Central nervous system neuropeptide Y signaling via the Y1 receptor partially dissociates feeding behavior from lipoprotein metabolism in lean rats

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Rojas JM, Stafford JM, Saadat S, Printz RL, Beck-Sickinger AG, Niswender KD. Central nervous system neuropeptide Y signaling via the Y1 receptor partially dissociates feeding behavior from lipoprotein metabolism in lean rats. Am J Physiol Endocrinol Metab 303: E1479–E1488, 2012. First published October 16, 2012; doi:10.1152/ajpendo.00351.2012.—Elevated plasma triglyceride (TG) levels contribute to an atherogenic dyslipidemia that is associated with obesity, diabetes, and metabolic syndrome. Numerous models of obesity are characterized by increased central nervous system (CNS) neuropeptide Y (NPY) tone that contributes to excess food intake and obesity. Previously, we demonstrated that intracerebroventricular (icv) administration of NPY in lean fasted rats also elevates hepatic production of very low-density lipoprotein (VLDL)-TG. Thus, we hypothesize that elevated CNS NPY action contributes to not only the pathogenesis of obesity but also dyslipidemia. Here, we sought to determine whether the effects of NPY on feeding and/or obesity are dissociable from effects on hepatic VLDL-TG secretion. Pair-fed, icv NPY-treated, chow-fed Long-Evans rats develop hypertriglyceridemia in the absence of increased food intake and body fat accumulation compared with vehicle-treated controls. We then modulated CNS NPY signaling by icv injection of selective NPY receptor agonists and found that Y1, Y2, Y4, and Y5 receptor agonists all induced hyperphagia in lean, ad libitum chow-fed Long-Evans rats and hypertriglyceridemia in the absence of increased food intake and body fat accumulation compared with vehicle-treated controls. Next, we found that at equipotent doses for food intake NPY Y1 receptor agonist had the most robust effect on VLDL-TG secretion, a Y2 receptor agonist had a modest effect, and no effect was observed for Y4 and Y5 receptor agonists. These findings, using selective agonists, suggest that the possibility that the effect of CNS NPY signaling on hepatic VLDL-TG secretion may be relatively dissociable from effects on feeding behavior via the Y1 receptor.

brain; obesity dyslipidemia; hepatic lipid metabolism

Models of obesity/diabetes dyslipidemia suggest that increased visceral fat mass and insulin resistance lead to elevated adipocyte lipolysis, which increases free fatty acid (FFA) delivery to liver, where it is efficiently cleared, reesterified to TG, and loaded onto a nascent apolipoprotein B (apoB) particle, ultimately resulting in VLDL maturation and secretion (32, 49). This process is ordinarily suppressed by integrated hepatic insulin action. With a rise in VLDL-TG in the circulation, cholesteryl ester transfer protein exchanges cholesterol esters from HDL and LDL particles with TGs from VLDL, ultimately lowering HDL-C levels and facilitating the accumulation of atherogenic LDL-C particles (reviewed in Refs. 1 and 22).

In the context of integrated energy homeostasis, short-term energy stores are provided by glucose and glycogen, whereas long-term needs are met by adipose and liver-derived lipids, a process that is tightly regulated by the central nervous system (CNS). Neuropeptide Y (NPY)-expressing neurons are widely distributed in the CNS, concentrated in the mediobasal hypothalamus, important in the regulation of feeding and energy homeostasis, and increasingly recognized as having a role in lipid homeostasis (59). NPY is a potent orexigenic peptide and, when delivered by chronic infusion directly into the brain of rats and mice, has been observed to promote hyperphagia, obesity, dyslipidemia, and metabolic syndrome (48, 65), similar to that of the leptin-deficient ob/ob mouse (25, 31, 71) and the genetically leptin-resistant fa/fa Zucker fatty rat (16, 43). These genetic models of obesity are characterized by high NPY mRNA and peptide levels in the hypothalamus, secondary to the absence of negative feedback regulation by leptin (9, 28, 54, 56). Rodent models of diet-induced obesity (made obese by feeding a highly palatable diet) and streptozotocin-induced diabetes (insulin deficient), which are more typical of human diabetes, are also characterized by elevated CNS NPY tone (57, 68).

Positive energy balance and obesity pathogenesis are thought to result from defects in inhibitory feedback signaling to the CNS (including neuronal insulin and leptin resistance and impaired nutrient sensing) (44, 45). We hypothesized that increases in NPY tone within these neural circuits may contribute to dyslipidemia in addition to obesity. We demonstrated previously that intracerebroventricular (icv) administration of NPY directly into the third ventricle of lean, fasted, wild-type rats increases hepatic VLDL-TG secretion independent of food intake (59). Peripherally administered NPY had no such effect, and taken together these findings suggest that NPY-regulated neural circuits may be involved in the regulation of TG metabolism in the liver (59).
NPY is a 36-amino acid neuropeptide member of the pancreatic polypeptide family that includes peptide YY (PYY) and pancreatic polypeptide (PP) (29). NPY affects a wide variety of physiological functions via the activation of a population of distinct G protein-coupled NPY receptor subtypes: Y1, Y2, Y4, and Y5 (29, 35). All NPY receptor subtypes are expressed in the hypothalamus (18, 60). The effects of NPY on feeding and energy homeostasis are thought to be mediated largely by hypothalamic Y1 and Y5 receptors (reviewed in Ref. 29). Y2 receptors, having affinity for both NPY and PPY (35), act in an inhibitory manner on both orexigenic NPY as well as anorexigenic proopiomelanocortin (POMC) neurons in the arcuate nucleus (ARC) (21). The hypothalamic Y4 receptor is highly selective for PP over NPY or PPY (35) and is thought to mediate anorexigenic effects by decreasing hypothalamic NPY (2). Yet the receptor subtype(s) involved in the central NPY regulation of lipoprotein metabolism is not well understood, nor is the relative effect of a given receptor on feeding vs. lipoprotein metabolism. In genetic models in which the NPY Y1, Y2, Y4, or Y5 receptor has been deleted on an ob/ob background (a rodent model of severe hypertriglyceridemia), the effect on TGs is not reported, except for the Y2 receptor deletion having no effect (37, 46, 52, 53).

Our study employed two approaches, the first determining whether CNS NPY retains the effect on hepatic VLDL-TG secretion chronically (3 days; twice daily icv administration) in the absence of increased food intake and fat mass and the second determining whether different CNS NPY receptors mediate feeding vs. VLDL-TG regulatory effects. We evaluated the effect of selective icv NPY Y1, Y2, Y4, and Y5 receptor agonists on both feeding and hepatic VLDL-TG secretion in lean, fasted rats to determine which receptor subtype(s) is involved in the central NPY regulation of lipoprotein metabolism in lean, fasted rats to determine which receptor subtype(s) is involved in the central NPY regulation of lipoprotein metabolism. In genetic models in which the NPY Y1, Y2, Y4, or Y5 receptor has been deleted on an ob/ob background (a rodent model of severe hypertriglyceridemia), the effect on TGs is not reported, except for the Y2 receptor deletion having no effect (37, 46, 52, 53).

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MATERIALS AND METHODS

Animal studies. Male Long-Evans rats (HsdBlu:LE) weighing 250–274 g were purchased from Harlan (Indianapolis, IN), maintained under temperature- and humidity-controlled conditions with a 12:12-h light-dark cycle (lights on at 6 AM), and given free access to water and a standard rodent chow diet (5001; 3.02 kcal/g, 58% carbohydrate, 28.5% protein, 13.5% fat; Lab Diet, Richmond, IN). Study protocols were approved by the Institutional Animal Care and Use Committee of the Tennessee Valley Veterans Affairs (VA) Healthcare System.

Surgical preparation. Rat surgeries were performed under inhalational isoflurane anesthesia, and Buprenex (0.05 mg/kg body wt) was administered postoperatively to mitigate pain and distress. Animals were prepared with stereotaxic implantation of a stainless-steel guide cannula (22 gauge; Plastics One, Ronkonkoma, NY) into the third cerebral ventricle (40) and a sterile MicroRenathane catheter (R-CAC-M37-R; Brain Tree Scientific, Braintree, MA) implanted into the carotid artery, as described (59).

Intracerebroventricular infusions. Studies were performed 5–7 days after surgery, when food intake and body weight curves returned to a presurgery trajectory. Recombinant NPY and a selective agonist for the Y2 receptor, human (h)PYY-(3-36), were purchased from GenScript (Piscataway, NJ). Selective agonists for the Y1 receptor, [F7, P34]-NPY, and the Y5 receptor, [Ala31, Aib32]-NPY, were synthesized as described previously (13, 58). A selective agonist for Y4, human PP (hPP), was purchased from Tocris Bioscience (Ellisville, MO). All receptor agonists were dissolved in 0.9% normal saline and freshly prepared. All icv compounds were administered in a 2-μl volume over a time period of 1 min.

Chronic NPY pair-feeding study. Recombinant NPY (1 nmol) was administered icv twice daily (8 AM and 5 PM) over 3 days in rats matched for body weight. To control for the orexigenic effects of NPY, the caloric intake of pair-fed NPY-treated rats was carefully matched to that of the icv vehicle (Veh; saline)-treated control rats. Plasma TG and cholesterol levels were measured daily from blood collected by tail prick. At the end of the study (day 3) 4-h-fasted rats were given either icv NPY (1 nmol) or Veh, and following a 120-min postinjection period, trunk blood was collected at study termination. Body weight and body composition using an EchoMRI-700 NMR spectrometer (Echo Medical Systems, Houston, TX) to determine lean and fat mass were measured at study termination.

NPY receptor agonist selectivity and dosing. The NPY EC50 values as measured in vitro are 2.6, 5.1, 814, and 4.9 nM for the Y1, Y2, Y4, and Y5 receptor subtypes, respectively (35). All peptide ligands used in our studies are selective compounds for their respective NPY receptor subtypes and bind with subnanomolar affinity (35). The Y1 selective peptide [F7, P34]-NPY has >3,000-fold selectivity for the Y1 receptor over that of either the Y2 or Y5 receptor (58). For Y2 receptor activation, we used hPYY-(3–36), which has a 181–>1,000-, and fivefold greater affinity for the Y2 receptor than for Y1, Y4, and Y5 receptor subtypes, respectively (69). To activate the Y4 receptor, we used hPP that has a 2.2–>56-, and 1.4-fold greater affinity for the Y4 receptor than for Y1, Y2, and Y5 receptor subtypes, respectively (35, 38). We also used a Y5 agonist, [Ala31, Aib32]-NPY, which has >77–, 12–, and >77-fold selectivity for the Y5 receptor than for the Y1, Y2, and Y4 receptor subtypes, respectively (35, 38). The dose of agonist utilized for each receptor subtype was estimated on the basis of in vitro receptor potencies relative to NPY (35, 38, 58, 69) for food intake and lipid studies. The dosages chosen for icv treatment were NPY (1 nmol), Y1 agonist [F7, P34]-NPY (1 nmol), Y2 agonist hPYY-(3–36) (1 nmol), Y4 agonist hPP (1 nmol), and Y5 agonist [Ala31, Aib32]-NPY (2 nmol). Food intake studies. Paired food intake responses to agonists of each receptor subtype compared with vehicle control, 12-h-fasted, and NPY-treated animals. Rats that had surgically implanted icv cannulae and were matched for body weight were studied. We administered the icv compounds NPY (1 nmol), [F7, P34]-NPY (1 nmol), hPYY-(3–36) (1 nmol), hPP (1 nmol), [Ala31, Aib32]-NPY (2 nmol), or Veh (saline) to ad libitum Chow-fed rats at 10 AM (lights on at 6 AM) and measured 2-h food intake postinjection.

Tyloxaolip (lipid production) experiments. For lipid production studies, rats with surgically implanted icv cannulae and carotid catheters were matched for body weight and fasted, with free access to water from 6 to 10 AM. We confirmed previously that 4-h-fasted rats are in a postabsorptive state and that chylomicrons do not contribute to the observed changes in TGs (59). A baseline blood sample was drawn through the carotid catheter, and then plasma TG clearance was blocked by an intravenous infusion of tyloxapol (300 mg/kg body wt) that at the dosage used potently inhibits lipoprotein lipase (LPL) activity (42, 55). Thirty minutes after tyloxapol infusion, icv compounds or Veh were injected at time 0 min. Also, at 0 min and at 30-min intervals, 200 μl of blood was collected in a tube with 2 μl of 50 mM EDTA (59). The TG secretion rate was determined as the slope of the concentration of plasma TGs over time using linear regression analysis (calculated from time 0 to 120 min).

Lipoprotein fractionation. Fast protein liquid chromatography (FPLC) analysis was performed as described previously (59). Plasma samples (150 μl) were size fractionated on a Superose-6 300 GL column (GE Healthcare Biosciences, Little Chalfont, UK), and 300-μl column fractions were collected in a 96-well plate.
Lipid assays. Lipids were assayed from plasma with enzymatic colorimetric assays using the following reagent kits for TG and total cholesterol from Raichem (San Diego, CA); nonesterified FFA from Wako Diagnostics (Richmond, VA) and glycerol from Sigma-Aldrich (St. Louis, MO).

Plasma hormones and metabolites. Plasma levels of insulin and glucagon were quantified using radioimmunoassays from Millipore (Billerica, MA); all assays were performed by the Vanderbilt Diabetes Center Hormone Assay & Analytical Services Core (Nashville, TN). Blood glucose concentration was measured using a glucometer from Accu-Chek Advantage, Indianapolis, IN) on trunk blood at study termination.

Western blot analysis. Liver proteins were extracted and protein concentrations determined using Pierce BCA Protein Kit (Thermo Scientific, Rockford, IL). For immunoblotting, liver protein extracts were separated on a 4–12% Bis-Tris XT gel using a Bio-Rad XT Criterion System (Bio-Rad, Hercules, CA), followed by electrophoretic transfer of proteins to nitrocellulose membranes. Primary antibodies used at a 1:1,000 dilution for immunodetection were as follows: microsomal TG transfer protein (MTP; sc-33116), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-25778), and actin (sc-1616) purchased from Santa Cruz Biotechnology (Santa Cruz, CA); acetyl-CoA carboxylase (ACC; 3662), phosphorylated ACC (Ser79; 3661), and fatty acid synthase (FAS; 3180) antibodies purchased from Cell Signaling Technology (Beverly, MA); heat shock protein 70 (HSP70; ADI-SPA-816) purchased from Enzo Life Sciences (Framingdale, NY); and apoB 100/48 (kindly provided by Larry Swift; Vanderbilt University, Nashville, TN) (61). Horseradish peroxidase-conjugated secondary antibodies used at a 1:7,500 dilution for immunodetection were donkey anti-goat IgG purchased from Santa Cruz Biotechnology and anti-rabbit IgG purchased from Promega (Madison, WI). Western blots were analyzed by densitometry utilizing Image J Software (National Institutes of Health).

RNA isolation and quantitative RT-PCR. Liver samples were homogenized, and mRNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). From these RNA samples, cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). RT-PCR was performed with IQ SYBR Green Supermix, as described previously (51). Quantification results for each RNA of interest were normalized to the housekeeping gene ribosomal protein L13a (RPL13a), and for comparative analysis RNA ratios of the treatment group were normalized to the Veh control group. Primer sets designed to detect and amplify stearoyl-CoA desaturase-1 (SCD-1) [as described previously (41)] and RPL13a (forward primer: 5′-TACTCTGAGGAGAAGC-GAG-3′ and reverse primer: 5′-GCCTGTTCCTTAGCCTCAA-3′) were used.

Statistical analysis. Data are presented as means ± SE. Two-group comparisons were performed using Student’s t-test (unpaired, 2-tailed) and three-group (or more) comparisons by one-way ANOVA with Bonferroni’s posttest analysis. Treatment vs. time was compared by two-way repeated-measures ANOVA with Bonferroni’s posttest analysis. All analyses were performed using GraphPad Prism, version 5.04, 2010 (GraphPad Software, San Diego, CA). Differences with P < 0.05 were considered statistically significant.

RESULTS

Chronic NPY treatment increases plasma TG levels independent of food intake, positive energy balance, and increased body adiposity. Previously, we demonstrated that icv administration of NPY into the third ventricle of lean rats increases hepatic VLDL-TG secretion independent of feeding, as the animals were denied access to food just prior to and during experimental procedures (59). Because exogenous administration of NPY to a lean animal can drive the development of obesity and metabolic syndrome (48, 65), we sought to determine whether a more chronic NPY administration increases plasma TGs independent of its orexigenic effects to increase food intake and body adiposity. NPY was administered ivc (1 nmol) twice daily over 3 days in chow-fed rats. Importantly, to prevent NPY-induced hyperphagia, the food intake of pair-fed NPY-treated rats was calorically matched to that of the Veh-treated controls. At the end of the study (day 3), 4-h-fasted rats were given either icv NPY (1 nmol) or Veh, and trunk blood was collected at study termination (120 min postinjection). Pair-feeding successfully matched the body composition of NPY-treated rats relative to Veh-treated animals, resulting in no differences in body weight, lean mass, or fat mass at study termination (Fig. 1A). However, we found that chronic NPY injections in pair-fed rats resulted in an ~50% increase in plasma TGs by treatment days 2 and 3 (P < 0.01, n = 6/group;
hepatic TG production rate to a similar extent [6.7 ± 0.4 mg·dl⁻¹·min⁻¹ for Y1 (n = 7) vs. Veh, P < 0.0001; Fig. 3, B and E]. The Y2 receptor agonist hPYY-(3–36) (1 nmol) stimulated TG production 1.5-fold [5.1 ± 0.3 mg·dl⁻¹·min⁻¹ for Y2 (n = 7) vs. Veh, P < 0.01; Fig. 3, C and E] and was less potent than the Y1 receptor agonist (Y1 vs. Y2, P < 0.01; Fig. 3E). Neither the Y4, hPP (1 nmol), nor Y5 [Ala³¹, Aib³²]-NPY (2 nmol) receptor agonists increased the rate of hepatic TG production beyond that of Veh treatment (P = NS; Fig. 3, D and E). The ability of NPY and its Y1 and Y2 receptor agonists to increase TG production over time was treatment dependent [F(6,50) = 11.75, P < 0.0001]. Collectively, our results suggest that although both Y1 and Y2 receptor agonists regulate plasma TG levels and food intake, an NPY signal mediated through a Y1 receptor increases hepatic TG production more potently, whereas one mediated through the Y2 receptor has a greater effect on food intake (compare Figs. 2 and 3E).

Intracerebroventriculally administered Y1 receptor agonist enhances hepatic secretion of TGs in the form of VLDL-lipoprotein. Since both Y1 and Y2 receptor agonists increased hepatic TG production, we next confirmed that modulation of CNS NPY signaling via these receptor subtypes increases TGs in the VLDL fraction. To avoid any nonspecific effects from the use of tyloxapol during the measurement of TG production rates, we performed these experiments in the absence of tyloxapol. NPY (1 nmol), Y1 receptor agonist (1 nmol), Y2 receptor agonist (1 nmol), and Veh were given icv, and then trunk blood was collected at study termination (120 min postinjection). NPY increased plasma TGs by ~200% (NPY vs. Veh, P < 0.001, n = 6–13/group; Fig. 4A). Similarly, the Y1 receptor agonist recapitulated the NPY effect by doubling plasma TG levels (Y1 receptor agonist vs. Veh, P < 0.001, n = 6–13/ group; Fig. 4A), whereas the Y2 receptor agonist had no effect on plasma TGs in the absence of tyloxapol (Y2 receptor

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**Table 1. Effects of chronic NPY administration to pair-fed chow-fed rats at 120 min post-icv injection on glucoregulatory hormones and metabolites**

<table>
<thead>
<tr>
<th></th>
<th>Veh</th>
<th>NPY-PF</th>
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<tbody>
<tr>
<td>Glucagon, pg/ml</td>
<td>63.1±4</td>
<td>107±14*</td>
</tr>
<tr>
<td>Glyceral, mg/dl</td>
<td>10.4±1.8</td>
<td>15.6±4.7</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.04±0.1</td>
<td>2.09±0.50</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>97.6±10.0</td>
<td>83.8±3.6</td>
</tr>
<tr>
<td>FFA, nmol/l</td>
<td>0.31±0.03</td>
<td>0.23±0.02</td>
</tr>
<tr>
<td>TG, mg/dl</td>
<td>80.5±5.0</td>
<td>156±32*</td>
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</tbody>
</table>

Data are means ± SD (n = 6/group). NPY, neuropeptide Y; icv, intracerebroventricular; Veh, vehicle; NPY-PF, NPY-pair-fed; TG, triglyceride; FFA, free fatty acid. *P < 0.05 for NPY-PF vs. Veh comparisons.

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Fig. 1B with no observed alteration in total plasma cholesterol levels (P = not significant (NS); Table 1). The effect of chronic NPY treatment to increase plasma TG over time was dependent on the treatment [F(1,22) = 7.99, P < 0.01]. The changes in TGs observed with chronic NPY treatment in pair-fed rats (P < 0.05; Table 1) were not due to elevated adipocyte lipolysis, as we did not observe any changes in FFA or glycerol at study termination (P = NS; Table 1), consistent with our previous findings (59). Although glucagon levels were significantly elevated compared with Veh treatment (P < 0.05; Table 1), insulin levels only trended higher (P = 0.09; Table 1). Individual plasma samples were size-fractionated by FPLC and quantified for TG content. As observed previously with an acute injection of icv NPY in lean fasted rats, chronic NPY treatment in pair-fed rats increased TG content in VLDL-containing fractions (fractions 10–20; Fig. 1C).

Selective activation of NPY receptor subtypes induces hyperphagia in lean, ad libitum chow-fed rats. We first sought to identify an icv dosage of NPY that would lead to a physiologically relevant feeding response. Lean, ad libitum chow-fed rats were given either icv NPY (1 nmol) or Veh, and the 2-h feeding response postinjection was compared with the 2-h refeeding response of 12-h-fasted rats. As expected, both icv NPY treatment and 12 h of fasting potently induced hyperphagia compared with Veh treatment (Fig. 2). We found that 1 nmol NPY given icv induced the same 2-h feeding response as observed after a 12-h fast (P = NS; Fig. 2). All of the selective NPY receptor agonists, Y1 ([F7, P34]-NPY; 1 nmol), Y2 [hPYY-(3–36), 1 nmol], Y4 (hPP; 1 nmol), and Y5 ([Ala³¹, Aib³²]-NPY; 2 nmol), induced hyperphagia in ad libitum chow-fed rats relative to Veh (Fig. 2). Therefore, all of the selective receptor agonists (Y1, Y2, Y4, and Y5) induced a 2-h feeding response similar in magnitude to 12 h of fasting (P = NS; Fig. 2). Of particular note, the Y2 receptor agonist, at a dose equivalent to NPY (1 nmol), stimulated feeding above all of the other compounds except for the Y5 agonist (Fig. 2). Collectively, these data confirm that we used physiologically relevant doses (1–2 nmol) of each selective receptor agonist for our food intake and lipid studies.

A Y1 receptor agonist increases plasma TG levels to the greatest extent relative to other NPY receptor subtype agonists. In corroboration with our previous findings (59), NPY increased the hepatic TG production rate significantly [6.4 ± 0.5 mg·dl⁻¹·min⁻¹ for NPY (n = 5) vs. 3.5 ± 0.1 mg·dl⁻¹·min⁻¹ for Veh (n = 26), P < 0.0001; Fig. 3, A and E]. We found that the Y1 receptor agonist [F7, P34]-NPY (1 nmol) also increased hepatic TG production rate to a similar extent [6.7 ± 0.4 mg·dl⁻¹·min⁻¹ for Y1 (n = 7) vs. Veh, P < 0.0001; Fig. 3, B and E]. The Y2 receptor agonist hPYY-(3–36) (1 nmol) stimulated TG production 1.5-fold [5.1 ± 0.3 mg·dl⁻¹·min⁻¹ for Y2 (n = 7) vs. Veh, P < 0.01; Fig. 3, C and E] and was less potency than the Y1 receptor agonist (Y1 vs. Y2, P < 0.01; Fig. 3E). Neither the Y4, hPP (1 nmol), nor Y5 [Ala³¹, Aib³²]-NPY (2 nmol) receptor agonists increased the rate of hepatic TG production beyond that of Veh treatment (P = NS; Fig. 3, D and E). The ability of NPY and its Y1 and Y2 receptor agonists to increase TG production over time was treatment dependent [F(6,50) = 11.75, P < 0.0001]. Collectively, our results suggest that although both Y1 and Y2 receptor agonists regulate plasma TG levels and food intake, an NPY signal mediated through a Y1 receptor increases hepatic TG production more potently, whereas one mediated through the Y2 receptor has a greater effect on food intake (compare Figs. 2 and 3E).

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Fig. 2. Effects of central nervous system (CNS)-administered NPY receptor subtype agonists on 2-h food intake. Food intake was measured in lean, ad libitum chow-fed rats for 2-h after they received an icv injection of Veh (n = 13), NPY (1 nmol; n = 4), or a selective NPY receptor subtype agonist for Y1 ([F7, P34]-NPY, 1 nmol), Y2 [human peptide YY-(3–36), 1 nmol], Y4 (human pancreatic polypeptide, 1 nmol), or Y5 ([Ala³¹, Aib³²]-NPY, 2 nmol) (n = 4–6/group). These 2-h food intake measurements compared with a 2-h refeeding response of 12-h-fasted lean rat are shown (fasted/refed; n = 5). Data are presented as means ± SE and were analyzed by 1-way ANOVA. *Significant differences (P < 0.05), as tested with Bonferroni’s posttest, for comparisons relative to Veh; and #significant differences (P < 0.05) for comparisons relative to Y2 receptor agonist.
The Y2 receptor agonist increased plasma glucose levels by 64%; therefore, we determined whether CNS NPY signaling via the Y1 or Y2 receptor increases lipolysis. Extending our previous findings (59), consistent with our previous findings (59). CNS NPY did not activate markers of lipolysis. Neither treatment with NPY nor the agonists for Y1 or Y2 receptor subtypes altered increased TG content in these fractions, whereas the Y2 receptor agonist had little effect (Fig. 4B). Neither treatment with NPY nor the agonists for Y1 or Y2 receptor subtypes altered total plasma cholesterol levels (data not shown).

**Increased CNS NPY Y1 or Y2 receptor signaling did not alter markers of adipocyte lipolysis or glucoregulatory hormones.** Current models suggest that increased adipocyte lipolysis during states of fasting or insulin resistance leads to increased substrate (FFA and glycerol) delivery to the liver for glucose and ketone body metabolism and sensitivity to insulin in fasted rats (12, 36, 63, 67, 70), and thus liver glucagon levels (P < 0.01; Table 2). Intracerebroventricularly administered NPY, Y1, and Y2 receptor agonists did not alter plasma insulin or glucagon levels (P = NS; Table 2).

**CNS NPY signaling via the Y1 receptor modulates liver SCD-1 mRNA levels.** Hypothalamic signaling via several hormones (especially leptin) and metabolites regulates hepatic SCD-1 gene expression (17, 34, 66, 67, 70), and thus liver expression of SCD-1 is a robust marker of known hypothalamic-liver regulatory pathways. First, we investigated whether increased CNS NPY signaling via the Y1 receptor modulated relevant liver regulatory targets. Lean, 4-h-fasted rats were given either icv NPY (1 nmol), the Y1 receptor agonist (1 nmol), or Veh, and at either 60 or 120 min postinjection, trunk blood and liver samples were collected. Similar to our previous findings at 120 min post-icv injection (Fig. 4A), we found at 60 min post-icv injection that treatment with either NPY or the Y1 receptor agonist doubles plasma TG levels with respect to Veh (NPY: 256 ± 60% vs. Veh: 100 ± 11%, P < 0.01; Y1 receptor agonist: 171 ± 21% vs. Veh: P < 0.01; n = 5–11/group). We then quantified expression levels of key lipid metabolic markers involved in VLDL assembly and secretion. At both 60 and 120 min, neither NPY nor the Y1 receptor agonist altered levels of phosphorylated ACC (Ser79), ACC, FAS, hepatic apoB-48, or MTP (P = NS, n = 5–7/group; Fig. 5, A and B), consistent with our previous findings (59). CNS NPY did not induce SCD-1 mRNA levels fourfold relative to Veh (P < 0.01; Table 2). In contrast, the Y2 receptor agonist increased plasma glucose levels by 13% (P < 0.05; Table 2), whereas neither the NPY nor the Y1 receptor agonist had any effect on glucose concentration (P = NS; Table 2). Intracerebroventricularly administered NPY, Y1, and Y2 receptor agonists did not alter plasma insulin or glucagon levels (P = NS; Table 2).

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metabolic regulation whose gene product is involved in lipid metabolism.

DISCUSSION

An atherogenic dyslipidemia characterized in part by elevated plasma TG levels is the major lipid abnormality associated with obesity, diabetes, and metabolic syndrome (22, 23, 26). Although peripheral factors (visceral adiposity and insulin resistance) clearly contribute to this disorder (32, 49), we hypothesized that increased CNS NPY action contributes to both the pathogenesis of obesity and the pathogenesis of obesity dyslipidemia. Here, we sought to determine whether the effects of NPY on feeding and/or weight gain are dissociable from the effects on hepatic VLDL-TG secretion. We employed two approaches, the first one asking whether NPY retains the effect on hepatic VLDL-TG secretion chronically in the absence of increased food intake and weight gain and the second one asking whether different NPY receptors mediate feeding vs. lipid effects. We found that chronic (3 days) icv injections of NPY in pair-fed animals compared with Vehicle-treated controls to maintain identical body composition also led to hypertriglyceridemia, which suggests that hyperphagia and accumulation of excess adiposity are not required for this effect. Moreover, using selective NPY receptor agonists, we demonstrate, within the limits of our model, that central NPY signaling via the Y1 receptor predominantly regulates effects on hepatic lipoprotein production. The Y2 receptor agonist modestly stimulated VLDL-TG production, albeit at a dose that had a profound effect on feeding. Neither the Y4 nor the Y5 agonist had an effect on plasma TGs. Conversely, all agonists stimulated feeding, with the NPY Y2 receptor agonist having a much more robust effect on feeding than TG production. In contrast, at the same dose used as the NPY Y2 receptor agonist, the NPY Y1 receptor agonist had a greater effect on TG production than feeding. Thus, we postulate that NPY regulates feeding and lipoprotein metabolism partially via separate NPY receptor systems and/or mechanisms.

Whereas our study and others (27, 62) reveal that central administration of the NPY Y2 receptor agonist PYY-(3-36) stimulates feeding under some conditions, Batterham et al. (8) reported that this Y2 receptor agonist has an inhibitory effect on food intake when administered by direct intra-arcuate injection or via peripheral administration. Because the inhibitory Y2 receptor is found on both the ARC NPY and POMC neurons, this adds an additional layer of complexity to the regulation of the NPY/POMC neural circuit by Y2 agonists. Both endogenous and exogenous Y2 agonist action in the ARC

Table 2. Effects of CNS NPY and agonists for Y1 and Y2 receptor subtypes at 120 min post-icv injection on glucoregulatory hormones and metabolites

<table>
<thead>
<tr>
<th>Veh</th>
<th>NPY</th>
<th>Y1 Agonist</th>
<th>Y2 Agonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFA, mmol/l</td>
<td>0.33 ± 0.03</td>
<td>0.39 ± 0.05</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>Glucagon, ng/ml</td>
<td>79 ± 7</td>
<td>76 ± 4</td>
<td>77 ± 10</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>137 ± 5</td>
<td>145 ± 6</td>
<td>20 ± 5</td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 6-7/group). CNS, central nervous system. *P < 0.05 for icv treatment vs. Veh comparison.

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Fig. 4. Effects of CNS NPY receptor subtypes Y1 and Y2 on hepatic VLDL-TG secretion. A and B: NPY (1 nmol), Veh, or receptor agonists for either Y1 ([F7, P34]-NPY, 1 nmol) or Y2 [human peptide YY-(3-36), 1 nmol] were given icv in the absence of tyloxapol to 4-h-fasted lean rats (n = 6-13/group), and trunk blood was collected at 120 min postinjection. Each receptor subtype agonist was tested in a separate cohort of animals, and each cohort was matched to its own Veh control group, so plasma TG levels of each treatment group were normalized to their matched Veh controls. A: plasma TG levels of each treatment group (%Veh control) are shown. Data are means ± SE. *Statistical significance (P < 0.05), as determined by 1-way ANOVA with Bonferroni’s posttest analysis, for all comparisons relative to Veh. B: the TG content of FPLC fractions after size fractionation of pooled plasma samples is illustrated. Column fractions 10–20, with the size range corresponding to VLDL, are illustrated to allow comparisons between the different icv treatment groups: Veh (□), NPY (●), Y1 receptor agonist (▲), and Y2 receptor agonist (◆).
is context dependent, as elegantly described by Ghamari-Langroudi et al. (21). An additional consideration is that icv administration of the Y2 agonist may result in its dispersion to other hypothalamic and nonhypothalamic regions. Finally, the potential activation of the Y5 receptor by PYY-(3–36) could also explain the increase in food intake.

A strength of our study was that we matched test compounds for potency on feeding behavior (similar to a 12-h fast), with the exception of the Y2 receptor agonist. A weakness is that we utilized only a single dosage of each test compound. It is conceivable that, at significantly higher or lower doses, opposite and/or differential effects on feeding relative to VLDL-TG secretion may have been observed. Indeed a “U-shaped” curve, where activation has a greater effect than activation of the Y2 receptor on resistance (44, 45). Because we show that Y1 receptor activation has a greater than 77-fold selectivity for the Y5 than for the Y1 receptor (3).

We reported previously that the NPY Y5 receptor agonist BWX-46 increased VLDL-TG secretion in lean, fasted rats (59), a finding not replicated here using [Ala$^{31}$, Aib$^{32}$]-NPY (Fig. 3, D and E). However, the NPY Y5 receptor agonist [Ala$^{31}$, Aib$^{32}$]-NPY (2 nmol icv) employed in our current study has greater than 77-fold selectivity for the Y5 than for the Y1 receptor (35, 38), whereas BWX-46 (12 nmol icv) employed in our previous study has less Y5 selectively and greater cross-reactivity with the Y1 receptor (3). Thus, at the dose used in our previous study (59), it appears likely that BWX-46 activated both the NPY Y1 and Y5 receptors, resulting in the observed increase in VLDL-TG production.

Elevated hypothalamic NPY tone (and a reduction of POMC tone) is associated with obesity and diabetes in both rodent models (57, 68) and humans (4, 5), likely due to defects in inhibitory feedback signals to the CNS (i.e., insulin and leptin resistance) (44, 45). Because we show that Y1 receptor activation has a greater effect than activation of the Y2 receptor on lipoprotein metabolism, it informs of potential structure-function relationships of the NPY-regulated neural circuitry involved. There is a reportedly close physical localization and apparent functional relationship between NPY Y1 and Y2 receptors in neurons found within the ARC, the lateral hypothalamic area, the dorsomedial hypothalamic nucleus, and the
paraventricular nucleus (PVN), whereas the ventromedial hypothalamic nucleus (VMN) contains only Y1 receptor-positive neurons (60). Furthermore, a study by Chee et al. (15) reports that NPY inhibits the excitatory (anorexigenic) outflow between the VMN and ARC POMC neural circuitry via the activation of the Y1 receptor subtype in the VMN. Moreover, studies in VMN-lesioned rats, which recapitulate a state of elevated NPY tone, have elevated plasma TGs (10), even as early as 10 days postoperatively, together with decreased plasma FFA and glucose levels (30). Finally, perfused livers from VMN-lesioned rats secrete more TGs than controls (30). Altogether, these data suggest that the VMN is a potential hypothalamic site in which NPY may regulate lipoprotein metabolism via selective activation of the Y1 receptor. Of course, our initial studies reported here employing icv injections cannot localize the effect. Thus, future studies will involve selective inhibition of the Y1 receptor in PVN compared with VMN with microinjection techniques in an obese, hypertriglyceridemic rodent model characterized by elevated NPY tone (i.e., fa/fa Zucker fatty rat). Collectively, these findings may lend plausibility that the brain is a potential therapeutic target to treat obesity dyslipidemia.

Our finding that the NPY Y1 receptor is most robustly coupled to lipoprotein metabolism is consistent with the conclusions from a genetic association study in severely obese human subjects matched for body mass index in which those individuals with the endogenous CC haplotype (relative to the TT/CT polymorphism) of the untranslated region of the NPY Y1 receptor gene had elevated fasting serum triglycerides and significantly lower high-density lipoprotein cholesterol concentrations (11). It is not yet clear whether this haplotype correlates with a relative gain of NPY Y1 receptor function, but we would hypothesize in the context of our findings that the CC haplotype is a relatively hyperfunctional allele and thus would confer increased TGs in the setting of obesity.

Several studies have investigated the effect of global deletion of the NPY Y1, Y2, Y4, and Y5 receptors on the background of the ob/ob mouse, which is a leptin-deficient, obese rodent model characterized by elevated CNS NPY tone and severe hypertriglyceridemia. Unfortunately, these studies report only on the effect of this genetic manipulation on energy homeostasis and not on whether deletion of the various NPY receptors attenuates hypertriglyceridemia, except for the Y2 receptor, which was noted to have no effect (37, 46, 52, 53). Because all of the NPY receptor subtypes are expressed both centrally and peripherally (except for the brain-specific Y5 receptor) (35), it would be interesting to determine whether brain-specific deletion of the Y1 receptor in the obese ob/ob mouse would attenuate hypertriglyceridemia.

Current models suggest that increased adipocyte lipolysis during states of fasting or insulin resistance leads to increased substrate (FFA and glycerol) delivery to liver, which can increase hepatic VLDL-TG secretion (32, 49). The observation that increased CNS NPY signaling via the Y1 receptor doubled VLDL-TGs, whereas plasma FFA and glycerol levels were unchanged, suggests that adipocyte lipolysis was not increased and thus would not account for the NPY-stimulated increase in hepatic VLDL-TG production. Moreover, we observed this same effect in rats given chronic NPY treatments under pair-fed conditions, leading to the doubling of VLDL-TG secretion independent of changes in adipocyte lipolysis. Although CNS Y2 receptor signaling did not alter markers of adipocyte lipolysis, the Y2 receptor agonist surprisingly had no effect on plasma TGs in the absence of tyloxapol (Fig. 4, A and B). That the Y2 receptor agonist did increase VLDL-TG secretion modestly in the presence of tyloxapol (Fig. 3, C and E) suggests that Y2 receptor activation may also have an effect to enhance TG clearance perhaps through the modulation of adipose tissue LPL activity (33).

SCD-1 catalyzes the desaturation of palmitic and stearic acids to palmitoleic and oleic acids, and its expression is known to be regulated by CNS leptin (17, 66), glucose (34), and melanocortin action (67) in the same hypothalamic feeding circuits engaged by NPY. Whereas leptin suppresses SCD-1 [and NPY tone (56)], we have observed a robust induction of hepatic SCD-1 expression in response to CNS NPY treatment. This effect is recapitulated by Y1 receptor activation, providing further evidence that NPY signaling via the Y1 receptor is mechanistically involved in hepatic lipid metabolism.

Provision of oleic acid or modulation of SCD-1 activity changes VLDL production rate by increasing triglyceride loading in the late maturation phase (17, 34). Presently, we demonstrate that the elevation in hepatic VLDL-TG secretion by increased CNS NPY signaling via the Y1 receptor is associated with a rapid (within 60 min) induction of hepatic SCD-1 gene expression, which would suggest that SCD-1 activation may contribute to changes in VLDL-TG secretion. This is supported by previous findings in which hypothalamic glucose (34) and glycerol (70) metabolism reduced hepatic SCD-1 mRNA and inhibited hepatic VLDL-TG secretion. Although the definitive mechanistic link between hepatic SCD-1 and the alteration of hepatic SCD-1 expression by CNS NPY signaling remains to be determined, changes in the formation rate of hepatic oleic acid may be an important mediator (34).

Our results show that neither the NPY nor the Y1 agonist influenced the level of key de novo lipogenic enzymes, phosphorylated ACC (Ser79), ACC, or FAS. Hepatic apoB is an essential component of liver-derived VLDL; if lipid is not loaded by MTP, apoB becomes a target for proteasomal degradation (19). In contrast to humans, the rat produces predominantly apoB-48 instead of apoB-100 in the liver (14). Our results show that there were no significant changes in hepatic tissue levels of apoB-48 detectable by Western blot from increased CNS NPY and Y1 receptor signaling (Fig. 5, A and B), although this method is not as sensitive as radiolabelling methods. Similarly, we found that MTP, which plays a pivotal role in VLDL maturation and secretion of triglyceride-rich lipoproteins (7, 19), was unaltered by NPY and Y1 receptor agonist treatment (Fig. 5, A and B). Collectively, these data suggest that CNS NPY acts primarily via the Y1 receptor to increase plasma TGs and likely does so by altering the late maturation step of VLDL assembly and secretion.

In conclusion, we demonstrate, within the limits of our model, that the effects of NPY on feeding and/or weight gain are relatively dissociable from the effects on hepatic VLDL-TG secretion. Three days of twice daily icv NPY injections in pair-fed animals compared with Veh-treated controls to maintain identical caloric intake and body composition led to hypertriglyceridemia. CNS NPY signaling via the Y1 receptor predominantly regulates effects on hepatic lipoprotein production, whereas the activation of the Y2 receptor has a greater effect on feeding. Altogether, these findings raise the possibility
that NPY regulates feeding and lipoprotein metabolism partially via separate NPY receptor systems and/or mechanisms.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.M.R., J.M.S., R.L.P., and K.D.N. did the conception and design of the research; J.M.R., J.M.S., and S.S. performed the experiments; J.M.R., J.M.S., and S.S. analyzed the data; J.M.R., J.M.S., R.L.P., and K.D.N. interpreted the results of the experiments; J.M.R. prepared the figures; J.M.R. drafted the manuscript; J.M.R., J.M.S., R.L.P., A.G.-B.-S., and K.D.N. edited and revised the manuscript; J.M.R., R.L.P., and K.D.N. approved the final version of the manuscript.

REFERENCES


