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Combined Deletion of Y1, Y2, and Y4 Receptors Prevents Hypothalamic Neuropeptide Y Overexpression-Induced Hyperinsulinemia despite Persistence of Hyperphagia and Obesity

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Neuropeptide Y (NPY) is a key regulator of energy homeostasis and is implicated in the development of obesity and type 2 diabetes. Whereas it is known that hypothalamic administration of exogenous NPY peptides leads to increased body weight gain, hyperphagia, and many hormonal and metabolic changes characteristic of an obesity syndrome, the Y receptor(s) mediating these effects is disputed and unclear. To investigate the role of different Y receptors in the NPY-induced obesity syndrome, we used recombinant adeno-associated viral vector to overexpress NPY in mice deficient of selective single or multiple Y receptors (including Y1, Y2, and Y4). Results from this study demonstrated that long-term hypothalamic overexpression of NPY lead to marked hyperphagia, hypogonadism, body weight gain, enhanced adipose tissue accumulation, hyperinsulinemia, and other hormonal changes characteristic of an obesity syndrome. NPY-induced hyperphagia, hypogonadism, and obesity syndrome persisted in all genotypes studied (Y1−/−, Y2−/−, Y2Y4−/−, and Y1Y2Y4−/− mice). However, triple deletion of Y1, Y2, and Y4 receptors prevented NPY-induced hyperinsulinemia. These findings suggest that Y1, Y2, and Y4 receptors under this condition are not crucially involved in NPY's hyperphagic, hypogonadal, and obesogenic effects, but they are responsible for the central regulation of circulating insulin levels by NPY.

Abbreviations: ***AAV*** *Adeno-associated viral vector,* ***ARC*** *arcuate nucleus,* ***bGHpA*** *bovine growth hormone poly-A,* ***DMN*** *dorsomedial nuclei,* ***ICV*** *intracerebroventricular,* ***NPY*** *neuropeptide Y,* ***NTS*** *nucleus tractus solitaries,* ***PVN*** *paraventricular nucleus,* ***PYY*** *preferential Y2/Y5 receptor agonist,* ***r*** *recombinant,* ***WAT*** *white adipose tissue,* ***WPRE*** *woodchuck posttranscriptional regulatory element*

NEUROPEPTIDE Y (NPY) IS a 36-amino-acid peptide that is highly expressed in the hypothalamus and implicated in the etiology of obesity and insulin resistance due to its hyperphagic and hormonal and metabolic effects (1–3). Within the hypothalamus, NPY is produced most abundantly in the arcuate nucleus (ARC), which innervates virtually the entire hypothalamus, including the paraventricular nucleus (PVN) and dorsomedial nuclei (DMN) (4). Intracerebroventricular or hypothalamic administration of NPY to normal rodents lead to defects characteristic of obesity including hyperphagia, accelerated body weight gain, hyperleptinemia, hypercorticosteronemia, hyperinsulinemia, decreased circulating concentrations of IGF-I, and increases in white adipose tissue weight (1, 3, 5–7). In addition, hypothalamic NPY content is significantly increased in genetically obese rodents such as *fa/fa* and *cp/cp* rats as well as *ob/ob* mouse (8–10).

NPY acts via the G protein-coupled Y receptors, of which there are five receptors cloned to date, denoted Y1, Y2, Y4, Y5, and y6 (11). In mouse hypothalamus, Y1 receptors are present in the PVN, DMN, ARC, medial preoptic nucleus, suprachiasmatic nucleus, the periventricular nucleus, the medial zone of the mamillary region, and the tuberal and perifornical areas (12). Distribution of Y2 receptors in the mouse hypothalamus was reported in the ARC, periventricular nucleus, DMN, parvocellular PVN, lateral hypothalamic area, medial and lateral preoptic areas,

bed nucleus of the stria terminalis, medial preoptic nucleus, and the tuberal and perifornical areas and zona incerta (12). Expression of Y4 receptors is less well studied and seems to be limited to the medial preoptic nucleus, PVN, and ARC (13, 14). In mouse hypothalamus, the presence of Y5 receptors was described only in the ARC (15). Interestingly, despite high level of Y5 receptor mRNA detected in the human ARC, no Y5 receptor binding sites were found in the human hypothalamus (16, 17). In the mouse brain, expression of the Y6 receptor mRNA has been reported in the hypothalamus (18); however, no functional analysis has been performed to clarify its role in the regulation of energy homeostasis.

Despite the large number of studies that document the effects of central NPY administration on energy homeostasis, the molecular mechanisms underlying these effects remain poorly understood. In fact, there is still considerable conflict in the literature about the role of different Y receptors in the regulation of body weight. For example, a recent report showed intracerebroventricular (ICV) administration of a Y1 receptor preferring agonist in mice increased body weight and adiposity but did not induce hyperphagia (19). Y1 receptor antagonists were able to inhibit NPY-induced and spontaneous feeding (20, 21). However, food intake studies using Y1 receptor antisense oligodeoxynucleotides reported variable findings (22–24). Interestingly, whereas Y1 receptor knockout mice exhibit reduced fasting-induced hyperphagia, they also develop significant increases in body weight, fat mass, and insulinemia in the absence of hyperphagia (25, 26). Similar controversies exist regarding the role of Y2 receptors in energy homeostasis. Hypothalamus-specific or germline deletion of Y2 receptors lead to marked reductions in the body weight of lean mice (27), and significant reductions in adiposity or body weight and the type 2 diabetic syndrome of *ob/ob* mice, in the absence of reductions in food intake (28, 29). In contrast, another germline Y2 receptor knockout model was shown to develop increased body weight, fat deposition, and hyperphagia (30). Moreover, food intake in obese human subjects was shown to be inhibited by a preferential Y2/Y5 receptor agonist (PYY)3–36 (31) and ICV administration of PYY3–36 or other Y2 receptor agonists has been shown to reduce feeding and body weight in rodents (19, 32, 33). The role of Y4 receptors in the regulation of energy homeostasis is less well studied. Y4 receptor knockout mice have a lean phenotype; however, deletion of the Y4 receptor on the *ob/ob* background had no effect on the hyperphagia, obesity, or type 2 diabetic phenotype of *ob/ob* mice (34). On the other hand, peripheral administration of pancreatic polypeptide (a Y4 preferring agonist) to mice increases metabolic rate and decreased food intake and body weight gain (35).

Compared with the many studies attempting to decipher the role of individual Y receptors in feeding and weight gain, studies examining the involvement of individual Y receptors in NPY-mediated neuroendocrine effects are sparse. Chronic infusion (7 d) of PYY3–36 in rodents induced increases in plasma insulin and corticosterone to the same extent as NPY infusion and increased plasma leptin to a lesser extent, compared with NPY (36). Acute ICV administration of NPY and its analogs that preferentially activate Y1 and Y5 receptors increased plasma insulin levels. In contrast, administration of Y2 and Y4 preferring analogs had no effect on plasma insulin level (37).

To identify the receptors mediating the NPY-induced obesity syndrome and accompanying hormonal perturbations, we used recombinant (r) adeno-associated viral vector (AAV) to chronically overexpress NPY in mice deficient of selective single or multiple Y receptors (including Y1, Y2, and Y4). Y5 receptor knockouts were not investigated in this study because the Y5 and Y1 receptors are localized only 20 kb apart on the same chromosome, and it is therefore extremely unlikely to obtain Y1Y5 receptor double-knockout mice by cross-breeding.

MATERIALS AND METHODS

Animals

Generation of the Y1, Y2, and Y4 knockout mice were published previously (27, 34, 38). Double- or triple-knockout mice were obtained by crossing Y1, Y2, or Y4 knockout mice, respectively. All mice were on a mixed C57BL/6–129/SvJ background. All research and animal care procedures were approved by the Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee and were in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose. Mice were housed under conditions of controlled temperature (22 C) and illumination (12-h light cycle, lights on at 0700 h). Mice were fed a normal chow diet *ad libitum* (6% calories from fat, 21% calories from protein, 71% calories from carbohydrate, 2.6 kcal/g; Gordon's Specialty Stock Feeds, Yanderra, New South Wales, Australia). Adult female mice

aged between 12 and 17 wk were used in the study. Male wild-type mice within the same age range were used for a pilot study.

Vector production

Human NPY cDNA was subcloned into an AAV expression cassette consisting of the rat neuron-specific enolase promoter, woodchuck posttranscriptional regulatory element (WPRE), and a bovine growth hormone poly-A (bGHpA) signal flanked by AAV2 inverted terminal repeats (pAM/NSE-NPY-WPRE-bGHpA). The same expression cassette without the transgene (pAM/NSE-empty-WPRE-bGHpA) was used as control. High-titer chimeric AAV vectors expressing a mix of

AAV serotype 1 and serotype 2 capsid proteins were generated as described previously (39). Briefly, HEK 293 cells were transfected with the AAV plasmid, together with the AAV helper plasmids pH21, pRV1, and pFΔ6 by calcium phosphate transfection methods. Forty-eight hours after transfection, cells were harvested and the vector purified by heparin affinity columns as described (40). Genomic titers were determined using the Prism 7700 sequence detector system (PerkinElmer-Applied Biosystem, Foster City, CA) with primers against the WPRE sequence and vector titer normalized to approximately 1 × 1013 genome copies/ml.

Vector administration

Adult mice were anesthetized with a single dose of ketamine/xylazine (100 mg/kg and 20 mg/kg; ip) and placed on a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA). One microliter rAAV1/2 vector was injected bilaterally into the hypothalamus at a rate of 0.1 μl/min using a 10 μl Hamilton syringe attached to Micro4 microsyringe pump controller (World Precision Instruments Inc., Sarasota, FL). The injection coordinates for hypothalamus were (from bregma): anterioposterior, −2.1 mm; mediolateral, ± 0.4 mm; and dorsoventral, −5.3 mm (41). Animals were kept on a heating pad during surgery.

Immunohistochemistry

To confirm NPY overexpression, a separate group of mice also injected with rAAV1/2-NPY vector was killed by sodium pentobarbitone overdose (15 μl Nembutal, ip) and perfused transcardially with 1× PBS followed by 4% paraformaldehyde. After cryoprotection in 30% sucrose, coronal brain sections of 40 μm were cut for immunohistochemistry. Briefly, sections were rinsed in PBS-Triton X-100 before being incubated in 1% (vol/vol) H2O2 in 50% (vol/vol) methanol for 10 min to remove endogenous peroxidase. After 2 × 5 min rinses in PBS-Triton X-100, sections were incubated overnight at room temperature with polyclonal NPY primary antibody (1:1000; Chemicon, Temecula, CA). Sections were then washed with PBS-Triton X-100, and antirabbit biotinylated secondary antibody (1:500; Sigma, St, Louis, MO) was applied. After a 3-h incubation, sections were washed with PBS-Triton X-100 and treated with ExtrAvidin peroxidase (1:500 dilution; Sigma) for 2 h before a final wash in PBS and staining with diaminobenzidine. Sections were mounted onto slides and left to dry overnight before being dehydrated in ascending concentrations of ethanol, immersed in xylene, and coverslipped. Immunostained brain sections were photographed using a digital camera (DC 480; Leica Microsystems GmbH, Wetzlar, Germany) attached to a Axiophot microscope (Carl Zeiss, Jena, Germany).

Determination of food intake and weight gain

The weight of food taken from the hopper and body weights were measured daily at a set time. Body weight gain at the end of the 3-wk period was calculated with reference to the initial body weight.

Tissue collection

Three weeks after vector injection, animals were culled between 1200 and 1400 h by cervical dislocation and decapitation for collection of trunk blood. Food was removed from cages 2–4 h before cull. Brains were immediately removed and frozen on dry ice. Inguinal, retroperitoneal, mesenteric, and epididymal white adipose tissue (WAT) depots as well as liver were dissected out and weighed. For a subset of animals (n = 3–6/group except for Y1−/−-NPY), the combined weight of both ovaries and fallopian tubes was also recorded as an index of effects of NPY on gonadal function.

Serum analyses
Serum hormone levels were determined with commercial RIA kits from Linco Research (St. Charles, MO) (leptin, insulin), ICN Biomedicals (Costa Mesa, CA) (corticosterone), and Bioclone Australia (Marrickville, Australia) (IGF-I). Basal serum glucose levels were determined with a glucose oxidase kit (Trace Scientific, Melbourne Australia).

In situ hybridization and densitometry
To assess the level of vector-mediated NPY overexpression, brains from four rAAV-NPY treated mice of each genotype were randomly selected for *in situ* hybridization and subsequent densitometry measurement. Mice were culled by cervical dislocation and brains were immediately removed and frozen on dry ice and stored at −80 C for subsequent *in situ* hybridization. Coronal sections (20 μm) were cut and thaw mounted on slides. An oligonucleotide probe complementary to mouse NPY (5′-GAGGGTCAGTCCACACAGCCCCATTCGC-TTGTTACCTAGCAT-3′) mRNA was labeled with [35S]thio-dATP (Amersham Biosciences, Buckinghamshire, UK) using terminal deoxynucleotidyltransferase (Roche, Mannheim, Germany). Slides were fixed in 2% paraformaldehyde followed by 2 × 20 sec washes in 1× PBS and 10 min in 1× triethanolamine buffer containing acetic anhydride. Sections were subjected to ascending ethanols (70, 80, 95, and 100%; 1 min each), chloroform (5 min), and then descending ethanol (100 and 95%; 1 min each) to remove lipid. Sections were air dried before overnight incubation with 50 μl labeled probe (5 × 105 dpm) in a humidity chamber at 42 C. Sections were washed in descending concentrations of sodium saline citrate (5×, 2×, and 1×) containing dithiothreitol followed by water and dehydration in ethanol. Slides were air dried before exposure against BioMax film (Kodak, Rochester, NY) and placed in a lightproof autoradiographic cassette until development using an M35-M X-OMAT processor (Kodak).

For each brain, six sections across the rostrocaudal axis of the hypothalamus were selected for analysis. Hybridization signals were semiquantified by density measurement using the National Institutes of Health Image 1.61 software with care taken to select the same area over the hypothalamus.

Statistical analysis
All data are expressed as means ± sem. Differences among groups of mice were assessed by ANOVA followed by Fisher's *post hoc* comparisons if appropriate (StatView, version 4.51; Abacus Concepts, Berkeley, CA). Body weight gain was also subjected to repeated-measure ANOVA. Statistical significance was defined as $P < 0.05$.

RESULTS

Vector-mediated NPY overexpression in the hypothalamus
rAAV-NPY injected mice exhibited a robust increase in NPY immunoreactivity in the hypothalamus, compared with the rAAV-empty injected controls, which exhibited NPY immunoreactive levels comparable with that of naïve mice (Fig. 1). Vector-mediated NPY expression was seen as early as 3 d (data not shown) and persisted for at least 2 months as described previously (39). Body tissue and serum collection in this study were performed in 3-wk treated animals when vector mediated transgene expression reached stable level.

In situ hybridization for NPY and density measurements of the hybridization signal revealed no significant difference in the hypothalamic expression level between the rAAV-NPY treatment groups (data not shown). The mRNA expression of NPY in the hypothalamus of all rAAV-NPY treated groups was approximately 10-fold greater than endogenous expression levels in wild-type mice (data not shown).

Effect of hypothalamic NPY overexpression on body weight and food intake
Using wild-type mice, we showed that hypothalamic rAAV-NPY treatment had a similar effect on body weight gain in male and female mice 3 wk after injection. However, there was a trend for greater body weight gain in females (178.6 ± 7.8% of initial body weight, n = 8), compared with males (157.6 ± 5.0%, n = 5; ANOVA, $P = 0.077$; Fig. 2B). Therefore, all subsequent studies were conducted in female mice.

In rAAV-NPY-treated mice, after an initial small drop in body weight due to surgery, body weight increased rapidly and steadily over the 3 wk after injection in all genotypes (Fig. 2A). A significant difference was observed in the rate of body weight gain between rAAV-NPY

injected wild-type and Y1Y2Y4−/− mice (Fig. 2A). Body weight gain 3 wk after vector injection was 6-fold higher in rAAV-NPY injected animals, compared with rAAV-empty counterparts in all genotypes (Fig. 2B). However, the degree of body weight gain in rAAV-NPY-treated mice was different between genotypes, with body weight gain being significantly less in Y1Y2Y4−/− mice, compared with wild-type mice (Fig. 2B).

Total food intake over the 3 wk study period was significantly increased over empty vector-treated control values by approximately 2-fold in rAAV-NPY-treated mice for all genotypes except for Y1Y2Y4−/− mice, which had a 1.5-fold increase, compared with their empty vector counterparts (Fig. 2C). There was also a significant difference, compared with rAAV-NPY-injected wild types, in the food intake of rAAV-NPY injected Y1−/− and Y2Y4−/− mice as shown in Fig. 2C (*black columns*). However, it should be noted that the rAAV-empty groups of Y1−/− and Y2Y4−/− mice already showed a greater food intake than wild-type mice.

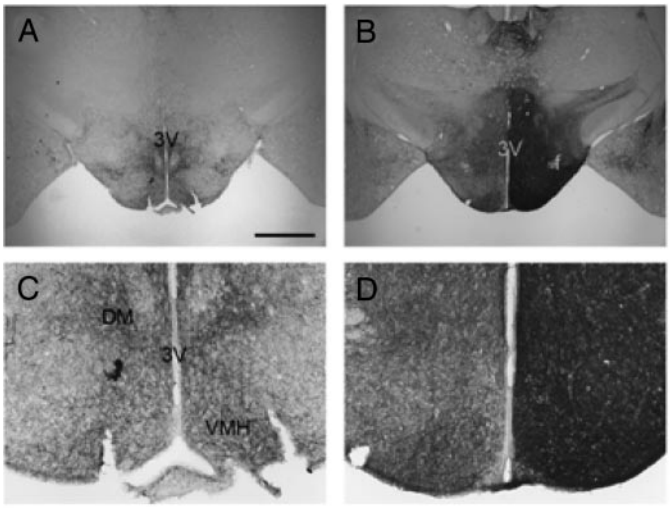

FIG. 1. rAAV-mediated NPY overexpression in mouse hypothalamus. A, Representative coronal brain section showing NPY immunoreactivity in the hypothalamus of naïve mice. B, Representative coronal brain section from mice unilaterally injected with rAAV1/2-NPY in the right hypothalamus shows more intense NPY immunostaining in the injected side, compared with noninjected side and naïve animals. C and D, Higher-magnification images of A and B, respectively. *Scale bar*, 1 mm (A and B) and 250 μm (C and D). 3V, Third ventricle; f, fornix; DM, dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus.

Effect of hypothalamic NPY overexpression on adipose tissues, liver, and reproductive organs

The rAAV-NPY-treated mice showed significant increases in both the absolute and relative weights of the various WAT depots studied, including inguinal, epididymal, mesenteric, and retroperitoneal (data not shown). To account for differences in body weight among groups, only relative weights (WAT weight as a percentage of body weight) were used for comparison. The effect of NPY overexpression on total WAT weight was similar across genotypes (Fig. 3A).

The increase in hypothalamic NPY expression levels by rAAV resulted in significant increases in absolute liver weight in all genotypes (Fig. 3B). The increase in liver weight was more than the proportional increase relative to body weight because the NPY groups exhibited higher relative liver weight, compared with empty vector controls, except for Y2−/− mice (Fig. 3C).

Treatment with rAAV-NPY significantly reduced the weight of female reproductive organs (ovaries and fallopian tubes) in wild-type mice, either when expressed as absolute weight (55 ± 5 mg *vs.* 117 ± 18 mg, means ± sem, $P < 0.05$) or when expressed as a percent of body weight (0.15 ± 0.03% *vs.* 0.55 ± 0.09%, means ± sem, $P < 0.01$). Similarly, rAAV-NPY injections in mice of all other genotypes studied (Y2−/−, Y2Y4−/−, Y1Y2Y4−/−) induced reductions in both absolute and relative reproductive organ weights, with no significant difference in the effect among genotypes (vector and genotype interaction effect, $P = 0.88$ and $P = 0.73$ for absolute and

relative weight respectively; data not shown).

Effect of hypothalamic NPY overexpression on hormone and metabolite levels

Serum leptin levels were significantly increased in rAAV-NPY-treated animals of all genotypes except for Y2−/− mice (Fig. 4A). Serum insulin levels were comparable among empty vector controls of the different genotypes (Fig. 4B). However, there was a significant difference among genotypes with respect to the effect of NPY overexpression on serum insulin. rAAV-NPY administration induced robust increases in serum insulin levels in the wild-type, Y1−/−, Y2−/−, and Y2Y4−/− mice, but it had no significant effect on serum insulin levels of Y1Y2Y4−/− mice (Fig. 4B). Serum glucose levels were significantly increased by rAAV-NPY treatment in the wild-type and Y1−/− mice but not in the Y2−/−, Y2Y4−/−, and Y1Y2Y4−/− mice (Fig. 4C). Notably, the serum glucose level in Y1Y2Y4−/−-NPY-overexpressing mice was significantly lower than that of wild-type-NPY-overexpressing mice (Fig. 4C). Basal plasma corticosterone levels were increased by rAAV-NPY administration in all genotypes under study, although statistical significance was reached only in wild-type and Y2−/− mice (Fig. 4D). rAAV-NPY in general reduced serum IGF-I levels, most notably in wild-type mice (Fig. 4E). Two-way ANOVA showed there was no impact of genotype on this effect of NPY (no significant genotype/vector interaction effect).

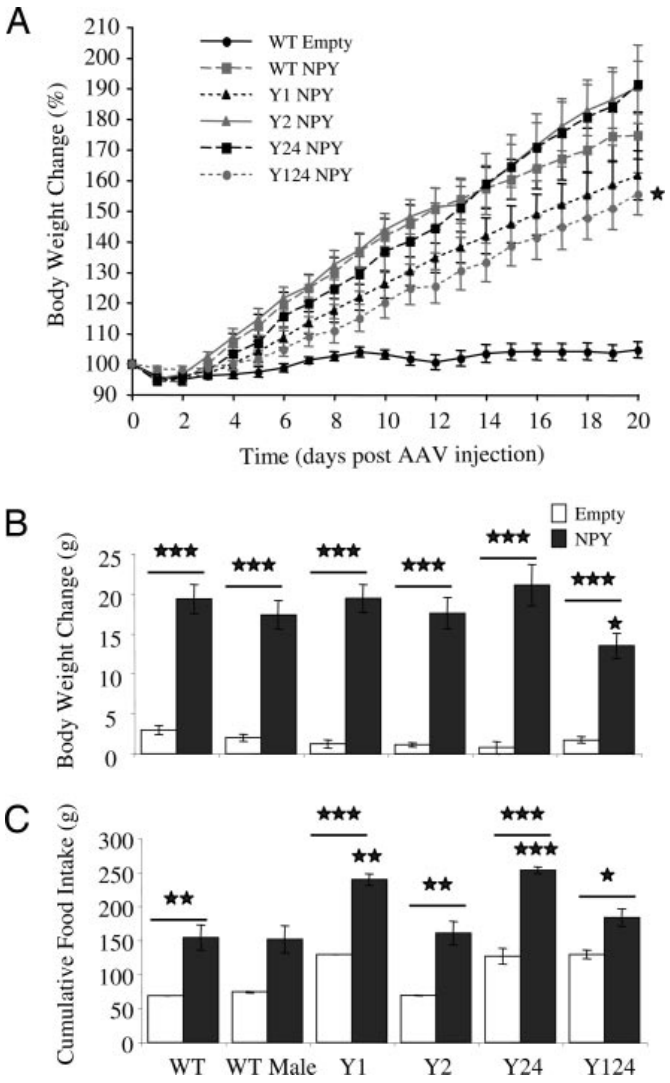

FIG. 2. Effect of Y receptor deficiencies on rAAV-NPY induced body weight gain and food intake. A, Body weight change as a percentage of initial body weight after rAAV vector injection. rAAV-empty-injected mice of all genotype showed comparable growth curve; therefore, only that for wild-type mice is shown. B, Body weight gain at 3 wk after rAAV vector injection. C, Cumulative food intake for 3 wk after rAAV vector administration. Data are means ± SEM of at least six mice per group. ⋆, $P < 0.05$; ⋆⋆, $P < 0.01$; ⋆⋆⋆, $P < 0.001$ *vs.* rAAV-NPY injected wild-type mice; or the comparison indicated by *horizontal bars above columns*. WT, Wild type; Y1, $Y1^{-/-}$; Y2, $Y2^{-/-}$; Y24, $Y2Y4^{-/-}$; Y124, $Y1Y2Y4^{-/-}$. Except for WT-male, all other groups consisted of female mice.

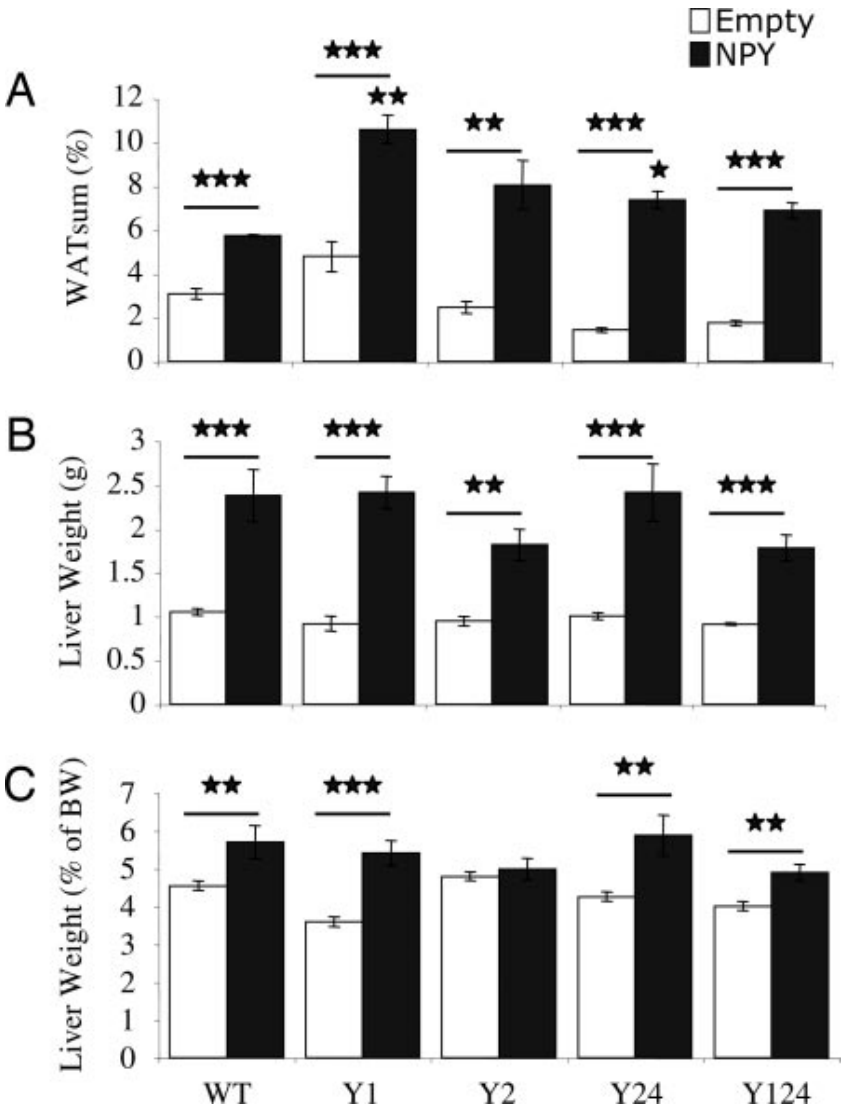

FIG. 3. Effect of Y receptor deficiencies on rAAV-NPY-induced obesity. A, Relative weight of total WAT (WATsum) as a percent of body weight 3 wk after rAAV-NPY administration. Absolute (B) and relative (C) liver weight 3 wk after rAAV-NPY administration. Data are means ± SEM of at least six mice per group. ★, $P < 0.05$; ★★, $P < 0.01$; ★★★, $P < 0.001$ *vs.* rAAV-NPY injected wild-type mice; or the comparison indicated by *horizontal bars above columns*. WT, Wild type; Y1, $Y1^{-/-}$; Y2, $Y2^{-/-}$; Y24, $Y2Y4^{-/-}$; Y124, $Y1Y2Y4^{-/-}$.

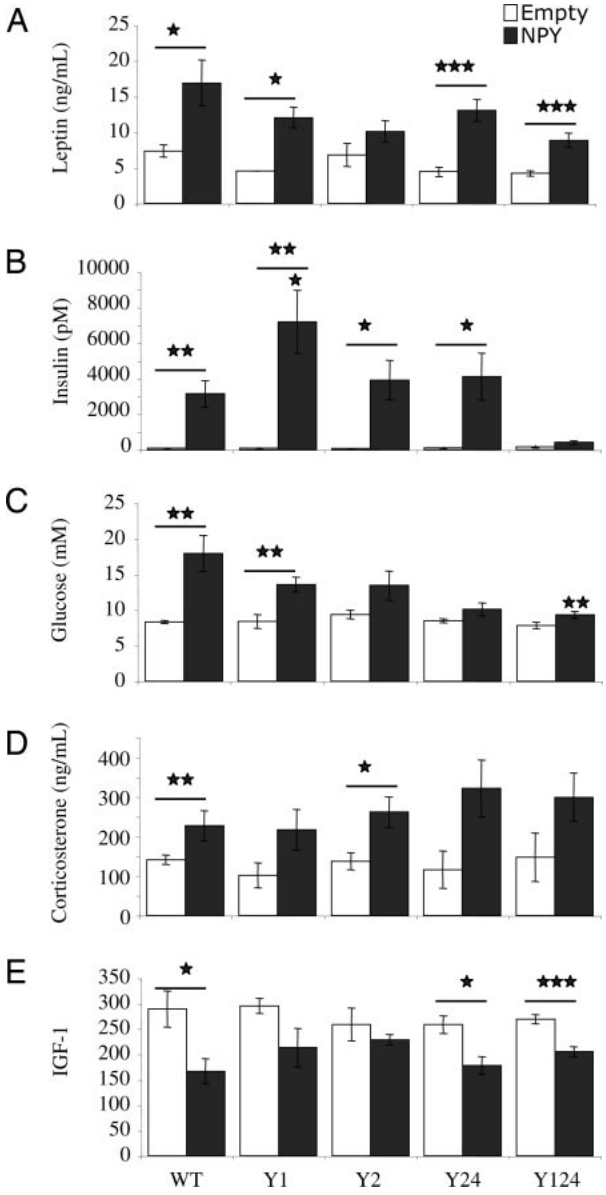

FIG. 4. Effect of Y receptor deficiencies on rAAV-NYP-induced neuroendocrine effects. Circulating levels of serum leptin (A), insulin (B), glucose (C), corticosterone (D), and IGF-I (E) 3 wk after rAAV vector administration. Data are means ± SEM of at least six mice per group. ⋆, $P < 0.05$; ⋆⋆, $P < 0.01$; ⋆⋆⋆, $P < 0.001$ *vs.* rAAV-NPY injected wild-type mice; or the comparison indicated by *horizontal bars above columns*. WT, Wild-type; Y1, $Y1^{-/-}$; Y2, $Y2^{-/-}$; Y24, $Y2Y4^{-/-}$; Y124, $Y1Y2Y4^{-/-}$.

DISCUSSION

This study is the first report using rAAV vectors to overexpress NPY in the mouse hypothalamus. Our data demonstrate that long-term hypothalamic overexpression of NPY resulted in marked hyperphagia, increased body weight, enhanced adipose tissue accumulation, hypogonadism, hyperinsulinemia, and other hormonal changes characteristic of an obesity syndrome, consistent with changes after central administration of exogenous NPY peptide (3, 5, 6, 42–44). Single deletion of the Y1 and Y2 receptors, double deletion of Y2 and Y4 receptors, or triple deletion of Y1, Y2, and Y4 receptors did not prevent the NPY-induced hyperphagia, hypogonadism, and obesity syndrome. However, triple deletion of Y1, Y2, and Y4 receptors prevented NPY-induced hyperinsulinemia. These findings suggest that whereas Y1, Y2, and Y4 receptors are not crucially

involved in NPY's hyperphagic, hypogonadal and obesogenic effects under these conditions of high NPY, they are responsible for the central regulation of circulating insulin levels by NPY.

Our data indicate that Y1, Y2, and Y4 receptors are only partially responsible for mediating NPY's obesogenic effects, and they may compensate one another because deletion of one receptor alone had no effect. One potential mediator for NPY's action is the Y5 receptor, which has been shown to be orexigenic. ICV administration of specific Y5 receptor agonists in mice increased body weight and adiposity and induced hyperphagia and hypogonadism (19, 36, 45). However, whereas there is evidence for an involvement of the Y5 receptor in mediating the orexigenic action of NPY, the degree of its involvement remains to be evaluated. A study using specific Y5 receptor antagonists showed a lack of effect on feeding (46). In addition, Y5 receptor knockout mice did not affect feeding response to either acute or chronic NPY infusion or abolish or attenuate the consequent obesity syndrome (47, 48). It has been suggested that biological redundancies are likely to exist between Y1 and Y5 receptor signaling in the NPY-mediated control of food intake and energy balance (48). A study using a specific Y5 receptor antagonist that had no affinity toward other known Y receptors reduced ICV NPY- or Y5 receptor agonist-mediated overfeeding in rats. However, a similar effect was observed in Y5 receptor knockout mouse, thus suggesting involvement of receptors other than the currently known Y receptors (49).

There is mounting evidence of the existence of such other yet-unidentified Y receptors, which may mediate NPY's orexigenic and obesogenic actions. Comparing the efficacies in stimulating food intake using prototypic peptide agonists for Y1-Y6 receptors and nonpeptide Y1 receptor antagonists, Iyengar *et al*. (50) concluded that the mediation of NPY-induced feeding cannot be unequivocally attributed to any one of the known Y receptors. They suggested that NPY-induced feeding is mediated by either a combination of more than one Y receptor or a unique but as-yet-unknown Y receptor subtype (50). Recently two independent studies using competition binding experiments in wild-type mice using specific Y receptor blockers and radioligand binding experiments in Y receptor knockout mice, both showed results suggestive of additional Y receptors (51, 52). Future studies using Y1Y2Y4Y5 receptor-deficient mice, which can be generated only by simultaneous targeted deletion of both the Y1 and Y5 receptor, or using Y1Y2Y4−/− mice in combination with specific Y5 receptor blockers may address this question. In addition, it remains possible that under conditions of excessive concentration of NPY, this ligand may bind and activate receptors for which it normally has no or only low affinities. Therefore, the effect of other non-Y receptors involved in energy homeostasis cannot be ruled out.

Intriguingly, despite the development of hyperphagia and an obesity syndrome in Y1Y2Y4−/− mice treated with rAAV-NPY, these animals had normal insulin levels. In contrast, hypothalamic NPY overexpression induced marked hyperinsulinemia in Y1−/−, Y2−/−, and Y2Y4−/− mice to an extent at least as great as that seen in wild-type animals. This suggests that the three receptors, Y1, Y2, and Y4, may be compensatory for one another in mediating NPY's central regulation of insulin because deletion of one or two of them did not alter the degree of NPY-induced hyperinsulinemia, compared with that observed for wild-type mice. Furthermore, the development of an obesity syndrome in the Y1Y2Y4−/−-NPY mice with normal insulin level suggests that hyperinsulinemia was not solely responsible for the observed obesity in NPY-treated animals. Conversely, the normal glucose level in Y1Y2Y4−/−-NPY-overexpressing mice demonstrated that the degree of obesity remaining in these mice was not sufficient to impair glucose homeostasis and implied that in this model of obesity, impairments in glucose homeostasis were not secondary to the obesity.

Central NPY peptide administration to normal rats has been shown to increase plasma insulin levels both acutely and chronically (53–55). This effect can be observed in animals under pair-fed conditions, indicating the existence of a central and direct NPY effect that is independent of hyperphagia (3, 55). Central NPY induces basal hyperinsulinemia through glucocorticoid-dependent parasympathetic activation (via the vagus nerve) to the pancreas (53, 56, 57). In addition to its central effect on insulin release, NPY also acts directly on the pancreas to inhibit both basal and stimulated insulin release (54). This suggests that the direct action of NPY on insulin release is inhibitory, whereas the central action of NPY indirectly results in an increase in plasma insulin. Because the mice studied were germline knockout models, it is possible that the deletion of Y1, Y2, and Y4 receptors in sites other than the hypothalamus may have contributed to the prevention of rAAV-NPY-mediated hyperinsulinemia. One potential brain region is the nucleus tractus solitarius (NTS) because NPY microinjected into the NTS was shown to induce significant increase in circulating insulin levels. Therefore, the NTS may be an important site in

NPY's central effect on insulin regulation (53). In support of this proposition, the NTS has been shown to contain Y1, Y2, and Y4 receptors (58). The amygdala represents another brain region that may be involved in insulin regulation because lesions to this region have been shown to induce hyperinsulinemia independent of hyperphagia and weight gain (59). The amygdala sends to and receives projections from the hypothalamus (60) and contains high levels of Y receptors (58), thus representing another site of action for hypothalamic NPY to influence insulin level.

The insulin regulatory effect by Y1, Y2, and Y4 is less likely to be mediated at the peripheral level because the direct effect of NPY on the pancreas is inhibitory. This inhibitory action on insulin secretion by NPY was suggested to be mediated via the Y1 receptor because ligand potency on insulin secretion inhibition in an insulinoma cell line closely resembles a Y1-like profile, and NPY mediated inhibition of insulin secretion was blocked by a Y1-specific antagonist (61). This may explain the aggravated hyperinsulinemia observed in rAAV-NPY-treated Y1−/− mice because the direct inhibition by NPY signaling via Y1 receptors is disabled, leaving only the central stimulatory effect, thus resulting in elevated insulin secretion. This is consistent with previous reports on naive germline Y1−/− mice, which developed late-onset obesity and hyperinsulinemia (25, 62). So far, Y1 is the only receptor subtype identified in the pancreas (61, 63); whether Y2 and Y4 receptors are also present remains to be tested.

In addition to the increased body weight, hypogonadism, hyperphagia, and hyperinsulinemia, hypothalamic NPY overexpression induced other neuroendocrine defects consistent with that seen in genetically obese rodents as well as obese humans. These changes include hyperleptinemia (42), hypercorticism (64), impaired GH secretion (44, 65), and increased fat accretion, which is likely a result of increased *de novo* lipogenesis in liver and adipose tissue (3, 66). Our data suggest that whereas the hyperinsulinemic effects of chronic central rAAV-NPY treatment is mediated via actions at Y1, Y2, and Y4 receptors, other effects of rAAV-NPY are not. This dissociation of mechanisms of action of NPY is reminiscent of leptin signaling, whereby specific interruption of leptin receptor-signal transducer and activator of transcription 3 signaling results in obesity, hyperphagia, and hyperinsulinemia, whereas complete absence of leptin receptors as in db/db mice results in these and other defects, notably infertility and impaired linear growth (67).

In this study, we observed significant reductions in the weight of female reproductive organs in NPY-overexpressing wild-type animals, as reported in a previous study whereby NPY was infused into the lateral ventricle of intact adult female rats for 7 d (6). The same study also demonstrated impaired ovulation as determined by daily vaginal smears in NPY-treated animals (6). In addition, in this study we observed similar NPY-induced reductions in the weight of female reproductive organs in all genotypes under study, so any variations in the effects of rAAV-NPY on metabolic and neuroendocrine parameters were unlikely to be influenced by variations in estradiol fluctuation among groups. Whereas NPY may exert both excitatory and inhibitory effects on gonadotropic function (68–70), it is clear from previous studies and present data that in intact rodents, chronic elevation of hypothalamic NPY leads to suppression of the reproductive axis (6, 36, 71).

In conclusion, our study demonstrated that deletion of Y1, Y2, and Y4 receptors prevented the development of NPY-induced hyperinsulinemia, despite manifestation of other obesity syndrome characteristics such as hyperphagia, hypogonadism, increase in body weight and fat deposits, hyperleptinemia, hypercorticism, and reduction in plasma IGF-I. This finding indicates that Y1, Y2, and Y4 mediate central NPY's effect on insulin level independent of other neuroendocrine changes. The stable overexpression of NPY by rAAV vector allows us to mimic a situation of chronically elevated hypothalamic NPY levels as observed in some forms of diabetes mellitus and obesity (72–75) and may provide a useful model for the study of perturbations associated with diabetes and obesity.

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nothing to declare.

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