A Novel Nociceptin Receptor Antagonist LY2940094 Inhibits Excessive Feeding Behavior in Rodents: A Possible Mechanism for the Treatment of Binge Eating Disorder

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ABSTRACT

Nociceptin/orphanin FQ (N/OFQ), a 17 amino acid peptide, is the endogenous ligand of the ORL1/nociceptin-opioid-peptide (NOP) receptor. N/OFQ appears to regulate a variety of physiologic functions including stimulating feeding behavior. Recently, a new class of aminospiro-piperidine-based NOP antagonists was described. One of these molecules, LY2940094 has been identified as a potent and selective NOP antagonist that exhibited activity in the central nervous system. Herein, we examined the effects of LY2940094 on feeding in a variety of behavioral models. Fasting-induced feeding was inhibited by LY2940094 in mice, an effect that was absent in NOP receptor knockout mice. Moreover, NOP receptor knockout mice exhibited a baseline phenotype of reduced fasting-induced feeding, relative to wild-type littermates. In lean rats, LY2940094 inhibited the overconsumption of a palatable high-energy diet, reducing caloric intake to control chow levels. In dietary-induced obese rats, LY2940094 inhibited feeding and body weight regain induced by a 30% daily caloric restriction. Last, in dietary-induced obese mice, LY2940094 decreased 24-hour intake of a high-energy diet made freely available. These are the first data demonstrating that a systemically administered NOP receptor antagonist can reduce feeding behavior and body weight in rodents. Moreover, the hypophagic effect of LY2940094 is NOP receptor dependent and not due to off-target or aversive effects. Thus, LY2940094 may be useful in treating disorders of appetitive behavior such as binge eating disorder, food choice, and overeating, which lead to obesity and its associated medical complications and morbidity.

Introduction

Nociceptin/orphanin FQ (N/OFQ) is a 17-amino acid neuropeptide with high homology to the opioid peptide dynorphin. N/OFQ was identified as the natural ligand for the orphan receptor ORL1 (today referred to as the NOP receptor) (Meunier et al., 1995; Reinscheid et al., 1995). The NOP receptor is a Class A G-protein-coupled receptor that is functionally coupled to inhibition of adenylate cyclase, activation of mitogen-activated-protein kinases, activation of K+ conductance and inhibition of Ca2+ conductance (see reviews by Mogil and Pasternak, 2001; New and Wong, 2002). The NOP receptor is widely expressed in the central nervous system (CNS) including the cortex, hippocampus, medial septum, amygdala, thalamus, hypothalamus, and the nucleus of the solitary tract. Moreover, NOP is expressed in the peripheral nervous system as well as in the gastrointestinal tract, smooth muscles, and in cells of the immune system. Initially, N/OFQ was found to produce hyperalgesia, leading to the naming the peptide nociceptin (Meunier et al., 1995); however, in addition to pain, N/OFQ modulates other CNS behaviors, including stress and anxiety, depression, substance abuse, prolactin secretion, locomotor activity, body temperature, memory, and feeding (Mustazza and Bastanzio, 2011; Gavioli and Calo’, 2013; Singh et al., 2013; Witkin et al., 2014).

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ABBREVIATIONS: ANOVA, analysis of variance; BED, binge eating disorder; CHO, Chinese hamster ovary; CNS, central nervous system; DIO, dietary induced obese; HED, high-energy diet (diet high in both fat and sucrose); 5-HT, 5-hydroxytryptamine or serotonin; J113397, 1-[(3R,4R)-1-cyclooctylmethyl-3-

phenalen-1-yl)-1-phenyl-1,3,8-triaza- spiro[5.5]decan-4-one; RQ, respiratory quotient; SB-612111, (1-

hexahydro-1-

peptide sequence); NOP, nociceptin opioid peptide receptor; ORL1, opioid-receptor-like-1; RO, receptor occupancy; Ro 64-6198, (1-

drochloride; KO, knockout; LY2940094, [2-

hydroxymethyl-4-piperidyl]-3-ethyl-1, 3-dihydro-2H-benzimidazol-2-one; JTC-801, (1-

pentane-6,7,8,9-tetrahydro-5H-benzocyclohepten-5-ol-oxygen consumption; WT, wild type.
Numerous studies have reported that activation of NOP receptors produces a robust orexigenic effect in rodents. Indeed, fasting regulates N/OFQ and NOP receptor gene expression in the CNS (Rodi et al., 2002; Przydzial et al., 2010). Highly potent and efficacious orexigenic effects are produced by administration of either N/OFQ peptide analogs or small molecule agonists (Pomonis et al., 1996; Polidori et al., 2000), leading the authors to hypothesize that the feeding effects of N/OFQ are mediated at the level of the forebrain and/or hypothalamus (Polidori et al., 2000). Micro-injection studies confirmed this hypothesis and identified the nucleus accumbens and ventromedial hypothalamic nucleus as specific sites of the orexigenic actions of N/OFQ (Stratford et al., 1997). It is postulated that the orexigenic effects of N/OFQ are the result of inhibition of anorexigenic signaling (Farhang et al., 2010). These data support the hypothesis that inhibitors to NOP receptor signaling will regulate food intake and might therefore be useful in the treatment of obesity and eating disorders.

Several NOP selective antagonists (J113397, JTC-801, and SB-612111) and agonists (Ro 64-6198 and W121293) have been developed (Zaveri et al., 2005; Largent-Milnes and Vanderah, 2010). Recently, we identified a novel series of NOP receptor antagonists based on a dihydrospiro(piperidine-4,7-thieno[2,3-c]pyran) scaffold (Toledo et al., 2014). These antagonists were found to have high affinity and antagonist activity for the human NOP receptor. In vivo, we found that the antagonists had good oral bioavailability and exhibited CNS activity. One of these molecules, LY2940094 (Toledo et al., 2014), exhibited subnanomolar antagonist potency (Kb = 0.17 nM) in vitro at the human NOP receptor. Moreover, LY2940094 was orally bioavailable in rats (F = 57%, T1/2 = 3.8 hours) in vivo and demonstrated sustained NOP receptor occupancy in the brain p24 hours receptor occupancy (RO = 62% at 10 mg/kg orally). When tested in vivo LY2940094 potently inhibited the hypothermic effects of exogenously administered NOP agonist Ro 64,6198, confirming its on-target activity.

Here we hypothesize that inhibition of NOP receptor signaling in the CNS will modulate feeding behavior. We demonstrate for the first time that a small molecule, orally bioavailable, CNS-penetrant NOP receptor antagonist, LY2940094, does indeed inhibit feeding behavior in rats and mice in a genotype-specific manner and stimulates lipid utilization in diet-induced obese mice.

### Materials and Methods

#### Compounds, Dose Selection, and Formulation

[2-[4-[2-Chloro-4,4-difluoro-spiro][5H-thieno][2,3-c]pyran-7,4′-’piperidine-1′-yl)methyl]-3-methyl-pyrazol-1-yl]-3-pyridyl]methylmethanol (LY2940094) as a free base or a tartrate salt was used for all studies and was synthesized at Lilly Research Laboratories (Indianapolis, IN) as previously described (Toledo et al., 2014). Doses of LY2940094 were selected that were previously shown to produce saturating in vivo occupancy of NOP receptors in the rat hypothalamus for at least 6 hours to ensure suitable receptor blockade over the testing period (Toledo et al., 2014; Barth et al., 2014). The serotonin 2C receptor agonist meta-chlorophenylpiperazine (mCPP) was used at an ED50 dose as a positive control for the NOP receptor knockout mouse study and was purchased from Sigma-Aldrich (St. Louis, MO). Naltrexone HCl was purchased from Sigma-Aldrich. [3H]-nociceptin was purchased from Perkin-Elmer Life and Analytical Sciences (Waltham, MA). A 20% Captisol in 25 mM PO4 buffer (pH 2) formulation was used in the studies, and all doses were corrected for the salt weight. This formulation permitted the compound to be completely dissolved in solution.

#### Animals

Male lean and DIO Long-Evans rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and male DIO C57Bl/6 mice were purchased from Taconic (Hudson, NY). Male NOP receptor knockout (KO) mice and wild-type littermates maintained on a 12:96 inbred background were generated by heterozygous breeding as previously described (Kest et al., 2001). All animals were singly housed in rooms using a 12-hour light/dark cycle and had ad libitum access to food and water unless otherwise stated. Lean animals were maintained on chow diet TD2014, whereas DIO animals were maintained on the high-energy diet TD95217 (Harlan Teklad, Madison, WI). All in vivo experiments were performed during the “lights on” photoperiod unless otherwise stated. Animals were maintained and experiments were conducted in compliance with the policies of the Animal Care and Use Committee of Eli Lilly and Company in conjunction with the American Association for the Accreditation of Laboratory Animal Care-approved guidelines.

#### In Vitro NOP Receptor Binding

A filtration-based [3H]-nociceptin binding assay was used to determine the affinity (Kd) of LY2940094 using previous conditions with minor modifications (Ardati et al., 1997). Assay incubations were performed in deep-well 96-well plates with [3H]-nociceptin (final assay concentration 0.2 nM) and 5–10 μg of membrane protein (isolated from CHO cells expressing cloned human NOP receptors) in a final volume of 0.5 ml of HEPES buffer (20 mM; pH 7.4) containing, 5 mM MgCl2, 1 mM EDTA, 100 mM NaCl, and 0.1% bovine serum albumin and incubated for 60 minutes at 25°C. Reactions were terminated by filtration on glass fiber filters GF/C Filtermat A (Perkin-Elmer Life and Analytical Sciences) pretreated with 0.3% polyethyleneimine. The filters were washed three times with 5 ml of ice-cold Tris-HCl buffer (50 mM, pH 7.4) and then dried and embedded with MultiLex scintillant (Perkin-Elmer Life and Analytical Sciences), and the bound radioactivity was counted in a Microbeta Trilux (Perkin-Elmer Life and Analytical Sciences). Specific binding was determined by displacement with 100 nM unlabeled nociceptin. Curves were plotted as the percent of specific inhibition versus LY2940094 concentration, and IC50 values were determined using four-parameter nonlinear regression (XLFit version 4.0, DDBS). Kd values were calculated from the IC50 according to Cheng and Prusoff, where \( K_d = IC_{50} \times (1 + D \times K_N)^{-1} \) (Cheng and Prusoff, 1973). Reported values for Kd are shown as geometric means ± the standard error of the mean (S.E.M.), from n = 3 independent assays. Geometric means are calculated by the equation GeoMean = 10(average [log K1 + log K2 + ...log Kn]/square root of the number of replicates, n).

#### In Vitro Opioid Receptor Binding

To determine receptor selectivity to classic opioid receptors, the binding affinity (Ki) of LY2940094 was determined in CHO or human embryonic kidney cell membranes expressing cloned human μ, κ, or δ receptors, as previously described (Emmerson et al., 2004). Briefly, membranes (4–6 μg protein per reaction) were added to buffer containing 50 mM Tris-HCl, 100 mM NaCl, 1 μM GDP, 5 mM MgCl2, and 1 mM EDTA (pH 7.4). The reactions were initiated by the addition of 0.2 nM [3H]- diprenorphine to yield a 500 nl final assay volume. The reaction was incubated for 120 minutes at 25°C. Specific binding was determined by displacement with 10 μM naltrexone. Reactions were terminated by rapid filtration through glass fiber filters, radioactivity was measured, and data were analyzed using procedures identical to those used for NOP receptor binding.
In Vitro Functional Activity on G-protein Activation

The NOP receptor antagonist affinity ($K_a$) of LY2940094 was measured in CHO membranes expressing cloned human NOP receptors with a GTP-$\gamma$S ([35S] binding assay, according to previously described protocols with minor modifications (Delapp et al., 1999; Ozaki et al., 2000). Assays were in a 200 $\mu$L volume with a 20 mM HEPES buffer containing 100 mM NaCl, 5 mM MgCl$_2$, 1 mM EDTA, 0.1% bovine serum albumin, 3 mM GDP, 0.5 mM GTP-$\gamma$S. NOP receptor membrane suspension was added at a concentration of 20 $\mu$g protein per well, and receptor stimulation was achieved with 300 nM nociceptin. Wheat germ agglutinin-coated scintillation proximity assay beads (Perkin-Elmer Life and Analytical Sciences) were added at 1 mg per well to detect membrane-bound GTP-$\gamma$S ([35S]). Plates were sealed and incubated for 2 hours at 25°C and then placed at 4°C overnight to allow the scintillation proximity assay beads to settle. Plates were then counted for radioactivity in a Microbeta TriLux instrument. Specific GTP-$\gamma$S ([35S]) binding was determined as the difference in counts per minute observed in the absence and presence of 10 $\mu$M unlabeled GTP-$\gamma$S. Data were plotted as the percent of specific GTP-$\gamma$S ([35S]) bound from which IC$_{50}$ values were determined using four-parameter nonlinear regression routines (XLFit version 4.0, IDBS). Antagonist affinity ($K_I$) was estimated according to Delapp et al. (1999) using a modification of the equation of Cheng and Prusoff (1973) where $K_I = IC_{50} \times (1 + D \times EC_{50}^{-1})^{-1}$. Reported values for $K_I$ are shown as geometric mean $\pm$ the standard error of the mean (S.E.M.) from three independent experiments.

Agonist activity was assessed using a functional GTP-$\gamma$S ([35S]) binding assay similar to those described for antagonist measurement, except that the agonist assay did not add nociceptin to stimulate NOP receptor-mediated G-protein activation. Basal activity was determined in the absence of added nociceptin. A control nociceptin concentration response curve (geometric mean EC$_{50}$ for nociceptin was 2.25 nM, S.E.M. = 0.41, $n = 18$) was included in each assay. The concentration range tested for LY2940094 in the agonist assay was 0.17 nM to 10 $\mu$M (final assay dimethylsulfoxide concentration in all wells was 1%). The percent efficacy was calculated relative to nociceptin, which was set at 100%.

Models of Hyperphagia

Fasting-Induced Food Intake Study in Mice. Experiments were performed on 12-week-old male NOP receptor knockout mice and wild-type (WT) littermates maintained on a 12:12 inbred background. Individually housed mice were acclimated to housing conditions for a minimum of 3 days before the onset of testing to eliminate stress effects due to the change from group to individual housing. Mice of each genotype ($n = 5$ per treatment group for each test day) were randomly assigned to each treatment group on each test day. Food was removed from cages just before the onset of the dark cycle. After a 15-hour fast, mice were administered LY2940094 at doses (3 or 30 mg/kg orally) that produce saturating levels of NOP receptor occupancy in the hypothalamus (Barth et al., 2014) or vehicle via oral gavage 1 hour before gaining ad libitum access to food during the “lights on” period (LabDiet 5058). An additional group of mice received the 5-HT$_2C$ receptor agonist mCPP (at an ED$_{90}$ dose of 30 mg/kg, i.p.), which is known to potently inhibit food intake to serve as a positive control for both genotype (to confirm that NOP receptor knockout mice do not exhibit an abnormal feeding response to a known anorectic agent) and compound treatment. Measurements of food intake were recorded 1 hour after access to food to the time during which mice are typically at rest and not normally eating. The first hour after food presentation represents a period of the most robust hyperphagia, after which the animals eat at a much slower normal rate. After initial testing, mice were rested for 1 week with ad libitum access to chow. After the week of rest, mice were retested after a Latin-square study design. Therefore, at the end of the study all mice received all drug (LY2940094 and mCPP) and vehicle treatments; thus each treatment group at the end of the study had an $n = 12$.

Hyperphagia Stimulated by Consumption of a Highly Palatable Diet in Lean Rats.

Male Long-Evans rats (376–445 g) at 9–10 weeks of age were individual housed and accustomed to a 12-hour light/dark cycle on a reverse light cycle (lights off at 10:00 AM) for at least 1 week before the start of experiment. All rats were maintained on a standard chow diet TD2014 (Harlan) ad libitum with free access to water. Before receiving drug treatment, a mean daily food intake was obtained for 3 days for each rat by dosing all rats with vehicle. Rats were divided into four treatment groups by block randomization according their average food intake ($n = 7$ rats per group). On the day of study, rats were treated with either LY2940094 (10 or 30 mg/kg) or vehicle by oral gavage. These doses of LY2940094 are known to saturate NOP receptors in the hypothalamus in vivo while maintaining target selectivity based on our study in NOP receptor KO mice (see above). Thirty minutes after drug administration, rats in three treatment groups (vehicle, 10 mg/kg, 30 mg/kg) were given access to a high-energy diet (HED) TD95217 (Harlan Teklad), whereas one group (vehicle treated) was maintained on the standard rat chow diet to measure the amount of hyperphagia stimulated by access to the HED. Food intake was monitored for 5 hours after access to the HED. Three-day cumulative food intake and body weight were also measured after daily treatment with LY2940094.

Hyperphagia Stimulated by a Mild Calorie Restriction Diet in DIO Rats.

Overeating commonly occurs when a person is dieting or immediately after cessation of the diet. To test how NOP receptor blockade would function in the context of dieting, male DIO Long Evans rats (520–631 g) at 14–15 weeks of age were placed under a mild caloric restriction designed to model the reduced calorie intake that occurs when obese people diet (generally a 20–30% reduction in daily caloric intake). All rats were fed the HED TD9521 (for 10–12 weeks before the start of the study for them to develop obesity. The rats were individually housed and accustomed to 12-hour light/dark on a reverse light cycle (lights off at 10:00 AM) at 24°C for at least 1 week before the start of the experiment. All rats were maintained on HED TD95217 (Harlan Teklad) with free access to water throughout the study. Before initiating the mild caloric restriction, the rats were dosed orally with vehicle, and food intake and body weight were monitored for 3 days so that average food intake was obtained for each rat. Rats were then grouped by block randomization according to a prediction that their body weight into 5 groups ($n = 6$ rats/group). During the experiment rats were treated orally with either vehicle or LY2940094 (10 mg/kg, a dose previously shown to inhibit food intake in rats) 30 minutes before the start of the dark cycle. Group A rats were treated with vehicle and fed ad libitum with TD 95217 diet throughout the entire 4-day study period. Groups B and C were treated with vehicle and fed with 70% of their average daily food intake for 3 days, whereas Groups D and E were treated with LY2940094 (10 mg/kg orally) and fed with 70% of their average food intake.
daily food intake for 3 days. On day 4, the forced caloric restriction was lifted, and all the groups of rats were returned to ad libitum feeding conditions. During day 4 ad libitum feeding, Groups B and D were treated with vehicle, whereas Groups C and E were treated with LY2940094 (10 mg/kg orally). Daily food intake and body weight were monitored for the entire period of the study. Six-hour and 24-hour food intake and body weight change after last dosing (day 4) were also measured. Body weight was not different between the groups on day 4, therefore, the groups were separated into days 1–3 (the period during the restricted feeding) and day 4 (when all animals were returned to ad libitum feeding conditions).

Model of Ad Libitum Feeding and Energy Utilization

Free Access Food Intake in DIO Mice. To determine if blockade of NOP receptors was effective in reducing free access feeding in the context of obesity we conducted a study in DIO mice. Three- to four-month-old male diet-induced obese (DIO) C57Bl/6 mice (40.3–44.1 g) were purchased from Taconic (Germantown, NY). Animals were individually housed in a temperature-controlled (24°C) facility with a 12-hour light/dark cycle (lights on 10:00 PM to facilitate animal dosing just before the start of the dark cycle) and had free access to a HED (Teklad TD95217) and water. Before the experiment started, mice were treated with vehicle by oral gavage and weighed for 2 days to acclimatize them to the handling procedures. The day before the study, mice were block randomized by body weight to treatment groups (n = 8/group) so that each group had mean body weight that did not significantly differ.

Study 1: The effect of LY2940094 on food intake and body weight in DIO mice. Just before the onset of the dark photoperiod, LY2940094 or vehicle was administered by oral gavage at 20 mg/kg (8 ml/kg) to DIO C57Bl/6 mice. This dose was chosen as doses within this range reduced fasting-induced food intake in mice while maintaining on target specificity (see Results). Food intake was measured at 0- to 2-, 2- to 4-, 4- to 6-, and 6- to 24-hour bins posttreatment with LY2940094. Animals received a second dose of LY2940094 treatment 24 hours later, and body weight was measured.

Study 2: The effect of LY2940094 on energy expenditure and fuel utilization in DIO mice. Indirect calorimetry was performed to examine the effect of LY2940094 on energy expenditure and fuel utilization. After 24 hours of acclimation to the 11 cm × 21 cm calorimetry chambers (Columbus Instruments International Corp., Columbus, OH), LY2940094 or vehicle was administered to mice (N = 5/group) at 20 mg/kg (8 ml/kg) by oral gavage. Oxygen consumption (VO₂) and CO₂ production were measured to calculate the respiratory quotient (RQ) and energy expenditure. RQ was calculated as the ratio of CO₂ production to VO₂. Energy expenditure was calculated as the product of caloric value (CV) and VO₂ per kilogram (kg) of body weight, where caloric value = 3.815 + 1.232*RQ (Elia and Livesey, 1992). Locomotor activity was monitored over 24 hours while the animals were in the calorimetry chamber by assessing laser beam breaks in the X-, Y-, and Z-planes.

Statistical Analysis

Data were expressed as mean ± standard error (S.E.M.). Fasting-induced feeding in mice was analyzed using a two-way analysis of variance (ANOVA) with genotype and drug treatment as factors followed with a Bonferroni’s multiple comparison post hoc test. The amount of HED consumed over 5 hours by lean rats after treatment with LY2940094 was measured and analyzed by one-way ANOVA with a Tukey post hoc test, whereas cumulative daily HED intake was analyzed using a two-way ANOVA with time and drug treatment as factors followed by a Bonferroni’s multiple comparison post hoc test. In calorie-restricted DIO rats, changes in food intake and body weight after treatment with LY2940094 were analyzed using a one-way ANOVA with Tukey post hoc test. In ad libitum-fed DIO mice, food intake was measured after LY2940094 treatment in time bins (0- to 2-, 2- to 4-, 4- to 6-, and 6- to 24-hour bins) and analyzed by two-way ANOVA with time and drug treatment as factors followed by a Bonferroni’s multiple comparison post hoc test, whereas changes in body weight were analyzed by a Student’s t test.

### Results

**LY2940094 Is a Potent and Selective Antagonist at NOP Receptors**

LY2940094 (Fig. 1) exhibits high affinity binding, high functional antagonist potency with no agonist activity, and high selectivity against other opioid receptors (Tables 1 and 2). The binding affinity for LY2940094 in membranes expressing recombinant human NOP receptors was Kᵢ = 0.1 nM. High binding affinity was found in membranes isolated from rat brain, Kᵢ = 0.7 nM, confirming that LY2940094 is potent at both the rodent and human NOP receptors. Antagonist potency of LY2940094 was similarly high at the human NOP receptor, Kᵢ = 0.17 nM. No agonist activity of LY2940094 at human NOP receptors was detected at concentrations up to 10 µM. Moreover, selectivity of LY2940094 over the other classic opioid receptors (mu, delta, and kappa) was greater than 1000-fold (Table 2).

**LY2940094 Inhibits Fasting-Induced Feeding in Mice**

We employed congenic NOP receptor knockout mice on a 129/S6 background to examine the effects of LY2940094 on fasting-induced feeding and to evaluate whether the effects were NOP receptor mediated and not the result of off-target or toxic effects. We found that both genotype and treatment with LY2940094 significantly reduced fasting-induced food intake [overall two-way ANOVA for interaction F(3, 88) = 4.0, P = 0.01; genotype F(1, 88) = 7.1, P = 0.009; and treatment F(3, 88) = 34, P < 0.001]. Interestingly in vehicle-treated mice, NOP KO mice consumed less food than WT littermate controls (Fig. 2). The reduction in fasting-induced feeding in NOP KO mice was robust, approximating a 41% reduction from controls (P < 0.01 WT vehicle versus KO vehicle). After the administration of LY2940094 (at doses that produce full RO) to WT mice, fasting-induced feeding was significantly inhibited (P < 0.005 WT vehicle versus WT at 3 mg/kg and P < 0.05 WT vehicle versus 30 mg/kg LY2940094). Contrary to the response in WT mice, LY2940094 did not affect feeding in NOP KO mice at either 3 or 30 mg/kg, doses that decreased feeding in WT littermates. To ensure that the NOP KO mice were capable of

### Statistical Analysis

Data were expressed as mean ± standard error (S.E.M.). Fasting-induced feeding in mice was analyzed using a two-way analysis of variance (ANOVA) with genotype and drug treatment as factors followed with a Bonferroni’s multiple comparison post hoc test. The amount of HED consumed over 5 hours by lean rats after treatment

<table>
<thead>
<tr>
<th>hNOP Binding Affinity Kᵢ ± S.E.M., n = 3</th>
<th>rNOP Binding Affinity Kᵢ ± S.E.M., n = 3</th>
<th>hNOP Antagonist Affinity Kᵢ ± S.E.M., n = 3</th>
<th>hNOP Agonist Activity Eᵢmax @ 10 µM, n = 3</th>
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<td>0.105 ± 0.0361</td>
<td>0.71 ± 0.1</td>
<td>0.166 ± 0.0352</td>
<td>−0.500 ± 2.52</td>
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In vitro pharmacology of the NOP receptor antagonist LY2940094 at human (hNOP) or rat (rNOP) receptor expressed in CHO cells

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exhibiting a reduction in food intake after the administration of an anorexigenic agent, we tested the 5-HT2C agonist mCPP. We found that 30 mg/kg mCPP produced a maximum and equivalent inhibition of 1-hour food intake in both WT and NOP KO mice (P < 0.001 WT vehicle versus WT and KO).

**LY2940094 Inhibits Consumption of Palatable Food Intake in Lean Rats.**

Another model of hyperphagia that we tested was feeding induced by the acute access to a highly palatable, high energy diet (HED). In this model lean rats given acute access to a HED will over consume the diet preferentially over chow, ingesting significantly more calories than animals given access to chow alone. We found that LY2940094 potently inhibited 5-hour HED-induced hyperphagia [overall one-way ANOVA treatment F(3, 20) = 5.536, P = 0.006]. As can be seen in Fig. 3A, access to a HED resulted in an approximately 36% increase in acute caloric intake over rats given access to chow alone (see vehicle-treated HED versus vehicle-treated chow groups). Interestingly when given a choice between chow and HED the rats will preferentially eat almost all of their calories from the HED and not consume the chow diet. Doses of 10 and 30 mg/kg of LY2940094 (doses that produce approximately 100% occupancy) produced a robust inhibition of caloric intake of the HED (P < 0.05). Interestingly, the reduction in caloric intake approximates that of the chow-only vehicle-treated groups. Thus, LY2940094 was able to completely prevent the hyperphagia induced by acute access to a HED. When administered for 3 days, LY2940094 reduced both cumulative food intake [overall two-way ANOVA was significant for interaction F(9, 80) = 2.329, P = 0.02; time F(3, 80) = 38.96, P < 0.0001; and treatment F(3, 80) = 14.6, P < 0.0001]. Three-day cumulative food intake was lower over the testing period in animals treated with LY2940094 compared with vehicle-treated rats on HED alone (P < 0.01) (Fig. 3B). Vehicle-treated animals eating HED gained a significantly greater amount of weight than the chow-fed animals over the testing period (P < 0.0001), whereas LY2940094-treated rats gained less weight (Fig. 3C) (P < 0.01 at 30 mg/kg dose). These data support that the N/OFQ system may be involved in macronutrient preference or palatable food consumption. Moreover, our findings support that this form of hyperphagia, and subsequent body weight gain can be inhibited by LY2940094.

**LY2940094 Inhibits Food Intake in Calorie-Restricted DIO Rats.**

To model dieting we placed DIO rats on a mild calorie restriction (animals were given access to 70% of free access eating) for 3 days. During the 3-day restriction period, animals in Groups B and C received vehicle treatment, whereas animals in Groups D and E received 10 mg/kg oral LY2940094 (Fig. 4). On day 4, Groups A, B, and D received vehicle and Groups C and E were treated with 10 mg/kg oral LY2940094. Immediately after the oral treatment on day 4, animals were returned to ad libitum food access. Figure 4A shows that in the first 6 hours after replenishment of food, calorie-restricted animals in Groups B and C significantly over consumed the diet compared with the vehicle-treated ad libitum-fed group A [overall one-way ANOVA F(4, 25) = 4.009, P < 0.05]. Continued treatment with LY2940094 (Group E) during restriction and ad libitum periods completely abolished this caloric restriction-induced hyperphagia. Moreover, administration of LY2940094 during restriction days and/or on the ad libitum day prevented animals from overeating in the 24-hours upon food replenishment [Fig. 4B; overall one-way ANOVA F(4, 25) = 2.937, P < 0.05]. Examination of changes in body weight during this study revealed that during the caloric restriction phase of the study (days 1–3) animals in all treatment groups lost body weight [Fig. 4C; overall one-way ANOVA F(4, 25) = 7.742, P < 0.001]. Interestingly, animals treated with LY2940094 tended to lose less weight than the vehicle-treated controls. However, when the animals were returned to ad libitum

![Fig. 2. LY2940094 inhibits fasting-induced food intake in male wild-type 129Sv mice (solid bars) but not NOP receptor knockout mice (open bars) after oral administration. Mice were fasted overnight, dosed with LY2940094 30 minutes before presentation of food during the lights-on period. Food intake was measured for 1 hour. Data were compared with a two-way ANOVA with genotype and treatment as factors followed by Bonferroni’s post hoc test. *P < 0.001 WT vehicle versus ORL KO vehicle; #P < 0.05 WT vehicle versus WT 3 and 30 mg/kg LY2940094; ^P < 0.001 WT vehicle versus WT and ORL1 knockout mCPP, 30 mg/kg.](image-url)
feeding conditions on day 4 (when the forced diet was lifted) animals treated with LY2940094 gained less weight than vehicle-treated controls ($P < 0.05$) [Fig. 4D; overall one-way ANOVA $F(4, 25) = 5.783$, $P = 0.002$]. Thus, LY2940094 is effective in preventing hyperphagia and weight regain after a period of mild caloric restriction resembling dieting.

**LY2940094 Inhibits 24-hour Free Access Food Intake and Stimulates Lipid Utilization in DIO mice**

The effect of LY2940094 on spontaneous 24-hour free-access food intake was measured in DIO C57Bl/6 mice maintained on a HED. Consistent with the previous findings, LY2940094 inhibited spontaneous free-access feeding in DIO mice. To
map the time course of the effects of LY2940094 over 24 hours, we measured food intake in 2-hour bins at 20 mg/kg oral dose of LY2940094. This was a dose expected to lack off-target effects based on our knockout mouse study and yet it is high enough to expect exposure to cover 24 hours based upon previous receptor occupancy data (Toledo et al., 2014). We found that 24-hour food intake was reduced [overall two-way ANOVA time $F(3, 56) = 20.51, P < 0.0001$; treatment $F(1, 56) = 13.35, P < 0.001$, although there was no interaction $F(3,56) = 2.530, P < 0.07$] in each of the time bins, reaching statistical significance in the 6- to 24-hour bin ($P < 0.001$) by 20 mg/kg LY2940094 (Fig. 5A). Cumulative 24-hour intake was significantly reduced [overall two-way ANOVA interaction $F(4, 70) = 5.647, P = 0.0005$; time $F(4, 70) = 60.23, P < 0.0001$; treatment $F(1,70) = 30.18, P < 0.0001$] by approximately 45% (Fig. 5B). Moreover, body weight was significantly reduced by treatment with LY2940094 (Fig. 5C, $P = 0.0217$). Consistent with the reduced food intake, the respiratory quotient (RQ) was significantly reduced by 20 mg/kg LY2940094, indicating a shift from carbohydrate to oxidation of fat to drive metabolism (Fig. 6). LY2940094 at this dose did not affect either energy expenditure or 24-hour locomotor activity (data not shown).

### Discussion

In the present study we tested the hypothesis that a potent and selective NOP receptor antagonist, LY2940094, would reduce overeating and consequently weight gain. We recently reported that LY2940094 occupies NOP receptors for at least 6 hours after oral administration (Toledo et al., 2014) and has low intrinsic plasma clearance, making it a good candidate to assess behavior over 24 hours. At doses that fully occupy NOP receptors, LY2940094 inhibited feeding behavior in a variety of rodent models. Critically, we demonstrated that the effects of LY2940094 are due to specific blockade of NOP receptors. NOP receptors are widely distributed in the rat brain. High receptor density has been identified throughout the rat CNS including the hypothalamus (Florin et al., 2000; Gehlert et al., 2006). Recently, we used a liquid chromatography-tandem mass spectrometry method (Pedregal et al., 2012; Toledo et al., 2014) to measure RO of novel NOP receptor antagonists in the hypothalamus of rats. After oral administration, LY2940094 potently occupied NOP receptors in the hypothalamus with an ED$_{80}$ of 3 mg/kg (E. Raddad et al., submitted manuscript). The duration of RO was reasonably long at a 10 mg/kg dose of LY2940094, providing 80% receptor coverage for at least 4 hours. Therefore, to adequately evaluate the efficacy of LY2940094 we chose doses for our feeding efficacy studies based on doses that would completely saturate NOP receptors. Using the RO data, we chose doses ranging from 3 to 30 mg/kg for our in vivo feeding studies.

The effects of N/OFQ on feeding are well documented (for review, see Witkin et al., 2014). Hyperphagia is consistently observed after intracerebroventricular administration of N/OFQ into either the lateral or third ventricle but not the fourth ventricle (Pomonis et al., 1996; Polidori et al., 2000). The

**Fig. 4.** LY2940094 inhibits food intake and body weight regain induced by 3 days of a mild calorie restriction (30%) in male DIO rats. On days 1–3 rats were either fed ad libitum (Group A) or were restricted to 70% of their free feeding (Groups B, C, D, E), during which time animals received either vehicle (Groups A, B, C) or 20 mg/kg oral LY2940094 (Groups D and E). On day 4 all groups were returned to ad libitum feeding, and food intake was monitored after either vehicle (Groups A, B, D) or 20 mg/kg oral LY2940094 (Groups C and E). (A) Day 4 6-hour cumulative food intake, (B) Day 4 24-hour cumulative food intake, (C) Day 1–3 cumulative body weight change, (D) Day 4 body weight change. *$P < 0.05$ versus Group A, **$P < 0.01$ versus Group A ***$P < 0.001$ versus Group A, #P < 0.05 versus Group E one-way ANOVA with Tukey’s post hoc analysis.
orexigenic effects of N/OFQ are speculated to arise in the hypothalamus, a brain region involved in regulating food intake and that expresses abundant NOP receptors (Stratford et al., 1997). Moreover, chronic administration of N/OFQ by minipump stimulated food intake, decreased energy expenditure, and produced an increase in body weight gain in mice (Matsushita et al., 2009). Interestingly, when controlled for food intake, N/OFQ infusion still stimulated an increase in fat mass due to a decrease in core body temperature and uncoupling protein 1 expression after N/OFQ infusion. These data support that N/OFQ effects on energy metabolism engages mechanisms other than stimulating food intake alone. Indeed, genetic deletion of NOP was reported to increase core body temperature in mice, supporting direct tonic actions of endogenous N/OFQ signaling on energy expenditure (Uezu et al., 2004). Interestingly, N/OFQ inhibits activation of anorexigenic proopiomelanocortin neurons in association with meal termination (Bomberg et al., 2006; Farhang et al., 2010). Moreover, both N/OFQ and the small molecule NOP agonist Ro 64-6198 inhibit CRF-induced anorexia (Ciccocioppo et al., 2002, 2004). A working hypothesis is that N/OFQ may stimulate feeding via inhibition of anorexigenic pathways, resulting in disinhibition of orexigenic mechanisms. Our data would support this hypothesis in that we found that LY2940094 primarily worked in models where the rodent exhibited hyperphagia, stimulated by frank fasting, mild caloric restriction, and weight loss (to mimic dieting), or after the acute presentation of a palatable high-energy diet. Thus a NOP antagonist such as LY2940094 may be most useful in the treatment of disorders associated with hyperphagia, such as binge eating disorder (BED).

Recently, Micioni di Bonaventura et al. (2013) reported in a rat model of stress-induced binge eating in animals with a history of calorie restriction, increased the sensitivity to the hyperphagic effects of N/OFQ. Consistent with these findings we found that blockade of NOP with LY2940094 was effective in reducing hyperphagia associated with mild calorie restriction as well as fasting. Moreover, the ability of LY2940094 to inhibit fasting-induced feeding was totally blocked in NOP receptor knockout mice, whereas the hypophagic effect of the 5HT2C agonist mCPP was preserved in NOP receptor knockout mice. These data demonstrate that 1) genetic deletion of NOP receptors reduces fasting-induced feeding, 2) LY2940094 inhibits fasting-induced feeding in WT mice, 3) the anorexigenic effect of LY2940094 is dependent on NOP receptor expression, 4) the effects of LY2940094 were not the result of producing aversive actions or other side effects influencing feeding, and 5) NOP receptor knockout mice are capable of responding maximally to other anorectic agents such as a 5HT2C agonist. We found a similar effect to inhibit fasting-induced feeding in a genotype-dependent manner using the NOP receptor antagonist SB612111 (Witkin et al., 2014).
Mice and rats lacking the NOP receptor have been generated (Nishi et al., 1997; Küster et al., 1999; Clarke et al., 2001; Kest et al., 2001; Homberg et al., 2009; Rizzi et al., 2011). Phenotyping of the mouse and rat NOP receptor knockout supports a role for endogenous N/OFQ and NOP receptor signaling in the control of numerous CNS functions; however, little data currently exist regarding a feeding or metabolic phenotype after deletion of the NOP receptor (Higgins et al., 2002; Depner et al., 2003, Gavioli et al., 2003; Marti et al., 2004; Uezu et al., 2004; Chung et al., 2006). NOP receptor knockout mice displayed a reduced preference for sucrose and a lower intake of high-fat diet under no-choice conditions (Koizumi et al., 2009). The deletion of the NOP receptor had no effect on conditioned place preference or operant responding, leading the authors to conclude that reward responses and motivation for food was unaltered in NOP receptor knockout mice. Moreover, we recently reported that NOP knockout mice exhibit a reduction in fasting-induced feeding (Witkin et al., 2014). We found a similar effect of deletion of NOP receptor on fasting-induced feeding in the present study, thus replicating the earlier findings. Interestingly, the degree of inhibition of feeding produced by LY2940094 was nearly identical to the reduction in fasting-induced feeding produced by the deletion of the NOP receptor itself. Therefore, in agreement with our RO data, the doses of LY2940094 that we used likely saturated the NOP receptors in key CNS structures associated with food intake. Based on the current findings a more detailed examination of the metabolic phenotype of NOP receptor knockout mice is warranted.

Literature supports that N/OFQ does not appear to mediate palatability/reward-based feeding in rodents eating a preferred diet; however, N/OFQ produces hyperphagia in fat-preferring rats (Olszewski et al., 2002). These data led us to choose studying the effect of the nociceptin antagonist LY2940094 on animals eating a highly palatable diet high in fat and sucrose (HED). In the present study, consistent hyperphagia was induced in rats given short-term access to a HED. We found that blockade of NOP receptors with LY2940094 effectively inhibited feeding stimulated by acute access to a HED. Moreover, we found that LY2940094 reduced the caloric intake of animals eating HED to that of Chow-fed controls, supporting that LY2940094 completely blocked the hyperphagia induced by limited access to a HED in lean rats. The reduction in 5-hour caloric intake was maintained for 24 hours and observed after several days of dosing. So it appears that the animals did not rapidly develop tolerance to the inhibitory effects of LY2940094 on overconsumption of HED. Consistent with the effect in lean rats, LY2940094 was effective in reducing spontaneous feeding over a 24-hour period in DIO mice maintained on a HED. Thus, LY2940094 is effective in reducing both free-access feeding in DIO mice and hyperphagia induced by limited access to a highly palatable HED in lean rats. Our data somewhat contrast from those of previous investigators that did not find N/OFQ mediates palatability-based feeding (Olszewski et al., 2002). One explanation for the difference could be that we used a nutritionally complete diet, high in both fat and carbohydrates, whereas the previous study used diets specifically formulated to be high in either carbohydrates (sucrose) or fat (vegetable shortening and corn oil) to identify the diet preference of the animal. Our diet uses sucrose as the primary source of carbohydrate and lard as the principal source of fat. Thus, differences in the composition of the diet may have influenced the results between studies.

Binge eating disorder (BED) affects roughly 1–4% of the U.S. population. Currently, atomoxetine, fluoxetine, topiramate, and baclophen have demonstrated limited efficacy in clinical studies for BED (McElroy et al., 2007a,b; Broft et al., 2007; Grilo et al., 2012). The present work demonstrated that, in preclinical models, the novel NOP receptor antagonist LY2940094 effectively inhibited feeding behavior. Several of these models exhibited a significant hyperphagia, a hallmark of BED that is often associated with obesity. These findings raise the intriguing possibility that LY2940094 may be effective in the treatment of patients with BED and/or obesity. Given the existence of a PET ligand for NOP receptors (Pike et al., 2011; Pedregal et al., 2012) and the demonstrated safety of LY2940094 in human clinical investigation (Post et al., 2014), LY2940094 can be used to study the role of NOP receptors in eating disorders and metabolic complications.

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Authorship Contributions

Participated in research design: Statnick, Chen, and Pintar.
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Contributed new reagents or analytical tools: LaFuente, Jimenez, Benito, Diaz, Martinez-Grau, Toledo, and Pintar.

Performed data analysis: Statnick, Chen, Suter, Song, Hu, and Pintar.

Wrote or contributed to the writing of the manuscript: Statnick, Chen, Witkin, Rorick-Kehn, Martinez-Grau, Toledo, and Pintar.

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