PHARMACOLOGY OF THE ACQUISITION AND EXPRESSION OF FLAVOR PREFERENCE CONDITIONING TO FRUCTOSE: ROLES OF DOPAMINE AND OPIOID RECEPTORS IN RATS

by

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Abstract

Pharmacology of the Acquisition and Expression of Flavor Preference Conditioning to Fructose; Roles of Dopamine and Opioid Receptors in Rats

By

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For omnivores, such as humans and rats, learning to associate flavor with postingestive consequences of palatable food is essential for survival. Research into the neurochemical substrate(s) mediating this adaptive behavior consistently demonstrates the involvement of dopamine, whereas the role of opioids is equivocal. The effects of dopamine and opioid receptor antagonists on the acquisition and expression of flavor preferences conditioned (CFP) by the sweet taste of fructose were examined in real-feeding rats. Rats were trained to drink fructose paired with one novel flavor and less-preferred saccharin paired with another flavor. Groups of rats received either vehicle, D1 (SCH23390, 200 nmol/kg, sc) or D2 (raclopride, 200 nmol/kg, sc) antagonists, or vehicle with intakes matched to the dopamine groups (D1-yoked, D2-yoked). Preferences were assessed in two-bottle tests with both flavors presented in saccharin following vehicle or antagonist doses (50-800 nmol/kg).

Acquisition of a significant conditioned flavor preference (CFP: ~75%) to fructose observed in the control and yoked groups was eliminated in D1-trained and D2-trained rats. SCH23390, and to a lesser degree raclopride, also blocked the expression of an existing conditioned flavor preference. Raclopride also attenuated fructose preferences in rats exposed to two-bottle training with fructose and saccharin solutions. Thus, both D1 and D2 antagonists block the acquisition of fructose CFP, whereas D1, and to a lesser extent D2, antagonists attenuate fructose CFP expression. The ability of the general opioid antagonist, naltrexone to alter the acquisition and expression of fructose CFP was also examined. Although naltrexone (0.1-5.0 mg/kg) reduced fructose and saccharin intakes during training, it failed to interfere with the acquisition of a fructose CFP. Further, although naltrexone (0.1-5 mg/kg) reduced saccharin intake in two-bottle tests, it again failed to alter the expression of an established fructose flavor preference. Thus, dopamine D1 and D2 receptor antagonism, but not general opioid receptor antagonism, reduces flavor-flavor conditioning by fructose in free-feeding rats. These results strongly suggest that dopamine mediates the association of flavor cues with ingestive consequences, whereas opioids appear to mediate the hedonics of consummatory behavior, playing a very minor role in CFP learning for simple carbohydrates.

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This dissertation, and the educational journey that has preceded it, is dedicated to my father, whose love for, and pursuit of, learning provided the model that I have belatedly followed, to my mother, whose emotional and financial support for six decades made this achievement possible, and to my wife, who gave me the motivation to become somebody worthy of her love and companionship.

Table of Contents

List of Figures

CHAPTER ONE: INTRODUCTION

Food stimuli, and especially sugars, are ingested for nutritive (postingestive mechanisms) purposes and also for hedonic (orosensory mechanisms) value. Whereas a great deal of research has focused upon the innate mechanisms governing the selection of food to be ingested, it has become increasingly clear that selection of intake is also subject to environmental manipulations and learning. The Conditioned Flavor Preference (CFP) paradigm has been used to demonstrate the role of learning in the development of food preferences (see reviews: Capaldi, 1992; Sclafani, 1995, 1997). Numerous studies have found that animals have a preference for ingestive stimuli that have a sweet taste, and will develop strong preferences for a flavor paired with a sweet taste over a flavor paired with a less sweet taste (e.g., Holman, 1975; 1980, Yu et al., 1999). In general, there are two particular types of these preferences defined by their site of action. When a preference develops for a flavor that has been paired with a sweeter taste (e.g., saccharides such as sucrose and fructose) relative to a second flavor paired with a less sweet taste (e.g., saccharin), this preference is referred to as a flavor-flavor conditioned preference. When a preference develops for a flavor paired with a positive (e.g., nutritive) post-ingestive consequence relative to a second flavor paired with a neutral (e.g., water) post-ingestive consequence, this preference is referred to as a flavor-nutrient conditioned preference. These allow us to distinguish between preferences mediated by orosensory relative to post-ingestive (stomach and intestines) mechanisms. The most common procedure used in the experimental study of acquired food preferences uses two different cue flavors (e.g. cherry and grape) that are added to a nutritive food (e.g. carbohydrate or fat) and non-nutritive food (saccharin or mineral

oil). Rats or other laboratory animals are trained to consume the two flavored foods during separate sessions to facilitate their associating the cue flavors with the post-ingestive consequences. Flavor-nutrient preference learning is then assessed in a two-choice test. In this test, the two flavors are presented in identical solutions to assure that any differential intakes can be attributed to a learned response to one of the two cue flavors (Sclafani, 1995).

This training procedure is considered to be a form of Pavlovian learning, or classical conditioning, with some innately reinforcing substance considered to be the unconditioned stimulus (UCS), with its associated flavor referred to as the conditioned stimulus (CS+). In contrast, the flavor paired with the non-nutritive food is the less-preferred conditioned stimulus (CS-). Two techniques have been developed to distinguish between the oral and post-ingestive mechanisms involved in learning a CFP. The sham-feeding preparation involves the surgical placement of a stainless steel fistula in the stomach of an anesthetized animal. The fistula can be opened or closed by the removal or insertion of a stainless steel screw. During sham-feeding training or testing the screw is removed, allowing the passage of ingested solutions from the mouth through the esophagus and out of the gastric fistula, thereby minimizing the post-ingestive effects of the nutrients in the test or training solutions. With this preparation, if a preference develops, it is considered to be a flavor-flavor preference (Yu, 1999, 2000a, 2000b). An intragastric infusion procedure focuses on the contribution of the post -ingestive mechanisms. One flavor (CS+) is paired with an intragastric infusion of the studied nutrient (e.g. sucrose), and a second flavor (CS-) is paired with an intragastric infusion of water, which controls for gastric volume, yet has no inherent

nutritional value in terms of caloric content (Ackroff and Sclafani, 1991; Sclafani and Ackroff, 1993; Azzara et al, 2001, 2001). Any subsequent flavor preference conditioned to the CS+ is considered to be a flavor-nutrient preferences, primarily mediated by gastrointestinal mechanisms.

The disaccharide, sucrose, and, one of its constituents, glucose, are both able to act as a UCS, producing a CFP in real-feeding animals (e.g., Ackroff and Sclafani, 1990; Hsaio and Smith, 1995; Sclafani et al., 1999) acting primarily at either the orosensory or the postingestive level to produce, respectively, a flavor-flavor or a flavor-nutrient CFP (e.g., Azzara et al., 2000; Yu et al., 1999). Additionally, a flavor-flavor CFP in real-feeding animals can be developed using a UCS (e.g., fructose) that possess weak or absent post-ingestive consequences (Sclafani et al, 1994; Sclafani et al., 1999).

The underlying pharmacology of flavor-flavor and flavor-nutrient CFPs has been a recent subject of study. Two neurochemical candidates, the endogenous opioid and dopamine systems, have each been implicated in the intake of sweet palatable solutions (see reviews: Benninger 1993; Cooper et al., 1985; Levine et al., 1985; Morley et al, 1982; Smith, 1995; Terry, 1996). Because of their respective well-established roles in these ingestive responses, it was therefore hypothesized that they would play an important role in the acquisition and expression of both flavor-flavor and flavor-nutrient CFP for sucrose. In sham-feeding animals, it was established that the D-1 receptor antagonist, SCH23390, and to a lesser degree the D-2 receptor antagonist, raclopride, dose-dependently interfered with the expression, but not the acquisition of a flavor-flavor CFP for sucrose (Yu et al., 2000a,b). In contrast, the general opioid antagonist, naltrexone failed to alter either the acquisition or expression of the same flavor-flavor

sucrose CFP (Yu et al., 1999). In animals receiving intragastric infusions, it was definitively established that the D-1 receptor antagonist, SCH23390, but not the D-2 receptor antagonist, raclopride interfered with the acquisition, and secondarily, reduced the expression of a flavor-nutrient CFP for sucrose (Azzara et al., 2001). Again, the general opioid antagonist, naltrexone failed to alter either the acquisition or expression of an identical sucrose flavor-nutrient CFP (Azzara et al., 2000). However, it should be noted that place preferences already conditioned by sucrose relative to water availability are significantly reduced by naltrexone pretreatment, yet acquisition of this place preference was unaffected by opioid antagonism (Delamater et al., 2000). Thus, there are situation-specific roles for the opioid system in learning situations involving sucrose.

The foregoing research was conducted with perhaps one of the strongest sugars (sucrose) to elicit a CFP. This raises several questions about the presence and potency of pharmacological effects in these CFP paradigms, and whether their effects extend to other sugars. With respect to the latter issue, Sclafani and co-workers (Ackroff and Sclafani, 1991, 1993, 1994; Drucker et al., 1994; Lucas and Sclafani, 1999a,b) systematically compared the relative preferences and the relative strengths of preferences among such different carbohydrates as sucrose, glucose, fructose, galactose and polycose. Fructose, a component of sucrose (glucose + fructose) was found to elicit flavor-flavor CFP in real-feeding animals, but failed to elicit a flavor-nutrient CFP following intragastric infusions (Sclafani et al, 1993). Therefore, the use of fructose as the UCS in a flavor-flavor CFP paradigm with real-feeding animals can extend our ability to determine whether dopamine and/or the endogenous opioids mediate

orosensory neural substrates involved in the acquisition and expression of this flavor-flavor CFP.

The potency of sucrose as a reinforcer in CFP paradigms was confirmed by the markedly greater consumption of the CS+ (16% sucrose) solutions than of the CS- (0.2% saccharin) solutions during training of sham-feeding rats (Yu et al., 1999, 2000a, 2000b). Therefore, it could be argued that the failure of opioid and dopamine antagonists to interfere with the acquisition of a sucrose CFP reflected the greater familiarity of the animals with the CS+ because the animals did not have sufficient exposure to the CS-. Because fructose has minimal post-ingestive consequences (Ackroff and Sclafani, 1993, 1999) a fructose-conditioned CFP may be more sensitive to the influence of pharmacologic perturbations than a sucrose-conditioned CFP.

Thus, the **first specific aim** of the dissertation is to examine the role of dopamine D-1 and D-2 receptors in the acquisition and expression of a flavor preference conditioned by fructose by dose-dependently and time-dependently assessing the effects of antagonists for the D1 (SCH23390) and D2 (raclopride) dopamine receptor subtypes. Further, the **second specific aim** of the dissertation is to examine the role of opioid receptors in the acquisition and expression of a flavor preference conditioned by fructose by dose-dependently and time-dependently assessing the effects of the general opioid antagonist, naltrexone. A novel and distinctive flavor (e.g., cherry) paired in solution with the UCS fructose (8%: CS+/F) will be presented in four one-bottle training sessions that alternate with four one-bottle training sessions with a different flavor (e.g., grape) paired with a less preferred saccharin solution (0.2%: CS-/S) over an 8-day training period. Separate groups of animals receive pretreatment

either with vehicle (Control) or the antagonist across a range of doses. Rats are then exposed to two bottles of saccharin (0.2%), one containing the fructose-paired flavor (now identified as CS+/S rather than CS+/F) and the other the saccharin-paired flavor (CS-/S) for two days. A significantly greater intake of the CS+/S relative to the CS-/S solution suggests CFP. If the antagonist interferes with learning a fructose CFP, the percentage of the total intake that is CS+/S should be reduced. The elimination, or significant reduction, of the acquisition of a fructose CFP following pretreatment with an antagonist relative to animals pretreated with vehicle would suggest a role for that neurotransmitter system in the acquisition of a fructose conditioned flavor preferences. Similarly, among animals with an established fructose conditioned flavor preference, if pretreatment with an antagonist eliminates or significantly reduces the expression of that preference relative to the expression of the preference on days when they are pretreated with vehicle, that would suggest that the neurotransmitter system antagonized plays a role in the expression of fructose conditioned flavor preferences.

To establish the procedures and concepts utilized in this study, the following background will summarize literature examining: a) establishment of conditioned flavor preferences, b) roles of orosensory and postingestive factors in preference conditioning, c) neurochemical and pharmacological mediators of palatable intake, d) opioid and dopaminergic systems as a potential candidates mediating CFP, and e) specific aims and rationale of the present studies.

A. Conditioned Flavor Preferences

Behavioral studies demonstrate the importance of learning in the development of food preferences (see reviews, Capaldi, 1992; Sclafani, 1995, 1997). When an animal

ingests a distinctive flavor that is followed by a nutritive postingestive consequence, an association is made between that flavor and the positive postingestive consequence, resulting in a preference for that flavor over a different flavor that has not been paired with a positive nutritive outcome. Studies attempting to identify the neurochemical substrates underlying the acquisition and expression of that flavor preference have consistently found that dopamine plays a role in this behavior (Azzara et al., 2001). The role of the endogenous opioids in the mediation of the intake of palatable foods has been consistently demonstrated, with opioid agonists increasing palatable intake, and general and selective opioid receptor subtype antagonists reducing palatable intake (see reviews: Bodnar, 1996, 2004; Gosnell et al, 1986; Gosnell and Levine, 1996). Interestingly, the evidence for the involvement of the opioid sytem in CFP appears to be more equivocal. Mehiel (1996) found that the general opioid antagonist naloxone blocked both the acquisition and expression of a preference for a flavor paired with glucose in free-feeding rats. Yet several other studies (Azzara et al., 2000;Yu et al., 1999) using either sham-feeding or intragastric feeding preparations have been unable to demonstrate that a wide range of naltrexone doses altered either the acquisition or expression of flavor-flavor or flavor-nutrient CFPs for sucrose. One possible explanation for this discrepancy in the results may be that Mehiel (1996) only exposed the animals in his study to the antagonist on the day that they were given access to the CS+ solution, while only administering vehicle injections on the days that the CS- solution was available. Therefore, it is possible that the absence of a learned or expressed preference reflected a conditioned avoidance to the aversive effects of naloxone rather than a failure to respond to the CS+ paired flavor. The sham-feeding

and intragastric studies cited above sought to eliminate this potential confound by administering the antagonist prior to exposure to both the CS+ and CS- solutions. This approach has been adopted in the present studies as well. The following sections will now examine: i) the learning or acquisition of a flavor preference and ii) the expression of a flavor preference.

i. Learning or Acquisition of a Flavor Preference

The most frequently utilized procedure for developing a CFP is the Classical Conditioning technique of pairing a previously neutral flavor stimulus (CS), with another stimulus that is known to have an innately reinforcing effect (UCS) (e.g., Capaldi, 1992, 1996; Fanselow and Birk, 1982; Sclafani, 1995, 1997). An innate preference (across certain concentration gradients) has consistently been found in animals for the sweet taste of the nutritive natural sugars, such as sucrose, and its components (glucose and fructose), relative to the taste of non-nutritive artificial sweeteners, such as saccharin (Yu et al., 1999, 2000a,b). However, it is important to note that animals will prefer a flavor paired with a high saccharin concentration over a flavor paired with a low saccharin concentration (Holman, 1975, 1980), indicating that saccharin is not aversive, only less preferred than sucrose or either of its components. Whether presenting a solution of a natural sugar (UCS) flavored with one distinctive flavor cue (CS+) in one bottle every other day, and a solution of saccharin flavored with a different distinctive flavor cue (CS-) in one bottle on the intervening days i.e. one-bottle training), or, alternatively, making both flavors simultaneously available (i.e. two-bottle training), it has been repeatedly demonstrated that rats exhibit a strong and consistent preference for the CS+ (sugar) flavor over the CS- (saccharin) flavor (Yu et

al., 1999, 2000a,b). Evaluation of differences in responses to the flavors can be accomplished using either an acceptance or a preference paradigm. When the one-bottle testing method is used to demonstrate that an association has been learned for the flavor cue that has been paired with the UCS, increased intake over successive one-bottle testing days is referred to as acceptance. Thus, acceptance indicates whether the CS+ flavor is becoming more attractive when compared to itself on previous occasions, but this paradigm is inherently devoid of choice. Alternatively, preference paradigms provide a choice, measuring relative intakes of the CS+ flavor compared with a CS- flavor in a common base with two-bottle testing. The strength of the preference can be determined either in terms of absolute intake of CS+ versus absolute intake of CS-, or in terms of CS+ intake as a percentage of combined total intake. Hence preference indicates the potency of the CS+ in relation to another comparator (the CS-), and is a truer measure of the "preference" of the animal.

Conversely, a conditioned taste aversion (CTA) can be learned for an otherwise preferred taste such as sucrose when it is paired with lithium chloride infused directly into the stomach (flavor-toxin: Gaston, 1978; Sclafani et al, 1999).

Some previous studies have presented the CS+ and CS- in training sessions on alternate days, while in other studies, they have both been available at the same time (see reviews: Capaldi, 1992; Sclafani, 1995). The duration of training to the CS+ and CS- stimuli that has resulted in a CFP has varied from as little as a few minutes (Elizalde and Sclafani, 1990; Hsaio and Smith, 1995) to unlimited access over days (Sclafani, 2002). Although it has not been possible to condition a flavor-flavor CFP when there is any appreciable delay between the presentation of the tasted CS and the

tasted UCS, it has been possible to condition a flavor-nutrient preference despite a delay between presentation of the tasted CS and the IG infusion of the UCS (Sclafani, 1995).

Evaluation of brain structures that might be mediating or modulating ingestive behavior has indicated that the lateral hypothalamus (Teitelbaum and Stellar, 1954), area postrema (Garcia and Koelling, 1966), nucleus of the solitary tract, parabrachial nucleus and the vagus nerve (Calingasan and Ritter, 1993; Ritter et al, 1994) play important roles in feeding behavior in general and conditioning in particular. That the area postrema is essential to the development of a conditioned flavor aversion has been demonstrated in lesion studies (Garcia and Koelling, 1966). Touzani and Sclafani (2002) demonstrated that lesions placed in the area postrema disrupt a CTA , but fail to alter the development of a CFP in the same animals, suggesting that the brain structures involved in CTA may be different than those mediating a flavor-nutrient CFP. In contrast, Knight (1996, 1997) observed that area postrema lesions disrupted flavor-nutrient conditioning. Another series of lesion studies indicated that there was no evidence that vagal afferents are necessary for flavor-nutrient conditioning (Sclafani and Lucas, 1996;Sclafani et al., 2003), but it was demonstrated that lesions placed in the lateral hypothalamus disrupted flavor-nutrient CFP learning, especially when there is a short (15-20 minutes) delay between CS and UCS. Finally, the parabrachial nucleus has been found to be essential for associating taste cues with aversive (CTA), and positive taste consequences, but has less effect on flavor discrimination when lesioned (Sclafani et al, 2001). The overall results of these studies suggest that, although these structures are all involved in feeding behavior, the location of the neural substrate that mediate CFPs may be located elsewhere in the brain.

Another variable in protocols used to study CFPs is the type of macronutient used as the UCS, and has been found to account for some differences in the characteristics of the CFP (Sclafani, 1995). For example, Lucas and Sclafani (1999a) found that IG infusions of carbohydrates (16% maltodextrin) paired with one saccharin flavored solution conditioned a preference for that flavor over a flavor in a saccharin solution paired with an IG infusion of fats (7.1% corn oil). However, the amount of training exposure to the macronutrient produced differential results (Lucas and Sclafani, 1999b). Whereas a carbohydrate-paired flavor is preferred over a fat-paired flavor in 30 minute testing sessions, the reverse was true when testing measured relative consumption over the course of 22 hours. The reinforcing effects of carbohydrates and fats were found to be equivalent when they were consumed orally, whereas carbohydrates were more effective when introduced via IG infusion (Mehiel and Bolles; 1988; Perez et al, 1998). This difference may be due to some aversive effects of the corn oil when infused directly into the gut, an effect that doesn't occur when the corn oil is consumed orally (Deutsch et al, 1976; Ramirez, I., 1984). Other studies (Van Vort and Smith, 1983) have demonstrated that strength of flavor preference varies as a function of the length of time of the training session and also the nutritional state of the animal (Drucker et al.,1994). It also has been observed that, when given a choice, rats initially prefer a sweeter (25% concentration) relative to a less sweet (10%) solution (Booth et al, 1972). However, within a few days, animals will reverse this preference. A similar time-dependent pattern of preference and reversal has been observed when comparing sucrose and polycose solutions (Ackroff and Sclafani, 1991).

ii. Expression of a Conditioned Flavor Preference

As indicated above, preference learning is typically assessed in a two-choice test, usually with the two cue flavors presented in a common base. When assessing a CFP, the CS+ and CS- flavors are presented in either a solution that is a mixture of the two different solutions with which they had been paired (i.e. a solution that has both saccharin and the natural sugar: Yu, 1999, 2000a,b), or in a solution that is only sweetened with non-nutritive saccharin (Holman, 1975). Studies have found significant preferences ranging from moderate (60%) to strong (97%) for the CS+ flavored solution over the CS- flavored solution. This preference has been demonstrated to be resistant to extinction for long periods of time (days to weeks), even when the CS+ flavor continues to be presented in the absence of the UCS (Drucker et al, 1994). These effects are observed across a range of taste stimuli, including sucrose and fructose (Ackroff et al, 2001). This learned preference is so powerful that it has even been capable of converting normally avoided flavors (e.g., sour and bitter) into preferred flavors (see review: Sclafani, 1995, 1997). Preference conditioning has also produced increased acceptance (defined by total intake in one-bottle tests), sometimes termed conditioned acceptance, of the CS+ over the CS-, which is an alternative measure of the strength of the CFP (Drucker et al, 1994). Conditioned acceptance of a CS+, however, is more susceptible to extinction when there is non-reinforcement of the CS+, and does not develop with all types of flavors in non-deprived animals (Ramirez, 1996). This is the reason that the one-bottle training and two-bottle choice testing method rather than the one-bottle acceptance method was chosen to evaluate dose-response relationships on a fructose CFP in these pharmacology studies.

B. Roles of orosensory and postingestive factors in preference conditioning

Carbohydrates, fats and proteins, both individually and in combination, frequently, but not always, will act as a UCS in preference learning (e.g., Capaldi, 1992,1996). Ethanol, with its relatively high caloric density, is also capable of conditioning a flavor preference (Ackroff and Sclafani, 2002, 2003; Cunningham and Niehus, 1997). Carbohydrates have been demonstrated to be a more powerful UCS than fats (e.g. Lucas and Sclafani, 1989), and among the carbohydrates, sucrose is preferred by rats compared to fructose as a UCS (Sclafani and Mann, 1987). In fact, experiments with intragastric infusion preparations have found that fructose has minimal and insignificant postingestive consequences relative to the development of a CFP (Ackroff and Sclafani, 1999). When one flavor is paired with an intragastic infusion of fructose, and a different flavor is paired with water, no significant preference develops for the flavor paired with fructose over the flavor that was paired with water. The texture alone of an ingestate has also been demonstrated to be innately reinforcing, with a CFP developing for a flavor paired with nonnutritive mineral oil over a flavor paired with equally nonnutritive, but less viscous solutions (Elizalde and Sclafani, 1990a). By combining previously neutral flavors with solutions that are innately reinforcing, apparently due to their sweet taste or viscous consistency, preferences can be conditioned for ingestates with no postingestive consequences (Ackroff and Sclafani, 1999; Sclafani and Ackroff, 1994), providing a means of dissociating orosensory mechanisms from postingestive mechanisms, thereby enabling examination primarily of the role of orosensory mechanisms.

Van Vort and Smith (1983) first used the sham feeding preparation to study both flavor preference and place preference conditioning. In their study rats were presented with milk paired with a cue flavor on alternating days with the fistula open (sham feeding) in one location, and on other days were presented the milk in a different location, with the fistula closed (real feeding), and paired with a different cue flavor. After 12 training cycles the rats developed a preference for the flavor that had been paired with the real feeding over the other flavor. Another interesting finding in that study was that the rats sham fed six times more milk than they did in the real feeding session, suggesting that the mechanisms that sustain the continuing ingestion of food may be different from the mechanisms involved in the development of a food preference.

Flavor-nutrient conditioning is possible with a delay between the presentation of the CS and the UCS, whereas in flavor-flavor conditioning the CS and UCS must be closely associated temporally, suggesting that the mechanisms mediating flavor-flavor preferences may be different than the mechanisms involved in flavor-nutrient conditioned preferences (Sclafani, 1999).

D. Neurochemical and Pharmacological Mechanisms of Palatable Intake: Role of Dopamine and Endogenous Opioids

i. Dopamine: The potent reward value of sweet taste may be mediated by a several different central neurochemical systems, among which dopamine and the endogenous opioids are two potential candidates. The evidence that mesolimbic dopamine circuits are involved in the mediation of reward, which in turn leads to the repetition of behavior, such as feeding, is that these behaviors are associated with

activation of those circuits (see reviews: Berridge, 1996; Berridge and Robinson, 1998; Salamone, 1991; Salamone et al,1997; Wise and Rompre, 1989). The dopamine system has been implicated in reinforcement mechanisms related to food and water intake (Agmo et al., 1993; 1995; Berridge and Robinson, 1998; Nakajima, 1989). Specifically, it can be inferred that both D-1 and D-2 receptors are involved in the ingestive response to sweet solutions because D-1 and D-2 dopamine antagonists suppress the intake of sugar and saccharin solutions in rats (Geary and Smith, 1985; Muscat and Wilner, 1989; Schneider et al., 1986a, 1986b, 1988, 1989; Smith and Schneider, 1988; Wise and Rompre, 1989; Xenakis and Sclafani, 1981). Furthermore, eating caused an increase in nucleus accumbens dopamine release that persisted until its termination in food-deprived animals (Radhakishun et al., 1988). Specific evidence for a role of dopamine in flavor reactivity is supported by an increase of dopamine release within the nucleus accumbens by naïve rats in response to an intraoral infusion of saccharin (Mark et al., 1991). In contrast, when the saccharin solution was administered to rats with a conditioned aversion to that taste, accumbens release of dopamine was significantly reduced. Dopamine release is also modified by positive ingestive consequences (Mark et al., 1994) such that consumption of a CS+ flavor paired with intragastric infusions of polycose were associated with increased dopamine release, but this increase in dopamine release was not found following exposure to the CS- flavor cue that had been paired with IG infusions of water. Untrained animals consuming the CS solutions showed no change in dopamine release between the CS+ and CS- flavors. Learning about other food-related cues may also influence dopamine release. Richardson and Gratton (1996) trained rats to lever-press for milk rewards, and in early training

sessions, nucleus accumbens dopamine release occurred in temporal conjunction with the presentation of the milk reward. However, over sessions, the release shifted forward in time to become associated with the cue signaling the start of the session. Studies have also consistently found that dopamine plays a role in the incentive salience of reward-related stimuli (Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999).

It is important to note that dopamine plays a rather complicated role in these processes since both general dopamine receptor agonists and antagonists decrease food intake (see review: Terry, 1996). Two major subfamilies of dopamine receptor subtypes have been examined to identify what receptor-specific role they may play in mediating food reward. D1 and D2 receptors activate and inhibit, respectively, the cellular second-messenger enzyme, adenyl cyclase (Sibley and Monsma, F. Jr., 1992). D1 agonists (e.g. SKF-38393) and D1 antagonists (e.g. SCH23390) dose-dependently and time-dependently significantly decrease intake under palatable, deprivation, sham feeding and ad libitum conditions (e.g., Hobbs et al, 1994;Terry, 1996). The changes in intake following drug manipulations of the D2 receptor have produced more varied results. Both the D2 agonist N-0437 and the D2 antagonist pimozide produced a biphasic effect on food intake, with low doses increasing food intake, and higher doses decreasing food intake (Terry, 1996). One potential explanation for the biphasic effect is that D2 agonists and antagonists may act at presynaptic autoreceptors at lower doses, and at traditional postsynaptic receptors at higher doses (Terry, 1996). This evidence therefore suggests that the dopaminergic system is involved in modulating feeding, and particularly intake of palatable substances, making it an appropriate neurochemical target in pharmacological studies examining CFP.

ii. Endogenous opioids: The role of opioids in feeding behavior is also well documented (see reviews: Bodnar, 1996, 2004; Morley et al., 1982), making it a second obvious neurochemical candidate for a role in CFP. The general opioid antagonist naloxone significantly reduces food intake in food-deprived rats (e.g., Holtzman, 1974), while opioid agonists such as morphine increase food intake (Levine and Morley, 1983; Sanger and McCarthy, 1980, 1981). The brain sites associated with opioid modulation of feeding behavior was demonstrated by the stimulation of food intake following central intracerebral administration of opioid peptides and receptor agonists into such traditional feeding-related sites as the hypothalamus, nucleus accumbens, ventral tegmental area, amygdala, parabrachial nucleus and nucleus of the solitary tract, among others (e.g., Bakshi and Kelley, 1993; Gosnell et al., 1986, 1996; Grandison and Guidotti, 1977; Majeed et al., 1986; Mucha et al., 1986; Stratford et al., 1997). The characterization of specific opioid receptor subtypes, mu, kappa and delta, has enabled the identification of relationships between these receptor subtypes and specific types of ingestive behaviors mediated by the endogenous opioids. Spontaneous and deprivation-induced intakes seem to be modulated primarily by mu receptors, which also seem to be involved in regulating long-term intake and body weight (Arjune and Bodnar, 1990a,b; Cole et al., 1995, 1997, 1998; Levine et al., 1983,1985, 1995; Mann et al., 1988; Simone et al., 1985; Ukai and Holtzman, 1988), while opioid control of feeding induced by glucoprivation or lipoprivation seems to be mediated by both mu and kappa receptors (Arjune et al., 1990a,b; Stein et al., 2000). Opioid antagonists appear to reduce the hedonic qualities of sweet substances since they reduce the intake of sucrose and saccharin more potently than the intake of water (Levine et al, 1982; Sclafani et al,

1982), and opioid antagonists also block feeding of sweet solutions driven by food restriction (Levine et al, 1995). Sucrose intake is reduced in sham-fed rats by the general opioid antagonist naloxone in a manner that is behaviorally equivalent to the reduction of palatability that results from diluting the concentration of test solutions, and this effect can be reversed by increasing the concentration of the solutions (Kirkham and Cooper, 1988). Moreover, mu and kappa opioid receptor antagonists reduce sucrose intake in both real-feeding (Beczkowska et al., 1992) and sham-feeding (Leventhal et al., 1996) animals, suggesting an orosensory component in this response. Further, these studies indicate that both general and specific opioid antagonism reduce palatable intake by specifically interfering with the maintenance, but not the initiation of feeding. Naloxone also reduces the positive hedonic effects of sucrose in taste-reactivity paradigms (e.g., Parker et al., 1992). For these reasons, it has been suggested that the opioid system is involved in the mediation of the hedonics of eating that sustain feeding, referred to as "liking" by Berridge (1996), who makes a distinction between that pleasurable component of appetitive behavior and that which is driven by the "wanting" aspect, or incentive salience. Further evidence for the involvement of opioids in feeding behavior is derived from studies that have found that the ingestion of palatable foods, such as sucrose, increase the density and synthesis of opioid receptors and opioid peptides. A diet rich in sucrose increased hypothalamic Dynorphin A1-17 and Prodynorphin mRNA levels, while caloric restriction of the same carbohydrate decreased their levels (Welch, Kim, Grace, Billington, and Levine, 1996). Therefore, a reciprocal relationship emerges such that stimulation and blockade of opioid receptors alters palatable intake, and correspondingly, palatable intake alters opioid peptides and

receptor message levels. However, these studies do not address whether endogenous opioids mediate the orosensory mechanisms involved in learning the association between flavor cues and innate species-specific taste preferences for sugars such as fructose. This would be suggested if antagonism of the opioid system by the general antagonist naltrexone significantly altered the acquisition or expression of a CFP for fructose.

D) Opioid and Dopaminergic systems as potential candidates mediating CFP

While CFPs have been studied extensively at the behavioral level, there have been fewer studies of the neurochemical and pharmacological mechanisms underlying this behavior. Food stimuli are ingested for nutritive purposes and also for hedonic value. The latter can be governed by innate heritage and species-specific factors, but is also subject to environmental manipulations (i.e. learning). As previously mentioned, two learning paradigms—conditioned taste aversion (CTA) and conditioned flavor preferences (CFP) have been used to study the role of learning in food choices. Although the physiology and pharmacology of CTA has been extensively studied, the underlying neurochemical and pharmacological substrates of CFP are not as well understood.

As previously mentioned, the involvement of the endogenous opioid and dopamine neurotransmitter systems in feeding behavior is well-documented. Because the role of these neurotransmitter systems has been demonstrated in other types of feeding behavior, they are obvious candidates for a role in CFP. Using dopamine antagonists, studies examining the role of the dopamine system have consistently

demonstrated the involvement of dopamine in both the acquisition and the expression of a CFP that is governed by flavor-flavor conditioning in sham-feeding preparations (Yu et al., 2000a,b) as well as one that is governed by flavor-nutrient conditioning in intragastric infusions (Azzara et al., 2001). However, the results have been mixed with respect to the role of the endogenous opioids in either the acquisition or expression of a CFP. The use of the flavor-flavor and flavor-nutrient paradigms that were procedurally identical to the positive dopamine antagonist studies revealed that naltrexone failed to alter either acquisition or expression of a CFP elicited by sucrose in sham-feeding rats (Yu et al., 1999), and that naltrexone failed to alter either acquisition or expression of a CFP elicited by intragastric sucrose infusions (Azzara et al., 2000). In contrast, a study of intact, freely-feeding rats (Mehiel, 1996) indicated the ability of naloxone to prevent both the acquisition and expression of a CFP. Although this contradictory evidence might suggest that the surgeries disrupted the involvement of the opioids in the development of a CFP, a more likely explanation for this difference may be procedural. As previously discussed, Mehiel (1996) administered the opioid antagonist on those days when they were presented with the CS+, and not on the days that they were exposed to the CS-. This raises the possibility that the aversive effects of the antagonists were a more significant conditioning stimulus than the positive effects of the intended UCS, thereby canceling out the conditioning effects that would otherwise result from associating positive ingestive consequences with the absence of those consequences. For this reason the present study continues to employ the administration of antagonists during both CS+ and CS- training. And, since fructose has strong conditioning

properties only in flavor-flavor paradigms, but not flavor-nutrient paradigms, we can use a similar paradigm in freely-feeding rats to produce a flavor-flavor fructose CFP.

E. Rationale and Specific Aims:

This section will examine the central features and methodology of the present series of studies with respect to hypotheses for: i) the study of the acquisition and expression of a fructose-paired flavor through the pharmacological analysis of conditioned flavor preferences, ii) the role of motivational state in the pharmacological analysis of conditioned flavor preferences, iii) the choices of specific palatable substances in the pharmacological analysis of conditioned flavor preferences, iv) the pairing of artificial flavors and bottle position in the pharmacological analysis of conditioned flavor preferences, v) the use of a dose-response curve for the antagonist studies and the use of equimolar doses in the D_1 and D_2 antagonist studies, and vi) the choice of dependent measures for analysis. This will be followed by a description of the Specific Aims and Hypotheses of the present study (Part vii).

i. The Study of Acquisition and Expression of Conditioned Flavor Preferences: In the acquisition phase of flavor-flavor conditioning, rats learn to associate the taste of fructose with an artificial flavor. This association should occur during the one-bottle training phase. In the expression phase of flavor-flavor conditioning, rats maintain an already learned preference. In the present experiments evaluating the pharmacological effects on the expression of flavor preferences, rats will be trained to drink different training solutions, and preference learning will then assessed in a two-choice test with vehicle or drug injections administered prior to the session and the two flavors presented in a common base. In the present experiments

evaluating the pharmacological effects on the acquisition of the flavor preferences, rats will be administered vehicle or drug injections before each of the one-bottle training sessions. Since dopamine antagonists have been previously shown to reduce intakes during similar training paradigms (Yu et al., 2000a,b), a yoked-control group will receive vehicle injections during training with intakes limited to the mean of the drug groups that do not acquire a preference. The data from this group will be used to determine if any drug effect on preference conditioning is secondary to reduced intakes of the training solutions. Preference drug effects on the expression of the CS+ preference will also be measured by injecting the rats with drug prior to the CS+ versus CS- choice tests because drug experience and ingestive experience may interact to determine the effect of drug (Lynch and Burns, 1990). Therefore, any significant variations in the acquisition of a flavor preference would suggest pharmacological effects upon the learning of this preference, and significant variations in the expression of a flavor preference would suggest pharmacological action upon the maintenance of an already-learned or acquired preference.

ii. The Role of Motivational State in the Pharmacology of Conditioned Flavor Preferences: Before the onset of training, the rats will be placed on a food restriction schedule that maintained their body weights at 85-90% of their ad libitum level for the duration of food-restricted training and testing conditions. This restriction regimen will ensure that rats will readily consume with short latency the flavored solutions during the daily 2 h training sessions conducted during the light cycle when intake is typically minimal. However, it is important to note that there is evidence that food restriction, particularly prolonged food restriction, produces a variety of

physiological and behavioral changes including: the enhancement of the hedonic response to food taste, and the sensitization of animals to the reinforcing properties of food, drugs of abuse and lateral hypothalamic self-stimulation (see review, Carr, 1996; also Carr and Wolinsky, 1993). The sensitivity to rewarding brain self-stimulation is inversely related to body weight (Carr, 1996). Studies on food restriction and brain neurochemical changes from Carr's laboratory suggest that these changes in sensitization of reward is mediated by opioid receptors, since the effect can be reversed by central administration of the general opioid antagonist, naltrexone, μ antagonist, TCTAP and the κ antagonist, nor-binaltrophimine. Carr and coworkers have also demonstrated that chronic food restriction altered prodynorphin-derived peptides in local regions, increased the levels of prodynorphin mRNA in the lateral hypothalamus and central amygdala, and induced elevated hypothalamic opioid secretion in naltrexone treated rats that is unique to the state of food restriction (Carr et al, 1998). Chronic food restriction also altered μ and κ binding in the bed nucleus of the stria terminalis, the basolateral/basomedial amygdala, and the parabrachial nucleus structures, which have strong anatomical and functional connections related to taste reactivity and/or taste aversion learning (Wolinsky et al, 1994; 1996).

The dopamine system has also been implicated in the behavioral changes in response to food restriction since restriction increases the sensitivity of neural substrates for rewarding and stimulant effects of drugs that are administered systemically and centrally. For example, food restriction potentiated the threshold-lowering effect of amphetamine in hypothalamic self-stimulation and increased its locomotor-stimulating

effects (de Vaca and Carr, 1998). Food restriction also augmented a cellular immediate early gene response (c-fos) to acute amphetamine in the cingulate cortex and caudal caudate-putamen) which are known to mediate rewarding and other behavioral effects of psychostimulants (Carr and Kutchukhidze, 2000).

In the experimental studies, animals are always tested under food-restricted conditions because there is evidence indicating an interaction between hunger state and drug potency (Beczkowska et al, 1993; Carroll et al, 1979; Lynch and Libby, 1983; Terry and Katz, 1992). There is also evidence indicating an interaction between motivational state and preference. Sclafani and coworkers have demonstrated that food deprivation caused rats to switch their preference from sucrose to either corn starch or corn oil (Sclafani, Ackroff, 1993), and from saccharin to mineral oil (Lucas and Sclafani, 1996). Food restriction selectively increased the CS+ intake but had no effect on the CS- intake during short preference tests, whereas water deprivation enhanced both CS+ and CS- intakes (Drucker et al, 1994). Berridge (1991) showed that food deprivation enhanced hedonic reactivity to sweet taste as well as the palatability of water.

iii. The Choice of Palatable Substances During Initial Habituation and Main Training Conditions: For initial habituation to the feeding procedure, an 8% maltodextrin solution will be used (BioServ, Frenchtown, NJ), which has a distinctive taste to rats and is the most preferred carbohydrate at low concentrations (Sclafani, 1987). This ensures that the rats will readily learn to sample the solutions early in the short test period, yet there will be no pre-exposure to either the fructose or saccharin

tastes during the initial pretraining period; this approach proved extremely effective in the flavor-flavor paradigms using sham-feeding rats (Yu et al., 1999, 2000a,b).

Actual Training Solutions: The present study will use the following training solutions: a palatable 8% Fructose solution (CS+/F or UCS), and a less palatable 0.2% saccharin solution (CS-/S). This concentration of fructose is chosen because it has been demonstrated to produce a strong flavor-flavor preference. A 0.2% saccharin solution is clearly palatable to rats since they consumed 98.9 ml of saccharin, but only 4.4 ml of water in 24-hour two-bottle preference test (Sclafani and Nissenbaum, 1985). Although saccharin and sugar are both sweet, and both are highly preferred when paired with water (Sclafani and Nissenbaum, 1985), rats prefer concentrated sugar solutions to saccharin solutions in choice tests (Collier and Novell, 1967; Young and Madsen, 1963). Prior work also indicates that rats learn to prefer a flavor mixed into concentrated sugar solutions over a flavor mixed into a saccharin solution (Mehiel, 1996; Sclafani, Ackroff, 1994). Both orosensory (taste) and postingestive (nutritive) effects of sugars appear to reinforce this preference (Sclafani, Ackroff, 1994). However, studies have demonstrated that fructose, unlike sucrose or glucose, does not act postingestively to condition a flavor preference, which should enable this study to identify the role of oral mechanisms to the minimization of postingestive factors.

The Testing solution: In the present series of studies both cue flavors will be presented in a 0.2 % saccharin solution during the two bottle choice tests. This base solution was chosen to insure that any differential intake between the two flavors can be attributed to a learned response to the two cue flavors rather than consumption of the preferred fructose over the less preferred saccharin solution. That is a difference

between this and the previous flavor-flavor studies (Yu et al., 1999, 2000a,b) that used a combined sucrose (8%) and saccharin (0.1%) testing solution after using sucrose (16%) and saccharin (0.2%) training solutions. This overall nutritive solution in these studies was mitigated by the sham-feeding preparation. The use of a base saccharin solution rather than a combined fructose-saccharin solution is better suited in the present study because the animals were freely-feeding, and the non-nutritive saccharin solution would minimize post-ingestive satiety signals.

iv. The Choices of Artificial Flavors, Bottle Position and Other Variables: For half of the rats an artificial flavor (e.g., cherry, unsweetened powdered Kool-Aid drink mixes, CS+) will be paired with the 8% fructose solution (US), and a second artificial flavor (e.g., grape, CS-) will be paired with the 0.2% saccharin solution during alternating one-bottle training sessions (120 minutes each day). The other half of the rats will have the grape flavor as the CS+ and the cherry flavor as the CS-. All of the flavors used in the present series of studies are less preferred to water in choice tests but have not shown any aversive properties per se (Yu et al., 1999, 2000a,b).

When two bottles are introduced during training, the position of the CS solution and water will be counterbalanced across days using a left-right-right-left pattern to ensure the differential intake is not due to any possible position preference. During testing, the position of the CS+ and CS- solutions are to be counterbalanced in an identical manner; this approach was successful in our prior studies (Yu, 1999, 2000a).

Rats in all experiments will be initially exposed to 8 consecutive days of one-bottle training sessions (120 min/day) with the CS+ (fructose) training solution presented on odd-numbered days, and the CS- (saccharin) presented on even-numbered

days. Initial exposure to the more preferred fructose solution insured greater initial responses that would persist throughout training. It should be noted that intakes typically increase over the four days, but asymptote by the fourth day, because exposure is limited to a maximum of 24 ml of the CS solutions. The 120-minute training and testing session is adopted to provide optimal time to develop and express conditioned preferences.

In all expression experiments, on training days 5-8, the rats will receive vehicle treatment (1 ml normal saline/kg body weight, s.c.) 30 min prior to the training session, during which they had access to two sipper tubes, one containing the CS- or CS+ solution, and the other containing water. This procedure acclimates the rats both to the injection procedure and the presence of two sipper tubes during the choice tests. The position of the CS and water sipper tubes will vary across days, using a left-right-right-left pattern. In the acquisition experiments, rats are to receive drug or vehicle treatment prior to all of the training sessions, a second sipper tube containing water will also be introduced on day 5 to acclimate the rats to the presence of two bottles during the choice tests.

v. The Uses of Equimolar Dose Response Curves in the Antagonist Studies: During the expression phase of the experiments, multiple doses will be given to rats to study the functional relationship between the drug dosage and their effects (dose response curve). Each rat will receive a subcutaneous injection of several doses of Naltrexone (0, 0.1, 1.0, 2.5 and 5 mg/kg). Raclopride and SCH23390 will be administered at equimolar (equivalent) doses (0, 50, 200, 400 or 800 nmol/kg) to obtain dose response curves. Equimolar doses (0, 50, 200, 400 or 800 nmol/kg) of the D_2

antagonist, raclopride and the D_1 antagonist, SCH23390 are to be used to evaluate if the dopaminergic D_1 and D_2 receptors play functionally equivalent roles in the acquisition and expression of flavor preferences conditioned by the sweet taste of sucrose. The drug doses are counterbalanced across animals with each dose followed by a saline injection.

To study the role of opioid and dopaminergic system in the acquisition of conditioned flavor preferences, naltrexone doses of 0.1, 1.0 and 5.0 mg/kg, as well as raclopride and SCH23390 doses (200 nmol/kg) have been chosen so that the drug doses are high enough to maximize the desired pharmacological effect yet minimize the possibility of eliminating training intake, which would have affected preference acquisition per se (Azzara et al., 2000, 2001; Yu et al., 1999, 2000a,b).

vi. Dependent Measures: One-bottle intakes of CS+ and CS- solutions will be ascertained daily over the four days of each type of training. This will allow determination of increased intakes of over training as well as overall differences during training between solutions. Total intakes are to be recorded during the test sessions to evaluate the overall effect of antagonists on intake of the solutions because both opioid and dopamine antagonists are expected to dose-dependently reduce overall intake. Given the lower hedonic potency of fructose, combined with the satiety effect of fructose, the intake of fructose and saccharin should be more equivalent, especially over the 2 h intake protocol.

One measure of preference will be used in this study. This measure computes percent CS+ intake by dividing CS+ intake by total intake, systematically comparing changes in each form of intake following pharmacological treatment relative to vehicle

treatment across the dose range. In this analysis, selective reductions in the CS+ intake, but not the CS- intake, are expected to effectively eliminate expression of a conditioned flavor preference.

vii. Specific Aims and Hypotheses of the present study:

Specific Aim 1A: It is hypothesized that the D_1 receptor antagonist, SCH23390 will dose-dependently reduce the expression of conditioned flavor preferences for fructose in free-feeding rats that were food restricted during one-bottle training and two-bottle testing, and maintained at 90% of their initial weight. This hypothesis is based upon the ability of SCH23390 to significantly reduce the expression of flavor-flavor conditioning to sucrose in sham-feeding rats (Yu et al., 2000a,b).

Specific Aim 1B: It is hypothesized that the D_2 receptor antagonist, raclopride will dose-dependently reduce the expression of conditioned flavor preferences for fructose in free-feeding rats that were food restricted during one-bottle training and two-bottle testing, and maintained at 90% of their initial weight. This hypothesis is based upon the ability of raclopride to significantly reduce the expression of flavor-flavor conditioning to sucrose in sham-feeding rats (Yu et al., 2000a,b). It is further hypothesized that SCH23390 will exert greater and more consistent reductions of the expression of the fructose flavor-flavor preference than raclopride because of the latter's lesser potency in the sham-feeding study (Yu et al., 2000a,b). The alternative hypothesis is that raclopride will not affect the expression of the fructose flavor-flavor preference because of the lesser potency of fructose relative to sucrose.

Specific Aim 1C: It is hypothesized that the D_1 receptor antagonist, SCH23390 will dose-dependently reduce the acquisition of conditioned flavor preferences for

fructose in free-feeding rats that were food restricted during one-bottle training and two-bottle testing, and maintained at 90% of their initial weight. This hypothesis is based upon the ability of SCH23390 to significantly reduce the acquisition of flavor-nutrient conditioning to sucrose in intragastric preparations (Azzara et al., 2001). Although SCH23390 did not interfere with the acquisition of a flavor-flavor preference conditioned by sucrose, it is possible that because fructose is a less preferred UCS than sucrose, a fructose-conditioned preference may be more sensitive to pharmacological perturbation. it will be The alternative hypothesis is that SCH23390 will not affect the acquisition of the fructose flavor-flavor preference because of its inability to alter the acquisition of sucrose flavor-flavor preferences in sham-feeding rats (Yu et al., 2000a,b).

Specific Aim 1D: It is hypothesized that the D_2 receptor antagonist, raclopride will dose-dependently reduce the acquisition of conditioned flavor preferences for fructose in free-feeding rats that were food restricted during one-bottle training and two-bottle testing, and maintained at 90% of their initial weight. The alternative hypothesis is that raclopride will not affect the acquisition of the fructose flavor-flavor preference because of its inability to alter the acquisition of sucrose flavor-flavor preferences in sham-feeding rats (Yu et al., 2000a,b) or the acquisition of sucrose flavor-nutrient preferences in intragastric preparations (Azzara et al., 2001).

Specific Aim 1E: Like Specific Aim 1D, it is hypothesized that the D_2 receptor antagonist, raclopride will dose-dependently reduce the acquisition of conditioned flavor preferences for fructose in free-feeding rats that were food restricted during two-

bottle training sessions allowed them access to both the CS+ fructose solution and the CS- saccharin solutions.

Specific Aim 2A: It is hypothesized that the opioid receptor antagonist, naltrexone will dose-dependently reduce the expression of conditioned flavor preferences for fructose in free-feeding rats that were food restricted during one-bottle training and two-bottle testing, and maintained at 90% of their initial weight because fructose is a less-potent hedonic stimulus than sucrose. The alternative hypothesis is that naltrexone will not affect the expression of the fructose flavor-flavor preference because of its inability to alter the expression of sucrose flavor-flavor preferences in sham-feeding rats (Yu et al., 1999) or the expression of sucrose flavor-nutrient preferences in intragastric preparations (Azzara et al., 2000).

Specific Aim 2B: It is hypothesized that the opioid receptor antagonist, naltrexone will dose-dependently reduce the acquisition of conditioned flavor preferences for fructose in free-feeding rats that were food restricted during one-bottle training and two-bottle testing, and maintained at 90% of their initial weight because fructose is a less-potent hedonic stimulus than sucrose. The alternative hypothesis is that naltrexone will not affect the acquisition of the fructose flavor-flavor preference because of its inability to alter the acquisition of sucrose flavor-flavor preferences in sham-feeding rats (Yu et al., 1999) or the acquisition of sucrose flavor-nutrient preferences in intragastric preparations (Azzara et al., 2000).

CHAPTER TWO: GENERAL METHODS

Subjects: Adult male albino Sprague-Dawley rats (300-380 g) were purchased from Charles River Laboratories, Wilmington, MA and were housed individually in wire mesh cages and maintained on a 12 h light/12 h dark cycle with Purina rat chow and water available ad libitum. All experimental protocols are 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. Two weeks before testing began, the rats were placed on a food restriction schedule that maintained their body weights at 85-90% of their ad libitum level throughout the entire experiment.

Test Solutions: For initial exposure, a maltodextrin solution (8%: BioServ, Frenchtown, NJ), which has a distinctive taste from fructose to rats was used (Sclafani A. 1987). The training solutions consisted of either fructose (8%: Sigma, St. Louis, Mo.) or sodium saccharin (0.2%: Sigma Chemical Co., St. Louis, MO). Unsweetened grape and cherry Kool-Aid flavors (0.05%: General Foods, White Plains, NY) were used as the conditioned stimuli. Half of the rats had a cherry flavor added to the fructose solution (CS+) and a grape flavor added to the saccharin solution (CS-); with the two flavors reversed for the remaining rats. In the two-choice preference tests, the CS+ and CS– flavors will each be presented in a 0.2% saccharin solution. The fructose-paired flavor is referred to as the CS+ and the saccharin-paired flavor as the CS- because 8% fructose is preferred to 0.2% saccharin (Sclafani and Ackroff, 1994). CS+/F refers to the flavored fructose solution used in training, and CS+/S refers to the same flavor

presented in saccharin during choice testing. The CS-/S refers to the flavored saccharin solution used in training and testing.

Drugs: To examine effects of opioid, D1 and D2 dopamine receptors upon acquisition and expression of conditioned flavor preferences conditioned by fructose, the general opioid antagonist, Naltrexone (NTX: Sigma Chemical Company, St. Louis, MO), the selective D1 antagonist, SCH23390 (Research Biochemicals Intl., Natick, MA: Waddington and O'Boyle, 1989), and the selective D2 antagonist, Raclopride (Research Biochemicals Intl., Natick, MA: Christensen et al., 1984), will be dissolved in 0.9% normal saline, and administered subcutaneously in a 1ml/kg volume.

Initial Training: All rats in the present experiments will initially be trained to drink a maltodextrin solution (8%) from calibrated sipper tubes (100 ml, 1 ml gradations) under initial 23-hour water deprivation and subsequent ad libitum conditions for water intake.

One-Bottle Training: All rats in the experiments will undergo one-bottle training with the CS+/F (Days 1, 3, 5, 7) and CS-/S (Days 2, 4, 6, 8) solutions, limited to 24 ml, and presented on alternating days over an eight day paradigm. This procedure maximized the possibility of equal CS+/F and CS-/S intake.

Two-Bottle Testing: All rats in the experiments will undergo two-bottle testing with the CS+ and CS- flavors presented individually in a saccharin solution (0.2%).

Statistical analyses: For the vehicle treatments in the expression studies, CS+ and CS- intakes will be recorded to the nearest ml after 30 and 120 minutes of the 2 h session. Intakes during training will be evaluated by a two-way repeated-measures analysis of variance with the CS- and CS+ conditions as one variable, and the five days

of exposure to vehicle as the repeated variable. Tukey corrected comparisons ($p<0.05$) will be used to detect significant effects. For drug expression studies, separate randomized-blocks analyses of variance will evaluate any alterations in CS+ and CS- intake as a function of pooled vehicle and drug dose treatments, and alterations in total intake as a function of vehicle and drug treatment. For acquisition studies, the CS+ and CS- intakes will be recorded to the nearest ml. Data are to be analyzed as describe above except for the following differences: Intakes during training will be evaluated by a randomized-block analysis of variance with the Control and Drug groups as a between-subject variable, the CS- and CS+ conditions as one repeated-measure variable, and the four days of exposure as the second repeated-measure variable. Tukey corrected comparisons ($p<0.05$) will be employed to detect significant effects.

CHAPTER THREE: DOPAMINE RECEPTOR SUBTYPE ANTAGONISTS AND FLAVOR PREFERENCES CONDITIONED BY FRUCTOSE

Introduction

Animals use flavor cues (taste, odor, texture) to guide their selection of nutritious foods and avoidance of toxic foods (Capaldi, 1996). Flavor preferences and aversions, in turn, are based in part on learned associations between the various flavor elements in the foods, flavor-flavor conditioning, and between the flavor cues and postingestive consequences, flavor-nutrient and flavor-toxin conditioning. A primary example of flavor-flavor conditioning is the acquired preference for an arbitrary flavor cue (e.g., banana extract) added to a sweet solution (e.g., saccharin solution) (Holman, 1975). The naturally-preferred sweet taste is considered to be an unconditioned stimulus (US) that reinforces the animal's preference for the added flavor, which represents the conditioned stimulus (CS).

The potent reward value of sweet taste may result, in part, because sweet taste activates mesolimbic dopamine circuits that are implicated in the mediation of natural as well as drug rewards. It has long been known that dopamine (DA) antagonists suppress the intake of sweet solutions in rats (Geary and Smith, 1985; Muscat and Willner, 1989; Xenakis and Sclafani, 1981). Various findings suggest that this suppression results, in part, because DA antagonists reduce the reward value of sweet taste (Schneider, 1989; Smith, 1995) although other explanations have been proposed to account for drug effects on food intake and reinforcement (Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999; Salamone et al., 1997). In addition to reducing the

intake of sweet solutions, DA antagonists may also alter the ability of sweet solutions to reinforce the preference for other flavors. In particular, Hsiao and Smith (1995) reported that rats showed a reduced preference for a flavored 10% sucrose solution previously consumed while they were treated with the D_2 antagonist raclopride compared to a differently flavored sucrose solution previously consumed while they were treated with saline.

Sucrose can reinforce flavor preferences based on its sweet taste as well as its postingestive nutritive actions through the processes of flavor-flavor and flavor-nutrient conditioning, respectively (Sclafani, 1995). Hsiao and Smith (1995) used brief (5 min) training sessions to minimize postingestive factors, but the amount of sucrose consumed in the training sessions may have had a postingestive reinforcing action. To separate the effects of drugs on flavor-flavor and flavor-nutrient learning, our laboratories have used sham-feeding and intragastric (IG) infusion procedures, respectively (Azzara et al., 2000, 2001; Yu et al., 1999, 2000a, 2000b). With sham-feeding, the ingested sucrose solution drains out of an open gastric fistula thereby minimizing postingestive nutrient actions (Weingarten and Watson, 1982). With the IG procedure, on the other hand, the sucrose is infused into the stomach thereby eliminating the sugar's taste as a conditioning factor (Sclafani, 1995). Yu et al. (2000a, 2000b) used the sham-feeding procedure to determine the effects of DA antagonists on the acquisition and expression of flavor conditioning by the sweet taste of sucrose. Rats were treated with a D_1 antagonist (SCH23390), a D_2 antagonist (raclopride), or saline during sham-feeding training trials with different flavors added to a 16% sucrose solution or a less preferred 0.2% saccharin solution. In subsequent drug-free choice tests with both flavors

presented in sucrose+saccharin solutions, the D_1 and D_2 groups displayed comparable preferences for the sucrose-paired flavor to those of saline-control rats that had their training intake limited to that of the drug groups, indicating a negligible effect on acquisition (Yu et al., 2000b). However, both antagonists dose-dependently reduced the preference for the CS+ flavor when administered prior to the choice test, indicating strong expression effects (Yu et al., 2000a).

The finding of Yu et al. (2000b) that DA antagonists did not block the acquisition of flavor conditioning by the sweet taste of sucrose would appear inconsistent with Hsiao and Smith's (1995) conditioning results as well as other studies suggesting that DA antagonists reduce the reward value of sweet taste (see: Schneider, 1989; Smith, 1995). There are several procedural differences between the two conditioning studies that may account for the discrepant findings. In particular, Hsiao and Smith (1995) used a higher dose of raclopride and exposed their rats to less sucrose during training compared to the Yu et al. (2000b) study. In addition, the rats in the Hsiao and Smith study (1995) were given matched amounts of the raclopride-paired flavor and vehicle-paired flavor, whereas the drug-exposed rats in the Yu et al. (2000b) study were given unlimited access to the CS+ and CS- flavors, and both flavors were paired with raclopride.

In view of these considerations, the present study further investigated the effect of DA antagonism on flavor preference learning produced by sweet taste. In this case, a conditioning procedure developed by Sclafani and Ackroff (1994) was used in which rats are trained to drink matched amounts of differently flavored 8% fructose and 0.2% saccharin solutions. This training procedure produces a robust preference for the

fructose-paired flavor in a two-bottle choice test when both flavors are presented in 0.2% saccharin. Fructose, rather than sucrose or glucose, is used in this conditioning procedure because, unlike these other sugars, fructose has little or no postingestive reinforcing action during the short-term sessions. This is demonstrated by the failure of IG fructose infusions to condition a CS+ preference as well as by other findings (Sclafani and Ackroff, 1994; Sclafani et al., 1993, 1999). Thus, the preference for a flavor that is mixed into a fructose solution is considered to be a form of flavor-flavor learning based on the more preferred taste of 8% fructose relative to 0.2% saccharin (Sclafani and Ackroff, 1994). This paper has been published in Pharmacology, Biochemistry and Behavior (75 (2003) 55-65).

Experiment 1

The first experiment determined if treating rats with a D_1 or D_2 antagonist (200 nmol/kg SCH23390 or raclopride: Yu et al., 2000b) during one-bottle training with flavored fructose and saccharin solutions attenuated their learning of a preference for the fructose-paired flavor. In addition to a vehicle-treated control group, which was trained like the two drug groups, two additional vehicle-treated groups had their training intakes matched to those of the D_1 and D_2 groups, respectively. Drug treatment was expected to reduce training intake of the flavored solutions, and the yoked-control groups allowed for a determination of the effect of reduced training intakes on flavor preference learning. Following training, flavor preferences were compared among the five groups with all rats treated with vehicle. The rats were then treated with various doses (50-800 nmol/kg) of SCH23390 or raclopride prior to the flavor preference tests. In this way, drug effects on both the acquisition and expression of fructose-conditioned

flavor preferences could be assessed. An important feature of this experiment was that the rats in the drug groups were treated with SCH23390 or raclopride on both fructose and saccharin training days, so that any potential adverse drug effects would be associated with both flavors.

Methods

The subjects and test solutions are described in the General Methods Section.

Rats were initially trained to drink an 8% maltodextrin solution from calibrated sipper tubes (100 ml, 1 ml gradations) while food and water restricted, and then while food was restricted with water available ad libitum except during daily 2 h sessions. The sipper tube was mounted on the front of the cage held by a taut steel spring, and was positioned so that the spout(s) entered the cage about 3-6 cm above the cage floor. This training procedure was repeated daily until all rats approached the sipper tubes with short (< 1 min) latency, typically within three days. The limited food rations were given after each training session.

Three groups of rats were given 8 one-bottle training sessions (2 h/day) with 24 ml of the CS+/F solution presented on odd-numbered days, and 24 ml of the CS-/S solution presented on even-numbered days. On days 5-8, the rats had access to two sipper tubes adjacently attached to the front of the cage, one containing the CS+/F or CS-/S solution, and the other containing water. This acclimated the rats 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 days using a left-right-right-left pattern. Intakes were measured to the nearest 1 ml at 0.5 and 2 h during each session.

The rats in the first group (Control group, n=15) received a vehicle injection (1 ml normal saline/kg body weight, s.c.) 30 min prior to the one-bottle training trials, while rats in the second (D_1 group, n=7) and third (D_2 group, n=7) groups received the D_1 antagonist, SCH23390 (Research Biochemicals Intl., 200 nmol/kg, s.c.), and the D_2 antagonist, raclopride (Research Biochemicals Intl., 200 nmol/kg, s.c.), respectively. These equimolar doses were chosen based upon their effects on sucrose-saccharin conditioned flavor preferences in sham-feeding rats (Yu et al., 2000a, 2000b). Two additional groups (D_1 Yoked group, n=8; D_2 Yoked group, n=9) received vehicle injections throughout one-bottle training, but their intakes of CS+/F and CS-/S solutions were limited to the mean 2 h intakes of the D_1 (11 ml) and D_2 (19 ml) groups.

Following training, all groups were given 10 two-bottle choice test sessions (2 h/ day) with the CS+/S and CS-/S solutions; intakes were unlimited in these tests. The positions of the two sipper tubes were counterbalanced as described above, and intake was measured after 0.5 and 2 h. The five groups received vehicle injections 30 min prior to the first 2 test sessions. Over the next eight days, half of the Control group (n=7), the D_1 group, and the D_1-Yoked group received SCH23390 at ascending doses of 50, 200, 400 and 800 nmol/kg 30 min prior to the test sessions on odd-numbered days. The remainder of the Control group (n=8), the D_2 group, and the D_2-Yoked group received raclopride at ascending doses of 50, 200, 400 and 800 nmol/kg 30 min prior to the test sessions on the odd-numbered days. All groups were given vehicle injections on even-numbered test days.

Intakes during training were evaluated using analysis of variance for the Control, D_1, and D_2 groups; the yoked-control groups were not included in this analysis

because their intakes were matched to the drug groups. Preliminary analysis of the two-bottle data failed to reveal significant differences over the 6 vehicle sessions, and therefore the vehicle data were averaged over these sessions. A between-group analysis of the averaged vehicle data was performed to determine if the different conditions during training affected the expression of the CS+ vs. CS- preference. To determine if D_1 antagonism during two-bottle testing altered total intake or CS preference, analyses of variance was performed with the Control, D_1 and D_1-yoked training groups across SCH23390 doses. Similar analyses were performed with the Control, D_2 and D_2-yoked training groups across raclopride doses. Tukey corrected comparisons ($p<0.05$) detected significant effects. The pattern of results for the 0.5 and 2 h intake measurements were generally similar. To simplify presentation, only the 0.5 h data are presented in detail; 2 h data are mentioned only when they differ from the 0.5 h results.

Results

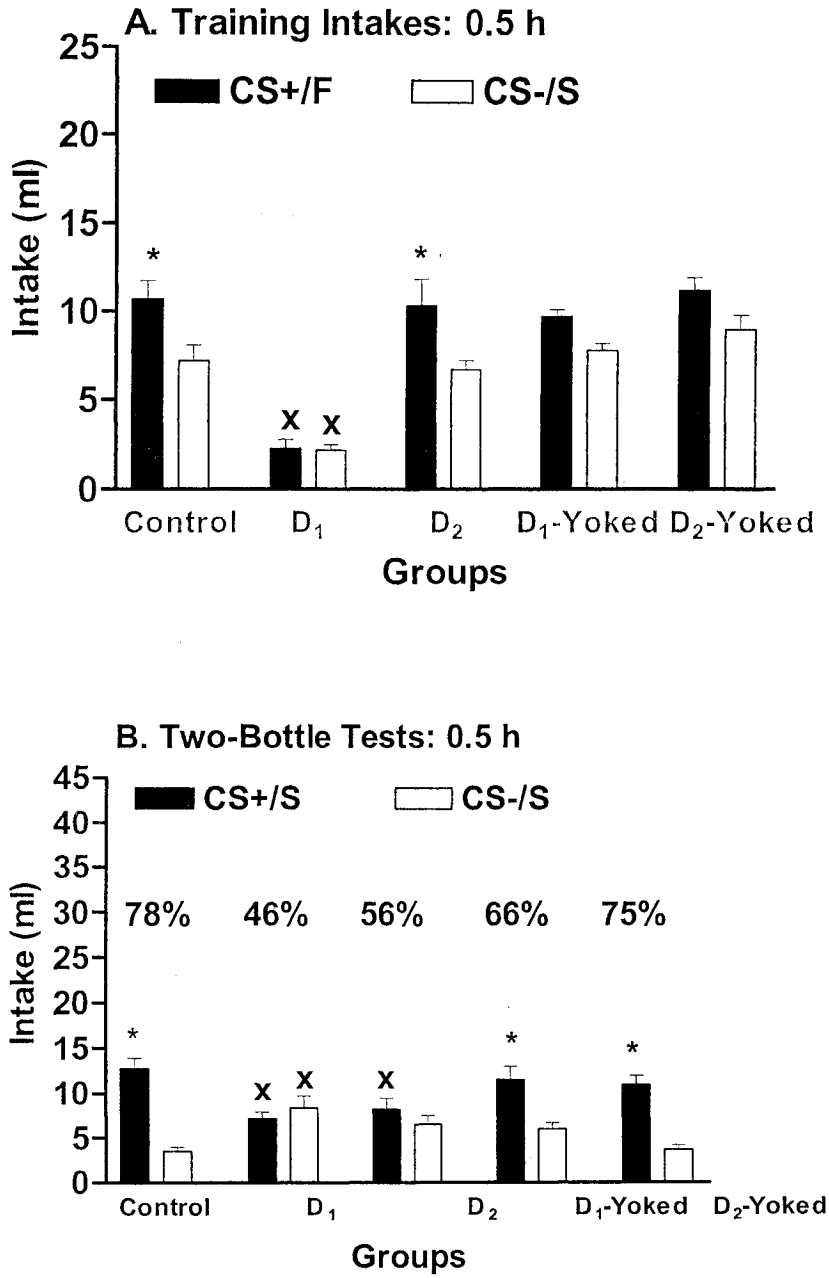
Drug effects on Training Intakes. Figure 1A presents the one-bottle training intakes of the CS+/F and CS-/S averaged over the 4 sessions with each solution. Analysis of the 0.5 h data indicated that, overall, the groups differed in their CS intakes ($F(2,28)= 45.42$, $p<0.0001$), that CS+/F intakes exceeded CS+/S intakes ($F(1,14)= 39.32$, $p<0.0001$), and there was an interaction between group and CS conditions ($F(2,28)= 16.52$, $p<0.0001$). Individual comparisons revealed that Control and D_2 groups did not differ in their CS intakes, and both groups consumed significantly more than did the D_1 group. Furthermore, both the Control and D_2 group, but not the D_1 group consumed more CS+/F than CS-/S at the 0.5 h time point. There were no differences between the 2 h intakes of CS+/F and CS-/S across groups, and the D_1 group

Figure 1. A: Upper Panel (One-Bottle Training Data): Intakes (mean +SEM) in one-bottle training sessions of a 8% fructose solution containing one flavor (CS+/F; four odd-numbered days) and a 0.2% saccharin solution containing a different flavor (CS-/S; four even-numbered days) after 0.5 h. The flavors were 0.05% grape or cherry Kool Aid. The Control group received systemic administration of saline (1 ml/kg, i.p.) 30 min prior to each training session. The D_1 and D_2 groups received 200 nmol/kg doses of SCH23390 and raclopride, respectively, 30 min prior to the training sessions. The Yoked control groups received saline injections 30 min prior to training, but solution intakes were limited to the amounts consumed after 2 h by the D_1 (D_1-Yoked: 11 ml) and D_2 (D_2-Yoked: 19 ml) groups. **B: Lower Panel (Two-Bottle Acquisition of Preference):** Intake (mean +SEM) of CS+/S and CS-/S saccharin following vehicle treatment in the two-bottle tests in the five training groups after 0.5 h. The numbers atop the bars represent the percent of total intake consumed as CS+/S.

Notes: * denotes significant differences between CS+/F and CS-/S intakes within a given group (Tukey comparisons, $p<0.05$).

x denotes significant differences in either CS+/F intake or CS-/S intake relative to the corresponding vehicle control group (Tukey comparisons, $p<0.05$).

Figure 1.



continued to drink less of the CS solutions than did the Control and D_2 groups ($F(2,28)= 23.94$, $p<0.0001$). Note that while the D_1-yoked rats consumed more than the D_1 rats during the initial 0.5 h of the session (Figure 1A), the 2 h intakes of the two groups were well matched.

Drug effects on CS+ Preference Acquisition. In assessing whether the different training regimens altered the acquisition of the fructose-conditioned flavor preference, the two-bottle CS+/S and CS-/S intakes of the five groups were compared following vehicle treatment (Figure 1B). Analysis of the 0.5 data indicated that, overall, the rats consumed more CS+/S than CS-/S ($F(1,14)= 86.06$, $p<0.0001$) and there were significant group x CS interactions ($F(4,56)= 25.56$, $p<0.0001$), but no overall group effect on CS intakes. Individual comparisons revealed that the Control, D_1-Yoked, and D_2-Yoked groups, but not the D_1 or D_2 groups, consumed significantly more CS+/S than CS-/S. Although the CS+/S preferences were somewhat weaker in the D_1-Yoked and D_2-Yoked groups (66% and 75%) compared to the Control group (78%), these three groups did not differ in their CS+ or CS- intakes. The significant CS+/S preferences displayed by the Yoked groups indicates that the lack of a CS+/S preference in the D_1 and D_2 groups was not due to their reduced CS intakes during training.

Drug effects on total test intakes. Analysis of total CS intakes during the two-bottle tests following SCH23390 treatment revealed significant differences across doses ($F(4,28)= 55.91$, $p<0.0001$) and for the interaction between groups and doses ($F(8,56)= 2.14$, $p<0.047$), but not among groups (Figure 2A). SCH23390 significantly reduced total CS intakes in the Control group following all doses. The D_1 and D_1-yoked groups

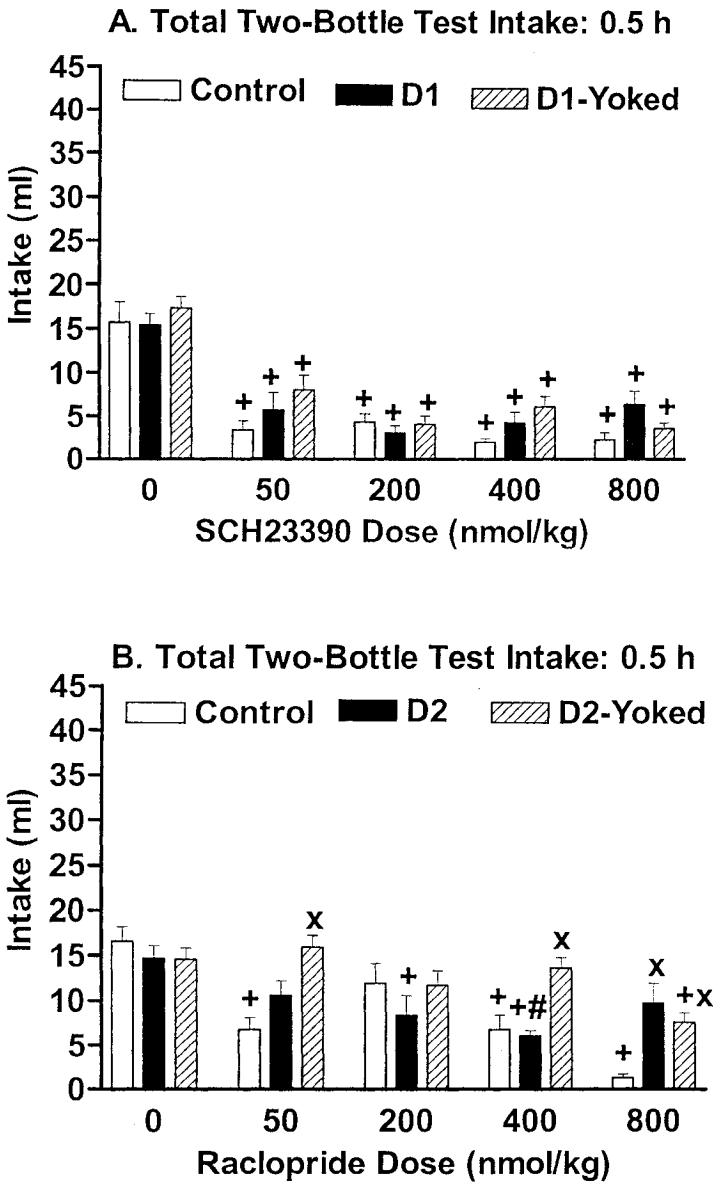
Figure 2: Total Intake in Two-Bottle Tests. Intake (mean +SEM) after 0.5 h of both CS+/S and CS-/S (total) solutions during two-bottle tests following pretreatment with vehicle or SCH23390 doses (50-800 nmol/kg) in the vehicle Control, D_1 and D_1-Yoked training groups (**A: Upper Panel**) and following pretreatment with vehicle or raclopride doses (50-800 nmol/kg) in the Control, D_2 and D_2-Yoked training groups (**B: Lower Panel**). One caveat about the presentation of this figure is that it takes scalar data on the abscissa and presents it as linear. This was done because bar graphs showing differences between CS+/S and CS-/S intakes were the best presentation for data comparison.

Notes: **x** denotes significant differences in total (CS+/S intake and CS-/S intake) relative to the vehicle control training group (Tukey comparisons, $p<0.05$).

+ denotes significant antagonist dose effects for a particular intake condition relative to its corresponding vehicle control (Tukey comparisons, $p<0.05$).

# denotes significant antagonist dose effects for a particular intake condition relative to its yoked control group (Tukey comparisons, $p<0.05$).

Figure 2.



displayed significant reductions in total CS intakes following all SCH23390 doses at 0.5 h.

Analysis of D_2 drug effects on the total intakes of CS intakes during the two-bottle tests revealed significant differences across doses ($F(4,32)= 23.73$, $p<0.0001$), for the interaction between groups and doses ($F(8,64= 9.43$, $p<0.0001$), and among groups at the 0.5 h time point ($F(2,16)= 4.70$, $p<0.025$) (Figure 2B). Raclopride significantly reduced total CS intake in the Control group at the 50, 400 and 800 nmol/kg doses. The D_2 group displayed significant reductions in total CS intakes following the 200 and 400 nmol/kg raclopride doses, whereas the D_2-Yoked group displayed a significant intake reduction only following the 800 nmol/kg dose.

Drug effects on expression of CS+ preference. Since the Control, D_1-yoked and D_2- yoked groups showed a CS+/S preference, whereas the D_1 and D_2 groups failed to display CS+/S preferences, each of the groups were analyzed separately at each of the two time points.

Figure 3 presents the two-bottle CS+/S vs. CS-/S intakes of the Control, D_1 and D_1-yoked training groups following treatment with SCH23390. In Control rats, significant differences were observed among SCH23390 doses ($F(4,30)= 21.30$, $p<0.0001$), between CS+/S and CS-/S solutions ($F(1,30)= 5.33$, $p<0.028$), and for the dose x CS interaction ($F(4,30)= 8.46$, $p<0.001$). The CS+/S preference (77%) of the Control group expressed following vehicle was eliminated by all doses of SCH23390 (Figure 3A). This was caused by significant drug-induced reductions in CS+/S, but not CS-/S intake. In the D_1-Yoked group, significant differences were observed among SCH23390 doses ($F(4,35)= 21.90$, $p<0.0001$), between CS+/S and CS-/S solutions

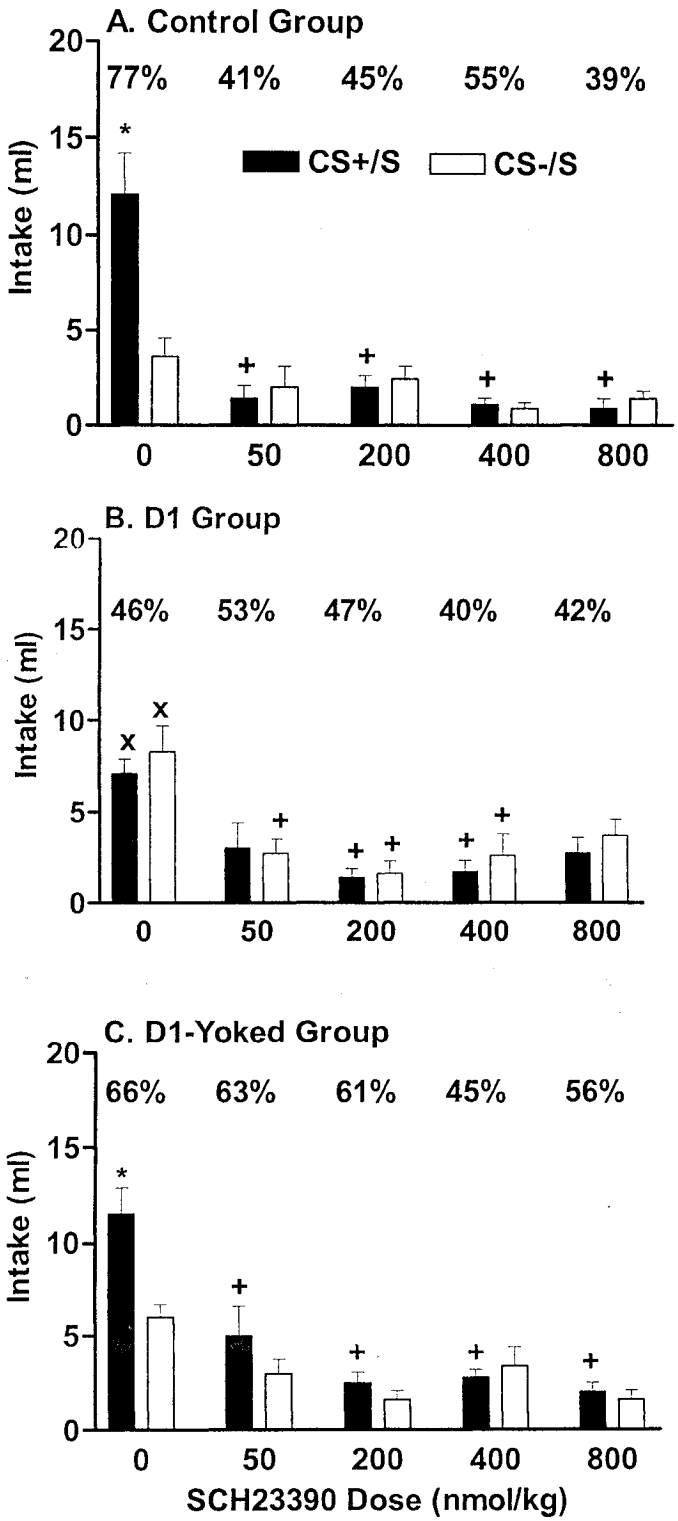
Figure 3 (Expression of Flavor Preferences). Intake (0.5 h mean +SEM) of CS+/S and CS-/S solutions during two-bottle tests following treatment with vehicle and SCH23390 in the Control (**A: Upper Panel**), D_1 (**B: Middle Panel**) and D_1-Yoked (**C: Lower Panel**) groups. The numbers atop the bars represent the percent of total intake consumed as CS+/S. One caveat about the presentation of this figure is that it takes scalar data on the abscissa and presents it as linear. This was done because bar graphs showing differences between CS+/S and CS-/S intakes were the best presentation for data comparison.

Notes: * denotes significant differences between CS+/S and CS-/S intakes within a given group (Tukey comparisons, $p<0.05$).

x denotes significant differences in either CS+/S intake or CS-/S intake relative to the corresponding vehicle control group (Tukey comparisons, $p<0.05$).

+ denotes significant antagonist dose effects for a particular intake condition relative to its corresponding vehicle control (Tukey comparisons, $p<0.05$).

Figure 3.



($F(1,35)= 7.54$, $p<0.0095$) and for the dose x CS interaction ($F(4,35)= 3.19$, $p<0.025$). The CS+/S preference (66%) of the D_1-Yoked group following vehicle treatment was blocked by all SCH23390 doses (Figure 3C). This was caused by significant drug-induced reductions in CS+/S, but not CS-/S intake. In contrast, the D_1 -trained rats did not display a CS+/S preference following vehicle treatment in two-bottle testing (46%), and significant differences were observed only among SCH23390 doses ($F(4,30)= 11.61$, $p<0.0001$). SCH23390 treatment did not alter their relative preference for the two CS flavors but significantly reduced CS+/S (200-400 nmol/kg) and CS-/S (50-400 nmol/kg) intakes (Figure 3B). Thus, D_1 antagonism prior to two-bottle testing eliminated the expression of the fructose-conditioned CS+/S preference in those training groups (Control and D_1-yoked) showing such a preference, and reduced both CS+/S and CS-/S intakes in the training group (D_1) that failed to acquire a preference.

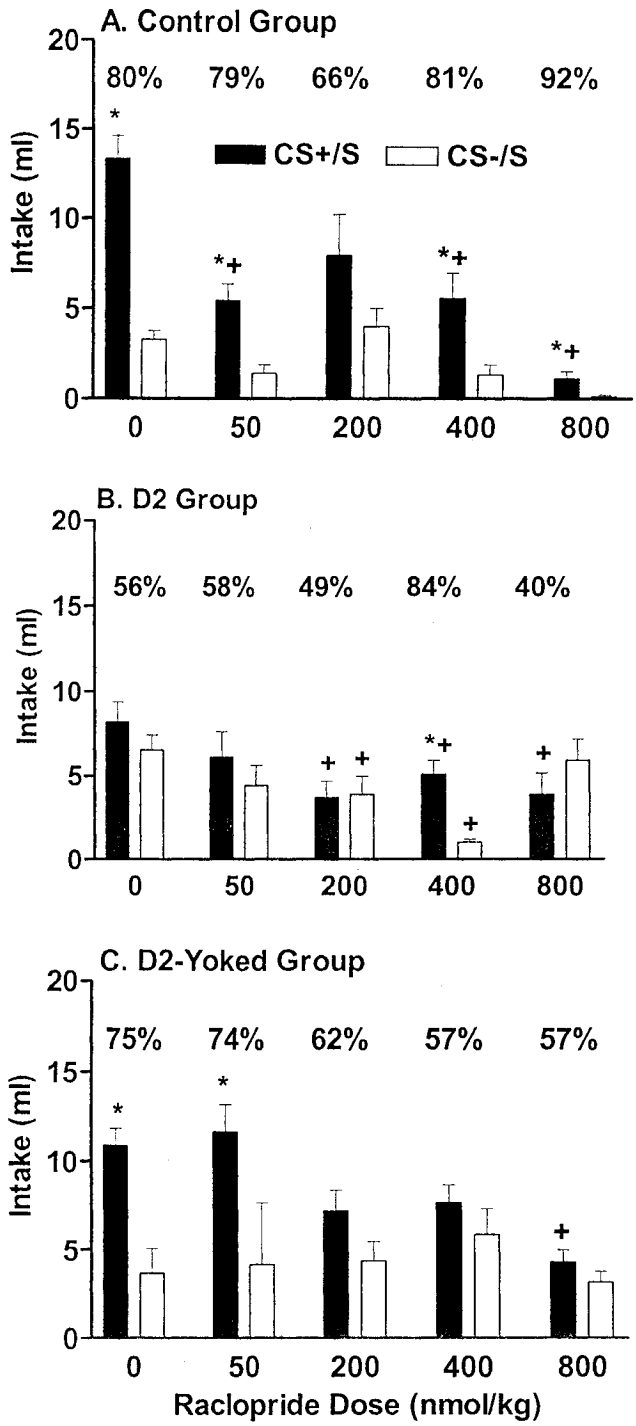
Figure 4 presents the two-bottle CS+/S vs. CS-/S intakes of the Control, D_2 and D_2-yoked training groups following treatment with raclopride. In Control rats, significant differences were observed among raclopride doses ($F(4,35)= 14.62$, $p<0.0001$), between CS+/S and CS-/S solutions ($F(1,35)= 41.63$, $p<0.0001$), and for the dose x CS interaction ($F(4,35)= 4.16$, $p<0.007$). As illustrated in Figure 4A, the CS+/S preference of the control group persisted over most raclopride doses; only at the 200 nmol/kg dose did the control rats fail to drink more CS+/S than CS-/S. Raclopride significantly reduced CS+/S, but not CS-/S intake following the 50, 400 and 800 nmol/kg doses. In the D_2-Yoked group, significant differences were observed among raclopride doses ($F(4,40)= 5.41$, $p<0.0014$), between CS+/S and CS-/S solutions ($F(1,40)= 34.62$, $p<0.0001$), and for the dose x CS interaction ($F(4,40)= 3.08$, $p<0.027$).

Figure 4 (Expression of Flavor Preferences). Intake (0.5 h mean +SEM) of CS+/S and CS-/S solutions during two-bottle tests following treatment with vehicle and raclopride in the Control (**A: Upper Panel**), D_2 (**B: Middle Panel**) and D_2-Yoked (**C: Lower Panel**) groups. Differences (Tukey comparisons, $p<.05$) between intakes of the CS+ and CS- solutions within a test are indicated by crosses. The numbers atop the bars represent the percent of total intake consumed as CS+/S. One caveat about the presentation of this figure is that it takes scalar data on the abscissa and presents it as linear. This was done because bar graphs showing differences between CS+/S and CS-/S intakes were the best presentation for data comparison.

Notes: * denotes significant differences between CS+/S and CS-/S intakes within a given group (Tukey comparisons, $p<0.05$).

+ denotes significant antagonist dose effects for a particular intake condition relative to its corresponding vehicle control (Tukey comparisons, $p<0.05$).

Figure 4.



The CS+/S preference (75%) of the D_2-Yoked rats observed following vehicle treatment persisted following the 50 nmol/kg raclopride dose, but at the higher doses the rats no longer consumed more CS+/S than CS-/S (Figure 4C). Raclopride significantly reduced CS+ intake only at the highest dose after 0.5 h, and its effects largely dissipated at 2 h such that CS+/S preferences were observed at the 50 and 400 nmol/kg doses. In contrast to the Control and D_2-Yoked groups, the D_2 group did not display a CS+/S preference following vehicle treatment in two-bottle testing (56%), and significant differences were observed only among raclopride doses at 0.5 ($F(4,30)= 4.50$, $p<0.006$), and not after 2 h. Raclopride treatment significantly reduced CS+/S (200-400 nmol/kg) and CS-/S (200-400 nmol/kg) intakes at 0.5 h (Figure 4B); these effects dissipated after 2 h. The D_2 rats showed no preference for the CS+/S at the 50, 200 and 800 nmol/kg doses, but unexpectedly they consumed more CS+/S than CS-/S at the 400 nmol/kg dose. The reason for this isolated preference is not clear. Thus, D_2 antagonism prior to two-bottle testing reduced intakes, and had inconsistent effects on CS+/S preference in the two groups that acquired a preference for the fructose-paired flavor: the Control group continued to prefer the CS+/S at all raclopride doses except 200 nmol/kg, whereas the D_2-Yoked group displayed a CS+/S preference only at the 50 nmol/kg dose. The potency, duration and magnitude of raclopride-induced effects upon these preferences thus appeared smaller than those induced by D_1 antagonism.

Experiment 2

Experiment 1 revealed that SCH23390 or raclopride treatment during one-bottle training blocked the acquisition of a fructose-conditioned flavor preference, however there is evidence that solution intakes in one-bottle tests may not always reflect

preferences as measured in two-bottle tests (Sclafani, 1987). Therefore, the training data of the first experiment do not provide a definitive assessment of dopamine antagonist-induced effects on fructose vs. saccharin preference. This issue was addressed in Experiment 2 in which control and D_2 groups received two-bottle access to flavored 8% fructose vs. 0.2% saccharin on half of the training sessions. Only the CS- was available during the remaining sessions to insure that the rats consumed sufficient amounts of CS- during training.

Methods

Male rats of similar strain, age, and source of those used in Experiment 1 were housed and pretrained with 8% maltodextrin as in the first experiment. One group (Control group, n=8) received a vehicle injection (1 ml normal saline/kg body weight, sc) 30 min prior to each of 8 daily training trials, while a second group (D_2 group, n=10) received the D_2 antagonist, raclopride (200 nmol/kg). On odd-numbered training days, two-bottle training sessions (2 h/day) occurred with the rats receiving 24 ml each of the CS+/F and CS-/S. The left-right position of the CS solutions systematically varied over days. On even-numbered days, the rats were given one-bottle training sessions (2 h/day) with 24 ml of the CS-/S available. Following training, the rats were given two-bottle test sessions (2 h/day) with the CS+/S and CS-/S solutions. The rats in both groups received vehicle injections 30 min prior to the two test sessions. As in first experiment, intakes were recorded at 0.5 and 2 h during training and test sessions. Only the 0.5 h data are presented in detail although 2 h data are described when they differ from the 0.5 h results.

Results

Drug effects on Training Intakes. Analysis of the two-bottle intakes on the odd-numbered training days revealed that, overall, the vehicle-treated control group consumed significantly more of the CS solutions than did the D_2 group after 0.5 h (0.5 h: $F(1,9)= 12.53$, $p<0.006$), the rats consumed more CS+/F than CS-/S ($F(1,9)= 46.01$, $p<0.0001$), and there was a significant interaction between groups and CS conditions ($F(1,9)= 7.72$, $p<0.021$) (Figure 5A). Individual tests indicated that the Control rats consumed more CS+/F than did the D_2 group in session 7. The groups did not differ in their CS-/S intakes. CS intakes increased over training sessions ($F(3,27)= 21.38$, $p<0.0001$) which was due primarily to an increase in CS+/F intake. Within-group comparisons revealed that the Control rats consumed more CS+/F than CS-/S during all two-bottle sessions except the first one. The D_2 rats consumed more CS+/F than CS-/S during the last three 2 h sessions, although at the 0.5 h time point, the difference was significant only during the final training session. Overall, the preference for CS+/F over CS-/S for Control rats (73% - 81%) was greater than that of the D_2 rats (53% - 75%) (Figure 5A).

Analysis of the one-bottle CS- training intakes indicated that, overall, the Control rats consumed more CS-/S than did the D_2 rats ($F(1,9)= 25.16$, $p<0.0007$), that intakes increased over training sessions ($F(3,27)= 32.00$, $p<0.0001$), and there was Group x Session interaction ($F(3,27)= 3.28$, $p<0.036$). In particular, Control rats consumed significantly more CS-/S than did D_2 rats during the last three sessions (Figure 5B). Over the 8 days of training, the rats consumed more CS-/S (intake totaled over one- and two-bottle training sessions at 2 h) then CS+/F, although this difference

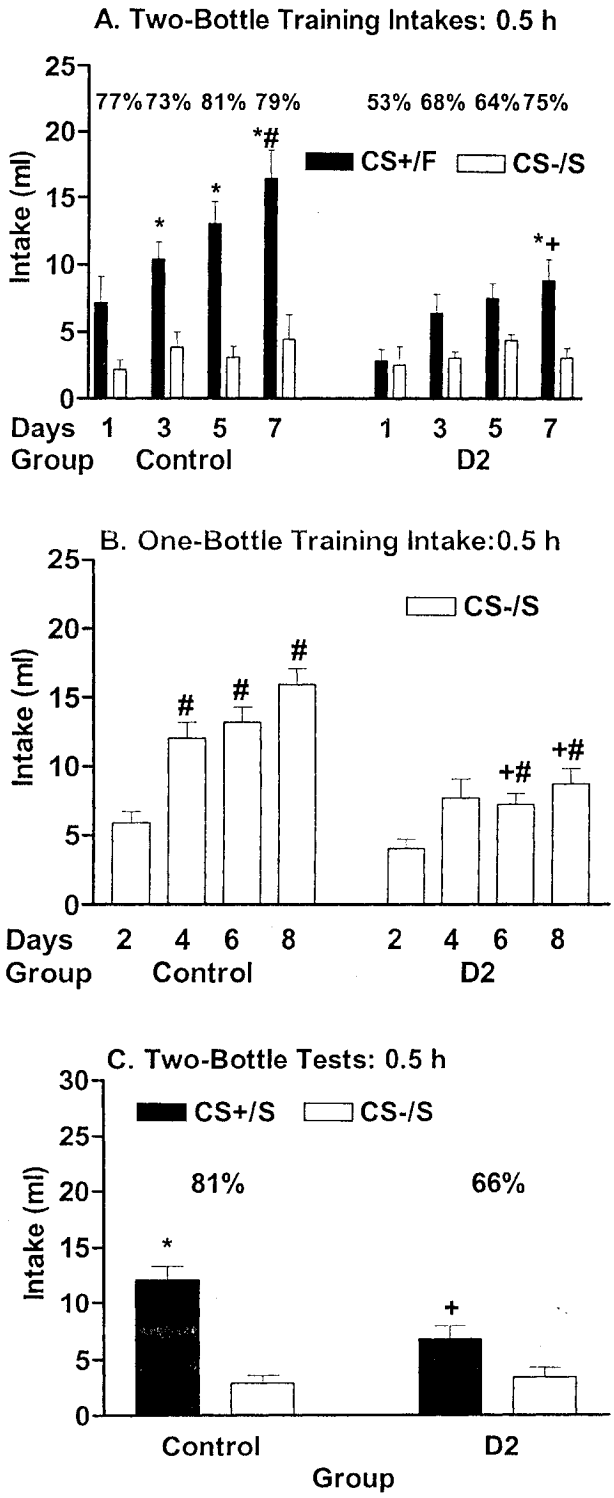
Figure 5 (Two-Bottle Training and Testing). Intakes (Mean ±SEM) in two-bottle training sessions of flavored 8% fructose (CS+/F) and 0.2% saccharin (CS-/S) solutions after 0.5 h during four odd-numbered days (**A: Upper Panel)** and of CS-/S in one-bottle training sessions after 0.5 h on four even-numbered days (**B: Middle Panel**). The Control group received saline (1 ml/kg, s.c.) 30 min prior to each training session, whereas the raclopride group received a 200 nmol/kg dose 30 min prior to the training sessions. The numbers atop the bars represent the percent of total intake consumed as CS+/F. **C. Lower Panel:** Intake (mean +SEM) of CS+/S and CS-/S solutions during two-bottle testing sessions in Control and D_2 groups after 0.5 h. The numbers atop the bars represent the percent of total intake consumed as CS+/S.

Notes: * denotes significant differences between CS+/S and CS-/S intakes within a given group (Tukey comparisons, $p<0.05$).

+ denotes significant antagonist dose effects for a particular intake condition relative to its corresponding vehicle control (Tukey comparisons, $p<0.05$).

# denotes significant antagonist dose effects for a particular intake condition relative to its yoked control group (Tukey comparisons, $p<0.05$).

Figure 5.



was significant only for the D_2 group (D_2: 100.9 (±8.6) vs. 62.5 (±5.9) ml; control: 106.5 (±7.1) vs. 82.8 (±3.8) ml).

Drug effects on CS+ Preference Acquisition. Figure 5C presents the results of the CS+/S vs. CS-/S two-bottle test which followed vehicle treatment. Analysis revealed significant differences between groups ($F(1,9)= 9.50$, $p<0.013$), between CS+/S and CS-/S intakes ($F(1,9)= 42.00$, $p<0.0001$) and for their interaction ($F(1,9)= 9.20$, $p<0.014$). Individual tests indicated that the Control rats consumed significantly more CS+/S than CS-/S; their percent CS+/S intakes were 81% (Figure 5C). In contrast, the D_2 group failed to consume significantly more CS+/S than CS-/S. Compared to the Controls, the D_2 rats drank less CS+/S; the groups did not differ in their intake of CS-/S.

Discussion

In confirmation of prior work (Sclafani and Ackroff, 1994), the control rats trained with flavored 8% fructose and 0.2% saccharin solutions displayed a significant (~80%) preference for the fructose-paired flavor in choice tests with both flavors presented in saccharin solutions. This preference is attributed to the rats associating the CS+ flavor with the sweet taste of fructose rather than the sugar's postingestive actions. This assumption is based on findings showing that fructose has a relatively weak postingestive reinforcing effect (Sclafani and Ackroff, 1994; Sclafani et al., 1993, 1999). Also consistent with many prior reports (see: Schneider, 1989; Smith, 1995), the control and drug groups reduced their intake of the fructose solution when injected with the dopamine receptor antagonists SCH23390 and raclopride prior to test sessions.

The new findings of the present study are that treating rats with SCH23390 or raclopride during one-bottle training blocked the development of the fructose-

conditioned CS+ preference. In the case of the D_1 group, SCH23390 treatment significantly reduced the intakes of the flavored fructose and saccharin solutions during training. This reduction did not account for the failure of the D_1 group to acquire a CS+ preference, however, because the D_1-yoked rats, which were limited to the training intakes of the D_1 rats, displayed a significant CS+ preference. In Experiment 1, the D_2 and control groups consumed similar amounts of CS solutions during training, and therefore reduced CS intakes were not a factor in the D_2 group's failure to display a CS+ preference. In Experiment 2, the CS training intakes of the D_2 rats were somewhat less than that of the controls at the 0.5 h time point, but the 2 h intakes of the two groups did not differ. Taken together, these results indicate that the D_1 and D_2 antagonists did not prevent the acquisition of CS+ flavor conditioning because they reduced the exposure to the flavored fructose or saccharin solutions. Instead, the data suggest that the acquisition of flavor conditioning was inhibited because the drugs attenuated the reward value of the fructose taste.

Experiment 2 revealed that vehicle-treated control rats significantly preferred the CS+/F solution to the CS-/S solution during the two-bottle training sessions which confirms prior findings that rats prefer 8% fructose to 0.2% saccharin in short-term "taste" tests (Sclafani and Ackroff, 1994). The D_2 rats injected with raclopride (200 nmol/kg) throughout training also preferred the CS+/F to the CS-/S during the training sessions, although their preference was attenuated relative to that of the control rats. This supports the idea that D_2 antagonism reduced the rats' attraction to the sweet taste of sugar. Nevertheless, the fact that the D_2 rats consumed more fructose than saccharin during training, but did not reliably prefer the fructose-paired flavor in testing indicates

that the acquisition of a flavor preference conditioned by sweet taste is more susceptible to D_2 drug antagonism than is the unconditioned preference for the sweet taste itself.

The present results contrast with the findings of Yu et al. (2000b) that SCH23390 and raclopride did not prevent rats from acquiring a preference for a CS+ flavor paired with a sucrose solution over a saccharin-paired flavor. These discrepant results may be accounted for by the different conditioning procedures used in the two studies. In particular, while both studies paired the CS- flavor with a 0.2% saccharin solution, the CS+ flavor was paired with 16% sucrose (0.47 M) in our earlier work and with 8% fructose (0.44 M) in this study. Taste tests indicate the rats prefer sucrose to fructose over a range of isomolar concentrations (Sclafani and Mann, 1987). Furthermore, the rats in the earlier study sham-fed the sucrose solution whereas the present rats real-fed the fructose solution. This may have further increased the difference in the reward value of the two sugar solutions because some data suggest that postingestive satiety, experienced by a real-feeding but not a sham-feeding animal, attenuates the rat's attraction to carbohydrate solutions (Sclafani et al., 1994; Warwick and Weingarten, 1996). Another consequence of the sham-feeding procedure is that it allowed the rats to consume substantially more of the flavored sucrose solution than of the flavored saccharin solution during training, whereas the real-feeding rats of the present study consumed equivalent amounts of the fructose and saccharin training solutions. Thus, while treatment with the dopamine antagonists during training may have reduced the reward value of the flavored sugar solutions in both studies, the drug effect may have been more pronounced with the real-fed fructose solution used in the present experiments than with the sham-fed sucrose solution used by Yu et al.(2000b).

This interpretation predicts that DA antagonists would prevent flavor conditioning using a sucrose sham-feeding training procedure if a less concentrated, and therefore less preferred sucrose solution was used.

In addition to preventing the development of an acquisition of a CS+ preference in the D_1 group, SCH23390 treatment blocked the expression of the CS+ preference in the Control and D_1-Yoked groups. This resulted from a reduction in CS+/S but not CS-/S intake during the two-bottle test sessions. Raclopride also selectively reduced CS+/S intake in the Control and D_2-Yoked groups, but this reduction was not as pronounced as that produced by SCH23390. Consequently, the Control and D_2-Yoked groups continued to prefer the CS+ flavor at some dose levels. This pattern of results, in general, agrees with previous data showing the D_1 and D_2 antagonism attenuated the expression of a flavor preference conditioned by sucrose in sham-feeding rats (Yu et al., 2000a, 2000b). The studies differed in that SCH23390 and raclopride had greater and weaker effects, respectively, on two-bottle test intakes and preferences in the current study than in our prior studies involving sucrose. Also, whereas the two drugs produced similar reductions in the expression of CS+ preference in the Yu et al. (2000a, 2000b) studies, SCH23390 suppressed CS+ preference to a greater degree than raclopride in the present experiment. These different drug effects on two-bottle preference may be related to the fact that Yu et al. (2000a, 2000b) tested flavor preferences using a sucrose + saccharin solution which is more palatable than the plain saccharin solution used in the present study.

The present results extend the findings of Hsiao and Smith (1995) that DA receptor antagonism reduced the flavor preference conditioning action of a sweet

solution. In their study, rats preferred a flavored sucrose solution previously paired with vehicle injection over a different flavored sucrose solution previously paired with raclopride injection. A limitation of their conditioning procedure is that only one flavor is associated with the drug and thus adverse drug effects may contribute to the reduced flavor preference. In the present study, however, both CS+ and CS- flavors were paired with drug treatment making it unlikely that any adverse drug effects during training influenced the outcome of the flavor preference test. An earlier study by Ettenberg and White (1981) observed that the D_2 antagonist pimozide blocked flavor preference conditioning by lateral hypothalamic (LH) self-stimulation. In this experiment, rats that drank a coffee-flavored solution followed by the opportunity to bar press for LH self-stimulation subsequently preferred the flavored solution to plain water, whereas control rats not allowed to self-stimulate preferred water to the coffee solution. Other rats treated with pimozide during flavor/self-stimulation training failed to show a preference for the coffee solution. This finding may be relevant to the effects of D_2 antagonism on sugar-conditioned flavor preferences obtained in the present study and by Hsiao and Smith (1995) because LH self-stimulation and food, sugars in particular, appear to act on the same (or highly similar) neural reward system(s) (Coons and White, 1977; Ono et al., 1985). Assuming that common systems are involved, the present findings predict that a D_1 antagonist would block flavor preference conditioning by LH self-stimulation, and that both D_1 and D_2 antagonists would interfere with the expression of the flavor preference based on LH self-stimulation.

As noted in the introduction, flavor preferences can be reinforced not only by the sweet taste of sucrose, but also by its postingestive actions. Azzara et al. (2001)

investigated the role of dopamine receptors in postingestive nutrient conditioning by pairing the intake of CS+ and CS- flavors presented in saccharin solutions with IG infusions of sucrose and water, respectively. Separate groups of rats were treated with SCH23390, raclopride, or vehicle during the conditioning sessions. Both dopamine antagonists reduced intake during one-bottle training, but only the D_1 antagonist blocked the development of a CS+ preference as revealed in two-bottle flavor tests conducted in the absence of the drugs. This contrasts with the effectiveness of both the D_1 and D_2 antagonists to block flavor conditioning by orally-consumed fructose in the present experiment. Taken together, these findings indicate a differential involvement of DA receptors subtypes in the reinforcing actions of sweet taste and postingestive sugar reinforcement.

Other investigators have reported selective involvement of D_1 receptors in flavor aversion learning. In this case, water-restricted rats were trained to drink a sweet solution (saccharin or sucrose) which was followed by LiCl-induced toxicosis. SCH23390 applied systemically or microinjected into either the lateral hypothalamus or nucleus accumbens retarded the development of a sweet taste aversion (Caulliez et al., 1996; Fenu et al., 2001). In contrast, treatment with D_2 antagonists (raclopride or sulpiride) did not attenuate taste aversion learning. These findings along with those of Azzara et al. (2001) suggest that D_1 receptors are involved in learning about both positive and negative postingestive consequences. However, some data suggest that different processes my be involved in preference and aversion learning. That is, in the Azzara et al. (2001) and present studies, flavor preference conditioning was blocked by SCH23390 injected prior (15 or 30 min) to consumption of the CS solutions during

training. In contrast, Fenu et al. (2001) reported that SC23390 administered before (0 or 30 min) consumption of the CS solution was ineffective, and only SC23390 injections given 5 min after CS intake (but before LiCl treatment) blocked taste aversion learning. The preference and aversion conditioning procedures differed in many respects which may account for these discrepant results. Further work using common procedures is needed to elucidate D_1 receptor involvement in flavor preference and aversion learning.

The present experiments add to a growing literature implicating dopamine receptors in flavor learning. These findings indicate that both D_1 and D_2 antagonists retard the acquisition of flavor preference conditioning by the sweet taste of fructose. D_1 and, to a lesser extent, D_2 antagonists also attenuated the expression of a previously acquired flavor preference. The results are consistent with the idea that DA antagonists reduce the rewarding properties of sweet taste (Schneider, 1989; Smith, 1995) although they do not exclude other interpretations of DA function. Also, as mentioned above, different DA subsystems may be involved in flavor-flavor and flavor-nutrient learning, and possibly in flavor preference and flavor aversion learning. Berridge and Robinson (1998) have dichotomized food reward into hedonic ("liking") and incentive ("wanting") components. They proposed that DA is primarily involved in incentive aspects of food reward, whereas brain opioid systems mediate hedonic aspects of food reward. According to their model, DA is not critical for hedonic reward learning although this view is challenged by the findings that SCH23390 blocked flavor aversion learning as measured by intake and taste reactivity tests (Fenu et al., 2001). The present results also raise questions concerning the role of DA in hedonic reward learning since flavor-flavor conditioning is thought to involve hedonic processes (Breslin et al., 1990).

This issue requires further investigation because the two-bottle choice tests used in the present study do not necessarily distinguish between hedonic and incentive components of learned flavor preferences.

CHAPTER FOUR: OPIATE RECEPTOR ANTAGONISM AND FLAVOR PREFERENCES CONDITIONED BY FRUCTOSE

Introduction

Blockade of the endogenous opioid system with the general opioid antagonists naloxone and naltrexone potently reduces intake of palatable fluids, including sucrose and saccharin (e.g., Cooper, 1983; Levine et al., 1982; Lynch, 1986; Lynch and Libby, 1983; Siviy and Reid, 1983). Opioid antagonists appear to reduce the hedonic qualities of sweet substances because they: (a) suppress intake of sweet solutions more than plain water (Cooper, 1983; Le Magnen et al., 1980; Sclafani et al., 1982); (b) block that portion of feeding that appears driven by sweet taste in food restricted animals (Levine et al., 1995); (c) reduce sucrose's positive hedonic qualities in a taste-reactivity paradigm (Parker et al., 1992); and (d) reduce sucrose intake in sham-fed rats (Kirkham, 1990; Kirkham and Cooper, 1988a; Rockwood and Reid, 1982) in a manner behaviorally equivalent to reductions in palatability obtained by diluting the test solution (Kirkham and Cooper, 1988b). Mu- and kappa-, but not delta-selective opioid antagonists also reduce sucrose intake in both real-feeding (Beczkowska et al., 1992) and sham-feeding (Leventhal et al., 1995) tests. Consistent with these results, central infusions of opioid agonists increase the intake of a saccharin solution but not plain water (Zhang and Kelley, 2002), and consumption of sweet solutions increase brain β-endorphin levels more than does the consumption of plain water (Yamamoto et al., 2000).

The opioid system has also been implicated in flavor preference conditioning by sweet taste. In particular, Mehiel (1996) trained rats to drink different flavors mixed

into a preferred glucose solution and a less preferred saccharin solution and then measured their preferences for the flavors alone. Treatment with naloxone during the flavor+glucose training sessions prevented the rats from acquiring a preference for the glucose-paired flavor. However, these same rats were not treated with naloxone during the flavor+saccharin sessions, and thus they may have associated the glucose-paired flavor with mild aversive effects of the drug. More recently, Yu (Yu et al., 1999) trained rats with different flavors mixed into sucrose and saccharin solutions. One group was was treated with naltrexone (0.1 mg/kg BW) during both sucrose and saccharin training sessions, while the second group was treated with saline throughout training. Even though the naltrexone group consumed much less flavored sucrose during training than did the saline group, both groups displayed significant preferences for the sucrose-paired flavor over the saccharin paired flavor. Naltrexone treatment during two-bottle testing also had little or no effect on the expression of the sucrose-conditioned preferences (Yu et al., 1999). The rats in these experiments sham-fed the sucrose and saccharin solutions throughout training and testing so that the learned flavor preferences were attributed to the sweet taste rather than the postingestive nutritive actions of sucrose. In another study, Azzara (Azzara et al., 2000) trained rats with flavored saccharin solutions paired with intragastric infusions of sucrose or water. Significant preferences for the sucrose-paired flavor were observed in rats treated with naltrexone or saline throughout training. Thus, flavor conditioning by both the sweet taste and postingestive actions of sucrose was not blocked by the opioid antagonist.

In contrast to the failure of naltrexone to block flavor conditioning, other studies from our laboratory revealed that dopamine D1 and D2 receptor antagonism attenuated

the expression of sucrose-conditioned flavor preferences in sham-feeding rats (Yu et al., 2000a, 2000b). The first experiment conclusively demonstrated that D1 and D2 antagonists blocked both the acquisition and expression of flavor preferences conditioned by the sweet taste of fructose (Baker et al., 2003). In this study, saline and drug-treated rats drank matched amounts of flavored fructose and flavored saccharin solutions during one-bottle training sessions and were then given two-bottle choice tests with both flavors presented in saccharin solutions. The saline-treated Control group displayed a significant preference for the fructose-paired flavor in the two-bottle tests which confirmed prior results (Sclafani and Ackroff, 1994). On the other hand, groups treated with D1 or D2 antagonists failed to acquire a preference for the fructose-paired flavor. The rats "real-fed" the solutions during training and testing, and were thus exposed to the postingestive nutritive actions of fructose as well as its sweet taste. Nevertheless, the learned flavor preference displayed by the Control group was attributed specifically to the sweet taste of fructose. This interpretation was based on the results of other studies showing that intragastric fructose infusions, unlike glucose or sucrose infusions, do not condition flavor preferences in rats trained 30 min/day (Sclafani et al., 1999).

The dopamine drug effects obtained in the fructose conditioning study were more pronounced than those obtained in the sucrose conditioning studies; this may be due to procedural differences between the conditioning studies (e.g., real- vs. sham-feeding, matched vs. unmatched training intakes of sugar and saccharin solutions, 8% fructose vs. 16% sucrose sugar solutions, use of mixed sucrose/saccharin solutions vs. only saccharin solutions in two-bottle choice tests). These data suggest that the use of

fructose as the unconditioned stimulus produces a conditioned flavor preference that is more sensitive to the effects of pharmacological perturbation of the putative neurochemistry that mediates the learning and expression of this behavior. Therefore, the present experiment used the fructose conditioning method to further investigate the impact of naltrexone on flavor conditioning by sweet taste. This was of interest because the failure of opioid antagonism to block sugar-conditioned preferences seems inconsistent with the ability of general and selective opioid antagonists to attenuate the intake of sweet solutions per se. An additional feature of the present study was that naltrexone's effect on sweet taste conditioning was evaluated at doses of 0.1, 1.0 and 5.0 mg/kg body weight whereas only the 0.1 mg/kg dose was examined in our prior sucrose study. The combination of a more sensitive test method and expanded dose range was designed to increase the likelihood of obtaining a drug effect if, in fact, the opioid reward system is involved in flavor conditioning by sweet taste. This experiment has been accepted for publication in Pharmacology, Biochemistry and Behavior.

2. Methods

The subjects and test solutions are described in the General Methods Section.

Rats were initially trained (2 h/day) to drink the maltodextrin solution from calibrated bottles (100 ml, 1 ml gradations; Lab Products) while food and water restricted, and then while food was restricted with water available ad libitum except during the daily training sessions. The bottle was mounted on the front of the cage held by a spring, and was positioned so that the sipper spout entered the cage about 3-6 cm above the cage floor. This training procedure was repeated daily until all rats

approached the sipper spouts with short (<1 min) latency, typically within three days. The limited food rations were given after each training session.

Four groups of rats were given 8 consecutive daily one-bottle training sessions (2 h/day) with 24 ml of the CS+/F solution presented on odd-numbered days, and 24 ml of the CS-/S solution presented on even-numbered days. On days 5-8, the rats had access to two bottles, one containing the CS+/F or CS-/S solution, and the other containing water. This acclimated them to the presence of two bottles during the choice tests. Water intake was negligible in these training trials. The position of the CS and water bottles varied across days using a left-right-right-left pattern. Intakes were measured to the nearest 1 ml at 0.5 and 2 h during each session.

The rats in the first group (Control group, n=20) received a saline injection (1 ml normal saline/kg body weight, sc) 30 min prior to each of the one-bottle training trials. The general opioid antagonist, naltrexone (NTX: Sigma Chemical Co.) was administered subcutaneously 30 min prior to the one-bottle training trials to the three drug groups at doses of 0.1 (NTX 0.1 group, n=10), 1.0 (NTX 1.0 group, n=10) or 5.0 (NTX 5.0 group, n=10) mg/kg. Following training, all rats were treated with saline 30 min before being given two-bottle access (2 h/day) to unlimited amounts of CS+/S and CS-/S solutions on two consecutive days.

To determine the effect of naltrexone treatment on the expression of the CS+ preference, the control rats were given another 7 two-bottle test sessions following treatments with naltrexone or saline. Half of rats were injected with NTX at ascending doses of 0.1, 1.0, 2.5 and 5.0 mg/kg 30 min prior to the test sessions on the four-odd

numbered days. The remaining rats were treated with the NTX doses in a descending order. All rats were given saline injections on the three even-numbered days.

Mean intakes averaged over the four one-bottle training sessions with each CS were evaluated using a two-way analysis of variance for the Control and three NTX groups as a between-subject variable and the CS+/F and CS-/S conditions as a within-subject variable. Mean intakes during the two post-training CS+/S vs. CS-/S choice tests were similarly analyzed with groups and CS solutions as between and within factors, respectively. The effect of naltrexone on the expression of the CS preference was determined by analyzing the CS+/S vs. CS-/S intakes of the Control rats following saline or drug treatment. Saline data were based on the average of the five saline tests. Preliminary analysis revealed no differences between the control rats tested with ascending and descending doses and therefore only the combined data are presented. CS+ intakes during the two-bottle choice sessions were expressed as a percent of total intake, and these data were evaluated using analysis of variance.

Results

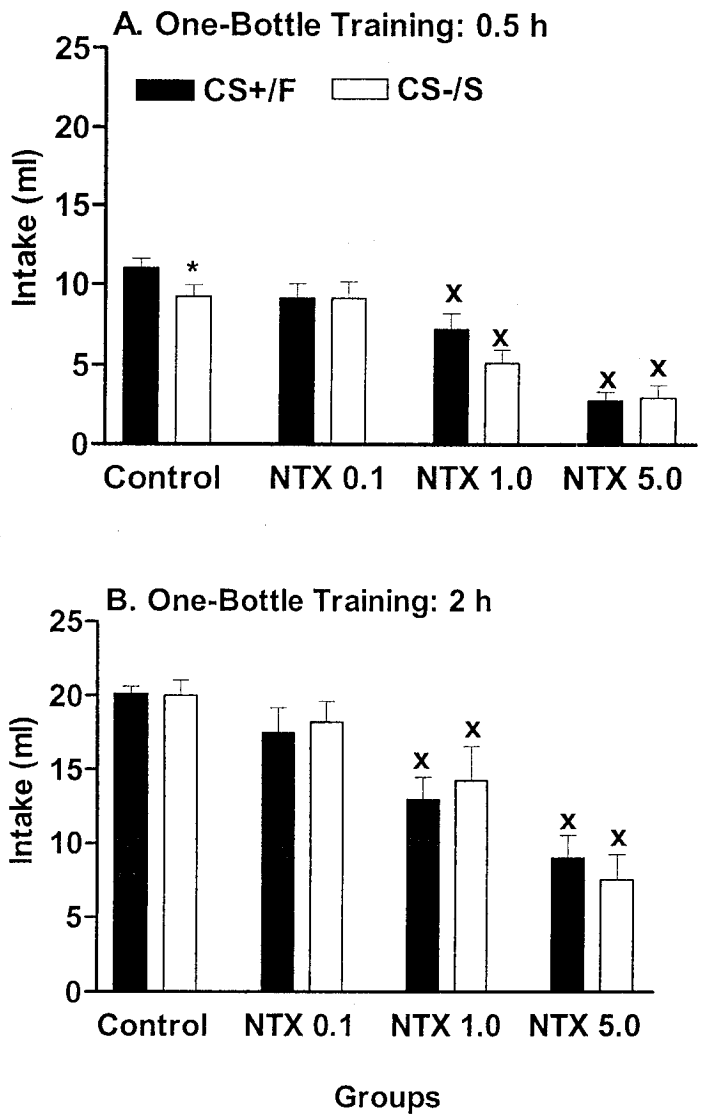
Naltrexone Effects on Training Intakes: Figure 6 presents the one-bottle training intakes of the CS+/F and CS-/S solutions of the four groups averaged over the four training days with each solution. Analysis of the 0.5 h data indicated that, overall, the groups differed in their CS intakes ($F(3,57)= 67.07$, $p<0.0001$), that CS+/F intakes were significantly higher than CS-/S intakes ($F(1,19)= 31.46$, $p<0.0001$), and that there was an interaction between groups and CS solutions ($F(3,57)= 6.32$, $p<0.001$). Individual comparisons revealed that the CS+/F and CS-/S intakes of the Control and NTX 0.1 groups were comparable, and significantly higher than those of the NTX 1 group which

Figure 6 (One-Bottle Training). Mean intakes (+SEM) during one-bottle training sessions of flavored 8% fructose solution (CS+/F) and flavored 0.2% saccharin solution (CS-/S) after 0.5 h (Panel A) and 2 h (Panel B). The Control group were injected with saline and the Ntx 0.1, Ntx 1.0 and Ntx 5.0 groups were injected with naltrexone doses of 0.1, 1 and 5 mg/kg, respectively, 30 min prior to the training sessions. One caveat about the presentation of this figure is that it takes scalar data on the abscissa and presents it as linear. This was done because bar graphs showing differences between CS+/S and CS-/S intakes were the best presentation for data comparison.

Notes: * denotes significant differences between CS+/F and CS-/S intakes within a given group (Tukey comparisons, $p<0.05$).

x denotes significant differences in either CS+/F intake or CS-/S intake relative to the corresponding vehicle control group (Tukey comparisons, $p<0.05$).

Figure 6.



were in turn significantly higher than those of the NTX 5 group. CS+/F intake exceeded CS-/S intake only in the Control group. Analysis of the 2 h data indicated that, overall, the groups continued to differ in their CS intakes ($F(3,57)= 57.18$, $p<0.0001$) and there was an interaction between groups and CS solutions ($F(3,57)= 2.83$, $p<0.05$), but there was no difference between CS+/F and CS-/S intakes. Individual comparisons showed that the CS+/F and CS-/S intakes of the Control and NTX 0.1 groups were comparable, and significantly higher than those of the NTX 1 group which were in turn higher than those of the NTX 5 group.

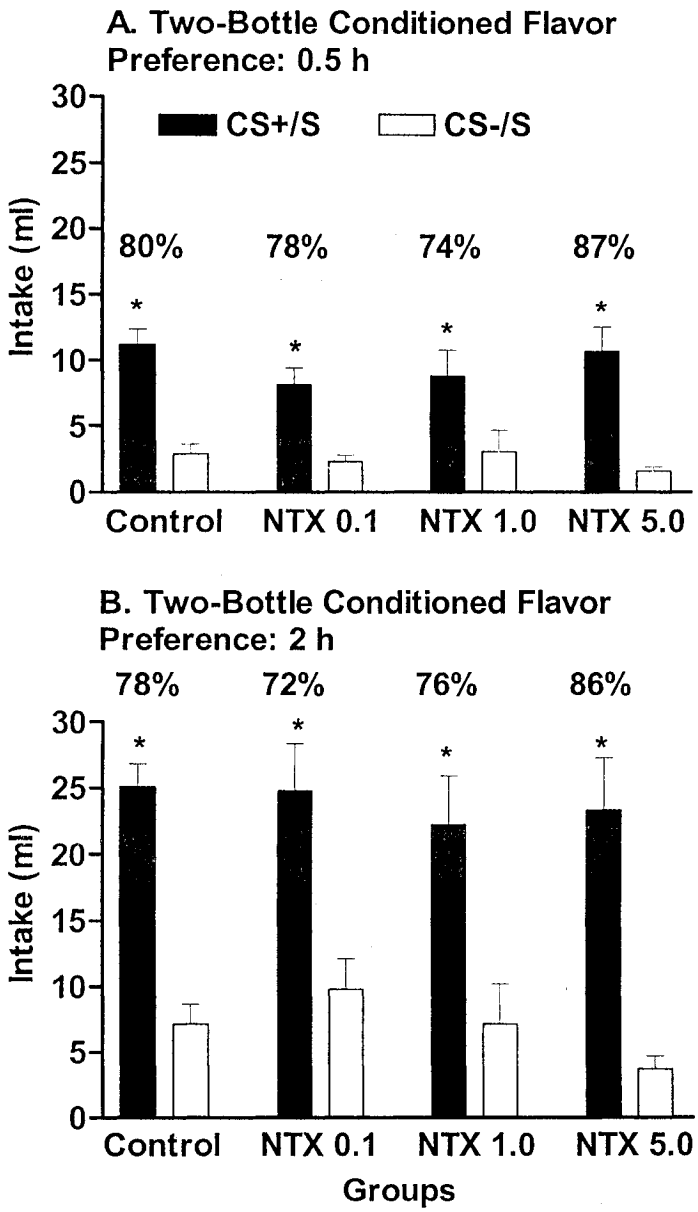
Naltrexone Effects on CS+ Preference Learning: The effect of the different drug treatments during training on preference conditioning was evaluated by comparing the two-bottle CS+/S vs. CS-/S intakes of the four groups following saline treatment (Figure 7). Analysis of the 0.5 and 2 h data indicated that, overall, the rats consumed more CS+/S than CS-/S (0.5 h: $F(1,46)= 46.45$, $p<0.0001$; 2 h: $F= 58.45$ $p<0.0001$), and there were no group differences or group by CS interactions. The four groups also did not significantly differ in their percent CS+ intakes (Figure 7). The percent CS+ intakes of the control, NTX 0.1, and NTX 1 groups ranged from 72 - 80% and were slightly higher in the NTX 5 group (87%). Thus, despite the dose-dependent reduction in overall CS+ and CS- intakes during one-bottle training, the 1.0 NTX and 5.0 NTX groups displayed fructose-conditioned preferences indistinguishable from those of the Control and 0.1 NTX groups.

Naltrexone Effects on Expression of CS+ Preference: The effects of naltrexone treatment on the expression of the CS+ flavor preference were evaluated in a series of two-bottle tests conducted with the control rats. These were extinction tests in that the

Figure 7 (Two-Bottle Acquisition of Preference). Mean intake (+SEM) after 0.5 h (Panel A) and 2 h (Panel B) of CS+/S vs. CS-/S solutions in the Control, Ntx 0.1, Ntx 1.0 and Ntx 5.0 groups during two-bottle preference tests. All groups were treated with saline prior to tests. The numbers atop the bars represent the percent of total intake consumed as CS+/S. One caveat about the presentation of this figure is that it takes scalar data on the abscissa and presents it as linear. This was done because bar graphs showing differences between CS+/S and CS-/S intakes were the best presentation for data comparison.

Notes: * denotes significant differences between CS+/S and CS-/S intakes within a given group (Tukey comparisons, $p<0.05$).

Figure 7.



CS+ flavor was no longer paired with the fructose solution. A preliminary analysis revealed that half of the control rats (n=10) displayed a robust CS+ preference that persisted from the first to the last two-bottle tests following saline treatment (90% to 83%). The remaining rats displayed a weaker CS+ preference in the initial two-bottle test and lost their preference by the last saline test (68% to 53%). Only the subset (n=10) of rats that showed a persistent conditioned flavor preference following saline treatment were included in the analysis of the drug effect on the expression of the CS+ preference.

As illustrated in Figure 8, overall the rats consumed more CS+ than CS- at the different naltrexone doses and the drug suppressed total CS intakes. Analysis of variance confirmed that there were CS main effects at both the 0.5 and 2 h time points (0.5 h: $F(1,9) = 60.467$ $p<.0001$; 2 h: $F(1,9) = 76.464$, $p<.0001$) as well as a main effect of naltrexone dose on total intakes (0.5 h: $F(4,36) = 4.546$ $p<.0001$; 2 h: ($F(4,36) = 9.944$, $p<.0001$). There were also significant CS x Dose interactions at both time points (0.5 h: $F(4,36) = 5.001$ $p<.001$; 2 h: ($F(4,36) = 3.984$, $p<.01$). This occurred because naltrexone reduced the intake ($p<.05$) of the CS+ but not the CS-. Nevertheless, CS+ intake exceeded ($p<.05$) CS- intake at all drug doses. During the first 0.5 h of testing, all doses of naltrexone suppressed ($p<.05$) total CS intake with respect to saline treatment, and intakes following the different doses did not differ from each other (Figure 9). All drug doses also suppressed CS intake at 2 h and, in addition, intakes were lower ($p<.05$) after the 5.0 mg/kg dose compared to the lower naltrexone doses. Percent CS+ intakes fluctuated following the various naltrexone doses (Figure 8), but

Figure 8 (Expression of Preference). Mean intake (+SEM) after 0.5 h and 2 h of CS+/S vs. CS-/S solutions during two-bottle tests following treatment with saline and naltrexone (0.1 - 5 mg/kg) in the ten control rats that displayed a robust CS+ preference that persisted from the first to the last two-bottle tests following saline treatment. Significant main effects for CS solution and drug dose were observed, whereas the CS x dose interaction was not significant. The numbers atop the bars represent the percent of total intake consumed as CS+/S. One caveat about the presentation of this figure is that it takes scalar data on the abscissa and presents it as linear. This was done because bar graphs showing differences between CS+/S and CS-/S intakes were the best presentation for data comparison.

Notes: * denotes significant differences between CS+/S and CS-/S intakes within a given group (Tukey comparisons, $p<0.05$).

Figure 8.

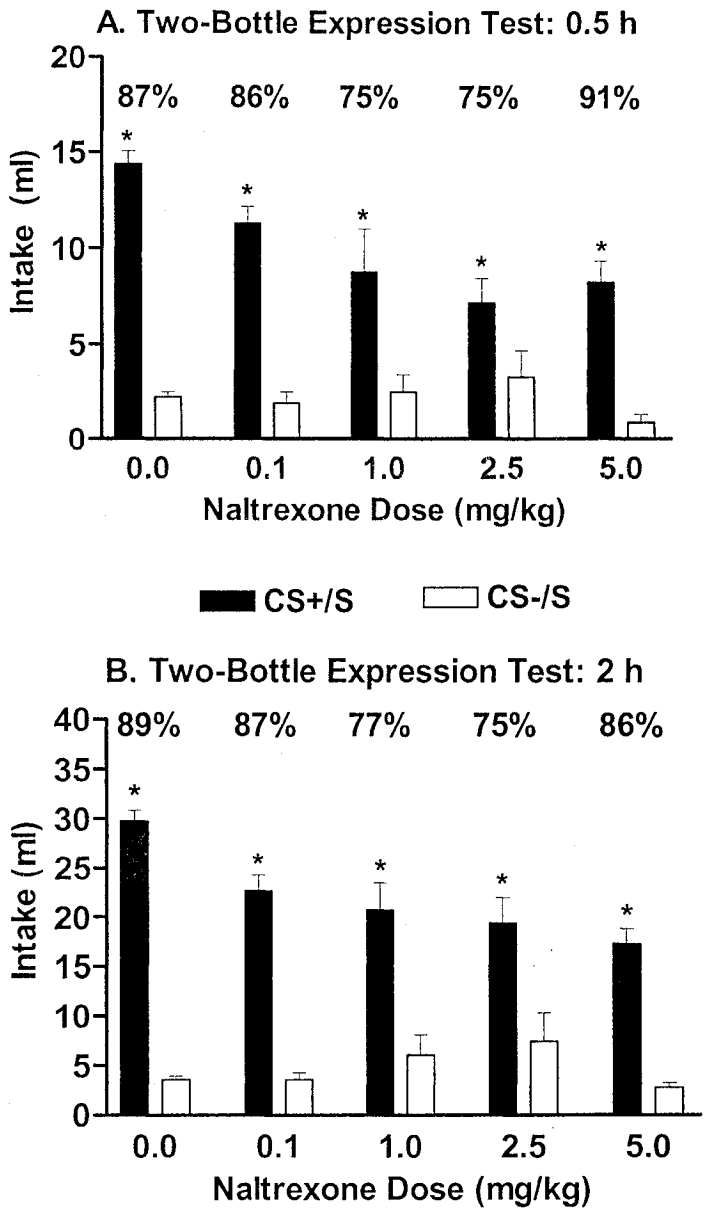
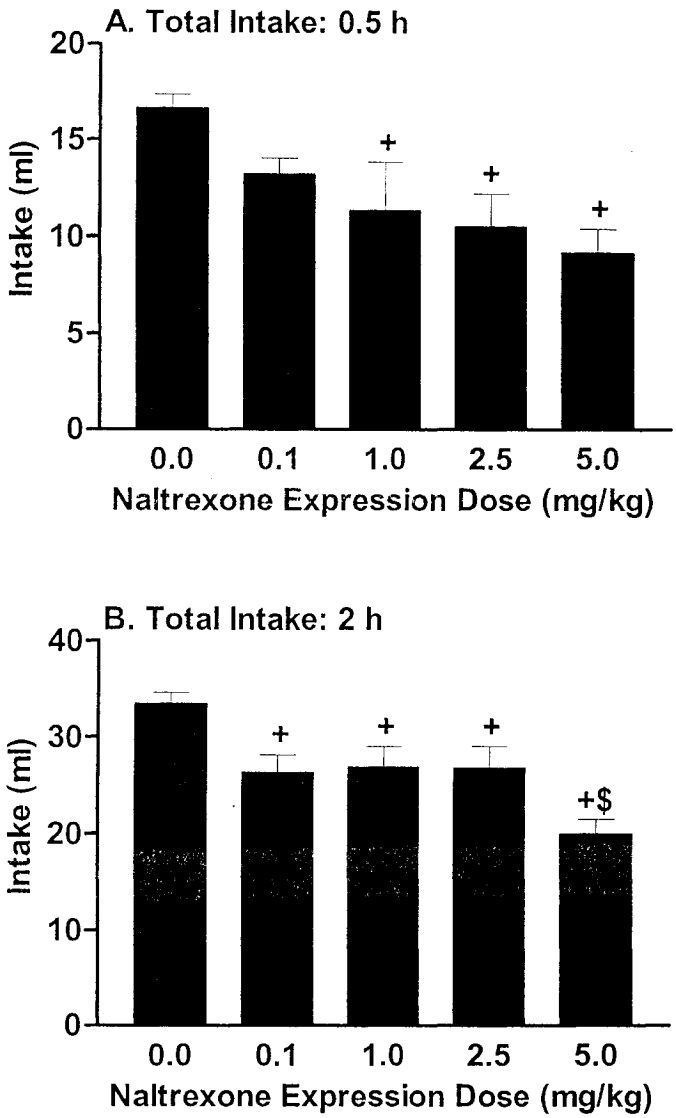


Figure 9 (Total Intake). Mean total intake (+SEM) of CS+/S and CS-/S after 0.5 h (Panel A) and 2 h (Panel B) during two-bottle tests following treatment with saline (0 mg/kg) or naltrexone (0.1-5 mg/kg) in the Control group. Asterisks denote significant intake differences between saline and all drug doses; crosses denote significant differences between 5 mg/kg dose and lower doses (Tukey comparisons, $p<0.05$). One caveat about the presentation of this figure is that it takes scalar data on the abscissa and presents it as linear. This was done because bar graphs showing differences between CS+/S and CS-/S intakes were the best presentation for data comparison.

Notes: + denotes significant antagonist dose effects for a particular intake condition relative to its corresponding vehicle control (Tukey comparisons, $p<0.05$).

$ denotes significant antagonist dose effects relative to other lower doses of the antagonist (Tukey comparisons, $p<0.05$).

Figure 9.



there were no significant effects of naltrexone dose on this measure of CS+ preference at the 0.5 or 2 h time points. Doses also suppressed CS intake at 2 h and, in addition, intakes were lower ($p<.05$) after the 5.0 mg/kg dose compared to the lower doses. Percent CS+ intakes fluctuated following the various naltrexone doses (Figure 8), but there were no significant effects of naltrexone dose on this measure of CS+ preference at the 0.5 or 2 h time points.

Discussion

The sweet taste of sugar is a potent reward for rats and many other species and there is a considerable evidence that the "sweet tooth" is mediated in part by the brain opioid system. This was first suggested by reports that naloxone and naltrexone suppressed the intake of sugar and saccharin solutions in rats (Le Magnen et al., 1980; Sclafani et al., 1982), which is confirmed by the present results. Naltrexone reduced the intakes of the flavored fructose and saccharin solutions throughout training at 1 and 5 mg/kg doses, and reduced flavored saccharin intakes during two-bottle testing at doses of 0.1 to 5 mg/kg. As briefly reviewed in the Introduction and in greater detail elsewhere (Kelley et al., 2002; Levine et al., 2003; Yamamoto, 2003;Yeomans and Gray, 2002), a variety of findings indicate that the opioid system is specifically involved in the hedonic evaluation of sweet tasting foods and fluids.

Sweet tastants, in addition to being primary rewards that elicit avid ingestive responses, can also condition preferences for associated flavors, i.e., they can condition secondary rewards. This was first demonstrated by Holman (1975) who trained rats with distinctively flavored sweet (0.32%) and less sweet (0.065%) saccharin solutions. In subsequent choice tests with both flavors presented at the same saccharin

concentration, the rats preferred the flavor that had been paired with the sweeter solution. Other studies have conditioned flavor preferences with the sweet taste of sucrose (Breslin et al., 1990; Myers and Hall, 2000; Yu et al., 1999). These later studies minimized the postingestive actions of sucrose as a conditioning factor by limiting the amount of sucrose consumed during training (Breslin et al., 1990; Myers and Hall, 2000) or by using a gastric sham-feeding procedure during training and testing (Yu et al., 1999). The present study used fructose rather than sucrose as the sweet reward because fructose, unlike other sugars, has little or no postingestive reinforcing action during short-term training sessions (Sclafani and Ackroff, 1994; Sclafani et al., 1999). In confirmation of prior reports, the control rats displayed a significant preference for the fructose-paired flavor (CS+) over the saccharin-paired flavor (CS-) when both were presented in saccharin solutions (Sclafani and Ackroff, 1994; Baker et al., 2003). This CS+ preference is attributed to rats associating the CS+ flavor with the sweet taste of the fructose solution consumed during training.

Like controls, the rats in the three naltrexone groups displayed significant preferences for the fructose-paired CS+ flavor even though the drug, at the 1 and 5 mg/kg doses, suppressed the intake of the flavored fructose and saccharin solutions during training. Furthermore, pretreating the control rats with naltrexone (0.1 - 5.0 mg/kg) did not the block their expression of the CS+ preference during the two-bottle choice tests. That is, although naltrexone selectively reduced the intake of the CS+ during the preference tests, the rats continued to consume more CS+ than CS- at all dose levels. The percent CS+ preference was lower (75-77%) at some naltrexone doses, compared to the saline baseline (87-89%), but these differences did not achieve

statistical significance. These findings confirm and extend the prior report of Yu (Yu et al., 1999) that a 0.1 mg/kg naltrexone dose did not prevent the establishment of a flavor preference conditioned by the sweet taste of sucrose in sham-feeding rats nor did the drug (0.1 - 5 mg/kg) block the expression of the sucrose-conditioned flavor preference when administered before the two-bottle tests.

The present results contrast with the report of Mehiel (1996) that naloxone (4 mg/kg) blocked the development of a preference for a flavor mixed into a glucose solution over a flavor paired with a less preferred saccharin solution. As noted in the Introduction, however, the rats in their experimental group were treated with naloxone only on CS+/sucrose training days and their lack of preference for the CS+ flavor may be related in part to an association between the CS+ flavor and potential aversive effects of the drug. Naltrexone (0.5 mg/kg) was also reported to block a sucrose-induced preference for a orange odor in 6-day old rat pups as measured in a modified place preference paradigm (Shide and Blass, 1991). There was no CS- odor in this study and the possibility that an odor-drug association interfered with an odor-sucrose association can not be ruled out. In adult rats, naltrexone (0.1 - 5.0 mg/kg) did not prevent the development of a sucrose-conditioned place preference when the drug was administered on training trials with the sucrose-paired place and water-paired place, but did attenuate the expression of an already formed place preference (Delamater et al., 2000). Another recent study reported that chronic naltrexone treatment inhibited the redevelopment of a sucrose-diet preference in animals offered the choice of a sucrose-diet vs. starch-diet but had little effect on the expression of an established preference (Levine et al., 2002). This latter study did not investigate flavor preferences conditioned by sweet taste,

therefore the results, while interesting from a "diet" relapse perspective, are not relevant to the present conditioning data.

The failure of naltrexone to block sucrose or fructose conditioned flavor preferences appears to be inconsistent with the ideas that (a) opioid antagonists suppress the hedonic evaluation of sweet taste and/or that (b) sweet taste reinforces flavor preferences through a hedonic conditioning process. These apparent inconsistencies are discussed below.

While the opioid modulation of taste hedonics is well supported, other neurochemical systems are implicated in the hedonic response to sweet and other palatable tastants. In particular, several studies indicate that benzodiazepine receptors participate in the palatability evaluation of foods and fluids (see reviews: Berridge and Peciña, 1995; Cooper and Higgs, 1996). This may explain why opioid antagonists suppress, but typically do not completely block the consumption of sweet solutions. A related point is that opioid antagonists typically do not reduce the initial response to sweet rewards during the first minutes of testing in experiments involving ingestive, operant, or taste reactivity measures (Beczkowska et al., 1992; Ferraro et al., 2002; Frisina and Sclafani, 2002; Kirkham and Cooper, 1988a, b; Leventhal et al., 1995; Schwarz-Stevens et al., 1992; Higgs and Cooper, 1998). These results suggest that the opioid system is not involved in all aspects of taste hedonics, but may be primarily involved in the response-sustaining effect of palatable foods and fluids. Thus, naltrexone may fail to prevent flavor conditioning by sugar solutions because the drug has little effect on the animal's hedonic evaluation of the sugar and saccharin solutions early in the training sessions, which may be the basis for the conditioned flavor

preference. Consistent with this view, intraoral infusion studies indicate that flavor preferences can be conditioned by small amounts of a sugar solution (Myers and Hall, 2000; Breslin et al., 1990). This interpretation may also explain why naltrexone has relatively little effect on the expression of the conditioned CS+ preference. That is, the rats' choice of the CS+ over the CS- in the two-bottle tests may be determined by their evaluation of the flavors early in the test session while their hedonic evaluation was unaffected by naltrexone.

The role of hedonics in flavor conditioning also requires consideration. According to Berridge (1996), food reward involves two separate processes: a hedonic component and an incentive salience component. The hedonic value of food is inferred by taste reactivity tests which measure orofacial responses evoked by intraoral infusions of tastants whereas the incentive value is inferred by instrumental approach responses to foods and fluids (Berridge, 1996). Berridge (1996) further proposes that opioids are primarily involved in the hedonic process while dopamine systems are primarily involved in the incentive process. Manipulations that influence food reward typically impact on both food hedonics and incentive, but in some situations only one or the other process may be affected. With respect to flavor conditioning, it is possible that conditioned preferences reflect increased incentive value in addition to, or instead of increased hedonic value. Support for this view is provided by taste reactivity (TR) analysis of flavor preferences conditioning by IG sugar infusions. In one study, rats were trained with flavored saccharin solutions as conditioned stimuli and they displayed a strong CS+ preference in two-bottle tests and an increased hedonic response to the CS+ in TR tests compared to the CS- (Myers and Sclafani, 2001). In a second study

with bitter and sour CS solutions, rats also showed a strong CS+ preference in the two-bottle test, yet their TR responses to the CS+ and CS- did not differ (Myers and Sclafani, 2002). These data indicate that the conditioning of a strong flavor preference does not necessarily require a shift in the hedonic evaluation of the flavor as measured by TR responses. Whether a similar situation exists in the case of flavor preferences conditioned by sweet taste is not certain. Pairing a bitter or sour CS+ with intraoral infusions of sucrose has been reported to increase the hedonic TR response to the CS+ (Breslin et al., 1990). It is not known, however, whether this hedonic shift is necessary for the establishment or expression of a sweet taste-conditioned CS+ preference. It is possible that treating rats with naltrexone during training with flavored fructose and saccharin solutions may have blocked the conditioning of a hedonic response to the CS+ flavor, yet the rats may have still preferred the CS+ in two-bottle tests because of a conditioned incentive response to the CS+. This interpretation can be tested by comparing the effects of naltrexone on CS+ preference and TR responses.

In contrast to the minimal effect of naltrexone on the acquisition and expression of the fructose-conditioned flavor preference observed here, we recently reported that D1 (SCH23390) and D2 (raclopride) antagonists completely blocked both CS+ preference acquisition and expression of fructose-conditioned flavor preferences (Baker et al., 2003). The same dopamine antagonists interfered with sucrose-conditioned flavor preferences in sham-feeding rats which are unaffected by naltrexone (Yu et al., 1999, 2000a, 2000b). Together, these data indicate that flavor conditioning by the sweet taste of sugar is modulated by dopamine but not opioid receptors. Other findings suggest a similar receptor pharmacology of flavor preference learning produced by the

postingestive actions of sugar. That is, dopamine antagonism, but not opioid antagonism prevented rats from developing a preference for a CS+ flavor paired with IG infusions of sucrose (Azzara et al., 2000, 2001). In this case, however, only the D1 antagonist blocked preference learning. According to the model of Berridge (1996), these drug actions suggest that conditioned flavor preferences, at least as measured by two-bottle tests, are modulated by a dopamine incentive salience system rather than by an opioid-based hedonic system. This interpretation, as well as the role of other neurotransmitter systems in flavor learning, requires further investigation.

CHAPTER FIVE: GENERAL DISCUSSION

The ability to produce pronounced conditioned flavor preferences in these experiments using fructose as the unconditioned stimulus and the more preferred solution and saccharin as the less-preferred solution in real-feeding rats was consistent with previously-described findings (e.g., Sclafani and Ackroff, 1994) of acquired preferences for a sugar-paired flavor over a saccharin-paired flavor. The present study conclusively demonstrated that the D-1 receptor antagonist, SCH23390 interfered with both the acquisition and expression of flavor preferences conditioned by fructose. The "non-specific" explanation that SCH23390-treated animals during training consumed less CS+/F and CS-/S solutions and failed to exhibit preferences due to less exposure to the two flavored solutions was not supported by our observation that vehicle-treated rats matched for CS+/F and CS-/S intake (the D-1-yoked group) displayed significant flavor preferences. The D-2 receptor antagonist, raclopride also interfered with the acquisition and expression of flavor preferences conditioned by fructose, though to a lesser degree than that produced by D-1 receptor antagonism. Additionally, raclopride also reduced the acquisition of fructose flavor preferences when animals were exposed to both CS+/F and CS-/S solutions during the same training trial. Again these effects were specific since the D-2-yoked group treated with vehicle displayed normal fructose flavor preferences. These data extend and support the ability of D-1 and D-2 antagonists to reduce the expression of sucrose-conditioned flavor preferences in sham-feeding rats (Yu et al., 2000x), and the ability of D-1 antagonists to reduce the acquisition of flavor-nutrient conditioned preferences by sucrose in intragastric feeding preparations (Azzara et al., 2000). The present study also replicated the inability of opioid antagonism with

naltrexone to interfere with either the learning or expression of a preference for a sugar-paired flavor over a saccharin-paired flavor, supporting previous findings using different approaches (Yu et al., 1999; Azzara et al., 2000). The implications of these findings are discussed in turn below.

Fructose as an Unconditioned Stimulus: By the use of fructose as the unconditioned stimulus in these experiments, as well as restricting the total intake of the solution with the US-paired flavor, it was possible to demonstrate that the preference for the US-paired flavor was not due to the relative unfamiliarity of the rats with the CS-paired flavor as a result of a disproportionate consumption of that flavored solution. This was in contrast to the original studies in sham-feeding rats in which sucrose was consumed at a 3-4-fold greater rate than saccharin in both restricted and unrestricted animals (Yu et al., 1999; 2000a, 2000b). Indeed, when both CS+/F and CS-/S solutions were provided to animals on odd-day training days and CS-/S solutions were provided to animals on even-day training days (Study 1, Experiment 2), overall training intakes of CS-/S solutions actually exceeded overall training intakes of CS+/F solutions. Yet the CS+/S preference was as pronounced, and the D-2 antagonist, raclopride produced the same degree of interference with acquisition of the preference. These important controls highlight the strength of the flavor paired with the unconditioned stimulus and preferred solution, and not necessarily the amount of the exposure per se. This finding has relevance for further studies potentially employing intracerebral microinjection of the antagonists into specific brain structures putatively involved in mediating these forms of learning. By necessity, one would have to reduce the number of training days

to accomplish these acquisition studies, and our findings suggest that such an approach will work.

Summary of Dopamine Antagonist Efficacy: The effect of dopamine antagonism at the D1 and D2 receptor subtypes was to prevent the acquisition of a preference for the flavor that had been paired with an 8% fructose solution over the flavor that had been paired with a 0.2 % saccharin solution. In an earlier study (Baker et al, 2001) in which the same doses of the D1 (SCH23390) and D2 (Raclopride) antagonists were administered to determine whether dopamine antagonism would reduce the intake of a highly palatable diet in rats, the absence of any effect provides evidence that reductions observed in the present study do not reflect motor impairment. As described in detail earlier, the failure to acquire a preference by the rats that were pretreated with 200 nmol/kg of either the D1 antagonist SCH23390 or the D2 antagonist Raclopride could not be explained by the reduced intake of the CS+/F solution because yoked-control groups that were limited to the intake of the D1 and D2 groups did demonstrate a preference comparable to the group of rats treated with a saline solution prior to training. These results differ from those of the sham-feeding studies conducted by Yu et al (2000a,b) in which neither SCH23390 or Raclopride at the same dose interfered with the acquisition of a US-paired flavor preference. However, in the study by Azzara et al (2001), pretreatment with SCH23390, but not Raclopride, did prevent the acquisition of a preference for a flavor paired with an intragastric infusion of sucrose over a flavor paired with an intragastric infusion of water. The expression of an already established flavor preference in the vehicle trained rats was blocked at all doses of SCH23390. The elimination of the preference resulted from reduced consumption of

CS+/S solution, but not the CS-/S solution, during two-bottle testing. Raclopride only reduced the intake of CS+/S sufficiently at 200 nmol/kg to eliminate the established preference. Although it did not eliminate the preference at the other 3 dosage levels, the preference was attenuated. Thus SCH23390 and Raclopride had greater and weaker effects, respectively, on two-bottle intakes and established preferences for fructose in real-feeding rats than were demonstrated in our previous studies that had used sucrose as the US in sham-feeding rats.

The differences in the drug effect on preferences in the present study as compared to the previous studies that used sucrose as the US may be due to the fact that those studies used a test solution that was a mixture of sucrose and saccharin, which is more palatable than the saccharin solution used in this group of studies. We demonstrated that if the rats were trained with both the CS+/F and CS-/S solutions available, although they would not acquire a preference for the CS+/S solution over the CS-/S in two-bottle testing, they would consume more of the CS+/F than CS-/S during training, suggesting that the innately reinforcing hedonics are mediated separately from the conditioned association with the cue flavor. There are other possible explanations for the differences in the effect on the acquisition of a conditioned flavor preference between this study and that of Yu et al (2000a,b), such as the use of free-feeding rats in this study versus sham-feeding rats in that study, which might have affected the developing preference in some as yet unappreciated manner. It may also be that because fructose is less preferred than sucrose (Ackroff and Sclafani, 1993, 2001), the general effects of any type of antagonism might disrupt the learning of a flavor preference, and that therefore the failure to acquire a CFP by the rats pre-treated with dopamine prior to

training was not specific to dopamine. However, if that were true, it would be expected that antagonism of the opioid system with naltrexone would have effects on the flavor preference conditioned by fructose similar to the disruptions observed with dopamine antagonism. As summarized below, this was not the case.

Summary of Lack of Opioid Antagonist Efficacy: Hedonics play a major role in explaining opioid modulation of ingestive behavior since opioid agonists (e.g., Clarke & Parker, 1995; Doyle et al, 1993; Parker et al, 1992; Pecina & Berridge, 1995) and antagonists (e.g., Glass et al, 1996; Levine et al, 1995; Parker et al, 1992; Shabir & Kirk, 1999) respectively increase or decrease palatable intake in a manner consistent with their effects on measures of taste reactivity. In the literature, the inhibitory effects of naloxone and naltrexone became most apparent in studies examining their effects upon palatable solutions and diets, with both antagonists decreasing intake of sucrose and saccharin solutions as well as high-fat or high-sugar diets (e.g., Apfelbaum & Mandenhoff, 1981; Cooper et al, 1985a,b; Lynch & Libby, 1983; Mandenhoff et al, 1987; Sclafani et al, 1982). Correspondingly, saccharin intake was also reduced in opioid-deficient CXBK mice (Yirmaya, 1988). This inhibition was more potent for palatable intake than for normal diets and water in rodents (Levine et al, 1982) and in humans (Fantino & Brinnel, 1986). Although naloxone blocked saccharin intake in one-bottle tests, this demonstrated an interference with saccharin acceptance rather than any underlying preference. Naloxone reduced sucrose intake without affecting eating latency, suggesting effects upon the maintenance, rather than the initiation of intake (Kirkham & Blundell, 1984, 1986). Therefore, one reason as to why general opioid antagonists fail to alter conditioned preferences to either sucrose or fructose is that their

effects appear to be delayed, thereby allowing for the possibility that the pairing of the flavor (CS) and sugar (UCS) will take place. One possible study to address this possibility is to present animals with unflavored fructose and saccharin solutions during the first 10-15 minutes of exposure, times where naltrexone is presumably ineffective, and then presenting thereafter the two flavors paired with fructose and sacchrin respectively.

The hedonic and orosensory characteristics of the sucrose solutions were postulated as those aspects of palatable intake at which opioid antagonists produced their effects in part because naloxone continued to inhibit sucrose intake in vagotomized rats (Clarkson et al, 1982), suggesting that antagonism of hedonic rather than vagally-mediated post-ingestive factors were producing naloxone's inhibitory effects. Thus, general opioid antagonists significantly and stereospecifically reduced sucrose intake by shifting the sucrose concentration threshold to the right in sham-fed rats, a procedure that minimizes the influence of gastric and intestinal factors that normally lead to satiation (Kirkham & Cooper, 1988a,b; Rockwood & Reid, 1982). In contrast, other laboratories suggested that naloxone acted to modify nutrient selection by selectively reducing fat intake (Marks-Kaufman and Kanerek, 1981), or by reducing intake of the preferred macronutrient (Gosnell et al., 1986).

Conditioned flavor preferences in animals are observed for novel flavors paired with sucrose relative to novel flavors paired with saccharin (flavor-flavor conditioning). Naloxone disrupted the acquisition and expression of a preference for a CS+ flavor added to the nutrient solution of glucose (Mehiel, 1996). Although naltrexone was capable of reducing intake of both flavored solutions, it failed to alter either the

acquisition or expression of flavor-flavor conditioning in sham-feeding rats, indicating that consumption of the nutrient may be an important factor in producing naltrexone's effects (Yu et al, 1999). In examining conditioning of post-ingestive effects of sucrose consumption, naloxone interfered with acceptance of flavors conditioned by intragastric carbohydrate infusions (Ramiriz, 1997). However, using preference measures in flavor-nutrient conditioning, naltrexone failed to alter either the acquisition or expression of flavor preferences conditioned by intragastric sucrose (Azzara, 2000). In contrast, animals displaying a place preference for a compartment previously paired with sucrose display dose-dependent reductions in the expression, but not the acquisition of this preference following naltrexone pretreatment (Delamater et al, 2000). Although naloxone can reduce the acquisition of a conditioned place preference for sucrose, effects occur at doses that also produce a conditioned place aversion (Agmo et al, 1995).Because of the well documented effect of opioid agonists to increase the intake of sugar solutions, and of opioid antagonists to reduce the intake of those solutions in a manner that is behaviorally equivalent to reducing the concentration of sugars in the solutions, the opioid neurotransmitter system has been a prime candidate for the mediation of conditioned flavor preferences.

However, most of the subsequent research, with a few exceptions (e.g. Mehiel, 1996), have failed to disrupt either the learning or expression of conditioned flavor preferences using opioid antagonists. In short, there has been no variability in the outcomes in our laboratory regarding the effects of opioid blockade on either the acquisition or the expression of conditioned flavor preferences. Whether the US is sucrose or fructose, whether the intakes of CS+ and CS- are unequal or equivalent, and

whether the rats acquire their flavor preference in a free-feeding paradigm or through sham-feeding or gastric infusion, despite the significant effect that naloxone has with respect to the reduction of intake of CS+ and CS- solutions per se relative to controls, opioid blockade has no distinguishable effect on any aspect of conditioned flavor preferences.

Preference Studies and their relevance to Reward Models: Since the serendipitous discovery of the nucleus accumbens septi to support intracranial self-stimulation by Olds and Milner (1954) 50 years ago there has been extensive research to identify the neurochemistry of reward, as well as the relationship between reward and motivation. Reward has frequently been characterized as being synonymous with pleasure, thereby attributing the reinforcement potency of a stimulus to its intrinsic hedonic value. This concept of motivation was described 300 years ago by the British Empiricists Thomas Hobbes and John Locke (Hergenhahn, 1997), who suggested that the basis of all learning is the hedonistic pursuit of pleasure, and is still believed to be mechanism that underlies the development of a conditioned preference (Wise and Rompre, 1986). The involvement of mesolimbic and neostriatal projections have been identified as a neural substrate that is involved in reward behavior by electrophysiological and microdialysis techniques that quantify both increased activity in these systems and increased release of dopamine when animals engage in activities known to be pleasurable such as eating or engaging in sexual intercourse (Martel and Fantino, 1996). Other studies have demonstrated that dopamine blockade reduces reward-directed consumatory behavior (Wise and Rompre, 1986). This concept is consistent with the Anhedonia Hypothesis, which postulates that brain dopamine

systems mediate the pleasure produced by food, sexual intercourse and other unconditioned incentives (Wise, 1982). Wise suggested that, "the dopamine junctions represent a synaptic way station where sensory inputs are translated (in the nucleus accumbens) into the hedonic messages that we experience as pleasure".

However, more recently, some researchers (e.g. Berridge and Robinson, 1998) have suggested that reward is not a unitary process, but rather, "a constellation of multiple processes, many of which can be separately identified in behavior, especially after the component processes are dissociated by brain manipulations". Evidence of this would be the finding that once nutrient-conditioned flavor preferences are established they are very resistant to extinction, persisting during repeated testing in absence of the reinforcer (Myers and Sclafani, 2003). However, alternative explanations as to the role of dopamine in reward began to emerge with the realization that the dopamine function in reward often seems to be more involved with the anticipatory and approach phases of motivated behavior than with the consummatory phase, when hedonic activation is maximal (Berridge and Robinson, 1998). The Anhedonia Hypothesis predicts that dopamine systems should be maximally aroused during the period of maximal pleasure, but studies using microdialysis find that the dopamine systems are often activated prior to the meal to the same extent or even to a greater extent than during food consumption (Martel and Fantino, 1996). Therefore some behavioral neuroscientists have suggested that the aspect of reward mediated by dopamine systems is learning associations that predict a hedonic event, or, in the words of Berridge and Robinson (1998), "the attribution of salience to otherwise neutral events". This would suggest that the motivation to consume a food may be something

that has been learned as a result of the nutritive consequences associated with having consumed that food in the past, yet the actual innate pleasure derived from consumption may be mediated by a neural substrate that is distinct from, and can be dissociated from the neural substrate mediating that pleasure. Those researchers proposed that there is a distinction between "wanting" and "liking", with wanting being an innate response, and liking being learned. This interpretation of motivation implies that the development of a conditioned preference does not necessarily require or produce an increase in the hedonic value of that stimulus, but rather an increased "wanting" of it due to the learned association with the unconditioned stimulus that is "liked". This possibility is supported by a study (Myers and Sclafani, 2003) that found that conditioned flavor preferences, as demonstrated by both two-bottle choice testing and one-bottle acceptance testing, did not seem to alter the hedonics of the flavor when evaluated before and after training by taste reactivity testing. The animals "wanted" the conditioned flavor more than they had prior to training that involved pairing one flavor with an intragatric infusion of glucose and another flavor with an intragastric infusion of water. What made this study particularly compelling was that the two different flavors that were paired with the infusions were both inherently avoided nonsweet flavors used, which enabled the taste reactivity responses to demonstrate that the animals did not "like" the flavors any more after they developed a demonstrated preference for them than they did prior to training.

The results of the experiments conducted in the present study are consistent with the conception of reward consisting of two components: A hedonic component involving the consummatory phase of a necessary biological behavior such as eating

that is mediated by the opioid system, while the association of relevant cues that signal the availability of food being mediated by the dopamine system. This would explain why opioid antagonism reduces intake, because the pleasure driving that behavior has been reduced, but not the ability to make the association between the unconditioned stimulus (the sweet taste of fructose) and the cue flavor. This distinction between the role of the opioids and the role of dopamine is even more clearly demonstrated in the second experiment with dopamine antagonism that utilized two-bottle training. The rats that were pretreated with raclopride had access to both CS+/F and the CS-S bottles simultaneously during training, yet they did not demonstrate a preference when tested with both flavors available in a saccharin solution. This would make sense if the opioid system, which was not perturbed, was driving consumption during training, causing them to drink more of the UCS solution, but because of interference with the dopamine system, they never learned to make the association between the cue flavor paired with fructose. Our data would thereby support the concept that the incentive salience, or "wanting" component, is mediated by dopamine (among other neurotransmitters), and that the hedonic, or "liking" component, is mediated by the endogenous opioids (among other neurotransmitters). This conceptual approach as well as other issues is the rationale for proposed further studies as detailed in the next section.

Future Directions: The further understanding of the mechanisms involved in the acquisition and expression of conditioned flavor preferences has benefited from the use of many different types of carbohydrates (i.e. various sugars such as sucrose, glucose and fructose, and complex carbohydrates such as polycose and galactose) in comparison preference studies (Ackroff et al, 1993; Sclafani and Mann, 1987).

Therefore, one line of research is to continue to determine whether dopamine continues to produce a primary role in preferences caused by all carbohydrates (e.g., polycose, maltose), and not just those considered as sweet (e.g., sucrose, fructose). A second obvious candidate is the development of preferences for particular types of fats (e.g. saturated and unsaturated). It would be important to discover whether dopamine will have interfere with flavor preferences conditioned to both the taste and post-ingestive consequences of lipids. Therefore, one could test the hypothesis that dopamine is acting in a macronutrient-specific manner, or rather is acting on all palatable substances in the same manner. As indicated earlier, fat intake per se (Apfelbaum and Mandenhoff, 1981;Marks-Kaufman and Kanerek, 1981) or preferences for fat (Gosnell et al, 1986) are major macronutrients upon which the opioid system exerts effects. If this concept is correct, then one would begin to expect reductions in preferences conditioned by the taste and/or post-ingestive consequences of fat by general and selective opioid antagonism. Another type of study of opioid antagonism that would be of interest, as previously described in the summary of opioid effects, would be to allow the animals to consume some unflavored solution for 10 – 15 minutes before making the flavored solutions available since the effect of opioid antagonism does not have any appreciable effect on the initial phase of consumption. It would also be important to do central administration of specific opioid receptor subtype antagonists (e.g. mu, kappa and delta) to rule out the possibility that the action of one of these may negate the action of one of the others. Studies using sham-feeding, intragastric infusions and real-feeding animals would address this issue. Very little work has been performed evaluating whether flavors associated with proteins (e.g. animal and plant) condition preferences. If they

do, the same logic would prevail for dopamine and opioid analysis of any observed effect.

The rationale provided a coherent series of reasons for studying dopamine and opioids as candidates for these conditioning effects. However, by doing so, we do not subscribe to the belief that they are the ONLY candidates to produce such effects. This latter concept would fly in the face of emerging genetic evidence linking a wide array of genes to the mediation of such important effects. Therefore, learning the possible role in the conditioning of flavor preferences by using antagonists of such "orexigenic" transmitter systems, including NPY, cannabinoids and benzodiazipines, and by using agonists of such "anorectic" transmitter systems, including CCK-8 and 5HT would contribute to a more complete understanding of the neural substrates involved in this type of learning.

Additionally, an understanding of the site(s) of action in the central nervous system mediating acquisition and expression of conditioned flavor preferences in the same way that lesion techniques elucidated the structures involved in the acquisition and expression of conditioned flavor aversions (Touzani and Sclafani, 2002b) and conditioned flavor preferences (Touzani and Sclafani, 2001a). Given the effectiveness of antagonist studies, one could propose an intracerebral microinjection technique whereby physiologically-relevant doses of the antagonists could be applied to candidate structures(e.g., nucleus accumbens or amygdala) to determine the extent of their involvement.

In conclusion, the results of the experiments in this study provide important information regarding the neurochemistry of flavor preferences conditioned by

carbohydrates. The effects of antagonism of the dopaminergic system at the D1 and D2 sub-receptors with SCH23390 and raclopride, respectively, and of the opioid receptors with the general opioid antagonist naltrexone, adds evidence to the growing body of literature suggesting that two different neurotransmitter systems mediate the motivation to pursue unconditioned reward. Our results are consistent with the conceptual distinction between the hedonics that drive consumption being mediated by endogenous opioids, while learning to associate the significance of cues such as taste are mediated by dopamine, which, unlike opioids, is essential for omnivores like rats to be able to recognize the incentive salience of those cues necessary to acquire and express conditioned flavor preferences.

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