THE ACTIVATION OF MESOLIMBIC DOPAMINE NEURONS DURING NOVEL AND DAILY, LIMITED ACCESS TO PALATABLE FOOD IS BLOCKED BY THE OPIOID ANTAGONIST, LY255582


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Running Title: LY255582 Inhibits Feeding-Induced DA Release
Abstract

An analog of the 3,4-dimethyl-4-(3-hydroxyphenyl)piperidine series, LY255582, exhibits high in vitro binding affinity and antagonist potency for the opioid receptors: mu, delta, and kappa. In vivo, LY255582 exhibits potent effects in reducing food intake and body weight in several rodent models of obesity. In the present study we evaluated the effects of LY255582 to prevent the consumption of a highly palatable (HP) diet (a high fat/high carbohydrate diet) both when the food was novel and following daily, limited access to the HP diet. Additionally, we examined the effects of consumption of the HP diet, and of LY255582 treatment on mesolimbic dopamine (DA) signaling by in vivo microdialysis. Consumption of the HP diet increased extracellular DA levels within the nucleus accumbens (NAc) shell. Increased DA in the NAc shell was not related to the quantity of the HP diet consumed, and the DA response did not habituate following daily scheduled access to the HP diet. Interestingly, treatment with LY255582 inhibited consumption of the HP diet and the HP diet-associated increase in NAc shell DA levels. Moreover, the increased HP diet consumption observed following daily, limited access to the HP diet was completely prevented by LY255582 treatment. LY255582 may be a useful tool in understanding the neural mechanisms involved in the reinforcement mechanisms regulating food intake.

Keywords: opiate, nucleus accumbens, obesity, food intake, microdialysis
Introduction

The involvement of the central opioid system in the regulation of food intake is well established (for review see (22, 49). Many studies have demonstrated that agonists at all three opioid receptor subtypes, mu, delta, and kappa, increase food intake (26, 44), whereas antagonists decrease food intake (22, 49). More specifically, manipulations of the opioid system regulate hedonic feeding or the intake and response to highly palatable (HP) macronutrients (high fat and/or high sucrose) (5, 6, 33, 37). The acquisition of hedonic feeding appears to involve activation of the mesolimbic dopamine (DA) system, the dopaminergic projection from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (8, 11, 17, 21, 41, 42). This pathway is a key component of the brain reward circuit (33, 60) which consists of neural pathways that are strongly implicated in reward and reinforcement mechanisms of drugs of abuse, as well as vital behaviors, including feeding and sexual behavior. Several neurotransmitter and neuropeptide systems that modulate feeding do so, at least in part, by acting through the mesolimbic DA pathway (1, 36, 59). Endogenous opioids regulate the mesolimbic DA projection at both the level of the VTA and NAc (30, 54) providing a potential mechanism by which opioids may alter hedonic feeding.

The trans-3,4-dimethyl-4-(3-hydroxyphenyl)piperidine opioid receptor antagonist, LY255582, exhibits high binding affinity for the mu, delta and kappa opioid receptors in vitro (36). Additionally, we recently reported that LY255582 displays inverse agonist activity in cell lines expressing the cloned human delta receptor (19). In vivo this molecule is a pure antagonist at mu and kappa opioid receptors blocking morphine- and
U50,488-mediated antinociception, and bremazocine-induced diuresis (43, 64).

Importantly, LY255582 has also been shown to potently decrease food intake after central or peripheral administration, and produces a sustained weight loss in several rodent models of obesity (38, 52, 53, 55).

Our laboratory has developed a hedonic feeding paradigm for investigating compound effects on HP food consumption. In this model, rats are fed regular chow *ad libitum*, and are given daily, limited access (one hour) to an HP diet during the light portion of the light-dark cycle when food intake is normally low. Under these conditions, rats progressively increase their intake of the HP diet following several days of HP diet access resulting in a large bout of feeding during the one hour access period. The objectives of the present studies were to determine 1) the effect of LY255582 on consumption of the HP diet in animals given daily, limited HP diet access, 2) the effect of LY255582 on HP diet induced increases in NAc shell DA levels in animals consuming the HP diet for the first time and 3) the effect of LY255582 on the progressive increase in HP diet intake and the accompanying alterations in NAc shell DA levels that occur during the escalation phase of HP diet intake. The NAc shell subregion was targeted, as several studies have demonstrated a preferential increase in DA in the NAc shell versus core in response to the consumption of palatable diets (9, 58).
Materials and Methods

General Methods

Subjects: Male, Sprague Dawley rats (250-270 g; purchased from Harlan Sprague Dawley, Indianapolis, IN) were single housed in plastic shoebox cages in a temperature and humidity controlled colony maintained on a 12/12 light/dark cycle [lights on at 0400 hours for Experiment 1 (Effect of LY255582 on Consumption of an HP Diet) and lights on at 0600 hours for Experiments 2a, 2b and 2c (Microdialysis Experiments)]. In their homecage, animals were maintained on normal rat chow (LabDiet 5001, Purina Mills Inc., Richmond, IN) and water ad libitum. The normal rat chow consisted of 59.4 % carbohydrate, 28.4 % protein, 12.3 % fat, (3.3 kcal/g). The HP diet that was used in all experiments was Teklad 95217 (Harlan Teklad, Madison, WI) consisting of 41.3 % carbohydrate (primarily as sucrose: 157g/100g food), 18.7 % protein, 40 % fat (4.25 kcal/g). All experiments were conducted during the light portion of the light cycle between 0700-1500 hours, when baseline feeding is normally low. HP diet access occurred an average of six hours into the light cycle. All animal protocols used in the study were in accordance with the NIH Guide for Care and Use of Laboratory Animals (NIH Publication No. 86-23, 1985) and approved by the Lilly Research Labs Institutional Animal Care and Use Committee. All efforts were made to minimize animal discomfort and to use the minimum number of animals required to obtain appropriate statistical power.
Compounds: LY255582 was synthesized at Lilly Research Laboratories according to Mitch et al., 1993. An in vivo formulation of LY255582 was prepared in water acidified with 1% lactic acid (v/v) and administered subcutaneously (s.c.) in a 1 ml/kg dose volume.

Experiment 1: Effect of LY255582 on Consumption of an HP Diet

Experiment 1 examined the effect of acute LY255582 treatment on food intake in animals given daily limited access to the HP diet. On Days 1-3, all rats (n = 7/group) were dosed (s.c.) with vehicle (1 ml/kg) and returned to their home cage. One hour after dosing, rats were given one hour access to the HP diet (at approximately 1130) in addition to having their regular chow in their home cage. On Day 4, rats received either vehicle or 0.1-3.0 mg/kg, s.c., LY255582. One hour following compound treatment rats were given access to the HP diet in the same manner. Daily one hour HP diet intake and 24 hour chow intake were measured throughout the study.

Experiments 2a, 2b, and 2c: Microdialysis Experiments

Common Methods

Stereotaxic Surgery: Rats were anesthetized with 2-3% isoflurane anesthesia and placed in a stereotaxic apparatus with the incisor bar set at -3.3 mm (David Kopf Instruments, Tujanga, CA). Briefly, the skull was exposed by removing a small piece of scalp and the tissue was retracted. Two to three pilot holes were drilled into the skull for anchor
screws, and a separate hole was made to allow the insertion of the guide cannula over the NAc shell using the following coordinates: AP = + 1.7 mm, ML = + 0.8 mm, DV = - 6.0 mm, relative to Bregma (46). The guide cannula was fixed into place with Duralay dental adhesive (BASi, West Lafayette, IN). Following surgery, the animals were allowed two-five days to recover before any experimentation. Twenty four hours prior to testing, microdialysis probes (BR-2 probes, MWCO = 30,000 daltons, BASi, West Lafayette, IN) were inserted into the guide cannulae. The active membrane portion of the probe was 2 mm in length.

General Microdialysis Procedure: On the morning of the experiment, animals were moved to the testing room at approximately 0700 and placed in round bottom cages and tethered to a counterbalance arm. The probe inlet was connected to a syringe pump that delivered Ringer’s solution (in mM: 145 NaCl, 2.7 KCl, 1.0 MgCl2, 2.5 CaCl2, 2.0 Na2HPO4, pH 7.2-7.4) at a flow rate of 1.5 µL/min. Animals were perfused for 2.5 hours before sample collection began. Following this equilibration, samples were collected every 10 min. All microdialysates were collected into vials containing 5 µl of 0.2 N perchloric acid and maintained at 4° C in a fraction collector. Water was not available during microdialysis testing. Food was not available in the microdialysis cages until the presentation of the HP diet (approximately four hours after rats were placed in the cages and five hours into the light cycle).

Specific Methods
Experiment 2a: Microdialysis Following Novel Access to an HP Diet

This study examined the effect of LY255582 on the mesolimbic DA response to the HP diet in animals that had no previous history of HP diet consumption (i.e. when the HP diet was novel). Following set up and equilibration, three baseline samples were collected and animals were injected s.c. with either vehicle or 0.3 mg/kg LY255582 (n = 6-7/group). The dose of 0.3 mg/kg was chosen based on ex vivo binding studies which showed that 0.3 mg/kg of LY255582 produced greater than 80% occupancy of opioid receptors in the CNS, ensuring that the dose of LY255582 was sufficient to fully antagonize opioid receptors throughout the testing period (data not shown). One hour after treatment, animals were given a preweighed amount of the HP diet on the floor of the testing bowl. After one hour, the food remaining was removed and weighed so that the amount eaten could be calculated. A “pair-fed” vehicle treated control group was given the amount of HP diet consumed by LY255582 treated rats (0.7 g). The “pair-fed” animals consumed all 0.7 g of the HP diet during the one hour access period.

Experiment 2b: Microdialysis Following Repeated Access to an HP Diet

This study examined the effect of LY255582 on the intake of the HP diet and the changes in NAc shell DA levels in response to the HP diet during daily, one hour limited HP diet access sessions. The study employed a between subjects design with groups run concurrently (n = 5-9/group) as probe integrity can begin to deteriorate 24 hours after implantation. Each day, rats were taken to the testing room at approximately 0700,
placed in the testing bowls and tethered to the counterbalance arm. On Day 1 all animals were given vehicle injections (1 ml/kg, s.c.) and presented with HP diet one hour later. On Days 2-4, animals were given either vehicle or LY255582 (0.3 mg/kg, s.c.) and presented with HP diet one hour later. On Day 5, all animals were given vehicle injections one hour prior to HP diet presentation. Microdialysis sampling occurred on either the third day (Day 3, during the period when HP diet intake is increasing) or the fifth day (Day 5, to determine if HP diet intake and changes in DA levels occurs following cessation of LY255582 treatment) of the one hour HP diet access. The procedure of the microdialysis testing was identical in both experiments 2a and 2b with a 2.5 hour equilibration period, a 30 minute baseline sampling period occurring before drug treatment, and HP diet presentation occurring approximately four hours after the animals were placed in the cages (approximately five hours into the light cycle). For clarity, Fig. 1 graphically illustrates the experimental timeline.

**Experiment 2c: Microdialysis Following Access to a Regular Chow Diet**

To test whether the changes in consumption of the HP diet and subsequent increase in NAc shell DA levels were the result of the animals being in a mild calorie restricted state resulting in a deprivation-induced feeding response (since they were placed in the microdialysis bowls for four hours prior to food availability) we examined if access to regular chow under similar conditions elicited similar increases in feeding and NAc DA levels. Experimental conditions were identical to Experiment 2a and 2b except that a preweighed amount of regular rodent chow diet (LabDiet 5001) was provided to the rats
for one hour during the microdialysis sampling (occurring approximately four hours after 
the animals were placed in the cages and approximately five hours into the light cycle).

**Histology:** At the end of microdialysis experiments, animals were euthanized by CO2 
inhalation and tetrazolium red dye (5 mg/ml; Sigma Chemical, St. Louis, MO) was 
perfused through the probe to mark the probe track. The brain was removed and frozen 
at - 20° C and sliced on a cryostat at 40 μm to verify probe location. Only animals with 
approximately 80 % of the dialysis membrane in the shell of the NAc were included in 
the data analyses (data not shown).

**HPLC:** An ESA 542 pump delivered MD-TM mobile phase (ESA, Chelmsford, MA; pH 
adjusted to 5.0) through a reverse phase column (3.2 x 150 mm; C-18, 3 μM particle size) 
at 0.6 ml/min. Samples (15 μl) were injected onto the column with the aid of an 
autosampler and DA was quantified using a Coulachem III detector equipped with a 
guard cell and a 5014B analytical cell. The potential of the guard cell was set at + 350 
mV. The potential of the first electrode in the analytical cell was set at + 220 mV and the 
second electrode (E2) was set at - 290 mV. DA was detected on E2 set at a sensitivity of 
5 nA. DA was quantified based on peak area using a standard curve generated from DA 
standards run the day samples were analyzed. The software used to run the 
instrumentation and acquire data was EZChrom Elite version 2.8.3.

**Data Analysis:**

*Food intake data*
For experiment 1, to analyze the change in food intake across the four days, one hour HP and 24 hour chow intakes from the vehicle group were analyzed separately using one-way repeated measures ANOVAs. To analyze the effect of LY255582 treatment, one hour HP and 24 hour chow intakes on Day 4 were analyzed separately using one-way ANOVAs with Dunnett’s post hoc tests. In the microdialysis studies, food intake data were compared using a two-tailed t-test (Experiment 2a) or a two-way (Time X Group) repeated measures ANOVA with a compound symmetry matrix (Experiment 2b). Significant interactions were followed up using contrast effects and Fisher’s least significant difference (Fisher’s) test. Significance was set at p < 0.05.

**Microdialysis data:** All microdialysis data were normalized to percent of baseline (baseline DA = average of the three samples prior to injection). Baseline DA levels were analyzed using a one-way ANOVA (Experiment 2a) or a two-tailed t-test (Experiment 2b) on the untransformed data. Normalized DA levels were analyzed with a two-way mixed ANOVA (Time X Group) with either a heterogeneous Toeplitz (Experiment 2a) or a Toeplitz matrix (Experiment 2b). In Experiment 2c normalized DA levels were analyzed with a one-way ANOVA using a heterogeneous compound symmetry matrix. In order to determine the effect of LY255582 treatment on NAc shell DA levels, the first portion of the data were analyzed from -20 min through 60 min of data collection. In order to determine the effects of HP diet access on NAc shell DA levels and the effect of LY255582 treatment on this measure, a separate analysis was performed on the latter part of the data by analyzing -20 min to 140 min, excluding time points from 10 to 60 min.
Statistically significant interactions were followed up using contrast effects and a post hoc Fisher’s test. Statistical significance was set at $p < 0.05$. 
Results

**Experiment 1: Effect of LY255582 on Consumption of an HP Diet**

In vehicle treated rats, daily one hour access to the HP diet produced a progressive increase in the amount of the HP diet consumed over the four days of the study (Fig. 2a). A one-way repeated measures ANOVA revealed a significant main effect of Day $[F(3,18) = 23.68; p = 0.0001]$ on HP diet intake. Treatment on Day 4 with LY255582 produced a significant decrease in HP diet consumption $[F(4,30) = 9.17; p = 0.001]$. Post hoc analysis revealed that all doses of LY255582 decreased HP diet intake ($p < 0.01$). Interestingly, as HP diet intake increased 24 hour regular chow intake decreased $[F(3,18) = 6.14; p = 0.005]$ over the four day access period (Fig. 2b). A main effect of treatment was observed with administration of LY255582 $[F(4,30) = 6.31; p = 0.0008]$. In comparison to HP diet intake, LY255582 had a reduced effect on 24 chow intake on Day 4 in that only the highest dose (3 mg/kg) of LY255582 decreased 24 hour chow intake ($p < 0.01$ vehicle versus 3mg/kg,).

**Experiment 2a: Microdialysis Following Novel Access to an HP Diet**

In the group of animals tested during their first exposure to the HP diet, LY255582 (0.3 mg/kg, s.c.) significantly suppressed one hour HP diet intake during the access period $[t(11) = -7.69; p = 0.014]$ (Fig. 3 inset). Vehicle treated animals consumed
5.9 ± 0.6 grams of HP diet during the one hour access, whereas LY255582 treated animals ate only 0.7 ± 0.2 grams of the HP diet.

Raw baseline DA, which was defined as the three time points prior to injection, did not differ between any of the three groups \([F(2, 17) = 0.78; \ p = 0.47; \text{overall mean} = 1.01 \pm 0.17 \text{nM}]\). Furthermore, normalized DA levels were stable during the hour immediately following injection of vehicle or LY255582 (Fig. 3). This was confirmed by a mixed ANOVA run on the data from Time -20 through 60 min which demonstrated no significant main effects or interactions: Group \([F(2,13) = 0.75; \ p = 0.49]\), Time \([F(8,23) = 0.88; \ p = 0.55]\) Group x Time \([F(16, 32) = 0.96; \ p = 0.52]\).

Analysis of DA levels during the hour of HP diet availability indicated main effects of Group \([F(2,31) = 8.04; \ p = 0.002]\), Time \([F(11,30) = 3.22; \ p = 0.005]\), and a significant Group X Time interaction \([F(22,42) = 2.85; \ p = 0.002]\) (Fig. 3). Individual comparisons demonstrated that vehicle treated rats exhibited a significant increase in DA levels during the HP diet access period at 80, 90, 100 and 110 min compared to Time 0. The group labeled “pair-fed” had the amount of HP diet yoked to that consumed by rats treated with LY255582 and was given only 0.7 g of HP diet. The pair-fed group consumed all 0.7 g of the HP diet during the one hour access period. Moreover, the increase in DA levels in the pair-fed group demonstrated a significant increase in DA at all time points during the hour of HP diet availability and was similar to the vehicle control group. Interestingly, the group of animals treated with 0.3 mg/kg LY255582, which consumed 0.7 g of the HP diet, failed to display an increase in mesolimbic DA levels during the HP diet access period. Indeed, significant decreases in DA levels
compared to baseline were observed at two timepoints (70 and 100 min) in animals treated with LY255582 (Fig. 3).

**Experiment 2b: Microdialysis Following Repeated Access to HP Diet**

Experiment 2b was designed to examine the effect of LY255582 on the progressive increase in HP diet intake and the accompanying alterations in NAc shell DA levels that occur during the escalation phase of HP diet intake. Microdialysis sampling occurred on either Day 3 (Fig. 4) or Day 5 (Fig. 5) of HP diet access. In the group of animals tested on Day 5, LY255582 administration was terminated on Day 4 and groups differed only in their treatment histories (See Fig. 1 for timeline).

**Day 3 HP Diet Intake and Microdialysis:**

As shown in Fig. 4 (inset), following three days of HP diet access, rats showed a progressive increase in HP diet intake. Interestingly, the progressive intake in HP diet consumption was prevented in rats treated with LY255582. A two-way mixed ANOVA performed on the HP food intake data demonstrated a main effect of Treatment \(F(1,13) = 20.25; p = 0.0006\), Day \(F(2,26) = 7.39; p = 0.003\) and a significant Treatment x Day interaction \(F(2,26) = 12.83; p = 0.0001\). Individual comparisons supported that LY255582 treated animals showed a reduction in HP diet intake relative to vehicle treated animals on Days 2 and 3. Moreover, intake did not increase with repeated HP diet access in the LY255582 treated group, while intake was significantly higher on Days 2 and 3 compared to Day 1 in vehicle treated animals.
Analysis of the raw baseline NAc shell DA levels on Day 3 demonstrated no difference between the vehicle and LY255582 treated groups [$t(13) = 1.623; p = 0.129$]; overall mean $0.92 \pm 0.13$ nM. During the first hour post injection (Fig. 4) there was a slight but significant decrease in DA levels over time [$F(8,53) = 4.46; p = 0.0003$], however there was neither a main effect of Treatment [$F(1,26) = 0.0007; p = 0.98$], nor a significant Treatment x Time interaction [$F(8,53) = 1.89; p = 0.08$]. During the HP diet access period, NAc DA levels increased significantly in vehicle treated animals, but did not increase in animals treated with LY255582. The statistical analysis revealed no main effect of Treatment [$F(1,20) = 0.73; p = 0.4$], but significant main effects of Time [$F(11, 53) = 3.12; p = 0.003$] and a Treatment x Time interaction [$F(11, 53) = 3.79; p < 0.0005$]. Detailed analysis of each time point during the HP diet access period revealed an increase in NAc shell extracellular DA levels in vehicle treated animals at Time 70, 80, and 90 min (relative to Time 0). LY255582 treated animals did not show a change in DA levels at any time point during the HP diet access period on Day 3.

**Day 5 HP Diet Intake and Microdialysis:**

Analysis of food intake in the group of animals undergoing microdialysis testing on Day 5 (Fig. 5 inset) revealed main effects of Treatment [$F(1,12) = 35.52; p < 0.0001$], Day [$F(4,48) = 18.49; p < 0.0001$] and a Treatment x Day interaction [$F(4,48) = 6.69; p = 0.0002$]. Following up on the significant interaction revealed that animals treated with LY255582 had significantly lower food intakes compared to vehicle treated animals on Days 2-5 suggesting that animals treated with LY255582 did not progressively increase intake of the HP diet over the days of limited access. On Day 5 when all animals were given vehicle injections, those animals previously treated with LY255582 consumed 6.8
± 1.5 g of HP diet. The amount of HP diet intake in animals with LY255582 treatment history was significantly more than this group consumed on previous days (during treatment with LY255582), however, it was still significantly less than the amount consumed on Day 5 (11.7 ± 0.7 g) by animals treated with vehicle over the entire testing period. Interestingly, on Day 5 animals previously treated with LY255582 ate a similar amount to vehicle treated animals on Day 2. Therefore, the effects of LY255582 on HP diet intake were reversible following cessation of drug treatment.

On Day 5, raw baseline DA levels did not differ between the two groups with different treatment histories, but there was a trend for the baseline values in rats with a LY255582 treatment history to be higher compared to vehicle \([t(12) = 2.104; p = 0.057; \text{vehicle mean: } 0.66 \pm 0.13\text{nM}; \text{LY255582 mean: } 1.1 \pm 0.18 \text{nM}].\) However, normalized DA values were stable following the injection of vehicle as analysis of the first part of the microdialysis data (-20 min to 60 min; Fig. 5) revealed no main effects of Group, Time, nor an interaction between the two variables: Treatment \([F(1,12) = 0.067; p = 0.8],\) Time \((F(8,28) = 1.3; p = 0.27),\) Treatment x Time \((F(8,28) = 0.49; p = 0.85).\) During the period of HP diet access, both groups of animals exhibited an increase in NAc shell DA levels (Fig. 5) regardless of their prior treatment history. This conclusion was supported by a finding of only a main effect of Time \([F(11,30) = 2.11; p = 0.05]\) during HP diet access (i.e., excluding 10 min to 60 min). There was neither a significant main effect of Treatment \([F(1,23) = 0.66; p = 0.42]\) nor a Treatment x Time interaction \([F(11,30) = 0.39; p = 0.95].\) These data suggest that following cessation of LY255582 treatment, both HP diet consumption and extracellular DA levels in the NAc shell increase when animals are given access to an HP diet.
Experiment 2c: Microdialysis Following Access to a Regular Chow Diet

In order to understand if changes in consumption of the HP diet and subsequent increases in NAc shell DA levels were the result of the animals being in a mild calorie restricted state (since they were placed in the microdialysis bowls for four hours prior to food availability), we examined if access to regular chow under similar conditions elicited similar increases in feeding and NAc DA levels. In this separate group of animals treated identically to previous HP diet groups, one hour access to regular rodent chow resulted in very little food consumption. Rats ate an average of 0.05 ± 0.02 g of chow (Fig. 6 inset), with several animals consuming none of the chow diet. Normalized baseline NAc shell DA levels (-20 min to 60min; Fig. 6) revealed no effect of Time [$F(8,12.2) = 1.81; p = 0.169$] in the first hour following vehicle injection. Moreover, following presentation of regular chow DA levels remained unchanged as indicated by no effect of Time [$F(10,10.9) = 0.81; p = 0.629$] during the chow access period (i.e., excluding 10 min to 60 min; Fig. 6).
Discussion

The present study demonstrated that with daily limited access rats increased HP diet intake while concomitantly decreasing their regular chow consumption. Additionally, extracellular DA levels in the NAc shell increased during access to the HP diet. Acute administration of LY255582 inhibited both the HP diet intake and the corresponding increase in NAc shell DA levels. In animals with repeated access to the HP diet, LY255582 prevented the progressive increase in HP diet consumption and prevented the increase in extracellular NAc DA levels that occurred during HP diet access. However, when LY255582 treatment was stopped rats increased their consumption of HP diet and there was an associated increase in DA levels in the NAc shell. Moreover, animals given access to a regular chow diet under identical conditions ate very little and exhibited no change in NAc shell DA levels.

Rats given daily limited access to the HP diet over several days demonstrated an increase in HP diet consumption resulting in a large bout of feeding during the one hour access period. This bout of feeding represented more than 1/3 of the animal’s total daily caloric intake. Additionally, 24 hour regular chow intake decreased over this time period. Administration of LY255582 significantly reduced HP diet consumption under limited access conditions at all doses tested (0.1-3.0 mg/kg). Interestingly, it appeared to be somewhat selective for reducing HP diet intake since only the highest dose (3.0 mg/kg) decreased 24 hour chow consumption. Although LY255582 administration only occurred once per day, this finding is not likely due to insufficient levels of LY255582 during the 24 hour chow feeding period. Previous studies have demonstrated that 18
hours after oral administration of LY255582, opioid receptor occupancy in the CNS (measured by ex vivo receptor binding) is still significantly high. Furthermore, the level of opioid receptor occupancy at 18 hours is significantly correlated with the reduction in 24 hour food intake (55). Moreover, our finding of a preferential effect on palatable food intake is consistent with previous studies suggesting that opiate receptor antagonists primarily alter the intake of palatable food or a preferred macronutrient (23, 25, 34, 37).

Rats that were given access to the HP diet for the first time demonstrated an increase in DA levels in the NAc shell during the HP diet access period. This finding is consistent with other reports using in vivo microdialysis demonstrating an increase in NAc DA levels in response to palatable food (4, 7, 9, 10, 21, 27, 40-42, 47, 58). The response is somewhat selective for the HP diet since we found that rats given access to a regular chow diet under identical conditions ate very little and exhibited no alteration in NAc DA levels. In animals treated with LY255582, HP diet intake was decreased significantly and there was no corresponding rise in mesolimbic DA levels. Interestingly, animals that had their HP diet intake yoked to the group treated with LY255582 (i.e., “pair-fed” group in Fig. 3) exhibited a robust increase in NAc shell extracellular DA levels. The increase in extracellular DA levels in the NAc shell of pair-fed animals was similar to that seen in the vehicle treated animals, even though these rats consumed only 0.7 g of the HP diet compared to 5.9 ± 0.6 g in the vehicle group. This finding suggests that the lack of increase in DA levels seen in the LY255582 treated group was a pharmacological one and not merely a consequence of the decreased HP diet consumption. The DA response to the small quantity of food given to the “pair-fed” animals was somewhat unexpected. A few reports indicate that the DA response to
palatable food is correlated with the amount of food consumed (28, 39, 51, 61).

Methodological differences in the cited studies, including the use of within subject microdialysis, different types of food (nutritionally complete versus macronutrient specific diets), sham-feeding and/or food deprivation may have contributed to these differences. Within subject microdialysis studies are generally ill-advised due to damage induced by the probe over time (12, 13, 50). Additionally, food deprivation and restricted scheduled feeding are known to influence the “amount-dependent” rise in NAc DA; in that food deprived animals appear to exhibit this phenomenon whereas ad libitum fed animals do not (4, 47, 61).

When animals underwent microdialysis testing during the escalation phase of the HP diet access, vehicle treated animals exhibited an increase in NAc shell DA levels in response to the HP diet. Rats treated with LY255582 on Days 2-4 consumed less HP diet compared to vehicle treated animals, and did not progressively increase their intake of HP diet from Days 2-4. Moreover, LY255582 treated rats showed no change in NAc shell DA levels during this time. When LY255582 treatment was lifted on Day 5 of the testing period, rats consumed significant quantities of HP diet and demonstrated a significant rise in NAc shell DA levels during HP diet access. Although the animals that had been treated on Days 2-4 with LY255582 consumed less than those that had been treated with vehicle (6.8 ± 1.5 vs 11.7 ± 0.7), the increase in the extracellular NAc DA level was not statistically different from five day vehicle treated animals. These results are consistent with our findings in Experiment 2a with the pair-fed animals suggesting that consumption of the HP diet stimulated mesolimbic DA neurons independent of the quantity of the HP diet consumed.
Collectively, the results from our microdialysis studies suggest that LY255582 decreased HP diet intake via an inhibition of the mesolimbic DA pathway. In support of this hypothesis, it has been demonstrated that mu and delta opioid receptors are located on inhibitory GABAergic neurons within the VTA (18, 30). Activation of mu and delta opioid receptors through exogenous administration of opioid agonists results in increased DA release within the NAc shell (16, 29, 31, 54). Moreover, consumption of a high fat diet increases expression of endogenous opioid peptides (14), providing a mechanism whereby consumption of the HP diet (which contains 40% of calories/g from fat) may stimulate DA release in the NAc shell. Therefore, blockade of opioid receptors by LY255582 would prevent the increase in DA produced by HP diet access; a hypothesis that is supported by microinjection studies of other opioid antagonists (57, 58). The opioid receptor subtype responsible for the effects seen in the present study is unknown, since LY255582 binds to all three major opioid receptors with high affinity. A likely candidate is the mu opioid receptor since germ line deletion of the mu opioid receptor gene was found to reduce body weight gain in mice fed a high fat diet (56). Moreover, it is doubtful that these results arise from non-opioid receptor-mediated activity of LY255582 (such as activity at dopamine receptors), since autoradiographic binding studies with [3H]-LY255582 revealed a nearly identical opioid receptor-like binding pattern in wild-type mice compared to naltrexone, and no specific binding in triple opioid-receptor (mu, delta and kappa) knockout mice throughout the entire neural axis (20).

It is interesting that our animals did not exhibit habituation of the NAc shell DA response to the HP diet, since we saw a robust increase in DA on Day 3 and Day 5 of HP
diet access. Bassareo and Di Chiara (8) have shown that NAc shell DA response to palatable foods decreases following repeated exposure. However, this does not appear to be the case in food deprived animals (10, 61) nor in rats on a schedule of intermittent sucrose access (4, 47). An important point is that our rats did not have access to any food once they were placed in the microdialysis bowls until HP diet was presented (approximately four hours) which may have contributed to a semi-restricted and/or intermittent schedule. Therefore, it is possible that our rats are in a mild negative energy balance before the HP diet access period in the microdialysis sessions contributing to the lack of habituation seen in our studies. However, the HP diet access and microdialysis studies were conducted during the lights on period of the light/dark cycle, a time when chow intake is normally low. Moreover, we found that in animals given access to a regular chow diet under identical conditions, food intake was very low suggesting that the period when the animals are in the microdialysis bowls is not inducing a generalized deprivation-induced feeding response. Thus, it is unlikely that the increase in NAc shell DA found in the present study arises by an energy deficit that lends salient properties to any ingestant, but rather encodes salient properties of the HP diet itself.

Taken together, our data suggest that an increase in DA release in the NAc shell during the HP diet access period may be involved in the progressive increase in HP diet intake. This finding is consistent with other studies demonstrating that DA transmission in the NAc shell is important in the acquisition of motivated behaviors and motivational learning in particular (8, 17, 21, 41, 42). Our findings demonstrated that animals treated with the opioid antagonist LY255582 did not show a rise in NAc shell DA levels in response to the same amount of HP diet that elicited an increase in DA levels in the pair-
fed animals. These findings suggest that LY255582 may be preventing the DA response to the HP diet, and thus altering the incentive salience of the food. Moreover, the fact that the increase in DA is prevented by an opioid antagonist argues that an increase in endogenous opioid peptides are at least partially involved in regulating HP diet intake. Finally, our data suggest that the pan opioid antagonist, LY255582, may be a useful tool in understanding the neural mechanisms involved in processing the saliency of palatable food and the reinforcement mechanisms regulating food intake.
Perspectives and Significance

The activation of the mesolimbic DA system comprises a critical part of the neural network regulating reward and reinforcement mechanisms for substances of abuse. Indeed, many drug classes with addictive properties (including ethanol, nicotine, opiates, cannabinoids, and psychostimulants) activate the mesolimbic DA pathway, increasing DA levels within the nucleus accumbens (35, 62). However, the neural circuitry encoding salience and hedonic properties of addictive drugs likely did not evolve from the substances described above, but rather from natural reinforcers such as palatable, calorically-dense food (which primitive animals would require to maintain energy balance in an environment where food availability was limited) and engaging in sexual behavior (essential for continuation of the species). Given the results of the present study with the opioid receptor antagonist, LY255582, it appears that similar to ethanol intake (45, 48) activation of the mesolimbic DA system by consumption of palatable food is, in part, mediated by endogenous opioids. Indeed, microinjection of mu opioid receptor agonists into the nucleus accumbens increases the preferential intake of palatable food (63). This raises the question: does chronic and excessive consumption of palatable foods, which often occur for reasons other than maintaining energy balance, produce neurochemical and behavioral effects resembling addiction to drugs of abuse? Evidence supporting this hypothesis is mounting. Recently, Colantuoni et al., 2002, reported that rats given an intermittent access schedule to glucose develop a binge-like feeding pattern, and exhibit somatic signs of withdrawal and anxiety following administration of the opioid antagonist naloxone. Moreover, rats bingeing on sugar exhibit increases in mu-
opioid and DA D₁ receptor binding and decreases in DA D₂ receptor binding in the nucleus accumbens (15). These findings are particularly relevant since similar changes in striatal D₂ receptor binding have been reported in PET studies of both human substance abusers and patients with chronic obesity (60). Similar to other drugs of abuse, sugar bingeing rats exhibit locomotor cross-sensitization to amphetamine (3) and cocaine (24) and when forced to abstain from sugar access show an enhanced intake of unsweetened alcohol (2). Interestingly, patients with alcohol dependence exhibit an enhanced sucrose preference when compared to non-alcoholic controls (32). Given these findings it is reasonable to speculate that following repeated, over consumption of palatable food alterations in brain DA and opioid systems may support mechanisms and behaviors associated with addiction similar to the development of addiction to drugs of abuse. Indeed, many people exhibit cravings for and an uncontrollable urge to eat high fat, high sugar foods much in the same way that addicts crave and seek drugs of abuse. Together, these observations offer an interesting corollary to drug addiction. Whether food addiction exists in humans and contributes to the growing epidemic of obesity will be an important topic for future study.
Figure Legends

Figure 1. Experimental timeline depicting the temporal design of Experiment 2b. Separate groups of animals (i.e., between subjects design) were taken through the protocol to be tested on either Day 3 or Day 5.

Figure 2. The effect of LY255582 on HP diet intake (A) and concomitant 24 hour normal chow intakes (B). Data are expressed as the mean ± SEM (n = 7/group). HP diet increased over the first four days when only vehicle injections were administered. On the fourth day of HP diet access, animals were treated with LY255582 and all doses (0.1-3.0 mg/kg, s.c.) caused a significant decrease in HP diet intake compared to vehicle (★p < 0.01, Dunnett’s multiple comparison test). Only the highest dose tested (3.0 mg/kg) caused a reduction in 24 hour chow intake, suggesting that the effect of LY255582 was selective for the HP diet (★p < 0.01, Dunnett’s multiple comparison test).

Figure 3. The effect of acute LY255582 (0.3 mg/kg, s.c.) on one hour HP diet intake in animals tested during their first exposure to the HP diet (inset) and the accompanying change in NAc shell DA levels (n = 6-7/group). Data are expressed as the mean ± SEM (food intake data) or are expressed as a percent of baseline defined using the three time points prior to injection (microdialysis data; mean ± SEM). Acute LY255582 significantly reduced HP diet intake compared to vehicle treated animals (★; p = 0.014; Student’s t-test). Pair-fed animals were presented with and consumed the amount of HP diet ingested by animals treated with LY255582 (0.7 g). HP diet access caused a
significant increase in extracellular DA levels in the NAc shell in both the vehicle and
pair-fed groups regardless of the amount of HP diet consumed (★ p < 0.05 compared to
time 0 in vehicle labeled group; # p < 0.05 compared to time 0 in pair-fed labeled group;
post hoc Fisher’s test). Treatment with LY255582 inhibited HP diet intake and the
increase in DA levels observed during the HP access period. Indeed, significant
decreases in DA levels compared to baseline were observed at two timepoints in animals
treated with LY255582 (% p < 0.05; post hoc Fisher’s test

Figure 4. The effect of LY255582 (0.3 mg/kg, s.c.) on the escalation of HP diet intake
(inset) and on NAc shell DA levels following 3 days of the HP diet limited access
schedule. (n = 7-8/group). Data are expressed as the mean ± SEM (food intake data) or
are expressed as a percent of baseline defined using the three time points prior to
injection (microdialysis data; mean ± SEM). Vehicle treated animals exhibited an
increase in HP diet intake across Days (# p < 0.01 compared to Day 1; % p < 0.001
compared to vehicle; post hoc Fisher’s test), whereas LY255582 treated animals did not.
NAc shell DA levels increased in response to the HP diet in vehicle treated animals only
(Time points 70, 80 and 90 min; ★ p < 0.02; post hoc Fisher’s test). NAc shell DA levels
did not change in response to HP diet access in animals treated with LY255582.

Figure 5. The effect of cessation of LY255582 (0.3mg/kg, s.c.) treatment on HP diet
intake (inset; n = 5-9/group) and NAc shell DA levels on Day 5 of the limited HP diet
access schedule. On Day 5 both groups of animals were treated with vehicle and differ
only in their treatment histories. Data are expressed as the mean ± SEM (food intake
Vehicle treated animals exhibited an increase in HP diet intake across Days 1-4 (# p < 0.001 compared to Day 1; post hoc Fisher’s test), whereas in LY255582 treated animals HP diet intake remained low. On Day 5 when LY255582 treatment was stopped, HP intake increased significantly (# p < 0.001 compared to Day 1; post hoc Fisher’s test). NAc shell DA levels increased significantly during the HP diet access period on Day 5 in both groups regardless of prior treatment history ( p < 0.05; two-way repeated measures ANOVA).

**Figure 6.** Food intake and NAc shell DA levels following one hour access to a normal chow diet (n = 6 animals per group). Data are expressed as the mean ± SEM (food intake data) or are expressed as a percent of baseline defined using the three time points prior to injection (microdialysis data; mean ± SEM). Rats consumed an average of 0.05 ± 0.02 g of chow during the one hour access period (inset). No changes in NAc DA levels were observed over the experimental protocol period indicated by no observed effect of Time following vehicle injection (p = 0.169; one-way ANOVA) or during one hour access to a chow diet (p = 0.629; one-way ANOVA).
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Day 1: Rats receive HP diet access (no treatment).

Days 2-4: Rats are given vehicle or 0.3 mg/kg LY255582, s.c. One hour later they are given access to HP diet.

Day 5: All rats given vehicle one hour prior to HP diet access.

Microdialysis D3

Microdialysis D5
Fig. 2

A.

HP Intake (g)

Day 1 Veh  Day 2 Veh  Day 3 Veh  Day 4 Trt

Vehicle  0.1 mg/kg LY255582  0.3 mg/kg LY255582  1.0 mg/kg LY255582  3.0 mg/kg LY255582

B.

Chow Intake (g)

Day 1 Veh  Day 2 Veh  Day 3 Veh  Day 4 Trt

Vehicle  0.1 mg/kg LY255582  0.3 mg/kg LY255582  1.0 mg/kg LY255582  3.0 mg/kg LY255582
**Fig. 3**

* p < 0.05 compared to time 0 (Vehicle)
# p < 0.05 compared to time 0 (PF)
% p < 0.05 compared to time 0 (LY255582)
Fig. 4

![Graph showing changes in DA (% of baseline) over time with Microdialysis and HP Diet interventions.]

- * p < 0.05 compared to time 0 (Vehicle)
- # p < 0.05 compared to Day 1
- % p < 0.05 compared to Vehicle

- Vehicle (n=8)
- LY255582 (n=7)
Fig. 5

[Graph showing changes in DA (% of Baseline) over time with different interventions and vehicle comparisons.]

# p < 0.05 compared to Day 1
% p < 0.05 compared to vehicle
Chow intake: 0.05 ± 0.02 grams

n=6

Veh Injection

Chow

DA (% of Baseline)