Stress- and diet-induced fat gain is controlled by NPY in catecholaminergic neurons

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ABSTRACT

Neuropeptide Y (NPY) and noradrenaline are commonly co-expressed in sympathetic neurons. Both are key regulators of energy homeostasis and critical for stress-coping. However, little is known about the specific function of NPY in the catecholaminergic system in these regulations. Here we show that mice with NPY expression only in the noradrenergic and adrenergic cells of the catecholaminergic system (catNPY) exhibited exacerbated diet-induced obesity, lower body and brown adipose tissue temperatures compared to WT and NPY⁻/⁻ mice under a HFD. Furthermore, chronic stress increased adiposity and serum corticosterone level in WT but not NPY⁻/⁻ mice. Re-introducing NPY specifically to the catecholaminergic system in catNPY mice restored stress responsiveness associated with increased respiratory exchange ratio and decreased liver PACC to TACC ratio. These results demonstrate catecholaminergic NPY signalling is critical in mediating diet- and chronic stress-induced fat gain via effects on diet-induced thermogenesis and stress-induced increases in corticosterone levels and lipogenic capacity.

Keywords Neuropeptide Y; Catecholaminergic neurons; Stress; Energy homeostasis; Adiposity

1. INTRODUCTION

Neuropeptide Y (NPY) is 36-amino acid peptide that plays a critical role in the regulation of energy homeostasis [1]. It is well known for its potent orexigenic and obesogenic actions, with increased NPYergic activity being found in the brain under many obesifying conditions [1,2]. NPY has a wide distribution in the mammalian central and peripheral nervous system with highest expression in the hypothalamus, particularly the arcuate nucleus [3,4]. Hypothalamic NPY neurons are responsive to changes in circulating hormones such as leptin and insulin, and activated under the condition of negative energy balance to increase feeding and decrease energy expenditure [1]. In addition, NPY is also the predominant co-existing transmitter in catecholaminergic nerves. In the brain, NPY is extensively co-contained within brain stem catecholaminergic neurons such as the C1, C2 and C3 adrenergic cell groups and A1 noradrenergic cell group [5]. In the periphery, NPY is mainly co-stored and co-released with noradrenaline from sympathetic nerve terminals [6] and also produced by catecholamine-producing chromaffin cells of the adrenal medulla [7]. In contrast to the large amount of knowledge gained for hypothalamic NPY, limited information is available with regards to the specific role of NPY in the catecholaminergic neurons and their involvement in the regulation of energy homeostasis. Importantly, NPY in the peripheral sympathetic nerves has been shown to also be critical in the regulation of energy metabolism and adiposity, particularly under stressful conditions. Thus, it was shown that NPY release from noradrenergic sympathetic nerve terminus occurs primarily under conditions of prolonged sympathetic activation and chronic stress [8]. In addition, this stress-induced release of NPY has a local action in the adipose tissue to promote angiogenesis and adipogenesis that lead to an exacerbation of diet-induced obesity under these conditions [9]. However, many questions remain such as whether and how NPY in central catecholaminergic neurons participate the regulation of energy homeostasis, both under normal as well as under stressful conditions. In an effort to shed light on this question, Savontaus and colleagues generated a transgenic mouse model where NPY is over-expressed in the catecholaminergic system by using the dopamine-β-hydroxylase promoter to drive NPY over-expression. Mice with NPY over-expression in the catecholaminergic system develop obesity on a chow diet that is more pronounced in male mice, and exhibits an exacerbated obese phenotype in female mice on a high-fat diet [10,11]. These studies clearly demonstrated that increases in NPY activity in the catecholaminergic system promote fat gain. However, the potential interactions of NPY signalling in catecholaminergic and non-catecholaminergic neurons in this wild-type background catecholaminergic NPY over-expression mouse model may have limited the capacity to reveal the specific effects of catecholaminergic NPY on energy homeostasis. In addition, considering the critical role of catecholaminergic system in maintaining homeostasis during stress [12], the involvement of catecholaminergic...
NPY in energy metabolism under chronic stress would be an important and perhaps also an intrinsic aspect of NPY action warranting further investigation. To this end, we generated a transgenic mouse model that expresses NPY selectively only in the catecholaminergic system utilizing the same dopamine-β-hydroxylase gene promoter to drive NPY expression in otherwise NPY deficient mice. We then investigated parameters of energy homeostasis in wild type (WT), NPY deficient (NPY+/−), and catecholaminergic system-only NPY expressing (catNPY) mice under normal chow, a high-fat diet or chronic stress conditions. The NPY+/− mice reveal the impact of losing both catecholaminergic and non-catecholaminergic NPY signalling on various aspects of energy homeostasis, whereas catNPY mice will show how replenishing NPY specifically back to the catecholaminergic neurons modulate the changes induced by NPY global deletion, and whether some changes induced by NPY global deletion may be restored by replacing NPY back to catecholaminergic system.

2. MATERIALS AND METHODS

2.1. Animals

All research and animal care procedures were approved by the Garvan Institute/St. Vincent’s Hospital Animal Ethics Committee and were in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose. Male mice were used for all experiments. Mice were housed under conditions of controlled temperature (22 °C) and illumination (12:12 light cycle, lights on at 07:00 h). All mice were fed a normal chow diet (8% calories from fat, 21% calories from protein, 71% calories from carbohydrate, 2.6 kcal/g; Gordon’s Speciality Stock Feeds, Yanderra, NSW, Australia) and were given water ad libitum. Details of generation of the germline NPY-deficient mice (NPY−/−) and the transgenic mice overexpressing NPY under dopamine-β-hydroxylase promoter (OE-NPYβH) were published previously by Karl et al. [13] and Ruohonen et al. [10], respectively. The transgene construct contains a LacZ gene that allows the detection of transgene NPY expression by β-galactosidase staining [10]. Crossing OE-NPYβH mice onto the NPY−/− mice led to the generation of a mouse model with a specific NPY expression only in the noradrenergic and adrenergic nerves of the catecholaminergic system (catNPY). Littermate wild type (WT) and NPY+/− mice were studied with catNPY mice. All mice were on a mixed C57BL/6J-129/SvJ background.

2.2. Bacteria β-galactosidase expression

To determine the transgene expression, we stained for β-galactosidase whose expression is under the same promoter as the transgene. Coronary brain (25 μm) and adrenal (25 μm) fresh frozen sections were mounted on slides and processed for β-galactosidase histochemistry by 15-min fixation in LacZ Fix solution (0.2% glutaraldehyde, 0.1 M phosphate buffer pH 7.3, 2 mM MgCl2 and 5 mM EGTA), followed by 2 times of 5-min wash in LacZ Wash solution (0.1 M phosphate buffer pH 7.3, 2 mM MgCl2, 0.1% Na Decyoxicholate, and 0.02% NP40). Subsequently, slides were incubated in a β-galactosidase staining solution containing 1 mg/ml X-Gal (Promega, Alexandria, NSW, Australia), 0.03 mM spermidine, 0.2 mM NaCl, 5 mM K4Fe(CN)6 and 5 mM K3Fe(CN)6 in LacZ Wash solution overnight at 37 °C. Slides were fixed again in LacZ Fix solution for 10 min at 4 °C, followed by 2-min wash in LacZ Wash solution and 2-min wash in dH2O. Finally slides were counterstained with eosin (Eosin Y, Sigma-Aldrick, St Louis, MO, USA), dehydrated through serial concentrations of ethanol to xylene before coverslipping.

2.3. Chow and high-fat diet (HFD) studies

WT, NPY−/− and catNPY mice fed on chow were examined for body weight and body composition at 9 weeks of age. At 12 weeks of age, mice were examined for food intake. Briefly, mice were transferred from group housing on soft bedding to individual cages with paper towel bedding and allowed to acclimatize for 3 nights. Basal daily food intake was determined as the average of duplicate readings taken over 2 consecutive days. At 13 weeks of age, mice were examined for intraperitoneal glucose tolerance as described in Section 2.4. Body temperature and brown adipose tissue temperature were measured in mice at 14—15 weeks of age by using a non-invasive high-sensitivity infrared imaging [4,14] as described in Section 2.5. At 16 weeks of age, animals were culled between 13:00 and 16:00 h by cervical dislocation followed by decapitation. White adipose tissue depots (inguinal, epididymal, mesenteric and retro-peritoneal) were removed and weighed as published previously [15,16]. A subset of 9-week-old WT, NPY−/− and catNPY mice were fed on a high-fat diet (HFD, 43% calories from fat, 17% calories from protein and 20 MJ/kg; Specialty Feeds, Glen Forrest, WA, Australia) for 7 weeks. Parameters examined for chow-fed cohort described above were examined for HFD-fed cohorts at a similar age following the same procedures.

2.4. Intraperitoneal glucose tolerance test

Food was removed from cage hoppers at 08:30 h, and 6 h later a dose of glucose (1 g/kg body weight) was injected into the peritoneal cavity. Tail vein blood was collected at 0, 15, 30, 45, 60 and 90 min after the glucose bolus injection for the determination of glucose concentrations using a glucometer (Accu-Check Go, Roche) and insulin levels using a RIA kit from Millipore (Millipore, Billerica, MA, USA). Glucose tolerance curves for glucose and insulin are presented as absolute values. Area under the glucose or insulin concentration curves between 0 and 90 min after glucose ingestion were calculated, subtracting glucose or insulin concentrations prior to glucose ingestion.

2.5. Measurement of body and brown adipose tissue temperatures by infrared imaging

One day before the measurement, mice were shaved at interscapular and lumbar back regions under light isoflurane anaesthesia to expose the skin of these areas for temperature reading. Infrared imaging experiments were carried out on the following 2 consecutive days and readings from the 2 measurements were averaged. Briefly, a high-sensitivity infrared camera (ThermoCAM T640, FLIR, Danderyd, Sweden) was used. Sensitivity = 0.04 °C fixed on a tripod was placed at 90 cm above the freely moving mice to record the surface temperatures of the mice in a 30—60 s video. Thermo-frames that had mouse body naturally extended with both shaved skin regions vertical to camera lenses were extracted from the video. The hottest pixels of the lumbar back and interscapular regions from each extracted frames were obtained simultaneously and used as indicators of body and brown adipose tissue temperature, respectively.

2.6. Analysis of body composition

Upon completion of indirect calorimetry and physical activity measurements, animals were anesthetized with isoflurane and then scanned for whole-body lean and fat mass using Dual-energy X-ray absorptiometry (DEXA, Lunar Piximus2 mouse densitometer; GE Healthcare, Waukesha, WI, USA). The head and the tail were excluded from DEXA analyses.

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2.7 Cold stress study

Mice at 10 weeks of age were assigned to either non-stress group, or stress group where mice were exposed to cold stress 3 times per week for 10 weeks. This study ran across several cohorts and each cohort was comprised of all genotypes with both treatment groups in each genotype. Cold stress involved placing mice in 0.5 cm ice-cold water for 1 h as described previously [17]. At 19 weeks of age, we examined food intake, energy metabolism and physical activity in an oXyam system as described in Section 2.8. At 20 weeks of age, animals of different experimental groups were culled in alternate order between 13:00 and 16:00 h by cervical dislocation followed by decapitation and immediate blood collection. Plasma samples were stored at −20 °C until use. Corticosterone assay was performed using a Corticosterone Double Antibody RIA kit from MP Biomedicals (MP Biomedicals, LLC, Orangeburg, NY, USA). White adipose tissue depots (inguinal, epididymal, mesenteric and retroperitoneal) were removed and weighed. Liver tissue was snap frozen and stored at −80 °C till Western analysis as described in Section 2.9 was performed.

2.8 Examination of energy metabolism, respiratory exchange ratio and physical activity

Metabolic rate was measured by indirect calorimetry using an eight-chamber open-circuit calorimeter (Oxymax Series; Columbus Instruments, Columbus, OH, USA) as described previously [16]. Briefly, mice were housed individually in specially built Plexiglas cages (20.1 × 10.1 × 12.7 cm). Temperature was maintained at 22 °C with airflow of 0.6 l/min. Mice were singly housed for at least 3 days prior to transferring into plexiglass cages and were acclimatized to the cages for 24 h before recordings commenced. Mice were subsequently monitored in the system for 24 h. Pellet food was used when mice were in metabolic chambers, and excess amount food was given prior to the data collection so that no food needs to be re-introduced during the recording. Pellet food was weighed before and after the 24 h recording together with spillage collected at the bottom of the cage at the end of the run to calculate food intake. Oxygen consumption (VO₂) and carbon dioxide production (ICO₂) were measured every 27 min. The respiratory exchange ratio (RER) was calculated as the quotient of ICO₂/VO₂ with 100% carbohydrate oxidation giving a value of 1, and 100% fat oxidation giving value of 0.7 [18]. Energy expenditure (kcal heat produced) was calculated as Calorific Value (CV) × VO₂, where CV is 3.815 + 1.232 × RER [20]. Data for the 24-h monitoring period was averaged for 1-h intervals for energy expenditure and RER. Ambulatory activity of individually-housed mice was evaluated within the metabolic chambers using an OPTO-M3 sensor system (Columbus Instruments, Columbus, OH, USA), whereby ambulatory counts were a record of consecutive adjacent photo-beam breaks. Cumulative ambulatory counts of X and Y directions were recorded every minute and summed for 1-h intervals.

2.9 Western analysis

Western analysis was performed on liver samples following procedures described previously [16] in order to determine protein levels of acetyl-CoA carboxylase (ACC) levels, the key enzymes involved in fatty acid synthesis and overall energy metabolism in lipogenic tissues. Briefly, liver samples were resuspended in radioimmunoprecipitation assay buffer (PBS, pH 7.5; 1% nonidet NP-40; 0.5% sodium deoxycholate; and 0.1% SDS), supplemented with protease and phosphatase inhibitors (10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mmol/l Na3VO4, and 10 mmol/l NaF) and solubilized for 2 h at 4 °C. Equal amounts of tissue lysate (20 μg protein) were resolved by SDS-PAGE and immunoblotted with antibodies against phospho-Ser79 acetyl-CoA carboxylase (pACC), total acetyl-CoA carboxylase (IACC) (Cell Signalling Technology, Danvers, MA, USA), carnitine palmitoyltransferase-1 (CPT-1) (Alpha Diagnostic Int., San Antonio, Texas, USA), fatty acid synthase (FAS) (Cell Signalling Technology, Danvers, MA, USA), glyceroldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signalling Technology, Danvers, MA, USA). Immunob labelled bands were quantitated by densitometry and pACC to IACC protein level ratio was used as indication of ACC activity. The pACC to IACC ratio and the expression of CPT-1 and FAS relative to GAPDH from stressed mice were presented as percentage of that of non-stressed control mice.

2.10 In situ hybridization

In situ hybridization was performed on brain sections from wild type and catNPY mice to examine NPY expression following procedures described previously [4]. Briefly, coronal brain sections (25 μm) were cut on a cryostat and thaw-mounted on Superfrost slides (Menzel-Glaser, Braunschweig, Germany). Matching sections from same coronal brain level were assayed together using DNA oligonucleotides complementary to mouse NPY (5′-GAGGGCTGTCACACAG CCCCATTCGGTTGTACATGC-3′). The oligonucleotides were labelled with [35S]thio-dATP (Amersham Pharmacia) using terminal deoxynucleotidyltransferase (Roche, Mannheim, Germany) and hybridized with sections. Hybridization signals were visualized by exposing sections to βmax film (Kodak, Rochester, NY, USA) for 5–10 days. The film was then scanned to obtain the digital images of the hybridization signals on the sections.

2.11 Statistical analyses

All data are expressed as means ± SEM. Differences among different groups were assessed by ANOVA or repeated-measures ANOVA, followed by Tukey’s post hoc test if significant difference was found among genotypes. RER and physical activity over the continuous 24-h period were averaged for the whole 24-h period, as well as for the light and dark periods. Comparisons of energy expenditure (kcal/h) and respiratory exchange ratio were carried out by analysis of covariance (ANCOVA) with lean body mass (g) and energy balance (kcal/day) as the covariate, respectively. Energy balance was calculated as energy intake subtracted by energy expenditure. The adjusted means of energy expenditure at a common lean mass, and respiratory exchange ratio at a common energy balance for the comparisons were generated by ANCOVA. Statistical analyses were performed with SPSS for Mac OS X, version 16.0.1 (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as P < 0.05.

3. RESULTS

3.1 Generation of catecholaminergic NPY transgenic mouse model

In order to generate a transgenic mouse model with specific NPY expression only in catecholaminergic neurons, we crossed our germline NPY knockout line [13] with a transgenic line expressing NPY under the control of the mouse Dopamine β-Hydroxylase promoter [10]. Double heterozygous (NPYtg/–; NPY−/−) mice were then crossed again with either germline NPY−/− or NPY−/– mice to obtain the NPY−/−;NPYtg/− (catNPY) line as well as NPY−/−;NPY−/− and WT littermates, respectively, which served as controls. The transgene construct also contains a LacZ gene allowing for the determination of transgene expression by β-galactosidase staining. Figure 1 shows blue staining specifically in neurons of the locus coeruleus nuclei (LC) (Figure 1A and B), nucleus of solitary tract region in the brain stem (Figure 1C and D) and the medulla of the adrenal gland (Figure 1E and F).
3.2. NPY in catecholaminergic neurons promotes weight gain and fat gain in mice on HFD

WT, NPY−/− and catNPY mice when fed a chow diet have comparable growth curves from 9 to 16 weeks of age (Figure 2A). Body composition analysis by DEXA scan on 9-weeks-old mice showed no significant differences in whole body lean and fat masses among genotypes (Figure 2D and E). However, at 16 weeks of age, whereas whole body lean mass was comparable among genotypes (Figure 2D), whole body fat mass showed a significant increase in NPY−/− mice compared to that in WT or catNPY mice (Figure 2E). In addition, tissue analysis at the end of the study also showed significant increases in the summed weights of dissected white adipose tissue depots in NPY−/− mice compared to the other two genotypes (Figure 2F), consistent with a late-onset obesity in NPY−/− mice. Interestingly, body composition parameters measured in catNPY mice, i.e. whole body lean and fat masses and weights of dissected white adipose tissues, were all comparable to those in WT (Figure 2D–F), suggesting that the late-onset obesity in NPY−/− mice may be elicited by lack of NPY in the catecholaminergic neurons and replenishing NPY back to these neurons reverses this phenotype.

In order to investigate the role NPY in catecholaminergic neurons in the regulation of body weight and body composition under obesogenic conditions, we challenged mice with a high-fat diet (HFD) from 9 weeks of age for 7 weeks. All mice on HFD showed steady weight gain with a clear elevation in body weight in catNPY mice compared to WT and NPY−/− mice, significantly so from the 4th week on HFD onwards (Figure 2B and C). Body composition analysis showed significant increases in whole body fat mass but no significant changes in whole body lean mass in HFD-fed versus chow-fed groups (Figure 2D and E), suggesting HFD feeding primarily promotes weight gain by increasing fat mass. Importantly, whole body fat mass was significantly increased in HFD-fed catNPY compared to WT and NPY−/− mice (Figure 2E), although the summed weight of dissected white adipose tissue depots did not significantly differ among genotypes (Figure 2F). These results suggest that NPY in the catecholaminergic neurons under the condition of an obesogenic diet has a stimulatory role to promote weight gain that is primarily contributed by increases in fat mass.

We also examined glucose tolerance in WT, NPY−/− and catNPY mice under both chow and HFD conditions to investigate whether catecholaminergic neuron derived NPY is involved in the regulation of glucose homeostasis. On chow, WT, NPY−/− and catNPY mice exhibited comparable basal blood glucose and serum insulin levels, and similar area under the glucose and insulin curves after the glucose injections (Figure 2G and I). After 4-weeks of HFD, whereas not significantly altered in basal glucose levels, NPY−/− and catNPY mice showed improved glucose tolerance evidenced by reduced area under the glucose curves after the glucose bolus compared to WT mice on the same diet (Figure 2H and J). Basal insulin level and area under

Figure 1: Expression of dopamine-β-hydroxylase-LacZ transgene by β-galactosidase staining in neurons of the locus coeruleus nuclei (LC) around the 4th ventricle (A and B), nucleus of solitary tract region (NTS) in the brain stem (C and D; AP: area postrema; cc: central canal) and the medulla of the adrenal gland (E and F) of catNPY mice. Scale bar = 100 or 50 μm as indicated. Autoradiograms of Neuropeptide Y (NPY) mRNA expression by in situ hybridization in the LC and the arcuate nucleus of the hypothalamus (Arc) around the 3rd ventricle (3V) of wild type (G and I) and catNPY (H and J) mice.
the insulin curves after glucose injection were similar among HFD-fed WT, NPY−/− and catNPY mice (Figure 2K and L), suggesting the improved glucose tolerance in NPY−/− and catNPY mice on HFD may be due to enhanced insulin action. These results show that whereas NPY signalling contributes to HFD-induced impairment in glucose tolerance, the equally improved glucose tolerance and similar insulin response to glucose challenge between HFD-fed NPY−/− and catNPY mice suggest the effect of NPY on glucose homeostasis on HFD may be mediated by NPY in the non-catecholaminergic neurons.

3.3. NPY in the catecholaminergic neurons reduces body temperature and thermogenesis under HFD condition

To investigate the role of NPY in the catecholaminergic neurons in the regulation of energy homeostasis, we firstly examined energy intake.

Figure 2: Role of catecholaminergic NPY signalling in the regulation of energy metabolism and glucose homeostasis under chow and high-fat diet (HFD) conditions. Male wild type (WT), NPY−/− and catNPY at 9 weeks of age were fed on either chow or HFD for 7 weeks during which body weight (A, B and C) was monitored weekly. Body composition, i.e. whole body lean (D) and fat (E) masses were determined by dual-energy X-ray absorptiometry in mice at 9 weeks and 16 weeks of age. Individual white adipose tissue depots (namely the inguinal, epididymal, mesenteric, and retroperitoneal depot) were dissected, weighed at tissue collection with summed weights presented in F. Blood glucose levels (G and H) and area under the resultant glucose curve (I), serum insulin levels (J and K) and area under the resultant insulin curve (L) during a 90-min intraperitoneal glucose tolerance test (1 g/kg) in mice fed on either chow or a HFD. Caloric intake was determined at either chow or HFD conditions (M). Body temperature (N) at the lumbar back region and interscapular brown adipose tissue temperature (O) were determined by infrared imaging. Data are means ± SEM of 8–12 mice per group. *P < 0.05 versus WT mice on the same diet. †P < 0.05 versus NPY−/− mice on the same diet. ‡P < 0.05 versus chow-fed group of the same genotype.
Mice on HFD had significantly higher energy intake than their chow-fed counterparts (Figure 2M). However, there was no significant genotype effect on energy intake in WT, NPY−/− and catNPY mice fed on a same diet (Figure 2M), suggesting NPY in the catecholaminergic neurons may not play a key role in the regulation of feeding. We next determined body temperature and brown adipose tissue temperature by infrared imaging as an indication of thermogenesis. Body temperatures measured at the back region were not significantly different among genotypes, although there was a trend towards an increase in NPY−/− and catNPY mice on a chow diet (Figure 2N). However, brown adipose tissue temperature was significantly increased in NPY−/− and catNPY compared to WT mice (Figure 2O), suggesting an inhibitory role of NPY on brown adipose tissue thermogenesis and that this is likely mediated by NPY originating from non-catecholaminergic neurons.

HFD-fed WT mice at 5 weeks after commencement of the diet showed significantly higher body and brown adipose tissue temperatures than those fed on chow (Figure 2N and O), consistent with the known effect of HFD on thermoregulation [21,22]. In contrast, NPY−/− mice fed a HFD exhibited no increase in body or brown adipose tissue temperatures compared to their chow-fed counterparts (Figure 2N and O), suggesting that diet-induced thermogenesis requires NPY. In addition, there was no significant difference in body or brown adipose tissue temperatures between HFD-fed NPY−/− and WT mice, which is in contrast to findings under chow conditions (Figure 2N and O). Strikingly, catNPY mice on HFD showed significantly reduced body and brown adipose tissue temperatures compared to their chow-fed counterparts (Figure 2N and O), as well as compared to HFD-fed WT mice (Figure 2N and O). There results highlight an important and specific role of NPY in the catecholaminergic neurons under the HFD diet condition to reduce body temperature and brown adipose tissue thermogenesis, which is likely a key contributor to the exacerbate diet-induced weight gain observed in the catNPY mice.

3.4. Catecholaminergic NPY mediates stress-induced fat gain
Both NPY and the central catecholaminergic system are critically involved in stress-coping, with the later responding to an acute stressor to mediate the fight-or-flight response and the former playing a major role under chronic stress. It is therefore conceivable that NPY in the catecholaminergic neurons has an important and intrinsic role under stressful condition to regulate energy homeostasis. To investigate this we challenged mice with a 10-week cold stress protocol from 10 weeks of age and examined the hormonal and metabolic impacts of stress in WT, NPY−/− and catNPY mice. Since corticosterone is a key stress hormone having profound effects on metabolism and contributing to obesity as seen in Cushing’s syndrome, we assessed the level of corticosterone in serum samples of stressed and non-stressed mice where truck blood samples were collected between 13:00 and 16:00 h. We found that whereas the 10-weeks stress paradigm increased circulating corticosterone levels in WT mice (Figure 3A), it failed to do so in NPY−/− mice (Figure 3A). Importantly, re-introduction of NPY into catecholaminergic neurons restored this effect evidenced by the significant increase in serum corticosterone levels in stressed compared to non-stressed catNPY mice (Figure 3A). Investigating the effect of 10 weeks of stress treatment on body weight showed that WT mice weighed significantly more than their non-stressed counterparts (Figure 3B). This chronic stress-induced increase in body weight in WT mice was associated with significantly increased weights of the individual and summed white adipose tissue depots as well as whole body fat mass (Figure 3C–E), demonstrating chronic repeated cold stress promotes fat storage. Interestingly, the stress-induced significant weight gain and fat gain in WT mice were absent in NPY−/− mice (Figure 3F–I). Body weight in stressed NPY−/− mice in fact showed a trend towards a decrease compared to the non-stressed control group (Figure 3F).

Moreover, catNPY mice exhibited a significant increase in fat gain in response to chronic stress similarly to that observed in WT mice (Figure 3K–M), demonstrating a restoration of the effect stress has on adiposity by re-introduction of NPY specifically into catecholaminergic neurons. Considering the impact of corticosterone on metabolism and its contribution to the development of obesity, the parallel responses in corticosterone levels and adiposity to stress in WT, NPY−/− and catNPY mice suggest that increased corticosterone is a main contributor to the stress-induced fat gain observed in WT and catNPY mice. On the other hand, re-introduction of NPY specifically into catecholaminergic neurons does not restore the effect of stress to promote weight gain. In the contrary, body weight of stressed catNPY mice was in fact significantly lower compared to that of the non-stressed group (Figure 3J). This suggests that the effect of NPY on body weight and non-fat tissue masses during stress is likely dependent on NPY from non-catecholaminergic neurons.

3.5. NPY in catecholaminergic and non-catecholaminergic neurons has different roles in the regulation of energy expenditure and physical activity during chronic stress
To investigate the role of catecholaminergic NPY in the regulation of energy balance during chronic stress, we examined spontaneous food intake and energy expenditure by indirect calorimetry after 9 weeks of repeated cold stress. Importantly, daily food intake was significantly reduced by stress in WT, NPY−/− and catNPY mice (Figure 4A–C). Energy expenditure in stressed WT mice was significantly decreased during the dark period (21:00–06:00 h) compared to that in the non-stressed group (Figure 4D) and this may be an important contributor to the weight gain and fat gain (Figure 3B–E) observed in these mice. This stress-induced decrease in energy expenditure in WT mice (Figure 4D) was not seen in NPY−/− mice (Figure 4E), indicating the reduction in energy expenditure during stress is mediated by NPY. However, this effect of NPY on energy expenditure during stress may not involve catecholaminergic NPY, since the reduction in energy expenditure by stress was not observed in catNPY mice (Figure 4F). Physical activity was comparable between stressed and non-stressed WT mice (Figure 4G), whereas stressed NPY−/− mice exhibited a lower activity than that seen in the non-stressed group, significantly so during the light phase (Figure 4H). This reduction in physical activity by stress in NPY−/− mice was abolished by re-introduction of NPY into catecholaminergic neurons (Figure 4I), suggesting catecholaminergic NPY up-regulates physical activity during stress. Taken together, these results demonstrate that NPY not only plays important roles in regulating energy expenditure and physical activity during stress, but also that NPY derived from catecholaminergic or non-catecholaminergic neurons has different roles in these regulations, i.e. non-catecholaminergic NPY is primarily involved in regulating energy expenditure, whereas catecholaminergic NPY is more critical for regulating physical activity during stress.

3.6. Catecholaminergic NPY regulates oxidative fuel metabolism during chronic stress
In order to get more mechanistic insight on the observed alterations in body weight and body composition we also investigated aspects of fuel selection using the respiratory exchange ratio (RER) by indirect calorimetry after 9-week repeated cold stress exposure. RER was not significantly altered by chronic stress in WT or NPY−/− mice (Figure 4J
Interestingly however, stressed catNPY mice showed a significant increase in RER during the dark phase, indicative of increased lipogenesis and/or decreased lipid oxidation, compared to the non-stressed group (Figure 4L). Since stressed mice showed reduced food intake compared to their genotype-matched non-stress counterparts (Figure 4A–C), and since energy balance itself impacts on RER [23], we have employed energy balance as a covariate to control for this confounding factor for RER, whereby energy balance was calculated as daily energy intake subtracted by 24-h energy expenditure [23]. RER adjusted for energy balance showed similar pattern, i.e. no significant effects of stress on RER in WT or NPY−/− mice but significantly increased RER in catNPY mice (Figure 4M–O), suggesting that the increased RER in stressed catNPY mice is a direct effect of stress rather than secondary to stress-induced changes in food intake (Figure 4C). Importantly, the increase in RER by stress in catNPY mice suggest that during chronic stress catecholaminergic NPY may direct energy towards fat formation and storage instead of fat usage and ultimately promote fat gain. In addition, a lack of effect of stress on RER in NPY−/− mice suggests NPY in non-catecholaminergic neurons may have opposing effects on oxidative fuel metabolism to those of catecholaminergic NPY during stress.

To further analyse the mechanisms underlying the increase in RER by stress in catNPY mice, we determined the liver protein levels of acetyl-CoA carboxylase (ACC), the key enzyme that has a strong impact on
fatty acid synthesis as well as overall energy metabolism in lipogenic tissues including the liver. Since the phosphorylated ACC (pACC) is the inactive form, the pACC to total ACC (tACC) ratio was used to indicate the ACC activity with a lower ratio indicating a higher activity. Stress had no significant effects on ACC activity in WT or NPY<sup>−/−</sup> mice (Figure 4P and S), but significantly reduced the pACC to tACC ratio in catNPY mice (Figure 4P), suggesting a higher lipogenic capacity in stressed-catNPY mice. Consistent with a reduced pACC to tACC ratio, fatty acid synthase (FAS) in stressed catNPY mice showed a 1.3-fold increase compared to the non-stressed group (Figure 4O), whereas no such change was seen in WT or NPY<sup>−/−</sup> mice (Figure 4Q). These results suggest that an enhanced lipogenic capacity in stressed catNPY mice may contribute to the increased RER in these mice (Figure 4L and O). Since a reduced lipid oxidation may also result in an increased RER, we determined the liver protein levels of carnitine palmitoyltransferase-1 (CPT-1), the mitochondrial transmembrane enzyme controlling entry of fatty acid into mitochondria and the rate-limiting enzyme for fatty acid oxidation [24,25]. There was no significant difference in liver CPT-1 protein expression between non-stressed and stressed WT, NPY<sup>−/−</sup> or catNPY mice (Figure 4R and S), indicating that stress has little influence on fatty acid oxidative capacity in these mice. In summary, these results suggest that NPY in the catecholaminergic neurons plays an important role in the regulation of oxidative fuel metabolism during chronic stress and may mediate stress-induced fat gain via increasing liver lipogenic capacity.

4. DISCUSSION

In this study we demonstrate that NPY produced by catecholaminergic neurons is critical in the regulation of energy homeostasis particularly under conditions of chronic stress. Using a mouse model with specific NPY expression only in the noradrenergic and adrenergic cells of the catecholaminergic neuronal system, we show that NPY promotes weight gain and fat gain under the condition of an obesogenic diet and this effect is likely achieved by inhibiting diet-induced thermogenesis. Furthermore, we show that NPY in the catecholaminergic system promotes fat gain during chronic stress and that this is likely due to increased circulating corticosterone levels, a potential direct action of NPY released from catecholaminergic neurons on peripheral tissues and an increased respiratory exchange ratio. The underlying mechanism for the increased respiratory exchange ratio can be attributed to an increased lipogenic capacity, with stress significantly reducing the liver pACC to tACC ratio, indicative of an increased ACC activity, and FAS levels in catNPY, but not WT or NPY<sup>−/−</sup> mice. Food intake however, seems not to involve the catecholaminergic NPY system, since there was no genotype effect on feeding at basal chow, HFD feeding or chronic stress conditions. Finally, our study show that NPY signalling in the non-catecholaminergic neurons may play an important role in the regulation of glucose homeostasis in that the HFD-induced impairment in glucose tolerance seen in WT mice was similarly diminished in both NPY<sup>−/−</sup> and catNPY mice.
Results from this study show that NPY signalling in both catecholaminergic and non-catecholaminergic neurons plays an important role in the regulation of brown adipose tissue thermogenesis. The higher brown adipose tissue temperature in NPY−/− versus WT mice suggests that NPY may exert a tonic inhibition on brown adipose tissue activity at basal condition. This tonic inhibition by NPY on brown adipose tissue activity appears coming from NPY signalling in non-catecholaminergic neurons, since replacing NPY back to catecholaminergic neurons of NPY−/− mice had no effect on the increase in brown adipose tissue temperature due to NPY global deletion. This finding is in keeping with the recent report that NPY in the arcuate nucleus of the hypothalamus plays a critical role in the control of brown adipose tissue function [4]. Interestingly, under the condition of an obesogenic diet, whereas WT mice showed significantly increased body and brown adipose tissue temperatures, consistent with a diet-induced thermogenesis that primarily takes place in the brown adipose tissue [21,22], no such changes were observed in NPY−/− mice, suggesting a necessity of NPY signalling in this process. Considering the basal tonic inhibition by NPY signalling on brown adipose tissue function, a reduction in this tonic inhibition would lead to an enhanced brown adipose tissue activity. Indeed, a reduction by 45% in NPY expression has been shown in the arcuate nucleus in mice after a short term of HFD feeding [26]. Intriguingly, catNPY mice with NPY expressed only in the catecholaminergic system showed significant reduction in body and brown adipose tissue temperatures under the obesogenic diet compared to their chow-fed counterparts. This suggests that the catecholaminergic NPY signalling may be activated under the HFD condition to exert an inhibition on brown adipose tissue thermogenesis. Since diet-induced adaptive thermogenesis does occur in WT mice, the inhibition on brown adipose tissue thermogenesis by catecholaminergic NPY signalling on HFD may have been overwritten by the stimulatory effect from decreased non-catecholaminergic NPY signalling. It is interesting to note that the exacerbated diet-induced obesity was not observed in male mice that overexpress NPY in the catecholaminergic system on a WT background [11], suggesting that concurrent NPY activity in the non-catecholaminergic neurons in the transgenic mouse model [11] may have masked the specific action of catecholaminergic NPY signalling on parameters involved in energy homeostasis such as brown adipose tissue thermogenesis. Our model now demonstrates that NPY signalling in the catecholaminergic and non-catecholaminergic systems have important but different roles in the control of brown adipose tissue thermogenesis. Whereas NPY signalling in the non-catecholaminergic neurons may exert a tonic inhibition on brown adipose tissue function, NPY signalling in the catecholaminergic system is activated upon HFD feeding to inhibit brown adipose tissue adaptive thermogenesis leading to exacerbation of diet-induced obesity.

Stress is one of the key environment factors affecting metabolism and has been increasingly linked to obesity [27,28]. In this study, we used repeated cold stress since this stress paradigm has been shown to have profound effects on metabolism leading to exacerbated diet-induced obesity in mice [9,17]. The concomitant increases in adiposity and serum corticosterone level in stressed WT mice shows an activation of hypothalamo-pituitary-adrenal (HPA) axis and is in keeping with many other reports suggesting corticosterone being a link between stress and obesity [29—32]. Indeed, clinical conditions characterized by high cortisol levels, as seen in patients with Cushing’s or Prader—Willi syndrome, are also associated with abdominal obesity [33,34]. In contrast, abolishing corticosterone action in experimental animals by removing adrenal glands or administering glucocorticoid receptor inhibitor reduce body weight and adiposity [17,32,35,36]. Importantly, the finding that re-introducing NPY specifically into the catecholaminergic system on otherwise NPY deficient mice restores stress-induced fat gain and increases serum corticosterone level that are missing in NPY−/− mice demonstrates a prominent action of catecholaminergic NPY signalling in mediating stress-induced effects on energy metabolism and HPA axis activation. Consistent with this anatomical studies lend support to a role of catecholaminergic NPY signalling in the regulation of HPA axis, with the hypophysiotropic corticotropin-releasing-hormone (CRH) neurons in the paraventricular nucleus of the hypothalamus (PVN) receiving dense NPY innervations [37,38], and two thirds of the NPY innervations to CRH neurons originate from brain stem catecholaminergic neurons [37].

In our stress study, we used indirect calorimetry to investigate effects of chronic stress on parameters involved in energy metabolism in different genotypes. It is noteworthy that in contrast to the comparable caloric intake among genotypes measured in their home cage the caloric intake of non-stressed NPY−/− mice when measured in the metabolic chamber is lower than that of non-stressed WT or catNPY mice. A reason for this could be the different environment with the metabolic chambers being smaller and having a grid floor and a fan on the lid, represents a novel/environment to the mice compared to their home cage condition. NPY is an important stress-coping molecule and NPY−/− mice show an anxiogenic phenotype [13]. Although all mice were allowed to acclimatize for 24 h prior recording, a higher susceptibility to novel environmental stress may explain the reduced food intake in non-stressed NPY−/− compared to other non-stressed genotypes. Indeed, a recent study reported similar results, i.e. similar food intake between WT and NPY−/− mice at basal non-stress condition, and reduced food intake in NPY−/− but not WT mice when exposed to novel environment stress [39]. Importantly, in the stress study, both treatment groups went through the same procedure revealing the effect of chronic stress on energy metabolism in each genotype, and the subsequent comparisons of chronic stress-induced responses cross genotypes allow us to dissect the genotype differences in responses to chronic stress with minimum interference from the impacts of the anxiogenic phenotype due to NPY deletion. Using this strategy, we demonstrate that the restoration of stress-induced fat gain in catNPY mice was associated with a significant increase in respiratory exchange ratio, increased liver FAS levels and reduced liver pACC to tACC ratio, indicative of an increased ACC activity and an increased liver lipogenic capacity [40,41], whereas fatty acid oxidation pathways may not be greatly impacted on evidenced by the unaltered liver CPT-1 levels in response to stress in all genotypes. Catecholaminergic NPY signalling may influence liver lipogenic capacity via central as well as peripheral mechanisms. In keeping with the later hypothesis, mice with Y1 receptor knockdown in the periphery exhibited reduced respiratory exchange ratio and increased lipid oxidative capacity and exhibit ameliorated diet-induced obesity [16]. In addition, a direct action of NPY released from sympathetic nerve terminus in white adipose tissue to promote adipogenesis [17] may also contribute to the increase in respiratory exchange ratio. On the other hand, the lack of change in respiratory exchange ratio by stress in WT or NPY−/− mice suggests NPY derived from non-catecholaminergic neurons may have opposing actions on oxidative fuel metabolism compared to that of catecholaminergic NPY. Finally, our results show that energy expenditure is significantly reduced by stress in WT mice. Whereas this reduction in energy expenditure is consistent with reports of reduced thyroid activity by repeated stress [42,43] and may contribute to the stress-induced weight gain in WT mice, lack of changes in energy expenditure by stress in NPY−/− or catNPY mice suggest this effect of stress may not be elicited by NPY signalling in catecholaminergic neurons.
Our data also show that lack of NPY signalling confer protection against diet-induced impairment in glucose tolerance likely via improving insulin action in insulin-sensitive tissues. This beneficial effect of NPY deletion on glucose homeostasis during HFD feeding occurs without significant effects on diet-induced obesity, suggesting a direct control of NPY on glucose metabolism. Indeed, studies have shown that NPY has direct central effects to reduce hepatic insulin sensitivity thereby increasing endogenous glucose production [44–46]. In addition, central NPY induces hepatic insulin resistance via activation of sympathetic output to the liver [47]. Our finding that catNPY and NPYβ3- mice had comparable glucose tolerance on HFD extends previous reports to suggest that NPY in the non-catecholaminergic system is likely to be involved for the regulation of glucose metabolism. For instance, NPY neurons in the arcuate nucleus have been shown to have projection to pre-autonomic neurons in the PVN and regulate sympathetic output which would in turn control hepatic glucose production [44–47].

In summary, this work utilized a novel mouse model with NPY specifically expressed only in the adrenergic and noradrenergic cells of the catecholaminergic system to dissect out the specific role of catecholaminergic NPY signalling in the regulation of energy and glucose homeostasis. Our results show that catecholaminergic NPY activity has significant impact on body weight and adiposity under both an obesogenic diet and stressful conditions. Importantly, catecholaminergic NPY signalling is critical in the regulation of brown adipose tissue adaptive thermogenesis under obesogenic conditions and controls stress-induced increases in corticosterone levels and lipogenic capacity under chronic stress.

ACKNOWLEDGEMENTS

We thank the staff of the Garvan Institute Biological Testing Facility for facilitation of these experiments. This research was supported by the National Health and Medical Research Council, Australia, a Postgraduate Scholarship to JL, a Career Development Award to PAB, and a Senior Research Fellowship to HH. We thank Felicity Forsyth for secretarial assistance.

CONFLICT OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this paper and there has been no significant financial support for this work that could have influenced its outcome.

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