Chapter 4

Injection of rAAV-NPY in the paraventricular nucleus results in obesity

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INJECTION OF RAAV-NPY IN THE PARAVENTRICULAR NUCLEUS RESULTS IN OBESITY

ABSTRACT

Chronic central administration of NPY has dramatic effects on energy balance, however, the exact role of the hypothalamic paraventricular nucleus (PVN) in this is unknown. The aim of this study was to further unravel the contribution of NPY signaling in the PVN to energy balance. Recombinant adeno-associated viral particles containing NPY (rAAV-NPY) were injected in the rat brain with coordinates targeted at the PVN. For three weeks, body weight, food intake, endocrine parameters, body temperature and locomotor activity were measured. Furthermore, effects on insulin sensitivity and expression of NPY, AgRP and POMC in the arcuate nucleus were studied. Food intake was increased specifically in the light period. Furthermore, dark phase body temperature and locomotor activity were reduced. This resulted in obesity characterized by increased fat mass, elevated plasma insulin, leptin and adiponectin, decreased AgRP expression in the arcuate nucleus and decreased insulin sensitivity, whereas plasma corticosterone was unaffected. These data suggest that increased NPY expression targeted at the PVN is sufficient to induce obesity. Interestingly, plasma concentrations of leptin and insulin were elevated prior to a rise in food intake, suggesting that NPY in the PVN influences leptin and insulin secretion independent from food intake. This strengthens the role of the PVN in regulation of energy balance by NPY.

INTRODUCTION

Neuropeptide Y (NPY), which is widely distributed throughout the brain, has a robust feeding effect when injected centrally (193;220). Moreover, it decreases metabolic rate and thermogenesis (206;208;335). One week of chronic intracerebroventricular (ICV) administration of NPY results in an obese phenotype characterized by hyperphagia, elevated plasma concentrations of corticosterone, leptin and insulin, and increased lipogenesis in liver and adipose tissue (121;201;203;212;214).

Neurons in the arcuate nucleus that express NPY have been implicated in the regulation of energy balance and project to a variety of hypothalamic nuclei, including the paraventricular (PVN), dorsomedial (DMH) and lateral (LH) hypothalamic nuclei and the medial preoptic area (56;177-179). Although all these nuclei may be involved in this regulation, acute injections of NPY in the PVN and the perifornical area result in the largest increase in food intake (217;223). Furthermore, repeated daily injections of NPY in the PVN of female rats result in increased (high fat) food intake and body weight, however whether this increased weight gain is solely due to food intake or also to decreased energy expenditure remains to be determined (225). The role of NPY in the PVN for thermogenesis is contradictory; Currie *et al.* found a hypothermic effect within 3 hours after NPY injection in the PVN (223), Jolicoeur *et al.* found the opposite (205). To further unravel the role of NPY in the PVN in the regulation of energy balance, infusion of NPY in the PVN over longer

periods is required. It is, however not feasible to reliably infuse ligands locally in the brain for weeks.

One way of approaching this problem is genetic overexpression of NPY. However, results obtained using genetic overexpression of NPY are not consistent with data from pharmacological NPY administration. Global overexpression of NPY in transgenic mouse and rat models results in normal body weight and food intake when fed a regular chow diet (230;231;233). This indicates that in these transgenic models there is either a developmental change, compensation by counter-regulatory mechanisms, or only limited overexpression. By ablation of NPY/AgRP neurons, it was shown recently that, although these neurons are necessary for normal feeding in adult mice, they can be ablated without affecting body weight gain or food intake in neonates (155;156). Furthermore, a 4-fold NPY overexpression of NPY did not affect body weight regulation (232), suggesting an enormous plasticity of these systems during development.

Taken together, there are discrepancies between the strong effects after chronic icv administration of NPY and the modest effects of transgenic NPY overexpression on food intake and body weight. Furthermore, the specific involvement of the PVN in the obese phenotype is not clear. In order to bypass developmental compensation and address the role of long-term NPY signaling specifically in the PVN in the regulation of energy balance, we used viral gene transfer. Recombinant adeno-associated viral (rAAV) particles containing NPY cDNA were injected to induce local overexpression of NPY in the PVN, that is still present three weeks after injection. Rather than overexpressing NPY in the Arc, being the major source of NPY released in the PVN, we injected AAV-NPY directly in the PVN. A feature of AAV2 transduction in the brain is that the vector remains confined to the injection site (336). Indeed our earlier findings, using a similar strategy to overexpress agouti, showed that local immunostaining was limited to the PVN, and not observed in projection areas of the PVN (337). Thus, we and others (338) have demonstrated that AAV-mediated overexpression is suitable to induce local delivery of gene products. The effect of long-term overexpression of NPY in the PVN was evaluated by daily recording of body weight and food intake, and repeated analysis of hormonal changes and glucose tolerance. Finally, we also measured body temperature and locomotor activity.

MATERIAL AND METHODS

Animals

Male Wistar rats weighing 220-250 g were purchased from Charles River (Crl-Wu, Germany). They were individually housed in filtertop cages with *ad libitum* access to food and water. Animals were kept in a temperature and humidity controlled room (21±2 °C) under a 12h/12h light/dark cycle (lights on at 0700 h). All experimental procedures were

approved by the Committee for Animal Experimentation of the University of Utrecht, Utrecht, The Netherlands.

Surgical procedures

Rats were anesthetized with 0.1 ml/100 g im fentanyl/fluanisone (Hypnorm®, Janssen Pharmaceutica, Beerse, Belgium) and 0.05 ml/100 g ip midazolam (Dormicum®, Hoffman-LaRoche, Mijdrecht, The Netherlands). A silicone catheter was implanted in the right jugular vein and attached behind the shoulders with a backmount pedestal (Bilaney Consultants, Dusseldorf, Germany). Transmitters (TA10TA-F40 Data Science International, St Paul, Minnesota, USA) were placed in the abdominal cavity. Rats were left to recover for three weeks.

Seven days after baseline recordings, rats were anesthetized again as described above. Using a stereotax, rAAV-NSE-NPY (n=10) or rAAV-NSE-empty (used as control) (n=6) was injected bilaterally into the PVN (rAAV-NPY-PVN and rAAV-contr-PVN, coordinates AP: -1.8 mm from bregma, ML: ±0.3 mm from bregma, and DV: -8.0 mm below the skull). Another control group was injected bilaterally with rAAV-NSE-NPY into the thalamus (rAAV-NPY-thal, coordinates AP: -1.8 mm from bregma, ML: ±0.5 mm from bregma, and DV: -7.0 mm below the skull) (n=5). Production of rAAV-NPY has been described previously (339;340). 1 μl of virus ($2x10^8$ particles) was injected per site over five minutes, after which the needle was kept in place for ten minutes before removal.

After each surgery, rats received an injection with 0.05 ml/100 g sc buprenorphin (Temgesic®, Schering-Plough, Maarssen, The Netherlands).

Data analysis

One week before, till three weeks after viral injections, body weight, food intake, body temperature and locomotor activity were recorded. Body weight gain and food intake were measured daily at 1400 h. Feed efficiency (FE) of the rats was calculated as the ratio of body weight gain over four days and the total food intake of that period (day 8-11 and day 18-21).

Locomotor activity and body temperature were measured via the transmitters that send digitized data via radio frequency signals to a nearby receiver. The data were automatically recorded every ten minutes, and averaged per hour using DSI software (DSI, St Paul, MN).

Naso-anal length and waist circumference were measured at day 22 in awake animals.

Glucose tolerance test

An intravenous glucose tolerance test was performed at day 17 after injection of the AAV, between 1000h and 1100h. Rats were fasted for 1 hour and injected with glucose (150 mg in 0.5ml saline) via the jugular vein cannula, followed by 0.5 ml saline. 200 µl blood samples were taken via the jugular vein cannula just before, and 1, 5, 10, 20, 30 and 45 minutes after glucose administration. Blood glucose and plasma insulin were measured as described under plasma analysis.

Collection of blood and tissues

Blood samples were collected at ± 1000 h at seven and fourteen days after injection. A PE tube was connected to the backmount pedestal, catheters were rinsed with heparinized saline, blood samples of 2 ml were taken and collected in tubes containing 0.1 M EDTA, after which rats received 2 ml of saline. At day 23 after injection, rats were decapitated, trunk blood was collected in heparinized tubes after adding 83 µmol EDTA and 1 mg aprotinin, and immediately placed on ice. Plasma samples were stored at -20 °C until further analysis.

Brains were immediately removed after decapitation, quickly frozen in cold isopentane (-35 °C) and stored at -80 °C. Retroperitoneal and epididymal white adipose tissue (WAT), interscapular brown adipose tissue (BAT), liver and adrenals were isolated and weighed.

Localization of rAAV in the brain

16 µm coronal sections of the hypothalamus were sliced using a cryostat (Leica, Rijswijk, The Netherlands), thaw-mounted onto RNAse free Superfrost slides (Menzel, Germany) and stored at -80 °C. Viral infection was localized by in situ hybridization (ISH) with a digoxigenin (DIG)-labeled woodchuck post-transcriptional regulatory element (WPRE) probe. The WPRE sequence is part of the expression cassette of all vectors used here. ISH procedure has previously been described (341).

Immunohistochemistry

16 µm coronal sections of the hypothalamus were fixated in 4% paraformaldehyde. After pretreatment with 0.3% H_2O_2, slides were incubated overnight at 4 °C with a rabbit anti-NPY antibody (Niepke, 1:2000, a kind gift of Prof. Dr R.M. Buijs, Amsterdam, the Netherlands). Sections were then incubated for one hour with a secondary goat anti-rabbit-biotinylated antibody (1:100) (Jackson ImmunoResearch). After incubation for one hour with ABC (1:500) (Vector Laboratories, Burlingame, California, USA) sections were treated with diaminobenzidine (Sigma, Saint Louis, Missouri, USA, 0.5 mg/ml in tris buffered saline, with 30% H_2O_2) for ten minutes. All immunohistochemistry steps described above were followed

by a three times rinse with tris-buffered saline. Slides were then dehydrated in ethanol, cleared with xylene and coverslipped.

Plasma analysis

Plasma leptin, insulin, adiponectin and corticosterone were analyzed in duplicate using radioimmunoassay kits, (Linco Research, St Charles Missouri, USA for leptin, insulin and adiponectin, ICN Biochemicals, Costa Mesa, California, USA for corticosterone). Plasma adrenocorticotropic hormone (ACTH) was measured in duplicate using a specific rabbit antiserum directed to the midportion of ACTH, which was kindly provided by Dr G.B. Makara (Budapest, Hungary). Synthetic human $ACTH_{(1-39)}$ (Peninsula Laboratories, Belmont, California, USA) was labeled with ^{125}I and used as tracer (342). Plasma glucose was measured in duplicate using a Medisense glucosesensor (Abbott, Amersfoort, The Netherlands). Plasma free fatty acids (FFA), triglycerides (TG) and glycerol were measured in triplicate using an Acyl-CoA synthetase-acyl-CoA oxidase method (FFA, Roche Diagnostics, Penzberg, Germany) or a serum triglyceride determination kit (TG and glycerol, Sigma, Saint Louis, Missouri, USA).

Quantitative in situ hybridization

16 μm coronal sections were used for raISH. 33P-labeled antisense RNA probes were made using AgRP (396 bp mouse (343)), NPY (286 bp rat (344)) and POMC (350 bp rat (343)) cDNA fragments. raISH procedure has previously been described (345). Expression of AgRP, NPY and POMC was analyzed in the arcuate nucleus using Image J software (National Institutes of Health, Bethesda, Maryland, USA).

Statistical analysis

Data are presented as group means ± SEM. Differences in body weight gain, food intake, body composition and plasma levels were assessed using one-way ANOVA, followed by Tukey's post-hoc test. Body temperature and locomotor activity were analyzed by T-tests. Differences were being considered significant at $p<0.05$.

RESULTS

Localization of rAAV in the brain

The location of microinjection of rAAV vectors in PVN or thalamus (which served as a control group) was confirmed by post mortem WPRE *in situ* hybridization (Fig. 1). For rAAV-NPY-PVN and rAAV-contr-PVN, rats were only included in the study when WPRE expression was bilaterally in the PVN (Fig. 1A). In some of the rats that were included in these groups there was also staining of cells in the periventricular nucleus and in the thalamus surrounding the injection shaft. Three rats, where the PVN was hit unilaterally,

and/or only partly, were excluded from the rAAV-NPY-PVN group and were considered separately as controls (i.e. indicated as *missed* in Fig. 2) . From rAAV-NPY-thal rats (Fig. 1B), only one rat was excluded, because no WPRE expression was found. We found no WPRE positive cells in any hypothalamic nucleus in any of the rats included in this group.

At the end of the study (day 23), punctate NPY staining was observed in the thalamus of the rAAV-NPY-thal rats, as well as NPY-positive cell bodies. In rAAV-NPY-PVN rats, total NPY staining in the PVN, but not outside the injection area, was increased when compared to controls, as shown by NPY immunohistochemistry (Fig. 1C).

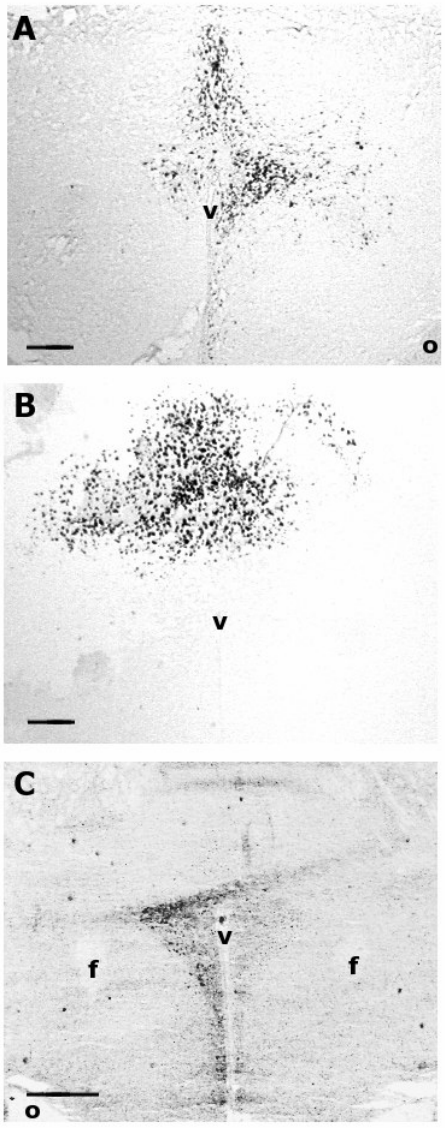

Figure 1: Localization of WPRE RNA expression in hypothalamic (A) and thalamic (B) sites injected with rAAV-NPY. (C) Photomicrograph of immunohistochemistry for NPY in the PVN in a rat injected unilaterally (left side) in the PVN with rAAV-NPY. Note the increased staining at the infection side. f = fornix, o = optic tract, v = third ventricle. Scalebar = 500 μm.

Effects of rAAV-NPY on body weight and food intake

The first three days after injection, there was a similar decrease in body weight and food intake in all groups of rats. Seven days after injection, all rats had recovered their initial body weight and food intake.

Food intake increased in rAAV-NPY-PVN rats and reached a plateau at fifteen days after injection (Fig. 2A). In the last week of the experiment, rAAV-NPY-PVN rats ate over 75% more food as compared to rAAV-contr-PVN and rAAV-NPY-thal rats. Food intake of the 'missed' injection group of rats was intermediate between that of rAAV-NPY-PVN 'hit' rats and controls.

Interestingly, food intake of rAAV-NPY-PVN animals was increased in the light phase, but not in the dark phase. Light phase feeding increased over time in rAAV-NPY-PVN 'hit' rats, but was stable in controls (Fig. 2B).

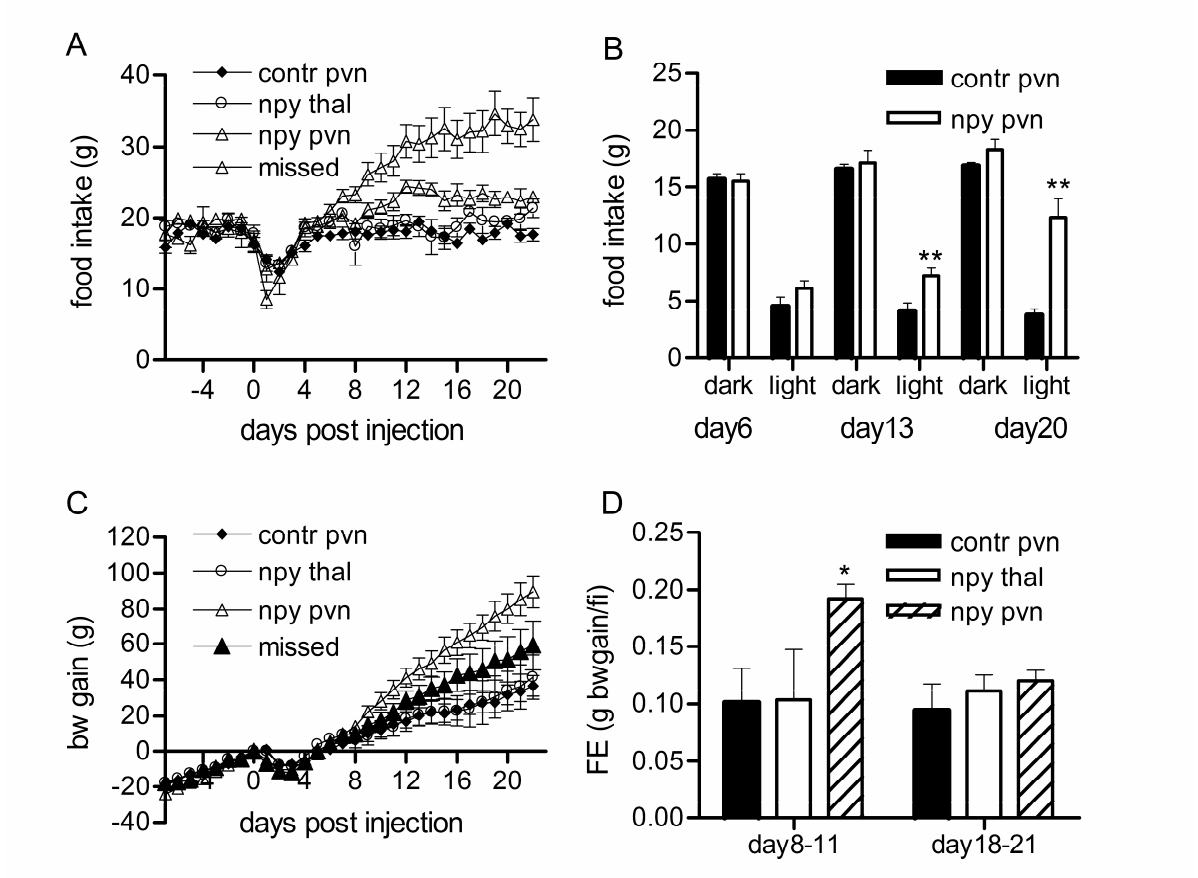

Figure 2: Effects of rAAV-NPY injections on daily food intake (A and B) and cumulative body weight gain (C) compared to rAAV-contr-PVN rats, rAAV-NPY-thal rats, and rats of the 'missed' injection group. D. The body weight gain per grams of food intake (Feed efficiency (FE)) calculated between day 8-11 and 18-21 after rAAV injection. * $p<0.05$, ** $p<0.01$.

Whereas rAAV-contr-PVN and rAAV-NPY-thal rats showed a slow gain of body weight after having recovered from the injection, similar to body weight gain before injection, rAAV-NPY-PVN rats showed a strong increase in body weight gain, starting at day 9 following injection (Fig. 2C). This led to an accumulated body weight gain of 240% compared to rAAV-contr-PVN rats on day 22. Body weight gain of the 'missed' injection group of rats was intermediate between rAAV-NPY-PVN rats and control groups.

To determine whether the body weight increase observed in rAAV-NPY-PVN rats was only due to food intake changes, we measured feed efficiency (FE), calculated as the ratio of body weight gain per total food intake over four days in individual rats. Since the food intake curve (Fig. 2A) was increasing until day 15 and was stable afterwards, these two phases were analysed separately. FE was significantly increased between day 8-11 after injection; however, between day 18-21 there was no difference anymore in FE (Fig. 2D).

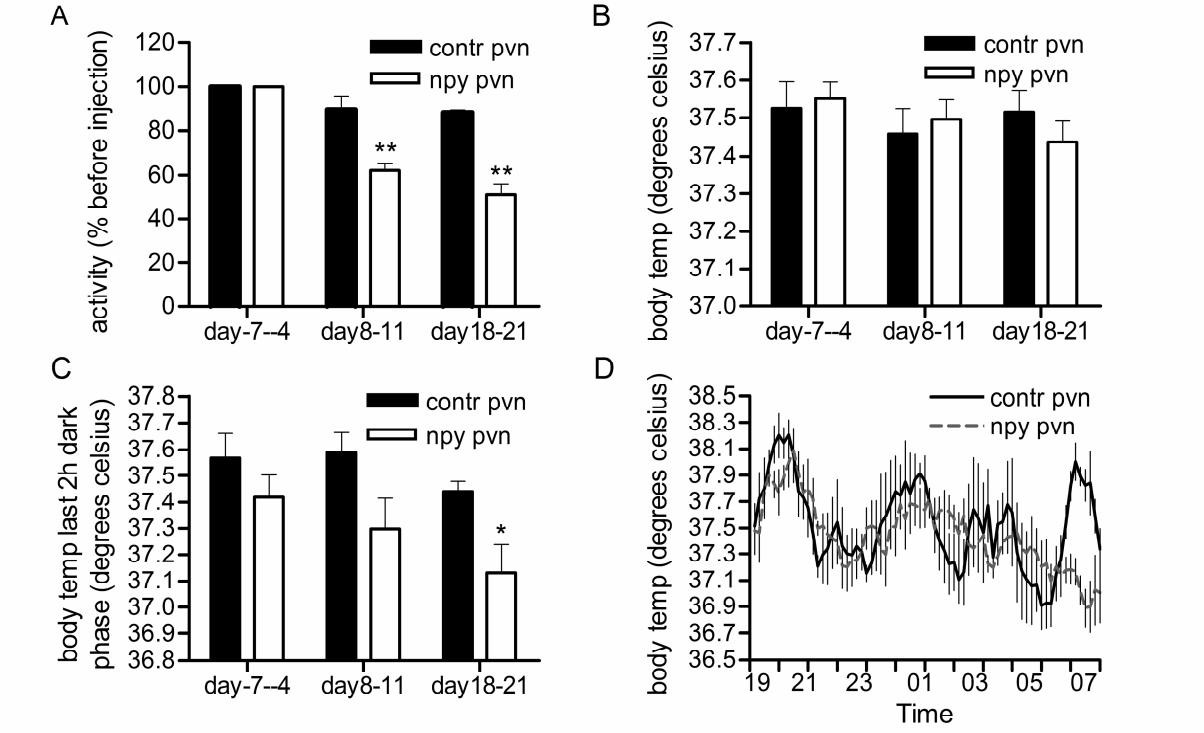

Figure 3: Effects of rAAV-NPY injections on locomotor activity (A) and body temperature (B) in the total dark phase compared to rAAV-contr-PVN rats. C Effects of rAAV-NPY injections on body temperature in the last two hours of the dark phase. D. Example of temperature rhythm in the dark phase (day 18). Activity data are presented as percentage of values before injection. * $p<0.05$, ** $p<0.01$.

Effects of rAAV-NPY on body temperature and locomotor activity

Because an increase in FE suggests a reduction in energy expenditure, we examined body temperature and locomotor activity at the same days as FE was measured. Locomotor activity in the light period was similar in both groups (data not shown). However, during the dark period, rAAV-NPY-PVN rats showed a reduction of locomotor activity. One week after injection night-time activity of rAAV-NPY-PVN rats was only 62% of the activity individual rats displayed before injection, which was further reduced to 51 ± 5% at day 18-21 (versus 88 ± 1% for rAAV-contr-PVN rats, $p<0.01$) (Fig. 3A).

Besides a reduced locomotor activity in the dark period, rAAV-NPY-PVN rats tended to have a decline in body temperature in the dark phase. Although in the second week after injection there was no difference in body temperature of rAAV-NPY-PVN and rAAV-contr-PVN rats, at the end of the study there was a trend towards hypothermia in rAAV-NPY-PVN rats (Fig. 3B), which was significant at the last two hours of the dark phase ($p<0.05$) (Figs 3C and D). There was no significant effect of rAAV-NPY injection on body temperature in the light phase.

Effects of rAAV-NPY on endocrine parameters

Table 1 and Figure 4 summarize plasma levels of endocrine parameters measured at three time points after rAAV-NPY injections.

rAAV-NPY-PVN injections resulted in a significant elevation in basal concentrations of plasma leptin and insulin seven days after injection (Figs 4A and B). Plasma leptin concentrations in rAAV-NPY-PVN rats increased drastically over time, resulting in five times higher levels compared to control injected rats at the end of the study.

Plasma insulin concentrations were higher than controls in the first period after injection, resulting in three fold higher levels two weeks after injection. In the last week of the study, plasma insulin concentrations were still increased as compared to control groups. However, plasma insulin concentrations had also increased in the control groups as expected in sedentary rats fed ad libitum at this age.

Plasma adiponectin concentrations were not significantly changed seven days after rAAV-NPY injections. However, both two and three weeks after injections, rAAV-NPY-PVN rats showed increased circulating adiponectin concentrations (Fig. 4C).

rAAV-NPY-PVN injections did not produce any change in glycemia in the first two weeks after injection. However at day 23, these rats were slightly hyperglycemic when compared to rAAV-contr-PVN rats.

Plasma concentrations of FFA, TG, corticosterone and ACTH of rAAV-NPY-PVN rats were not significantly different from control values at any time point (Table 1).

Table 1: Effects of rAAV-NPY on endocrine parameters 7, 14 and 23 days after injections

	Day 7			Day 14			Day 23		
	contr-pvn	**npy-thal**	**npy-pvn**	**contr-pvn**	**npy-thal**	**npy-pvn**	**contr-pvn**	**npy-thal**	**npy-pvn**
gluc (mmol/l)	10.8±0.26	10.3±0.40	10.5±0.2	9.8±0.41	9.3±0.10	10.3±0.49	10.4±0.22	11.0±0.60	11.7±0.22#
FFA (mg/dl)	3.7±0.47	3.6±0.77	2.9±0.60	3.6±0.78	4.1±0.71	2.9±0.53	0.9±0.08	1.1±0.19	0.7±0.10
TG (mg/dl)	85.0±14.9	97.5±12.0	118.6±10.5	78.3±8.9	63.1±5.1	75.0±7.9	106.5±18.4	91.1±16.3	102.3±6.5
glyc (mg/dl)	32.5±5.1	33.2±9.8	26.5±6.9	37.1±13.2	30.2±5.6	20.3±3.8	8.6±1.4	6.3±1.2	7.3±0.9
cort (μg/dl)	10.6±2.53	4.0±1.57	12.8±3.03	10.7±5.86	6.9±4.44	17.8±5.35	18.3±2.44	14.6±3.20	13.8±3.00
ACTH (pg/ml)	48.2±4.76	42.0±2.34	41.8±2.05	27.8±3.10	37.5±21.0	49.6±10.1	39.6±2.96	53.3±12.7	36.9±8.11

Gluc, glucose; FFA, free fatty acids; TG, triglycerides; glyc, glycerol; cort, corticosterone; ACTH, adrenocorticotropic hormone. #p<0.05 vs contr-pvn

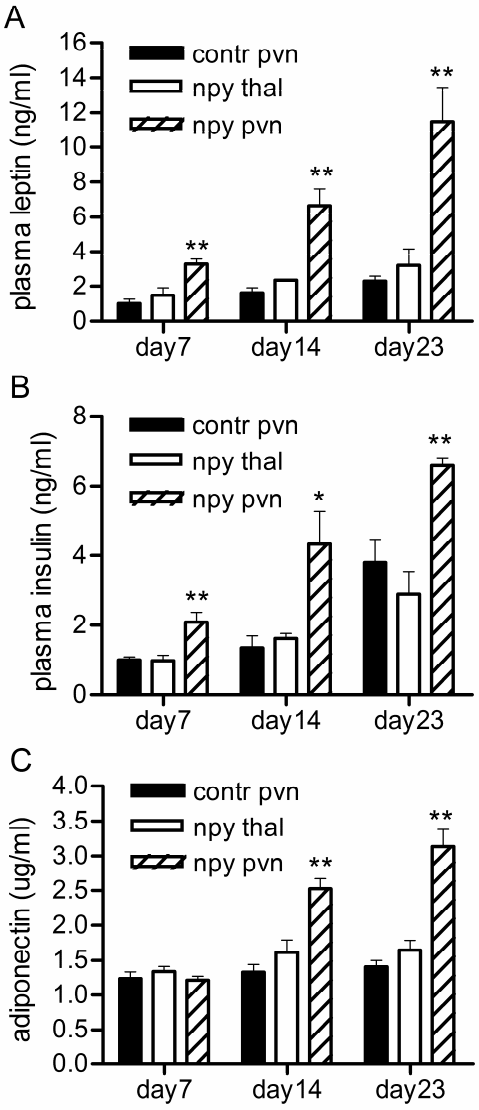

Figure 4: Effects of rAAV-NPY injections on basal plasma leptin (A), insulin (B) and adiponectin (Apn) (C) compared to rAAV-contr-PVN rats or rAAV-NPY-thal rats 7, 14 and 23 days after injection. * $p<0.05$, ** $p<0.01$.

Effects of rAAV-NPY on glucose tolerance

On day 17 after viral injections, we performed a glucose tolerance test. As shown in Figure 5A, glucose clearance after an intravenous glucose challenge was normal in rAAV-NPY-PVN rats. However, the insulin response to the glucose challenge was markedly increased (Fig. 5B), indicating a decreased insulin sensitivity.

Effects of rAAV-NPY on body composition

Table 2 and Figure 6 summarize body composition 23 days after viral injections. rAAV-NPY-PVN injections had a significant effect on the waist circumference of the rats, without influencing naso-anal length (Fig. 6A).

Total white adipose fat tissue (WAT) mass (retroperitoneal and epididymal) was significantly increased in rAAV-NPY-PVN rats as compared to both control groups (Fig. 6B). Additionally, interscapular BAT mass was also increased in rAAV-NPY-PVN rats (Table 2). Finally, rAAV-NPY-PVN rats had smaller adrenals as compared to control groups, but there was no significant change in thymus (Table 2).

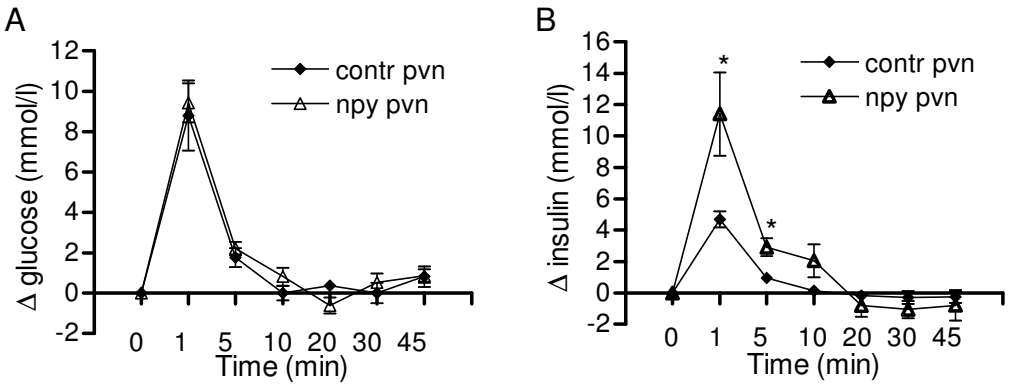

Figure 5: Effects of rAAV-NPY injections on blood glucose (A) and plasma insulin levels (B) compared to rAAV-contr-PVN rats following intravenous administration of glucose. * p<0.05.

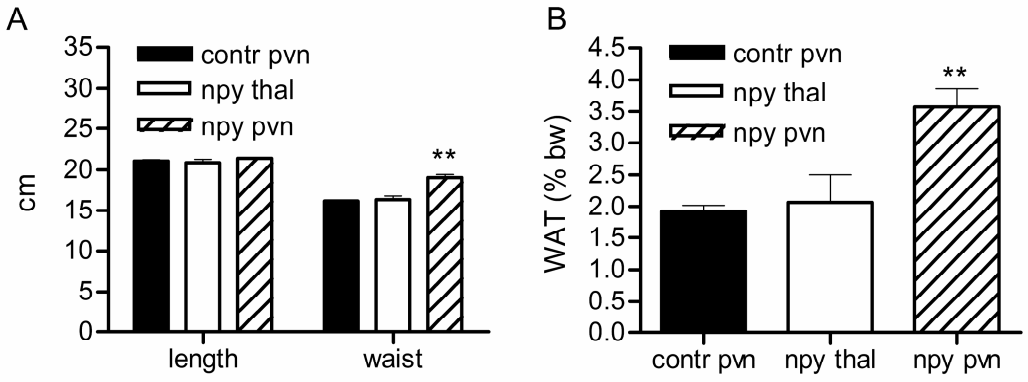

Figure 6: Effects of rAAV-NPY injections on naso-anal and waist size (A), retroperitoneal WAT depot (B), and liver weight (C) 23 days after injection compared to rAAV-contr-PVN rats or rAAV-NPY-thal rats. * p<0.05, ** p<0.01.

Table 2: Effects of rAAV-NPY on body composition 23 days after injections.

	Day 23		
	contr-pvn	**npy-thal**	**npy-pvn**
adrenals (‰bw)	0.14±0.01	0.15±0.01	0.11±0.01**
thymus (%bw)	0.10±0.022	0.13±0.011	0.12±0.010
BAT (%bw)	0.15±0.01	0.18±0.02	0.27±0.03**

** p<0.01 vs. contr-pvn and npy-thal.

Effects of rAAV-NPY on neuropeptide expression in the arcuate nucleus

mRNA expression of NPY, AgRP and POMC in the arcuate nucleus three weeks after rAAV injections was measured by raISH. AgRP expression of rAAV-NPY-PVN rats was significantly reduced to 40% of control values, while POMC and NPY expression were unchanged (Fig. 7).

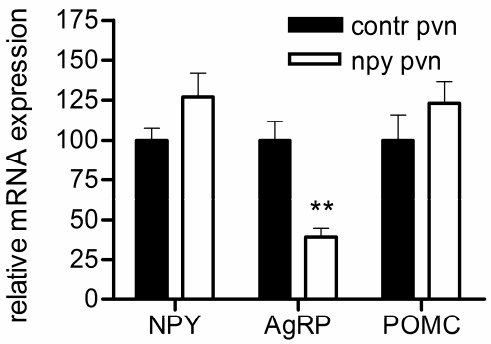

Figure 7: Effects of rAAV-NPY injections on arcuate nucleus expression of NPY, AgRP and POMC mRNA 23 days after injection compared to rAAV-contr-PVN rats. ** $p<0.01$.

DISCUSSION

In this study we show that rAAV-mediated overexpression in the PVN results not only in hyperphagia in the light phase, but also in changes in body temperature and locomotor activity in the dark phase. These changes together lead to an obese phenotype characterized by increased fat mass, elevated plasma concentrations of insulin, leptin and adiponectin, and decreased AgRP expression in the arcuate nucleus.

Interestingly, our data clearly show that the increased body weight gain is not solely due to increased food intake. In the first days when food intake was increased in rAAV-NPY-PVN rats, the rats showed a higher body weight gain than expected, compared to the amount of food eaten. This increased feed efficiency (FE) suggests that NPY overexpression in the PVN does not only stimulate food intake, but also reduces energy expenditure. Indeed, dark phase locomotor activity was reduced in rAAV-NPY-PVN rats, indicating that long-term elevated NPY signaling in the PVN is sufficient to reduce locomotor activity. This is a novel finding, since so far there are no reports of NPY administration into the PVN on locomotor activity in the home cage, although activity in the home cage or open field can be suppressed after ICV administration (209),. The fact that FE is no longer increased at the end of the experiment, in spite of a continuously lower activity, may be explained by the fact that rAAV-NPY-PVN rats had a significantly increased body mass, which requires more energy from food to maintain.

Another factor that can contribute to energy expenditure is thermogenesis. Several studies have clearly demonstrated that ICV NPY administration, or local NPY injections in

the PVN, decreases thermogenesis, by suppressing sympathetic activity to (208) and reducing UCPI mRNA in interscapular BAT (206;207). Although this suggests a NPY induced reduction in body temperature, results of NPY injections in the PVN are contradictory (205;223). Although we did not find effects of NPY on mean day time or night time body temperature, there was a significant reduction in body temperature in the late dark phase in rAAV-NPY-PVN rats. Our data show an increase in food intake restricted to the light period, whereas body temperature was only changed during the last 2h of the dark phase.

Others already reported that chronic icv NPY induces hyperphagia and obesity accompanied with high plasma concentrations of insulin and leptin (201;203;214). Interestingly, plasma concentrations of leptin and insulin rose before an effect of NPY on food intake and body weight could be observed. Already at day 7, rAAV-NPY-PVN rats showed elevated concentrations of plasma leptin and insulin, while food intake and body weight were still normal. Earlier acute ICV studies also showed this direct effect of NPY on insulin secretion (199-203), however, the direct involvement of the PVN was not shown before. In addition, the adipose tissue derived hormone adiponectin was not altered at this time point, suggesting a differential regulation of leptin and adiponectin.

At a later time point, rats injected with rAAV-NPY in the PVN showed insulin resistance; glucose clearance was normal after an intravenous glucose tolerance test, performed 17 days after virus injection, however, this was accompanied by an increased insulin response. Whether this is due to an increase in insulin secretion or a decrease in insulin clearance remains to be determined.

Although the effects of NPY on insulin secretion largely depend on food intake, with the largest effects occurring when food is available (199-201;203), NPY can also increase plasma insulin concentrations when eating is prevented (199;202;203). This food-independent increase in circulating insulin probably involves NPY-induced stimulation of the parasympathetic nervous system. Indeed, ICV NPY administration has been shown to activate the parasympathetic nervous system (346). Additionally, Buijs *et al.*, has injected pseudorabies virus in rats with a sympathetic denervation of the pancreas, and demonstrated the presence of vagal innervation from the PVN to the pancreas (347). Furthermore, it is known that the PVN is an important nucleus in the regulation of sympathetic nerve outflow (348). The PVN also innervates adipose tissue (349;350), and reduced sympathetic versus parasympathetic activity contributes to an increase in leptin concentrations (351-353). Therefore, it is plausible that the increase in leptin and insulin in the first period after rAAV-NPY-PVN injections results from NPY-induced vagal stimulation of the pancreas and WAT.

Despite the fact that others reported NPY-induced increases in HPA axis activity (203;354), long-term overexpression of NPY in the PVN did not alter plasma concentrations

of ACTH and corticosterone. The significant reductions in adrenal gland weight that we found even suggest decreased HPA axis activity. Although increased HPA axis activity has been associated with increased food intake and obesity (355), we did not find this to contribute to the obese phenotype described here.

rAAV-NPY-PVN rats showed a strong reduction in AgRP expression levels in the arcuate nucleus, presumably as a result of increased leptin signaling in this nucleus, since plasma levels of leptin were increased. The fact that the reduction in AgRP did not reduce food intake suggests that the elevated (viral mediated) NPY signaling in the PVN acts downstream of AgRP signaling.

Even though obesity is associated with low circulating adiponectin concentrations (352), there are no reports on the effects of central NPY administration on plasma adiponectin. Meanwhile, it has been shown recently that mice with increased adiponectin levels due to a mutation or transgenic overexpression indeed are obese (356;357). Since plasma adiponectin was increased in the rAAV-NPY-PVN rats at the end of the study, as well as WAT mass, it may be possible that the elevated adiponectin levels reflect increased WAT mass, which secretes adiponectin in the bloodstream.

We did not find a significant change in concentrations of FFA or TG at any time point of the study. This is consistent with previous studies which have shown that effects of chronically ICV infused NPY on fatty acids are transient (121;214).

While we show that long-term overexpression of NPY does have a phenotype, others reported that global overexpression of NPY from embryonic stage on did not result in changes in food intake and body weight gain in rats fed a normal chow (230;231;233). Compensatory changes during development or low NPY overexpression of NPY transgenic animals however may explain the lack of effect on body weight and food intake.

In summary, injection of rAAV-NPY in the PVN results in obesity, characterized not only by hyperphagia and increased fat storage, but also by elevated plasma leptin, insulin and adiponectin, decreased body temperature and hypoactivity. Although our earlier study with AAV-Agouti showed that rAAV-derived Agouti remained local (337), we cannot exclude the possibility that some of the viral-delivered NPY used in this study is transported to target sites of the PVN, explaining some of the described effects. The results suggest that NPY-induced obesity is not only the result of increased food intake, but also of increases in leptin and insulin that are partly food independent, and changes in locomotor activity and thermogenesis. This indicates that increased NPY expression in the PVN is sufficient to produce all parameters associated with obesity caused by increased NPY signaling in the brain, and strengthens the role of the PVN in regulation of energy balance by NPY.

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