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Opioid effects on palatability in the nucleus accumbens

Author
Woolley, Joshua

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Opioid Effects on Palatability in the Nucleus Accumbens

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Joshua Woolley

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Opioid Effects on Palatability in the Nucleus Accumbens

Abstract

Taste information is used to guide decisions about food consumption. In foraging animals, these determinations are critical for survival. Animals must consume a variety of nutritive items and avoid toxins. The palatability of a food item (i.e. the reward value of a food, as signaled by orosensory cues[1]) is critical to decision-making (e.g. what and how much of an item will be consumed), but the neural mechanisms underlying assessment of food reward are poorly understood. One process that powerfully modulates palatability is sensory specific satiety (SSS), whereby the perceived pleasantness of a food decreases as orosensory and olfactory features specific to that food are repeatedly experienced[2].

Testing opioid effects in a SSS paradigm allows us to distinguish between possible mechanisms underlying opioid effects on feeding. If opioids merely potentiate an existing preferences, then, following a SSS procedure, DAMGO should selectively increase consumption of the flavor that was not pre-fed because it would be non-sated, non-devalued, and therefore, relatively preferred. Alternatively, if 1) the mechanism of SSS is that opioid responses to the pre-exposed food decrease as the food is eaten to satiety or 2) MOP receptor activation reinforces a taste preference, MOP agonists should reverse the effect of satiation (i.e. increase consumption of the pre-fed food). Furthermore, if NAcc muscimol induces feeding independent of palatability, we hypothesized that its action would be unaffected by pre-feeding (i.e. SSS).

We found that infusion of a MOP-specific agonist into the NAcc eliminated SSS; this effect occurred through a preferential increase in consumption of the pre-fed food. Conversely, blockade of endogenous opioid signaling in the NAcc by naltrexone infusion increased SSS; this effect was mediated by a selective decrease in consumption of the pre-fed food. Additionally, if a

delay is introduced between pre-feeding and micro-injection, DAMGO no longer blocks SSS indicating that concomitant food-DAMGO pairing is critical for this effect. Taken together, these effects demonstrate that endogenous opioid signaling within the NAcc condition flavor preferences. However, intra-NAcc DAMGO does not cause long term changes in flavor preference; after a three hour delay no effect remains. Finally, micro-injection of DAMGO into an offsite control in the dorsal striatum, systemic injection of opioid agonists and antagonists and infusion of a GABA agonist into the NAcc modulate feeding but have no effect on SSS highlighting the importance of intra-NAcc opioid receptors for flavor conditioning.

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Introduction

Taste information is used to guide basic decisions about food consumption. In foraging animals, these determinations are critical for survival. Animals must consume nutritive items and avoid harmful toxins. The palatability of a food item (i.e. the reward value of a food, as signaled by orosensory cues[1]) is critical to decision-making (e.g. what and how much of an item will be consumed), but the neural mechanisms underlying assessment of food reward and choice are poorly understood. One process that powerfully modulates palatability and enhances the variety of foods consumed is sensory specific satiety (SSS). SSS is the phenomenon whereby the perceived pleasantness of a food decreases as the gustatory and olfactory features specific to that food are repeatedly experienced. For example, when people are pre-fed a particular food, they rate the pleasantness of the taste of that food lower relative to foods that they have been pre-fed[2]. Furthermore, when people or animals are presented with a variety of foods they tend to eat more than when only a single food is presented. The increased consumption due to variety can be quite large; in rats increases of 50% to 72% have been reported[3] while cats fed three differently flavored foods consume 47% more than when fed a singly flavored food[4]. This "variety effect" is thought to be due to SSS[5]. In humans, increases in caloric intake due to variety have ranged from 15% to 60%[6-8]. Dysregulation of SSS has been implicated in obesity and binge associated bulimia (for review see [6]).

The phenomenon of SSS has recently been exhaustively reviewed [5, 6, 9] but several salient features are relevant to the research presented in this thesis. First, variety induced increases in consumption still occur when the foods are controlled for composition. For example, presenting a variety of flavored versions of the same food (e.g. salt, lemon or curry flavored cream cheese sandwiches) increases consumption over a presentation of an identical volume of only one flavor in rats[10-12] and humans[8, 13]. Furthermore, rats trained to press levers for lemon flavored or salt

flavored polycose, when tested in extinction after pre-feeding, will perform the action that previously rewarded them with the non-pre-fed flavor[14].

Second, the difference in palatability between pre-fed and non-pre-fed foods develops rapidly (less than 2 minutes post-feeding) and lasts for at least one hour[15]. This indicates that it is not post-ingestive feedback that leads to SSS and favors the idea that sensory qualities of the food are the primary determinant of SSS. Furthermore, in addition to taste[8], variety induced increases in feeding have been found for foods with different textures (hard vs. soft)[16] as well as shapes (differently shaped pastas)[8]. In a study using differently colored chocolates there was no increase in consumption in the variety condition although subjects did report that chocolates of the pre-fed color did not taste as delicious[8]. Additionally, the pleasantness of the sight[17] and the odor[2] of foods eaten to satiety are decreased relative to non-eaten foods. Simply chewing a food without swallowing or even just smelling a food for 5 minutes is sufficient to decrease its pleasantness compared to non-sampled foods[17]. Taken together these results indicate that sensory features (odor, taste, and texture) are sufficient to produce SSS.

Third, energy density and macronutrient composition do not appear to affect SSS. In studies using high and low calorie versions of tomato soup or orange jello, no interaction between energy density and SSS was observed[18, 19]. Subjects reported liking the pre-fed food less irrespective of calorie content. In a similar study using high and low calorie versions of the same pudding, no effect of caloric density was found on SSS[20]. Similarly, decreases in palatability were higher when the pre-fed and test foods were matched for flavor than when they were matched for macronutrient composition[21, 22]. These studies indicate that SSS depends on sensory qualities and is insensitive to caloric density or macronutrient composition.

There is an extensive body of animal and human research implicating the orbito-frontal cortices (OFC) in the regulation of palatability. In particular, neurons in the caudolateral OFC of Macaques dynamically encode the reward value of smells, tastes, textures and visual cues. For example, OFC neural firing driven by consumption of a particular food decreases as that food is eaten to satiety but remains high to a food not eaten to satiety[1]. This neural property is not found at earlier stages of the gustatory system such as the nucleus of the solitary tract or the primary gustatory cortex. This satiety-driven decrease in neural firing is thought to contribute to SSS. Brain imaging studies in humans have also implicated the OFC in SSS[23, 24]. For example, when people are presented with vanilla and banana odors before and after being sated on bananas, there is a specific decrease in activation in the OFC in response to the banana odor after satiation[23]. Responses to the vanilla odor remain unchanged. SSS can easily be studied in the rat and is an ideal paradigm for investigating the neural mechanisms of reward modulation since it allows the reward value of a food (i.e. palatability) to be dissociated from its sensory properties.

Opioid receptors and endogenous opioid peptides are well positioned to modulate various aspects of feeding behavior. Systemic morphine (a μ opioid (MOP) receptor agonist) induces robust feeding in the rat[25, 26], while microdialysis experiments indicate that palatable foods stimulate release of endogenous opioids in the hypothalamus and other brain regions[27]. Naltrexone (a non-selective opioid receptor antagonist) decreases preference for sweet and fatty foods in rats without affecting total caloric or water intake[28]. The "taste reactivity test" (a test that examines the orofacial-affective responses of the rat) shows that morphine enhances taste palatability[29]. Importantly, daily pre-test naltrexone injections block normal preference acquisition for a saccharin solution[30]. In sham-feeding experiments where an animal has an intragastric fistula that allows food consumption without gastric filling or nutrient absorption, naltrexone decreases consumption[31, 32](for review see [33]). The behavioral effects of naltrexone appear to mimic sucrose dilution, i.e. rats given naltrexone treat 10% sucrose as if it were 5% sucrose. Similarly,

morphine causes rats to exhibit facial displays to sucrose solutions as though they are more concentrated[29]. When humans take naltrexone they report that sweet solutions do not taste as delicious although they report no change in sweetness[27]. Furthermore, studies with systemic opioid antagonist administration imply that it is critical for the animal to experience the taste in the presence of the drug in order to see a behavioral effect. For example, naltrexone does not change the speed with which a rat will run down a ramp for food or the latency to start eating but does decrease the rate and amount eaten[34]. Additionally, naltrexone does not change the speed with which a rat will push a lever 80 times in order to receive the first pellet but does change the rate of responding for subsequent pellets which were earned by three lever presses each[35]. Similarly, opioid agonists fail to increase conditioned responding on a lever for food, when tested in extinction, while amphetamine does[36]. Finally, in sham feeding paradigms, naltrexone induced decreases in consumption are not immediate; in one study 30 mls of sucrose had to be consumed before a change in lick rate was observed[32]. These studies indicate that the pairing of food intake with either an exogenous or endogenous opioid enhances continued consumption of that food.

Systemic administration of κ opioid (KOP) agonists also increases feeding in rats and other animals[37-40] (for review see[41]). For example, systemic ketocyclazocine, ethylketocyclazocine[37] or U50488[38] (all selective KOP receptor agonists) increase consumption of condensed milk in satiated rats. Similarly, systemic ethylketocyclazocine or bremazocaine[42] (another selective KOP receptor agonist) and intracerebroventricular (ICV) injections of dynorphin (Dyn)[40] (an endogenous KOP receptor agonist) significantly increase consumption of regular chow. Dyn was more effective than equimolar doses of D-Ala^2,N,Me-Phe^4,Gly-ol^5-enkephalin, (DAMGO) (a MOP receptor agonist) or DSLET (delta receptor selective agonist) at increasing feeding.

While KOP receptor agonists consistently increase consumption of chow, there are some discrepancies regarding their effects on consumption of sucrose and saccharin. Ten days of daily pre-test systemic U50488 administration increases consumption of a 20% sucrose solution when only this solution and water are available[43]. This effect appears on the first day of testing and continues for at least five days after the cessation of U50488 injections. In a study specifically investigating dose-response characteristics of opioid agonists on feeding, intracerebroventricular (ICV) U50488 injection decreases consumption of a 0.5% sucrose solution, has no effect on 2.5% sucrose solution and increases consumption of a 10% sucrose solution. Furthermore, when only saccharin or saline and water are available, ICV administration of U50488 does not increase consumption of palatable saccharin or saline solutions unlike the robust increases in consumption seen after ICV DAMGO injection[44, 45].

Unlike MOP agonists, KOP agonists are not thought to increase consumption by increasing the palatability of foods. In a particularly elegant series of experiments, Badiani and Stewart demonstrated that systemic KOP receptor agonists increase consumption by altering satiation[46]. When given simultaneous access to ground chow and three sucrose concentrations (1, 4 and 20%), systemic U50488 significantly and selectively increased consumption of the bland ground chow leaving sucrose consumption unchanged. This is markedly different than the effects of MOP agonists in similar choice paradigms (e.g. systemic morphine selectively increases sucrose consumption[47]). The lack of a KOP agonist induced increase in the highly palatable sucrose solutions argues against a palatability-specific effect.

Badiani and Stewart further investigated the effects of U50488 on consumption by examining the effects of KOP agonists on consumption during access only to sucrose[46]. When saline-treated rats were given daily 30 minute access to one of four concentrations of sucrose solutions (1, 4, 16 and 32%), consumption levels exhibit an inverted U-shaped curve. Consumption levels peak at

16%, and decrease for 32%. When consumption is plotted as a function of total grams of sucrose consumed, it is clear that consumption levels approach a common value asymptotically (around 2.2 grams). Consumption levels rise with increasing sucrose concentrations but plateau at 16% (i.e. even though total volume intake is lower for the highest concentration of sucrose, total sucrose intake is identical between 16% and 32% sucrose solutions). This has been interpreted to mean that at low sucrose concentrations palatability limits intake but at high sucrose concentrations it is the development of satiation that sets a homeostatic limit on intake. Supporting the hypothesis that KOP agonists do not affect palatability, systemic U50488 administration before 30 minute access to one of five concentrations of sucrose (1, 4, 16, 32, or 40%) only increased consumption of the two most concentrated solutions. U50488 decreased consumption of the least concentrated solution and left consumption of the two intermediate concentration solutions unaffected. When this data is expressed as total grams of sucrose consumed, it is clear that the normal plateau of sucrose consumption was not reached. Untreated and saline treated animals consume roughly 2.2 grams of sucrose when they are given access to 16, 32 or 40% sucrose solutions. U50488 treated animals did not show this plateau; sucrose intake doubled to 4.5 g[46]. This effect is similar to the differences in consumption of sucrose solutions between normally-fed and sham-fed animals. Removing post-ingestive consequences by sham feeding rats does not increase consumption of low concentration sucrose solutions but does increase consumption of highly concentrated sucrose solutions[48]. Sham-feeding should not affect the rat's experience of palatability, only that of satiation. Again, these effects are different from those of morphine, which increases consumption of all sucrose concentrations, and does so by increasing palatability[47]. These results suggest that, when given systemically, KOP receptor agonists increase feeding by altering hunger mechanisms (e.g. U50488 delays meal termination), not palatability.

The nucleus accumbens (NAcc) is a critical site for opioids to influence palatability. It contains high densities of MOP and KOP receptors as well as enkephalinergic and dynorphinergic fibers[49, 50]. Micro-injection of DAMGO (a MOP receptor agonist) into the NAcc selectively increases consumption of calorie dense (sucrose and lard[51]) and flavorful (saccharin and salt[52]) palatable items while leaving consumption of chow and water unchanged[26]. Furthermore, opioid antagonists injected into the NAcc can block consumption of palatable foods, indicating that endogenous opioid release plays a role in modulating feeding at this site[53]. Despite abundant KOP receptors and dynophinergic fibers, KOP agonists reportedly do not typically alter consumption when micro-injected into the NAcc[53-56].

The NAcc is well positioned to participate in the control of food intake (for reviews see [57, 58]). First, the NAcc receives taste and visceral information directly from the nucleus of the solitary tract (NTS) in the brainstem and the gustatory (insular) cortex as well as indirectly from these regions through the parabrachial nucleus (PB) via the gustatory thalamus (VPO). Furthermore, there are two pathways for gustatory information to reach the NAcc through the amygdala; the NTS-PB-central amygdalar nucleus-VTA-NAcc pathway and the gustatory cortex-basolateral amygala-NAcc pathway. Interestingly, the NAcc receives a particularly dense innervation from the ventral OFC, an area strongly implicated in reward modulation that also receives highly processed gustatory, olfactory and somatosensory information. Additionally, the lateral hypothalamus (LH), a critical nucleus in energy homeostasis and hunger mechanisms, sends melanin-concentrating hormone (MCH) containing projections directly to the medial shell region of the NAcc. Anatomical, physiological and genetic data support the hypothesis that MCH is critically involved in feeding behavior (e.g. ICV injections of MCH increase food intake and MCH knockout mice are hypophagic and lean) (for review see [59]).

NAcc outputs include classic basal ganglia motor circuits as well as the lateral hypothalamus and VTA. In turn, the LH projects to brainstem nuclei and spinal cord, which are critical for feeding behavior. Additionally, hormonal signals are critical for intake regulation and the NAcc is influenced by these signals in several ways. Insulin is a critical signal of energy stores, rapidly crosses the blood brain-barrier by a receptor mediated transport mechanism and is present in cerebral spinal fluid in concentrations proportional to levels in the periphery. A large body of evidence supports the hypothesis that circulating insulin enters the brain to produce anorexic responses (e.g. ICV injection of insulin inhibits food intake) (for review see [60]). Interestingly, there are high levels of insulin receptors in the NAcc[26]. Furthermore, the NAcc receives information from the arcuate nucleus of the hypothalamus (a critical site for sensing peripheral metabolic signals such as leptin, insulin, glucocorticoids and glucose) indirectly through the LH. In summary, the NAcc has appropriate inputs (e.g. gustatory, visceral and hormonal information) and outputs (skeletal and autonomic) to be a critical node in the feeding circuit.

In addition to opioids, other signaling systems known to be involved in feeding are well represented within the NAcc. The NAcc receives rich dopaminergic input from the VTA and substantia nigra pars compacta. Dopamine signaling is critical for motivated behavior and has been implicated in several aspects of feeding[47, 61, 62]. Furthermore, there are substantial levels of serotonin[63], cannabinoid[64] and somatostatin[65] (all implicated in the modulation of feeding) receptors in the NAcc. Interestingly, activation of all three of these receptors, as well as opioid receptors, can modulate DA signaling in various paradigms[63, 65-68] providing a possible common mechanism for their effects on feeding. The presence of these signaling systems in the NAcc further supports a critical role for this nucleus in the integration and execution of feeding behavior.

It is clear that opioid agonists increase, while antagonists decrease, consumption of palatable foods. Furthermore, endogenous opioids are released following consumption of palatable items. The question concerning endogenous opioid signaling that remains unanswered is exactly which aspects of the taste experience are modulated by opioid stimulation. If the magnitude of endogenous opioid release following palatable food consumption determines its rewarding properties (as supported by antagonist studies), why do exogenous agonists selectively increase consumption of already palatable items and not all foods equally or even less palatable foods more than highly palatable foods? In other words, why are palatable items more sensitive to opioid manipulations? Furthermore, there is debate regarding whether intra-NAcc DAMGO micro-injections increase consumption primarily by increasing palatability or by increasing consumption of a certain calorie dense macronutrient, in particular fat. Intra-NAcc DAMGO increases fat consumption irrespective of baseline preference[51] but also increases consumption of highly palatable non-caloric (i.e. fat-free) solutions like saccharin and salt. Thus, opioid stimulation within the NAcc must be at least partially related to palatability. Since in all previous studies macronutrient composition and palatability were confounded, the question of how these two different features of opioid stimulation interact remains open.

The present studies were designed in order to investigate which aspects of the taste experience are modulated by opioid stimulation within the NAcc. By using differently flavored versions of the same food item, macronutrient composition could be held constant while palatability was systematically varied using a SSS procedure. We investigated NAcc opioidergic effects on consumption when only a single flavor was available, when two flavors were available simultaneously and when two flavors were available but the palatability of one had been modulated by pre-feeding. By varying both the route of opioid delivery (e.g. systemic versus intra-NAcc) and receptor subtype specificity (MOP versus KOP) in these behavioral paradigms we were able to elucidate multiple distinct effects of opioid manipulations on flavor choice. To

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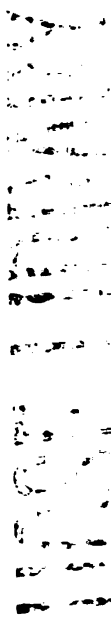

Chapter 1

The Effects of Opioid Signaling within the Nucleus Accumbens on Flavor Choice

Introduction

The flavor of food is an important determinant of what and how much we eat. Flavor is a component of the orosensory qualities of food that determine its hedonic value, or palatability. Among the molecular signaling systems that robustly regulate palatability are the opioid peptides and receptors. Opioid agonists induce robust feeding in the rat,[1, 2] by increasing the consumption of palatable food[3, 4] and microdialysis experiments indicate that palatable foods stimulate release of endogenous opioids in the hypothalamus and other brain regions involved in feeding[5]. Importantly, opioid antagonists decrease preference for sweet and fatty foods in rats without affecting total caloric or water intake[6]. Furthermore, the "taste reactivity test" (a test that examines the orofacial-affective responses of the rat) indicates that morphine enhances the positive hedonic effect of palatable food consumption[7]. Consistent with this interpretation, human subjects given the opioid antagonist naltrexone report that food does not taste as delicious, although taste intensity and recognition thresholds are not affected[5]. In fact, none of these opioid effects on consumption have been associated with any change in the ability to discriminate tastes (e.g. naltrexone does not affect a rat's ability to discriminate between sucrose and water[8]).

The nucleus accumbens (NAcc) is a critical site for opioid regulation of palatability. Microinjection of D-Ala^2,N,Me-Phe^4,Gly-ol^5-enkephalin, (DAMGO) (a μ opioid (MOP) receptor selective agonist) into the NAcc selectively increases consumption of calorie dense (sucrose and lard[9]) and flavorful (saccharin and salt[10]) palatable items while leaving consumption of chow and water unchanged[2]. Furthermore, opioid antagonists injected in the NAcc can block consumption of palatable foods, indicating that endogenous opioid release modulates feeding at

this site[11]. These data illustrate the importance of the NAcc for opioid-induced feeding and suggest that palatability is particularly relevant to the effects of opioid signaling on feeding.

In addition to opioids, glutamatergic and GABAergic signaling within the NAcc play a role in feeding. Within the shell region of the NAcc, blockade of glutamate receptors increases, while activation of glutamate receptors decreases, feeding[12, 13]. This manipulation probably alters tonic excitatory input from corticolimbic and thalamic circuits. Additionally, both $GABA_A$ (muscimol) and $GABA_B$ (baclofen) agonists elicit robust, dose-related increases in chow intake when micro-injected into the shell but not the core of the NAcc[14]. Furthermore, increasing levels of endogenous GABA by blocking GABA breakdown also increases feeding. These effects are specific to food; gnawing and water consumption are unaffected. Thus, it appears that decreases in neural firing in the shell of the NAcc increase consumption.

There are several important differences between GABA and opioid induced feeding. While muscimol induced feeding is insensitive to palatability or macronutrient composition[15], intra-NAcc DAMGO selectively increases consumption of foods that are either highly palatable or contain a particular macronutrient, namely fat. Many studies have shown increases in fat consumption with systemic morphine administration (for review see [9]). However, when baseline preferences are taken into account these data appear equivocal since foods with high fat content are also among the most palatable food items. For example, systemic morphine increases consumption of a food previously determined to be preferred irrespective of its macronutrient content[16]. Intra-NAcc DAMGO, however, increases fat consumption irrespective of baseline preference[9] suggesting that opioid stimulation in the NAcc is selective for the macronutrient fat. This cannot be the whole story, however, since consumption of highly palatable non-caloric solutions like saccharin and salt are also increased by intra-NAcc opioid stimulation. Therefore, opioid stimulation must be at least partially related to the palatability of a particular taste. In all

previous studies of the role of NAcc opioids in food choice and consumption, macronutrient composition and palatability were confounded, so it remains unclear exactly which parameter is regulated by opioids. The present study was specifically designed to investigate the relationship between NAcc opioid manipulation and taste preference. By using differently flavored versions of the same food item, macronutrient composition can be held constant while taste determined palatability is systematically varied.

Methods

Animals: A total of 38 male rats (Long Evans, Charles River Laboratories, Wilmington, MA) weighing between 270 and 480 g were used in the present studies. Animals were individually housed in conventional hanging cages in a temperature- and humidity-controlled room on a 12:12 hour light:dark cycle. Animals had *ad lib* access to water at all times and *ad lib* access to chow at all times except during testing.

Surgery: Animals were anaesthetized with isoflorane, their heads placed in a stereotaxic device and then following a small craniotomy, bilateral guide cannulae were stereotactically placed and then secured to the skull with stainless steel screws and dental cement. Coordinates for the target sites were 1.5 mm anterior, 1.1 mm lateral and 5.5 mm ventral from Bregma. For this study, the cannulae were not directed specifically at the core or the shell regions of the NAcc. For control microinjections, coordinates for the target sites were 1.5 mm anterior, 1.1 mm lateral and 3.5 mm ventral from Bregma. Animals were allowed 4 days recovery post surgery.

Drugs and injections: For micro-injections, DAMGO, the selective μ receptor agonist and muscimol, the selective $GABA_A$ receptor agonist, were obtained from Sigma Pharmaceuticals. Both of these drugs were dissolved in 0.9% sterile saline (for DAMGO 0.25 μg per side, for muscimol 50 nanograms per side). First, the stylet was removed from the guide cannulae and the

injector cannulae were inserted. The injector cannulae protruded 2.2 mm past the end of the guide cannula for a final distance of 7.7 mm ventral to Bregma. The drugs, in a volume of 0.5 μl of saline, were infused through injector cannulae connected to a microdrive pump by polyethylene tubing. The rate of infusion was 0.25 μl/min. The injector cannulae remained in place an additional minute after the infusion in order to allow for diffusion. Injectors were then removed and the stylets were replaced.

Behavioral testing and experimental design: After recovery from surgery (four days), animals were extensively handled. In order to overcome taste neophobia, rats were brought into the testing room on four separate days and given one hour simultaneous access to both flavors of pellets (chocolate and banana). After this initial exposure, all rats avidly consumed the pellets when available. The two types of flavored 1 gram pellets were made from the same meal substrate and were thus matched for all macro- and micro- nutrients (bio-serv, Frenchtown, New Jersey). Table 1 shows the composition of the flavored pellets. Pellets were always delivered in test tube dispensers. Rats were required to bite the pellets and pull them from a hole in the bottom of the tube. This level of effort encouraged the rats to only take what they would eat and greatly facilitated consumption quantification. Every fifteen minutes post injection, the number of pellets remaining in the dispenser was counted and a visual inspection of the cage for dropped pellets was made.

Experiment 1: In order to determine whether DAMGO had differential effects on consumption of chocolate or banana pellets, animals (n=14) were micro-injected with DAMGO or saline into the NAcc and then given 1.5 hour *ad lib* access to either banana or chocolate pellets. All rats underwent all four conditions and individual rats were randomly assigned to groups randomized for the order of injection and flavor.

Experiment 2: In order to determine whether DAMGO affects flavor consumption differently when rats are allowed to choose between flavors, rats (n=7) were micro-injected with DAMGO or saline and given 1.5 hour simultaneous *ad lib* access to both chocolate and banana flavored pellets. All rats underwent both conditions and injection and flavor orders were randomized.

Experiment 3: To determine whether GABA receptor activation within the NAcc affects consumption when rats have a choice between flavors, rats (n=17) were microinjected with muscimol or saline and given 1.5 hour simultaneous *ad lib* access to both chocolate and banana flavored pellets. All rats underwent both conditions and injection and flavor orders were randomized.

Data analysis: All data are expressed as mean ± s.e.m. Data were analyzed using repeated measures analysis of variance (ANOVA) with pharmacologic manipulation as a within subject factor and flavor as a between subject factor. *Post-hoc* comparisons were made using the Student-Newman-Keuls method.

Histology: After the completion of testing, rats were anaesthetized deeply with sodium pentobarbital and transcardially perfused with a 0.9% isotonic saline solution followed by 10% formalin solution. Brains were removed and stored in 10 % formalin for several days followed by an overnight immersion in 10% sucrose solution. Brains were sliced into 45 mm sections, mounted and stained with a neutral red stain. Sections were examined under the microscope in order to determine placement of micro-injector tips.

Results

Experiment 1: Intra-NAcc micro-injection of a MOP selective agonist in a non-choice paradigm.

DAMGO in the NAcc increases consumption of either flavor when it is presented alone. One-way repeated measure ANOVA indicates that DAMGO significantly increased consumption [$F(1,27)=21.548$, $P<0.001$] (fig 1a, b) with no significant drug × flavor interaction. Further analysis indicated that these differences are significant from the 60 minute time point onward. In the absence of choice there were no differences in consumption between chocolate or banana pellets following either saline or DAMGO micro-infusion.

Experiment 2: Intra-NAcc microinjection of a MOP selective agonist in a choice paradigm.

Intra-NAcc DAMGO increases chocolate consumption when the alternative flavor is banana. One-way repeated measure ANOVA indicated that DAMGO significantly increased consumption [$F(1,13)=10.500$, $P<0.01$] and there was a significant drug × flavor interaction [$F(1,13)=5.88$, $P<0.05$] (fig 2a). Further analysis indicated that these differences were significant from the 15 minute time point onward. *Post Hoc* mean contrasts conducted on data from the 90 minute time point indicated that rats ate significantly more chocolate than banana pellets with DAMGO ($P<0.001$). This finding was due to a significant DAMGO induced increase in chocolate consumption ($P<0.001$) (fig 2b). In contrast to the large increase produced by DAMGO in consumption of banana when it is the only taste available, in the choice paradigm with chocolate as the alternative, there was no significant DAMGO induced increase in banana pellet consumption but a large DAMGO induced increase in chocolate pellet consumption ($P<0.001$).

Experiment 3: Effect of intra-NAcc microinjection of a $GABA_A$ selective agonist on consumption of flavored pellets in a choice paradigm.

Unlike intra-NAcc DAMGO, intra-NAcc muscimol increases consumption of both chocolate and banana pellets equally when they are presented simultaneously. Repeated measure ANOVA on

the 90 minute time point showed a significant muscimol induced increase in feeding [$F(1,33)=11.151$, $P<0.005$] (fig 3a, b). There was no significant drug × flavor interaction. Further ANOVA's indicated that the significant drug effect emerged by 15 minutes post-injection. While intra-NAcc DAMGO selectively increases consumption of chocolate and has virtually no effect on banana consumption, intra-NAcc muscimol increases consumption non-selectively with regard to flavor.

Discussion

The main purpose of this study was to determine the contribution of taste to the enhancement of food intake by opioid and GABA agonists. We found that when food pellets of different flavors were presented separately, intra-NAcc DAMGO enhanced consumption of either flavored pellet to the same extent. When both flavors were presented at the same time in a choice paradigm, DAMGO selectively increased chocolate consumption. Since chocolate is slightly preferred by rats in the absence of opioid treatment (data not shown) DAMGO appears to selectively increase consumption of a food with a preferred (i.e. more palatable) flavor. Thus DAMGO enhances intake of palatable foods in general and, in addition, it selectively enhances preference in a choice paradigm.

The effects of intra-NAcc DAMGO on consumption when the two types of flavored pellets were presented alone and when they were presented together are consistent with previous work. Intra-NAcc DAMGO significantly increases consumption of both bland chow and palatable sucrose when these are presented alone in non-deprived rats [17-19]. When given a choice between foods, however, micro-injection of DAMGO into the NAcc selectively increases consumption of calorie dense (sucrose and lard[9]) and flavorful (saccharin and salt[10]) palatable items while leaving consumption of chow and water unchanged[2]. Similarly, Kelley and colleagues have shown that intra-NAcc DAMGO enhances consumption of either a carbohydrate or fatty mash when they are

presented alone (although fat consumption is increased significantly more), but only increases consumption of the fatty mash when both foods are presented simultaneously irrespective of the animals' baseline preferences (i.e. carbohydrate preferring rats still only increase their fat consumption after DAMGO micro-injection)[9]. The present experiments confirm and extend those findings by showing that when given a choice between foods with identical macronutrient content (and texture) but different flavors, DAMGO selectively increases consumption of a the food with the preferred taste. These two actions of MOP agonists in the NAcc are related but distinct. If food of only one flavor is present, MOP agonists will enhance feeding relative to other competing behaviors. On the other hand, if a flavor choice is presented, MOP agonists will selectively enhance intake of the preferred flavor.

Opioid signaling in different brain regions may have different effects on feeding. For example, naltrexone selectively decreases consumption of a preferred diet when injected into the central nucleus of the amygdala but nonspecifically reduces consumption after micro-injection into the paraventricular nucleus[20] Taken together with the findings that systemic morphine injection (which activates opioid receptors throughout the brain and periphery) increases consumption based on animals' baseline preference[16], while intra-NAcc DAMGO increases consumption of fat irrespective of baseline preference[9], it has been proposed that opioid signaling may be involved in macronutrient choice as well as palatability depending upon the brain region[21]. This highlights the importance of using brain-region specific manipulations to understand the role of opioid signaling in feeding, specifically with regard to its influence on palatability versus macronutrient choice behavior. The current study, in conjunction with those of Dr. Kelley[9], suggests that opioid signaling within a single neural structure (NAcc) can have both palatability and macronutrient specific effects.

In order to investigate the role of GABAergic signaling within the NAcc on palatability, we micro-injected muscimol into the NAcc and then gave rats a choice of flavored pellets. Muscimol increased consumption non-specifically; consumption of chocolate and banana pellets were increased equally. This is distinctly different from the selective increase in chocolate consumption induced by intra-NAcc DAMGO. These results are in agreement with previous findings that unlike intra-NAcc DAMGO, intra-NAcc muscimol increases consumption without altering the preference between fat and carbohydrate meals when presented together[15]. In further support of GABAergic feeding being independent of palatability, intra-NAcc muscimol only increases consumption of caloric foods leaving intake of highly palatable saccharin and saline solutions unchanged[15]. Finally, the flavor independent enhancement of food intake by muscimol is similar to the type of feeding induced by lateral hypothalamus stimulation[14, 22, 23]. Taken together, these previous results and the current findings indicate that muscimol induced feeding is not dependant upon palatability.

Previous work has shown that the effects of NAcc micro-injections of muscimol[14, 15, 23] or glutamate antagonists[24, 25] are anatomically restricted to the medial anterior shell. Furthermore, within the shell of the NAcc there appears to be a rostral-caudal gradient in motivational effects of these manipulations. Muscimol[22, 23] or glutamate antagonists[25] elicit feeding rostrally and defensive behaviors caudally. The anatomical specificity of muscimol induced feeding is distinctly different from the widespread sensitivity of circuits within the striatum to DAMGO manipulations[26]. DAMGO can induce feeding when injected throughout the NAcc and the dorsal striatum (at higher doses). Additionally, the time courses of drug induced feeding for these two compounds are different; muscimol induced feeding is immediate and short lasting (1 hour) while DAMGO induced feeding is slower in onset and can last for several hours. Taken together, these results indicate that despite the fact that both DAMGO and muscimol within the NAcc

inhibit neurons and increase feeding; their effects are separate and distinguishable. Receptor distribution patterns and local microcircuit anatomy may account for these differences.

A possible confounding issue in the present study is the fact that opioids may have had an effect on hunger or general motivation for food. If opioids induced changes in either of these properties, the results from this study would be difficult to interpret. In one study a progressive ratio paradigm where the number of lever presses needed for the delivery of food reward is systematically increased, the breakpoint (the number of times the animal will press the lever for a reward before it stops responding and "gives up") is robustly increased by intra-NAcc DAMGO[27]. This suggests that DAMGO can increase motivation for a food reward. While these data could be interpreted as being contradictory to the hypothesis that opioid systems modulate the hedonic value of tastes (so called "liking") and not the motivation (or "wanting") for food, it is also clear that increased liking for a food can drive increased wanting. Ann Kelley articulates this position most clearly: "This makes intuitive sense in a biological framework; clearly, if the hedonic impact of the currently available reward is high, responses aimed at obtaining that reward will be invigorated."[2] It is important to note that in this experiment the rats were able to taste the food while under the effects of DAMGO, since when tested in extinction, DAMGO does not increase breakpoint[28]. Furthermore, while hunger establishes a condition under which novel food-reinforced behaviors are acquired more readily, opioid stimulation does not[29] (i.e. opioid stimulation does not increase the rate of learning new behaviors in order to acquire food). Naltrexone does not diminish rats' motivation to start feeding but does decrease feeding rate and overall consumption during a single meal[30, 31]. Finally, human subjects taking naltrexone report that food does not taste as delicious but do not report decreases in hunger and do not show long term decreases in food intake[5]. Thus it is clear that opioid stimulation of feeding is not synonymous with a behavioral state induced by food deprivation. Instead, opioids seem to selectively increase the rewarding value of already palatable foods.

In summary, the present study demonstrates that MOP receptor stimulation within the NAcc can have at least two separate effects: DAMGO 1) increases consumption of palatable foods in general and 2) selectively increases consumption of a preferred flavor. This preference cannot be accounted for by macro-nutrient content because the pellets used in the present study are nutritionally identical. Additionally, intra-NAcc DAMGO increased consumption of both flavors equally when presented alone proving that chocolate consumption is not more sensitive to opioid manipulation in general. While a $GABA_A$ receptor agonist micro-injected into the NAcc also increased feeding, this effect was independent of relative palatability; consumption of the more and less preferred flavors was increased equally. These differences highlight important functional differences between opioid and GABAergic signaling systems within the NAcc and support a specific role for opioid signaling in preference based exclusively on flavor.

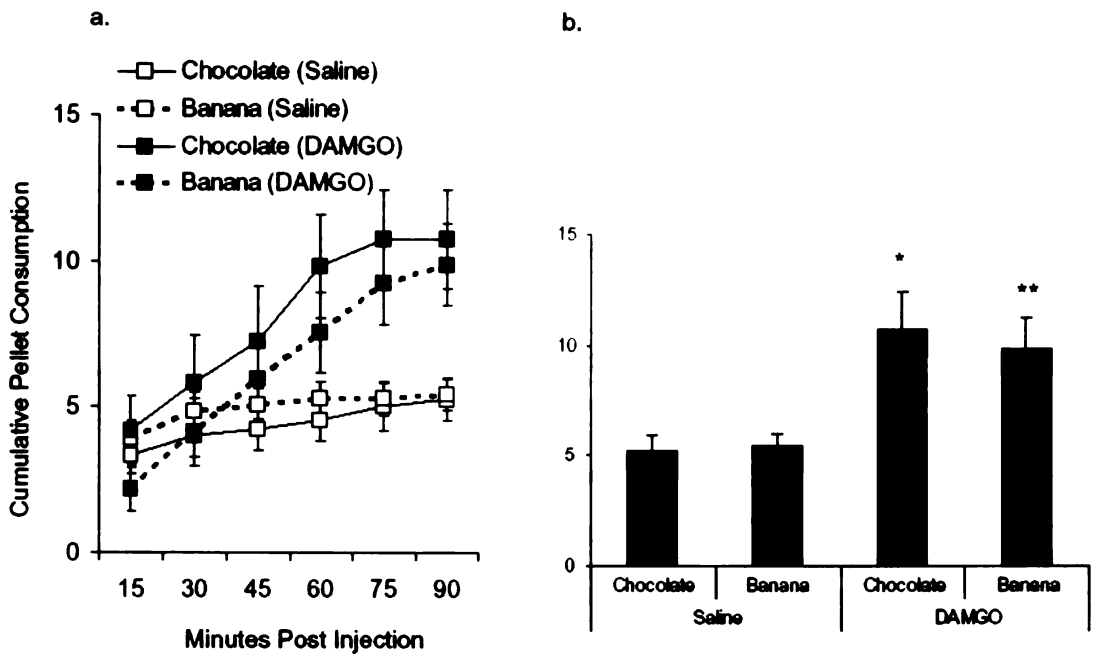

Figure 1.

Effect of intra-NAcc μ opioid receptor stimulation on consumption in a non-choice paradigm. **a.** The cumulative number of flavored pellets consumed following saline or DAMGO micro-injection is shown for each 15 minutes post injection. Since only one flavor was available after micro-injection each line represents consumption on a separate day of testing. **b.** Cumulative consumption at the 90 minute time point. * indicates ($p<0.05$) ** indicate ($p<0.01$) from saline condition for the same flavor.

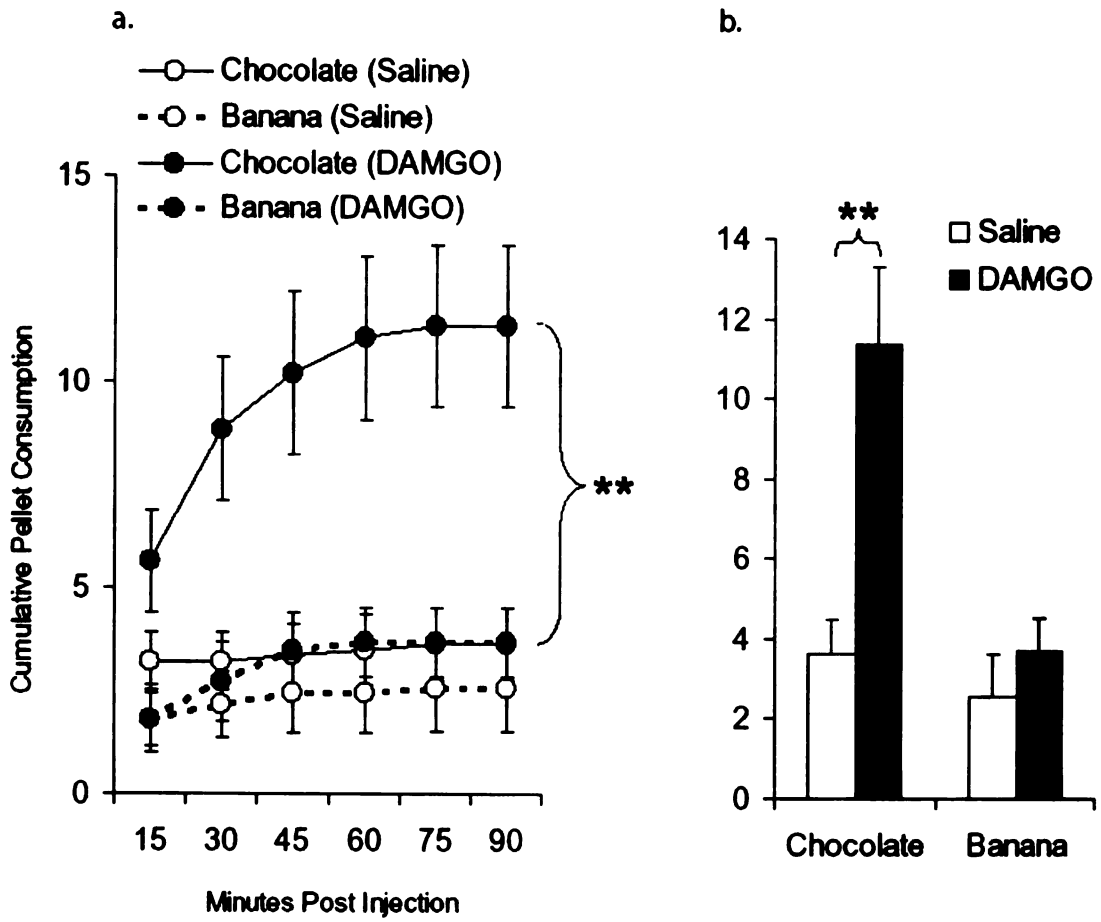

Figure 2.

Effect of intra-NAcc μ opioid receptor stimulation on consumption in a choice paradigm. **a.** The number of flavored pellets consumed following saline or DAMGO micro-injection is shown for each 15 minutes post injection. Since both flavors were available after micro-injection, closed circles represent data from one test session while open circles represent data from a separate test session. **b.** Cumulative consumption at the 90 minute time point. * indicates ($p<0.05$) ** indicate ($p<0.01$).

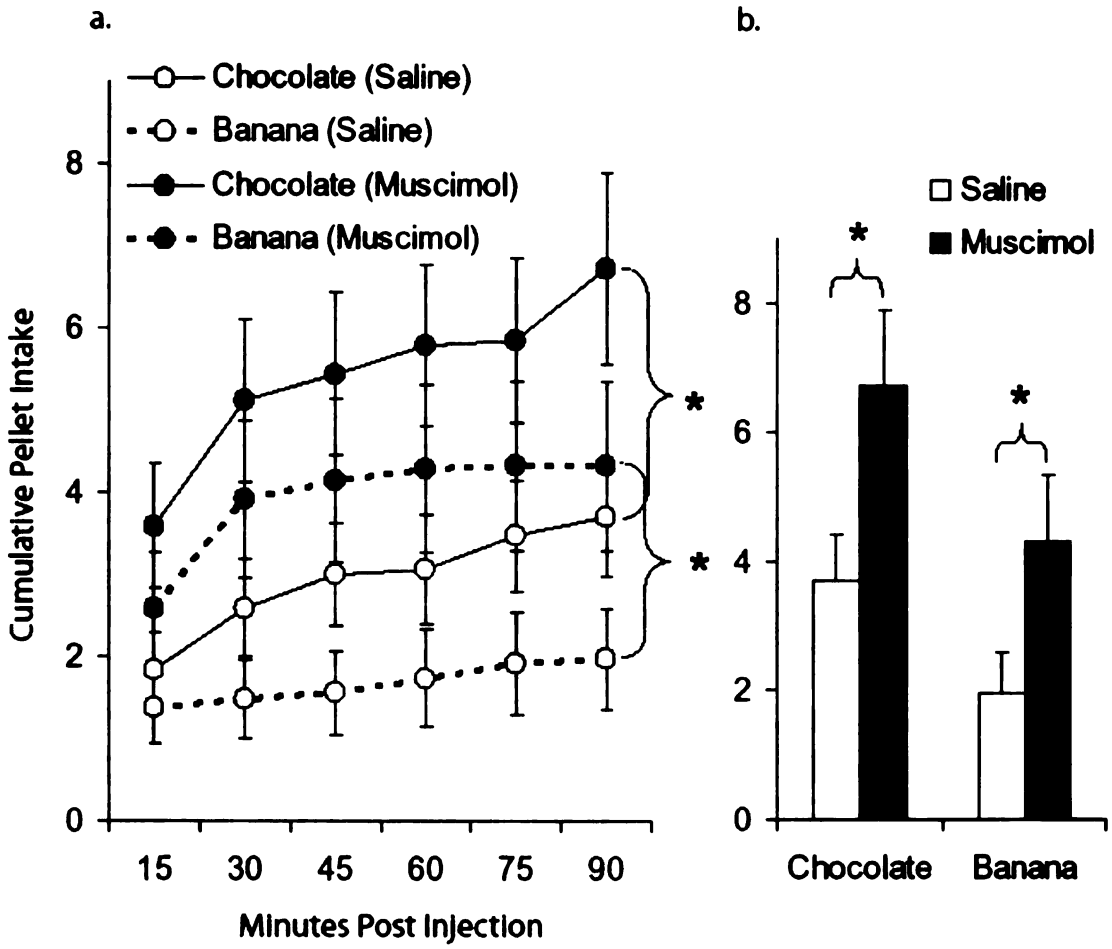

Figure 3.

Effect of intra-NAcc $GABA_A$ receptor stimulation on consumption in a choice paradigm. **a.** The number of flavored pellets consumed following saline or muscimol micro-injection is shown for each 15 minutes post injection. **b.** Cumulative consumption at the 90 minute time point.

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Chapter 2

The Effects of Opioid Modulation within the Nucleus Accumbens on Taste Conditioning

Introduction

Taste information is used to guide decisions about food consumption. In foraging animals, these determinations are critical for survival. Animals must consume a variety of nutritive items and avoid toxins. The palatability of a food item (i.e. the reward value of a food, as signaled by orosensory cues[1]) is critical to decision-making (e.g. what and how much of an item will be consumed), but the neural mechanisms underlying assessment of food reward are poorly understood. One process that powerfully modulates palatability is sensory specific satiety (SSS), whereby the perceived pleasantness of a food decreases as orosensory and olfactory features specific to that food are repeatedly experienced. For example, when people are pre-fed a particular food, they rate the pleasantness of its taste lower relative to foods that they have not just eaten[2]. Furthermore, in rats[3-5] and humans[6, 7]consumption of a variety of flavored versions of the same food is higher than when an identical volume of a singly flavored version is presented.. The increased consumption due to variety can be quite large[6, 8-10]; in rats increases of 50% to 72% have been reported[11]. SSS is thought to contribute to this "variety effect"[8].

SSS induced increases in consumption occur even when food variety is controlled for composition. For example, when tested in extinction, rats trained to press one lever for lemon flavored Polycose and a different lever for salt flavored Polycose, will perform the action that previously provided the non-pre-fed flavor[12]. Additionally, variety induced increases in consumption have been found for foods that differed only in texture (hard vs. soft)[13], colors, or shape[6]. Similarly, the pleasantness of the sight[14] and the odor[2] of foods eaten to satiety are decreased relative to non-eaten foods. Satiety effects based on flavor do not require that food actually be consumed. Simply chewing a food without swallowing or even just smelling a food is

sufficient to decrease its pleasantness compared to non-sampled foods[14]. Finally, neither energy density nor macronutrient composition affect SSS[15-19]. Taken together these results indicate that sensory features (odor, taste, and texture) are sufficient to produce SSS. SSS can easily be studied in the rat and is an ideal paradigm for investigating the neural substrates of food reward since it allows the reward value of a food (i.e. palatability) to be changed while holding its sensory properties constant.

There is extensive literature implicating opioid signaling in consumption, especially palatability driven feeding[20-23]. Opioid agonists increase, while antagonists decrease, consumption of palatable foods much more than bland chow[24-26]. Microinjection of D-Ala^2,N,Me-Phe^4,Gly-ol^5-enkephalin, (DAMGO) (a μ opioid (MOP) agonist) into the nucleus accumbens (NAcc) selectively increases consumption of calorie dense (sucrose and lard[27]) and flavorful (saccharin and salt[28]) items while leaving consumption of bland chow and water unchanged[20]. Furthermore, opioid antagonists injected in the NAcc can significantly reduce consumption of palatable foods, indicating that endogenous opioid release plays a role in modulating feeding at this site[29]. These data implicate the NAcc in opioid-induced feeding and raise the possibility that this area is critical for opioid regulation of SSS.

In addition to opioids, non-specific GABAergic inhibition of NAcc neurons also increases feeding. Both $GABA_A$ (muscimol) and $GABA_B$ (baclofen) agonists elicit robust, dose-related increases in chow intake when micro-injected into the shell of the NAcc[30]. There are several important differences between muscimol and DAMGO induced feeding, however, including time course, anatomical specificity, and effects on palatability[30-32]. Importantly, unlike intra-NAcc DAMGO, intra-NAcc muscimol only increases consumption of caloric foods leaving saccharin and saline intake unchanged[32]. Similarly, muscimol does not alter the preference between fat and carbohydrate meals when presented together[32] which is different from DAMGO's selective

increase of fat consumption in a similar paradigm[27]. These findings have been interpreted to indicate that muscimol enhances feeding behavior non-selectively, in contrast to the palatability or macronutrient composition dependent increases seen with DAMGO.

We have previously investigated the roles of intra-NAcc MOP and $GABA_A$ receptor agonists in a flavor choice paradigm using nutritionally identical highly palatable flavored pellets. When injected into the NAcc, DAMGO selectively increases, while naltrexone selectively decreases, consumption of the more preferred chocolate pellets. In contrast, muscimol increases consumption of the two flavors equally. Given the similarities and differences between the effects on feeding of these two signaling systems within the NAcc, we decided to further investigate their roles in reward modulation using a SSS paradigm.

If endogenous opioids modulate the reward value of food, then exogenous opiates should alter the choice behavior of the rat. If it is only the current palatability (or preference) for a food that determines the ability of DAMGO to increase its consumption, then, following a SSS procedure, DAMGO should selectively increase consumption of the flavor that was not pre-fed because it would be non-sated, non-devalued, and therefore, relatively preferred. Alternatively, if 1) the mechanism of SSS is that opioid responses to the pre-exposed food decrease as the food is eaten to satiety or 2) MOP receptor activation reinforces a taste preference, MOP agonists should reverse the effect of satiation (i.e. increase consumption of the pre-fed food). Furthermore, if NAcc muscimol induces feeding independent of palatability, we hypothesized that its action would be unaffected by pre-feeding (i.e. SSS).

Methods

Animals: A total of 117 male rats (Long Evans, Charles River Laboratories, Wilmington, MA) weighing between 270 and 480 g were used in the present studies. Animals were individually

housed in conventional hanging cages in a temperature- and humidity-controlled room on a 12:12 hour light:dark cycle. Animals had *ad lib* access to water at all times and *ad lib* access to chow at all times except during testing.

Surgery: Animals were anaesthetized with isoflorane, their heads placed in a stereotaxic device and then following a small craniotomy, bilateral guide cannulae were stereotactically placed and then secured to the skull with stainless steel screws and dental cement. Coordinates for the target sites were 1.5 mm anterior, 1.1 mm lateral and 5.5 mm ventral from Bregma. For this study, the cannulae were not directed specifically at the core or the shell regions of the NAcc. For control microinjections, coordinates for the target sites were 1.5 mm anterior, 1.1 mm lateral and 3.5 mm ventral from Bregma. Animals were allowed 4 days recovery post surgery.

Drugs and injections: For micro-injections, DAMGO, the selective MOP agonist, naltrexone, the non-selective opioid antagonist, and muscimol, the selective $GABA_A$ receptor agonist, were obtained from Sigma Pharmaceuticals. All drugs were dissolved in 0.9% sterile saline (DAMGO: 0.25 μg per side, naltrexone: 20 μg per side, and muscimol: 50 nanograms per side). First, the stylet was removed from the guide cannulae and the injector cannulae were inserted. The injector cannulae protruded 2.2 mm past the end of the guide cannula for a final distance of 7.7 mm ventral to Bregma. The drugs, in a volume of 0.5 μl of saline, were infused through 12.5 mm injector cannulae connected to a microdrive pump by polyethylene tubing. The rate of infusion was 0.25 μl/min. The injector cannulae remained in place an additional minute after the infusion in order to allow for diffusion. Injectors were then removed and the stylets were replaced. For subcutaneous injections, morphine and naltrexone were diluted in 0.9% sterile saline at concentrations of 2.5 mg/kg for morphine and 1 mg/kg for naltrexone and injected subcutaneously with a 1 cc syringe.

Behavioral testing and experimental design: After recovery from surgery (four days), animals were extensively handled. In order to overcome taste neophobia, rats were brought into the testing room on four separate days and given one hour simultaneous access to both flavors of pellets (chocolate and banana). After this initial exposure, all rats avidly consumed the pellets when available. The two types of flavored 1 g pellets were made from the same meal substrate and were thus matched for all macro- and micro- nutrients (bio-serv, Frenchtown, New Jersey). Table 1 shows the composition of the flavored pellets. Pellets were always delivered in test tube dispensers. Rats were required to bite the pellets and pull them from a hole in the bottom of the tube. This amount of effort encouraged the rats to only take what they would eat and greatly facilitated consumption quantification. Every fifteen minutes post injection, the number of pellets remaining in the dispenser was counted and a visual inspection of the cage for dropped pellets was made.

Experiment 1: To determine whether dynamically altering the palatability of a flavor will affect DAMGO's ability to increase consumption, a sensory specific satiety (SSS) paradigm was used. Rats (n=16) were given one hour *ad lib* access to either banana or chocolate pellets. At the end of this hour, rats were micro-injected with either DAMGO or saline. Rats were then given 1.5 hour simultaneous *ad lib* access to both chocolate and banana flavored pellets. All rats underwent all four conditions and injection and flavor orders were randomized.

Experiment 2: In order to determine whether the MOP receptors specifically within the NAcc are critical for the effects of DAMGO on SSS, DAMGO was injected 2 mm dorsal to the injection site within the NAcc using the same SSS paradigm (n=8).

Experiment 3: In order to determine whether the effects of the non-specific opioid agonist morphine delivered systemically has similar effects to DAMGO delivered directly into the NAcc,

Data analysis: All data are expressed as mean ± s.e.m. Data were analyzed using repeated measures ANOVA with pharmacologic manipulation as a within subject factor and flavor as a between subject factor. *Post-hoc* comparisons were made using the Student-Newman-Keuls method.

Histology: After the completion of all testing, rats were anaesthetized deeply with sodium pentobarbital and transcardially perfused with a 0.9% isotonic saline solution followed by 10% formalin solution. Brains were removed and stored in 10 % formalin for several days followed by an overnight immersion in 10% sucrose solution. Brains were sliced into 45 mm sections, mounted and stained with a neutral red stain. Sections were examined under the microscope in order to determine placement of micro-injector tips.

Results:

Experiment 1: Interaction between SSS and intra-NAcc micro-injected μ opioid selective agonist induced consumption.

In the SSS choice paradigm, control rats (intra-NAcc saline) ate significantly less of whichever flavor of pellet had been available during the pre-feeding period. When DAMGO was given in the NAcc following pre-feeding, consumption of the sated food was increased more than the non-sated food eliminating the SSS effect (fig1a). One-way repeated measures ANOVA indicate that DAMGO significantly increased consumption [$F(1,29)=14.004$, $P<0.001$] and there is a significant drug × satiation interaction [$F(1,29)=4.460$, $P<0.05$] (fig 1a). *Post hoc* mean contrasts performed on the 90 minute time point indicated that there is a significant difference between consumption of the sated and non-sated foods only during saline administration. Furthermore, DAMGO significantly increases consumption of both the sated and non-sated foods ($P<0.05$) (fig 1b). While there were significant differences between consumption of chocolate and banana

pellets with saline and with DAMGO administration ($P<0.05$) (rats ate more chocolate pellets), there was no interaction between pharmacological manipulation and flavor (fig 1c). Further repeated measures ANOVA's indicate that the significant effect of DAMGO on consumption emerged by 15 minutes and the interaction between satiation and pharmacologic manipulation became significant after 45 minutes of testing.

t-tests of the DAMGO induced increases in consumption indicate that DAMGO increased consumption of the sated food significantly more than the non-sated food ($P<0.05$) but increased chocolate and banana consumption roughly equally (fig 1c). Similarly, the pattern of DAMGO induced increases in consumption of the sated and non-sated foods looks similar irrespective of which flavor is pre-fed (fig 1d). In this SSS paradigm, DAMGO selectively increased consumption of the pre-fed food but did not differentially increase consumption of either flavor.

Experiment 2: Interaction between SSS and DAMGO micro-infusion dorsally to the NAcc.

DAMGO 2 mm dorsal to our previous injections increased consumption only for the non-sated food and this effect did not appear until 75 minutes after micro-injection. One-way repeated measures ANOVA at the 90 minute time point indicated that DAMGO at this site significantly increased consumption [$F(1,15)=5.322$, $P<0.05$] and there were trends for a drug × satiation interaction [$F(1,15)=3.091$, $P<0.1$] and drug × satiation × flavor interaction [$F(1,15)=3.740$, $P<0.1$] (fig 5). *Post hoc* mean contrasts performed on the 90 minute time point indicated that there was a significant difference in consumption between saline and DAMGO conditions only for the non-sated food ($P<0.05$). Further repeated measures ANOVAs indicate that the significant effect of DAMGO on consumption emerged by 75 minutes and the trend for an interaction between satiation and pharmacologic manipulation appeared after 75 minutes of testing.

Experiment 3: Interaction between SSS and subcutaneous injection of morphine induced consumption.

Systemic morphine non-selectively increased consumption. One-way repeated measures ANOVA at the 90 minute time point indicated that systemic morphine increased consumption non-specifically [$F(1,29)=15.251$, $P<0.01$]. There were no drug × satiation or drug × flavor interactions (fig 4a, b). Further repeated measure ANOVA's indicate that the significant effect of morphine on consumption emerged by 30 minutes.

Experiment 4: Interaction between SSS and naltrexone micro-infusion into the NAcc.

Surprisingly, naltrexone increased consumption of the non-sated food at 15 minutes after injection. One-way repeated measures ANOVA at the 15 minute time point indicated that naltrexone significantly increased consumption [$F(1,43)=4.106$, $P<0.05$] and there is a significant drug × satiation interaction [$F(1,43)=5.891$, $P<0.05$] (fig 2a). *Post hoc* mean contrasts performed on the 15 minute time point indicated that there is a significant difference in consumption between saline and naltrexone conditions only for the non-sated food ($P<0.05$). These effects disappeared at later time points. At 30 minutes post injection, there was a trend for naltrexone to increase non-sated consumption and for there to be a drug × satiation interaction ($P<0.1$).

By the end of the testing session, however, a significant inhibitory effect of naltrexone on consumption of the sated food emerged. One-way repeated measures ANOVA at the 90 minute time point indicated significant drug × satiation [$F(1,43)=5.191$, $P<0.05$] (fig 2a) and drug × satiation × flavor [$F(1,43)=4.199$, $P<0.05$] interactions. *Post hoc* mean contrasts performed on the 90 minute time point indicated that there is a significant naltrexone induced decrease in consumption only for the sated food ($P<0.05$). This effect was primarily accounted for by a significant naltrexone induced decrease in consumption of chocolate when it is the sated food (fig 2b) ($P<0.01$).

Experiment 5: Interaction between SSS and subcutaneous injection of naltrexone.

Similar to its action in the NAcc, systemically administered naltrexone induced a preferential and late emerging reduction in chocolate intake when rats were pre-fed chocolate. In contrast to NAcc naltrexone, however, when given systemically, it also reduced chocolate intake when banana was the pre-fed flavor. One-way repeated measures ANOVA indicated that subcutaneous naltrexone significantly decreased consumption [$F(1,47)=11.160$, $P<0.01$] (fig 3a). There was a significant drug × flavor interaction [$F(1,47)=5.748$, $P<0.01$] but no significant drug × satiation interaction. This significant interaction was due to a larger effect of naltrexone on chocolate consumption (Fig. 3b). Further repeated measure ANOVA's indicate that the significant effect of systemic naltrexone on consumption emerged by 60 minutes and the significant interaction between naltrexone and flavor emerged by 75 minutes. In summary, systemic naltrexone decreased consumption equally for the sated and non-sated foods.

Experiment 6: Interaction between SSS and intra-NAcc DAMGO micro-injection with an introduced delay.

One-way repeated measure analysis of variance showed no significant effects of DAMGO (fig 6a) including no significant drug × flavor or drug × satiation interactions (fig 6b). In this paradigm, DAMGO had no effects on consumption.

Experiment 7: Interaction between SSS and intra-NAcc micro-injection of a $GABA_A$ selective agonist.

Unlike DAMGO, intra-NAcc muscimol increased consumption non-specifically with regard to which food was pre-fed. One-way repeated measures ANOVA indicate that muscimol significantly increased consumption [$F(1,33)=53.322$, $P<0.001$] and there was no significant drug × satiation interaction (fig7a). There was a significant drug × flavor interaction [$F(1,33)=8.70$,

P<0.005] due to a significantly larger effect of muscimol on chocolate consumption (fig 7b, c). Further repeated measures ANOVA's indicate that the significant effect of muscimol on consumption and the significant interaction between flavor and pharmacologic manipulation both emerged by 15 minutes post injection.

Discussion:

The main purpose of this study was to further elucidate the role of NAcc opioid signaling in flavor based choices of food consumption. We used a SSS paradigm to devalue specific flavors and determine how exogenous and endogenous opioids contribute to the orosensory reward value of food. Using this paradigm, MOP receptor agonism in the NAcc increases consumption of a pre-fed food such that the SSS effect (devaluation) of pre-feeding is eliminated. Although rats exhibit a natural preference for chocolate, the effect of DAMGO on satiety is similar irrespective of flavor.

The equally high consumption of the sated and non-sated flavors in the SSS paradigm following intra-NAcc DAMGO micro-injection is surprising since consumption of the pre-fed, devalued food is increased more than the non-pre-fed, and therefore non-devalued, food. This finding is at odds with the hypothesis that DAMGO increases consumption based on palatability or preference. The DAMGO enhancement of consumption of a pre-fed flavor is not due to a reduced ability to discriminate taste differences since our previous experiments have shown that rats maintain a clear preference for chocolate over banana pellets following DAMGO administration. Furthermore, in the SSS paradigm rats still prefer chocolate to banana pellets after DAMGO micro-injection.

A previous Fos (an immediate early gene whose expression is well correlated with neural activity) mapping study found that DAMGO increased consumption when micro-injected

throughout the NAcc in a broad gradient with ventral and lateral sites being the most effective[33]. The study also found that micro-injecting DAMGO into sites in the dorsal striatum was also effective at increasing consumption, albeit at higher doses. To investigate the specificity of the effects of intra-NAcc DAMGO micro-injection on consumption in a SSS paradigm, we micro-injected DAMGO 2 mm dorsal to our previous microinjections. This manipulation selectively increased consumption of the non-sated food. The fact that there was no increase in sated food consumption supports the importance of the NAcc for the present findings. Furthermore, there was no increase in consumption until 75 minutes post-injection. This is distinctly different from intra-NAcc DAMGO which significantly increases consumption by 15 minutes post-injection. This difference between the intra-NAcc and control micro-injections suggests that DAMGO has to diffuse farther to be effective when injected into the control region. Finally, one possible confound with the intra-NAcc DAMGO micro-injections is that the DAMGO could diffuse back along the cannulae tracks into the ventricle. This is unlikely to be the cause of the increased feeding in the present study since intracerebroventricular injections of DAMGO failed to increase consumption of bland chow at doses six times the current dose[34]. Also, our control injections (i.e. injections 2mm dorsal) address this concern since they are placed much closer to the ventricles. Any effects of DAMGO diffusing into the ventricles should be heightened in the control micro-injections. The fact that DAMGO micro-injected in this dorsal region had only minimal effects rules out this possibility.

The present findings and those of others have suggested a role for MOP receptor stimulation in taste preference conditioning. There is also substantial evidence, however, that injecting morphine (which has high affinity for the μ receptor) systemically following access to a novel taste will produce a robust conditioned taste aversion (CTA) in adult rats[35-38]. There are several important features, however, that make these CTA experiments less relevant to the present study. CTA induced by morphine, and drugs of abuse in general, is substantially different from CTA

induced by lithium chloride and other truly emetic agents[39, 40]. For example, pairing LiCl with sucrose both decreases the amount ingested and the number of positive facial displays in response to sucrose infusion, while pairing morphine with sucrose only decreases the amount ingested[39]. Furthermore, as opposed to LiCl induced CTA, morphine induced CTA requires multiple pairings (for review see [41]). This evidence has led several investigators to argue that CTA induced by drugs of abuse is fundamentally different from LiCl induced CTA. In particular, Patricia Grigson has proposed that the decreased consumption of a novel food which has been paired with morphine is not due to CTA at all, but is instead due to a reward comparison or negative contrast phenomenon[41]. In this view, animals decrease consumption of the morphine paired food not because the food becomes associated with some aversive outcome, but instead, because the food comes to predict morphine availability. Since morphine is so much more rewarding, the paired food becomes less rewarding by virtue of the contrast to morphine. As a result, animals preemptively decrease intake of the paired food. Another difference between LiCl and morphine induced CTA is much higher concentrations of morphine must be used to induce CTA than to induce odor preference (4-40 times as much) [35-38]. While low doses of morphine can robustly induce odor preference, in neonatal rats, high doses of morphine have also been shown to induce an aversion to paired odors[42, 43]. Similarly, in a study looking specifically at dose-response effects of i.p. μ agonists on taste conditioning in adult rats, an inverted U-shaped curve was found for morphine and other μ agonists; low doses induced a taste preference while high doses caused taste aversion[44]. This suggests the involvement of opioid receptors with different affinities in taste conditioning. Despite apparent conflict with previous findings, the aforementioned features of morphine induced taste and odor aversion allow for the possibility that intra-NAcc DAMGO plays a role in conditioning flavor preference.

An additional difference between the present study and previous studies implicating opioid agonists in CTA is the method of delivery. Clearly, systemic morphine injections that activate

opioid receptors throughout the body will have significantly different effects than selectively activating μ receptors within the NAcc. In order to explore the specificity of the DAMGO effect on consumption in the SSS paradigm, we repeated the SSS paradigm replacing intra-NAcc DAMGO microinjections with systemic morphine injections. We found that morphine significantly increased consumption non-specifically. Morphine increased consumption equally for chocolate and banana pellets as well as for sated and non-sated flavors. The discrepancy between the effects of intra-NAcc DAMGO and systemic morphine administration on consumption could be due to morphine's effects either at other receptor types besides μ (such as κ and δ) or at other sites in the brain (i.e. the amygdala or the hypothalamus) or even the periphery. Either way, the fact that systemic morphine administration does not selectively increase consumption of the sated food explains why previous studies using systemic morphine have found mixed effects of opioid modulation on taste conditioning.

The data from the SSS paradigm indicates that NAcc MOP agonists participate in the computation of flavor preference and rules out the possibility that NAcc MOP agonists simply enhance a predetermined flavor preference. Two possible mechanisms for the enhancement of preference for a recently consumed flavor by NAcc DMAGO are: 1) SSS is due to progressively decreasing taste-induced opioid release with repeated sampling of the same flavor. In this scenario, MOP agonist administration then washes out the differential opioid levels between the sated and non-sated flavors thereby rendering consumption of the two flavors equal. 2) DAMGO acts to enhance the reward value (i.e. reinforces) the immediately proceeding flavor in addition to its known enhancement of incentive to consume any palatable food items. In other words, micro-injecting DAMGO into the NAcc after pre-feeding with either flavor enhances the value of that flavor relative to all un-reinforced tastes including those that would have been preferred to it. Consequently, consumption levels are equalized between the sated (but reinforced) and non-sated (unreinforced) flavors.

While there is no direct evidence that opioid release in the NAcc changes as a food is eaten to satiety, two lines of experimentation support this hypothesis. Firstly, neurons in the caudolateral orbito-frontal cortex (OFC) (an area that has strong projections to the NAcc) of Macaques dynamically encode the reward value of smells, tastes, textures and visual cues. For example, OFC neural firing driven by consumption of a particular food decreases as that food is eaten to satiety but remains high to a food not eaten to satiety[1, 45]. This neural property is not found at earlier stages of the gustatory system such as the nucleus of the solitary tract or the primary gustatory cortex. This satiety-driven decrease in neural firing may contribute to SSS and could cause decreases in opioid release in the NAcc.

Secondly, presentation of a palatable food causes dopamine release in the ventral tegmental area (VTA)[46], the OFC and the NAcc[47]. Dopamine release in these latter two brain areas decreases for a food eaten to satiety, but not to a food not eaten to satiety. Given that non-selective opioid and D1 dopamine antagonists in the VTA block intra-NAcc DAMGO induced feeding, as well as blocking intra-VTA DAMGO induced feeding when injected into the NAcc [48, 49], a reduction in release of an endogenous MOP agonist in the NAcc could indirectly mediate the reduced DA release in the NAcc with satiety. Consistent with this idea, inactivation of the VTA with a locally microinjected GABA agonist does not affect feeding by itself, but it does block the increased feeding induced by DAMGO microinjection into the NAcc[50]. These data demonstrate that the VTA is critical for the feeding effects of opioid manipulations in the NAcc and are consistent with the idea that decreasing taste-induced opioid release contributes to SSS.

As an alternative to a reversal of a satiety effect, the relatively greater increase in consumption of the pre-fed flavor could be due to a conditioned preference for the flavor consumed just prior to DAMGO administration. MOP agonists can condition animals to prefer certain smells and

flavors. For example, in neonatal rats pairing intra-peritoneal (i.p.) morphine injections with saccharin intake increases saccharin consumption five days later[42]. Furthermore, a novel odor paired with i.p. morphine increases preference for that odor in a place preference paradigm[42]. Similarly, pre-training or pre-test naltrexone administration prevents the acquisition or expression, respectively, of a sugar and fat-conditioned odor preference[51]. In addition, injecting naltrexone 15 minutes prior to saccharin consumption prevents the development of a preference for this solution. This is not due to conditioned taste aversion since injecting naltrexone following access to saccharine has a much smaller effect on the development of a preference [52]. Finally, one study found that animals with repeated (3) DAMGO micro-injections into the NAcc but not the ventromedial or ventrolateral striatum showed increased consumption of chow on days when saline was micro-injected[53]. This effect was not observed when animals were micro-injected with saline on the first day of testing and DAMGO on the next day. This suggests that DAMGO micro-injection into the NAcc was causing a long-term change in feeding behavior that could possibly be due to a conditioned taste preference. These studies all support a role for opioid signaling in preference acquisition and expression which could explain some of the present findings.

To distinguish between these two hypotheses we investigated the effects of intra-NAcc naltrexone micro-injection during an SSS paradigm identical to the one used in previous experiments. If the first hypothesis is correct and repeated consumption is associated with a decrease in taste-induced opioid release, then intra-NAcc naltrexone micro-injection should selectively decrease consumption of the non-sated flavor. If, alternatively, opioid signaling in the NAcc reinforces consumption of the previously sampled food then intra-NAcc naltrexone should selectively decrease consumption of the sated flavor. In fact, we found that intra-NAcc naltrexone selectively decreased overall consumption of the sated food. The decrease was only significant when chocolate was the sated flavor. However, given the small quantities of banana pellet

consumed when banana is the sated food (saline treated animals consumed an average of 0.32 (±0.13) while naltrexone treated animals consumed 0.21 (±0.10) banana pellets in 90 minutes), this flavor specific consumption difference is probably due to a floor effect. This data supports the hypothesis that in the SSS paradigm opioid signaling in the NAcc reinforces preference for a food that has just been consumed, preventing the satiety induced flavor specific devaluation.

Surprisingly, we also found that naltrexone transiently, but significantly increased consumption of the non-sated food at the 15 minute time point. This effect may be due to naltrexone actions at other opioid receptors other than μ (such as κ and δ) or to an enhanced palatability contrast between the non-sated and sated flavors (i.e. a devaluation of the pre-fed flavor relative to the non-sated flavor). Alternatively, there are some reports that naltrexone can act as a partial agonist[54, 55]. Since most studies do not measure consumption at such short latencies following injection, these hypothesized early agonist effects could have previously been masked by slower acting antagonist effects. It is interesting to note that while consumption was increased by naltrexone at the 15 minute time point, naltrexone treated animals consumed very little during the remainder of the testing. Given that saline treated animals continued to eat at a steady rate, consumption levels between the groups for the non-sated food were equal by the 90 minute time point. This early increase followed by an overall decrease in feeding following naltrexone administration is consistent with the finding that systemic naltrexone administration actually *decreased* the latency to approach and initiate consumption of food while still accelerating the termination of feeding and thereby decreasing overall consumption[56].

Our experiments discussed so far support a role for opioid signaling in taste conditioning. Studies from the Bodnar laboratory, however, argue against a role of endogenous opioids in taste preference conditioning. In one study, different flavors were mixed into a preferred glucose solution and a less preferred saccharin solution. Animals were given ten days of alternating

access to these flavored solutions (five days of each) and were allowed to sham feed for 30 minutes (they had gastric fistulas that allowed the solutions to flow onto the ground and not enter the stomach). This training reliably induced a preference for the glucose paired flavor over the saccharin paired flavor when both were presented in mixed glucose-saccharin mixtures. Systemically administered naltrexone either before training trials or before testing trials failed to prevent this preference although it did significantly decrease overall consumption[57]. Similarly, rats will learn to prefer a flavor paired with an 8% fructose solution compared to a different flavor paired with a less preferred 0.2% saccharin solution. Systemic naltrexone also fails to prevent the acquisition or expression of this preference[58]. In contrast to naltrexone's failure to affect conditioned taste preference in this paradigm, dopamine antagonists block both the acquisition and expression of taste preferences conditioned by fructose[59] or sham feeding sucrose[60, 61]. As for why their results differ from the studies showing that naltrexone prevents development of taste preferences mentioned previously (i.e. [51]), Bodnar and colleagues correctly note that in those studies, naltrexone was only paired with one flavor thus allowing for the possibility of conditioning due to aversive properties of naltrexone distinct from its effects on opioid signaling.

There are several important differences between Bodnar's studies and the present study. Firstly, in all of Bodnar's studies post-ingestive consequences were minimized; either by using sham-feeding or fructose (intra-gastric fructose infusion fails to condition flavor preferences[62]) as compared to the present study where animals "real" fed on pellets that contain both carbohydrates and fat. This difference could be critical if it is the conditioning to the post-ingestive consequences of sugars and fats that is sensitive to opioid manipulation. Secondly, the time scale is different between the two sets of experiments. In Bodnar's experiments naltrexone was administered on training days and then the animal was tested for preferences on a separate day. In the present experiments, the effects of naltrexone were observed immediately by using a choice paradigm. It is possible that, in our paradigm, the conditioning effects of naltrexone, like

DAMGO (discussed later), are short lived. If that is the case, the effects seen in the present experiments illustrate the importance of opioid signaling in short-term maintenance of consumption. Finally and most importantly, while Bodnar's experiments always manipulated opioid signaling by systemically injecting naltrexone, the present study delivered naltrexone directly into the NAcc. There are opioid receptors throughout the brain[63] and periphery[64] and our results indicate that the delivery method is crucial for the observed behavioral effects.

We repeated the SSS paradigm but instead of micro-injecting naltrexone into the NAcc we administered naltrexone systemically. We found that systemic naltrexone decreased consumption independently of which flavor was pre-fed (i.e. sated and non-sated consumption were decreased equally). Intra-NAcc naltrexone micro-injection selectively reduced consumption of the pre-fed flavor in an identical paradigm. This discrepancy between systemic and intra-NAcc naltrexone must be due to activation of opioid receptors elsewhere throughout the brain and periphery. It illustrates that the conditioning effects we observed for intra-NAcc naltrexone are specific to the NAcc although the possibility exists that micro-injection of naltrexone into other brain regions may also induce taste conditioning. These findings might also explain why previous studies using systemic naltrexone failed to find consistent opioid induced taste conditioning. We also found that systemic naltrexone had a greater effect on consumption of chocolate than banana pellets. This is not surprising given the differential effects of naltrexone administration on foods of different palatability. Since chocolate is the more preferred flavor, this observation is consistent with the idea that endogenous opioid agonists enhance consumption of preferred flavors. This does not alter the finding, however, that systemic naltrexone decreased chocolate consumption both when it was the pre-fed and non-pre-fed flavor whereas intra-NAcc naltrexone only decreased chocolate consumption when it was the pre-fed flavor. In other words, even though there are flavor specific effects, intra-NAcc naltrexone selectively decreases consumption of the sated (pre-fed) food while systemic naltrexone does not.

An additional issue addressed in the current experiments is the role of NAcc GABA signaling in modulation of feeding. GABA agonists in the NAcc robustly increase feeding[30] but this feeding has several significant differences from opioid induced feeding. In order to examine the specificity of the opioid effects on feeding, we micro-injected muscimol into the NAcc in a SSS paradigm. Muscimol increased consumption non-selectively with regard to which flavor was pre-fed; consumption of the sated and non-sated foods was increased equally. This is distinct from the effects of intra-NAcc DAMGO preferentially increasing consumption of the pre-fed food. This discrepancy highlights important functional differences between activation of these two signaling systems. MOP receptor activation appears to be involved in palatability and relative taste preference, while $GABA_A$ receptor activation induces increased consumption without changing relative taste preference. Additionally, we found that following pre-feeding, muscimol increased consumption of chocolate pellets significantly more than banana pellets. This was unexpected given our previous results that, in the absence of pre-feeding, intra-NAcc muscimol increased consumption of both flavors equally. We do not currently have an explanation for this finding. Further research is needed.

There are several other differences between GABA and opioid agonist induced feeding. While intra-NAcc DAMGO increases breakpoint in a progressive ratio paradigm muscimol does not[65]. While intra-NAcc DAMGO increases consumption of both caloric (lard and sucrose) and non-caloric (saline and saccharin) palatable foods[21], intra-NAcc muscimol only increases consumption of caloric foods leaving saccharin and saline intakes unchanged[32]. Furthermore, muscimol did not alter the preference for fat or carbohydrate meals when presented together[32] which is different from DAMGO's selective increase of fat consumption in a similar paradigm[27]. These findings have been interpreted to indicate that muscimol induced feeding is not dependant

upon palatability or macronutrient composition. These results are in good agreement with the present finding that muscimol induced feeding is insensitive to SSS induced reward devaluation.

The primary output neuron of the NAcc is the GABAergic medium spiny neuron. These neurons exhibit extremely low spontaneous discharge rates; relying on excitatory inputs to drive them to threshold for firing. GABAergic inputs from interneurons and collaterals from other medium spiny neurons further modulate their firing patterns (for review see [66]). Opioid peptides presynaptically inhibit both glutamatergic and GABAergic synaptic transmission in the NAcc without altering resting membrane properties[67]. Despite the fact that both DAMGO and muscimol within the NAcc increase feeding, their behavioral effects are not identical. Differences in receptor distribution patterns, local microcircuit anatomy or cellular physiological effects must account for these differences. It is likely that the predominant effect of GABA agonists in the NAcc is inhibition of medium spiny neurons, whereas inhibition of GABAergic inputs by opioid agonists would likely disinhibit many medium spiny neurons.

Additional differences between GABA and opioid induced feeding are their distinct sites of action in the ventral striatum . Muscimol induced feeding is restricted to medial anterior sites within the shell of the NAcc[31, 32] while DAMGO can induce feeding when injected throughout the NAcc and the dorsal striatum (at higher doses)[33]. The muscimol sensitive region of the NAcc is the only portion of that nucleus that projects to the lateral hypothalamus, and medial hypothalamic nuclei show Fos expression after muscimol but not DAMGO micro-infusion into the NAcc[21].

It is also possible that the different drug effects are due to actions on different subsets of NAcc neuron. In fact, when rats have access to liquids of different palatability two distinct classes of NAcc neurons have been described[68]. One relatively large group of neurons showed inhibitions

preceding licking bouts that were unaltered by the palatability of the consumed liquid. Interestingly, these inhibitions began before the lick bout was initiated. Finally, the magnitude of these inhibitions were inversely correlated with the probability of unreinforced licking. These properties led to the proposal that the activity of these neurons inhibited licking behavior regardless of palatability or preference. A second class of neurons showed excitations that were correlated with the relative palatability of the solutions. One interesting possibility is that muscimol releases ingestive behavior non-selectively by inhibiting the first class of neurons (inhibitions), while DAMGO selectively disinhibits the second class of neuron and enhances their palatability driven excitations.

In order to investigate the time course of opioid induced taste conditioning we micro-injected DAMGO into the NAcc in a SSS paradigm with an introduced delay. In contrast to its effect when tested immediately after administration, by three hours the DAMGO enhancement of a pre-fed flavor was no longer observed. These results illustrate that MOP agonist induced taste conditioning is short-lived. Thus it appears that under the conditions of this experiment intra-NAcc DAMGO enhances the reward value of a flavor held in short term memory without a long term change in flavor preferences.

These short-term conditioning effects of opioids are consistent with a large body of evidence implicating opioid signaling specifically in meal maintenance (i.e. regulation of meal duration as opposed to meal initiation). In sham-feeding experiments where an animal is outfitted with an intragastric fistula that allows food consumption without gastric filling or nutrient absorption, opioid antagonists decrease consumption[69, 70](for review see [71]). However, this naltrexone induced decrease in consumption is not immediate; in one study 30 mls of sucrose had to be consumed (20 minutes of continuous drinking) before a change in lick rate was observed[70]. Furthermore, systemic naltrexone does not change the speed with which a rat will run down a

ramp for food or the latency to start eating but does decrease the rate and amount eaten after several trials[72]. Additionally, systemic naltrexone decreases meal length and extends the post-meal interval but does not affect meal frequency or feeding rate in freely-feeding rats[73]. Similarly, in a study of obese and lean Zucker rats trained to press a lever for food, systemic naltrexone decreased first meal size and duration although meal frequency was not effected[74]. In a different study, systemic naltrexone administration actually decreased the latency to approach and initiate consumption of food while still accelerating the termination of feeding[56]. These studies all support a role of opioid signaling in meal maintenance. The short-term conditioning effects of opioid modulation found in the present study may explain how opioid signaling affects meal maintenance.

There is overwhelming evidence that opioid signaling plays a more substantial role in non-homeostatic (incentive) feeding than in deprivation induced (hunger) feeding. Systemic naltrexone decreases consumption in non-deprived animals at significantly lower doses than in deprived animals. For example, systemic naloxone (another opioid antagonist similar to naltrexone) is more effective in reducing consumption of a sweet solution[29] or chocolate chip cookies[75] in less deprived animals than in more deprived animals. Furthermore, in ad libitum-fed rats, naloxone at doses as low as 0.3 mg/kg reduce consumption of cornstarch, sucrose or polycose. However, in chronically restricted animals (80% of free-feeding weight) naloxone failed to reduce cornstarch intake and only reduced Polycose intake at a dose of 3 mg/kg. Sucrose consumption was still reduced in food restricted rats. Since sucrose was the most preferred of these three solutions the authors conclude that behavioral sensitivity to opioid modulation depends on palatability and deprivation status. In a similar study, naloxone at doses as low as 0.3 mg/kg decreased consumption of regular chow in 24 and 48 hour deprived rats but failed to do so in chronically deprived rats[76]. Similar to the previous study, providing sweet chow increased the sensitivity to naloxone induced hypophagia in both deprived and non-deprived animals.

Additionally, when tested in a progressive ratio paradigm, naloxone showed a similar dose-deprivation relationship only decreasing breakpoint for non calorie restricted animals. Finally, systemic morphine decreases and increases consumption in food-deprived and non-deprived rats respectively at similar doses[77]. Taken together these findings illustrate that opioid modulation of feeding is strongly dependent upon energy status.

It is important to note that the effect of naltrexone on meal maintenance can be explained by a naltrexone induced decrease in the reward value of taste stimuli. Consequently, naltrexone induced decreases in consumption may require experiencing the taste of food while naltrexone is present in the relevant brain structures. In this situation, presumably, the same flavor would be experienced as less palatable. In support of this hypothesis, while naltrexone does not change the speed with which a rat will push a lever 80 times in order to receive the first pellet, it does change the rate of responding for subsequent pellets which were earned by three lever presses each[78]. This hypothesis would explain why measures of motivation prior to food sampling (i.e. latency to eat, approach speed) are not affected by systemic naltrexone, but meal duration and overall intake are.

The sensitivity of opioid signaling to deprivation status and food palatability, coupled with the finding that opioid antagonists specifically affect meal maintenance, raises the possibility that endogenous opioid release is in fact necessary for palatability driven feeding beyond immediate energy needs. Such a mechanism may be necessary to enhance consumption of relatively rare energy dense foods when these foods are available. The current finding that opioid signaling within the NAcc can produce short term changes in flavor preferences is consistent with this hypothesis. Normally, olfactory, gustatory and somatosensory (texture) features of a palatable food may cause opioid release. This release allows continued consumption of this food that may be independent of energy homeostatic (hypothalamic) needs. When DAMGO is micro-injected

into the NAcc following pre-feeding, this artificial stimulation mimics natural opioid release and promotes over-consumption of the food just eaten compared to the non-pre-fed food. When the DAMGO levels fall, however, the increased preference subsides.

Opioid agonists increase while antagonists decrease consumption of palatable foods. Furthermore, endogenous opioids are released following consumption of palatable items. The question concerning endogenous opioid signaling that remains unanswered is exactly which aspects of the taste experience are modulated by opioid stimulation. If the magnitude of endogenous opioid release following palatable food consumption determines its rewarding properties (as supported by antagonist studies), why do exogenous agonists selectively increase consumption of already palatable items and not all foods equally or even less palatable foods more than palatable foods? In other words, why are palatable items more sensitive to opioid manipulations? One possibility, which remains to be tested, is that opioid release both signals the reward value of a recently sampled taste and sensitizes the system to react more strongly to that taste in the future. This feed-forward loop could explain why antagonists prevent the development and expression of taste preferences while agonists enhance already established preferences. The present results support this hypothesis by demonstrating that intra-NAcc MOP receptor stimulation can condition a taste preference. Further testing is warranted to investigate these issues more extensively.

In conclusion, the present findings, in concert with our previous results, demonstrate that intra-NAcc DAMGO has at least three effects on consumption: 1) increases consumption of palatable foods, 2) selectively increases consumption of a more preferred flavor, and 3) increases consumption of a recently sampled palatable food. Specifically, MOP agonists transiently increase, while non-selective opioid antagonists decrease, consumption of a recently sampled food. These effects are not replicated by systemic opioid manipulations or intra-NAcc GABA

agonist injections which produce non-selective alterations in consumption. Pairing consumption of a flavor with subsequent MOP agonist release in the NAcc promotes continued consumption of food with that specific flavor during a bout of feeding. These data highlight the role of NAcc opioid receptors in flavor based decisions about food consumption when choice is available.

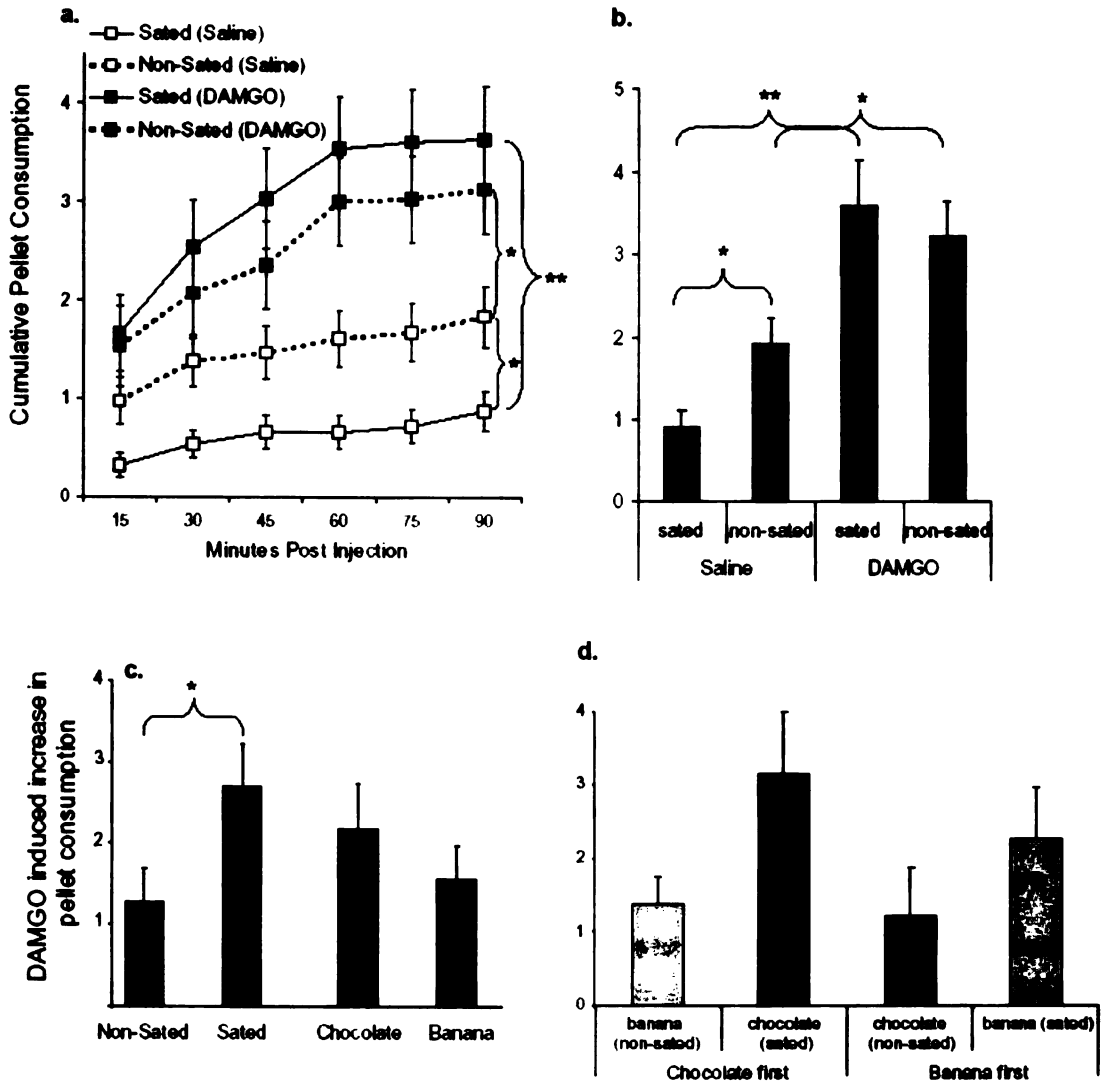

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Figure 1.

Effects of an intra-NAcc μ opioid agonist on consumption in a SSS paradigm. "Sated" represents the flavor which was pre-fed to the animal prior to micro-injection. "Non-sated" represents the flavor that was not pre-fed to the animal for all graphs. **a.** The cumulative number of pellets consumed following saline or DAMGO micro-injection is shown for each 15 minutes post injection. **b.** Bars indicate the total number of flavored pellets consumed following saline or DAMGO micro-injections at 90 minutes. **c.** DAMGO induced increases in consumption is shown. Bars indicate the total number of flavored pellets consumed following DAMGO minus the number consumed following saline micro-injections at 90 minutes. Chocolate and banana consumption are averaged across different sated and non-sated conditions i.e. when chocolate is pre-fed and when banana is pre-fed. **d.** Intra-NAcc opioid effects displayed by which flavor is pre-fed. Bars indicate number of flavored pellets consumed at 90 minutes following DAMGO minus the number consumed following saline micro-injections. The two columns in the "Chocolate first" section come from trials where chocolate was the pre-fed food. The two columns in the "Banana first" section come from trials where banana was the pre-fed flavor. Labels in brackets indicate which flavor was sated. Brown and yellow colors denote chocolate and banana pellet consumption respectively. (**) $p<0.01$, (*) $p<0.05$, (†) $p<0.1$ for all graphs.

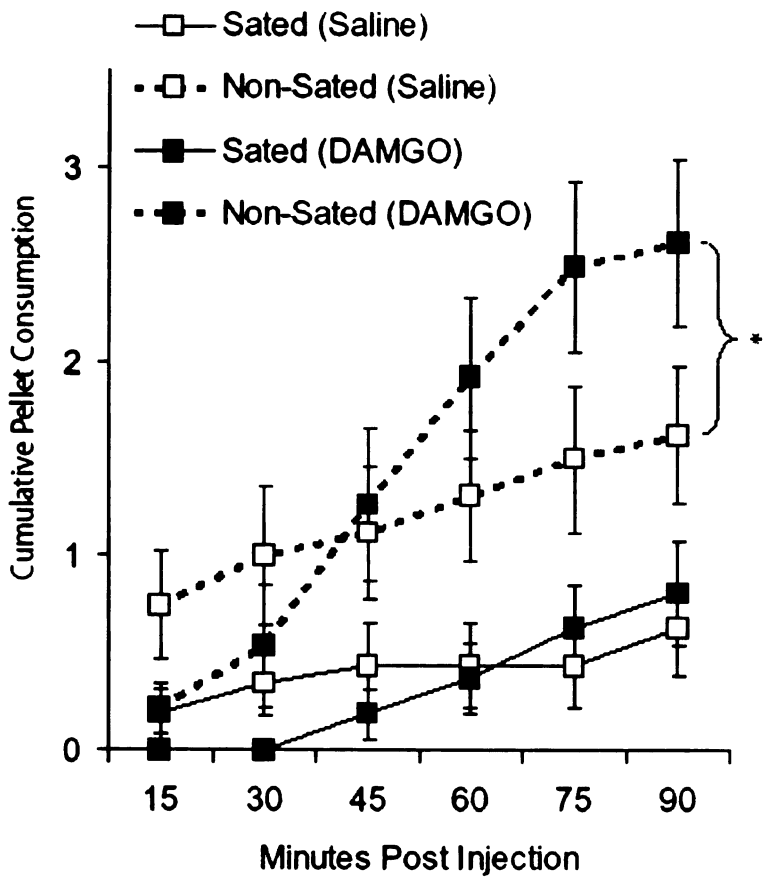

Figure 2.

Effect of DAMGO micro-injection 2 mm dorsal to previous micro-injections on consumption in a SSS paradigm. The cumulative number of pellets consumed following saline or DAMGO micro-injection is shown for each 15 minutes post injection.

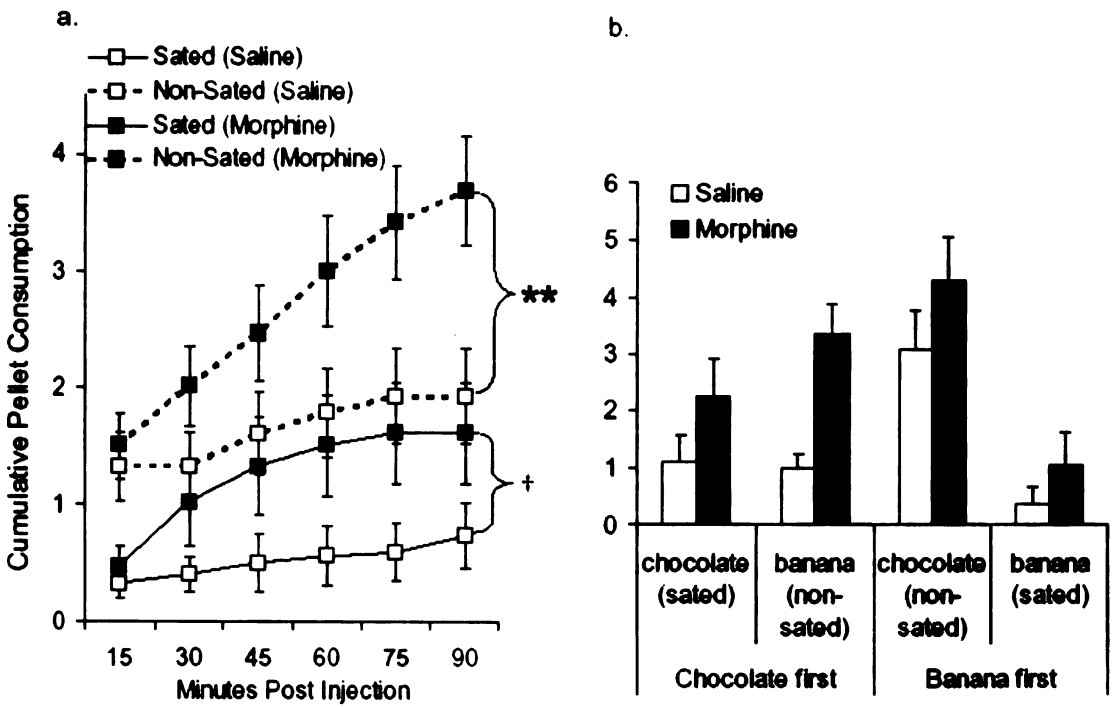

Figure 3.

Effect of systemic morphine on consumption in a SSS paradigm. **a.** The cumulative number of pellets consumed following saline or morphine subcutaneous injection is shown for each 15 minutes post injection. **b.** Systemic morphine effects displayed by which flavor is pre-fed. Bars indicate cumulative number of flavored pellets consumed at 90 minutes following saline or morphine subcutaneous injection. The two columns in the "Chocolate first" section come from trials where chocolate was the pre-fed food. The two columns in the "Banana first" section come from trials where banana was the pre-fed flavor. Labels in brackets indicate which flavor was sated.

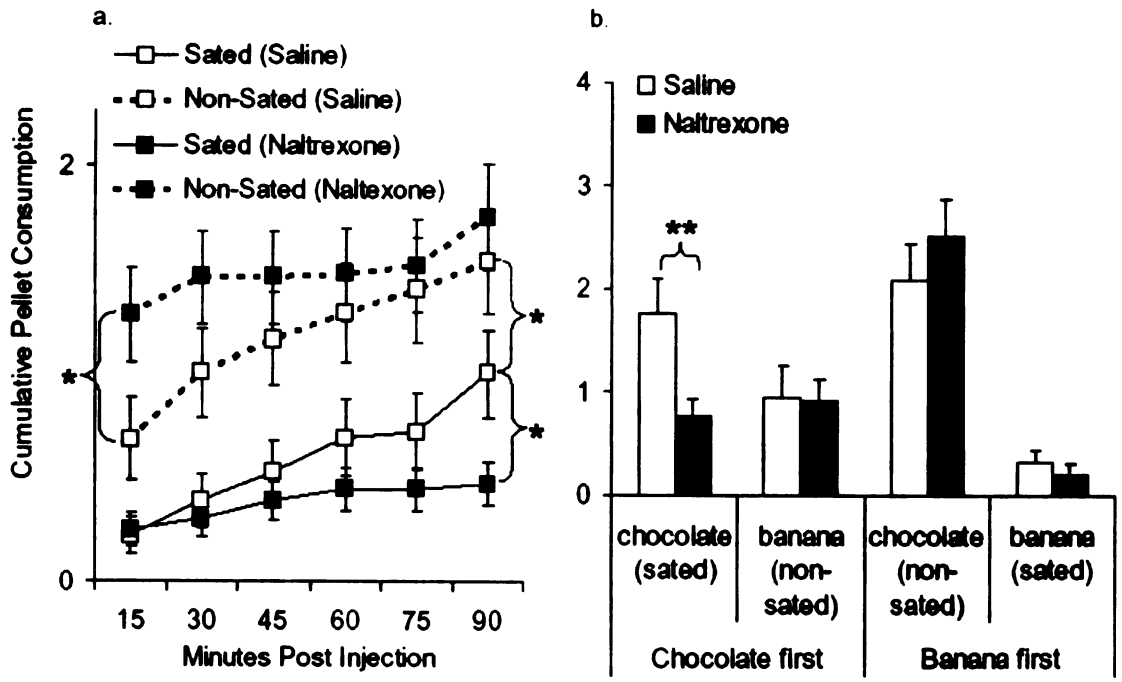

Figure 4.

Effects of intra-NAcc opioid antagonism on consumption in a SSS paradigm. **a.** The cumulative number of pellets consumed following saline or naltrexone micro-injection is shown for each 15 minutes post injection. **b.** Intra-NAcc naltrexone effects displayed by which flavor is pre-fed. Bars indicate cumulative number of flavored pellets consumed at 90 minutes following saline or naltrexone micro-injections. The two columns in the "Chocolate first" section come from trials where chocolate was the pre-fed food. The two columns in the "Banana first" section come from trials where banana was the pre-fed flavor. Labels in brackets indicate which flavor was sated.

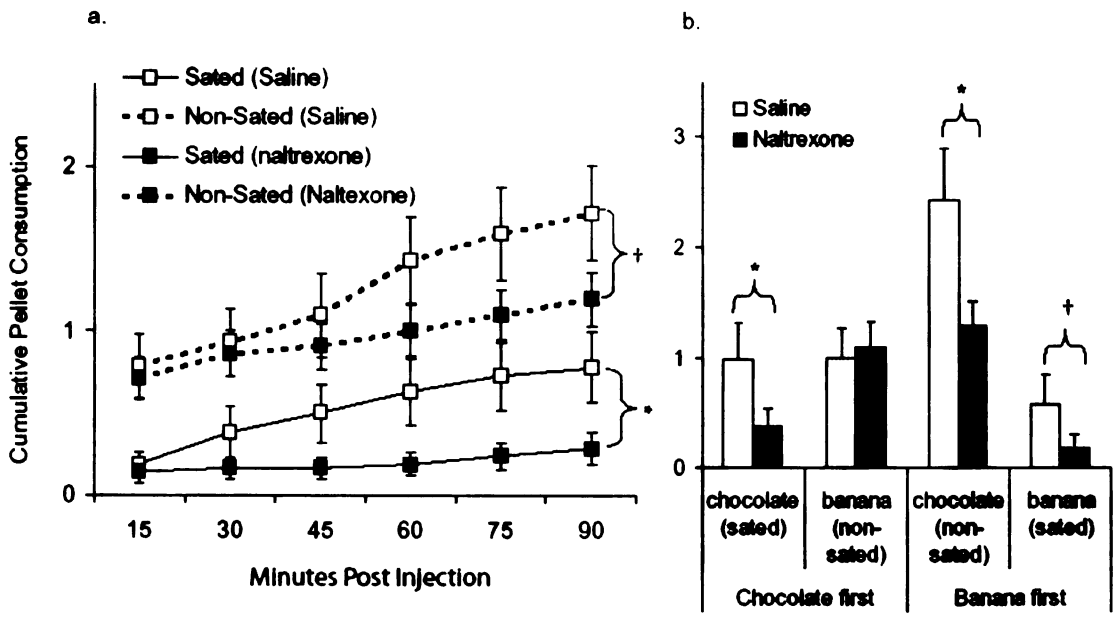

Figure 5.

Effects of systemic opioid antagonism on consumption in a SSS paradigm. **a.** The cumulative number of pellets consumed following saline or naltrexone subcutaneous injection is shown for each 15 minutes post injection. **b.** Systemic naltrexone effects displayed by which flavor is pre-fed. Bars indicate cumulative number of flavored pellets consumed at 90 minutes following saline or naltrexone subcutaneous injection. The two columns in the "Chocolate first" section come from trials where chocolate was the pre-fed food. The two columns in the "Banana first" section come from trials where banana was the pre-fed flavor. Labels in brackets indicate which flavor was sated.

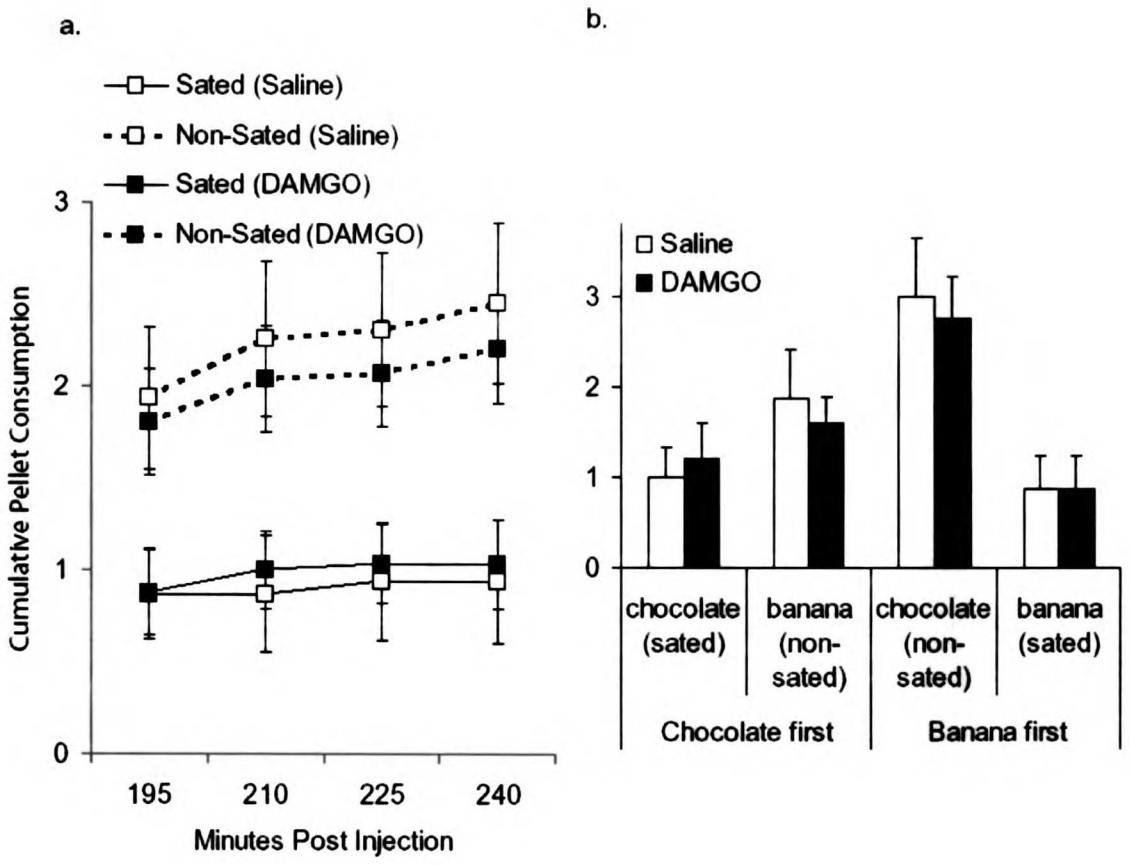

Figure 6.

Effects of an intra-NAcc μ opioid agonist on consumption in a SSS paradigm with an introduced delay. **a.** The cumulative number of pellets consumed following saline or DAMGO micro-injection is shown for each 15 minutes after flavored pellets were introduced to the cage. There was a 180 minute delay between micro-injection and pellet access. **b.** Intra-NAcc DAMGO effects displayed by which flavor is pre-fed. Bars indicate cumulative number of flavored pellets consumed at 240 minutes (180 minute delay plus 60 minutes of pellet access) following saline or DAMGO micro-injections. The two columns in the "Chocolate first" section come from trials where chocolate was the pre-fed food. The two columns in the "Banana first" section come from trials where banana was the pre-fed flavor. Labels in brackets indicate which flavor was sated.

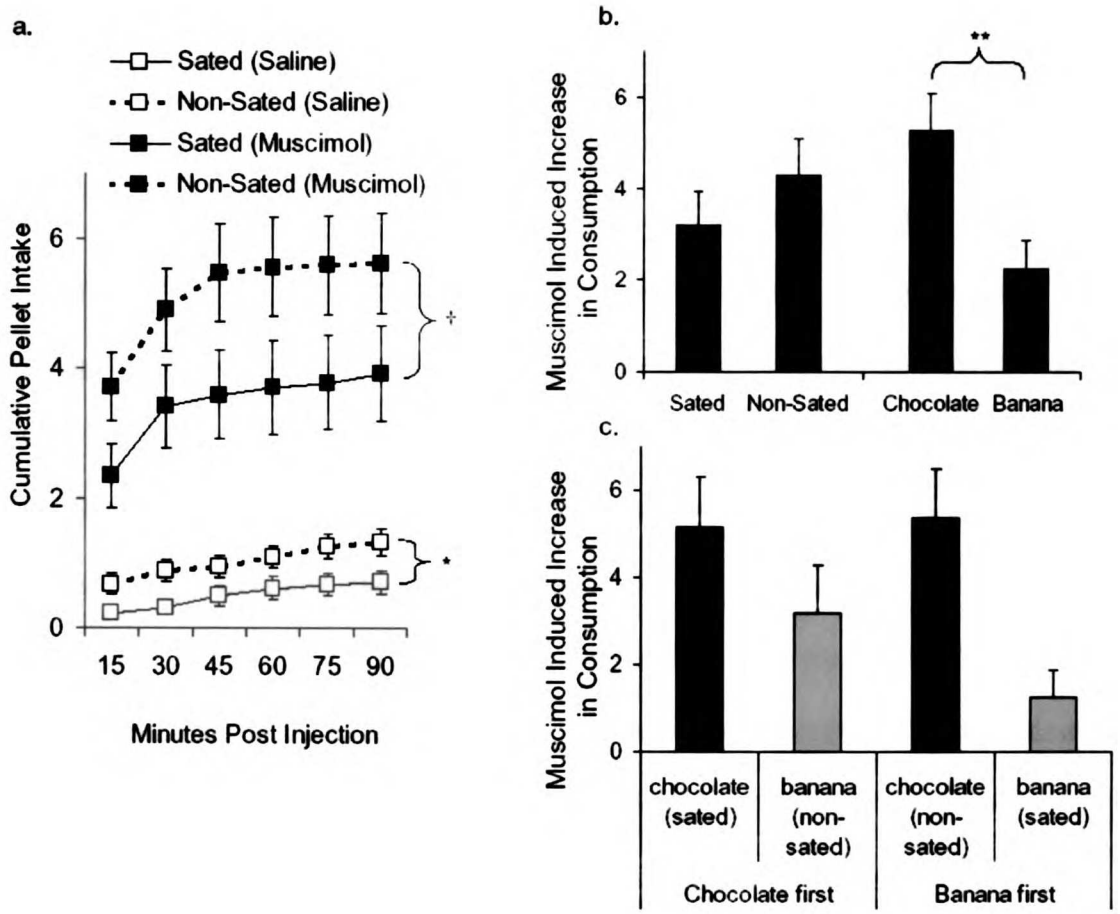

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Figure 7.

Effects of an intra-NAcc $GABA_A$ receptor agonist on consumption in a SSS paradigm. **a.** The cumulative number of pellets consumed following saline or muscimol micro-injection is shown for each 15 minutes post injection. **b.** Muscimol induced increases in consumption is shown. Bars indicate the total number of flavored pellets consumed following muscimol minus the number consumed following saline micro-injections at 90 minutes. Chocolate and banana consumption are averaged across different sated and non-sated conditions i.e. when chocolate is pre-fed and when banana is pre-fed. **c.** Intra-NAcc muscimol effects displayed by which flavor is pre-fed. Bars indicate number of flavored pellets consumed at 90 minutes following muscimol minus the number consumed following saline micro-injections. The two columns in the "Chocolate first" section come from trials where chocolate was the pre-fed food. The two columns in the "Banana first" section come from trials where banana was the pre-fed flavor. Labels in brackets indicate which flavor was sated. Brown and yellow colors denote chocolate and banana pellet consumption respectively.

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Chapter 3

Opposing regulation of satiety by Mu and Kappa Opioids

Introduction

Flavor information guides decisions about food consumption that are critical for survival. In the setting of choice, the palatability of a food item (i.e., the reward value of a food, as signaled by orosensory cues[1]) is essential to decision-making (e.g. what and how much of an item will be consumed), but the neural mechanisms underlying food preference are poorly understood. The opioid system is critical for the rewarding action of palatable foods. μ opioid receptor agonists induce robust feeding in the rat[2] by increasing the palatability of food[3]. Accordingly, in humans, non-selective opioid antagonists reduce the positive hedonic effect of food but leave taste recognition thresholds unaffected[4].

The NAcc is a critical site for opioid actions on palatability. It contains high densities of both μ opioid (MOP) and κ opioid (KOP) receptors as well as enkephalinergic and dynorphinergic fibers[5, 6]. Micro-injection of D-Ala^2,N,Me-Phe^4,Gly-ol^5-enkephalin (DAMGO, a MOP receptor selective agonist) into the NAcc selectively increases consumption of palatable items[7] leaving consumption of chow and water unchanged[8]. Additionally, opioid antagonists in the NAcc reduce consumption of palatable foods, indicating that endogenous opioid release modulates feeding[9]. We have previously shown that MOP agonists within the NAcc can condition taste preferences but the effect of KOP agonists at this site is unclear. KOP antagonists in the NAcc can alter consumption of under certain conditions but the range of effects differs from those of MOP antagonists (e.g. both MOP and KOP antagonists reduce deprivation and glucoprivic induced feeding but only MOP antagonists reduce consumption of sucrose in non-deprived rats)[10]. On the other hand, KOP agonist action within the NAcc reportedly does not alter

consumption of chow or a sucrose solution[11-13]. Furthermore, KOP agonists often have behavioral actions that are quite different than MOP agonists when injected into the same brain region[14]. To address this issue, we used a short term flavor conditioning paradigm to compare the actions of MOP and KOP agonists injected in the NAcc on the reward value of specific tastes.

Methods

Animals: A total of 33 male rats (Long Evans, Charles River Laboratories, Wilmington, MA) weighing between 270 and 450 g were used in the present studies. Animals were individually housed in conventional hanging cages in a temperature- and humidity-controlled room on a 12:12 hour light:dark cycle. Animals had *ad lib* access to water at all times and *ad lib* access to chow at all times except during testing.

Surgery: Animals were anaesthetized with isoflorane, their heads placed in a stereotaxic device and then following a small craniotomy, bilateral guide cannulae were stereotactically placed and then secured to the skull with stainless steel screws and dental cement. Coordinates for the target sites were 1.5 mm anterior, 1.1 mm lateral and 5.5 mm ventral from Bregma. For this study, the cannulae were not directed specifically at the core or the shell regions of the NAcc. For control microinjections, coordinates for the target sites were 1.5 mm anterior, 1.1 mm lateral and 3.5 mm ventral from Bregma. Animals were allowed 4 days recovery post surgery.

Drugs and injections: For micro-injections, U50488, the selective KOP receptor agonist was obtained from Sigma Pharmaceuticals. U50488 was dissolved in 0.9% sterile saline (3.25 μg per side). First, the stylet was removed from the guide cannulae and the injector cannulae were inserted. The injector cannulae protruded 2.2 mm past the end of the guide cannula for a final distance of 7.7 mm ventral to Bregma. U50488, in a volume of 0.5 μl of saline, was infused through 12.5 mm injector cannulae connected to a microdrive pump by polyethylene tubing. The

rate of infusion was 0.25 μl/min. The injector cannulae remained in place an additional minute after the infusion in order to allow for diffusion. Injectors were then removed and the stylets were replaced.

Behavioral testing and experimental design: After recovery from surgery (four days), animals were extensively handled. In order to overcome taste neophobia, rats were brought into the testing room on four separate days and given one hour simultaneous access to both flavors of pellets (chocolate and banana). After this initial exposure, all rats avidly consumed the pellets when available. The two types of flavored 1 gram pellets were made from the same meal substrate and were thus matched for all macro- and micro- nutrients (bio-serv, Frenchtown, New Jersey). Table 1 shows the composition of the flavored pellets. Pellets were always delivered in test tube dispensers. Rats were required to grab the pellets with their teeth and forcibly remove them from a hole in the bottom of the tube. This small amount of effort encouraged the rats to take only what they would eat and greatly facilitated consumption quantification. Every fifteen minutes post injection, the number of pellets remaining in the dispenser was counted and a visual inspection of the cage for dropped pellets was made.

To determine whether U50488 affects consumption when rats are allowed to choose between flavors, rats (n=9) were microinjected with U50488 or saline and given 1.5 hour simultaneous *ad lib* access to both chocolate and banana flavored pellets. All rats underwent both conditions and injection and flavor orders were randomized.

To determine whether intra-NAcc U50488 will alter consumption of a flavor to which the rat has just been exposed, a sensory specific satiety (SSS) paradigm was used. Rats (n=16) were given one hour *ad lib* access to either banana or chocolate pellets. At the end of this hour, rats were micro-injected with either U50488 or saline. Rats were then given 1.5 hour simultaneous *ad lib*

access to both chocolate and banana flavored pellets. All rats underwent all four conditions and injection and flavor orders were randomized. As a site control to confirm that the KOP receptors within the NAcc are critical for the effects of U50488 on SSS, U50488 was injected 2 mm dorsal to the injection site within the NAcc (n=8) using the same behavioral paradigm.

Results

Similar to previous reports, in the absence of pre-feeding, U50488 had no effect on *ad lib* consumption of either flavor (fig 1a, b). In contrast, in the SSS paradigm, KOP agonist micro-injection in the NAcc selectively increased consumption of whichever flavor had not been pre-fed to the rats. One-way repeated measures ANOVA indicated that U50488 significantly increased consumption [$F(1,31)=10.601$, $P<0.005$] and there is a significant drug × satiation interaction [$F(1,31)=8.085$, $P<0.01$] (fig 2a). *Post hoc* mean contrasts performed on the 90 minute time point indicated that U50488 significantly increased consumption of only the non-sated foods ($P<0.001$) (fig 2b) irrespective of which flavor was pre-fed (fig 2c). Further repeated measures ANOVA's indicate that the significant effect of U50488 on consumption emerged by 15 minutes and the interaction between satiation and pharmacologic manipulation became significant after 30 minutes of testing.

Since ICV KOP agonists increase consumption, one possible confound of the present study is leakage of U50488 back along the guide cannulae into the ventricles. In order to control for this possibility, we implanted cannulae 2 mm dorsal to our previous micro-injections. Micro-injecting U50488 at this control site had minimal effects on consumption. Repeated measures ANOVA on the 90 minute time point showed no significant effects of U50488 (fig 3). There was a trend for a drug × satiation interaction ($p<0.1$). This trend was due to a significant drug induced increase in consumption of the sated food ($p<0.05$). Repeated measures ANOVA on the 75 minute time

point indicated that there was a trend for a main effect of drug ($p<0.1$). Further repeated measures ANOVA's indicated that the trend for the interaction emerged by 75 minutes while the trend for a U50488 induced increase in consumption emerged by 45 minutes. In contrast, when micro-injected into the NAcc, U50488 increased consumption significantly after only 15 minutes. Since the control injections were closer to the ventricles, these behavioral differences rule out the possibility of significant effects of U50488 at other brain sites when injected into the NAcc. Finally, the control micro-injections tended to increase consumption of the sated food, which is opposite to the selective increase in non-sated food consumption after intra-NAcc U50488 micro-injections. This discrepancy further supports the specificity of this effect.

Comparison of the effects of MOP and KOP receptor activation on consumption in a SSS paradigm.

Data from a previous study looking at the effects of micro-injecting DAMGO (a MOP receptor agonist) into the NAcc on consumption in a SSS paradigm identical to the one used in the present study is shown for comparative purposes (fig 4a). DAMGO selectively increases consumption of pre-fed (sated) food. Although both intra-NAcc U50488 and DAMGO increased overall consumption of food pellets compared to saline following a period of pre-feeding, they had opposing effects on flavor preference; DAMGO increases, while U50488 decreases, preference for the pre-fed flavor. In other words, intra-NAcc DAMGO significantly decreases while U50488 significantly increases SSS (fig 4b).

Discussion

Pre-feeding with a given flavor produces a reduction of palatability specific for that flavor leading to increased preference for an alternative flavor when a choice is available. We have previously shown that intra-NAcc DAMGO reverses this effect so that rats preferentially consume more of the pre-fed food. In contrast, in the present study, intra-NAcc micro-injection of the KOP

receptor agonist U50488 enhances the SSS effect of pre-feeding so that rats preferentially consumed the food they had not previously experienced. Additionally, in the absence of pre-feeding, unlike DAMGO, micro-injection of U50488 into the NAcc did not increase consumption. These results suggest that KOP receptor agonists in the NAcc have a taste conditioning effect on flavor preference that is opposite to that of MOP agonists.

Systemic and intracerebroventricular (ICV) administration of KOP receptor agonists increase[15-18], and antagonists decrease[19, 20], feeding in the rat and other animals (for review see[21]). Given these robust effects on consumption it is tempting to attribute them to KOP receptor effects on palatability. However, systemic KOP receptor agonists have mixed effects on consumption of sucrose, increasing consumption of high sucrose concentrations and decreasing consumption of low concentrations[22, 23]. Additionally, ICV injection of the KOP agonist U50488 fails to increase consumption of palatable saline or saccharin solutions, whereas MOP receptor agonists are effective for these items[24]. Because of these and other findings[25-28] (e.g. systemic U50488 significantly and selectively increases consumption of bland ground chow leaving sucrose consumption unchanged[25]) systemic KOP receptor activation has been proposed to increase feeding by decreasing satiation (i.e. delayed meal termination), not by increasing palatability.

Investigations of the distribution of KOP agonist sensitivity has revealed that many sites where MOP agonists potently increase feeding are insensitive to KOP receptor activation. For example, intra-ventral tegmental area U50488 micro-injections fail to affect feeding[29]. Dynophin (Dyn, an endogenous KOP agonist) injection into the nucleus of the solitary tract[30] or the amygdala[31] did not affect consumption of chow while DAMGO micro-injections at these sites robustly increase feeding. On the other hand, Dyn micro-injected into the paraventricular and ventromedial hypothalamic nuclei, but not the globus pallidus, striatum or lateral hypothalamus, did increase intake of chow[31, 32]. Furthermore, lesions of the globus pallidus and striatum attenuate systemic

ketocuclazocine (a KOP receptor agonist) induced feeding[33] indicating that these sites are important for KOP receptor mediated feeding. However, given the connections between the hypothalamus and the NAcc, these results do not prove that the striatum is a target for KOP agonist action since the striatum may only be a necessary downstream node in the feeding circuit)[34]. In summary, KOP receptor agonists induce feeding most potently in the medial hypothalamus but have been reported to be relatively inactive at other brain sites.

We found that intra-NAcc U50488 increases consumption in a choice paradigm but only when rats have been pre-fed. The lack of U50488 effects on feeding in the absence of pre-feeding is in agreement with previous studies showing intra-NAcc KOP receptor agonists generally fail to increase consumption[11-13, 35]. For example, U50488 and bremazocine (a KOP receptor agonist) micro-injected into the NAcc were completely ineffective[11, 12] while Dyn only increased consumption of bland chow at high concentrations (10 nmol)[11]. Importantly, neither intra-NAcc U50488 nor Dyn altered consumption of a sucrose solution[13]. Intra-NAcc nor-binaltorphimine (a KOP receptor antagonist) also failed to change consumption of chow in food-deprived animals or sucrose in non-deprived animals[35]. These results suggest that behavioral context and orosensory properties of the available food are critical for opioidergic effects. In particular, it appears that a taste must be temporally paired with KOP agonist action in the NAcc in order to have an effect on consumption.

MOP and KOP receptors within the NAcc have opposing roles in several paradigms. For example, systemic MOP agonists increase dopamine release from the NAcc and are reinforcing while KOP agonists decrease dopamine release from the NAcc[25, 36] and are aversive[14]. Similar effects on intra-NAcc dopamine release are also found when these agonists are directly applied to the NAcc[37]. Additionally, in the NAcc, MOP agonists promote, while KOP agonists antagonize,

capsaicin induced antinociception and KOP receptor agonists completely block the ability of MOP agonists to promote antinociception in the NAcc[38].

We have previously demonstrated that the MOP receptor agonist DAMGO, when injected into the NAcc, selectively increased consumption of a food flavor that had just been consumed in a pre-feeding period. This effect was not reproduced by systemic morphine or control DAMGO micro-injections in the dorsal striatum indicating that the NAcc is critical for the effect. Furthermore, naltrexone in the NAcc selectively decreased consumption of the pre-fed food leaving consumption of the non-sated food unaffected. In contrast, systemic naltrexone decreased consumption non-selectively. Finally, if after pre-feeding, a 3 hour delay is introduced between micro-injection of DAMGO and simultaneous access to chocolate and banana pellets, DAMGO has no effects on consumption. These results illustrate that MOP agonists within the NAcc can condition taste preferences that are transient. Taken together with the present results, intra-NAcc DAMGO decreases while U50488 increases SSS. These findings point to a novel opposing role of MOP and KOP receptors within the NAcc on taste conditioning.

In conclusion, our previous work has shown that the MOP agonist DAMGO in the NAcc enhances the reward value (i.e. reinforces) the immediately proceeding flavor enhancing its reward value relative to other tastes. In contrast, U50488 in the NAcc reduces the preference for the pre-fed flavor, thus enhancing SSS as measured by the increased consumption of the relatively novel non-pre-fed flavor. Furthermore, as reported by others and unlike its effects in the SSS paradigm, U50488 has no effect on overall consumption or flavor preference in the absence of pre-feeding. These data point to the importance of pairing opioid stimulation immediately following consumption of food with a particular flavor and highlights the robust and opposing role of NAcc MOP and KOP opioid receptors in flavor reinforcement.

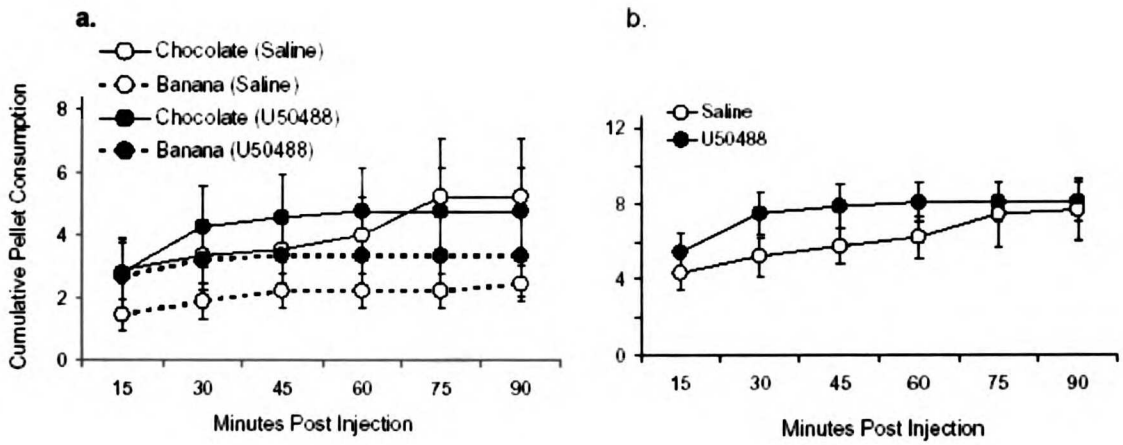

Figure 1.

Effect of intra-NAcc KOP receptor agonist on consumption in a choice paradigm. **a.** The number of flavored pellets consumed following saline or U50488 micro-injection is shown for each 15 minutes post injection. Since both flavors were available after micro-injection, closed circles represent data from one test session while open circles represent data from a separate test session. **b.** Total cumulative consumption (chocolate plus banana) following micro-injection of saline and U50488 is shown.

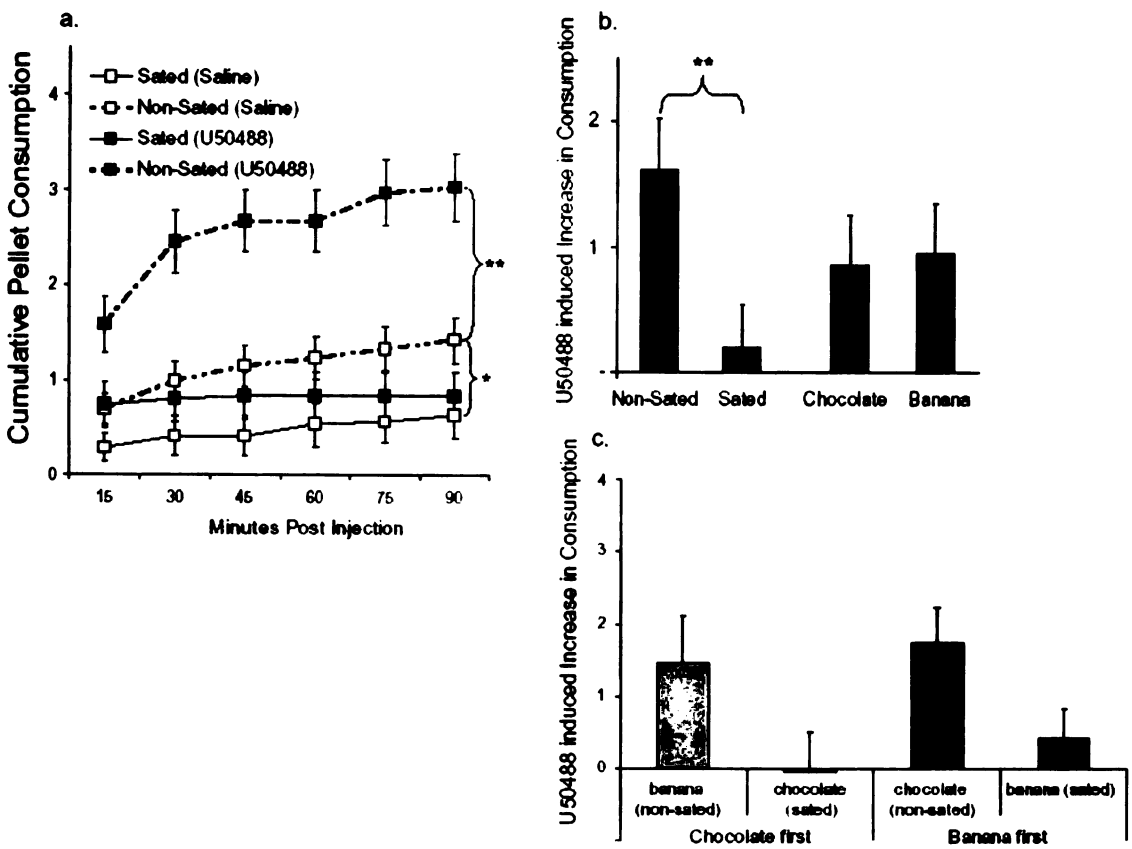

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Figure 2.

Effects of KOP receptor agonist on consumption in a SSS paradigm. "Sated" represents the flavor which was pre-fed to the animal prior to micro-injection. "Non-sated" represents the flavor that was not pre-fed to the animal for all graphs. **a.** The cumulative number of pellets consumed following saline or U50488 micro-injection is shown for each 15 minutes post injection. **b.** U50488 induced increases in consumption is shown. Bars indicate the total number of flavored pellets consumed following U50488 minus the number consumed following saline micro-injections at 90 minutes. Chocolate and banana consumption are averaged across different sated and non-sated conditions i.e. when chocolate is pre-fed and when banana is pre-fed. **c.** Intra-NAcc opioid effects displayed by which flavor is pre-fed. Bars indicate number of flavored pellets consumed at 90 minutes following U50488 minus the number consumed following saline micro-injections. The two columns in the "Chocolate first" section come from trials where chocolate was the pre-fed food. The two columns in the "Banana first" section come from trials where banana was the pre-fed flavor. Labels in brackets indicate which flavor was sated. Brown and yellow colors denote chocolate and banana pellet consumption respectively. (**) $p<0.01$, (*) $p<0.05$.

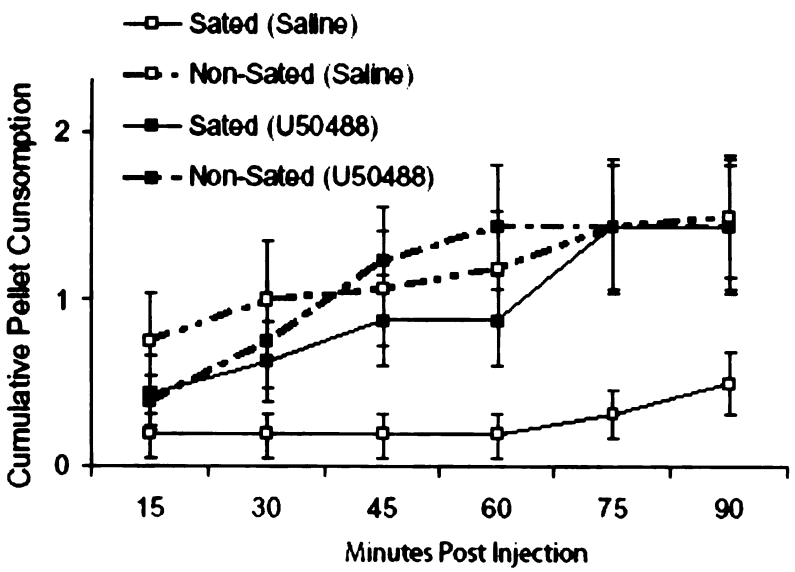

Figure 3.

Effect of U50488 micro-injection 2 mm dorsal to previous micro-injections on consumption in a SSS paradigm. The cumulative number of pellets consumed following saline or U50488 micro-injection is shown for each 15 minutes post injection.

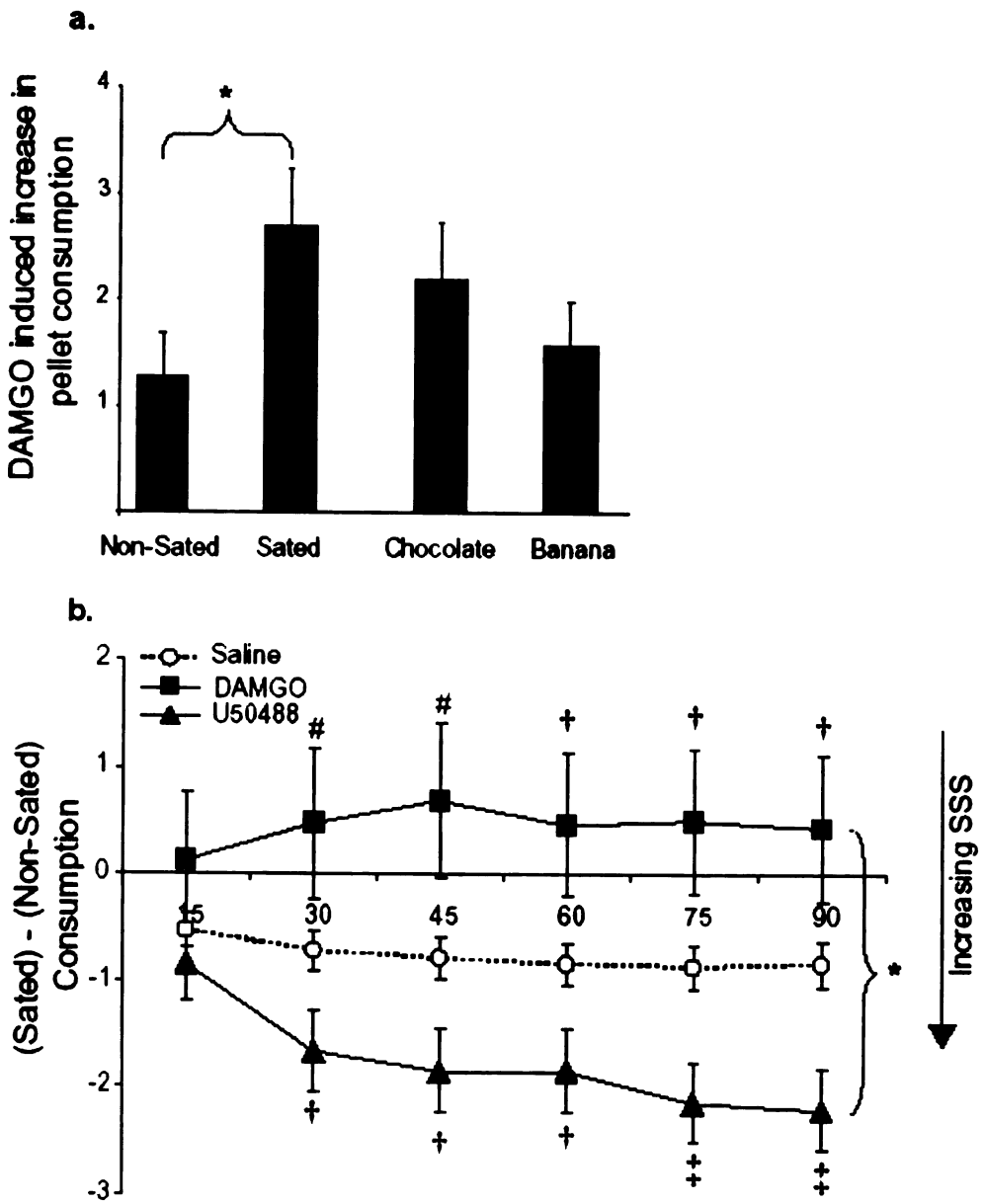

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Figure 4.

Effects of MOP and KOP receptor agonists in a SSS paradigm. **a.** DAMGO induced increases in consumption is shown. Bars indicate the total number of flavored pellets consumed following DAMGO minus the number consumed following saline micro-injections at 90 minutes. Chocolate and banana consumption are averaged across different sated and non-sated conditions i.e. when chocolate is pre-fed and when banana is pre-fed. **b.** DAMGO decreases while U50488 increases the magnitude of FSS. The difference between sated and non-sated consumption is shown for DAMGO, U50488 and saline. A negative value denotes greater relative consumption of the non-sated food. (‡) $p<0.005$, (†) $p<0.01$, (#) $p<0.05$ all compared to saline.

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Chapter 4

Neural Correlates of Eating Disturbances in Patients with Dementia

Preliminary Findings

There is extensive research in animals and humans implicating the orbito-frontal cortices (OFC) in the regulation of palatability In particular, neurons in the caudolateral OFC of Macaques have been shown to dynamically encode the reward value of smells, tastes, textures and visual cues. For example, OFC neural firing driven by consumption of a particular food decreases as that food is eaten to satiety but remains high to a food not eaten to satiety. This neural property is not found at earlier stages of the gustatory system such as the nucleus of the solitary tract or the primary gustatory cortex[1]. This satiety-driven decrease in neural firing may be the neural substrate of sensory specific satiety (SSS).

Patients with Fronto-Temporal Dementia (FTD), a slowly progressing neurodegenerative disease that affects the frontal (including OFC) and temporal cortices, have been reported to exhibit a wide range of behavioral abnormalities including disinhibition, apathy, loss of insight, and mood changes. Anecdotal and questionnaire studies have indicated FTD patients have particularly striking abnormalities in eating behaviors [2-4]. In particular, carbohydrate cravings have been found in FTD patients (in over 60% of FTD patients compared to 11% of Alzheimer's disease (AD) patients in one study)[2-5]. There are at least two clinical variants of FTD with distinct patterns of brain atrophy, frontal variant FTD (FvFTD) and temporal variant FTD (TvFTD). Patients with FvFTD have been reported to have significantly higher incidences and earlier onset of problems with appetite change, food preference, eating habits and other oral behaviors compared to patients with TvFTD and AD[5]. Another study reported gluttony and indiscriminate eating as a prominent feature of FvFTD, while eating inedible objects was much more common in TvFTD.[2]

Given the differences in the patterns of atrophy and eating disturbances between the two subtypes of FTD and AD,[6] studying feeding behavior and quantifying brain atrophy in these patients presents a unique opportunity to study the neural substrates underlying human feeding and food choice. Furthermore, the substantial heterogeneity in the patterns of brain atrophy which occurs in these disorders provides an opportunity to map the specific neural substrates underlying the feeding disturbances in these patients. Finally, by measuring circulating satiety signals in these patients, e.g. insulin and leptin, and correlating them with feeding disturbances, we can better define changes in peripheral signaling driven by central dysfunction. These data will contribute to our understanding of the reciprocal CNS connections with the periphery, which are integral to appropriate caloric homeostasis.

I have designed and implemented a study to quantify the feeding and food choice behaviors, as well as the satiety signaling profiles of patients with neurodegenerative disease, their caregivers, and healthy controls Additionally, any abnormalities can be correlated with specific areas of brain atrophy as determined by structural MRI. The questions to be answered include:

1. Is atrophy of orbito-frontal or other neural circuits (insula, nucleus accumbens, hypothalamus) associated with diminished sensory-specific satiety and loss or suppression of consumption that is potentiated by food variety?
2. Is atrophy of particular cortical and subcortical circuits associated with increased caloric intake, altered macronutrient preferences or altered food/flavor preferences?
3. Are there differences in circulating levels of peripheral satiety signals during a standardized meal in patients with different patterns of brain atrophy and/or altered feeding behavior?

Due to the nature of human research, this study is still ongoing, though we already have some interesting preliminary results. I will now describe the rationale, procedures and preliminary data for this project.

Methods

Patients with various forms of dementia and age-matched normal controls receive in-patient care at the UCSF General Clinical Research Center (GCRC) for four to five days; during this time, the patients' normal caregivers also stay at the GCRC. While at the GCRC, patients and normal controls receive extensive cognitive and behavioral testing, as well as a high quality structural MRI. I am attempting to investigate eating-related behaviors during the consumption of two lunches and one breakfast, as well as total caloric and macronutrient consumption, in demented patients, normal controls and caregivers staying in the GCRC over four days.

Experiment 1

Dementia patients (n=9 FvFTD, 2 TvFTD, 12 AD, 3 progressive aphasia (PA), 1 progressive supra-nuclear palsy (PSP) to date), their caregivers (n=13 to date) and normal controls (n=2 to date) are currently studied in the following paradigm. During the course of their stay in the GCRC, two successive lunches are manipulated in order to investigate the neural correlates of SSS. At the first lunch, subjects are allowed to pick their favorite type of sandwich among seven different types of sandwiches. The subject is given as many of their favorite type of sandwich as they wish until either they are full or we run out of time. Preliminary studies indicate that the lunch period is lengthy enough for subjects to eat to satiety. At the second lunch, subjects are given access to all seven types of sandwiches and are allowed to eat as much as they like. For both lunches, the total volume of food (i.e. the number of sandwich quarters) available at any given time is kept constant in order to minimize the effect of food availability on consumption. The amount consumed at both lunches is calculated by quantifying the leftover food. While the

lunches are labeled "lunch 1" and "lunch 2", in fact, their temporal order is randomized to prevent order effects.

All subjects will also undergo a separate taste test where they are required to taste and name the sandwiches to control for gross taste or memory deficits. Similarly, subjects rank basic taste solutions (salt, sugar, quinine and sour) on intensity and palatability in order to determine whether they have normal taste sensitivity.

The total amount consumed at lunch 2 relative to lunch 1 is a measure of the variety effect: any increase in intake at the second lunch must be due to variety and not to having access to a more preferred sandwich. By allowing subjects to pick their favorite sandwich at the first lunch, we minimize the impact of personal preference. Since the sandwiches have varying macronutrient content, we can also begin to elucidate any disease specific macronutrient preferences. All food eaten at other times during their stay is quantified and compared with their lunchtime eating behavior.

All patients and normal controls (but not caregivers) receive a high quality structural MRI. These images are analyzed using Voxel-based Morphometry (VBM) in collaboration with the Memory and Aging Center. VBM is a recently-introduced technique for the detection of regional brain atrophy by voxel-wise comparison of grey matter volumes between groups of subjects[7, 8]. Any behavioral deficits can be correlated with patterns of brain atrophy as determined by VBM.

Predictions

If the OFC is critical for SSS then OFC atrophy will prevent variety-induced increases in consumption. Furthermore, if SSS is critical for proper curtailment of eating then impaired SSS should lead to hyperphagia. Therefore, I predict that patients with atrophy of the OFC should

have increased consumption at both lunches compared to controls, but will not have a further increase in consumption when given a variety of sandwiches from which to choose. Additionally, patients with atrophy of the OFC have been reported to prefer and crave sweets. Therefore, I predict that our patients will consume more of the sweet sandwiches. Furthermore, when FvFTD patients rank the taste solutions on palatability, I predict that they will prefer the higher concentrations of sucrose.

Preliminary data

In our paradigm, we continued to bring sandwiches out to the subjects irrespective of their requests until the time ran out. We told the subjects that they did not have to eat the sandwiches but we always kept a standard volume of available food in the testing room until time ran out. Of the 27 patients investigated to date, four FvFTD patients (three men and one woman) had eating behaviors which were distinctly different from the others. They spontaneously told the experimenter that they were full after eating a quantity of sandwiches similar to the amount eating by other subjects. After saying that they were full, however, they continued to eat large quantities of sandwiches (fig 1a, b). For example, one subject ate five more sandwiches after telling the experimenter that she was full. Furthermore, all four patients still showed a sensitivity to variety, eating more when allowed to choose amongst all seven sandwiches than when only presented with their favorite sandwich. This disproves the hypothesis that FvFTD patients who over-ate would not be sensitive to variety. Two of these "over-eaters" returned at one year to be studied again in the same paradigm (data only shown for one returning "overeater"). Both showed identical behavioral patterns, saying they were full but continuing to eat despite remembering the experimenters and test procedures. Interestingly, these four patients were not particularly more obese than other subjects and the two returning subjects had not gained weight during the intervening year. Additionally, further analysis of eating behaviors revealed that FvFTD patients

were more likely to eat the jelly sandwiches than caregivers or patients with other diagnoses, supporting the prediction that patients would prefer the sweet sandwiches.

Preliminary VBM analysis comparing the atrophy patterns of these four patients to those of the other patients in the study revealed that these four patients have significantly more atrophy in the right fronto-insular cortex (figs 2a-e and 3a-f) ($p<0.05$ corrected for multiple comparisons within the region of interest). This area has been implicated in feeding and gustatory processing[1].

Experiment 2:

Subjects will receive a standard 550 kCal breakfast and will have serial blood samples taken in order to determine circulating levels of peripheral satiety signals before and after the meal. By using a standard sized breakfast we can dissociate hyperphagia from dysregulated peripheral signaling. Circulating levels of glucose, insulin, leptin, peptide YY (PYY), oxytocin, ghrelin, cortisol, Adrenocorticotropic Hormone (ACTH), and glucagon-like peptide 1 (glp-1) will be determined. In particular, we will try to capture the cephalic phase of insulin release which is mediated by the central nervous system through the vagus nerve by measuring insulin shortly after food presentation.

At a separate session, subjects will have their body dimensions measured in order to determine their fat distribution and body mass index (BMI). This data will be correlated both with the endocrine measurements and with the consumption data.

Outcomes and Predictions

Since the hyperphagia seen in FTD is presumably due to cortical atrophy, I predict that when the amount of food is externally controlled (i.e. a standard sized breakfast) the peripheral signaling profile will be normal or will indicate a hyperphagic state, i.e. high levels of leptin and insulin

with low ghrelin levels. Thus, the patients' hyperphagia may be due to an insensitivity to peripheral satiety signals. Alternatively, since many of these signals are driven by central mechanisms, patients with hyperphagia may have a signaling profile indicative of starvation. In particular, ghrelin levels, which are typically high in starvation, have been shown to be chronically high in conjunction with hyperphagia in other neurodegenerative diseases. All comparisons will be controlled for BMI.

Preliminary data

The blood samples for the subjects who have already undergone this paradigm are currently being analyzed by the laboratory of Dr. Peter Havel of UC Davis. Preliminary data should be available in the coming weeks.

Conclusions:

Obesity is a growing pandemic and is associated with significant health risks. Binge (compulsive) eating as well as palatability driven eating are major causes of excessive caloric intake. These proposed experiments will provide a deeper understanding of the neural circuits that underlie palatability and over-consumption. Understanding the neural mechanisms of non-homeostatic (excessive) eating patterns may lead to therapeutic interventions.

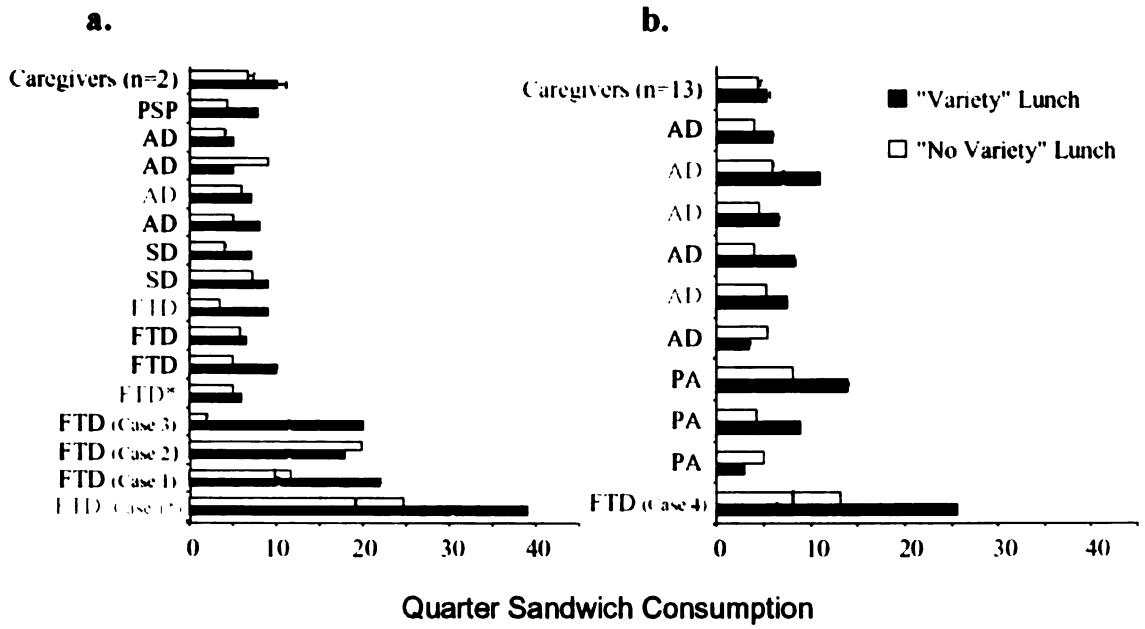
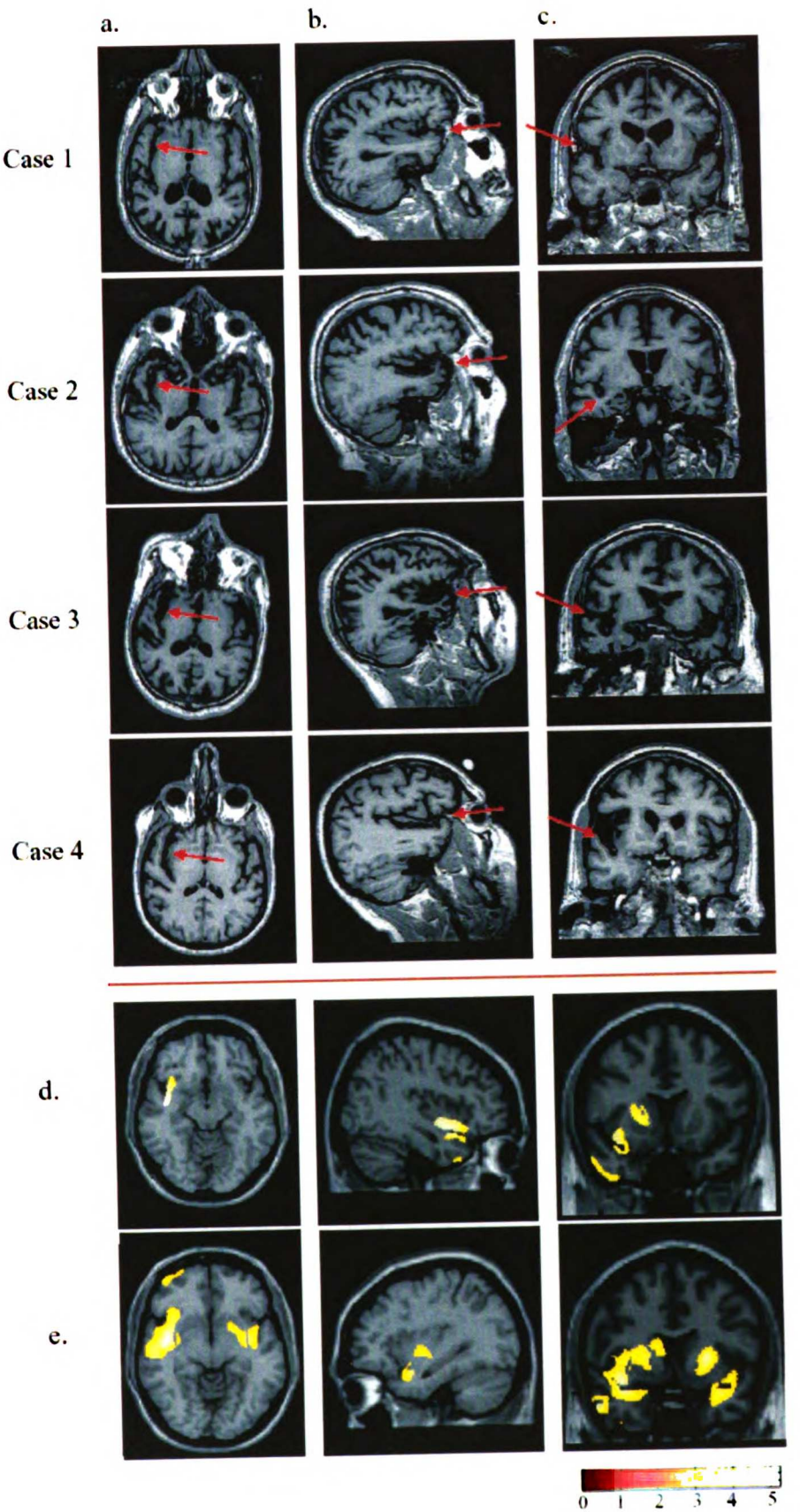


Figure 1

Some FTD patients show compulsive overeating. Number of sandwiches consumed during lunches with and without variety is shown for **a.** men and **b.** women. Total number of quarter sandwiches is shown on the x-axis and subjects are arranged on the y-axis by diagnosis. Red lines indicate a spontaneous claim of fullness. The place on the x-axis of these lines indicates how many sandwiches had already been consumed when the claim was made. Subjects were never asked if they were full or if they wanted more food. Subjects labeled by case number refer to FTD patients described in the text. A "*" indicates a second visit for that subject after a one year interval. Subject labels in red denote individuals who were excluded from the imaging analysis due to lack of an appropriate scan or a second visit. "Caregivers" are cognitively normal caregivers of patients with dementia. Their consumption data is presented as a mean. Error bars signify standard error. "FTD" stands for frontal variant fronto-temporal dementia. "SD" stands for Semantic Dementia which is otherwise known as temporal variant fronto-temporal dementia. "AD" stands for Alzheimer's disease while "PSP" stands for progressive supra-nuclear palsy.


a.
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c.
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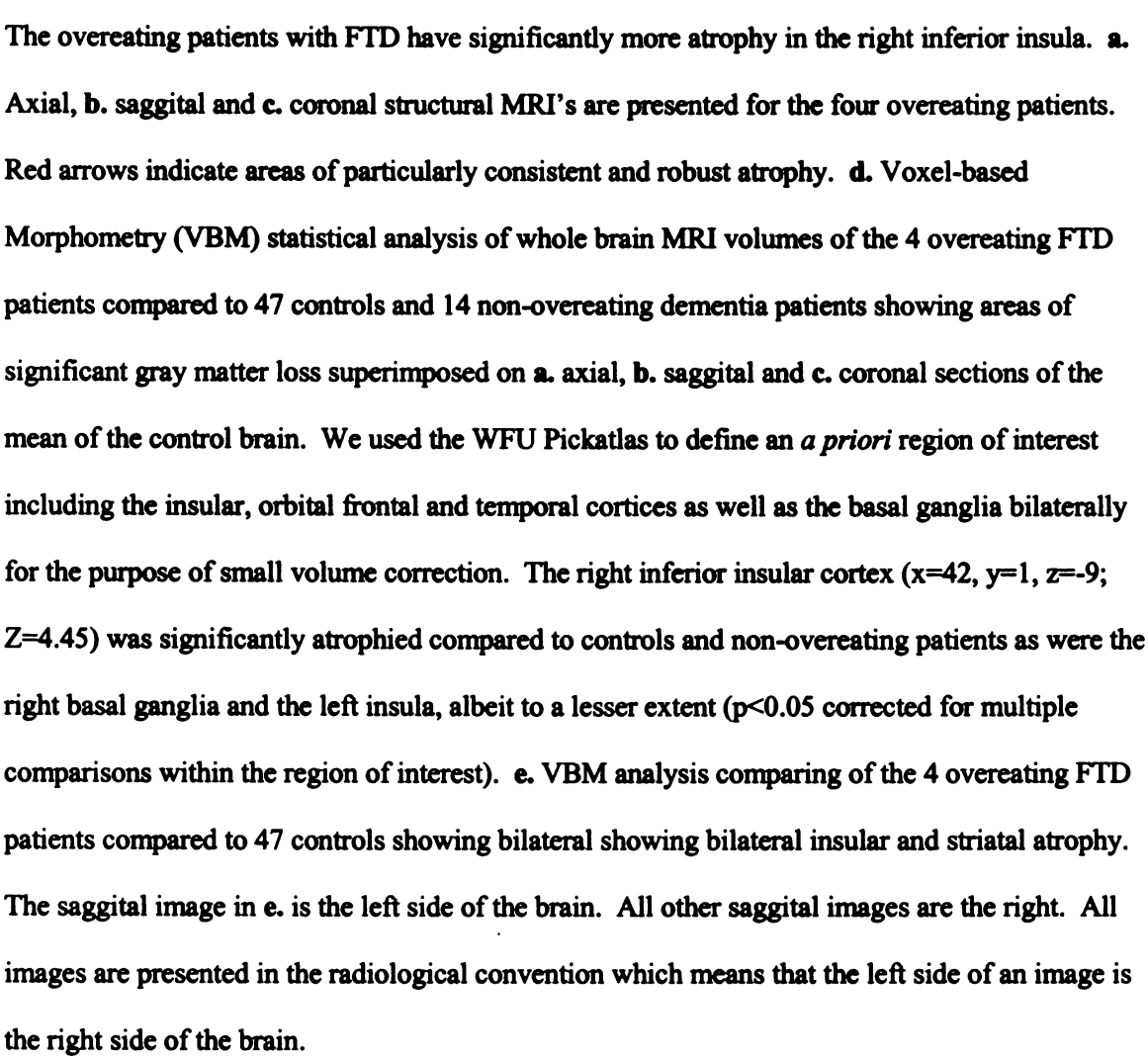
Figure 2



The overeating patients with FTD have significantly more atrophy in the right inferior insula. **a.** Axial, **b.** saggital and **c.** coronal structural MRI's are presented for the four overeating patients. Red arrows indicate areas of particularly consistent and robust atrophy. **d.** Voxel-based Morphometry (VBM) statistical analysis of whole brain MRI volumes of the 4 overeating FTD patients compared to 47 controls and 14 non-overeating dementia patients showing areas of significant gray matter loss superimposed on **a.** axial, **b.** saggital and **c.** coronal sections of the mean of the control brain. We used the WFU Pickatlas to define an *a priori* region of interest including the insular, orbital frontal and temporal cortices as well as the basal ganglia bilaterally for the purpose of small volume correction. The right inferior insular cortex (x=42, y=1, z=-9; Z=4.45) was significantly atrophied compared to controls and non-overeating patients as were the right basal ganglia and the left insula, albeit to a lesser extent ($p<0.05$ corrected for multiple comparisons within the region of interest). **e.** VBM analysis comparing of the 4 overeating FTD patients compared to 47 controls showing bilateral showing bilateral insular and striatal atrophy. The saggital image in **e.** is the left side of the brain. All other saggital images are the right. All images are presented in the radiological convention which means that the left side of an image is the right side of the brain.

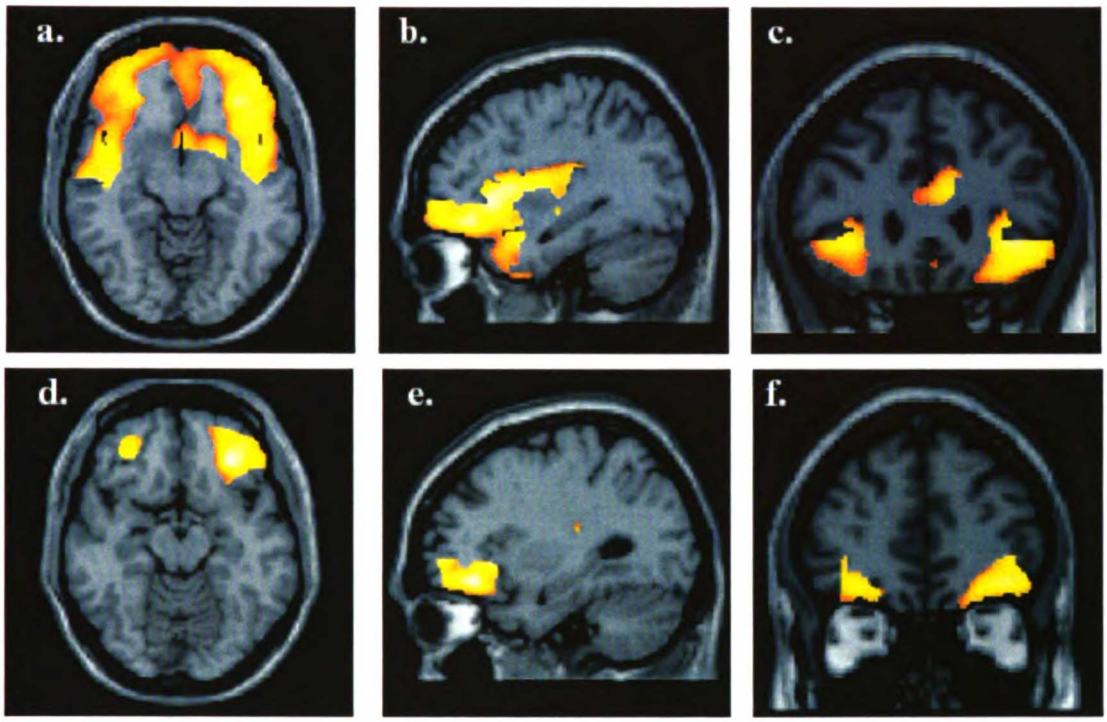


Figure 3

VBM statistical analysis of whole brain MRI volumes of the 14 non-overeating patients compared to 47 controls showing areas of significant gray matter loss superimposed on **a.** axial, **b.** saggital and **c.** coronal sections of the mean of the control brain. We used the WFU Pickatlas to define an *a priori* region of interest including the insular, orbital frontal and temporal cortices as well as the basal ganglia bilaterally for the purpose of small volume correction. The orbital frontal, temporal, anterior cingular cortices were atrophied bilaterally and there was significant atrophy in the left insular cortex and basal ganglia. VBM analysis comparing MRI volumes of the 14 non-overeating patients compared to 47 controls and the 4 overeating FTD patients is shown for **d.** axial, **e.** saggital and **f.** coronal sections of the mean of the control brain. There was significant atrophy only in the orbital cortices bilaterally and the left temporal lobe.

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Conclusions, caveats and future directions

The present studies show that MOP receptor agonists within the NAcc have at least three separate effects on consumption; they 1) increase consumption of palatable foods, 2) selectively increase consumption of a more preferred flavor, and 3) increase consumption of a recently sampled palatable food without altering long term preferences. This last effect is opposed by KOP receptor agonists in the NAcc which increase consumption of a relatively novel (non-pre-fed) food. These effects are specific to NAcc opioid receptors (i.e. opioid modulation at other brain sites and non-specific inhibition of NAcc neurons do not reproduce these effects). This work demonstrates for the first time that opioid signaling in the NAcc is involved in short-term flavor preference conditioning, and provides a possible mechanism for opioidergic effects on meal maintenance.

One potential limitation of the present study is that we only used two pellet flavors. We found that all rats slightly preferred chocolate to banana. Both pellets are made from the same mash, and the only difference between them is the addition of different artificial flavorings. At present, we have no explanation for the rats' preference. I have tasted both pellets and I also find the chocolate flavor more palatable. Presumably this is due to many previous associations of this flavor with the fatty and sweet features of real chocolate. The rats, however, have not had these experiences. Chocolate is not generally accepted as an addictive substance but it is, by a large margin, the most craved food in North America, accounting for nearly half of all food cravings in one study (for review see [1]). Why this is so has been hotly debated, although one hypothesis is that chocolate contains biologically active compounds. Chocolate has been reported to contain psychoactive compounds such as biogenic amines (e.g. phenylethylamine which is structurally and pharmacologically similar to catelcholamines and amphetamine), methylxanthines (e.g. caffeine and theobromine) and cannabinoid-like fatty acids (e.g. N-acylethanolamines which are

chemically and pharmacologically related to anandamide, an endogenous cannabinoid agonist). If these compounds were present in sufficient quantities only in the chocolate pellets, some of the effects in the present studies could be explained by direct pharmacological interactions between these compounds and the exogenous opioid agonists.

Most serious reviewers of the subject have decided that given the concentrations and oral bio-availability of these compounds in chocolate, there is little evidence that these constituents play any role in chocolate "addiction" or craving or even in appetite or liking for chocolate[2-4]. For example, even assuming that the orally consumed cannabinoid-like fatty acids are as potent as THC, the most psychoactively potent plant-derived cannibinoid, calculations show that a 70-kg person would need to eat 25 kg of chocolate to achieve a noticeable high[2]! Similarly, in a study of self-described chocolate cravers, subjects were given, in sealed boxes, a bar of milk chocolate, a bar of white chocolate (which contains none of the pharmacological components of chocolate), the pharmacological equivalent of chocolate in capsules containing powdered cocoa, placebo capsules, a white chocolate bar plus cocoa capsules or no treatment. They were instructed to consume the content of one box in a random order when they experienced a chocolate craving. The study found that only the chocolate bars (i.e. both milk and white) reduced cravings for chocolate indicating there is "no role for pharmacologic effects in the satisfaction of chocolate craving."[4] Furthermore, the flavorings added to the pellets are synthetically produced and thus should not contain many of the naturally occurring compounds in cocoa pods. More importantly, in this study DAMGO increases consumption of both chocolate and banana flavored pellets equally when they are presented alone. This rules out the possibility of a direct interaction between the MOP agonist and pharmacologically active compounds that are present only in the chocolate pellets. A potentially useful future direction would be to repeat the present experiments using alternatively flavored pellets (e.g. marshmallow and pina-colada).

Another limitation of the present study is that it did not differentiate between the core and shell sub-regions of the NAcc. These two sub-regions can be separated on the basis of their histochemical, cytoarchitectonic and hodological profiles (for review see [5]). Furthermore, there are important differences in the responses to certain pharmacologic manipulations between the two sites (for review see [6]). However, since previous studies have found that DAMGO was capable of increasing feeding throughout the NAcc[7], micro-injecting DAMGO into the border region between the shell and core appears justified. A more detailed mapping of the effects of opioid modulation in the current paradigms could explain some of the variability seen in the present studies, and may provide valuable insights into the functional specializations of these two sub-regions.

Other potentially interesting avenues for future research would be to investigate the roles of other signaling systems in similar behavioral paradigms. For example, delta opioid receptors are present in the NAcc and have been implicated in feeding[8]. Similarly, there are high levels of cannabinoid receptors in the NAcc, and it has previously been shown that anandamide, an endogenous cannabinoid agonist, robustly increases feeding when injected into the NAcc. Additionally, levels of endogenous cannabinoid agonists within the NAcc can be modulated by hunger [9]. There is some debate as to how cannabinoids increase feeding (i.e. do they increase feeding by altering palatability or hunger mechanisms)[10, 11], and there is evidence that opioid and cannabinoid signaling have synergistic and interacting effects on feeding[12]. These features make studying the effects of cannabinoid modulation on intake in behavioral paradigms identical to the ones used in the present studies potentially illuminating. Furthermore, studying the effects of pharmacologic manipulation within brain regions that project to the NAcc such as the prefrontal cortex and amygdala are obvious future avenues of investigation.

Finally, directly measuring endogenous opioid levels within the NAcc after presentation of palatable and bland foods could help elucidate the in vivo functions of opioid signaling. Similarly, the effects of exogenous opioids on NAcc neural firing could be investigated using in vivo electrophysiological recording techniques in taste responsive neurons before and after opioid modulation.

In conclusion, the present studies have elucidated several functions of NAcc opioid signaling on feeding. Future research avenues that could prove fruitful include manipulating opioid as well as other signaling systems (e.g. cannabinoids) in sub-regions of the NAcc and brain regions that send projections to the NAcc (e.g. the prefrontal cortex). Furthermore, utilizing additional pellet flavors, while maintaining identical nutritional composition, could further elucidate the roles of palatability and macronutrient content in opioid induced feeding.

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