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Hypothalamus specific re-introduction of Snord116 into otherwise Snord116 deficient mice increased energy expenditure

Qi[1], Y., Purtell[2], L., Fu[1], M., Zhang[1], L., Zolotukhin[3], S., Campbell[2], L. & Herzog[1], H.

[1]Neuroscience Division, [2]Diabetes Division, Garvan Institute of Medical Research, Sydney AUSTRALIA

[3]Department of Pediatrics, College of Medicine, Center for Smell and Taste, University of Florida, Gainesville, Florida 32610, USA

Corresponding Author:

Dr. Yue Qi

Neuroscience Division, Garvan Institute of Medical Research

384 Victoria Street, Darlinghurst NSW 2010

Sydney, Australia

Tel 61 2 9295 8301; Email: y.qi@garvan.org.au

Abstract

The Snord116 gene cluster has been recognized as a critical contributor to the Prader-Willi Syndrome (PWS) with mice lacking Snord116 displaying many classical PWS phenotypes including low postnatal body weight, reduced bone mass and increased food intake. However, these mice do not develop obesity due to increased energy expenditure. To understand the physiological function of Snord116 better and potentially rescue the altered

metabolism of Snord116$^{-/-}$ mice, we used an adeno-associated viral (AAV) approach to reintroduce the Snord116 gene product into the hypothalamus in Snord116$^{-/-}$ mice at different ages. Our results show that mid-hypothalamic re-introduction of Snord116 in 6-week old Snord116$^{-/-}$ mice leads to significantly reduced body weight and weight gain, associated with elevated energy expenditure. Importantly, when the intervention targets other areas such as the anterior region of the hypothalamus or the reintroduction occurs in older mice the positive effects on energy expenditure are diminished. These data indicate that the metabolic symptoms of PWS develop gradually and the Snord116 gene plays a critical role during this process. Furthermore, when we investigated the consequences of Snord116 re-introduction under conditions of thermo-neutrality where the mild cold stress influences are avoided, we also observed a significant increase in energy expenditure. In conclusion, the rescue of mid-hypothalamic Snord116 deficiency in young Snord116 germline deletion mice increases energy expenditure, providing fundamental information contributing to potential virus-mediated genetic therapy in PWS.

Introduction

Prader-Willi syndrome (PWS) is the most common human genetic obesity syndrome caused by mutations within an imprinted locus on chromosome 15 (q11.2-13)(1, 2). It is a lifelong disease and there is no cure. The PWS critical region contains a number of known protein-coding genes as well as six non-coding small nucleolar RNAs (snoRNAs) (3, 4). Previous studies using gene deletion models have investigated the contributions of the coding genes to the PWS phenotype but none could replicate the obesity causing effects seen in PWS subjects. Human case studies focus our attention on the Snord116 gene cluster, one of the snoRNAs, as micro-deletion of the SNORD116 gene cluster leads to a classical PWS phenotype including hypotonia and hyperphagia, consistent with a major role of this snoRNA in the development of PWS (3, 5-8).

Clinically, PWS infant suffer from low birth weight, muscular hypotonia and failure to thrive (9), followed by developing hyperphagia and extreme food-seeking behavior, which starts around 2-6 years of age (9). These characteristics can lead to morbid obesity, as well as metabolic morbidities (10), if they are not controlled. In the progress of the investigation, several PWS knockout mouse models have been generated (reviewed in (11)). Among them, germline Snord116$^{-/-}$ mice showed a significantly lower birth weight, which persists into late adulthood (12, 13), with increased food intake, reduced bone mineral density (BMD) and bone mineral content (BMC), higher energy expenditure and lower physical activity than WT counterparts (13, 14). Interestingly, when raised at thermo-neural temperature (30°C), these mice can normalise some of these phenotypes (15), indicating that mild stress caused by lower temperature can influence the development of these phenotypes.

In human and mice, SNORD116 is expressed in the central nervous system and particularly high in the hypothalamus area known to be critical in controlling appetite (16). This is also the area where the major controller of appetite neuropeptide Y (NPY) is located. Considering that NPY expression is strongly influenced by stress, alleviating this stress will reduce NPY levels and through this mechanism may help to normalise some of the altered metabolic parameters caused by the Snord116 deletion (15). Consistent with that the upregulated expression of the NPY and proopiomelanocortin (POMC) in the hypothalamus under room temperature conditions is one of the likely main drivers of the increased feeding in this model of Snord116 deficiency(12). This is also consistent with the phenotypic study showing that Snord116 specific deletion only in NPY neurons develops the identical metabolic alteration as seen in the germline deletion mice (15). Mechanically, this is likely due to the disrupted communication between NPY and POMC neurons that control appetite and energy homeostasis regulation (12). Furthermore, the significantly altered gene set of 'feeding behavior' and up-regulated expressions of appetite-regulating neuropeptides such as orexin and melanin concentrating hormone (MCH) in the lateral hypothalamic area were also observed in Snord116$^{-/-}$ mice (12), caused by the direct effect or secondary effect of Snord116 deletion.

Building on these findings, we hypothesised that the re-introduction of *Snord116* into the hypothalamus in Snord116-deficient mice could reverse the observed phenotype in this model. To test this hypothesis, we generated adenovirus associated virus (AAV)-Snord116 viral particles and injected them into the hypothalamus of Snord116$^{-/-}$ mice. Furthermore, we aimed to identify primary and secondary effects of replacement of this gene by conducting this arm of the study both at normal room temperature and under thermo-neutral conditions.

Materials and Methods

Animals

Research procedures and animal care for this study were carried out following the approvement from 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. Genetic mutant mice and their littermates (wild type mice) were housed at either 22±1°C as room temperature or 30±1°C for thermo-neutrality. Standard illumination (12-hour light-dark cycle, with lights on at 07:00 am) was applied to these mice with *ad libitum* access to water and 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).

Phenotype studies

For the purpose of comparison between this study and our previous published results on Snord116$^{-/-}$ mice, the same multi-measure metabolic profiling procedure in the different set of mice was used (12). Briefly, weekly body weight was measured from the beginning of neuro-surgery at the age of either 6 or 10 weeks old. Nasal-anal length was measured at the age of 12, 15 and 20 weeks old. The body composition was examined at the same age by *in vivo* dual energy X-ray absorptiometry (DXA; lean mass, fat mass, BMD and BMC), as well as by tissues collection of fat mass (interscapular brown adipose tissue (BAT), white adipose tissue (WAT) from the inguinal, epididymal, retroperitoneal and mesenteric deposits) and organs (gonads, spleen, pancreas, kidney, liver and heart). For tissue collection, these tissues were all excised and weighed, and expressed as a percentage of body weight.

To investigate energy intake, Nalgene metabolic cages (Medtex, Notting Hill, VIC) were used, which are designed for recording food intake, water consumption, faeces and urine production. 3-day single housed mice with powdered diet were acclimatised in these cages for 24 hours, followed by measurement of those parameters over three consecutive days. The average of 24-hour spontaneous food intake was expressed as calorie intake as a percentage of body weight of individual mice. Body weight was also monitored daily during the process. Fasting-induced food intake and relative body weight were also measured in the same cage at the time points of 1, 2, 4, 8, 24 and 48 hours after 18 hours fast. Mice were restrained and the rectal probe was inserted to measure rectal temperature at 9:00 am in the morning for three consecutive days. The average reading was presented as basal body temperature. Oxymax system (Oxymax Series; Columbus Instruments, Columbus, OH, USA) was used to measure the energy expenditure, physical activity and respiratory exchange ratio (RER) over a period of 4 days 11 weeks after the AAV Snord116 re-introduction. For insulin and glucose tolerance test (ITT and GTT), mice that had been fasted for 5 hours or 22 hours respectively were injected intraperitoneally with either insulin (0.75 IU/kg) (Novo Nordisk Pharmaceuticals, Baulkham Hills, NSW, Australia) or D-glucose (1.0g/kg) (Astra Zeneca, North Ryde, NSW, Australia), respectively. Glucose levels in blood collected from the tip of the tail were examined using an Accu-chek® Go glucometer (Roche, Dee Why, NSW, Australia) at 0, 15, 30, 60 and 90 minutes post injection.

***In situ* hybridisation and densitometry**

Briefly, the probe of DNA oligonucleotides of mouse Snord116 (5'-GTTCAGCTTTTCCAAGGAATGTTTGACTGGGAATCATCATAGATCC-3') with [35S] thio-dATP (Amersham Pharmacia Biotech, Buckinghamshire, UK) was used to incubate the pre-washed 30 µm fresh-frozen brain sections crossing the hypothalamus over night. The brain sections were then treated with post-rinse and the radioactive signals were detected by Biomax® MR film (Sigma-aldrich, New York, USA). The silver grain densities of labelled mRNAs were counterstained and the microscopic photos were taken by Carl Zeiss microscope. The more detailed protocol for *in situ* hybridisation can be found in (17).

Biochemical analyses

The serum insulin-like growth factor 1 (IGF-1) was detected by Enzyme-linked immunosorbent assay (ALPCO Diagnostics, Salem, NH, USA).

AAV-SNORD116 virus production

A 94 bp SNORD116-encoding sequence was inserted clockwise downstream of the CBA-GFP-poly (A) expression cassette in the AAV vector pTR-UF11 under the control of the human U6 promoter (18). The transgene cassette was pseudotyped into an AAV9 capsid using helper plasmid pAAV2/9 (Dr. J. Wilson,UPenn) and the viral vector was produced as described previously (19). After iodixanol step gradient purification, the vector was dialysed and concentrated to obtain the final stock of $5x10^{12}$ drp/ml.

AAV mediated Snord116 expression in the hypothalamus

The anaesthetised mouse was placed on a stereotaxic frame with ear bars (David Kopf Instruments, Tujunga, CA, USA). A 0.5 cm excision along the midline was made rostrally to lambda. A 2µl Hamilton MicroliterTM injection syringe attached to a Micro4 Micro Syringe Injection Unit (World Precision Instruments Inc., Sarasota, USA) was adjusted over the desired injection site. A 25G stainless needle was used to drill a hole in the skull to expose the brain, and the injection syringe was then lowered to the injection site in the hypothalamus. The co-ordinates used for injection were calculated by the size of the mouse brain according to the maps in The Mouse Brain in Stereotaxic Coordinates (20) (2 mm rostrally to the interaural line, 1 mm off the midline and 5.75 mm deep from the surface of the brain). 0.5 µl AAV-Snord116 in saline was injected over 5 min into the targeted hypothalamic area. The syringe was left in place for 10 min after completion of the injection to avoid reflux along the needle before being slowly taken out. The injection of the virus was made bilaterally. One suture was used to close the wound. The animal was kept on a heating pad until waking up and then it was transferred to single housing.

Statistical analyses

Experimental results were presented as means ± SEM. SPSS for Mac OS X version 16.0.1 (SPSS Inc, Chicago, IL, USA) was used to analyse the statistical significance. A significant difference can be concluded when $P<0.05$.

Results:

In an attempt to reverse the impaired growth in Snord116$^{-/-}$ mice we employed a viral reintroduction strategy. An AAV-virus expressing either Snord116 or an empty vector was injected bilaterally into the mid-region of the hypothalamus, including the lateral area of the hypothalamus, the ventromedial nuclei, and the arcuate nuclei (Arc), of Snord116$^{-/-}$ mice at

6 weeks of age. Animals then underwent a multi-measure metabolic profiling procedure at 22°C as previously described (12, 13). The successful reintroduction of Snord116 in germline deletion mice (Fig 1C and D), compared to the matched brain sections of the WT (Fig 1A) and the empty-vector injected mice (Fig 1B), was confirmed by *in situ* hybridisation.

Re-introduction of Snord116 into the hypothalamus of otherwise Snord116 deficient mice resulted in lower body weight gain over the monitoring period of 14 weeks compared to the empty virus injected control mice (Fig 1E and F). Similarly, nasal-anal length of the Snord116 gene rescued mice was shorter after 14 weeks post-injection (Fig 1G). Assessment of body composition showed that there was no difference in normalized fat and lean mass throughout the monitoring period (Fig 1H and I), but BMC and BMD were significantly reduced at 14 weeks post-injection (Fig 1J and K). No difference in spontaneous or fasting-induced food intake was observed between the treatment groups when measured 10 weeks after Snord116 re-introduction (Fig 1L and M). Importantly, while rectal temperature was not different (Fig 1 N) and physical activity during the dark phase was reduced (Fig 1P), energy expenditure was significantly higher in the Snord116-injected group (Fig 1O), consistent with the observed lower body weight. Analysis of RER indicated that Snord116-injected mice utilise more carbohydrate as an energy fuel source than controls (Fig 1Q). In addition, a significantly increased blood glucose level in Snord116-injected mice during GTT was observed with no difference in blood glucose level during ITT (Fig 1R and S). There also was no difference in serum IGF-1 levels between Snord116-injected mice and their controls (Fig 1T).

To validate the effectiveness of the timing and location of the viral intervention, we also performed Snord116 injection into the anterior region of the hypothalamus of 6-week-old Snord116$^{-/-}$ mice (Fig. 2) as well as into the mid-region of the hypothalamus when the mice were 10 weeks old (Fig. 3). Interestingly, these two models showed no alteration in the observed phenotype, indicating the critical role of timing and intervening regions when attempting such gene therapy approaches.

The experiments with bilateral injection of Snord116-containing virus into the mid-region of the hypothalamus of Snord116$^{-/-}$ mice at 6 weeks of age were also repeated at the thermo-neutral temperature of 30°C to assess the primary effects of Snord116 reintroduction without compensatory thermogenetic influences. Under these conditions, body weight was not different throughout the monitoring period between the Snord116 and empty vector-injected mice (Fig 4A and B). All other metabolic parameters were similarly unchanged between groups (Fig 4C-G). However, a trend of increased food intake in Snord116-induced mice and a significant increased fasting-induced food intake at 48-hour time point were observed (Fig 4H and I). Interestingly, energy expenditure remained significantly elevated in the Snord116-injected group compared to the empty vector-injected controls at 30°C (Fig 4J). No difference in physical activity was seen between groups, and Snord116-injected mice

used more carbohydrate than controls in the dark phase, as indicated by higher RER at this time (Fig 4K and L). There was also no difference in GTT and ITT results between groups (Fig 4M and N).

Collectively, Snord116-injected Snord116$^{-/-}$ mice displayed differences from empty vector-injected controls at 22°C also when the intervention was conducted at 30°C. In contrast to the germline model, however, it was the increased energy expenditure in both light and dark phases that remained at 30°C when other effects were normalised. These suggest that Snord116 is critical for hypothalamic functions that control energy expenditure during early development and hypothalamic viral Snord116 reintroduction can help to elevate energy expenditure which would be beneficial for sufferers of PWS.

Discussion:

Currently there is no cure for PWS. Strategies to prevent the development of obesity (and its metabolic and cardiovascular complications) are limited to restricting access to food and promoting physical activity. While much of the research into appetite-suppressing pharmaceutical therapies in PWS has focused on hormone mimetics and centrally acting drugs, the potential for a future gene therapy approach has remained, until now, largely unexplored. To our knowledge the present study represents the first attempt to correct the phenotype caused by the deletion of the snoRNA116 gene cluster using a viral gene therapy approach. We demonstrate that re-introduction of Snord116 in Snord116$^{-/-}$ mice leads to increased energy expenditure when administered specifically in the mid-hypothalamus at an early age, suggesting that SNORD116-based therapies may have the potential to correct this metabolic problem in PWS.

As our previous study showed that hypothalamic communication between NPY and POMC neurons is the likely mediator of Snord116 activity (12), the hypothalamus was selected as the most appropriate site to target this viral reintroduction. While the viral reintroduction of Snord116 was restricted to the hypothalamic region of otherwise Snord116-deficient mice, this was sufficient to produce discernible differences compared to empty-vector-injected knockouts in energy expenditure and body weight, at 22°C. Injections were performed at 6 weeks of age, the earliest possible time for which established stereotaxic cranial co-ordinates would be applicable to produce reproducible effects. Performing the viral re-introduction at an even earlier stage, as well as targeting the entire brain rather than only the hypothalamus, may be even more advantageous. Future studies employing this method could identify functions of Snord116 beyond the direct control of feeding behaviour that nevertheless have indirect influences on this complex regulatory pathway.

However, it is also important to keep in mind that Snord116 is being produced as a cleavage product from intronic sequences of the large SNURF-SNRPN transcripts. Using HEK293T cells, it has been reported that the Snord115, one of the SnoRNAs downstream to Snord116, influenced the expression of Snord116 (21). On the other hand, the deletion of the Snord116 cluster removes approximately 150kb of genomic sequence, including some of the exons of the SNURF-SNRPN transcripts. While there was no obvious difference in the expression levels of these large non-coding RNA transcripts in Snord116$^{-/-}$ mice seen from array data, it is possible that the overall structure of these RNA molecules has been altered due to the missing sequences contained in the Snord116 cluster and, through that, has contributed to an altered metabolic phenotype.

A genome wide array described the transcription of about 200 genes regulated by Snord116 (22) and the functional RNAs influenced by Snord116 are mostly related to appetite regulation and bone formation (12). However, the biological target of Snord116 remains unknown. Despite this, Snord116 with remarkable high birth rates is subjected to birth-and-death evolution in mammals and rodents (23), indicating that Snord116 plays a critical role in neural development. Building on those outcomes, our Snord116 rescue project demonstrated that the highly expressed Snord116 gene only rescued in the mid-hypothalamus before the maturation of the nervous system contributed to this process, most likely via the regulation of NPY system (12).

In conclusion, we conducted the first *in vivo* viral re-introduction of a snoRNA and by utilising an environment specifically designed to be thermo-neutral for mice and shown that the mid-hypothalamic replacement of Snord116 in otherwise Snord116-deficient mice led to an up-regulation in energy expenditure. As this effect could theoretically prove beneficial in obesity-prone individuals with PWS, these results open the door for future research into gene therapy strategies in PWS.

Conflict of interest

We would like to declare no conflict of interest.

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Figure legend

Figure 1

Resultant phenotype from the viral overexpression of Snord116 in the mid-region of the hypothalamus of male Snord116$^{-/-}$ mice (n=8) vs. AAV-empty-injected Snord116$^{-/-}$ mice (n=11) after the bilateral injection at 6 weeks of age.

Representative images dipped sections counterstained with hemotoxylin showing the expression of Snord116 mRNA as silver grains (black dots) in: A) coronal brain section of a wild type mouse through the hypothalamus; B) coronal brain section of a AAV-empty-injected Snord116$^{-/-}$ mouse through the hypothalamus with the injection track; C) coronal brain section of an AAV-Snord116-injected Snord116$^{-/-}$ mouse through the hypothalamus; D) the hypothalamic injection site in an AAV-Snord116-injected Snord116$^{-/-}$ mouse. (3V: the third ventricle; Arc: the arcuate nucleus; LHA: the lateral hypothalamic area; and VMH: the ventromedial nucleus of the hypothalamus). E) Absolute weekly body weight (BW) from 0 to 16 weeks post injection (6 to 22 weeks of age); F) Body weight gain from 0 to 16 weeks post-injection normalised to BW at injection; G) Nasal-anal length at 6, 9 and 14 weeks post-injection (12, 15 and 20 weeks of age); H) Fat mass as a percentage of BW (%BW); I) Lean mass (%BW); J) Bone mineral content (BMC) at 6, 9 and 14 weeks post-injection (12, 15 and 20 weeks of age); K) Bone mineral density (BMD); L) Spontaneous 24-hour calorie intake (%BW) at 7 and 15 weeks post-injection (13 and 21 weeks of age); M) Fasting-induced food intake (%BW); N) Rectal temperature; O) The average of energy expenditure in light and dark phases; P) physical activity; Q) respiratory exchange ratio (RER); R) Glucose tolerance test (GTT); S) Insulin tolerance test (ITT); and T) Serum levels of insulin-like growth factor 1 (IGF-1).

(* $P<0.05$, and ** $P<0.01$)

Figure 2

The phenotype from the viral overexpression of Snord116 in the anterior region of the hypothalamus of male Snord116$^{-/-}$ mice (n=6; —●—; black bars) vs. AAV-empty-injected Snord116$^{-/-}$ mice (n=6; ···□···; white bars) after the injection at 6 weeks of age

A) Absolute weekly body weight (BW) from 0 to 14 weeks post injection (6 to 20 weeks of age); B) Body weight gain from 0 to 14 weeks post-injection normalised to BW at injection; C) Nasal-anal length at 5, 8 and 13 weeks post-injection (12, 15 and 20 weeks of age); D) Fat mass as a percentage of BW (%BW); E) Lean mass (%BW); F) Bone mineral content (BMC) at 5, 8 and 13 weeks post-injection (12, 15 and 20 weeks of age); G) Bone mineral density (BMD); H) Spontaneous 24-hour calorie intake as a percentage of body weight (%BW) at 6 and 12 weeks post-injection (13 and 19 weeks of age), expressed as the average of triplicate readings over three consecutive days; I) Fasting-induced food intake (%BW); J) Body temperature; K) The average of energy expenditure in the light and dark phases; L) The average of physical activity; M) The average of respiratory exchange ratio (RER); N) Glucose tolerance test (GTT); and O) Insulin tolerance test (ITT).

Figure 3

The phenotype from the viral overexpression of Snord116 in the mid-region of the hypothalamus of male Snord116$^{-/-}$ mice (n=11; —●—; black bars) vs. AAV-empty-injected Snord116$^{-/-}$ mice (n=8; ···□···; white bars) after the injection at 10 weeks of age

A) Absolute weekly body weight (BW) from 0 to 12 weeks post injection (10 to 22 weeks of age); B) Body weight gain from 0 to 12 weeks post-injection normalised to BW at injection; C) Nasal-anal length at 2, 5 and 10 weeks post-injection (12, 15 and 20 weeks of age); D) Fat mass as a percentage of BW (%BW); E) Lean mass (%BW); F) Bone mineral content (BMC) at 2, 5 and 10 weeks post-injection (12, 15 and 20 weeks of age); G) Bone mineral density (BMD); H) Spontaneous 24-hour calorie intake as a percentage of body weight (%BW) at 3 and 9 weeks post-injection (13 and 19 weeks of age); I) Fasting-induced food intake (%BW); J) Core body temperature; K) The average of energy expenditure in the light and dark phases; L) The average of physical activity; M) The average of respiratory exchange ratio (RER); N) Glucose tolerance test (GTT); and O) Insulin tolerance test (ITT).

Figure 4

Thermoneutral phenotype from the viral overexpression of Snord116 in the mid-region of the hypothalamus of male Snord116$^{-/-}$ mice (n=9) vs. AAV-empty-injected Snord116$^{-/-}$ mice (n=6) after the injection at 6 weeks of age

A) Absolute weekly body weight (BW) from 0 to 9 weeks post-injection (6 to 15 weeks of age); B) Body weight gain from 0 to 9 weeks post-injection normalised to BW at injection; C) Nasal-anal length at 15 weeks of age (9 weeks post-injection); D) Fat mass as a percentage of BW (%BW) at 15 weeks of age (9 weeks post-injection); E) Lean mass (%BW); F) Bone mineral content (BMC); G) Bone mineral density (BMD); H) Spontaneous 24-hour calorie intake (%BW) at 14 weeks of age (8 weeks post-injection), expressed as the average of triplicate readings over three consecutive days; I) Fasting-induced food intake (%BW); J) The average of energy expenditure in the light and dark phases; K) The average of physical activity; L) The average of respiratory exchange ratio (RER); M) Glucose tolerance test (GTT); and N) Insulin tolerance test (ITT).

(* P<0.05, and *** P<0.001)