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Ambient temperature modulates the effects of the Prader-Willi syndrome candidate gene *Snord116* on energy homeostasis

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ABSTRACT

Germline deletion of the Prader-Willi syndrome (PWS) candidate gene Snord116 in mice leads to some classical symptoms of human PWS, notably reductions in body weight, linear growth and bone mass. However, Snord116 deficient mice (*Snord116*$^{-/-}$) do not develop an obese phenotype despite their increased food intake and the underlying mechanism for that is unknown. We tested the phenotypes of germline *Snord116*$^{-/-}$ as well as neuropeptide Y (NPY) neuron specific *Snord116*$^{lox/lox}$/*NPY*$^{cre/+}$ mice at 30 °C, the thermoneutral temperature of mice, and compared these to previous reports studies conducted at normal room temperature. *Snord116*$^{-/-}$ mice at 30 °C still weighed less than wild type but had increased body weight gain. Importantly, food intake and energy expenditure were no longer different at 30 °C, and the reduced bone mass and nasal-anal length observed in *Snord116*$^{-/-}$ mice at room temperature were also normalized. Mechanistically, the thermoneutral condition led to the correction of the mRNA expression of NPY and pro-opiomelanocortin (POMC), which were both previously observed to be significantly up-regulated at room temperature. Importantly, almost identical phenotypes and NPY/POMC mRNA expression alterations were also observed in *Snord116*$^{lox/lox}$/*NPY*$^{cre/+}$ mice, which lack the Snord116 gene only in NPY neurons. These data illustrate that mild cold stress is a critical factor preventing the development of obesity in *Snord116*$^{-/-}$ mice via the NPY system. Our study highlights that the function of Snord116 in the hypothalamus may be to enhance energy expenditure, likely via the NPY system, and also indicates that Snord116 function in mice is strongly dependent on environmental conditions such as cold exposure.

1. Introduction

Prader-Willi syndrome (PWS), a leading cause of genetic obesity, is a neurodevelopmental disorder arising from genomic imprinting (Butler, 1990; Holm et al., 1993). PWS in infancy is characterised by low birth weight, muscular hypotonia and failure to thrive (Dunn, 1968). The second phase of the disease has an onset at around 2–6 years of age and involves the development of hyperphagia and extreme food-seeking behavior (Dunn, 1968). When not strictly controlled, this can lead to morbid obesity in childhood and adulthood, with associated metabolic and cardiovascular co-morbidities (Kobielowa et al., 1971). There is also evidence that temperature regulation is impaired in suffers of PWS with a study in children showing significant changes in thermoregulation in PWS affected individuals compared to siblings or other normal controls (Williams et al., 1994).

PWS arises from the loss of expression of a critical genetic region on chromosome 15 (q11.2-13), a region that is largely conserved on chromosome 7 in mice. The PWS locus is large and encompasses a number of known protein-coding genes as well as non-coding genes, including six small nucleolar RNAs (snoRNAs): SNORD64, SNORD107, SNORD108, SNORD109 (two copies), SNORD116 (29 copies) and SNORD115 (48 copies) (Cavaille et al., 2000; Gallagher et al., 2002). Absence of the SNORD116 gene cluster has been reported in individuals with microdeletions within this locus (Ding et al., 2008; Duker et al., 2010; Gallagher et al., 2002; Kim et al., 2012; Schule et al., 2005). These individuals exhibit PWS-like features, including hypotonia and hyperphagia, identifying SNORD116 as a potential target for intervention (Ding et al., 2008; Duker et al., 2010; Gallagher et al., 2002; Kim et al., 2012; Schule et al., 2005).

The high degree of homology between SNORD116 in humans and Snord116 in mice allows the development of mutant mouse models with which to investigate this gene cluster. Several Snord116 knockout

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mouse (*Snord116*$^{-/-}$) models have been generated (reviewed in (Bervini and Herzog, 2013)). Germline *Snord116*$^{-/-}$ mice kept at room temperature (22 °C) have lower birth weight than wild type (WT), and this smaller size persists throughout the reported monitoring periods of 32 weeks (Khor et al., 2016; Qi et al., 2016). Additionally, these mice have increased food intake, reduced bone mineral density (BMD) and bone mineral content (BMC), higher energy expenditure and lower physical activity than WT counterparts (Khor et al., 2016; Zieba et al., 2015). Of these effects, it is not clear which are direct effects of Snord116 gene deletion and which, if any, are secondary adaptive responses. For instance, do *Snord116*$^{-/-}$ mice gain less body weight than controls because they are expending more energy than they can compensate for, or is energy expenditure increased as an adaptive response to greater heat loss due to a higher body surface to body mass ratio? To address this question, we aimed to assess the physiological effects of Snord116 deletion independent of compensatory metabolic adaptations by eliminating the need for cold-environment-induced thermogenesis by testing *Snord116*$^{-/-}$ and WT mice under thermoneutral conditions (30 °C).

2. Materials and methods

2.1. Animals

Garvan Institute/St. Vincent's Hospital Animal Ethics Committee has approved animal care for this study and the research procedures 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 ± 1 °C as room temperature and 30 ± 1 °C for thermoneutrality) and illumination (12-hour light-dark cycle, with lights on at 07:00 am). Animals had ad libitum access to a normal chow diet (8% calories from fat, 21% calories from protein, 71% calories from carbohydrate, and 2.6 kcal/g; Gordon's Specialty Stock Feeds, Yanderra, NSW, Australia). Water was unrestrictedly available.

2.2. Phenotype studies

A multi-measure metabolic profiling procedure was implemented as previously described (Qi et al., 2016), including the studies of body weight, body composition (results from dual-energy X-ray absorptiometry (DXA) and tissue collection), energy intake and expenditure, physical activity, and respiratory exchange ratio (RER). Briefly, body weight was measured weekly from the beginning of housing in a room at thermoneutral temperature. DXA was performed in vivo for the estimation of body composition (lean mass, fat mass, BMD and BMC, followed by tissue collection. The examined tissue were interscapular brown adipose tissue (BAT) and white adipose tissue (WAT) from the inguinal, epididymal, retroperitoneal and mesenteric deposits, as well as gonads, spleen, pancreas, kidney, liver and heart, which were excised and weighed. The weights were expressed as a percentage of body weight. To investigate energy intake, 24-hour spontaneous food intake was measured in Nalgene metabolic cages (Medtex, Notting Hill, VIC), after 3 days of single housing with powdered diet and 24 h in the metabolic cages. The cages are specially designed for determining food intake, water consumption, faeces and urine production. Food intake was monitored over three consecutive days, averaged as 24-hour intake and presented as calorie intake as a percentage of body weight of individual mice. Body weight was monitored daily during the process. Energy expenditure, physical activity and RER were tested in an eight-chamber open-circuit Oxymax system (Oxymax Series; Columbus Instruments, Columbus, OH, USA) after 24 h acclimation.

2.3. In situ hybridisation and densitometry

The protocol for in situ hybridisation was previously described (Sainsbury et al., 2002). Briefly, DNA oligonucleotides of mouse neuropeptide Y (NPY; 5′-GAGGGTCAGTCCACACAGCCCCATTCGCTT GTTACCTAGCAT-3′) and mouse pro-opiomelanocortin (POMC; 5′-TGGCTGCTCTCCAGGCACCAGCTCCACACATCTATGGAGG-3′) were incubated at 37 °C and labelled with [35S] thio-dATP (Amersham Pharmacia Biotech, Buckinghamshire, UK), respectively. Fresh-frozen brain was cut into sections at 30 μm thickness and those spanning the hypothalamic arcuate nuclei (Arc) were selected according to the mouse brain atlas. After pre-treatment in 2% PFA, the selected sections were washed and dehydrated, followed by hybridisation with the specific probes at 42 °C over night and post-hybridization wash. The sections were then exposed to Biomax MR films (MKBT5368, Carestream Health, Inc. New York, USA), in which silver grain densities of labelled mRNAs were compared between WT mice and the genetic mutants using ImageJ software (US National Institutes of Health). The probe-labelled sections were then developed in photo emulsion and fixer, to visualize the staining for photographic purpose.

2.4. Biochemical analyses

The serum concentrations of insulin-like growth factor 1 (IGF-1) were analysed by enzyme-linked immunosorbent assay (ALPCO Diagnostics, Salem, NH, USA).

2.5. Statistical analyses

All data are presented as means ± SEM. *t*-Tests were used to analyse the difference when the comparisons were implemented on two groups with one time point. One-way ANOVA with Bonferroni post-hoc tests were used to compare the data when there were more than two groups. Two-way ANOVA with Bonferroni post-hoc tests was used to analyse the differences for data with multiple time points, namely body weight, physical activity and RER. ANCOVA (with lean mass as a co-variant) was used to analyse energy expenditure data. 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

To assess which aspects of the phenotype resulting from Snord116 deletion are primary genetic effects and which are adaptive responses, a cohort of *Snord116*$^{-/-}$ mice and their controls were kept under thermoneutral conditions over a period of 16 weeks, beginning at four weeks of age. These groups were compared to corresponding groups of *Snord116*$^{-/-}$ and WT mice housed at 22 °C. A multi-measure metabolic profiling procedure was performed as previously described (Khor et al., 2016; Qi et al., 2016).

Our previous studies showed that *Snord116*$^{-/-}$ mice exhibited lower body weight and smaller body size than WT mice throughout the monitoring period at 22 °C. In the current study, at 30 °C *Snord116*$^{-/-}$ mice remained slightly lighter than WT with no difference in nasal-anal length, indicating that reduced body weight is indeed a true effect of the absence of Snord116 expression (Fig. 1A–D).

Another prominent characteristic of *Snord116*$^{-/-}$ mice is reduced BMC and BMD at 22 °C. In the present study conducted at 30 °C, this effect disappeared, with no difference in either parameter between genotypes (Fig. 1E and F). In addition, there was no difference between *Snord116*$^{-/-}$ and WT mice with respect to fat and lean mass (Fig. 1G and H). The weight of dissected BAT, as a percentage of body weight, was also significantly increased in knockout versus WT mice at 30 °C compared to those at 22 °C, which is likely due to the lower body weight of knockouts at this temperature (Fig. 1I). Similarly, the hyperphagia of *Snord116*$^{-/-}$ mice previously seen at 22 °C was no longer observed due to the significant reduced energy intake of *Snord116*$^{-/-}$ mice at 30 °C, with no difference in daily food intake between *Snord116*$^{-/-}$ mice and WT under thermoneutral conditions (Fig. 2A). This suggests that the previous hyperphagia was at least in part related to comparatively

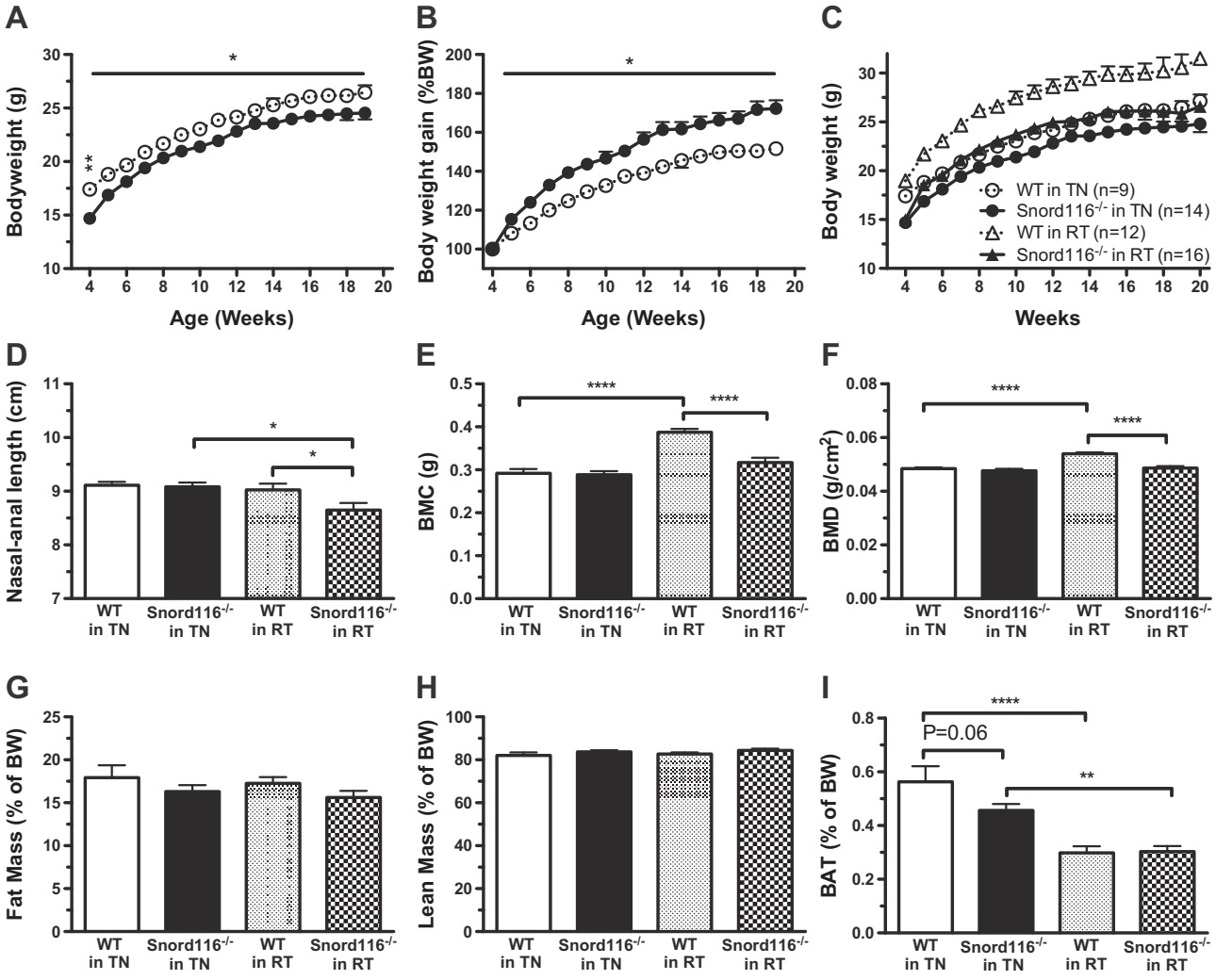

Fig. 1. The effect of thermoneutrality on the metabolic phenotypes of male *Snord116*$^{-/-}$ mice (n = 14) vs. control mice (n = 9). A) Absolute weekly body weight (BW) in thermoneutral conditions (TN) from 4 to 20 weeks of age; B) growth rate as a percentage of BW (%BW) at 4 weeks of age; C) absolute weekly BW of *Snord116*$^{-/-}$ mice vs. wild type at 30 °C and 22 °C (room temperature (RT)); D) nasal-anal length; E) bone mineral content (BMC); F) bone mineral density (BMD); G) fat mass (%BW); H) lean mass (%BW); and I) brown adipose tissue (BAT) mass (%BW). (* $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$).

smaller *Snord116*$^{-/-}$ mice needing to augment energy intake in proportion with cold-driven thermogenesis to maintain homeostasis, and that at 30 °C hyperphagia is no longer metabolically necessary. The energy expenditure phenotype in these animals supported this observation: despite *Snord116*$^{-/-}$ mice having higher energy expenditure than WT at 22 °C, this difference was no longer present at 30 °C (Fig. 2B). Physical activity also followed the same pattern, being not significantly different in *Snord116*$^{-/-}$ mice relative to WT at 30 °C (Fig. 2C). Interestingly, fuel source preference was altered in Snord116$^{-/-}$ mice under thermoneutral conditions, with Snord116$^{-/-}$ mice using more fat than WT mice during the dark phase, as indicated by a reduction in RER at this time (Fig. 2D).

These data show that while low body weight in *Snord116*$^{-/-}$ mice persists despite differences in environmental temperature, much of the observed phenotype of *Snord116*$^{-/-}$ mice at 22 °C—namely low BMD and BMC, high energy expenditure and low physical activity—was no longer discernible at 30 °C. Thus it is likely that growth impairment is a primary effect of *Snord116* deletion, with the other changes arising in response to these mice having high body surface to body weight ratio in a cold environment.

One of the likely main drivers of the increased feeding in this model of Snord116 deficiency is the upregulated expression of the feeding regulatory neuropeptides NPY and POMC in the hypothalamus under room temperature conditions (Qi et al., 2016). However, when exposed to a 30 °C environment, dysregulated expression of these neuropeptides was no longer observed (Fig. 2E–J). Considering that NPY expression is strongly influenced by stress and that low temperature represents a mild stress situation, alleviating this stress could have reduced NPY levels and, through this mechanism, helped to normalise some of the altered metabolic parameters caused by the Snord116 deletion. Furthermore, no difference in serum IGF-1 levels was seen between the knockout mice and their respective control at 30 °C (Fig. 2K), which is again consistent with the corrected body composition – notably lean mass, bone mass and nasal-anal length – under thermoneutral conditions.

Our previous study (Qi et al., 2016) also reported a mouse model with Snord116 gene selectively deleted in NPY neurons (*Snord116*$^{lox/lox}$/*NPY*$^{cre/+}$). These mice had an almost identical phenotype to that observed in the global *Snord116*$^{-/-}$ mice under conditions of room temperature, suggesting that the regulation of energy homeostasis by Snord116 involves the NPY system. To investigate whether the impact of temperature is also mediated by the NPY system, we also exposed *Snord116*$^{lox/lox}$/*NPY*$^{cre/+}$ mice to thermoneutral environment. Importantly, *Snord116*$^{lox/lox}$/*NPY*$^{cre/+}$ mice gradually increased their body weight and reached the same level of body weight as control mice after two weeks housed at 30 °C (Fig. 3A–C). Furthermore, these mice also showed the same phenotype as *Snord116*$^{-/-}$ mice at 30 °C,

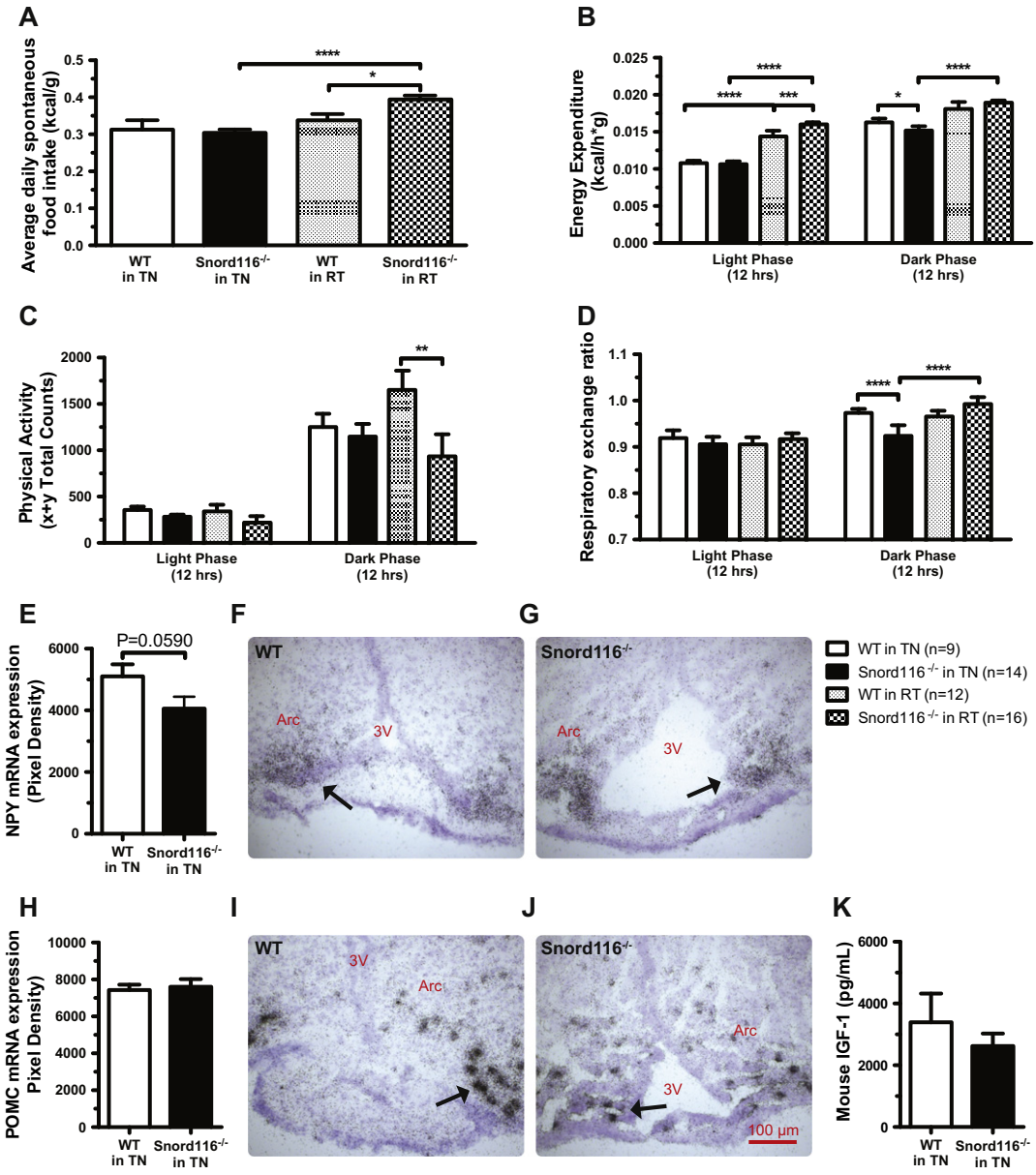

Fig. 2. Energy intake and expenditure of male *Snord116*$^{-/-}$ mice (n = 14) vs. control mice (n = 9) on the effect of thermoneutrality (TN) and room temperature (RT) and the altered pattern of neuropeptide expression. A) Spontaneous 24-hour food intake as a percentage of body weight (%BW); B) energy expenditure in light and dark phases; C) physical activity; D) respiratory exchange ratio; E) mRNA expression of neuropeptide Y (NPY) in the arcuate nuclei of the hypothalamus (Arc) of *Snord116*$^{-/-}$ mice vs. wild type; F and G) microscopic photographs of NPY mRNA expression (indicated by black arrows) in the Arc; H) mRNA expression of proopiomelanocortin (POMC) in the Arc; I and J) microscopic photographs of POMC mRNA expression (indicated by black arrows) in the Arc; (3 V: the third ventricle); and K) serum concentrations of insulin-like growth factor 1 (IGF-1). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$).

with no difference from controls in nasal-anal length, BMC, BMD, fat or lean mass, BAT mass (Fig. 3D–I), and spontaneous food intake or energy expenditure (Fig. 4A, B). Interestingly, however, those mice had increased physical activity and used more fat than WT as a fuel source in both the light and dark phases (Fig. 4C and D). Similar to what was observed in *Snord116*$^{-/-}$ mice, mRNA expression of NPY and POMC and serum concentration of IGF-1 were unchanged between *Snord116*$^{lox/lox}$/*NPY*$^{cre/+}$ and WT mice under thermoneutral conditions (Fig. 4E–K). Taken together, the majority of the observed phenotypes related to energy homeostasis and metabolism in *Snord116*$^{-/-}$ and *Snord116*$^{lox/lox}$/*NPY*$^{cre/+}$ mice were no longer present in these mice when housed at 30 °C, indicating a critical role for ambient temperature in the modulation of the function of Snord116 through the NPY system.

4. Discussion

This study provides in vivo evidence that ambient temperature has a substantial influence on the metabolic phenotype of mice. While previous reports showed that *Snord116*$^{-/-}$ mice housed at 22 °C exhibited low body weight, hyperphagia and changes in energy expenditure and physical activity compared to WT, we show that most of these features—with the exception of low body weight—were rescued when mice were housed at 30 °C, the thermoneutral temperature for rodents. This suggests that a direct effect of Snord116 deletion is impaired growth, with the perturbations in energy balance observed at 22 °C arising in response to the smaller size at an ambient temperature well below the thermoneutral threshold.

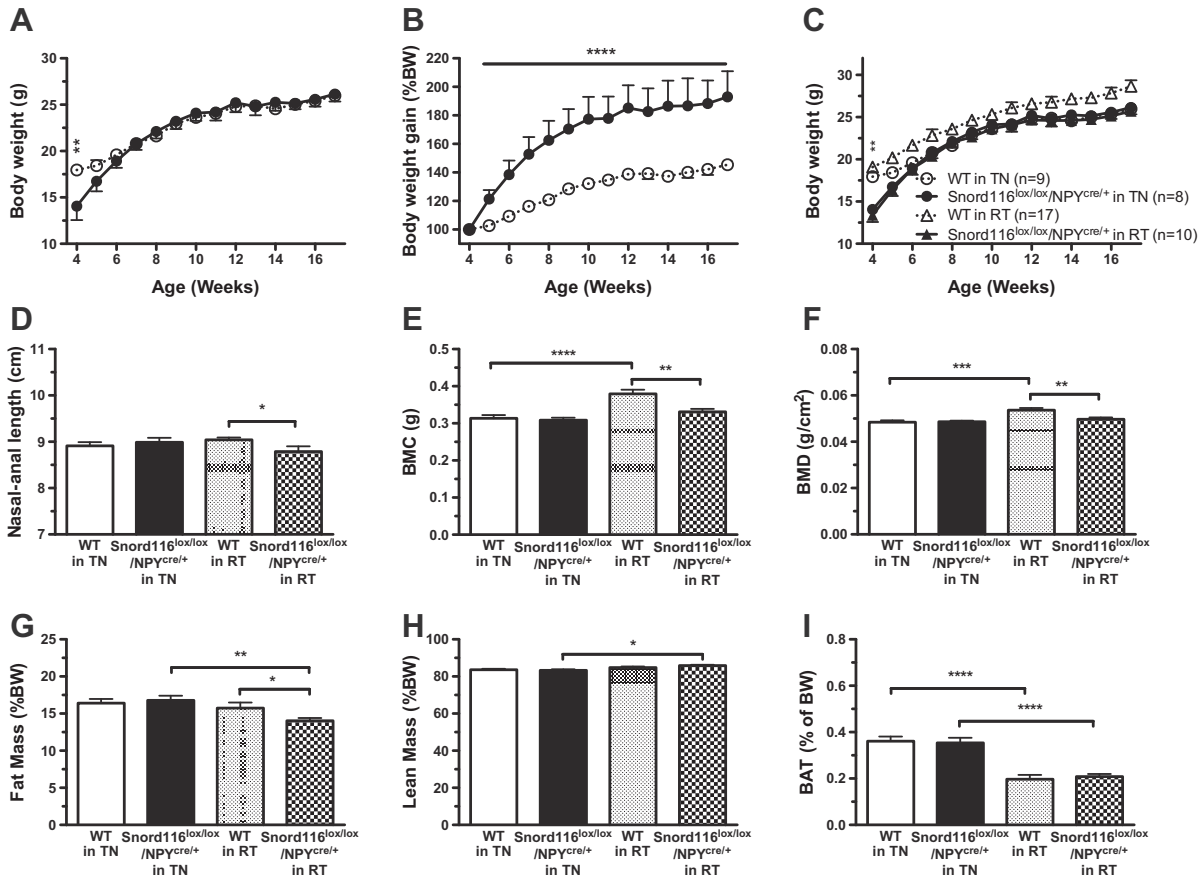

Fig. 3. The effect of thermoneutrality on the metabolic phenotypes of male *Snord116*$^{lox/lox}$/*NPY*$^{cre/+}$ (n = 8) vs. control mice (n = 9). A) Absolute weekly body weight (BW) in thermoneutral conditions (TN) from 4 to 20 weeks of age; B) body weight gain normalised to BW at 4 weeks of age; C) absolute weekly BW of *Snord116*$^{lox/lox}$/*NPY*$^{cre/+}$ mice vs. wild type at 30 °C and 22 °C (room temperature (RT)); D) nasal-anal length; E) bone mineral content (BMC); F) bone mineral density (BMD); G) fat mass (%BW); H) lean mass (%BW); and I) brown adipose tissue (BAT) mass (%BW). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$).

Mouse studies are commonly conducted at 22 °C, a condition that in terms of mouse physiology has to be considered as a mild cold stress (Karp, 2012). Smaller animals, whose surface to body weight ratio is higher than that of larger animals, lose comparatively more heat in a colder environment; therefore compensatory changes in energy balance to cover this additional expenditure are likely to take place. In *Snord116*$^{-/-}$ mice, this appears to occur via increased food intake and energy expenditure, and reduced physical activity. Conducting future rodent studies involving experimental groups likely to have lower (or higher) body weight than their associated control groups under thermoneutral conditions could therefore help to identify genetic effects without confounding compensatory factors.

Thermoneutrality is able to reduce the consumption of food in mice allowing them to still maintain their body weight while it increases the weight of BAT. However, the expression of uncoupling protein 1 (UCP-1) under this condition is actually decreased (Cui et al., 2016). This indicates that the function of BAT is not determined by its weight alone. Similar to this observation, we observed increased BAT weight under thermoneutral condition in both wild type and Snord116 knockout mice. More precise tests to understand the non-shivering heat produce, which is not the focus of this study, would be needed to investigate the function of BAT under thermoneutrality further. Thermoneutrality also altered the usage of energy source; it decreased the use of carbohydrates and increases that of fat. Interestingly, the lack of Snord116 gene exaggerates the usage of fat, which is most likely through decreased action of the NPY system.

We previously identified that dysfunction of NPY system is implicated in the critical role of Snord116 in the development of PWS-like phenotypes (Qi et al., 2016). Moreover, we demonstrated that, mechanistically, this dysfunction is likely caused by disrupted regulation of the two major populations of neurons that control appetite and energy homeostasis: the orexigenic NPY/agouti-related peptide (AgRP) neurons, and the anorexigenic POMC/cocaine and amphetamine regulated transcript (CART) neurons, which are located in the Arc. Under mild cold conditions (22 °C), the levels of NPY mRNA are significantly increased in the Snord116 deletion mice, but the normally observed concomitant down-regulation in POMC mRNA (reviewed in (Sohn, 2015)) did not occur, suggesting a lack of functional response in the *Snord116*$^{-/-}$ mice. However, one has to keep in mind that several peptide components can be produced from the POMC precursor due to alternative processing, and as such it is still possible that normal feeding control via the melanocortin system could be maintained through compensatory adjustments in producing alpha-melanocyte-stimulating hormone. Importantly, however, the deregulation of NPY mRNA in the Snord116 deficient mice is no longer observed when mice are held under thermoneutral conditions. This suggests that both the expression of NPY and the function of Snord116 are dependent on environmental temperature. This is also consistent with the findings in the NPY neuron-specific *Snord116*$^{lox/lox}$/*NPY*$^{cre/+}$ mice, where the normalisation in NPY mRNA expression is observed under thermoneutral conditions.

A human study (Williams et al., 1994) in patients with PWS showed a difference in body temperature related phenomena between PWS children and sibs of PWS patients or matched well children (but not other delayed children), suggesting that ambient temperature could have an influence on the clinical features of the disorder. This hypothesis is supported by current study's finding that lack of Snord116 function had a less severe effect when environmental temperature was higher.

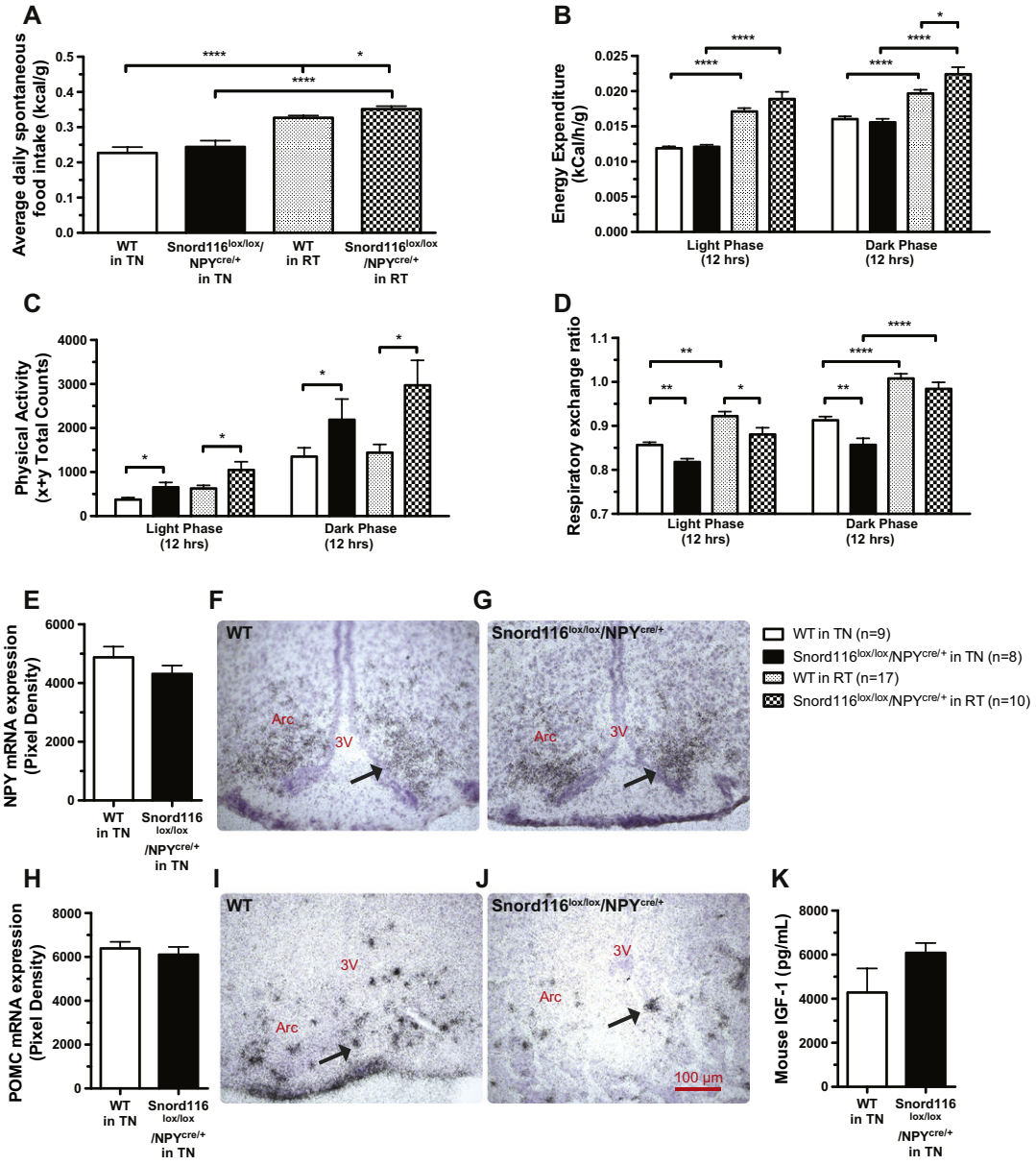

Fig. 4. Energy metabolism of male *Snord116*$^{lox/lox}$/*NPY*$^{cre/+}$ (n = 8) vs. control mice (n = 9) on the effect of thermoneutrality (TN) and room temperature (RT) and their neuropeptide expression under thermoneutrality. A) Spontaneous 24-hour food intake as a percentage of body weight (%BW); B) energy expenditure in light and dark phases; C) physical activity; D) respiratory exchange ratio; E) mRNA expression of neuropeptide Y (NPY) in the arcuate nuclei of the hypothalamus (Arc) of *Snord116*$^{lox/lox}$/*NPY*$^{cre/+}$ *mice* vs. wildtype; F and G) microscopic photographs of NPY mRNA expression (indicated by black arrows) in the Arc; H) mRNA expression of proopiomelanocortin (POMC) in the Arc; I and J) microscopic photographs of POMC mRNA expression (indicated by black arrows) in the Arc; (3 V: the third ventricle) and K) serum concentrations of insulin-like growth factor 1 (IGF-1). (* P < 0.05, ** P < 0.01,and **** P < 0.0001).

Thereafter, in daily care of PWS subjects, some adjustment of ambient temperature should be explored as a relatively painless part of therapy.

In conclusion, by utilising an environment specifically designed to be thermoneutral for mice, this study identified that several phenotypic features previously associated with the germline genetic deletion of Snord116 are likely to be secondary adaptations to low body weight rather than intrinsic genetic effects. In addition, conclusions drawn from several obese mouse models previously examined under mild cold stress conditions may need to be reassessed after thermoneutral studies.

Conflict of interest

The authors have no conflicts of interest to declare.

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