sensitivity to DA D1 and opioid antagonist effects on intrinsic sugar and fat intake. Whereas SCH produced comparable inhibition of sucrose intake in SWR and BALB/c mice (Dym et al., 2009), it produced potent inhibition of fat (Intralipid) intake in SWR, but no effects in BALB/c mice (Dym et al., 2010). It is unknown whether these effects are due to differences in DA D1 receptor binding between these two strains. Whereas NTX moderately inhibited sucrose intake in BALB/c mice, it was ineffective in SWR mice (Dym et al., 2007). Conversely, NTX suppressed Intralipid intake in SWR, but not BALB/c mice (Dym et al., 2010).

Although no studies have examined whether these two strains differ in opioid receptor binding, SWR mice display attenuated opioid-mediated responses to rewards as evaluated in morphine self-
administration (Belknap et al., 1993) and conditioned place preference (Gieryk et al., 2010; Solecki et al., 2009).

Because sugar-CFP studies also involve comparisons between sucrose and fructose as the CS+ on the one hand, and saccharin as the CS- on the other hand, it is important to determine whether any opioid-mediated or DA D1-mediated pharmacological effect on these learned responses paralleled their effects upon saccharin and fructose intake per se in SWR and BALB/c mice. Opioid control of saccharin intake has only been demonstrated in rats with NTX-induced suppression (Cooper, 1983; Lynch, 1986; Lynch and Libby, 1983), but DA D1 receptor mediation of saccharin intake is unknown. Conversely, DA D1, but not D2 receptor mediation of fructose intake has been demonstrated in rats with SCH, but not raclopride suppressing intake (Pritchett and Hajnal, 2011), but opioid receptor mediation of fructose intake is unknown. Therefore, the present study investigated whether DA D1 (SCH) and opioid (NTX) receptor antagonism altered intakes of fructose (8%) and saccharin (0.2%) solutions in these two inbred strains of mice over an identical dose range and time course as was performed for sucrose (Dym et al., 2007, 2009) and Intralipid (Dym et al., 2010) intake.

**Materials and Methods**

*Subjects:* Inbred BALB/c and SWR male mice (Jackson Laboratories, Bar Harbor, ME, 6 weeks of age) were acclimated to the Queens College vivarium for one week in group (5 per cage) housing. The animals were then housed individually for one-two weeks in plastic cages (30 x 20 x 15 cm) with stainless steel tops before testing in a room maintained on a 12 h light/12 h dark cycle (lights off at 2000 h) and constant temperature of 22°C. They were provided with chow (Lab Diet Mouse Chow 5015) and water *ad libitum* except as noted below. The experimental procedures were approved by the Queens College Institutional Animal Care and Use Committee certifying that all
subjects and procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

**Procedure:** At 3–7 h into the light cycle, chow and water were removed from the cage, and each animal was given 2-h access to either an 8% fructose solution or a 0.2% saccharin solution (Sigma Chemical Co., St. Louis, MO). The solution was presented in a 10-ml plastic syringe fitted with a stainless steel sipper tube (Dym et al., 2007, 2009, 2010). Intakes were measured to the nearest 0.1 ml at 5, 15, 30, 60, 90, and 120 min and then food and water rations were returned. The animals were trained to drink their sweetener solution until a criterion minimum of 1 ml was consumed over three consecutive exposures; three to five sessions were typically needed to reach this criterion. This criterion was employed to avoid “floor effects” of antagonist treatment.

Following this initial training period, each mouse received a session in which an intraperitoneal (ip) vehicle injection (0.3 ml distilled water/30 g body weight, 10 ml/kg) was administered 30 min prior to the presentation of the fructose or saccharin solution, and intake was measured as above for 2 h. On a subsequent test day, fructose or saccharin intake tests were then conducted 30 min following administration of either SCH (Sigma Chemical Co., St Louis, MO) at doses of 50, 200, 400, 800 and 1600 nmol/kg or NTX (Sigma Chemical Co.) at doses of 0.01, 0.1, 1.0 and 5.0 mg/kg. These dose ranges were identical to those used in strain surveys of DA D1 and opioid antagonist effects on sucrose and Intralipid intake (Dym et al., 2007, 2009, 2010). The group sizes were: a) BALB/c mice (SCH-fructose, n=9; NTX-fructose, n=9; SCH-saccharin, n=8; NTX-saccharin, n=8) and b) SWR mice (SCH-fructose, n=8; NTX-fructose, n=8, SCH-saccharin, n=8; NTX-saccharin, n=9). Half of the animals of each strain in each drug condition, matched for vehicle fructose or saccharin intake, received an ascending series of SCH or NTX doses, whereas the remainder received a descending series of SCH or NTX doses to control for drug-order effects. Exposure to ascending or descending dose orders failed to produce any differences in antagonist-
induced actions in any of the paradigms. A minimum of 72 h elapsed between injections to minimize carry-over effects as done previously (Dym et al., 2007, 2009, 2010); animals were not tested with "saccharin or fructose" during these intervening days.

Statistics: Separate two-way repeated-measures analyses of variance were performed on fructose or saccharin intake of BALB/c or SWR mice with drug dose as one within-subject variable, and the six intake time points as the second within-subject variable. Bonferroni comparisons (P<0.05) evaluated significant drug effects within groups. Post-drug intake difference scores were also calculated by subtracting fructose or saccharin intake 60 min following each SCH or NTX dose condition from corresponding vehicle intake for each animal in each strain. Then linear regression analyses were performed for each strain with SCH or NTX dose as the independent variable and the difference scores for each mouse in each strain as the dependent variable to determine the dose that would inhibit fructose or saccharin intake by 40% (ID40). A criterion of a dose necessary to produce a 40% inhibition of intake was chosen relative to the more typical ID50 criterion for the following reasons. First, in all cases, a 40% inhibition in intake by the antagonist was always significantly different from vehicle. Second, the use of the ID50 relative to the ID40 yielded effective doses that were invariably within and rarely outside of the actual 50-1600 nmol/kg SCH and the 0.01-5 mg/kg NTX dose ranges used, resulting in interpolated as compared to extrapolated data. To evaluate differences in baseline intake of fructose and saccharin, a three-way randomized-block analysis of variance compared vehicle intake of the two strains of the two solutions across the six intake times. Two-way analyses of variance examined solution and time effects for BALB/c and SWR mice.

Results

DA D1 Antagonist Effects on Fructose and Saccharin Intake in BALB/c and SWR Mice.

BALB/c mice: Significant differences in fructose intake were observed among SCH doses (F(5,40)= 6.48, p<0.0002), across test times (F(5,200)= 51.57, p<0.0001) and for the interaction
between doses and times (F(25,200)= 1.58, p<0.046). Significant reductions in fructose intake were noted following the 200 (5-30 min), 400 (5-120 min), 800 (5-120 min) and 1600 (15-60, 120 min), but not the 50 nmol/kg SCH doses (Figure 4A). Significant differences in saccharin intake were observed among SCH doses (F(5,42)= 15.70, p<0.0001), across test times (F(5,210)= 88.28, p<0.0001) and for the interaction between doses and times (F(25,210)= 8.58, p<0.0001). Significant reductions in saccharin intake were noted following the 50 (60 min), 200 (5-120 min), 400 (5-120 min), 800 (5-120 min) and 1600 (5-120 min) nmol/kg SCH doses (Figure 4B). Thus, BALB/c mice exhibit DA D1 dose-dependent antagonist-induced reductions in both fructose and saccharin intakes with the effects on saccharin observed at the lowest SCH dose and for more extended periods across the time course.

SWR mice: Significant differences in fructose intake were observed among SCH doses (F(5,42)= 8.24, p<0.0001), across test times (F(5,210)= 77.38, p<0.0001) and for the interaction between doses and times (F(25,210)= 3.27, p<0.0001). Significant reductions in fructose intake were noted following the 200 (5-120 min), 400 (5-120 min), 800 (15-120 min) and 1600 (5-120 min), but not the 50 nmol/kg SCH doses (Figure 5A). Significant differences in saccharin intake were observed among SCH doses (F(5,42)= 12.46, p<0.0001), across test times (F(5,210)= 68.54, p<0.0001) and for the interaction between doses and times (F(25,210)= 8.89, p<0.0001). Significant reductions in saccharin intake were noted following the 50 (30-120 min), 200 (5-120 min), 400 (5-120 min), 800 (15-120 min) and 1600 (5-120 min) nmol/kg SCH doses (Figure 5B). Thus, SWR mice exhibit DA D1 dose-dependent antagonist-induced reductions in both fructose and saccharin intakes with the effects on saccharin observed at the lowest SCH dose with comparable time course effects.
Figure 4. (Chapter 4) Alterations (mean, ±SEM) in fructose (8%, upper panel) and saccharin (0.2%, lower panel) intake following the five doses of the D1 dopamine receptor antagonist, SCH23390 in BALB/c mice. Significant differences in this and the next three figures in intake following specific drug doses relative to corresponding vehicle intake are denoted (*). The ID₅₀, calculated by subtracting fructose or saccharin intake 60 min following each dose condition from corresponding vehicle intake for each animal in each strain, and then performing linear regression analyses with dose as the independent variable and the difference scores for each mouse in each strain as the dependent variable, is indicated in this and all subsequent figures.
Figure 5. (Chapter 4) Alterations (mean±SEM) in fructose (8%, upper panel) and saccharin (0.2%, lower panel) intake following the five doses of the D1 dopamine receptor antagonist, SCH23390 in SWR mice.
Opioid Antagonist effects on Fructose and Saccharin Intake in BALB/c and SWR Mice.

**BALB/c mice:** Significant differences in fructose intake were observed among NTX doses (F(4,40)= 6.82, p<0.0003), across test times (F(5,200)= 180.68, p<0.0001) and for the interaction between doses and times (F(20,200)= 6.91, p<0.0016). Significant reductions in fructose intake were noted following the 0.1 (5 min), 1 (5 min), and 5 (5-15, 60-120 min), but not the 0.01 mg/kg NTX doses (Figure 6A). Significant differences in saccharin intake were observed among NTX doses (F(4,35)= 22.30, p<0.0001), across test times (F(5,175)= 188.75, p<0.0001) and for the interaction between doses and times (F(20,175)= 18.68, p<0.0001). Significant reductions in saccharin intake were noted following the 0.1 (15-120 min), 1 (15-120 min), and 5 (5-120 min), but not the 0.01 mg/kg NTX doses (Figure 6B). Thus, BALB/c mice exhibited opioid dose-dependent antagonist-induced reductions in saccharin intake, but NTX effects on fructose intake were limited to the highest drug dose.

Comparisons of baseline consumption and ID₅₀ effects of DA D1 and Opioid antagonists on saccharin and fructose intake in BALB/c and SWR Mice.

**Baseline consumption:** To evaluate if there were differences in the baseline intakes of fructose and saccharin in the two strains, intake following vehicle treatment was analyzed. Significant differences were observed over time (F(5,40)= 98.13, p<0.0001) and for the interaction between solutions and times (F(5,40)= 16.81, p<0.0001). Overall, the two strains did not differ in their fructose and saccharin intakes. BALB/c mice did consume significantly more saccharin (1.64 ml) than fructose (1.34 ml) at 120 min, and SWR mice consumed significantly more saccharin (1.36, 1.75 ml) than fructose (1.10, 1.36 ml) at 90 and 120 min.
Figure 6. (Chapter 4) Alterations (mean±SEM) in fructose (8%, upper panel) and saccharin (0.2%, lower panel) intake following the four doses of the Opioid receptor antagonist, Naltrexone in BALB/c mice.
**ID_{50} analyses:** (Table 1) The ID_{50} data for SCH-induced inhibition of *saccharin* intake was virtually identical (<50 nmol/kg) for both BALB/c (Figure 4B) and SWR (Figure 5B) mice. The ID_{50} data for NTX-induced inhibition of *saccharin* intake were also very low for BALB/c (0.9 mg/kg, Figure 6B) and SWR (0.02 mg/kg, Figure 7B). In contrast, effective inhibition of *fructose* intake by SCH was more than 4-fold greater in SWR (ID_{50} = 298 nmol/kg, Figure 5A) relative to BALB/c (ID_{50} = 1234 nmol/kg, Figure 7A) mice, revealing greater potencies of DA D1 signaling effects in the former strain. The effective inhibition by NTX on *fructose* intake was more than a 1.9-fold greater in SWR (ID_{40} = 2.59 mg/kg, Figure 4A) relative to BALB/c (ID_{40} = 4.99 mg/kg, Figure 3A) mice, revealing greater potencies of opioid signaling effects in the former strain.

**Discussion**

The present study investigated inbred strain differences between BALB/c and SWR mice in the suppressive effects on *fructose* and *saccharin* intake produced by DA D1 and opioid receptor antagonists over an identical dose range and time course as was previously performed for *sucrose* intake. Whereas *sucrose* intake was significantly reduced by SCH and NTX in BALB/c mice, only SCH was effective in SWR mice (Dym et al., 2007, 2009). Further, *Intralipid* intake was significantly reduced by SCH and NTX in SWR, but not in BALB/c mice (Dym et al., 2010). The present study demonstrated that although SCH and NTX significantly reduced *fructose* and *saccharin* intake in the two strains, they did so with differential strain-specific inhibitory magnitudes. Thus, although *saccharin* intake was similarly reduced by SCH and NTX in BALB/c and SWR mice, greater potencies of opioid (1.9-fold) and DA D1 (4-fold) receptor antagonism of *fructose* intake were noted in SWR relative to BALB/c mice, indicating strong strain differences. Before examining DA D1 and opioid antagonist effects, it must be noted that *saccharin* and *fructose* differ from *sucrose* on several
Figure 7. (Chapter 4) Alterations (mean, ±SEM) in fructose (8%, upper panel) and saccharin (0.2%, lower panel) intake following the four doses of the opioid receptor antagonist, naltrexone in SWR mice.
Table 1. (Chapter 4) Linear regression values (ID40) of SCH23390 (SCH, nmol/kg)- and naltrexone (NTX, mg/kg)-induced inhibition (60 min) of saccharin (0.2%), fructose (8%), sucrose (10%) and intralipid (5%) intakes in BALB and SWR mice. (Dym et al., 2007, 2009) and Intralipid (Dym et al., 2010)

<table>
<thead>
<tr>
<th>Condition</th>
<th>BALB</th>
<th>SWR</th>
<th>BALB</th>
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<td></td>
<td>SCH</td>
<td>SCH</td>
<td>NTX</td>
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<td>0.02 mg/kg</td>
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<tr>
<td>Fructose</td>
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<td>298 nmol/kg</td>
<td>4.99 mg/kg</td>
<td>2.59 mg/kg</td>
</tr>
<tr>
<td>Sucrose</td>
<td>638 nmol/kg</td>
<td>811.5 nmol/kg</td>
<td>3.61 mg/kg</td>
<td>6.86 mg/kg</td>
</tr>
<tr>
<td>Intralipid</td>
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<td>&lt;50 nmol/kg</td>
<td>11.85 mg/kg</td>
<td>0.88 mg/kg</td>
</tr>
</tbody>
</table>

- Data from Dym et al., 2007 and 2009
- Data from Dym et al., 2010
dimensions: 1) sweet taste intensity, 2) palatability, 3) nutritional/caloric value and 4) post-oral reinforcing effects. For C57BL/6 mice, the rank-order of preferences for comparable sweetener concentrations are sucrose > fructose ~ saccharin (Glendinning et al., 2010; Sclafani, unpublished data). This may also be true for SWR and BALB/c mice, but limited data are available to support this contention. The baseline intake finding that SWR and BALB/c mice consumed more saccharin than fructose after 90 and/or 120 min might suggest that saccharin was the more palatable sweetener, but one-bottle intakes do not necessarily reflect relative palatability. Rather, the mice may have consumed less fructose because it was more satiating than saccharin. The relatively similar baseline intakes of saccharin and fructose observed in BALB/c and SWR mice is somewhat surprising given the known differences in their sweet taste sensitivity. In particular, the two strains have different variants of the Tas1r3 taste receptor gene, which makes the SWR strain more sweet-sensitive than the BALB/c strain (Reed et al., 2004). Consistent with this difference, in a study of 24-h sucrose vs. water preferences, we observed greater sucrose preferences and intakes in SWR mice than BALB/c mice (Lewis et al., 2005). Strain differences in sweet taste sensitivity and 24-h sweetener intakes are not always reflected, however, in short-term intake measures. In a prior study, we observed that sweet sensitive and sub-sensitive mouse strains (including SWR and BALB/c) did not differ in their 1-h intakes of flavored 16% sucrose, 16% fructose or 8% fructose solutions (Pinhas et al., 2012). Implications of these findings are evaluated further in the General Discussion.
Chapter 5

DOPAMINE D1 AND OPIOID RECEPTOR ANTAGONISTS DIFFERENTIALLY REDUCE THE ACQUISITION AND EXPRESSION OF FRUCTOSE-CONDITIONED FLAVOR PREFERENCES IN BALB/c AND SWR MICE

Introduction

In addition to inborn preferences for sweet taste, learning plays an important role in modulating the appetite for sugar-rich foods. This is demonstrated in studies using the conditioned flavor preference (CFP) paradigm in which animals are trained to associate an arbitrary flavor (the conditioned stimulus, CS+) with a sugar solution and another flavor (the CS-) with water or a non-nutritive sweetener solution. Rats displayed a preference for a CS+ flavor mixed in a sucrose solution over a CS- flavor mixed in water (Mehiel & Bolles, 1988). Sucrose-CFP has been shown to be mediated by both orosensory and post-ingestive factors in rats with the orosensory component defined as flavor-taste conditioning produced by the sugar's sweet taste that is distinguished from flavor-nutrient conditioning produced by the sugar's post-ingestive effects (Sclafani, 1995). Flavor-nutrient learning has been extensively studied by training rats to associate a CS+ flavor with intragastric (IG) sucrose infusions and a CS- flavor with IG water infusions (Azzara et al., 2000, 2001). Flavor-taste learning has been studied by training rats to sham-feed a CS+ flavored sucrose solution and a CS- flavored saccharin solution; the sucrose, like the saccharin, drains out a gastric fistula and therefore has minimal post-ingestive nutritive actions (Yu et al., 1999, 2000a, 2000b). We have also investigated flavor-taste learning by training rats to "real-feed" a CS+ flavored fructose solution (Baker et al., 2003, 2004). The fructose-CFP was assumed to be reinforced by the sugar's sweet taste only because, unlike sucrose or glucose, IG fructose solutions failed to produce a CFP in rats using short daily training sessions (Sclafani et al., 1993, 1999; Ackroff et al., 2001).
Sugar intake in rats is mediated in part by brain dopamine (DA) (e.g., Geary and Smith, 1985; Muscat and Willner, 1989; Schneider et al., 1986; Sclafani et al., 1982) and opioid (e.g., Glass et al., 1996; Kirkham and Cooper, 1988; Levine et al., 1982, 1995; Rockwood and Reid, 1982; Sclafani et al., 1982) transmitter systems. In initial studies, we investigated the roles of DA and opioid systems in sugar-CFP. DA D1 or D2 receptor antagonism with SCH23390 (SCH) or raclopride, respectively, significantly attenuated the acquisition and expression of sucrose-CFP in sham-feeding rats (Yu et al., 2000a, 2000b), and the acquisition and expression of fructose-CFP in real-feeding rats (Baker et al., 2003), implicating these receptor subtypes in flavor-taste learning. DA D1, but not D2 receptor antagonism blocked CFP induced by IG sucrose in rats (Azzara et al., 2001), implicating this receptor subtype in flavor-nutrient learning. In contrast, opioid receptor antagonism with naltrexone (NTX) significantly reduced sugar intakes, but failed to affect the acquisition or expression of CFP in rats produced by sham-fed sucrose (Yu et al., 1999), real-fed fructose (Baker et al., 2004), or IG sucrose (Azzara et al, 2000).

In recent studies, we have extended our study of sugar intake and conditioning to inbred mouse strains. Pinhas et al. (2012) investigated strain differences in sucrose- and fructose-CFP. The food-restricted mice were trained (1 h/day) to real-drink CS+ flavored sugar and CS- flavored saccharin solutions in alternate one-bottle sessions and were then given two-bottle tests between the CS+ and CS- flavors both presented in saccharin solutions. SWR and BALB/c mice displayed the most robust and persistent CFP responses of the eight inbred strains tested. Other studies investigated the effects of DA and opioid receptor antagonists on sugar and saccharin intakes in these and other mouse strains. Whereas NTX significantly reduced sucrose intake in BALB/c mice, it was rather ineffective in SWR mice (Table 2: Dym et al., 2007). In contrast, SCH produced comparable reductions in sucrose intake in BALB/c and SWR mice (Table 2: Dym et al., 2007). Raclopride inhibited sucrose intake in these and other murine strains only at very high doses, and was not
examined in further studies (Dym et al., 2009). With respect to fructose, SCH and NTX were more effective in reducing intake in SWR than BALB/c mice (Kraft et al., 2015a). Both DA D1 and opioid receptor antagonists were very effective in reducing saccharin intake in both strains (Kraft et al., 2015a). Thus, selective strain differences were observed in the abilities of DA D1 and opioid antagonists to reduce nutritive and non-nutritive sweetener intakes in mice. Dym et al., (2012) examined the effects of DA D1 and opioid receptor antagonism on sucrose-CFP in BALB/c and SWR mice using the conditioning paradigm developed by Pinhas et al. (2012). The expression of a previously-learned sucrose-CFP was significantly reduced in both BALB/c and SWR strains by SCH and NTX injections. In contrast, the acquisition of a sucrose-CFP was significantly reduced by NTX, but not SCH in BALB/c mice, and by SCH, but not NTX in SWR mice, indicating a double dissociation in the pharmacological effects on sucrose-CFP in the two strains.

In the present study, we investigated the effects of DA D1 and opioid receptor antagonists on the expression and acquisition of fructose-CFP in BALB/c and SWR mice. This was of interest because, as mentioned above, prior studies indicated that sucrose and fructose differ in their conditioning actions. Real-fed sucrose involves both flavor-taste and flavor-nutrient learning. In contrast, fructose-CFP was assumed to be reinforced only by sweet taste (flavor-taste learning) because IG fructose solutions failed to produce a CFP in rats as well as in C57BL/6 mice (Sclafani & Ackroff, 2012; Zukerman et al., 2013a,b). Thus, the disruptive effects of DA and opioid antagonists on fructose-CFP may differ from those observed with sucrose-CFP.

Materials and Methods

Subjects: Inbred BALB/c (Stock #000651) and SWR (Stock #000689) male mice (Jackson Laboratories, Bar Harbor, ME, 6 weeks of age, both strains ~25 g at arrival) were acclimated to the
Table 2. (Chapter 5) Linear regression values (ID40) of SCH23390 (SCH, nmol/kg)- and naltrexone (NTX, mg/kg)-induced inhibition (60 min) of saccharin (0.2%), fructose (8%) and sucrose (10%) intakes in BALB/c and SWR mice.

<table>
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<th>BALB/c</th>
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<td>Saccharin</td>
<td>&lt;50 nmol/kg</td>
<td>&lt;50 nmol/kg</td>
<td>0.9 mg/kg</td>
<td>0.02 mg/kg</td>
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<tr>
<td>Fructose</td>
<td>1234 nmol/kg</td>
<td>298 nmol/kg</td>
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<td>6.86 mg/kg</td>
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</table>

*Data from Kraft et al., 2015a
*Data from Dym et al., 2007 and 2009
Queens College vivarium for one week in group (5 per cage) housing. The animals were then housed individually in plastic cages (30 x 20 x 15 cm) with stainless steel tops throughout the study, and maintained on a 12 h light/12 h dark cycle (lights off at 2000 h) at a constant temperature of 22°C. Chow (Lab Diet Mouse Chow 5015) and water were provided. Two weeks before testing began; the mice were placed on a food restriction schedule in which 2-3 g of chow was placed in their cages daily. The mice were weighed just prior to food-restriction, and their body weights were monitored until they gradually achieved 85-90% of their ad libitum level. The mice were maintained at that restricted level by limiting their intake throughout the experiment. The two strains maintained body weights between 28 and 35 g, and there were no weight differences between the strains. Individual mice were tested in one experimental paradigm. The experimental procedures were approved by the Queens College Institutional Animal Care and Use Committee certifying that all subjects and procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Fructose-CFP Expression Procedure: Food-restricted mice were initially trained (1 h/day) to drink an unflavored 0.2% saccharin solution from a stainless steel sipper tube connected to a 10 ml plastic syringe (Dym et al., 2012; Pinhas et al., 2012). This training procedure was repeated daily until all mice sampled the sipper tubes with short (< 1 min) latency, typically within three days. Then two training solutions, 8% fructose + 0.2% saccharin and 0.2% saccharin, flavored with 0.05% unsweetened grape or cherry Kool-Aid (Kraft Foods, White Plains, NY) were presented. For half of the mice in each strain, the CS+ flavor added to the fructose-saccharin solution was cherry and the CS- flavor added to the saccharin solution was grape; the flavor-sweetener pairs were reversed for the remaining animals. In the two-bottle preference tests, the CS+ and CS- flavors were each presented in 0.2% saccharin solutions as in our prior studies (Dym et al., 2012; Pinhas et al., 2012). All testing took place in each mouse’s home cage during the mid-light phase of the light:dark cycle. The limited
food rations were given 1 h after each training and testing session. Note that the 8% fructose concentration combined with 0.2 % saccharin used in this study differs from that of our prior sucrose conditioning study (16%) (Dym et al., 2012) because we found that that 8% fructose and 0.2% saccharin conditioned a stronger preference than 16% fructose in SWR and BALB/c mice (Pinhas et al., 2012).

Twenty-four BALB/c and 23 SWR inbred mice received ten one-bottle training sessions (1 h/day) with 8 ml of the CS+/fructose solution presented on odd-numbered days, and 8 ml of the CS-/saccharin solution presented on even-numbered days. On days 9 and 10, all mice had access to a second sipper tube containing water. This familiarized them to the presence of two sipper tubes used during the choice tests; water intake was negligible in these training trials. The position of the CS and water sipper tubes varied across the 2-bottle training and subsequent testing days using a left-right-right-left pattern. Solution intakes during training were measured by weighing (0.1 g) the sipper tubes before and after the 1-h sessions.

Following training, the mice were given six two-bottle choice test sessions (1 h/day) with access to the CS+ and CS- flavors mixed in 0.2% saccharin solutions. Thirty min prior to the first two days of two-bottle sessions, all mice were given an intraperitoneal (ip) vehicle (0.9% normal saline) injection. Then on subsequent days, 12 BALB/c and 10 SWR mice received 200 and 800 nmol/kg doses of SCH prior to two test sessions each as performed previously (Dym et al., 2012), with half tested with an ascending dose order and the remainder with a descending dose order. SCH was mixed at concentrations of 20 and 80 nmol/ml and administered at 10 ml/kg body weight. Additionally, 12 BALB/c and 11 SWR mice received 1 and 5 mg/kg doses of NTX as performed previously (Dym et al., 2012), with half tested with an ascending dose order and the remainder with a descending dose order. NTX was mixed at concentrations of 0.1 and 0.5 mg/ml and administered at 10 ml/kg body weight.
Fructose-CFP Acquisition Procedure: BALB/c and SWR mice received 10 one-bottle training sessions (1 h/day) with the CS+/Fructose solution presented on odd-numbered sessions, and the CS-/Saccharin solution presented on even-numbered sessions. Thirty min prior to each training session, intraperitoneal injections of vehicle (BALB/c: n= 6; SWR: n= 6), SCH (50 nmol/kg; BALB/c: n= 7; SWR: n= 7), or NTX (1 mg/kg; BALB/c: n= 6; SWR: n= 8) were administered as performed previously (Dym et al., 2012). To control for possible drug effects on CS training intake, which could influence conditioning, a fourth group (Limited Control: LTD CON) of BALB/c (n= 6) and SWR (n= 10) mice received vehicle injections throughout one-bottle training, but intakes of CS+/Fructose and CS-/Saccharin solutions were limited to the mean 1 h intakes of the SCH and NTX groups by exposing the animals during training to 15 min on the CS+ days and 20 min on the CS- days in each session. Following training, all mice were given six daily 2-bottle choice sessions (1 h/day) with access to CS+ and CS- flavored 0.2% saccharin solutions without injections. The positions of the CS+ and CS- solutions were counterbalanced across testing sessions, and the results were analyzed as mean 1 h intakes during successive pairs of sessions (referred to as Tests 1, 2 and 3).

Statistics: In expression, training intakes of the average of the five CS+/Fructose and five CS-/Saccharin sessions were determined for each strain, and evaluated for strain and CS differences using a two-way randomized-block analysis of variance (ANOVA). Expression of fructose-CFP in two-bottle preference tests was evaluated in a two-way ANOVA for strain differences following vehicle treatment. Vehicle intakes during the two-bottle preference tests were averaged over the two sessions and evaluated for strain differences in a two-way ANOVA. Subsequent two-ways ANOVAs evaluated drug effects within each strain (CS solution vs. Dose). Percent CS+ preference was calculated for each animal in each condition in the following manner: CS+ intake / Total Intake x 100. Mean percent CS+ preference was calculated for each group by averaging the individual scores. Separate ANOVAs evaluated percent CS+ intakes as a function of drug dose for each strain as well as
comparing strain differences. In acquisition, training intakes of the average of the five CS+/Fructose and five CS-/Saccharin sessions for the three (Veh, SCH, NTX) training groups were determined for each strain, and were evaluated for strain, drug group and CS differences using a three-way randomized-block ANOVA (Strain x Groups x CS). A three-way ANOVA compared the CS intakes of the four groups (Group x CS x Test). Separate two-way ANOVAs evaluated percent CS+ intakes of the four groups, whereas a 3-way ANOVA compared strain differences in percent CS+ intakes. When main or interaction effects were found, Tukey comparisons (p<0.05) were employed to detect significant effects. Drug-induced reductions in the acquisition or expression of fructose-CFP are operationally defined as a significant reduction in percent CS+ intakes and/or a failure to observe significant differences between CS+ and CS- intakes in the two-bottle preference tests.

Results

DA D1 and opioid receptor antagonism and Fructose-CFP expression in BALB/c and SWR mice. During training, the SWR mice consumed more of the CS solutions than did the BALB/c mice (F(1,23)= 182.54, p<0.0001). There was a CS x Strain interaction (F(1,23)= 86.48, p<0.0001), however, and the BALB/c mice consumed more CS+ than CS- (1.02±0.08 and 0.69±0.08 ml/h, respectively) while the SWR mice consumed less CS+ than CS- (1.42±0.08 and 2.24±0.12 ml/h, respectively). In the vehicle two-bottle tests, both BALB/c and SWR strains consumed more CS+ than CS- (F(1,23)= 123.10, p<0.0001). Note that the SWR mice consumed more CS+ than BALB/c (2.00±0.21 vs. 0.88±0.09 ml), while the two strains did not differ in CS- intakes (0.43±0.06 vs. 0.18±0.03 ml; CS x Strain interaction, (F(1,23)= 17.48, p<0.0001). The two groups, however, displayed similar CS+ percent preferences in the vehicle tests. Because the two strains showed dramatic differences in their CS+ two-bottle intakes, DA D1 and opioid antagonist effects on the expression of fructose-CFP were evaluated separately for each strain.
DA D1 Receptor Antagonism of Fructose-CFP Expression in BALB/c Mice. In the two-bottle tests following SCH, BALB/c mice overall consumed significantly more CS+ than CS- (F(1,33)= 53.29, p<0.0001), and there were significant dose (F(2,33)= 35.12, p<0.0001) and CS x Dose interaction (F(2,33)= 25.34, p<0.0001) effects. CS+ intakes significantly exceeded CS- intakes following vehicle, but not the 200 or 800 nmol/kg SCH doses (Figure 8A). BALB/c mice consumed significantly less CS+ at both SCH doses compared to vehicle; CS- intakes failed to differ as a function of SCH dose (Figure 8A). Total CS intakes were significantly (F(2,22)= 29.54, p<0.0001) lower following the 200 (0.28 ml) and 800 (0.25 ml) nmol/kg SCH doses relative to vehicle (1.1 ml). Percent CS+ intake was significantly reduced (F(2,22)= 5.83, p<0.01) by the 200 (59%) and 800 (42%) nmol/kg SCH doses relative to vehicle (86%) (Figure 8A). These data indicate that the magnitude of fructose-CFP expression was reduced by DA D1 receptor antagonism in BALB/c mice.

DA D1 Receptor Antagonism on Fructose-CFP Expression in SWR Mice. In the two-bottle tests following SCH, SWR mice overall consumed significantly more CS+ than CS- (F(1,27)= 37.20, p<0.0001) and there were significant dose (F(2,27)= 15.18, p<0.0001) and CS x Dose interaction (F(2,27)= 10.47, p<0.001) effects. CS+ intakes significantly exceeded CS- intakes following vehicle, but not the 200 or 800 nmol/kg SCH doses (Figure 8B). SWR mice consumed significantly less CS+ at both SCH doses compared to vehicle; CS- intake failed to differ as a function of SCH dose. Total CS intakes significantly (F(2,18)= 15.64, p<0.0001) declined following the 200 (1.14 ml) and 800 (0.74 ml) nmol/kg SCH doses relative to vehicle (2.76 ml). Percent CS+ intake was significantly reduced (F(2,18)= 4.01, p<0.05) by the 200 (71%) and 800 (63%) nmol/kg SCH doses relative to vehicle (85%) (Figure 8B). These data indicate that the magnitude of fructose-CFP expression was reduced by DA D1 receptor antagonism in SWR mice.
**Figure 8.** (Chapter 5) (Fructose-CFP Expression). Intakes (mean +SEM, g/1 h) of CS+ and CS- solutions in two-bottle tests in BALB/c (upper panel) and SWR (lower panel) inbred mice receiving systemic injections of the DA D1-like antagonist, SCH23390 at doses of 0, 200 and 800 nmol/kg 30 min prior to testing. Significant differences are denoted between CS+ and CS- intake within an injection condition (*) and between CS+ intake following a drug dose relative to the vehicle treatment (+). The percentages of CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted.
Strain Differences in DA D1 Antagonism of Fructose-CFP Expression. Analyses of strain differences in SCH-induced changes in percent CS+ intakes revealed significant differences among doses (F(2,22)= 12.52, p<0.001), but not between strains or for the interaction between strains and doses, indicating that percent CS+ intakes following SCH treatment did not differ between strains.

Opioid Receptor Antagonism in Fructose-CFP Expression in BALB/c Mice. In the two-bottle tests following NTX, BALB/c mice consumed significantly more CS+ than CS- (F(1,33)= 60.31, p<0.0001), and there were significant dose (F(2,33)= 3.34, p<0.048), but not CS x Dose interaction effects. CS+ intakes exceeded CS- intakes following vehicle and both NTX doses (Figure 9A). Total CS intakes significantly (F(2,22)= 4.55, p<0.05) declined following the 5 mg/kg NTX dose (0.6 ml) relative to vehicle (1.0 ml) or the 1 (1.0 ml) mg/kg NTX dose. Percent CS+ intakes failed to differ following the 0 (79%), 1 (82%) and 5 (87%) mg/kg NTX doses (Figure 9A). These data indicate that the magnitude of fructose-CFP expression failed to be affected by opioid receptor antagonism in BALB/c mice.

Opioid Receptor Antagonism on Fructose-CFP Expression in SWR Mice. In the two-bottle tests following NTX, SWR mice consumed significantly more CS+ than CS- (F(1,30)= 55.06, p<0.0001), but neither dose nor CS x Dose interaction effects were significant. CS+ intakes significantly exceeded CS- intakes following vehicle and both NTX doses (Figure 9B). Total CS intakes failed to differ among vehicle (2.1 ml) and the 1 (2.1 ml) and 5 (1.5 ml) mg/kg NTX doses. Percent CS+ intakes did not differ following the 0 (77%), 1 (73%) and 5 (75%) mg/kg NTX doses (Figure 9B). These data indicate that the fructose-CFP expression failed to be affected by opioid receptor antagonism in SWR mice.

Strain Differences in Opioid Antagonism of Fructose-CFP Expression. There were no strain differences in the effects of opioid antagonism on percent CS+ intakes among doses, between strains or for the interaction between strains and doses.
**Figure 9.** (Chapter 5) (Fructose-CFP Expression). Intakes (mean +SEM, g/1 h) of CS+ and CS- solutions in two-bottle tests in BALB/c (upper panel) and SWR (lower panel) inbred mice receiving systemic injections of the general opioid antagonist, naltrexone at doses of 0, 1 and 5 mg/kg 30 min prior to testing. Significant differences are denoted between CS+ and CS- intake within an injection condition (*) and between CS+ intake following a drug dose relative to the vehicle treatment (+). The percentages of CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted.
**DA D1 and Opioid receptor antagonism on Fructose-CFP acquisition in BALB/c and SWR mice.** The two strains were initially evaluated for differences in one-bottle training intakes in the acquisition paradigm. Overall, the SWR mice (2.34±0.08 ml/h) consumed significantly more of the CS solutions than did the BALB/c (1.54±0.04 ml/h; F(1,7)= 73.64, p<0.0001). Given this difference, the two-bottle preference test data in Vehicle, SCH, NTX and Limited Control groups were evaluated separately for each strain.

**Fructose-CFP Acquisition in BALB/c mice.** CS+/Fructose and CS-/Saccharin training intakes were significantly lower in the SCH and NTX groups relative to Vehicle, but not the Limited Control group (Figure 10A). In two-bottle tests, BALB/c mice overall consumed significantly more CS+ than CS- (F(1,6)= 63.32, p<0.0001), but significant differences were observed among groups (F(3,18)= 3.47, p<0.05), among tests (F(2,12)= 4.11, p<0.05), and for the interactions between groups and tests (F(6,36)= 6.54, p<0.05), groups and CS (F(3,18)= 4.50, p<0.05) and tests and CS (F(2,12)= 4.11, p<0.05). CS+ intake was significantly higher than CS- intake across all tests in the Vehicle (Figure 11A) and NTX (Figure 11B) groups. However, in the SCH group (Figure 11C), CS+ intake was significantly higher than CS- intakes in Test 1, but not Tests 2 and 3, suggesting that DA D1 antagonism hastened the extinction of an acquired fructose-CFP in BALB/c mice. In the Limited Control group (Figure 11D), CS+ intake was significantly higher in Tests 1 and 2, but not Test 3, suggesting that the lower CS+ and CS- training intakes reduced the duration of the fructose-CFP preference in this strain. Percent CS+ preference significantly differed across tests (F(2,12)= 4.14, p<0.05), but not among groups or for the interaction between groups and tests. Neither opioid nor DA D1 antagonism significantly altered percent CS+ preference across any test in BALB/c mice.
**Figure 10. (Chapter 5)** (Fructose-CFP Acquisition Training). Intakes (mean +SEM, g/1 h) of CS+/Fructose and CS-/Saccharin solutions in BALB/c (upper panel) and SWR (lower panel) mice pretreated 30 min earlier with vehicle (Veh), SCH23390 at a dose of 50 nmol/kg (SCH), naltrexone at a dose of 1 mg/kg (NTX) or vehicle and limited to drug-induced intake (LTD CON). Significant differences in CS+/Fructose or CS+/Saccharin intakes following SCH or NTX relative to corresponding Veh (+) are denoted. *Fructose-CFP Acquisition in SWR mice.* CS+/Fructose and CS-/Saccharin training intakes were significantly lower in the SCH and NTX groups relative to the Vehicle, but not the Limited Control group (Figure 10B).
Figure 11. (Chapter 5) (Fructose-CFP Acquisition). Intakes (mean +SEM, g/1 h) of CS+ and CS-solutions during two-bottle Tests 1, 2 and 3 in BALB/c mice receiving Veh (Panel A), naltrexone (Panel B), SCH23390 (Panel C) or the LTD CON condition (Panel D) during training. Significant differences are denoted between CS+ and CS- intake (*) and for drug treatment relative to corresponding Veh (+). The percentages of CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted.
In two-bottle tests, SWR mice overall consumed significantly more CS+ than CS- (F(1,9)= 107.08, p<0.0001), and significant differences were observed among tests (F(2,18)= 6.52, p<0.01), and for the interactions between groups and tests (F(6,54)= 8.65, p<0.001) and among groups, tests and CS (F(6,54) = 2.74, p<0.05). CS+ intakes significantly exceeded CS- intakes across all tests in the Vehicle (Figure 12A) and Limited Control (Figure 12D) groups. In the SWR NTX group, CS+ intakes were significantly higher than CS- intakes in Tests 1 and 2, but not Test 3 (Figure 12B), suggesting hastening of extinction. In the SCH group, CS+ and CS- intakes did not differ from each other in all three tests (Figure 12C), suggesting elimination of the preference. CS+ intake (Tests 1, 2 and 3) in the SCH group and CS+ intake (Test 3) in the NTX group were significantly lower than corresponding vehicle CS+ intakes. Percent CS+ preference was significantly different among groups (F(3,27)= 11.23, p<0.0001) and for the interaction between groups and tests (F(6,54)= 2.96, p<0.05), but failed to differ across tests. Percent CS+ preferences in the SCH group in Tests 1 (40%) and 2 (46%) (Figure 12C) were significantly lower than corresponding Tests 1 (81%) and 2 (93%) of the Vehicle group (Figure 12A), and Tests 1 (74%) and 2 (72%) of the Limited Control group (Figure 12D). These data indicate that DA D1 receptor antagonism blocked the acquisition of fructose-CFP in SWR mice, and that opioid receptor antagonism hastened the extinction of the fructose-CFP in SWR mice.

**Strain Differences in Fructose-CFP Acquisition.** Analyses of percent CS+ intakes in the BALB/c and SWR groups in the acquisition of fructose-CFP revealed significant differences among training drug conditions (F(3,27)= 14.21, p<0.0001) and for the interactions between strains and drug conditions (F(3,27)= 4.50, p<0.05), between strains and tests (F(2,18)= 5.65, p<0.05), between drug conditions and tests (F(6,54)= 4.14, p<0.01), and among strains, drug conditions and tests (F(6,54)= 2.66, p<0.05). SWR SCH mice displayed significantly lower percent CS+ intakes than BALB/c SCH mice (40% vs.75%) in Test 1, and the difference approached significance for in Test 2 (46% vs. 61%).
**Figure 12** *(Chapter 5)* (Fructose-CFP Acquisition). Intakes (mean ±SEM, g/1 h) of CS+ and CS-solutions during two-bottle Tests 1, 2 and 3 in SWR mice receiving Veh (Panel A), naltrexone (Panel B), SCH23390 (Panel C) or the LTD CON condition (Panel D) during training. Significant differences are denoted between CS+ and CS- intake (*) and for drug treatment relative to corresponding Veh (+). The percentages of CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted.
The SWR NTX mice displayed significantly lower percent CS+ intakes than BALB/c NTX mice (59% vs. 75%) in Test 3. In contrast, BALB/c Limited Control mice displayed significantly lower percent CS+ intakes than SWR Limited Control mice (57% vs. 78%) in Test 3, indicating a hastening of extinction of the preference in the BALB/c strain under limited training conditions.

**Discussion**

The present study revealed strain differences in the pharmacological effects of DA and opioid antagonists on fructose-CFP in mice. The following sections will first describe the fructose conditioning effects observed in the BALB/c and SWR mice, and then discuss the drug effects on fructose-CFP in the two strains in comparisons with previous findings obtained with sucrose-CFP (Dym et al., 2012).

*BALB/c and SWR Mice: Training and Preference Effects*: Overall, the SWR mice consumed more CS training solutions than BALB/c mice. However, BALB/c mice consumed more CS+/Fructose than CS-/Saccharin while the SWR mice consumed more CS-/Saccharin than CS+/Fructose. These findings are consistent with the strain differences originally reported by Pinhas et al. (2012). As noted by these authors, the differential sugar and saccharin training intakes of the BALB/c and SWR mice may be related to genetic differences in sweet taste sensitivity. SWR mice have the sensitive form of the T1r3 sweet receptor and generally drink more sugar and non-nutritive sweeteners than do BALB/c mice that have the sub-sensitive form of the sweet receptor (Reed et al., 2004). The SWR mice may drink more CS-/Saccharin than CS+/Fructose because their saccharin intake is not limited by satiating actions of the sugar, whereas the BALB/c mice may drink less CS-/Saccharin during training because of their reduced sweetener preferences. Although SWR mice also consumed more CS+/Fructose in training than BALB/c mice, the two strains displayed similar CS+ preferences in the two-bottle tests: the BALB/c mice had CS+ preferences of 79-86%, and the SWR had CS+ preferences of 77-85%. These preferences are comparable to the CS+sucrose preferences
displayed by BALB/c (74-85%) and SWR (75-78%) mice in our prior drug study (Dym et al., 2012), and confirm our original mouse CFP findings (Pinhas et al., 2012). Thus, comparisons between sugars and mouse strains are appropriate because the CFPs observed in the vehicle non-drug expression tests were very similar.

**DA D1 Receptor Antagonism and Fructose-CFP Expression:** In BALB/c mice, SCH eliminated fructose-CFP expression (42-59%) relative to vehicle (86%), an effect that was stronger than the ability of SCH (68-73%) to reduce the expression of sucrose-CFP relative to vehicle (85%) in BALB/c mice (Dym et al., 2012; Table 3A and B). Thus, the magnitude of DA D1 antagonist effects appeared more pronounced on fructose-CFP (using a 8% fructose + 0.2% saccharin CS+ stimulus) relative to sucrose-CFP (using a 16% sucrose CS+ stimulus) in BALB/c mice. This may be due to a greater reliability on flavor-taste (e.g., in fructose) processes relative to flavor-nutrient (e.g., observed in sucrose) in BALB/c mice.

In SWR mice, SCH induced comparable reductions of fructose-CFP expression (63-71%) relative to vehicle (85%) and sucrose-CFP expression (61-66%; Dym et al., 2012) relative to vehicle (75%). This contrasts with the BALB/c results and the notion that SCH differentially affects flavor-taste and flavor-nutrient conditioning effects. However, recent findings indicate that both fructose and sucrose have post-ingestive conditioning actions in SWR mice (Experiment 1). Previous studies in rats (Ackroff and Sclafani, 2001; Sclafani et al., 1993, 1999) and more recently in C57BL/6J mice (Sclafani & Ackroff, 2012; Zukerman et al., 2013a,b) indicated that fructose has little or no post-ingestive flavor conditioning actions. Subsequent experiments with FVB mice, however, revealed that this strain learns to prefer flavors based on post-ingestive fructose effects (Sclafani et al., 2014). For example, FVB mice, unlike C57BL/6J mice, learned to prefer a fructose-paired flavor over a different flavor paired with a "sweeter" non-nutritive sacralose-saccharin mixture. Using this paradigm, we observed that SWR mice, like FVB mice, display evidence of post-ingestive fructose conditioning.
(Huang et al., 2014). In contrast, BALB/c mice were like C57BL/6J mice in their failure to show post-ingestive fructose conditioning (Huang et al., 2014). These new findings suggest that SCH had similar effects on sucrose- and fructose-CFP in SWR mice because both sugars involve flavor-taste and flavor-nutrient learning processes. In contrast, the drug had dissimilar effects in BALB/c mice because in this strain, only sucrose involves both flavor-taste and flavor-nutrient learning processes.

**Opioid Receptor Antagonism and Fructose-CFP Expression:** In contrast to SCH, NTX failed to alter the expression of fructose-CFP in BALB/c or SWR mice (Table 3B). This contrasts with our prior finding that NTX promoted the extinction of a sucrose-CFP preference with repeated testing in BALB/c mice and even more so in SWR mice (Dym et al., 2012). These findings suggest that the opioid antagonist is more effective in suppressing the expression of sucrose-CFP than fructose-CFP. However, the present and prior study used different sugar concentrations during training (8% vs. 16%) which may have influenced drug effects on the persistence of the conditioned CS+ preferences. This issue requires further study.

**DA D1 receptor antagonism of Fructose-CFP acquisition:** Administration of the DA D1 receptor antagonist, SCH blocked the acquisition of a fructose-CFP in SWR mice in that they failed to significantly prefer the CS+ over the CS- in Tests 1-3 (40%-46%-64%). This pattern is similar to SCH-induced inhibition of sucrose-CFP acquisition in SWR mice although, as in the present experiment, the SWR-sucrose trained mice displayed an increase in CS+ preference in their last two-bottle test (61%-74%; Dym et al., 2012). In contrast, the SCH BALB/c mice displayed a significant 75% fructose-CFP preference in the first test that was similar to the previously-observed (Dym et al., 2012) initial 77% sucrose CS+ preference in the first test (). However, the SCH BALB/c mice subsequently lost the fructose-CFP preference in Tests 2 and 3 (61%-59%). This is in contrast to the sucrose-trained BALB/c mice which maintained their CS+ preference in their second (and last) test.
TABLE 3. (Chapter 5) Summary of dopamine (DA) D1 (SCH23390: SCH) and opioid (naltrexone: NTX) receptor antagonist effects upon the expression and acquisition of conditioned flavor preferences (CFP) elicited by sucrose and fructose in BALB/c and SWR mice.

<table>
<thead>
<tr>
<th>CFP Condition</th>
<th>SCH (nmol/kg) BALB/c</th>
<th>SCH (nmol/kg) SWR</th>
<th>NTX (mg/kg) BALB/c</th>
<th>NTX (mg/kg) SWR</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Sucrose-CFP</td>
<td>0: 85%</td>
<td>0: 75%</td>
<td>0: 74%</td>
<td>0: 78%</td>
</tr>
<tr>
<td>Expression Effects</td>
<td>200: 73%*</td>
<td>200: 66%*</td>
<td>1: 76%</td>
<td>1: 63%*</td>
</tr>
<tr>
<td></td>
<td>800: 68%*</td>
<td>800: 61%*</td>
<td>5: 63%*</td>
<td>5: 63%*</td>
</tr>
<tr>
<td>B. Fructose-CFP</td>
<td>0: 86%</td>
<td>0: 85%</td>
<td>0: 79%</td>
<td>0: 77%</td>
</tr>
<tr>
<td>Expression Effects</td>
<td>200: 59%*</td>
<td>200: 71%*</td>
<td>1: 82%</td>
<td>1: 73%</td>
</tr>
<tr>
<td></td>
<td>800: 42%*</td>
<td>800: 63%*</td>
<td>5: 87%</td>
<td>5: 75%</td>
</tr>
<tr>
<td>C. Sucrose-CFP</td>
<td>Veh: 85%-77%</td>
<td>Veh: 86%-89%</td>
<td>Veh: 85%-77%</td>
<td>Veh: 86%-89%</td>
</tr>
<tr>
<td>Acquisition Effects</td>
<td>SCH: 77%-80%</td>
<td>SCH: 61%*-74%</td>
<td>NTX: 54%*-66%</td>
<td>NTX: 83%-85%</td>
</tr>
<tr>
<td>D. Fructose-CFP</td>
<td>Veh: 71%-91%-77%</td>
<td>Veh: 81%-93%-91%</td>
<td>Veh: 79%-91%-77%</td>
<td>Veh: 81%-93%-91%</td>
</tr>
<tr>
<td>Acquisition Effects</td>
<td>SCH: 75%<em>-61%</em>-59%*</td>
<td>SCH: 40%<em>-46%</em>-61%*</td>
<td>NTX: 88%-87%-75%</td>
<td>NTX: 72%-76%-59%*</td>
</tr>
</tbody>
</table>

Significant drug effects are denoted in **bold** and with an asterisk (*). The expression effects indicate percent CS+ intake following vehicle (0) and drug doses. The acquisition effects for fructose-CFP show the percent CS+ intake in vehicle (Veh)-, SCH23390 (SCH)- and naltrexone (NTX)-trained mice in Tests 1, 2 and 3. The acquisition effects for sucrose-CFP (Dym et al., 2012) show the percent CS+ intake in vehicle (Veh)-, SCH23390 (SCH)- and naltrexone (NTX)-trained mice in Tests 1 and 2; a third test was not conducted.
These data suggest that SCH administered to BALB/c mice during training hastens extinction of a fructose-CFP, but not of a sucrose-CFP.

However, a potential caveat for this interpretation concerns the behavior of the Limited Control group of BALB/c mice included in this study. This group, which had their training intakes matched to the SCH and NTX groups, displayed significant CS+ preferences in the first (74%) and second (75%), but not the third (57%) tests, suggesting that the persistence of fructose-CFP in BALB/c mice was weakened by limiting their CS intakes during training. The BALB/c and SWR strains were chosen for their respective robustness and persistence in displaying sucrose- and fructose-CFP, but these effects were observed in animals that did not have profound restrictions on training intake (Pinhas et al., 2012).

**Opioid receptor antagonism of Fructose-CFP acquisition:** NTX failed to alter the acquisition of fructose-CFP in BALB/c mice in that they displayed significant CS+ preferences in Tests 1-3 (88%-87%-75%). In contrast, NTX BALB/c mice trained with sucrose failed to show a CS+ preference in Test 1 (54%) although a weak preference emerged in the second test (66%; Dym et al., 2012). The different NTX effects on fructose- and sucrose-CFP in BALB/c mice cannot be explained by any differential inhibitory actions of NTX on sucrose and fructose intake, per se (Dym et al., 2007; Kraft et al., 2015a). Conceivably, the stronger NTX inhibitory effect on sucrose-CFP than fructose-CFP may be related to sucrose, but not fructose having a post-ingestive conditioning action in this strain. Further studies should examine whether IG sucrose produces a CFP in BALB/c mice like those observed in 129P3 and C57BL/6J strains (Sclafani and Glendinning, 2005), and if so, whether IG sucrose-CFP acquisition in BALB/c mice is altered by opioid receptor antagonism.

The NTX SWR mice displayed significant CS+ preferences in Tests 1 and 2 but not in Test 3 (72%-76%-59%) unlike the persistent CS+ preferences displayed by the Vehicle and Limited Control groups (Figure 5). In contrast, NTX SWR mice trained with sucrose displayed strong and persistent
CS+ preferences (83%-85%), like their Vehicle controls (86%-89%), although note that these mice were not given a third choice test. Thus, it appears that NTX has opposite effects on sugar conditioning in SWR and BALB/c mice trained with fructose and sucrose, although this conclusion is not definitive given procedural differences in the two sugar studies.
Chapter 6

NMDA RECEPTOR ANTAGONISM DIFFERENTIALLY REDUCES ACQUISITION AND EXPRESSION OF SUCROSE- AND FRUCTOSE-CONDITIONED FLAVOR PREFERENCES IN BALB/c AND SWR

Introduction

The role of learning in modulating appetite for sugar-rich foods has been demonstrated using the conditioned flavor preference (CFP) paradigm in which rats and mice are trained to associate an arbitrary flavor (conditioned stimulus, CS+) with a sugar (e.g., sucrose, glucose or fructose) solution and another flavor (CS-) with a non-nutritive sweetener (e.g., saccharin) solution in short-term intake tests (e.g., Baker et al., 2003; Dela Cruz et al., 2014; Pinhas et al., 2012; Sclafani, 1995; Yu et al., 1999). Our laboratory (Pinhas et al., 2012) examined genetic variance in the magnitude and persistence of sucrose- and fructose-CFP in eight inbred and one outbred mouse strains by training food-restricted mice to consume CS+ flavored sugar and CS- flavored saccharin solutions in alternate one-bottle sessions followed by 2-bottle choice tests between the CS+ and CS- flavors both presented in saccharin solutions. Two inbred strains displayed particularly robust and persistent percent CS+ preferences defined as CS+ intake / Total Intake x 100% for sucrose-paired CS+ (SWR: 94%; BALB/c: 85%) and fructose-paired CS+ solutions (SWR: 75%; BALB/c: 78%) over six test sessions. Therefore, these two strains were selected for subsequent experiments on the pharmacology of sugar-conditioned flavor preferences.

Both sucrose and fructose were used in these pharmacological experiments because earlier studies of rats and C57BL/6J (B6) mice indicated that they engaged different conditioning processes. In particular, intragastric (IG) sucrose (and glucose) infusions conditioned flavor preferences in rats and B6 mice, via a process called flavor-nutrient conditioning, whereas IG fructose was ineffective (Sclafani & Ackroff, 2012; Zukerman et al., 2013a,b). Orally consumed fructose, however,
conditioned flavor preferences in rats and B6 mice (e.g., Baker et al., 2003; Sclafani & Ackroff, 2015; Pinhas et al., 2012), and this was attributed to a flavor-taste process in which the sweet taste of the sugar reinforced the preference for the CS+ flavor. Oral sucrose and glucose also conditioned flavor preferences thought to be mediated by both the sweet taste and post-oral actions of the sugars (e.g., Azzara et al., 2000; Dela Cruz et al., 2014; Yu et al., 1999). Recent studies (Sclafani et al., 2014, 2015) have revealed inbred strain differences in fructose-based flavor conditioning. That is, unlike in B6 mice, IG fructose was found to condition a flavor preference in FVB mice. In addition, FVB mice, but not B6 mice, acquired a preference for fructose over an initially more preferred non-nutritive sweetener (0.1% sucralose + saccharin, S+S) after experience with both sweeteners in long-term (24 h) tests. SWR mice, like FVB mice, learned to prefer fructose to S+S, while BALB/c mice, like B6 mice, strongly preferred S+S to fructose after experience with both sweeteners (Experiment 1). This indicated that fructose has both oral and post-oral reinforcing actions in SWR mice but only oral reinforcing effects in BALB/c mice. Despite this difference, SWR and BALB/c mice displayed similar fructose-CFPs in our original mouse study (Pinhas et al., 2012).

Our early pharmacological studies of flavor conditioning were conducted in rats, and revealed that a dopamine (DA) D1-like receptor antagonist (SCH23390) blocked the acquisition of flavor preferences produced by IG sucrose infusions and by orally consumed fructose (Azzara et al., 2001; Baker et al., 2003). SCH23390 also attenuated the expression of a previously learned fructose-CFP but had little effect on the expression of an IG sucrose-CFP (Azzara et al., 2001; Baker et al., 2003). Analyses of central sites of action revealed that acquisition, but not expression of IG glucose-CFP was blocked by a DA D1 antagonist administered into the nucleus accumbens, amygdala, medial prefrontal cortex and lateral hypothalamus (Touzani et al., 2008, 2009a, 2009b, 2010), whereas a DA D1 antagonist administered into these same sites differentially reduced acquisition and expression of fructose-CFP (Amador et al., 2014; Bernal et al., 2008, 2009; Malkusz et al., 2012). In contrast,
opioid receptor antagonism with naltrexone failed to affect the acquisition or expression of CFPs in rats produced by IG sucrose or orally-consumed fructose (Azzara et al., 2000; Baker et al., 2004). Studies of SWR and BALB/c mice revealed that the expression of a CFP produced by oral sucrose was attenuated by both DA D1-like and opioid antagonists (Dym et al., 2012). In contrast, acquisition of sucrose-CFP was reduced by opioid, but not DA D1 antagonism in BALB/c mice, and by DA D1, but not opioid antagonism in SWR mice, indicating a double dissociation in the pharmacological effects on sucrose-CFP in the two strains (Dym et al., 2012). A different pattern of drug effects were observed in mice trained with oral fructose (Kraft et al., 2015b). Thus, DA D1, but not opioid antagonism significantly reduced expression of fructose-CFP in both strains. However, BALB/c mice displayed hastened extinction of a fructose-CFP following DA D1, but not opioid antagonism during conditioning trials. DA D1 antagonism during training blocked the acquisition of a fructose-CFP in SWR mice, whereas opioid antagonism did not block acquisition but hastened the extinction of a fructose-CFP. Thus, these results indicate strain-specific, sugar-specific and transmitter-specific modulation of sugar-CFP.

The glutamatergic system has also been implicated in mediating sugar-CFP in rats such that the non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801, eliminated fructose-CFP acquisition, but not expression (Golden and Houpt, 2007). Further, the competitive NMDA antagonist, AP-5 administered into the rat amygdala eliminated acquisition, but not expression of a CFP induced by IG glucose (Touzani et al., 2013). In view of these rat findings, the present study examined whether NMDA receptor antagonism with MK-801 altered acquisition and/or expression of CFPs induced by orally consumed sucrose and fructose in BALB/c and SWR mice. NMDA antagonist-induced reductions in the acquisition or expression of fructose- or sucrose-CFP were operationally defined in terms of significant reductions in percent CS+ intakes and/or a failure to observe significant differences between CS+ and CS- intakes in 2-bottle preference tests.
Materials and Methods

Subjects and Initial Training Procedures: Inbred BALB/c (Stock #000651) and SWR (Stock #000689) male mice (Jackson Laboratories, Bar Harbor, ME, 6 weeks of age, both strains ~25 g at arrival) were acclimated to the Queens College vivarium for one week in group (5 per cage) housing. The animals were then housed individually in plastic cages (30 x 20 x 15 cm) with stainless steel tops throughout the entire study, and maintained on a 12 h light/12 h dark cycle (lights off at 2000 h) at a constant temperature of 22°C for two more weeks. Chow (Lab Diet Mouse Chow 5015) and water were provided, but two weeks before CFP training began, the mice were placed on a food restriction schedule in which 2-3 g of chow was placed in their cages daily with water available ad libitum. The mice were weighed just prior to the onset of food-restriction, and their body weights were maintained at 85-90% of ad libitum levels during training/testing. Individual mice were tested in one training paradigm. The experimental procedures were approved by the Queens College Institutional Animal Care and Use Committee certifying that all subjects and procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

While food-restricted, the mice were trained 1 h/day to drink unflavored 0.2% saccharin solution (Sigma-Aldrich Company, St. Louis MO) from a stainless steel sipper tube connected to a 10 ml plastic syringe (Dym et al., 2012; Kraft et al., 2015 a; Kraft et al., 2015b; Pinhas et al., 2012). This training procedure was repeated daily until all mice sampled the sipper tubes with short (< 1 min) latency, typically within three days.

Fructose-CFP and Sucrose-CFP Expression Procedure: Two training solutions were utilized in the fructose-CFP paradigm (8% fructose (Sigma-Aldrich Company) + 0.2% saccharin and 0.2% saccharin each flavored with 0.05% unsweetened grape or cherry Kool-Aid (Kraft Foods, White Plains, NY) (Kraft et al., 2015b). The sucrose-CFP paradigm used 16% sucrose (Domino Foods, Yonkers, NY) and 0.2% saccharin each flavored with 0.05% unsweetened grape or cherry Kool-Aid:
Dym et al., 2012). The 8% fructose + 0.2% saccharin solution was used instead of a 16% fructose solution because the former conditioned a stronger preference than the latter in SWR and BALB/c mice (Pinhas et al., 2012). For half of the mice, the CS+ flavor added to the sucrose (CS+/S) or fructose-saccharin (CS+/F) solutions was cherry, and the CS- flavor added to the saccharin solution was grape; the flavor-sweetener pairs were reversed for the remaining animals. In the 2-bottle preference tests, the CS+ and CS- flavors were each presented in a 0.2% saccharin solution (Dym et al., 2012; Kraft et al., 2015b; Pinhas et al., 2012). Training and testing took place in the home cages during the mid-portion of the light phase. The limited food rations were given 1 h after each training and testing session.

The mice received ten one-bottle training sessions (1 h/day) with 8 ml of the CS+/F or the CS+/S solution presented on odd-numbered days, and 8 ml of the CS-/saccharin solution presented on even-numbered days. On days 9 and 10, the mice also had access to a second sipper tube containing water, familiarizing them to the presence of two sipper tubes used during the choice tests. Water intake was negligible in these training trials. The position of the two sipper tubes varied across the 2-bottle training and subsequent testing days using a left-right-right-left pattern. Solution intakes during training were measured by weighing (0.1 g) the sipper tubes before and after the 1-h sessions.

Following training, the mice were given six 2-bottle choice test sessions (1 h/day) with the CS+ and CS- solutions. Thirty min prior to the first two sessions, an intraperitoneal (i.p.) vehicle (Veh: 0.9% normal saline) injection was administered to five BALB/c and five SWR mice in the fructose-CFP expression paradigm, and to five BALB/c and five SWR mice in the sucrose-CFP expression paradigm. Then on the subsequent test days, the mice received two sessions with each NMDA antagonist dose (100 and 200 µg/kg) with half tested with an ascending dose order and the remainder with a descending dose order. MK-801 was mixed at concentrations of 10 and 20 µg/ml and administered i.p. at 10 ml/kg body weight. The 100 µg/kg dose was chosen based on the testing
of fructose-CFP acquisition and expression in rats (Golden and Houpt, 2007), whereas a higher 200 µg/kg dose was chosen for its effectiveness in blocking food-anticipatory activity rhythms in rats (Ono et al., 1996).

**Fructose-CFP and Sucrose-CFP Acquisition Procedure:** BALB/c and SWR mice received 10 one-bottle training sessions (1 h/day) with either the CS+/F or CS+/S solution presented on odd-numbered days, and the CS-/saccharin solution presented on even-numbered days. In the fructose-CFP acquisition paradigm, i.p. injections of Veh (BALB/c: n= 8; SWR: n= 8) or MK-801 at doses of 100 (MK(100): BALB/c: n= 8; SWR: n= 8), or 200 (MK(200): SWR: n= 7) µg/kg were administered 30 min prior to the training session. In the sucrose-CFP acquisition paradigm, i.p. injections of Veh (BALB/c: n= 8; SWR: n= 8) or MK-801 at doses of 100 (MK(100): BALB/c: n= 8; SWR: n= 8), or 200 (MK(200): SWR: n= 8) µg/kg were administered. The SWR mice were tested at a higher 200 µg/kg NMDA antagonist dose because of the ineffectiveness of the 100 µg/kg MK-801 dose to eliminate fructose- and sucrose-CFP acquisition in this strain (see results). Following training, all mice were given six daily 2-bottle choice sessions (1 h/day) with the CS+ and CS- solutions without injections. The positions of the CS+ and CS- solutions were counterbalanced across testing sessions, and the results were analyzed as mean 1-h intakes during successive pairs of sessions (referred to as Tests 1, 2 and 3) to control for side position effects.

**Statistics:** In CFP expression, training intakes of the average of the five CS+ and five CS- sessions were determined, and evaluated for strain and CS differences using a two-way analysis of variance (ANOVA). Expression of fructose-CFP and sucrose-CFP in 2-bottle preference vehicle tests were evaluated in two-way ANOVAs between the two strains and CS+ vs. CS- intakes. Subsequent two-way ANOVAs evaluated drug effects within each strain (CS solution vs. Dose) for the sucrose-CFP and fructose-CFP expression paradigms. Percent CS+ preference was calculated for each animal in the following manner: CS+ intake / Total Intake x 100. Separate ANOVAs evaluated percent CS+
intakes as a function of drug dose for each strain as well as comparing strain (BALB/c vs. SWR) and sugar (sucrose vs. fructose). In acquisition, training intakes of the average of the five CS+/Fructose and five CS-/saccharin sessions were determined, and were evaluated for strain, drug group and CS using a three-way ANOVA (Strain x Groups x CS). A three-way ANOVA compared the CS intakes of the four groups (Group x CS x Test). Separate two-way ANOVAs evaluated percent CS+ intakes of the four groups, whereas a 3-way ANOVA compared strain differences in percent CS+ intakes. When main or interaction effects were found, Tukey comparisons (p<0.05) were employed to detect significant effects.

Results

Strain differences in training and testing without drug treatment. In the expression experiment, BALB/c mice consumed significantly more CS+/F than CS-during fructose-CFP training, whereas SWR mice displayed comparable CS+/F and CS- intakes (Figure 13A). In addition, SWR mice consumed significantly more CS-, but not CS+/F training solutions than BALB/c mice. Whereas BALB/c mice consumed significantly more CS+/S than CS- during sucrose-CFP training, SWR mice consumed comparable amounts of the CS+/S and CS- (Figure 13B). In addition, SWR mice consumed significantly more CS-, but not CS+/S during training than BALB/c mice. Strain differences in 2-bottle fructose and sucrose CS intakes also occurred following Veh treatment (F(1,11)= 87.16, p<0.0001) and for the interaction between strains and CS (F(1,11)= 12.82, p<0.043). SWR mice (Figure 13E) consumed significantly more CS+, but not CS- following Veh relative to BALB/c mice (Figure 13C). In sucrose-CFP expression, significant differences (F(1,13)= 4.82, p<0.047) following Veh occurred with SWR mice (Figure 13F) consuming more CS+ than BALB/c mice (Figure 13D). In the acquisition experiments the Veh-treated strains also displayed significant differences in one-bottle training intakes with sucrose (strain (F(1,7)= 10.36, p<0.015; interaction (F(1,7)= 36.38, p<0.0005), but not with fructose. Whereas BALB/c (Figure 14A) and SWR (Figure
16A) mice displayed similar CS+ and CS− intakes during CS+fructose training, CS− intakes of SWR mice (Figure 17A) were significantly higher than that of BALB/c mice (Figure 15A). Because strain-specific differences in intakes occurred, consistent with previous studies (Dym et al., 2012; Kraft et al., 2015b), NMDA antagonist effects on intakes in the expression and acquisition of fructose- and sucrose-CFP were evaluated separately for each strain. The strains were compared, however, in their percent CS+ preferences.

**NMDA receptor antagonism of fructose- and sucrose-CFP expression in BALB/c mice.** BALB/c mice consumed significantly more CS+ than CS− for fructose-CFP (F(1,12)= 39.37, p<0.0001; Figure 13C) and sucrose-CFP (F(1,12)= 74.67, p<0.0001; Figure 13D). CS+ intakes were significantly higher than CS− intakes following Veh and both MK-801 doses for fructose-CFP expression (Figure 13C), and for the 200, but not 100 µg/kg MK-801 doses for sucrose-CFP (Figure 13D). Percent CS+ intake was significantly reduced for fructose-CFP (F(2,8)= 5.94, p<0.026) following the 200, but not 100 µg/kg MK-801 dose relative to Veh (Figure 13C) and for sucrose-CFP (F(2,8)= 4.59, p<0.0047) following the 100, but not 200 µg/kg MK-801 dose relative to Veh (Figure 13D). Total CS intakes failed to differ among MK-801 doses relative to Veh for both fructose-and sucrose-CFP.

**NMDA receptor antagonism of fructose- and sucrose-CFP expression in SWR mice.** SWR mice consumed more CS+ than CS− for fructose-CFP (F(1,12)= 34.72, p<0.0001; Figure 13E) and sucrose-CFP (F(1,12)= 49.85, p<0.0001; Figure 13F). CS+ intakes were significantly higher than CS− intakes following Veh and the 100, but not 200 µg/kg MK-801 dose for fructose-CFP (Figure 13E), and for both doses for sucrose-CFP (Figure 13F). CS+ intakes were significantly higher than CS− intakes. MK-801 (200 µg/kg) also significantly increased CS+ sucrose intake relative to Veh (Figure 13F). Percent CS+ intake was significantly reduced for fructose-CFP (F(2,12)= 8.59, p<0.01) following the 200, but not 100 µg/kg MK-801 dose relative to Veh (Figure 13E), but not for sucrose-
**Figure 13. (Chapter 6)** Fructose- and Sucrose-CFP Expression Training and Testing: Intakes (mean +SEM, g/1 h) of solutions of fructose (CS+/F) and saccharin (CS-) (Panel A) and of sucrose (CS+/S) and saccharin (CS-) (Panel B) during one-bottle training. Significant strain differences in one-bottle training intakes were observed in the fructose-CFP (CS x Strain interaction, F(1,11)= 21.88, p<0.0007) and sucrose-CFP (CS x Strain interaction, F(1,13)= 4.81, p<0.047) expression experiments. Significant differences between CS+ and CS- training intake within strains (*) and between CS- intakes between strains (+) are denoted. Intakes (mean +SEM, g/1 h) of CS+ and CS-solutions in 2-bottle tests in the fructose- (Panels C and E) and sucrose- (Panels D and F) CFP experiments in BALB/c (Panels C and D) and SWR (Panels E and F) inbred mice receiving systemic injections of the NMDA antagonist, MK-801 at doses of 0, 100 and 200 µg/kg 30 min prior to testing. Significant differences are denoted between CS+ and CS- intake within an injection condition (*) and between CS+ intake following a drug dose relative to the vehicle treatment (+). The percentages of CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted.
Figure 14. (Chapter 6) Training intakes (mean +SEM, g/1 h) of CS+/Fructose and CS-/Saccharin solutions in BALB/c mice pretreated 30 min earlier with vehicle (Veh) or MK-801 at a dose of 100 µg/kg (MK(100)) (Panel A). Intakes (mean +SEM, g/1 h) of CS+ and CS- solutions during 2-bottle Tests 1, 2 and 3 in BALB/c mice receiving Veh (Panel B) or MK-801 (100 µg/kg, Panel C) during training. Significant differences are denoted between CS+ and CS- intake (*) and for drug treatment relative to corresponding Veh (+). The percentages of CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted.
Figure 15. (Chapter 6) Training intakes (mean +SEM, g/1 h) of CS+/Sucrose and CS-/Saccharin solutions in BALB/c mice pretreated 30 min earlier with vehicle (Veh) or MK-801 at a dose of 100 µg/kg (MK(100)) (Panel A). Intakes (mean +SEM, g/1 h) of CS+ and CS- solutions during 2-bottle Tests 1, 2 and 3 in BALB/c mice receiving Veh (Panel B) or MK-801 (100 µg/kg, Panel C) during training. Significant differences are denoted between CS+ and CS- intake (*) and for drug treatment relative to corresponding Veh (+). The percentages of CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted.
Figure 16. (Chapter 6) Training intakes (mean ±SEM, g/1 h) of CS+/Fructose and CS-/Saccharin solutions in SWR mice pretreated 30 min earlier with vehicle (Veh) or MK-801 at doses of 100 or 200 µg/kg (MK(100)) (Panel A). Intakes (mean ±SEM, g/1 h) of CS+ and CS- solutions during 2-bottle Tests 1, 2 and 3 in SWR mice receiving Veh (Panel B), MK-801 (100 µg/kg, Panel C) or MK-801 (200 µg/kg, Panel D) during training. Significant differences are denoted between CS+ and CS- intake (*) and for drug treatment relative to corresponding Veh (+). The percentages of CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted.
**Figure 17. (Chapter 6)** Training intakes (mean +SEM, g/1 h) of CS+/Sucrose and CS-/Saccharin solutions in SWR mice pretreated 30 min earlier with vehicle (Veh) or MK-801 at doses of 100 or 200 µg/kg (MK(100) (Panel A). Intakes (mean +SEM, g/1 h) of CS+ and CS- solutions during 2-bottle Tests 1, 2 and 3 in SWR mice receiving Veh (Panel B), MK-801 (100 µg/kg, Panel C) or MK-801 (200 µg/kg, Panel D) during training. Significant differences are denoted between CS+ and CS-intake (*) and for drug treatment relative to corresponding Veh (+) or the lower MK-801 dose (#). The percentages of CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted. CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted.
 Whereas total CS intakes failed to differ among doses for fructose-CFP, total CS intakes for sucrose-CFP were significantly (F(2,12)= 4.28, p=<0.05) higher following the 200 (3.4 g), but not 100 µg/kg MK-801 dose relative to Veh (2.2 g).

Whereas total CS intakes failed to differ among doses for fructose-CFP, total CS intakes for sucrose-CFP were significantly (F(2,12)= 4.28, p=<0.05) higher following the 200 (3.4 g), but not 100 µg/kg MK-801 dose relative to Veh (2.2 g).

Strain differences in NMDA receptor antagonism of fructose- and sucrose-CFP expression.

Significant differences in percent CS+ intakes were observed between fructose and sucrose experiments (F(1,48)= 5.81, p<02), among doses (F(2,48)= 7.17, p<0.002), and for the interactions between strains and sugars (F(1,48)= 10.48, p<0.0028) and sugars and doses (F(2,48)= 3.61, p<0.035). The strain x sugar interaction revealed comparable overall percent CS+ intakes for fructose (77%) and sucrose (75%) in BALB/c mice, but significantly higher percent CS+ intake for sucrose (85%) relative to fructose (71%) in SWR mice. The percent CS+ intake of fructose-CFP was significantly less following the 200 µg/kg MK-801 dose in SWR (56%, Figure 13E) relative to BALB/c (71%, Figure 13C) mice. In contrast, percent CS+ intake of sucrose-CFP was significantly greater following the 100 µg/kg MK-801 dose in SWR (89%, Figure 13F) relative to BALB/c (67%, Figure 1D) mice. Moreover, SWR mice treated with the 200 µg/kg MK-801 dose displayed significantly less percent CS+ intake for fructose-CFP (56%, Figure 13E) than for sucrose-CFP (84%, Figure 1F).

NMDA receptor antagonism of fructose- and sucrose-CFP acquisition in BALB/c mice.

Whereas fructose-CFP training intakes failed to differ between groups, CS conditions or their interaction (Figure 14A), sucrose-CFP training intakes were significantly higher (F(1,28)= 8.81, p<0.006) for CS+/S relative to CS- (Figure 15A). In 2-bottle tests, BALB/c mice, overall, consumed significantly more CS+ than CS- for both fructose-CFP (F(1,84)= 33.37, p<0.0001) and sucrose-CFP
(F(1,84)= 40.55, p<0.0001), and significant differences were observed for the group x CS interaction for both fructose-CFP (F(1,84= 51.28, p<0.00015) and sucrose-CFP (F(1,84)= 129.35, p<0.0002).

Whereas CS+ intake was significantly higher than CS- intake across all tests in BALB/c Veh groups for both fructose-CFP (Figure 14B) and sucrose-CFP (Figure 15B), BALB/c MK(100) groups failed to display any differences between CS+ and CS- intakes for both fructose-CFP (Figure 14C) and sucrose-CFP (Figure 15C). Indeed, CS+ intake of the BALB/c MK(100) group across tests in sucrose-CFP was significantly lower than corresponding Veh values, and CS- intake of this group across tests was significantly higher than corresponding Veh values (Figures 15B and C). Moreover, CS+ intake of the MK(100) group (Figure 14C) during the second test was significantly lower than the Veh group (Figure 14B) in fructose-CFP. Total CS intake failed to differ between the Veh and MK(100) groups in the fructose- and sucrose-CFP paradigms. The percent CS+ preferences of the BALB/c MK(100) groups were significantly lower than those of the Veh groups in the fructose-CFP (F(1,42)= 80.29, p<0.0001) and sucrose-CFP (F(2,14)= 5.51, p<0.017) experiments (Figures 14 and 15).

**NMDA receptor antagonism of fructose- and sucrose-CFP acquisition in SWR mice.** Fructose-CFP training CS- intakes exceeded CS+/F intakes in all groups (F(1,40)= 18.84, p<0.0001; Figure 16A). Sucrose-CFP CS- training intakes exceeded CS+/S intakes in the Veh and MK(100) groups but not the MK(200) group with significant main effects among groups (F(2,44)= 3.78, p<0.031) and between CS+/S and CS- conditions (F(1,44)= 15.33, p<0.0003) (Figure 17A). In 2-bottle tests, SWR mice, overall, consumed significantly more CS+ than CS- in the fructose-CFP (F(1,120)= 75.25, p<0.0001) and sucrose-CFP (F(1,132)= 174.44, p<0.0001) experiments. Significant differences were also observed among groups (fructose-CFP: F(2,120)= 6.39, p<0.0025; sucrose-CFP: F(2,132)= 20.34, p<0.0001), and for the group x CS (fructose-CFP: F(2,120)= 33.09, p<0.0001; sucrose-CFP: F(2,132)= 47.81, p<0.0001) and test x CS (sucrose-CFP: F(2,132)= 4.24, p<0.016) interactions. CS+
intake was significantly higher than CS- intake across all tests in the Veh and MK(100) groups in the fructose-CFP (Figures 16B and 16C) and sucrose-CFP (Figures 17B and 17C) experiments. In contrast, the SWR MK(200) group failed to display differences between CS+ and CS- intakes in the fructose-CFP (Figure 4D) and sucrose-CFP (Figure 5D) experiments. Indeed, CS+ intake of the MK(200) group was significantly lower than that of the Veh group in the fructose-CFP experiment and Veh and MK(100) groups in the sucrose-CFP experiment. Percent CS+ preference significantly differed among groups in the fructose-CFP (F(2,60)= 27.11, p<0.0001) and sucrose-CFP (F(2,66)= 31.77, p<0.0001) experiments. Whereas the SWR MK(200) group (Figure 16D) displayed significantly lower percent CS+ preferences across all tests relative to the Veh (Figure 16B) and MK(100) (Figure 16C) groups in the fructose-CFP experiment, the MK(200) group (Figure 17D) displayed significantly lower percent CS+ preferences across all tests relative to the Veh group (Figure 5B) in the sucrose-CFP experiment. Total CS intake was significantly lower in the MK(200) group than the Veh and MK(100) groups in the fructose-CFP (F(2,60)= 10.44, p<0.0001) and sucrose-CFP (F(2,66)= 26.34, p<0.0001) experiments.

Strain differences in fructose-CFP and sucrose-CFP acquisition: Analyses of percent CS+ intakes in the BALB/c and SWR groups in the fructose- and sucrose-CFP acquisition experiments revealed significant differences among drug conditions (F(4,210)= 72.33, p<0.0001). The fructose-CFP percent CS+ intakes in the MK(100) BALB/c mice (41-55%, Figure 14C) were significantly lower than those in MK(100) SWR mice (70-82%, Figure 16C), but comparable to those of the MK(200) SWR mice (41-44%, Figure 5C). The sucrose-CFP percent CS+ intakes in MK(100) BALB/c mice (40-56%, Figure 15C) were significantly lower than those in MK(100) SWR mice (65-83%, Figure 17C), but comparable to those in MK(200) SWR mice (44-58%, Figure 17D).
Discussion

The major finding of the present study was that the acquisition of fructose- and sucrose-CFP in BALB/c and SWR inbred strains was eliminated by systemic administration of the non-competitive NMDA antagonist, MK-801 during training. These data are highly consistent with the abilities of systemic MK-801 to eliminate the acquisition of fructose-CFP in rats (Golden and Houpt, 2007), and of amygdala administration of the competitive NMDA antagonist, AP-5 to eliminate the acquisition of CFP elicited by IG glucose (Touzani et al., 2013). The present study also demonstrated a clear strain difference in the potency of MK-801 to block the acquisition of sugar preferences. BALB/c mice failed to acquire fructose- and sucrose-CFP preferences when treated with a 100 µg/kg dose. In contrast, SWR mice displayed near-normal fructose- and sucrose-CFP preferences when treated with the 100 µg/kg dose, but failed to acquire fructose- and sucrose-conditioned CS+ preferences when treated with the 200 µg/kg dose. The enhanced potency of NMDA receptor antagonism to block acquisition of both forms of sugar-CFP in BALB/c mice is consistent with previous findings that BALB/c mice are more sensitive to lower MK-801 doses relative to other strains (AKR, C3H, C57BL/6, CD-1, DBA/2, MF1, NIH Swiss, Swiss-Webster) across a wide range of paradigms measuring anti-seizure activity, locomotion, stereotypy and learning and memory deficits (Akilloglu et al., 2012; Bado et al., 2011; Billingslea et al., 2003; Bradford et al., 2010; Burkett et al., 2010; Deutsch et al., 1997, 1998, 2011; Farley et al., 2012; Kalinichev et al., 2008; Mutlu et al., 2011a, 2011b, 2012; Perera et al., 2008). However, data are not available for SWR mice for these paradigms. The elimination of the acquisition of fructose- and sucrose-CFP in the BALB/c and SWR strains by MK-801 occurred in the absence of drug effects on CS+/F, CS+/S or CS- intakes during training (Figures 14A, 15A, 16A, 17A). Therefore, the elimination by MK-801 of the acquisition of sugar-CFP preferences cannot be explained by reduced CS intakes during training. Indeed, systemic and central administration of MK-801 and other NMDA antagonists typically increased sugar intake.
Expression of fructose- and sucrose-CFP in BALB/c mice was minimally affected by NMDA receptor antagonism with MK-801 administered after establishment of the preferences. In BALB/c mice, CS+ intakes were higher than CS- intakes following both MK-801 doses in both the fructose- and sucrose-CFP experiments. Whereas the magnitude of the fructose-CFP preference was marginally (72% vs. 85%) reduced by the highest (200 µg/kg) MK-801 dose in this strain, the magnitude of the sucrose-CFP preference was marginally (73-74% vs. 86%) reduced by the entire MK-801 dose range in BALB/c mice. SWR mice displayed a more potent and selective MK-801-mediated reduction in the expression of sugar-CFP. Whereas SWR mice failed to display any reduction in the expression of sucrose-CFP following MK-801, the highest (200 µg/kg) MK-801 dose blocked (56% vs. 82%) the expression of fructose-CFP. The minimal effects of MK-801 on expression of sucrose-CFP in both strains are consistent with the inability of amygdalar AP-5 to alter expression of CFP produced by IG glucose (Touzani et al., 2013). The strain-selective ability of high MK-801 doses to block expression of fructose-CFP in SWR, but not BALB/c mice is in contrast to the inability of NMDA antagonism to alter expression of fructose-CFP in rats (Golden and Houpt, 1997). The implications of these findings are discussed in the General Discussion.
DOPAMINE D1 AND OPIOID RECEPTOR ANTAGONISM EFFECTS ON THE ACQUISITION AND EXPRESSION OF FAT-CONDITIONED FLAVOR PREFERENCES IN BALB/c AND SWR MICE

Introduction

Dietary fat is recognized as contributing to the palatability of foods, overeating, and diet-induced obesity. Rodents are attracted to the flavor of fat (e.g., corn oil (CO)) from an early age, which may be mediated in part by taste receptors for fatty acids (Ackroff & Sclafani, 2009; Passilly-Degrace et al., 2009). In addition, the post-ingestive actions of fat are rewarding and can condition a preference for an arbitrary cue flavor (conditioned stimulus, CS+) in rats and mice (Ackroff et al., 2005; Ackroff & Sclafani, 2009; Sclafani, 1999; Sclafani and Glendinning, 2005). Dopamine (DA) mediation of the rewarding effect of fat flavor is suggested by the findings that CO sham-feeding promotes nucleus accumbens (NAc) DA release in rats (Liang et al., 2006), and DA D1 and D2 receptor antagonists suppress the sham-feeding response to CO and real-feeding of fats in rats (Baker et al., 2001; Davis et al., 2006; Rao et al., 2008; Weatherford et al., 1988; 1990). DA D2, but not D1 receptor antagonism suppressed operant responding for CO in mice (Yoneda et al., 2007), whereas DA D1, but not D2 receptor antagonism attenuated place preference conditioning by CO intake (Imaizumi et al., 2000). In inbred mice, strain differences were observed in the ability of the DA D1 antagonist, SCH23390 (SCH), but not the DA D2 antagonist, raclopride to differentially and significantly reduce intake of Intralipid, a stable soybean oil emulsion (Dym et al., 2010). In outbred rats, the expression of a fat conditioned flavor preference (CFP) was attenuated by DA D1, and to a lesser degree D2, antagonism, whereas both antagonists exerted minimal effects on the acquisition of the fat-CFP (Dela Cruz et al., 2012a).
A powerful role for the opioid system in fat appetite is supported by the significant reductions in fat intake produced by general and selective mu and kappa opioid receptor antagonists in rats (see reviews: Bodnar, 2004; Taha, 2010). Correspondingly, administration of the mu-selective opioid agonist, DAMGO into the NAc stimulated high-fat intake in rats (Zhang et al., 1998). Potent mouse strain-specific effects were also observed in the ability of naltrexone (NTX) to suppress Intralipid intake (Dym et al., 2010). Yet, NTX had only modest effects on the expression and no effect on the acquisition of a fat-CFP in rats (Dela Cruz et al., 2012b).

In the present study, we investigated the influence of DA D1 and opioid receptor antagonists on fat-CFP using inbred mouse strains. The BALB and SWR strains were selected because they are differentially responsive to the suppressive effects of DA and opioid receptor antagonism on fat intake. Specifically, whereas SWR mice displayed significant dose-dependent reductions in Intralipid intake following injection with SCH, the fat intake of BALB mice was refractory to DA D1 antagonism (Dym et al., 2010). In contrast, SWR mice displayed significant dose-dependent reductions in Intralipid intake following NTX treatment, whereas the fat intake of BALB mice was refractory to opioid antagonism (Dym et al., 2010). The BALB and SWR strains were also of interest because in a multi-strain study of sucrose-CFP, they were among the strains showing the highest CS+ preferences (Pinhas et al., 2012). A subsequent study (Dym et al., 2012) revealed that the acquisition of sucrose-CFP in the two strains was differentially affected by SCH and NTX treatment. In particular, in BALB mice, sucrose conditioning was impaired by NTX but not SCH treatment, whereas in SWR mice, the reverse drug pattern was observed.

Experiment 1 first established whether SWR and BALB mice would develop a fat-CFP by training them to drink a CS+ flavor (e.g., cherry) added to a more preferred 5% Intralipid solution, and a CS- flavor (e.g., grape) added to a less preferred 0.5% Intralipid solution. Preferences were then evaluated by two-bottle tests with both CS flavors presented in 0.5% Intralipid. The second and third
experiments examined the effects of SCH and NTX on the expression and acquisition of fat-CFP in SWR and BALB mice. To this end, the effects of SCH (200-800 nmol/kg) and NTX (1-5 mg/kg) on the expression of a previously-conditioned CS+ preference was evaluated by administering the drugs just prior to the two-bottle CS+ vs. CS- choice tests. Drug effects on the acquisition (learning) of this preference were evaluated by treating the mice with SCH (50 nmol/kg) or NTX (1 mg/kg) throughout one-bottle training, and subsequently conducting two-bottle choice tests in the absence of drug treatments. The procedures were similar to those used in the sucrose-CFP study (Dym et al., 2012) to allow comparisons of strain and drug effects on sugar- and fat-conditioned flavor preferences. If the neuropharmacological substrates of sugar-CFP and fat-CFP are identical in these strains, then SCH and NTX should both mildly reduce the expression of the fat-CFP in the two strains. Further, BALB mice would exhibit NTX-sensitive, and SCH-insensitive effects on acquisition of fat-CFP, whereas SWR mice would exhibit SCH-sensitive and NTX-insensitive effects on acquisition of fat-CFP. If, on the other hand, the neuropharmacological substrates of fat-CFP are identical to antagonist effects on fat intake per se (Dym et al., 2010), then SWR, but not BALB mice would show DA D1 and opioid antagonist-induced reductions in the acquisition and expression of fat-CFP.

Materials and Methods

Subjects: Inbred BALB and SWR male mice (Jackson Laboratories, Bar Harbor, ME, 6 weeks of age) were acclimated to the Queens College vivarium for one week in group (5 per cage) housing. The animals were then housed individually in plastic cages (30 x 20 x 15 cm) with stainless steel tops, and maintained on a 12 h light/12 h dark cycle (lights off at 2000 h) at a constant temperature of 22°C. All animals were provided with chow (Lab Diet Mouse Chow 5015) and water ad libitum, except when experimental testing was conducted. The experimental procedures were approved by the Queens College Institutional Animal Care and use Committee (Protocol 69) certifying that all
subjects and procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

**Test solutions:** The training solutions consisted of 5% and 0.5% Intralipid (Baxter Laboratories, Deerfield, IL) solutions flavored with 0.05% unsweetened grape or cherry Kool-Aid (Kraft Foods, White Plains, NY). For half of the mice in each strain, the CS+ flavor added to the 5% Intralipid solution was cherry and the CS- flavor added to the 0.5% Intralipid solution was grape; the flavor-fat solution pairs were reversed for the remaining animals. In the two-bottle preference tests, the CS+ and CS- flavors were each presented in the 0.5% Intralipid solution, a strategy used in our prior rat fat-CFP studies (Dela Cruz et al., 2012a, 2012b). These two Intralipid concentrations were chosen on the basis of consistent intake in the two strains in long-term (24 h: Lewis et al., 2007) and short-term (1 h: Dym et al., 2010) intake tests. All testing took place in each mouse’s home cage during the mid-light phase of the light: dark cycle. Two weeks before testing began, the mice were placed on a food restriction schedule that maintained their body weights at 85-90% of their ad libitum level. The mice were initially trained to drink an unflavored 0.2% saccharin (Sigma Chemical Co., St. Louis, MO) solution from a stainless steel sipper tube connected to a 10 ml plastic syringe (Pinhas et al., 2012). This training procedure was repeated daily until all mice sampled the sipper tubes with short (< 1 min) latency, typically within three days. Accurate measurement (±0.1 ml gradations) of this and all subsequent solutions was insured by using a retrofitted testing sipper tube described previously (Dym et al., 2007, 2009, 2010). Each sipper tube was firmly secured to the stainless steel top of the cage by a taut metal spring (100 mm) with clips at each end that affixed to the cage top so that the gradations and meniscus were easily visible. The sipper opening was occluded when placed on and then removed from the cage to minimize spillage in this and all subsequent conditions. Solution spillage was not observed during any of the training and test sessions. The limited food rations were provided 1 h after each training and testing session.
**Experiment 1: Establishment of Intralipid-CFP:** Eight BALB and nine SWR inbred mice received ten one-bottle training sessions (1 h/day) with 8 ml of the flavored CS+ 5% Intralipid solution presented on odd-numbered days, and 8 ml of the flavored CS- 0.5% Intralipid solution presented on even-numbered days. On days 9 and 10, all mice had access to a second sipper tube containing water. This familiarized them to the presence of two sipper tubes used during the choice tests; water intake was negligible in these training trials. The position of the CS and water sipper tubes varied across the two-bottle training and subsequent testing days using a left-right-right-left pattern. Solution intakes during training were measured by weighing (0.1 g) the sipper tubes before and after the 60-min sessions. Following training, the mice were given six two-bottle choice test sessions (1 h/day) with access to the CS+ and CS- flavors mixed in 0.5% Intralipid solutions. The positions of the CS+ and CS- solutions were counterbalanced across testing sessions.

**Experiment 2: Expression procedure:** A total of 12 BALB and 13 SWR mice were trained and tested as in Experiment 1. The mice of each strain were divided into SCH and NTX groups. The BALB SCH (n=6) and SWR SCH (n=7) mice were given subcutaneous (sc) vehicle (0.9% saline) injections 30-min prior to the first pair of two-bottle sessions. They were then injected sc with 200 and 800 nmol/kg doses of the DA D1 antagonist, SCH23390 (SCH, Sigma Chemical Co.) prior to two test sessions each, with half tested with an ascending dose order and the remainder with a descending dose order. SCH was mixed at concentrations of 20 and 80 nmol/ml and administered at 10 ml/kg body weight as in a prior study (Dym et al., 2009); these SCH doses were chosen based on their expression effects on sucrose-CFP (Dym et al., 2012). The BALB NTX (n=6) and SWR NTX (n=6) mice were given intraperitoneal (ip) vehicle injections prior to the first pair of two-bottle sessions. They were then injected ip with 1 and 5 mg/kg doses of the general opioid antagonist, naltrexone (NTX, Sigma Chemical Co) prior to two sessions each, with half tested with an ascending dose order and the remainder with a descending dose order. The drugs were mixed at concentrations of 0.1 and
0.5 mg/ml and administered at 10 ml/kg as in a prior study (Dym et al., 2007); these NTX doses were chosen based on their expression effects on sucrose-CFP (Dym et al., 2012). The positions of the CS+ and CS- solutions were counterbalanced across testing sessions.

**Experiment 3: Acquisition procedure:** Twenty-one BALB and 28 SWR mice were each divided into three groups (Veh, SCH, NTX) and were treated with vehicle, SCH, or NTX 30 min prior to the 10 one-bottle training sessions with the CS+ 5% Intralipid solution and the CS- 0.5% Intralipid solution. Five mice each in the BALB Veh and SWR Veh groups received sc vehicle injections; the remaining four BALB Veh and five SWR Veh mice received ip vehicle injections. Six BALB SCH and nine SWR SCH mice received a sc injection of SCH (50 nmol/kg), and six BALB NTX and nine SWR NTX mice received an ip injection of NTX (1 mg/kg). Following training, all mice were given six daily two-bottle choice sessions (1 h/day) with access to the CS+ and CS- solutions; no drugs were administered prior to these sessions. The positions of the CS+ and CS- solutions were counterbalanced across testing sessions. These doses of SCH and NTX were chosen based on their acquisition effects on sucrose-CFP (Dym et al., 2012).

**Statistics:** In the basic CFP and expression studies, training intakes were averaged over the five CS+ 5% Intralipid and five CS- 0.5% Intralipid sessions for each strain, and evaluated for differences using a t-test. Intakes during the preference tests of Experiment 1 were averaged over successive pairs of sessions (referred to as Tests 1, 2 and 3) and evaluated with two-way analyses of variance (ANOVA, CS solution vs. Test) for each strain. In Experiment 2, intakes during the two preference tests at each dose were averaged and evaluated with ANOVA (CS vs. Dose). Separate ANOVAs evaluated percent CS+ intakes as a function of test or drug dose for each strain.

In the acquisition studies, training intakes were averaged over the five CS+ 5% Intralipid and five CS- 0.5% Intralipid sessions for the three (Veh, SCH, NTX) BALB and SWR training groups and were analyzed with a two-way ANOVA (CS x Group). Intakes during the two sessions of
preference Tests 1, 2 and 3 were averaged. A three-way ANOVA compared the CS intakes of the Veh, SCH and NTX groups (Group x CS x Test). Separate two-way ANOVAs evaluated percent CS+ intakes of the three groups. When main or interaction effects were found, Neuman-Kuels comparisons (p<0.05) were used to detect significant effects. Drug-induced reductions in the acquisition or expression of Intralipid-CFP are operationally defined as a significant reduction in percent CS+ intakes and/or a failure to observe significant differences between CS+ and CS- intakes in the two-bottle preference tests.

Direct comparisons between the BALB and SWR strains were initially performed on the CS+ preferences of the BALB (n=29) and SWR (n=32) mice in the baseline CFP experiment, following Veh treatment in the expression experiment and following Veh training in the acquisition experiment using a t-test. For strain differences in the expression data, separate two-way ANOVAs evaluated the percent CS+ intakes between BALB and SWR mice (between-groups) and among Veh and the two drug dose conditions (within-groups) for DA D1 (SCH) and opioid (NTX) antagonists. For strain differences in the acquisition data, separate three-way ANOVAs evaluated the percent CS+ intakes between BALB and SWR mice (between-groups), training groups (Veh, drug, between-groups) and preference tests (Tests 1, 2 and 3, within-groups) for DA D1 (SCH) and opioid (NTX) antagonists.

Results

Fat-CFP in BALB and SWR mice. In BALB mice, the mean one-bottle training intake of the CS+ 5% Intralipid solution significantly exceeded that of the CS- 0.5% Intralipid solution (1.68 vs. 0.54 ml/1 h; t(7)= 8.96, p<0.0001). In the two-bottle tests, with both CS flavors presented in 0.5% Intralipid, overall, BALB mice consumed significantly more CS+ than CS- (F(1,21)= 12.14, p<0.002) and there were no significant Test (F(2,21)= 0.50, p=0.61) or CS x Test interaction effects (F(2,21)= 0.20, p=0.82). CS+ intakes significantly exceeded CS- intakes in each Test (Figure 18, upper panel). Percent CS+ intakes were similar (F(2,14)= 0.39, p=0.68) in Tests 1 (76%), 2 (70%) and 3 (77%)
(Figure 18, upper panel). Total CS intakes were also similar (F(2,14)= 0.74, p=0.50) in Tests 1 (0.73 ml), 2 (0.89 ml) and 3 (0.66 ml). Thus, Intralipid-CFP was stable across the three tests in BALB mice allowing for analysis of expression and acquisition drug effects.

In SWR mice, the mean one-bottle training intake of the CS+ 5% Intralipid solution significantly exceeded that of the CS- 0.5% Intralipid solution (2.46 vs. 1.65 ml/1 h; t(8)= 5.37, p<0.0007). In the two-bottle choice tests, SWR mice consumed significantly more CS+ than CS- (F(1,24)= 123.03, p<0.0001), and there were no significant Test (F(2,24)= 0.31, p=0.74) or CS x Test interaction effects (F(2,24)= 0.02, p=0.98). CS+ intakes significantly exceeded CS- intakes in each Test (Figure 18, lower panel). Percent CS+ intake were similar (F(2,16)= 0.52, p=0.60) in Tests 1 (84%), 2 (81%) and 3 (81%) (Figure 18, lower panel). Total Intralipid intakes were also similar (F(2,16)= 1.75, p=0.21) in Tests 1 (2.57 ml), 2 (2.83 ml) and 3 (2.82 ml). Thus, Intralipid-CFP was stable across three pairs of tests in SWR mice allowing for analysis of expression and acquisition drug effects.

**DA D1 antagonist effects on the expression of Fat-CFP in BALB and SWR mice.** In BALB mice, the mean one-bottle training intake of the CS+ 5% Intralipid solution significantly exceeded that of the CS- 0.5% Intralipid solution (1.98 vs. 0.60 ml/1 h; t(11)= 10.00, p<0.0001). In the two-bottle choice tests with the BALB SCH group, there were significant differences among drug doses (F(2,15)= 3.55, p<0.05), between CS conditions (F(1,15)= 7.51, p<0.015) and for CS x Dose interaction effects (F(2,15)= 8.92, p<0.003). CS+ intakes exceeded CS- intakes following vehicle, but not following the 200 and 800 nmol/kg SCH doses (Figure 19, upper panel). BALB mice consumed significantly less CS+ following the 200 and 800 nmol/kg SCH doses compared to vehicle; CS- intakes failed to differ as a function of SCH dose (Figure 19, upper panel). Total CS intakes were lower (F(2,10)= 4.58, p<0.039) following the 200 (0.58 ml), but not 800 (0.73 ml) nmol/kg SCH doses relative to vehicle (1.08 ml).
Figure 18. (Chapter 7) Following ten alternating one-bottle training trials with flavored 5% (CS+) and 0.5% (CS-) Intralipid solutions, intakes (mean +SEM, g/1 h) of CS+ and CS- flavors mixed in 0.5% Intralipid solutions were assessed in three pairs of two-bottle tests in BALB (upper panel) and SWR (lower panel) inbred mice. Significant differences are denoted between CS+ and CS- intakes for each strain across tests (*).
The reduction in percent CS+ intake following the 200 (44%) and 800 (58%) nmol/kg SCH doses relative to the vehicle dose (74%) approached, but failed to achieve significance (F(2,10)= 2.68, p=0.11) (Figure 19, upper panel).

In SWR mice, the mean one-bottle training intake of the CS+ 5% Intralipid solution was significantly higher than that of the CS- 0.5% Intralipid solution (2.66 vs. 1.94 ml/1 h, t(12)= 3.55, p<0.004). In the two-bottle preference tests with the SWR SCH group, there were significant differences among drug doses (F(2,18)= 25.55, p<0.0001), between CS conditions (F(1,18)= 4.34, p<0.0007) and for CS x Dose interaction effects (F(2,18)= 15.75, p<0.0001). CS+ intakes significantly exceeded CS- intakes following vehicle, but not following the 200 or 800 nmol/kg SCH doses (Figure 19, lower panel). SWR mice consumed significantly less CS+ at both SCH doses compared to vehicle; CS- intake failed to differ as a function of SCH dose (Figure 19, lower panel).

Total CS intakes significantly declined (F(2,12)= 33.06, p<0.0001) following the 200 (0.97 ml) and 800 (0.50 ml) nmol/kg SCH doses relative to vehicle (2.86 ml). The percent CS+ intake significantly declined (F(2,12)= 12.73, p<0.001) following the 200 (51%) and 800 (48%) nmol/kg SCH doses relative to vehicle (81%) in SWR mice (Figure 19, lower panel).

**Opioid antagonist effects on the expression of Fat-CFP in BALB and SWR mice.** In the two-bottle preference tests with the BALB NTX group, there were significant differences among drug doses (F(2,15)= 5.74, p<0.014), between CS conditions (F(1,15)= 5.83, p<0.029) and for CS x Dose interaction effects (F(2,15)= 7.37, p<0.006). CS+ intakes exceeded CS- intakes following vehicle, but not following 1 and 5 mg/kg NTX doses (Figure 20, upper panel). BALB mice consumed significantly less CS+ following the higher, but not lower NTX dose relative to vehicle; CS- intakes were not altered by NTX (Figure 20, upper panel). Total CS intakes significantly declined (F(2,10)= 5.00, p<0.031) following the 5 (0.38 ml), but not the 1 (0.47 ml) NTX doses relative to vehicle (0.8 ml).
Figure 19. (Chapter 7) (Expression Procedure). Following ten alternating one-bottle training trials with flavored 5% (CS+) and 0.5% (CS-) Intralipid solutions, intakes (mean +SEM, g/1 h) of CS+ and CS- flavors mixed in 0.5% Intralipid solutions were assessed in two-bottle tests in BALB (upper panel) and SWR (lower panel) inbred mice receiving systemic injections of the DA D1-like antagonist, SCH23390 at doses of 0, 200 and 800 nmol/kg 30 min prior to testing. Significant differences in this and the next figure are denoted between CS+ and CS- intake within an injection condition (*) and between CS+ intake following a drug dose relative to the vehicle treatment (+). The percentages of CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted.
Percent CS+ intakes significantly (F(2,10)= 6.17, p<0.018) declined following the 5 (33%) but not the 1 (68%) mg/kg NTX dose relative to the vehicle treatment (76%) (Figure 20, upper panel). In the two-bottle preference tests with the SWR NTX group, overall, the mice consumed

significantly more CS+ than CS- (F(1,15)= 12.86, p<0.0027), but drug dose (F(2,15)= 0.61, p=0.56) and CS x Dose interaction (F(2,15)= 0.89, p=0.43) effects failed to achieve significance (Figure 3, lower panel). CS+ and CS- intakes in SWR mice following NTX did not differ from corresponding vehicle values (Figure 20, lower panel). Total CS intakes also did not differ (F(2,10)= 1.06, p=0.38) following the 0 (2.63 ml), 1 (2.23 ml) and 5 (2.08 ml) mg/kg NTX doses. Percent CS+ intakes did not differ (F(2,10)= 1.17, p=0.35) although it was lower with the 5 (67%) mg/kg NTX dose than with the 0 (79%) and 1 (78%) mg/kg NTX doses (Figure 20, lower panel).

**DA D1 and opioid antagonist effects on the acquisition of Fat-CFP in BALB mice.** Overall, the BALB mice in the Veh, SCH and NTX groups consumed significantly more CS+ 5% Intralipid than CS- 0.5% Intralipid (2.03 vs. 0.49 ml/1 h, F(1,16)= 1752.20, p<0.0001) during one-bottle training. Total training intakes were significantly lower in the NTX (1.56 ml/1 hr.), but not SCH (3.1 ml/1 h) group relative to the Veh group (2.9 ml) (F(2,16)= 25.07, p<0.0001); the Group x CS interaction approached, but did not achieve significance (F(2,16)= 3.33, p=0.062) (Figure 21, upper left panel). In the two-bottle preference tests conducted without drug injections, overall, the BALB mice consumed significantly more CS+ than CS- (F(1,8)= 33.28, p<0.0004), and significant differences were also observed among tests (F(2,16)= 4.81, p<0.023) and for the Test x CS interaction (F(2,16)= 7.97, p<0.004), but not for groups (F(2,16)= 1.27, p=0.31) or for the Group x Test (F(4,32)= 2.75, p=0.14), Group x CS (F(2,16)= 1.82, p=0.19) or Group x Test x CS (F(4,32)= 0.22, p=0.93) interaction effects. CS+ intake was significantly higher than CS- intake in the first two tests, and approached significance (p = 0.06) in the third test of the vehicle-trained group (Figure 21, upper
Figure 20. (Chapter 7) (Expression Procedure). Following ten alternating one-bottle train trails with flavored 5% (CS+) and 0.5% (CS-) Intralipid solutions, intakes (mean+SEM, g/1 h) of CS+ and CS-flavors mixed in 0.5% Intralipid solutions were assessed in two-bottle tests in BALB(upper panel) and SWR(lower panel) inbred mice receiving systemic injections of the general opioid antagonist, naltrexone at doses of 0, 1 and 5 mg/kg 30 min prior to testing.
right panel). CS+ intake was significantly higher than CS- intake across all three tests in the SCH group (Figure 21, lower left panel), and in the first test in the NTX group (Figure 4, lower right panel). Overall, percent CS+ preferences significantly declined across Tests 1 (80%), 2 (74%) and 3 (64%) (F(2,16)= 3.77, p<.046), but the Veh (71%), SCH (75%) and NTX (71%) groups failed to display significant main (F(2,16)= 0.51, p=0.61) or interaction (F(4,32)= 1.08, p=0.38) effects.

*DA D1 and opioid antagonist effects on the acquisition of Fat-CFP in SWR mice.* Overall, the SWR groups treated with Veh, SCH or NTX during one-bottle training consumed significantly more CS+ 5% Intralipid than CS- 0.5% Intralipid (2.25 vs. 1.37 ml/1 h, F(1,9)= 103.63, p<0.0001; Figure 22, upper left panel). There were also significant group (F(2,18)= 5.95, p<0.01) and Group x CS interaction effects (F(2,18)= 3.48, p<0.05). Total training intakes was significantly lower in the SCH compared to the Veh (4.6 ml) and NTX (3.0 ml) groups than the Veh (4.6 ml) group (Figure 22, upper left panel). Specifically, the SWR SCH group consumed less (P< 0.05) CS- 0.5% Intralipid while the SWR NTX group consumed less (P < 0.05) CS+ 5% Intralipid than the Veh group (Figure 22, upper left panel).

*Strain comparisons between BALB and SWR mice.* Overall, the CS+ preferences of the BALB (n=29) and SWR (n=32) mice in the baseline CFP experiment, following Veh treatment in the expression experiment and following Veh training in the acquisition experiment did not significantly differ (77.1% vs. 80.3%, t(50)= 0.80, p=0.44), indicating that the strength and persistence of fat-CFP preferences were comparable in the two strains. Evaluation of strain differences in DA D1 antagonist effects upon expression of fat-CFP revealed significant differences among conditions (F(2,12)= 14.72, p<0.0006), but not between strains (F(1,6)= 0.30, p=0.87) or the strain x condition interaction (F(2,12)= 0.90, p=0.43). BALB and SWR strains failed to differ in percent CS+ intake following Veh or the 200 or 800 nmol/kg doses of SCH. Evaluation of strain differences in opioid antagonist effects upon expression of fat-CFP revealed significant differences among conditions (F(2,10)= 6.96, (3.1 ml) and NTX (3.0 ml) groups than the Veh (4.6 ml) group (Figure 22, upper left panel). Specifically, the SWR SCH group consumed less (P< 0.05) CS- 0.5% Intralipid while the SWR NTX group consumed less (P < 0.05) CS+ 5% Intralipid than the Veh group (Figure 22, upper left panel).
Figure 21. (Chapter 7) (Acquisition Procedure). Upper Left Panel: Training intakes (mean +SEM, g/1 h) of CS+ 5% and CS- 0.5% Intralipid solutions in BALB mice pretreated 30 min earlier with vehicle (Veh), SCH23390 at a dose of 50 nmol/kg (SCH) or naltrexone at a dose of 1 mg/kg (NTX). Significant differences in this and the next figure are denoted between CS+ and CS- intakes within each group (*) as are significant differences in CS+ or CS- intakes following SCH or NTX relative to corresponding Veh (+). Testing intakes (mean +SEM, g/1 h) of CS+ and CS- solutions during two-bottle Tests 1, 2 and 3 in BALB mice receiving Veh (upper right panel), SCH (lower left panel) or NTX (lower right panel) during training. Numbers atop bars represent the mean percent intakes of CS+ (%CS+). Significant differences in this and the next figure are denoted between CS+ and CS-intake and for %CS+ intake within each test (+).
Analysis of the two-bottle preference tests revealed, overall, that SWR mice consumed significantly more CS+ than CS- (F(1,9)= 27.14, p<0.0006) with a significant Group x CS (F(2,18)= 17.99, p<0.0001) interaction, but not for groups (F(2,18)= 1.24, p=0.31), tests (F(2,18)= 1.19, p=0.32) or for the Group x Test (F(2.21, p=0.17), Test x CS (F(2,18)= 1.94, p=0.17) or Group x Test x CS (F(4,36)= 0.28, p=0.89) interaction effects. CS+ intakes significantly exceeded CS- intakes in all three tests in the SWR Veh (Figure 5, upper right panel) and SWR NTX (Figure 5, lower right panel) groups whereas CS+ and CS- intakes did not differ in the SWR SCH group (Figure 5, lower left panel). The percent CS+ intake averaged over the three tests was significantly lower (F(2,18)= 16.39, p<0.0001) in the SCH group (48%) than in the NTX (80%) and Veh (75%) groups (Figure 5); tests (F(2,18)= 1.00, p=0.39) and the group x test interaction (F(4,36)= 0.29, p=0.88) failed to differ. p<0.013), but not between strains (F(1.5)= 1.38, p=0.29) or the strain x condition interaction (F(2,10)= 2.20, p=0.16). Whereas BALB and SWR strains failed to differ in percent CS+ intake following Veh or the 1 mg/kg NTX dose, the 5 mg/kg dose of NTX produced significantly greater inhibition of the expression of fat-CFP in BALB (33%) relative to SWR (67%) mice. Evaluation of strain differences in DA D1 antagonist effects upon acquisition of fat-CFP revealed significant differences between strains (F(1,9)= 6.80, p<0.028), between groups (F(1,9)= 8.99, p<0.015), across tests (F(2,18)= 5.77, p<0.012) and the strain x group interaction (F(1,9)= 10.51, p<0.0009), but not for the strain x test (F(2,18)= 1.30, p=0.30), group x test (F(2,18)= 0.48, p=0.62) or strain x group x test (F(2,18)= 0.67, p=0.53) interaction effects. BALB and SWR strains failed to differ in percent CS+ intake following Veh. However, SWR mice trained with SCH displayed significantly lower percent CS+ intake than BALB mice trained with SCH during the first (51% vs. 76%) and second (44 vs. 79%) tests, and approached significance during the third (48% vs. 69%) test.
Figure 22. (Chapter 7) (Acquisition Procedure). Upper Left Panel: Training intakes (mean +SEM, g/1 h) of CS+ 5% and CS- 0.5% Intralipid solutions in SWR mice pretreated 30 min earlier with vehicle (Veh), SCH23390 at a dose of 50 nmol/kg (SCH) or naltrexone at a dose of 1 mg/kg (NTX). Testing intakes (mean +SEM, g/60 min) of CS+ and CS- solutions during two-bottle Tests 1, 2 and 3 in SWR mice receiving Veh (upper right panel), SCH (lower left panel) or NTX (lower right panel) during training. Numbers atop bars represent the mean percent intakes of CS+ (%CS+).
Evaluation of strain differences in opioid antagonist effects upon acquisition of fat-CFP revealed significant differences between strains (F(1,9)= 5.62, p<0.042), across tests (F(2,18)= 7.93, p<0.003) and for the interaction between strains and groups (F(1,9)= 3.59, p<0.049), but not between groups (F(1,9)= 1.84, p=0.21), or for the strain x test (F(2,18)= 1.54, p=0.24), group x test (F(2,18)= 0.01, p=0.99) or strain x group x test (F(2,18)= 0.20, p=0.82) interaction effects. BALB and SWR strains failed to differ in percent CS+ intake in Veh-trained or NTX-trained groups.

Discussion

In this study, we investigated fat-CFP in two inbred mouse strains and the involvement of DA D1-like and opioid receptor signaling in this learning process. As noted above, the BALB and SWR mice acquired similar preferences, 77 vs. 80%, respectively, for the CS+ flavor added to a 5% Intralipid solution over the CS- flavor added to a 0.5% Intralipid solution. This occurred despite the fact that SWR mice consumed more of the flavored 5% Intralipid solution during training than did BALB mice. SWR mice also consumed more 5% and 0.5% Intralipid than BALB mice in 24 h intake tests (Lewis et al., 2007). In a recent drug analysis of sucrose-CFP (Dym et al., 2012), BALB and SWR mice displayed comparable preferences for the sucrose-paired CS+ flavor (82% vs. 79%, respectively), which were of similar magnitude to the fat-CFP obtained in the present study. These strong and similar sucrose-CFP responses occurred in BALB and SWR mice despite the facts that whereas SWR mice consumed significantly more sucrose than BALB mice in 24-h intake tests (Lewis et al., 2005), BALB mice consumed significantly more CS+ (sucrose) than CS- (saccharin) solutions during training, whereas SWR mice consumed more CS- (saccharin) than CS+ (sucrose) solutions during training (Dym et al., 2012; Pinhas et al., 2012). The robust and persistent fat-CFP observed in BALB and SWR mice using Intralipid as the fat source was very similar to the robust and persistent fat-CFP observed in outbred Sprague-Dawley rats using corn oil as the fat source (Dela
Cruz et al., 2012a, 2012b). These findings indicate that BALB and SWR inbred mouse strains are well-suited for pharmacological studies of fat and sugar conditioning.

*DA D1 signaling and modulation of fat-CFP learning in inbred mice.* In our earlier study (Dym et al., 2012) evaluating DA D1 antagonist effects upon the expression of sucrose-CFP, both BALB and SWR mice displayed significant reductions in their CS intakes and CS+ preferences. Further, SCH treatment during training prevented SWR, but not BALB mice from displaying an initial CS+ preference in the two-bottle test. Therefore, if the neuropharmacological substrates of sugar-CFP and fat-CFP were identical in these strains of mice, we would expect that SCH should reduce the expression of a fat-CFP in both strains, and that DA D1 antagonist-sensitive effects upon the acquisition of fat-CFP should be noted for the SWR, but not the BALB strain. In fact, SCH blocked the expression of fat-CFP in both BALB and SWR mice, and the two strains failed to differ in percent CS+ intake following Veh or the 200 or 800 nmol/kg doses of SCH in the expression paradigm. The fat-CFPs observed in the BALB and SWR mice were similar in magnitude to that previously observed in outbred Sprague-Dawley rats (~80%) trained with flavored corn oil emulsions (Dela Cruz et al., 2012a). As in the present experiment, the expression of the fat-CFP in rats was attenuated by SCH at 200 and 800 nmol/kg doses. Thus, identical DA D1 antagonist-sensitive reductions were observed for the expression of both fat- and sucrose-CFP in the two inbred mouse strains and in outbred rats.

The acquisition of fat-CFP was eliminated in the SWR mice receiving SCH during training, but was not at all impaired in the BALB mice receiving SCH during training. Thus, whereas BALB and SWR strains receiving vehicle during training failed to differ in the acquisition of fat-CFP, SWR mice trained with SCH displayed significantly lower % CS+ intake than BALB mice trained with SCH. Hence, identical patterns of the strain-specific sensitivity of SWR, but not BALB mice to DA D1 antagonist-induced elimination were observed for the acquisition of both fat- and sucrose-CFP.
Interestingly, DA D1 antagonism during training failed to affect the acquisition of fat-CFP in outbred rats (Dela Cruz et al., 2012a), which is similar to the pattern observed with BALB mice, but not SWR mice in the present study. These data suggest strain- and species-specific effects of SCH on the acquisition of fat-CFP. Whereas similarities are observed in the DA D1 antagonist-mediated reductions in expression of sucrose- and fat-CFP in both strains, and in acquisition of sucrose- and fat-CFP in SWR, but not BALB strains, DA D1 antagonism exerts differential effects on the suppression of fat and sucrose intake in the two strains. That is, SCH suppressed Intralipid intake more in SWR than BALB mice (Dym et al., 2010), but suppressed sucrose intake to similar degrees in both strains (Dym et al., 2009). Interestingly, the relative abilities of DA D1 antagonism to reduce fat intake in SWR, but not BALB mice were good predictors in assessing DA D1 antagonist-induced effects upon the acquisition, but not the expression of fat-CFP. It should be noted that BALB mice differed from 10 other inbred strains in their failure to self-administer cocaine, suggesting sub-sensitivity in the DA reward system (Thomsen & Caine, 2011). However, SWR mice were not included in this study so it is not clear that BALB and SWR mice differ in this respect.

**Opioid signaling and modulation of fat-CFP learning in inbred mice.** In our earlier study (Dym et al., 2012) evaluating opioid antagonist effects upon the expression of sucrose-CFP, both BALB and SWR mice displayed significant reductions in their CS intakes and CS+ preferences. Further, NTX treatment during training prevented BALB, but not SWR mice from displaying an initial CS+ preference for sucrose in the two-bottle tests. Therefore, if the neuropharmacological substrates of sugar-CFP and fat-CFP were identical in these strains of mice, we would expect that NTX should reduce the expression of a fat-CFP in both strains, and that opioid antagonist-sensitive effects on the acquisition of fat-CFP should be observed in BALB, but not SWR mice. In fact, BALB mice displayed NTX-induced reductions in the expression of fat-CFP, similar to that observed for sucrose-CFP (Dym et al., 2012). In contrast, SWR mice failed to display NTX-induced reductions in
the expression of fat-CFP. Indeed, inter-strain comparisons revealed that the 5 mg/kg dose of NTX produced significantly greater inhibition of the expression of fat-CFP in BALB (33%) relative to SWR (67%) mice. Like the SWR mice, the expression of fat-CFP in outbred rats was also insensitive to NTX administration (Dela Cruz et al., 2012b). Thus, different strain-specific patterns of opioid antagonist-induced effects were observed for the expression of fat- and sucrose-CFP.

In addition, the acquisition of fat-CFP was not impaired by NTX in SWR mice, whereas BALB mice displayed a marginally less persistent fat-CFP following NTX during training as compared to BALB-Veh and SWR-NTX groups. Like the SWR mice, the acquisition of fat-CFP in outbred rats was also insensitive to NTX administration (Dela Cruz et al., 2012b). In marked contrast to these preference acquisition effects, NTX significantly decreased fat intake in SWR mice but not in BALB mice (Dym et al., 2010). Thus, the ability (SWR) or inability (BALB) of NTX to suppress Intralipid consumption does not predict the drug’s effects on the acquisition and expression of fat-CFP.

SWR mice appear to have a selectively compromised opioid-mediated reward system. In contrast to 10 other inbred strains, a wide range of NTX doses failed to alter sucrose intake in SWR mice (Dym et al., 2007), corresponding to the failure of opioid antagonism to affect the acquisition of sucrose-CFP (Dym et al., 2012). Furthermore, SWR mice display significantly less morphine self-administration behavior as compared to 14 other inbred strains (Belknap et al., 1993), and display less morphine-induced conditioned place preferences (Gieryk et al., 2010; Solecki et al., 2009). Yet, when administered opioid drugs chronically, SWR mice display exaggerated responses. SWR mice are far more sensitive to naloxone-precipitated withdrawal as measured by far greater numbers of jumping responses than ten other inbred mouse strains, including BALB mice (Kest et al., 2002). The potential significance and mechanisms of action are presented in the General Discussion.
Chapter 8

ACQUISITION AND EXPRESSION OF FAT-CONDITIONED FLAVOR PREFERENCES ARE DIFFERENTIALLY AFFECTED BY NMDA RECEPTOR ANTAGONISM IN BALB/c AND SWR MICE

Introduction

Dietary fat stimulates overeating in outbred rats (e.g., Ackroff and Sclafani, 2009), and can condition a preference for an arbitrary cue flavor (Ackroff et al., 2005; Ackroff and Sclafani, 2009; Sclafani, 1999). Dopamine mediation of fat-related reward in outbred rats is supported by observations that sham-feeding of corn oil released nucleus accumbens dopamine (Liang et al., 2006), and that dopamine D1 and D2 receptor antagonists suppressed fat intake (e.g., Baker et al., 2001; Davis et al., 2006; Rao et al., 2008; Weatherford et al., 1988). Yet, dopamine D1 (SCH23390) and D2 (raclopride) receptor antagonism only attenuated expression (maintenance) and minimally affected acquisition (learning) of a fat (corn oil) conditioned flavor preference in outbred rats (Dela Cruz et al., 2012b). The use of inbred mouse strains allows for the study of genetic variance in the analysis of the pharmacological substrates of fat intake per se and the acquisition and expression of fat-conditioned flavor preferences. BALB/c and SWR mice in particular display robust fat intake and fat-conditioned flavor preferences (Dym et al., 2010; Kraft et al., 2013). Further, dopamine D1, but not D2 receptor antagonism differentially reduced intake of the soybean oil emulsion, Intralipid with SCH23390, but not raclopride reducing Intralipid intake in inbred SWR, but not BALB/c mice (Dym et al., 2010). The expression of Intralipid-conditioned flavor preference was significantly reduced by SCH23390 in both strains, whereas preference acquisition was significantly reduced by SCH23390 in SWR, but not BALB/c mice (Kraft et al., 2013).
General and selective opioid receptor antagonists also potently reduce fat intake in rats (see reviews: Bodnar, 2004; Taha, 2010) and outbred mice (Sakamoto et al., 2015a,b,c). Yet, naltrexone only modestly the reduced expression and failed to affect the acquisition of corn oil-conditioned flavor preferences (Dela Cruz et al., 2012b). Naltrexone potently suppressed Intralipid intake in SWR, but not BALB/c inbred strains (Dym et al., 2010). In contrast, naltrexone significantly reduced expression and mildly reduced acquisition of Intralipid-conditioned flavor preferences in BALB/c, but not SWR mice (Kraft et al., 2013).

The NMDA receptor has been implicated in the mediation of fat intake such that the non-competitive NMDA receptor antagonist, MK-801 produces short-term suppression of fat-enriched food intake elicited by the inhibitor of fatty acid oxidation, 2-mercaptoacetate (Duva et al., 2005). Intake of a high-fat source was suppressed by the NMDA antagonists, MK-801 (Buttigieg et al., 2014) and memantine (Popik et al., 2011), as well as the NMDA co-agonist, D-serine (Sasaki et al., 2015). Further, mice with deletion of NMDA receptors in AgRP neurons display reductions in body fat (Liu et al., 2012), MK-801 administered into the caudomedial nucleus of the solitary tract abolished duodenal lipid-induced activation of brown fat thermogenesis (Blouet and Schwartz, 2012). Moreover, a crucial role for glutamate signaling in food-related incentive learning is supported by findings that glutamate receptor antagonists administered into either the amygdala or nucleus accumbens impaired appetitive instrumental learning (Hernandez et al., 2005; Kelley et al., 1997) and conditioned taste avoidance (Yasoshima et al., 2000). Further, NMDA receptor antagonists administered into the ventral tegmental area impaired cue-sucrose learning and accumbal dopamine release (Stuber et al., 2008; Zellner et al., 2009; Zweifel et al., 2009). MK-801 completely blocked the acquisition, but minimally affected the expression of conditioned flavor preferences elicited by fat (corn oil: Dela Cruz et al., 2012b) and sugar (fructose: Golden and Houpt, 2007) in outbred rats. We (Kraft et al., 2016) recently found that whereas MK-801 minimally reduced expression of fructose-
and sucrose-conditioned flavor preferences in BALB/c mice, only a high dose of MK-801 blocked expression of fructose-, but not sucrose-conditioned flavor preferences in SWR mice. In contrast, MK-801 completely eliminated the acquisition of fructose- and sucrose-conditioned flavor preferences in both strains with lower antagonist doses more effective in BALB/c (100 µg/kg) relative to SWR (200 µg/kg) mice.

Therefore, the present study investigated whether systemic administration of MK-801 differentially affected expression and acquisition of fat-conditioned flavor preferences in BALB/c and SWR mice. The preference was induced by training mice to drink a CS+ flavor (e.g., cherry Kool Aid) added to a more-concentrated 5% Intralipid solution, and a CS- flavor (e.g., grape) added to a less-concentrated 0.5% Intralipid solution, and evaluating preferences in two-bottle tests with both CS flavors presented in 0.5% Intralipid in the two strains (Kraft et al., 2013).

Materials and Methods

Subjects: Inbred BALB/c (Stock #000651) and SWR (Stock #000689) male mice (Jackson Laboratories, Bar Harbor, ME, 6 weeks of age, both strains ~25 g at arrival) were acclimated to the Queens College vivarium for one week in group (5 per cage) housing. The animals were then housed individually in plastic cages (30 x 20 x 15 cm) with stainless steel tops throughout the entire study, and maintained on a 12 h light/12 h dark cycle (lights off at 2000 h) at a constant temperature of 22°C for two more weeks consistent with our previous studies (e.g., Kraft et al., 2013, 2016). It should be noted that such housing can constitute a stress for rodents, but the intake paradigms and maintenance of restricted body weight levels required individual housing. Chow (Lab Diet Mouse Chow 5015) and water were provided, but two weeks before preference training began, the mice were placed on a food restriction schedule in which 2-3 g of chow was placed in their cages daily with water available ad libitum. The mice were weighed just prior to the onset of food-restriction, and their body weights were maintained at 85-90% of ad libitum levels during training/testing. Individual mice were tested in
one training paradigm. The experimental procedures were approved by the Queens College Institutional Animal Care and Use Committee certifying that all subjects and procedures are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

**Test Solutions and Initial Training:** The training solutions consisted of 5% and 0.5% Intralipid (Baxter Laboratories, Deerfield, IL) solutions flavored with 0.05% unsweetened grape or cherry Kool-Aid (Kraft Foods, White Plains, NY). For half of the mice in each strain, the CS+ flavor added to the 5% Intralipid solution was cherry and the CS- flavor added to the 0.5% Intralipid solution was grape; the flavor-fat solution pairs were reversed for the remaining animals. In the two-bottle preference tests, the CS+ and CS- flavors were each presented in the 0.5% Intralipid solution, a strategy used in our prior rat fat-conditioned flavor preference studies (Dela Cruz et al., 2012a, 2012b). These two Intralipid concentrations were chosen on the basis of consistent intake in the two strains in long-term (24 h: Lewis et al., 2007) and short-term (1 h: Dym et al., 2010; Kraft et al., 2013) intake tests. All testing took place in each mouse’s home cage during the mid-light phase of the light: dark cycle. Two weeks before testing began, the mice were placed on a food restriction schedule that maintained their body weights at 85-90% of their ad libitum level. The mice were initially trained to drink an unflavored 0.2% saccharin (Sigma Chemical Co., St. Louis, MO) solution from a stainless steel sipper tube connected to a 10 ml plastic syringe (Pinhas et al., 2012). This training procedure was repeated daily until all mice sampled the sipper tubes with short (< 1 min) latency, typically within three days. Accurate measurement (+0.1 ml gradations) of this and all subsequent solutions was insured by using a retrofitted testing sipper tube described previously (Dym et al., 2007, 2009, 2010; Kraft et al., 2013). Each sipper tube was firmly secured to the stainless steel top of the cage by a taut metal spring (100 mm) with clips at each end that affixed to the cage top so that the gradations and meniscus were easily visible. The sipper opening was occluded when placed on and then removed from the cage to minimize spillage in this and all subsequent conditions.
Solution spillage was not observed during any of the training and test sessions. The limited food rations were provided 1 h after each training and testing session.

**NMDA Receptor Antagonism and Fat-Conditioned Flavor Preference Expression Procedure:** Ten BALB/c and 10 SWR male mice received ten one-bottle training sessions (1 h/day) with 8 ml of the flavored CS+ 5% Intralipid solution presented on odd-numbered days, and 8 ml of the flavored CS- 0.5% Intralipid solution presented on even-numbered days. On days 9 and 10, all mice had access to a second sipper tube containing water. This familiarized them to the presence of two sipper tubes used during the choice tests; water intake was negligible in these training trials. The position of the CS and water sipper tubes varied across the two-bottle training and subsequent testing days using a left-right-right-left pattern. Solution intakes during training were measured by weighing (0.1 g) the sipper tubes before and after the 60-min sessions. Following training, the male mice were given six two-bottle choice test sessions (1 h/day) with access to the CS+ and CS- flavors mixed in 0.5% Intralipid solutions. The positions of the CS+ and CS- solutions were counterbalanced across testing sessions. As conducted in previous expression studies (e.g., Kraft et al., 2013, 2016), thirty min prior to the first two test sessions, an intraperitoneal (i.p.) vehicle (Veh: 0.9% normal saline) injection was administered to the mice. Then on the subsequent test days, the mice received two sessions each with the two NMDA antagonist doses (100 and 200 µg/kg) with half tested with an ascending dose order and the remainder with a descending dose order. MK-801 was mixed at concentrations of 10 and 20 µg/ml and administered i.p. at 10 ml/kg body weight. The doses were chosen based on testing of fructose-conditioned flavor preference acquisition and expression in outbred rats (Golden and Houpt, 2007), and the testing of fructose- and sucrose-conditioned flavor preference acquisition and expression in these inbred mouse strains (Kraft et al., 2016). The use of a paradigm in which vehicle injections were assessed initially, and drug doses were assessed subsequently raises the possibility that any drug-induced decrease could theoretically be due to extinction of the CS+ preference due to
repeated testing. However, in initially assessing fat-conditioned flavor preferences in these strains (Kraft et al., 2013), BALB/c and SWR mice failed to display any reduction in CS+ preferences over three pairs of preference tests. Further, male mice were chosen because all previous studies were performed in males, thereby allowing direct comparisons of strain-specific NMDA antagonist expression effects on fat-conditioned flavor preferences with strain-specific NMDA antagonist effects on sugar-conditioned flavor preferences (Kraft et al., 2016), and with dopamine D. and opioid antagonist effects on fat-conditioned flavor preferences (Kraft et al., 2013). It should be noted that male (86%) and female (80%) C57BL/6 mice displayed similar preferences for a CS+ flavor paired with intragastric infusions of 6.4% Intralipid solutions (Sclafani et al., 2013). One other issue concerns strain-specific stress responses to the paradigm and to MK-801. As indicated previously, these strains were selected because they showed similar preference conditioning responses to fat in our earlier study (Kraft et al., 2013). The possibility that they display different stress response to MK-801 administration requires other types of behavioral tests in future studies.

**NMDA Receptor Antagonism and Fat-Conditioned Flavor Preference Acquisition Procedure:**

Seventeen additional BALB/c and 18 additional SWR male mice were each divided into two subgroups and were treated with Veh (BALB/c, n=8; SWR, n=9) or MK-801 (100 µg/kg: BALB/c, n=9; SWR, n=9) 30 min prior to the 10 one-bottle training sessions with the CS+ 5% Intralipid solution and the CS- 0.5% Intralipid solution. Following training, all mice were given six daily two-bottle choice sessions (1 h/day) with access to the CS+ and CS- solutions mixed in 0.5% Intralipid; no drugs were administered prior to these sessions. The positions of the CS+ and CS- solutions were counterbalanced across testing sessions, and the results were analyzed as mean 1-h intakes during successive pairs of sessions (referred to as Tests 1, 2 and 3) to control for side position effects.

**Statistics:** Significant differences in expression training intakes were observed between BALB/c and SWR mice (F(1,9)= 6.83, p<0.028), between the CS+ 5% and CS- 0.5% Intralipid
conditions (F(1,9)= 15.80, p<0.003), and for the strain x CS interaction (F(1,9)= 8.09, p<0.0193).

Therefore, each strain was evaluated separately for intakes during the two preference tests following each dose using a 2-way ANOVA (CS vs. Dose). Percent CS+ preference was calculated for each animal in the following manner: CS+ intake / Total Intake x 100. Separate ANOVAs evaluated percent CS+ intakes as a function of drug dose for each strain. Significant differences in acquisition training intakes were observed between BALB/c and SWR mice (F(1,8)= 24.82, p<0.001) and between the CS+ 5% and CS- 0.5% Intralipid conditions (F(1,8)= 788.11, p<0.0001). Again, each strain was evaluated in separate ANOVA’s for the two (Veh, MK801) BALB/c and SWR training groups and was analyzed with a two-way ANOVA (CS x Group). Intakes during the two sessions of preference Tests 1, 2 and 3 were averaged. A three-way ANOVA compared the CS intakes of the Veh and MK801 groups (Group x CS x Test). Separate two-way ANOVAs evaluated percent CS+ intakes of the three groups. When main or interaction effects were found, Newman-Kuels comparisons (p<0.05) were used to detect significant effects. Drug-induced reductions in the acquisition or expression of Intralipid-CFP are operationally defined as a significant reduction in percent CS+ intakes and/or a failure to observe significant differences between CS+ and CS- intakes in the two-bottle preference tests.

Results

NMDA Antagonism and Fat-Conditioned Flavor Preference Expression in BALB/c and SWR mice: During expression training, although intakes of the CS+ 5% Intralipid solution were similar between BALB/c (2.04 g) and SWR (2.08 g) mice, the SWR strain (2.10 g) consumed significantly more of the CS- 0.5% Intralipid solution than the BALB/c strain (1.06 g), consistent with a previous study (Kraft et al., 2013). Therefore, MK-801 effects on fat-CFP expression were evaluated separately for the two strains. In two-bottle testing of BALB/c mice, significant differences were observed among doses (F(2,27)= 5.07, p<0.014), between CS+ and CS- conditions (F(1.27)= 13.30,
p<0.001) and for the dose x condition interaction (F(2,27)= 9.80, p<0.0006). CS+ intakes were significantly higher than CS- intakes in BALB/c mice following Veh, but not following the 100 and 200 µg/kg MK-801 doses (*, Figure 23A). CS+, but not CS- intakes following Veh were significantly lower following both MK-801 doses in BALB/c mice (+, Figure 23A). Percent CS+ intake in BALB/c mice was significantly reduced for Intralipid-conditioned flavor preferences (F(2,18)= 125.49, p<0.00046) following the 100 (48%) and 200 (50%) µg/kg MK-801 doses relative to Veh (71%) (Figure 23A). Total CS intakes significantly differed (F(2,18)= 7.21, p<0.005) for the 100 (1.09 g) and 200 (1.01 g) MK-801 doses relative to Veh (1.79 g) in BALB/c mice.

In two-bottle testing of SWR mice, significant differences were observed between CS+ and CS-conditions (F(1,27)= 8.19, p<0.008) and for the dose x condition interaction (F(2,27)= 3.23, p<0.05), but not among doses (F(2,27)= 0.39). CS+ intakes were significantly higher than CS- intakes in SWR mice following Veh and the 200, but not the 100, µg/kg MK-801 doses (*, Figure 23B). Percent CS+ intake in SWR mice was significantly reduced for Intralipid-conditioned flavor preferences (F(2,18)= 6.84, p<0.006) following the 100 (48%), but not 200 (63%) µg/kg MK-801 dose relative to Veh (72%) (Figure 23B). Total CS intakes failed to differ (F(2,18)= 0.42) among Veh (2.44 g), and the 100 (2.24 g) and 200 (2.59 g) MK-801 doses in SWR mice. Strain comparisons of NMDA-induced fat-conditioned flavor preference expression effects evaluated percent CS+ preferences and found significant differences among vehicle and NMDA antagonist doses (F(2,54)= 11.26, p<.0001), but not between BALB/c and SWR mice (F(1,54)= 1.21, ns) or for the interaction between strains and doses (F(2,54)= 0.96, ns).

**NMDA Receptor Antagonism of fat-Conditioned Flavor Preference Acquisition in BALB/c and SWR mice:** During acquisition training, significant differences were observed between the BALB/c and SWR strains (F(1,8)= 24.82, p<0.001), between CS+/5% and CS-/0.5% training conditions.
Figure 23. (Chapter 8) Fat-Conditioned Flavor Preference Expression Testing: Intakes (mean +SEM, g/1 h) of flavored Intralipid solutions of 5% (CS+) and 0.5% (CS-) during one-bottle training in BALB/c (Panel A) and SWR (Panel B) inbred mice receiving systemic injections of the NMDA antagonist, MK-801 at doses of 0, 100 and 200 µg/kg 30 min prior to testing. Significant differences are denoted between CS+ and CS- intake within an injection condition (*). The percentages of CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted.
Overall, CS training intakes were significantly higher in SWR mice than BALB/c mice, and CS+/5% training intakes were significantly higher than CS-/0.5% training intakes in both strains (Figures 24A and 24D). CS+/5% training intakes following Veh and MK-801 were significantly higher in SWR mice than BALB/c mice, whereas CS-/0.5% training intakes following MK-801 were significantly higher in SWR mice than BALB/c mice. Therefore, MK-801 effects on fat-conditioned flavor preference acquisition were evaluated separately for the two strains.

In 2-bottle preference tests in BALB/c mice, significant differences were observed between Veh and MK-801 groups (F(1,8)= 5.24, p<0.05), among the three tests (F(2,16)= 5.02, p<0.02), between CS+ and CS- conditions (F(1,8)= 27.11, p<0.0008), and for the interaction between groups and conditions (F(1,8)= 100.69, p<0.0001). Whereas CS+ intake was significantly higher than CS- intake across all tests in the BALB/c Veh-trained group (Figure 24B), the BALB/c MK(100)-trained group failed to display any differences between CS+ and CS- intakes across tests (Figure 24C). The significant differences (F(1,8)= 80.29, p<0.0001) in percent CS+ preferences of BALB/c mice revealed lower preferences in the MK(100)-trained relative to the Veh-trained groups across all three tests (Figures 24B and 24C). Total CS intake was significantly higher (F(1,8)= 6.68, p<0.03) in Veh-trained (1.45 g) than MK(100)-trained (1.01 g) BALB/c mice across all three tests (F(2,16)= 4.23, p<0.03).

In 2-bottle preference tests in SWR mice, significant differences were observed only for the interaction between groups and conditions (F(1,8)= 70.61, p<0.0001). Whereas CS+ intake was significantly higher than CS- intake across all tests in the SWR Veh-trained group (Figure 24E), the SWR MK(100)-trained group displayed significantly greater CS- intake than CS+ intake in the
Figure 24. (Chapter 8) Training intakes (mean ±SEM, g/1 h) of CS+ and CS- Intralipid solutions in BALB/c (Panel A) or SWR (Panel D) mice pretreated 30 min earlier with vehicle (Veh) or MK-801 at a dose of 100 µg/kg (MK(100)). Significant differences between CS+ and CS- intake are denoted (*) as well as significant differences in CS+ and CS- training intakes between strains (#). Preference test intakes (mean ±SEM, g/1 h) of CS+ and CS- solutions during six days of three pairs of 2-bottle tests (1, 2 and 3) in BALB/c mice receiving Veh (Panel B) or MK-801 (100 µg/kg, Panel C) during training or in SWR mice receiving Veh (Panel E) or MK-801 (100 µg/kg, Panel F) during training. Significant differences are denoted between CS+ and CS- intake (*) and for drug treatment relative to corresponding Veh (+). The percentages of CS+ intake over total intake are denoted above each pair of values with significant differences relative to vehicle treatment (+) noted.
second and third tests (Figure 24F). Further, CS+ and CS- intakes were significantly lower across all three tests in the SWR MK(100)-trained group relative to the SWR Veh-trained group (Figures 24E and 24F). The significant differences (F(1.8)= 125.34, p<0.0001) in percent CS+ preferences of SWR mice revealed lower preferences in the MK(100)-trained relative to the Veh-trained groups across all three tests (Figures 24E and 24F). Total CS intake failed to differ in Veh-trained and MK(100)-trained SWR mice across all three tests.

Strain comparisons of NMDA-induced fat-CFP acquisition effects evaluated percent CS+ preferences and found significant differences between the Veh and MK(100) groups (F(1,93)= 307.17, p<0.0001) and for the interactions between strain and group (F(1,93)= 9.96, p<002), between strain and test (F(2,93)= 3.84, p<0.025), and among strain, group and test (F(2,93)= 3.12, p<0.049), but not between strains (F(1,93)= 1.22, ns) or among tests (F(2,93)= 1.05, ns). The BALB/c and SWR MK(100)-trained groups displayed significantly smaller preferences than their corresponding Veh-trained groups. The magnitude of the preference was significantly less in the SWR MK(100)-trained group (17%) relative to the BALB/c MK(100)-trained group (47%) on the third test; they showed non-significant reductions on the second test (BALB/c: 31%; SWR: 24%).

**Discussion**

The major finding of the present study was that NMDA receptor antagonism with MK-801 eliminated acquisition (learning) of fat (Intralipid)-conditioned flavor preferences in BALB/c mice (Table 4H). These data consistently parallel the recent finding that MK-801 eliminated acquisition of both sucrose- and fructose-conditioned flavor preferences in BALB/c mice (Table 4C) (Kraft et al., 2016) as well as blocking acquisition of fat (corn oil)-conditioned flavor preferences (Table 4G) and fructose-conditioned flavor preferences (Table 4B) in outbred rats (Dela Cruz et al., 2012a; Golden and Houpt, 2007). Moreover, the preference response for the flavor associated with the higher Intralipid concentration was not only eliminated in SWR mice treated with a 100 µg/kg dose of MK-
801 during training, but appeared to turn into an avoidance response during the second and third choice tests. This strain-specific effect appears to be unique to fat-conditioned flavor preferences as a higher 200, but not 100 µg/kg dose of MK-801 eliminated the acquisition of sucrose- and fructose-conditioned flavor preferences in SWR mice without producing an avoidance response (Kraft et al., 2016). It should be noted that this is the first instance in which a preference was turned into an avoidance response in either outbred rats or inbred mice by pharmacological manipulation (see review: Bodnar, 2017). Quinine-induced conditioned avoidance responses have been shown to be enhanced by NMDA (as well as D, and opioid) receptor antagonists in outbred rats (Rotella et al., 2014). However, it is not clear why MK-801 produces a strain-specific switching in the learning of a conditioned response from a preference to an avoidance response. In sum, it appears that NMDA receptor signaling is essential for the learning of preferences elicited by both sugars and fat in two distinct inbred mouse strains and outbred rats. This pattern differs from the relative inabilities of systemic dopamine D and opioid receptor antagonism to produce similar effects on acquisition of fat- and sugar-CFP in these inbred murine strains. Thus, whereas acquisition of fat-conditioned flavor preferences was eliminated in SWR mice by SCH23390 (Table 4I: Kraft et al., 2013), it was ineffective in altering fat-conditioned flavor preferences in BALB/c mice (Table 4H: Kraft et al., 2013) or outbred rats (Dela Cruz et al., 2012b).

Dopamine D receptor antagonism also exerted a different pattern of effects on acquisition of sugar-conditioned flavor preferences such that SCH23390 eliminated acquisition of sucrose-conditioned flavor preferences was eliminated in SWR, but not BALB/c (Dym et al., 2012). In contrast, SCH23390 eliminated acquisition of fructose-conditioned flavor preferences in SWR mice (Table 4F) and outbred rats (Table 4B), and hastened the extinction of fructose-conditioned flavor preferences in BALB/c mice (Table 4E) (Baker et al., 2003; Kraft et al., 2015b).
**TABLE 4.** Summary of NMDA (MK-801), dopamine (DA) D1 (SCH23390), and opioid (naltrexone) receptor antagonist effects on acquisition (learning) and expression (maintenance) of sugar (sucrose, fructose) and fat (corn oil, Intralipid) conditioned flavor preferences (CFP) in outbred male rats and inbred male mouse strains.

<table>
<thead>
<tr>
<th>CFP Paradigm</th>
<th>Strain</th>
<th>MK-801 Acq</th>
<th>NMDA Exp</th>
<th>SCH23390 Acq</th>
<th>DA D1 Exp</th>
<th>Naltrexone Acq</th>
<th>(Opioid) Exp</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Sucrose (Sham)</td>
<td>CD-1 Rat</td>
<td>n/a</td>
<td>n/a</td>
<td>Reduced</td>
<td>Eliminated</td>
<td>No Effect</td>
<td>No Effect</td>
</tr>
<tr>
<td>B. Oral Fructose</td>
<td>CD-1 Rat</td>
<td>Eliminated</td>
<td>No Effect</td>
<td>Eliminated</td>
<td>Eliminated</td>
<td>No Effect</td>
<td>No Effect</td>
</tr>
<tr>
<td>C. Oral Sucrose</td>
<td>BALB/c Mice</td>
<td>Eliminated</td>
<td>Reduced</td>
<td>No Effect</td>
<td>Reduced</td>
<td>Eliminated</td>
<td>Reduced</td>
</tr>
<tr>
<td>D. Oral Sucrose</td>
<td>SWR Mice</td>
<td>Eliminated</td>
<td>No Effect</td>
<td>Eliminated</td>
<td>Reduced</td>
<td>No Effect</td>
<td>Reduced</td>
</tr>
<tr>
<td>E. Oral Fructose</td>
<td>BALB/c Mice</td>
<td>Eliminated</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Reduced</td>
<td>No Effect</td>
<td>No Effect</td>
</tr>
<tr>
<td>F. Oral Fructose</td>
<td>SWR Mice</td>
<td>Eliminated</td>
<td>Eliminated</td>
<td>Eliminated</td>
<td>Reduced</td>
<td>No Effect</td>
<td>No Effect</td>
</tr>
<tr>
<td>G. Oral Corn Oil</td>
<td>CD-1 Rat</td>
<td>Eliminated</td>
<td>Reduced</td>
<td>No Effect</td>
<td>Reduced</td>
<td>No Effect</td>
<td>Reduced</td>
</tr>
<tr>
<td>H. Oral Intralipid</td>
<td>BALB/c Mice</td>
<td>Eliminated</td>
<td>Eliminated</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
</tbody>
</table>

**Note:** **Eliminated:** operationally defined as a failure to observe significant differences between CS+ and CS- intakes in 2-bottle choice tests, AND %CS+ intake at or approaching indifference (50%).

**Reduced:** operationally defined as significant reductions in CS+ intake AND/OR %CS+ intake relative to corresponding vehicle values. **No Effect:** operationally defined as a failure to observe significant differences between drug and corresponding vehicle values in intakes or %CS+ intake.
Naltrexone mildly reduced the acquisition of fat-conditioned flavor preferences in BALB/c mice (Table 4H), but not SWR mice (Table 4I) or outbred rats (Dela Cruz et al., 2012a; Kraft et al., 2013). Further, naltrexone eliminated acquisition of sucrose-conditioned flavor preferences in BALB/c (Table 4C), but not SWR (Table 4D) mice that received naltrexone during training (Dym et al., 2012). Moreover, naltrexone only hastened extinction of fructose-conditioned flavor preferences in SWR mice (Table 4F), but not in BALB/c mice (Table 4E) or outbred rats (Baker et al., 2004; Kraft et al., 2015b). Taken together, these data underscore the importance of NMDA receptor signaling in the formation of both major triggers (sugar and fat) of preference learning.

The present study also demonstrated that NMDA receptor antagonism was intimately involved in the expression (maintenance) of fat-conditioned flavor preferences in both strains. Thus, an already-acquired Intralipid-conditioned flavor preference was eliminated by MK-801 at doses of 100 and 200 µg/kg in BALB/c mice (Table 4H), whereas the lower, but not higher dose eliminated this response in SWR mice (Table 4I). The lack of clear dose-dependent effects of MK-801 upon expression of fat-conditioned flavor preferences in SWR mice was not due to dose order effects as the presentation of the MK-801 doses was counterbalanced across animals within this group, and could not account for the observed effects. Further, it should be noted that although the higher 200 µg/kg MK-801 dose did not eliminate preference expression, CS+ and CS- intakes failed to differ from each other (Figure 23). These data differ from the minimal reductions in expression of fructose- and sucrose-CFP in BALB/c mice (Table 4C, 4E) following MK-801, and the elimination of expression of fructose-, but not sucrose-conditioned flavor preferences by a high dose of MK-801 in SWR mice (Table 4D, 4F) (Kraft et al., 2016). The pattern of the present findings also differ from the ability of MK-801 to eliminate expression of corn oil-conditioned flavor preferences in outbred rats (Table 4G), but only at doses that markedly reduced overall intake (Dela Cruz et al., 2012a). Therefore, it appears that effects of NMDA receptor signaling on the maintenance of an already-acquired conditioned
flavor preference varies as functions of the nutrient (sugars or fat) and species (inbred mouse strains and outbred rats). The implications of these findings will be presented in the General Discussion.
Chapter 9

GENERAL DISCUSSION

The genetic differences observed in the BALB/c and SWR in all six studies is substantial in highlighting the potential variance of neuroanatomical, neurochemical and neurophysiological processes. The first study of this series demonstrated that SWR mice reversed their initial preference for non-nutritive S+S over fructose in Test 1 to a fructose preference over S+S in Test 4 after separate experience with the two sweeteners in Tests 2 and 3 strongly indicates that fructose exerts post-oral reinforcing actions in this inbred strain. In contrast to *ad-libitum* fed SWR mice, either *ad-libitum* fed or food-restricted BALB/c mice did not reverse their preference for S+S over fructose, but rather displayed strong S+S preferences in both Tests 1 and 4. Given that B6 mice in general are more sensitive to sweeteners than BALB/c mice (Glendinning et al., 2005; Ramirez and Fuller, 1976; Reed et al., 2004), the role of sweet taste sensitivity in fructose conditioning is questionable. Other data indicate that T1r3 sweetener sensitivity does not fully account for the hedonic or motivational response of inbred mice to sugars. For example, in short-term lick tests, which are thought to reflect taste hedonics, sweet sub-sensitive 129 mice licked at lower rates for dilute sucrose solutions than did sweet-sensitive B6 and SWR mice, but 129 mice licked as much or more for concentrated sucrose solutions as did B6 and SWR mice (Glendinning et al., 2005). Also, whereas 129 mice licked less on a progressive ratio schedule for a 4% sucrose solution than did B6 mice, they actually licked more for a 16% sucrose solution than did the B6 mice (Sclafani, 2006). These and other findings (Boughter and Bachmanov, 2007) indicate that multiple genetic factors contribute to variations in sugar intake, preference and motivation in inbred mouse strains. Thus, genetic and phenotypic differences other than those related to sweet taste sensitivity may be responsible for the differential flavor conditioning displayed by BALB/c and B6 mice in our prior study. Note that BALB/c mice also displayed a stronger sucrose-conditioned preference than did B6 mice (86% vs. 66%;) (Pinhas et al., 2012),
indicating that the strains differ in their response to sugars that have post-oral reinforcing actions as well. Future studies should compare flavor conditioning in BALB/c and B6 mice using other nutritive and non-nutritive solutions to further characterize the differences in flavor/sugar learning in these strains.

Further work is also required to explain why the post-oral actions of fructose are reinforcing in some inbred mouse strains (FVB, SWR) but not others (B6, BALB/c). Recent sugar conditioning studies in B6 mice indicate that intestinal glucose transporters/sensors SGLT1 and SGLT3 have an important role in glucose conditioning in B6 mice (Zukerman et al., 2013). These “transceptors,” which do not bind to fructose, presumably mediate glucose conditioning in FVB and SWR mice that also display fructose conditioning. Consistent with this view, our studies of sugar conditioning in FVB mice revealed that glucose was more potent than fructose in conditioning flavor preferences and had a more rapid time course (Sclafani et al., 2014). The present finding that SWR and food-restricted BALB/c mice, like FVB and B6 mice, strongly prefer glucose to fructose in direct choice tests also suggests that different physiological processes mediate post-oral fructose and glucose reinforcement in these strains. The availability of inbred strains that differ in their post-oral conditioning response to fructose should facilitate the study of post-oral fructose reinforcement. Finally, the present findings indicating that SWR and BALB/c mice differ in their post-oral conditioning response to fructose are relevant to our recent report of differential actions of dopamine and opioid antagonist drugs on fructose-conditioned flavor preferences in the two strains (Kraft et al., 2015b).

The second study of this series found that although SCH and NTX significantly reduced fructose and saccharin intake in the two strains, they did so with differential strain-specific inhibitory magnitudes. Thus, although saccharin intake was similarly reduced by SCH and NTX in BALB/c and SWR mice, greater potencies of opioid (1.9-fold) and DA D1 (4-fold) receptor antagonism of fructose
intake were noted in SWR relative to BALB/c mice, indicating strong strain differences. Dose-dependent and time-dependent comparisons of DA D1 and opioid receptor antagonist effects on saccharin and fructose intakes in BALB/c and SWR mice are important for the following reasons. First, DA D1 antagonist-induced decreases in sweet intake have only been reported using sucrose (Bello and Hajnal, 2006; Geary and Smith, 1985; Muscat and Willner, 1989; Schneider et al., 1986, 1990; Tyrka et al., 1992; Weatherford et al., 1990) or fructose (Pritchett and Hajnal, 2011) in rats. Therefore, it was not known if the pattern of DA D1 antagonist effects would be identical for fructose and saccharin intakes in inbred mice as determined previously for sucrose (Dym et al., 2009).

Second, although NTX has been found to decrease sucrose (Kirkham and Cooper, 1988a, 1988b; Levine et al., 1982, 1995; Rockwood and Reid, 1982; Weldon et al., 1996) and saccharin (Cooper, 1983; Lynch, 1986; Lynch and Libby, 1983) intakes in rats, it is not known if the pattern of opioid antagonist effects would be identical for fructose and saccharin intake in inbred mice as determined previously for sucrose (Dym et al., 2007). NTX has been demonstrated to antagonize mu-opioid receptors and activate kappa-opioid receptors. Striking differences emerge in the abilities, potencies and magnitudes of DA D1 and opioid receptor antagonists to reduce palatable solution intakes that vary as a function of strain and tastant. First, it is quite apparent that saccharin intake is most potently reduced by both DA D1 (BALB/c and SWR: ID₄₀: <50 nmol/kg) and opioid (ID₄₀: BALB/c: 0.9 mg/kg; SWR: 0.02 mg/kg) receptor antagonists in the two strains as compared to fructose intake, confirming roles for DA D1 and opioid receptors in mediating the intake of this non-nutritive sweetener in these two inbred strains, and supporting the 30-year old notion that flavor enhances the antidipsogenic effect of naloxone (Levine et al., 1982). The ID₄₀ values for saccharin relative to fructose intake were respectively 24.7-fold (BALB/c) and 6-fold (SWR) lower following SCH treatment and were respectively 5.5-fold (BALB/c) and 129-fold (SWR) following NTX treatment. Although robust baseline saccharin intakes were observed in both strains, a possible explanation of these drug effects
is that, at the concentration tested, saccharin was the least palatable solution based on its taste and/or post-oral actions, although this remains to be established for the SWR and BALB/c strains.

Second, fructose intake was more effectively inhibited (lower ID₄₀) by both DA D1 (4-fold) and opioid (1.9-fold) receptor antagonists in SWR (SCH: 298 nmol/kg; NTX: 2.59 mg/kg) relative to BALB/c (SCH: 1234 nmol/kg; NTX: 4.99 mg/kg) mice. Similar and more pronounced differences were previously observed with Intralipid: the SCH and NTX ID₄₀ doses for suppressing Intralipid intake were much lower for SWR than BALB/c mice (Dym et al., 2010). Together, these data suggest fundamental differences in the DA and opioid receptor systems controlling ingestion in the two mouse strains. Yet, an opposite pattern of drug effects was observed with sucrose: the SCH and NTX ID₄₀ doses for suppressing sucrose intake were higher for SWR than BALB/c mice (Dym et al., 2007). There is no obvious explanation for these differential drug sensitivities in the two strains. Sucrose and fructose are both sweet so taste quality apparently does not account for the differential drug sensitivities of SWR and BALB/c mice. The two sugars differ, however, in their post-ingestive reinforcing actions: intragastric infusions of sucrose but not fructose condition strong flavor preferences in C57BL/6 mice (Sclafani and Ackroff, 2012; Zukerman et al., 2013). Yet, Intralipid also has potent post-ingestive reinforcing actions in C57BL/6J mice (Ackroff and Sclafani, 2014; Sclafani and Glendinning, 2005; Zukerman et al., 2011), suggesting that differences in post-ingestive nutrient reinforcement do not explain why SWR and BALB/c mice display different drug response patterns to fructose, sucrose, and Intralipid. However, recent findings revealed strain differences in post-ingestive nutrient effects that may be relevant to the present drug findings (Sclafani et al., 2014). In particular, there is evidence that fructose has little or no post-ingestive reinforcement action in BALB/c mice, but does so in SWR mice (Huang et al., 2014). Whether BALB/c and SWR mice also differ in their sensitivity to the post-ingestive reinforcement actions of sucrose and Intralipid are not known. Thus, further study of the relative orosensory and post-ingestive reinforcing actions of the
sugars and fat in BALB/c and SWR mice are needed to evaluate their differential responses to DA and opioid receptor antagonists.

The third study of the series demonstrated distinct effects of DA D1 and opioid receptor antagonists upon fructose- relative to sucrose-CFP in BALB/c and SWR mice. In BALB/c mice, SCH eliminated fructose-CFP expression relative to vehicle, an effect that was stronger than the ability of SCH to reduce the expression of sucrose-CFP relative to vehicle in BALB/c mice. However, in SWR mice, SCH induced comparable reductions of fructose- and sucrose-CFP expression relative to vehicle. In contrast, NTX failed to alter the expression of fructose-CFP in BALB/c or SWR mice, an effect different from the ability of NTX to promote the extinction of a sucrose-CFP preference with repeated testing in BALB/c mice and especially in SWR mice.

The fourth study in this series demonstrated that that the acquisition of fructose- and sucrose-CFP in BALB/c and SWR inbred strains was eliminated by systemic administration of the non-competitive NMDA antagonist, MK-801 during training. In contrast, expression of fructose- and sucrose-CFP in BALB/c mice was minimally affected by NMDA receptor antagonism with MK-801 administered after establishment of the preferences. We previously (Dym et al., 2012; Kraft et al., 2015b [Experiment 3]) evaluated the roles of DA D1 and opioid receptor antagonism in mediating acquisition of sucrose- and fructose-CFP in BALB/c and SWR mice. Acquisition of sucrose-CFP was significantly reduced by opioid, but not DA D1 antagonism in BALB/c mice, and by DA D1, but not opioid antagonism in SWR mice, indicating a double dissociation in the pharmacological effects on sucrose-CFP in the two strains (Dym et al., 2012). However, DA D1 and opioid antagonist effects upon acquisition of fructose-CFP produced a different pattern. The persistence of fructose-CFP was shortened by DA D1, but not opioid antagonists administered during training in BALB/c mice. In contrast the acquisition of fructose-CFP in SWR mice was eliminated by DA D1 antagonism, and its persistence was shortened by opioid antagonism (Kraft et al., 2015b). Therefore, DA D1 and NMDA
receptors would appear to play a potential interactive role in mediating acquisition of both sucrose- and fructose-CFP in SWR mice. Our laboratories (Touzani et al., 2013) previously confirmed such a relationship in rats for acquisition of CFP elicited by IG glucose such that amygdalar co-administration of AP5 on one side and SCH23390 on the contralateral side eliminated a glucose-CFP. NMDA antagonism alters the acquisition of learned behaviors related to conditioned taste avoidance, discriminated approach to sucrose solution, fear conditioning and inhibitory avoidance learning (e.g., Burns et al., 1994b; Goosens and Maren, 2004; Yasoshima et al., 2000) and forebrain DA-glutamate interactions have been observed (e.g., Baldwin et al., 2002; Beninger and Gerdjikov, 2004; Burns et al., 1994a; Nai et al., 2010; Smith-Roe and Kelley, 2000). The loci at which such potential interactions occur in SWR mice require further investigation, but might include those sites (nucleus accumbens, amygdala, medial prefrontal cortex, lateral hypothalamus) at which DA D1 antagonism reduces both fructose-CFP and IG glucose-CFP in rats (Amador et al., Bernal et al., 2008, 2009; Malkusz et al., 2012; Touzani et al., 2008, 2009a, 2009b, 2012). In contrast, opioid and NMDA receptors would appear to play a potential interactive role in mediating acquisition of sucrose-, but not fructose-CFP in BALB/c mice. This highly-selective strain- and species-specific effect may be related to observations of interactions between these systems in place preference and environmental conditioning, behavioral sensitization and tolerance (Bespalov et al., 1998, 2001; Carlezon et al., 2000; Harris et al., 2004; Ma et al., 2006; Ribeiro Do Couto et al., 2004, 2005; Tzschentke and Schmidt, 1997).

In the present study the BALB/c and SWR mice displayed similar preferences for the fructose- and sucrose paired CS+ flavors (82-85%) in the expression experiments. This contrasts with the stronger sucrose-conditioned preferences observed in our original study (Pinhas et al., 2012) but is consistent with subsequent experiments in which similar preferences were conditioned by the two sugars (Dym et al., 2012; Kraft et al., 2015b). Overall, the two strains displayed comparable sugar-
conditioned preferences in these studies that are surprising given that the SWR mice have a more sensitive sweet taste receptor than BALB/c mice (Reed et al., 2004). The strain difference in sweet sensitivity is most evident in their response to saccharin solutions (Reed et al., 2004) which can explain why BALB/c mice generally consume less of the CS-/saccharin than the CS+/sugar solution while SWR mice drank as much or more CS-/saccharin as CS+/sugar solution during training (present study; Pinhas et al., 2012; Dym et al., 2012; Kraft et al., 2015b). These findings indicate that absolute CS+ and CS- intakes during training do not determine the magnitude of the CS+ preference expressed in the 2-bottle tests.

The fifth experiment in this series demonstrated that BALB and SWR mice acquired similar fat-conditioned preferences for the CS+ flavor added to a 5% Intralipid solution over the CS- flavor added to a 0.5% Intralipid solution. For DA D1 receptor antagonism, SCH blocked the expression of fat-CFP in both BALB and SWR mice. In contrast, acquisition of fat-CFP was eliminated in the SWR mice receiving SCH during training, but was not at all impaired in the BALB mice receiving SCH during training. For opioid receptor antagonism, BALB, but not SWR mice displayed NTX-induced reductions in the expression of fat-CFP. Further, the acquisition of fat-CFP was not impaired by NTX in SWR mice, whereas BALB mice displayed a marginally less persistent fat-CFP following NTX. Research conducted over the past 40 years conclusively demonstrates that opioid signaling underlies food reward, including reductions of fat and sugar intake following general, mu and kappa opioid receptor subtype antagonists (see reviews: Bodnar, 2004; Taha, 2010). To this end, systemic naloxone reduced sucrose intake and taste reactivity in both food-restricted and ad-libitum-fed animals (Apfelbaum and Mandenoff, 1981; Cooper et al., 1985; Levine et al., 1995; Parker et al., 1992). Whereas ventricular administration of mu and kappa, but not delta-1 opioid antagonists reduced fat or sucrose intake under real-feeding (Arjune et al., 1990a, 1990b, 1991; Beczkowska et al., 1992; Islam et al., 1990) and sham-feeding (Leventhal et al., 1995) conditions, general and mu, but not delta
opioid antagonism in the NAc shell produced modest decreases in sucrose intake (Bodnar et al., 1995; Kelley et al., 1996). Yet in multiple types of conditioning studies, opioid signaling has little effect on learned preferences. Despite dose-dependently reducing overall intake, systemic NTX persistently failed to alter the acquisition or expression of CFP elicited by oral sucrose in sham-feeding rats (Yu et al., 1999), oral fructose in real-feeding rats (Baker et al., 2004), oral corn oil in real-feeding rats (Dela Cruz et al., 2012b) or intragastric infusion of sucrose (Azzara et al., 2000). Moreover, NTX administered directly into either the shell or core regions of the NAc reduced fructose and saccharin intake, yet failed to alter the acquisition or expression of CFP elicited by oral fructose or intragastric glucose (Bernal et al., 2010). Thus, endogenous opioids, while critically involved in mediating fat and sugar intake per se in outbred rats and many inbred mouse strains, do not participate in preferences mediated by flavored sugar or fat solutions.

Orally-consumed fat conditions flavor preferences through both oral and post-oral mechanisms (Elizalde & Sclafani, 1990). It is possible that SWR mice were able to develop fat-CFP based on the post-oral mechanisms that depend on dopamine systems (see reviews: Sclafani et al., 2011; Touzani et al., 2010), but not on opioid systems (Azzara et al., 2000; Bernal et al., 2010). Because SCH failed to block the acquisition of fat-CFP in BALB mice, this would suggest that BALB mice utilize the palatable, but not post-ingestive actions of the fat to develop a fat-CFP. This interpretation predicts that BALB mice, unlike C57BL/6 mice (Zukerman et al., 2013), would not learn to prefer a CS+ flavor paired with intragastric fat infusions.

Brain opioid and DA systems have each been intimately related and sensitive to fat intake. Intake of fat or combined fat/sugar diets increase forebrain and hypothalamic mu opioid receptor binding (Barnes et al., 2006; Ong and Muhlhausler, 2011; Smith et al., 2002; Vucetic et al., 2010), striatal and hypothalamic enkephalin gene expression (Kelley et al., 2003; Leibowitz et al., 2009), hypothalamic dynorphin peptide and mRNA levels (Chang et al., 2007, 2008; Welch et al., 1996) and
central beta-endorphin (Mizushige et al., 2009). In contrast, decreased sensitivity to effects of fat intake and consumption are observed in mice lacking mu-opioid receptors (Zuberi et al., 2008) or dynorphin (Sainsbury et al., 2007). These neurochemical and molecular changes in endogenous opioid function as a function of fat intake are largely consistent with the pharmacological evidence reviewed above demonstrating significant reductions in fat intake produced by general and selective mu and kappa opioid receptor antagonists in rats (see reviews: Bodnar, 2004; Taha, 2010). These reductions reflect strong opioid mediation of acceptance of palatable fat sources. If opioid mediation of fat-conditioned flavor preferences acted in a similar manner, then general opioid antagonism should uniformly reduce the acquisition and expression of fat-CFP in rats and mice. The minimal actions of NTX upon the acquisition and expression of CO-CFP in rats (Dela Cruz et al., 2012b) and Intralipid-CFP in SWR mice in the present study show important differences between opioid mediation of fat intake per se, and fat-CFP. That BALB mice displayed NTX-induced reductions in the acquisition and expression of fat-CFP indicates a potential genetic difference in the mediation of this type of learning.

Changes in brain DA systems as a function of fat intake are more complex. Thus, maternal high-fat diets in the perinatal period increased tyrosine hydroxylase expression in the ventral tegmental area and nucleus accumbens (Naef et al., 2008, 2011), and up-regulated the DA reuptake transporter (Vucetic et al., 2010). DA receptor availability in the nucleus accumbens was lower in rats consuming more energy from fat during diet-induced obesity (van de Giessen et al., 2012a, 2012b), and tyrosine hydroxylase mRNA expression was lower in obese mice consuming a high-fat diet (Li et al., 2009). Epigenetic dysregulation induced by dietary obesity was related to increased hypothalamic DA and lowered ventral tegmental area-accumbal DA (Vucetic et al., 2012). Striatal DA levels were closely related to the amount of calories ingested as fat (Ferreira et al., 2012), but DA release from striatal slices were lower in rats exposed to a high-fat diet (York et al., 2010). Finally, up-regulation
of five genes related to DA was observed in mice consuming a high-fat diet (Lee et al., 2010). DA mediation of the rewarding effect of fat flavor is suggested by the findings that CO sham-feeding promotes nucleus accumbens (NAc) DA release in rats (Liang et al., 2006), and DA D1 and D2 receptor antagonists suppress the sham-feeding response to CO and real-feeding of fats in rats (Baker et al., 2001; Davis et al., 2006; Rao et al., 2008; Weatherford et al., 1988; 1990). Again, these studies reflect potential DA mediation of acceptance of a palatable fat source. If DA mediation of fat-conditioned flavor preferences acted in a similar manner, then DA D1 antagonism should uniformly reduce the acquisition and expression of fat-CFP in rats and mice. The minimal actions of DA D1 and D2 antagonism upon the acquisition and expression of CO-CFP in rats (Dela Cruz et al., 2012a) questioned this simple relationship. However, species and genetic variation may play a role such that the expression of fat-CFP was reduced by DA D1 antagonism in SWR and BALB mice, and the acquisition of this response was impaired by DA D1 antagonism in SWR, but not BALB mice. 

In summary, the present findings demonstrate that comparable, robust and persistent fat-CFP can be elicited in two inbred mouse strains using higher 5% and lower 0.5% concentrations of distinctly flavored Intralipid solutions. DA D1 antagonism significantly reduced the expression of fat-CFP in both BALB and SWR mice, whereas the acquisition of fat-CFP was eliminated in SWR, but not BALB mice in animals receiving SCH during training. The relative abilities of DA D1 antagonism to reduce fat intake in SWR, but not BALB mice (Dym et al., 2010) were good predictors in assessing DA D1 antagonist-induced effects upon the acquisition, but not the expression of fat-CFP. Opioid antagonism significantly reduced the expression of fat-CFP in BALB, but not SWR mice, and NTX-induced changes in the acquisition of fat-CFP were at best minimal. Thus, it appears that the pattern of DA D1, but not opioid antagonist effects upon the expression and acquisition of fat-CFP in the two strains of mice was quite similar to that observed for sucrose-CFP (Dym et al.,
2012), suggesting similarities in the DA D1 receptor substrates of preferences conditioned by sugar and fat in inbred murine strains.

The sixth and last experiment in this series demonstrated that NMDA receptor antagonism with MK-801 eliminated acquisition (learning) of fat (Intralipid)-conditioned flavor preferences in BALB/c mice. Moreover, acquisition of fat-CFP was not only eliminated in SWR mice treated with MK-801 during training, but appeared to turn into an avoidance response during the second and third choice tests. Expression of Intralipid-conditioned flavor preference was eliminated by MK-801 in BALB/c mice, whereas the lower, but not higher dose of MK-801 eliminated fat-CFP expression in SWR mice (Table 4I). This varied pharmacological pattern has also been observed in studies examining dopamine D1 and opioid receptor antagonists upon expression of fat- and sugar-conditioned flavor preferences. Thus, SCH23390 significantly reduced expression of fat-conditioned flavor preferences in both BALB/c and SWR mice (Table 4H, 4I: Kraft et al., 2013), but not in outbred rats (Table 4G: Dela Cruz et al., 2012b). SCH23390 reduced expression of both sucrose- and fructose-conditioned flavor preferences in both BALB/c and SWR mice (Table 4C-F: Dym et al., 2012; Kraft et al., 2015b) while eliminating expression of both responses in outbred rats (Table 4A, 4B: Baker et al., 2003; Yu et al., 2000). Naltrexone reduced expression of fat-conditioned flavor preferences in BALB/c mice, but not in SWR mice or outbred rats (Dela Cruz et al., 2012a; Kraft et al., 2013). In contrast, naltrexone reduced expression of sucrose-conditioned flavor preferences in both BALB/c and SWR mice, but not in outbred rats (Dym et al., 2012; Yu et al., 1999). Finally, naltrexone failed to affect the expression of fructose-conditioned flavor preferences in BALB/c mice (Table 4E), SWR mice (Table 4F) or outbred rats (Table 4B) (Baker et al., 2004; Kraft et al., 2015b).

These data clearly demonstrate the vital role of NMDA receptor signaling in preferences conditioned by fats and sugars, and are consistent with broader evidence implicating NMDA receptor signaling in reward-related and palatable food-related learning. Whereas MK-801 accelerated gastric
emptying of a 15% sucrose meal (Covasa et al., 2000a), it failed to affect the reduction of sucrose intake by intestinal nutrient infusions in sham-feeding rats (Covasa et al., 2000b). Fourth ventricular administration of MK-801 or inhibitors of the NR2 subunits of the NMDA receptor increased sucrose intake (Guard et al., 2009; Zheng et al., 1999). Although MK-801 increased sucrose intake, it produced effects similar to an animal model of negative symptoms of schizophrenia (Vardigan et al., 2010). Intake of a high-fat source was suppressed by the NMDA antagonists, MK-801 (Buttigieg et al., 2014) and memantine (Popik et al., 2011), as well as the NMDA co-agonist, D-serine (Sasaki et al., 2015). Memantine in turn also decreased intake of a sweet candy in a baboon model of binge eating (Bisaga et al., 2008), and reduced binge eating of sweets when microinfused in the nucleus accumbens (Smith et al., 2015). MK-801 also impaired the food-anticipatory activity rhythm in rats (Ono et al., 1996), as well as memory for nonspatial, socially transmitted food preferences (Roberts and Shapiro, 2002). The NMDA receptor is also responsible for plasticity in appetitive instrumental learning using a distributed corticostriatal network such that administration of the competitive NMDA antagonist, AP5 into the amygdala or medial prefrontal cortex impaired this form of learning (Baldwin et al., 2000; Kelley, 2004). NMDA and other glutamatergic receptors in the nucleus accumbens and ventral tegmental area are involved in various forms of food-motivated instrumental learning (Giertler et al., 2005; Hachimine et al., 2016; Ranaldi et al., 2011). Finally, this distributed brain network (ventral tegmental area – amygdala – nucleus accumbens – medial prefrontal cortex) appears to be activated by combinations of NMDA and dopamine D1 receptor systems (e.g., Smith-Roe and Kelley, 2000), and would be a likely candidate for NMDA-dopamine D1 receptor interactions in the acquisition and expression of fat-conditioned flavor preferences given its ability to mediate dopamine antagonist effects upon sugar-conditioned flavor preferences (see reviews: Sclafani et al., 2011; Touzani et al., 2010, 2013).
Future Implications:

The murine strain-specific differences in pharmacological substrates mitigating innate and learned responses to nutritive and non-nutritive sweeteners have often been attributed to underlying genetic variance. Two modalities can validate genetic variance; Mendelian randomization and identification of divergent quantitative trait loci (QTL) to potentially localize chromosomal regions, and ultimately genes. These two modalities will better elucidate the fundamental basis of these strain-specific differences. A small number of studies have identified QTLs for saccharin and sucrose intake, including Prp (Blizard et al., 1999), Sac (Lush, 1989), and TAS1r3 (Inoue et al., 2004) localized to distal chromosome 4 using C57BL/6J crosses with DBA/2J or 129P3/J mice. Mendelian randomization in inbred mouse studies typically employ the F2 generation of cross-breeding between two strains displaying intake characteristics. F2 generations of C57BL/6By and 129/J mice bred for sucrose preference failed to reveal corresponding changes in MSG relative to sucrose intake preferences, suggesting a unique genetic mechanism for this taste (Bachmanov et al., 2001b; Beauchamp et al., 1998). Loci on chromosomes 2, 4, 9 and 16 have been identified for body weight, body length, and adiposity in a genome scan of an F2 intercross between the 129P3/J and C57BL/6ByJ mouse strains (Reed et al., 2003; Reed et al., 2006). QTL studies of ethanol preference (3% and 10% concentrations) in C57BL/6By X 129P3/J F2 hybrids, identified two loci on distal chromosome 4 (Ap3q) and proximal chromosome 7 (Ap7q) with an identified male-specific locus on chromosome 8 (Ap8q) (Bachmanov et al., 2000). Thus, although murine strain-specific effects determine the ability of pharmacological antagonists to mediate sucrose-CFP, little is known as to whether these effects are directly mediated by underlying genetic variance. Thus, it would be advantageous for further experiments are to systematically evaluate whether the F2 generation of the two inbred strains discussed at length in this dissertation (BALB/c, SWR) display divergent acquisition of sucrose-CFP following pharmacological receptor antagonism.
As previously delineated, the acquisition of sucrose-CFP is blocked by DA D1, but not opioid receptor antagonism in SWR mice, and by opioid, but not DA D1 receptor antagonism in BALB/c mice, indicating a double dissociation between receptor antagonist effectiveness and inbred mouse strain (Dym et al., 2012) Examination of the F1 generation of SWR and BALB/c will determine if cross-bred mice will exhibit sensitivity (SWR) or insensitivity (BALB/c) to SCH23390; and sensitivity (BALB/c) or insensitivity (SWR) to Naltrexone during sucrose-CFP acquisition. It is hypothesized that subsets of these cross-bred F1 mice will respectively display elimination (25%), no effect (25%) and intermediate effects (50%) on sucrose-CFP acquisition. If this hypothesis were supported, this would set the stage for the performance of QTL analysis upon these mice to potentially identify underlying genetic changes.

Another major question that arises from this dissertation is whether the strain-specific differences are due to genetic or alternatively epigenetic factors. Genetics are the study of genes, genetic variation and heredity in organism. Epigenetics literally means "above" or "on top of" genetics. Epigenetics is the study of heritable changes in gene expression (active versus inactive genes) that do not involve changes to the underlying DNA sequence — a change in phenotype without a change in genotype — which in turn affects how cells read the genes. Epigenetic change is a regular and natural occurrence but can also be influenced by several factors including age, the environment/lifestyle, and disease state. As a result, are the strain specific differences we found genetic or epigenetic? Housing of animals, mother-child interaction, sibling interactions and the separation of the mice at 3 weeks are all factors that could affect our true understanding of the work comprised of this dissertation. Future studies should be designed to address this important distinction.

Another further direction of these studies is to see the effects of dopamine (D3) receptor antagonists. Peng et. al. 2009 found that the preferential dopamine D3 receptor antagonist S33138 inhibits cocaine reward decreased without affecting rotarod performance, nor locomotion. Additionally, the D3 receptor antagonist did not produce non-specific motoric effects, as it did not
affect spontaneous locomotor activity or PR responding for food (Galaj et al., 2014). Given that DA D1 receptor antagonist SCH23390 attenuated cocaine-induced locomotor activity (Adams et al., 2010), it is worthy of further study.
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