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Hypothalamic Expression of SnoRNA *Snord116* is Consistent with a Link to the hyperphagia and obesity symptoms of Prader-Willi Syndrome

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Abstract

The hypothalamus is integral to the regulation of body homeostasis, including food intake, energy balance, and blood pressure. Dysfunction of the hypothalamus has been associated with a broad range of disorders; many of which are sex-dependent in prevalence. Small nucleolar (sno) RNAs are a group of small RNAs located in nucleoli that modulate chemical modifications and maturation of ribosomal or other RNAs. Recent data suggest that snoRNA *Snord116* is important for the pathogenesis of Prader-Willi syndrome (PWS) characterized by hyperphagia and obesity. The current study was conducted to assess a potential cellular link between *Snord116* and phenotypes of PWS. Data from mice revealed *Snord116* expression in the medial hypothalamus, particularly within nuclei that are part of feeding circuitry. High expression of *Snord116* was evident in the paraventricular (PVN) and ventromedial (VMH) nuclei, but particularly prevalent in the arcuate nucleus (ARC) according to *in situ* hybridization. *Snord116* expression level in ventral hypothalamic dissections including ARC was significantly greater (by 2-fold) than that in cortex; and its expression level in dorsal hypothalamic dissections including PVN was double that in cortex. The enhanced expression pattern of *Snord116* in hypothalamic nuclei was observed at weaning and young adult stages, but less obvious neonatally when expression was significantly more widespread. Therefore the expression of *Snord116* likely is regulated developmentally. These results provide a new lead for understanding the mechanism(s) related to hyperphagia and obesity symptoms in PWS patients.

Keywords

snoRNA *Snord116*; hypothalamus; Prader-Willi syndrome (PWS); postnatal development; mouse

1. Introduction

The hypothalamus is located in the ventral part of the diencephalon. It functions as a master neuroendocrine control center by regulating the pituitary gland and controlling many

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functions throughout the body. The hypothalamus is implicated in a wide range of disorders. For instance, hypothalamic dysfunctions are associated with major depressive disorders (MDD) (Swaab et al., 2005) and metabolic disorders (e.g. hypothalamic obesity; Caqueret et al., 2005). Although diseases related to hypothalamic dysfunction often develop in later stages of life, the etiologic factors often are rooted in development (Tobet et al., 2009). Thus, studies of the hormonal and genetic factors that contribute to hypothalamic development are important in understanding disease mechanism, prevention, and treatment.

Much research on the molecular factors contributing to hypothalamic development is focused on signaling molecules (e.g., steroid hormones, transmitters, peptides, etc.) and transcription factors (McClellan et al., 2006; Morton et al., 2006; Tobet et al., 2009; Shimogori et al., 2010). The current study focuses on a different class of molecules: small nucleic acid polymers in the brain. Non-coding RNAs account for about half of transcribed RNAs (Mattick, 2005). However, it has not been recognized until recent years that transcribed non-coding RNAs may have a number of important cellular functions (Mattick, 2005). Studies of neurodevelopment indicate that small RNAs play roles in cell fate determination during neuronal differentiation (Li et al., 2010). On the other hand, in mature neurons small RNAs may regulate gene expression and be involved in cognitive brain functions (Schratt, 2009). Considering that non-coding RNAs can be just as important as protein-coding RNAs in the nervous system, it is important to consider the possible regulatory roles of small RNAs on hypothalamic development and function and their potential involvement in disease.

Among non-coding RNAs, one particular small nucleic acid sequence has been related to the pathogenesis of Prader-Willi syndrome (PWS) and its symptoms of appetite disorder and obesity. PWS is a disease caused by defects in certain imprinted genes. About 1 in 10,000-15,000 newborns are affected, and without intervention, it usually leads to morbid obesity through adolescence and in adulthood. PWS is mainly caused by deletion or imprinting defect of paternal genetic material located at chromosome 15q11-13. The SNURF-SNRPN locus at chromosome 15q11-13 is associated with PWS, which contains multiple protein coding genes and several non-protein-coding snoRNA genes (Cavaille et al., 2000; de los Santos et al., 2000; Gunay-Aygun et al., 2001; Butler et al., 2006). SnoRNAs are a group of small RNAs ranging 80 to 300 nucleotides in length that are located primarily in nucleoli and are required for the chemical modification and maturation of rRNA.

Studies from human data and cases (Bürger et al., 2002; Gallagher et al., 2002; Runte et al., 2005; Sahoo et al., 2008; de Smith et al., 2009; Duker et al., 2010) indicate that genes in the SNURF-SNRPN locus are etiologic factors in PWS, which were supported by genetically engineered mouse models (Skryabin et al., 2007; Ding et al., 2008). In particular, it was reported (Gallagher et al., 2002) that *SNORD116* potentially plays roles in the etiology of much or all of PWS symptoms. In recent years, this hypothesis was supported by the studies in several human cases showing that the deletion of the *SNORD116* gene cluster leads to PWS phenotypes including hyperphagia and obesity (Sahoo et al., 2008; de Smith et al., 2009; Duker et al., 2010). Furthermore, recent progresses using knockout mice have suggested that the snoRNA *Snord116* gene cluster, which has 27 copies in the SNURF-SNRPN locus, potentially was a critical element in PWS formation (Skryabin et al., 2007; Ding et al., 2008; Sahoo et al., 2008; de Smith et al., 2009; Duker et al., 2010). Other gene disruption studies in mice indicate that deletions of most protein-coding genes and snoRNA *Snord115*, another snoRNA found in this region, do not lead to PWS (Yang et al., 1998; Nicholls et al., 1999; Gerard et al., 1999; Tsai et al., 1999; Kozlov et al., 2007); while a region that mainly contains the *Snord116* gene cluster termed PWS critical region (PWS_{cr}) was important in PWS formation (Ding et al., 2005; Schule et al., 2005). Knockout mice

with deletion of only *Snord116* genes showed the phenotype of hyperphagia (Ding et al., 2008). Interestingly, the obesity phenotype did not develop due to changes in energy expenditure (Ding et al., 2008).

Previous studies showed that *Snord116* is primarily expressed in the embryonic brain, but its expression pattern in the brain remains unclear, especially at postnatal ages (Cavaillé et al., 2000; de los Santos et al. 2000; Lee et al., 2003). PWS symptoms, such as hyperphagia and obesity, are associated with the neuronal functions regulated by the hypothalamus (Holm et al., 1993; Gunay-Aygun et al., 2001). Based on the fact that a lack of *Snord116* leads to PWS, it seemed likely that this gene would be expressed in the hypothalamus related to circuits responsible for feeding and energy balance. We examined the expression of *Snord116* in the brain using in situ hybridization and quantitative reverse-transcription PCR. The result of these experiments provides a link between *Snord116* expression and PWS phenotypes at a cellular level.

2. Materials and methods

2.1. Animals

All animal procedures were performed according to the NIH *guide for the care and use of laboratory animal* and were approved by the Colorado State University Animal Care and Use Committee (ACUC). Mice were housed in the animal facility of the Lab Animal Resource (LAR) at the Colorado State University. Mice used in the current study were on a C57BL/6J background.

2.2. Probe generation

The *Snord116* probe was generated based on the sequence of *MBII-85* (alias of *Snord116*) C/D box snoRNA (Lee et al., 2003): AAT GAT GAT TCC CAG TCA AAC ATT CCT TGG AAA AGC TGA ACA AAA TGA GTG AAA ACT CTG TAC TGC CAC TCT CAT CGG AAC TGA. A duplex oligonucleotide of sense and antisense *MBII-85* was purchased from Integrated DNA Technologies (IDT, Inc.) and contained the above sequence and PstI overhang in the 3'-ends. The DNA generated from duplex oligonucleotide was then inserted into the PstI site of pBluescript plasmid. One insertion copy of *Snord116* into pBluescript plasmid was confirmed by sequencing. The linearized pBluescript plasmids containing the *Snord116* insertion were generated by restriction enzyme BamHI or EcoRI digestion. Antisense and sense riboprobes were then generated using T7 or T3 RNA polymerases and a Digoxigenin (DIG) RNA Labeling Kit (Roche, 11277073910).

2.3. In situ hybridization

Mice were examined at multiple ages including perinatal group (embryonic day (E) 17 and postnatal day (P)0), weaning age group (P19-P20), and adult group (6-10 weeks) for *in situ* hybridization (ISH). They were perfused intracardially with 4% paraformaldehyde/0.1M phosphate buffer (PB; pH= 7.4). The brains were post-fixed in 4% paraformaldehyde/0.1M PB overnight. The brains were then removed and embedded in 5% agarose prepared in 1% diethyl pyrocarbonate (DEPC; Sigma, D5758) treated H₂O. Coronal sections (100 µm) were cut through the hypothalamus using a vibrating microtome (Leica, VT1000S). The procedures for processing free-floating brain sections for ISH were carried out as described previously (Dellovade et al., 2000). Briefly, brain sections were treated with 6% hydrogen peroxide in PBT (0.05M PB, 1% Tween-20) for 1 h at RT, washed in PBT, then treated with 10 µg/ml proteinase K (Sigma, P2308)/PBT for 15 min at RT, 2mg/ml glycine/PBT for 10 min at RT. Brain sections were post-fixed in 4% paraformaldehyde/0.2% glutaraldehyde for 20 min at RT. Before hybridization, sections were incubated in pre-hybridization solution (50% formamide in saline-sodium citrate (SSC; Sigma, S6639) buffer containing 8.8 units/

ml heparin (Sigma, H9399), 0.05mg/ml yeast tRNA (Sigma, R5636), and 0.024mg/ml dextran sulfate (Sigma, D8906) for 1h at 60°C. Riboprobes were denatured at 85°C for 5 min and added to pre-hybridization incubation solution overnight at 60°C. On the second day, sections were washed three times for 30 min in a 50% formamide/25% SSC solution, then three times for 30 min in 50% formamide/10% SSC. Sections were washed in TBST (Tris buffered saline (TBS) containing 1% Tween-20) and pre-blocked in 10% sheep serum in TBST. Sections then were incubated with 1:2000 dilution of alkaline phosphatase conjugated anti-DIG antibody (Roche, 11093274910) in 1% sheep serum/TBST overnight at 4°C. On the third day, sections were washed in TBST to clear out primary antibody and stored at 4°C overnight. On the last day, sections were washed in a NTMT solution (0.1M NaCl, 0.1M Tris/pH=9.5, 0.05M MgCl₂, and 1% Tween-20) and incubated in a color detection mix solution (10% polyvinyl alcohol, 1 mM Levamisole (Vector Laboratories, SP5000), 2% NBT/BCIP (Roche, 11681451001), 0.1M Tris (pH=9.5), 0.1M NaCl, and 0.005M MgCl₂) in the dark at RT. The color detecting reaction was stopped by washing the sections in NTMT in the dark.

2.4. Hypothalamic dissection

Fresh hypothalami were dissected from P0, P19 and 6-10 weeks old adult mice. For P0 mice, brains were removed and placed in 0.05M phosphate buffered saline (PBS)/1% DEPC in a petri dish with ventral side up. Coronal cuts were made caudal to the optic chiasm and rostral to the brainstem. For P0 pups, triangular cuts were then made from the top of the third ventricle medially to the hypothalamic slice laterally at the base of the brain to further dissect out a region that included the PVN, VMH, and ARC. A piece of cortex also was removed from the same coronal slice as a control for brain region. For P19 and adult mice, brains were removed and dissected similarly to create a single coronal slice. However, at these older ages, a ventral hypothalamic piece that included the ARC and a dorsal hypothalamic piece that included PVN were dissected further from the same coronal slice in addition to a piece of cortex. The dissected pieces were snap-frozen or subjected to RNA extraction immediately.

2.5. Quantitative reverse-transcription PCR

Total RNA, including RNA smaller than 200bp, was purified using the mirVana™ miRNA isolation kit (Applied Biosystems, AM1560) according to the manufacturer's instructions. Total RNA was subjected to RNase-free DNase (Qiagen; Cat#79254) digestion for 10 minutes at room temperature. After DNase digestion, total RNA was precipitated and eluted in RNase free water. One microgram of total RNA was reverse transcribed using the qScript cDNA synthesis kit (Quanta Biosciences, Cat# 95047). cDNA was diluted 1:4 and used immediately for PCR amplification in 10 microliter reactions containing SYBR Green qPCR Master Mix (2X, Roche applied sciences). *Snord116* primers: forward 5'-TGATGATTCCAGTCAAACATTC-3'; reverse 5'-ACCTCAGTTCCGATGAGAGTGG-3'; *Gapdh* primers: forward-5'GGGAAGCCCATCACCATCTT-3'; reverse-5'GCCTTCTCCATGGTGGTGAA-3'. Primers were tested using PCR to ensure a single amplicon was produced. Real-time PCR was performed using a LightCycler480 PCR system (Roche Applied Sciences) with the following cycle conditions: 95°C for 5 minutes and 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Dissociation curve analysis was conducted to confirm amplification specificity.

2.6. Data analysis

To assess the expression pattern of *Snord116*, brain sections processed by ISH were examined under an Olympus BH2 microscope. Images of the hypothalamus were captured with 4x and 40x objectives and an Insight QE digital camera with Spot Advanced Software.

For comparisons the images were taken with the same illumination intensity and capture time. To quantify the relative level of *Snord116*, qRT-PCR was performed using the LightCycler 480 PCR system and Cp values were determined by the LightCycler 480 SW 1.5 software. Each reaction was performed in triplicate and averaged to determine final Cp values. The relative level of *Snord116* was obtained by normalizing the transcribed RNA signal of *Snord116* to the mRNA signal of *Gapdh* internal control (Schmittgen and Livak, 2008). One-way ANOVA was used for the statistical analyses.

3. Results

3.1. Expression of *Snord116* in newborn and adult brains

The brain expression pattern of *Snord116* was visualized by ISH at 3 different ages: perinatal (E17 and P0), weaning age (P19 and P20), and young adult (6-10 weeks) mice. The *Snord116* was more widely and evenly distributed before and after birth with little evidence of selective expression at both long and short incubation times with the substrate (representative images in Figure 1A and 1B). In negative control sections using either sense *Snord116* probe in ISH or hybridization buffer with no probe, no signals were observed (data not shown). This is consistent with a previous report that the expression of *Snord116* was more uniformly distributed in the embryonic brain (Lee et al., 2003). In contrast, the expression of *Snord116* in weaning and young adult mice was detectable more prominently in the hypothalamus, hippocampus, amygdala and pyriform cortex (Figures 2C, 2G, 2H, 2I); while most other brain areas, had a lower intensity signal (Figures 2D, 2E, 2F), with levels comparable to the area surrounding the hypothalamus (Figures 3A, 3B). These results suggest developmental regulation of *Snord116* expression in the hypothalamus. Other than that, the expression of *Snord116* in weaning and young adult mice appeared in cells throughout the brain if the substrate incubation time was relatively long (data not shown), suggesting its universal expression in the adult brain.

3.2. *Snord116* localization in hypothalamus

In weaning and adult stages, the expression of *Snord116* was prominent in the medial hypothalamic structures (Figures 3, 4), including the paraventricular nucleus (PVN), ventromedial nucleus of the hypothalamus (VMH) and particularly in the arcuate nucleus (ARC). Among those areas, the signal was greatest in the ARC (Figure 3B). At a higher magnification, *Snord116* was found in nuclear compartments particularly in nucleoli and adjacent to nuclear membranes (Figure 3C) in weaning and adult mice. In neonatal mice, *Snord116* was rarely detectable in nucleoli; in contrast, most *Snord116* signal was adjacent to nuclear membranes (Figure 1C). Except for PVN, VMH and ARC, other regions in the hypothalamus with prominent *Snord116* signal included the anteroventral periventricular nucleus (AvPV), rostral periventricular area of the third ventricle (RP3V) and posterior periventricular nucleus of the hypothalamus (PVp) (Figure 4).

3.3. *Snord116* expression according to qRT-PCR

To confirm the regional expression of *Snord116*, its relative levels were quantified by quantitative reverse-transcription PCR (qRT-PCR). The primers designed against *Snord116* were validated using PCR, and a single amplicon was detected of the expected size in brain samples, but barely detectable in liver samples as predicted (Cavaillé et al., 2000) (Figure 5A).

In P0 mice, qRT-PCR data revealed *Snord116* relative levels were similar between the cortex (n=5) and hypothalamus (n=5) (Figure 5B). The relative level of *Snord116* in the cortex was normalized to 1 and the level of *Snord116* in other regions was considered relative to the cortex. In contrast to the P0 mice (Figure 5B), at weaning *Snord116* relative

levels were different in the cortex, dorsal hypothalamus and ventral hypothalamus (Figure 5C, One-way ANOVA, $F(2,22)=46.8$, $p<0.0001$). The *Snord116* expression level in the dorsal hypothalamic dissection that included PVN ($n=10$) was almost double that in the cortex ($p<0.0001$, $n=10$); and in the ventral hypothalamic dissection including the ARC ($n=5$) it was approximately two times greater than in the cortex ($n=10$; $p<0.0001$) (Figure 5C). Between the hypothalamic areas, *Snord116* level in the ventral hypothalamic dissection that included ARC ($n=5$) was approximately twice as great as in the dorsal hypothalamic dissection that included PVN ($n=10$, $p<0.005$). Regional differences were similar between mice at weaning age and in adulthood. In adults, relative *Snord116* level in the hypothalamus was higher than in other brain regions, including the cortex, striatum and brain stem (data not shown).

3.4. Relative level of *Snord116* in female compared to male brains

Based on non-radioactive ISH, *Snord116* immunoreactivity was greater in females (4 of 6 female and male brains run in pairs; data not shown) compared to males, however, the variability of the ISH signals rendered the results unreliable. Therefore, we compared relative levels of *Snord116* between females and males by qRT-PCR. Data obtained from the experiment in figure 5C was broken down by sex. The relative level of *Snord116* in male cortex was normalized to 1 and the level of *Snord116* in other regions in males and in all regions in females was relative to the male cortex. The results revealed that *Snord116* levels were similar between females and males in weaning age mice in the cortex, dorsal hypothalamus and ventral hypothalamus in weaning age of mice (Figure 6).

4. Discussion

The current study examined the neuroanatomical distribution of *Snord116*, a factor in the pathogenesis of PWS related to overeating and obesity phenotypes (Sahoo et al., 2008; de Smith et al., 2009; Duker et al., 2010; Skryabin et al., 2007; Ding et al., 2008). Expression of *Snord116* was examined qualitatively using ISH to determine the localization and quantitatively in dissected tissues using quantitative reverse-transcription PCR. *Snord116* expression was prominent in hypothalamic regions that control food intake and regulate energy balance. Furthermore, expression of this gene became progressively more prominent in these hypothalamic areas after birth. The results support the view that *Snord116* may be related to appetite disorders and obesity symptoms in PWS patients.

The current study links *Snord116* expression to hypothalamic feeding circuitry at a cellular level. *Snord116* ISH signals were detected throughout the brain, however, they were prominent in the hypothalamus in both weanling and adult mice. Specifically, *Snord116* expression was high in the PVN and VMH, and particularly high in the ARC, all key components of hypothalamic feeding circuitry. The current data is consistent with prior evidence that *Snord116* is involved in CNS regulation of food intake and energy balance. First, *Snord116* is expressed in the brain (Cavaillé et al., 2000; de los Santos et al. 2000). Second, based on genetic studies in mice and human patients, *Snord116* is believed to be a key etiologic factor leading to the PWS and its associated phenotypes related to obesity and hyperphagia (Sahoo et al., 2008; de Smith et al., 2009; Duker et al., 2010; Skryabin et al., 2007; Ding et al., 2008). Within the brain, the hypothalamus is considered one region that may be responsible for the development of PWS (Swaab, 1997). Data in the current study provides direct evidence for the neuroanatomical distribution of *Snord116* in hypothalamic nuclei, including PVN, VMH and ARC that are associated with feeding.

In the current study, *Snord116* levels were most prominent in the ARC, suggesting its involvement in the function(s) of cells in the ARC. Dysfunction or damage of the hypothalamus has been associated with obesity and hypothalamic hyperphagia (Hochberg et

al., 2010). Among the nuclei in the hypothalamus, the ARC is one of the central structures controlling food intake and energy balance. In the ARC, two distinct cell groups related to the regulation of appetite and energy balance are the agouti-related peptide (AgRP) and the pro-opiomelanocortin (POMC)-expressing neurons. AgRP and POMC neurons are the receivers for peripheral hormone signals, such as leptin, insulin and ghrelin. AgRP neurons in the ARC project to the PVN, while POMC neurons project to additional nuclei including the PVN and VMH to further regulate feeding behaviour (Bouret, 2009). Determining that if AgRP or POMC neurons express *Snord116* may help determine the role of *Snord116* in feeding regulation. Based on the number of AgRP and POMC neurons compared to the number of *Snord116* positive cells in the ARC, it is not likely that *Snord116* is restricted to one population or the other.

At the cellular level *Snord116* is located inside cell nuclei, mostly in nucleoli and adjacent to the nuclear membrane in the older mice. Previous reports show that the accumulation of *Snord116* in nucleoli increased from perinatal to adult, correlating with maturation of nucleoli (Leung et al., 2009). During early postnatal development, *Snord116* was primarily located outside the nucleoli adjacent to the nuclear membrane. *Snord116* belongs to the C/D box family of snoRNA, which guide the 2'-O-methylation of ribosomal RNA (rRNA) by formation of RNA duplexes at target RNA modification site. However, increasing evidence suggests that snoRNAs may direct the chemical modification of or edit other types of RNA, such as snRNA, tRNA and even mRNA (Bachellerie et al., 2002). For example, snoRNA *Snord115*, another PWS related snoRNA, regulates the alternative splicing and RNA editing of the serotonin 2C receptor (5HT_{2C}R) (Flomen et al., 2004; Vitali et al., 2005; Kishore et al., 2006). Subsequent research showed that lack of *Snord115* was related to unusual behavior mediated by 5HT_{2C}R (Doe et al., 2009). Other than chemical modification and RNA editing, snoRNAs also likely play roles in gene regulation, such as gene silencing (Brameier et al., 2010). Collectively, snoRNAs potentially have complex roles and engage in multiple cellular functions. The extra-nucleolar localization of *Snord116* seen in the current study indicates the possibility that other than rRNA maturation, *Snord116* may have other cellular functions, especially during developmental stages.

Results of the current study show that, in contrast to adulthood, *Snord116* expression was not prominent in the hypothalamus at birth. This observation is consistent with a previous report (Lee et al., 2003). In that study *Snord116* expression was tested in the developing brains from embryo day 9.5 to 18.5 and *Snord116* was expressed at a low level throughout the brain. The current study extends these observations and reveals that the predominance of expression of *Snord116* in the hypothalamus occurs after birth, including at weaning and adult ages. The later development of this restricted expression may relate to the observation that over-eating in PWS patients is not present immediate after birth, but rather develops gradually during early childhood (McAllister et al., 2010). In addition, regulation of *Snord116* specific expression in the hypothalamus falls within the time window of postnatal programming of the hypothalamic feeding circuitry, which develops during the first few weeks after birth (Caqueret et al., 2005). During that time, ARC neuronal projections to other hypothalamic nuclei are maturing (Bouret et al., 2004; Bouret et al., 2006). *Snord116* may contribute to the development of hypothalamic feeding circuitry due to its timing and pattern of expression in development. Leptin is known to regulate food intake and energy expenditure in adults. However, leptin signals also play an important role in guiding postnatal development of hypothalamic feeding circuits (Bouret et al., 2004; Pinto et al., 2004). Without leptin signals, the number of axonal projections from ARC to PVN is reduced (Bouret et al., 2004; Pinto et al., 2004). It remains to be determined whether *Snord116* expression affects, or is affected by, leptin signaling during development.

Our results showed prominent expression of *Snord116* in hypothalamic nuclei associated with feeding regulation, indicating a linkage of *Snord116* neuroanatomical distribution and its involvement in the PWS formation. It will be important to further examine if and how these hypothalamic nuclei are functional molecular targets of *Snord116*. For feeding circuits, two potential mechanisms exist: 1) *Snord116* potentially affects feeding circuitry formation. It would be interesting to examine if axonal projections are defective in feeding circuitry when *Snord116* is deleted. 2) *Snord116* potentially indicates maturation of feeding circuitry. Thereafter, *Snord116* could play roles in feeding control by regulating downstream effectors, possibly through its chemical modification, editing or gene silencing functions (Bachellerie et al., 2002; Flomen et al., 2004; Vitali et al., 2005; Kishore et al., 2006; Brameier et al., 2010). Interestingly, a previous study showed similar gene expression profiling in the hypothalamus between wild type and *Snord116* deletion mice in the neonatal period including day 5 and day 13 (Ding et al., 2010). These results suggested *Snord116* does not play direct roles in feeding regulation in neonatal period. However, it might be more important to test the gene profiling at a later stage, such as after weaning or in adulthood. In the current study the expression of *Snord116* in hypothalamus became restricted at weaning age and into adulthood, while previously the hyperphagic symptoms appeared in *Snord116* deletion mice after 3 months of age (Ding et al., 2008). Other than simply looking at different time points, it might also be important to test other changes in mRNA, since *Snord116* plays roles in RNA modification and editing that may not be detected by standard gene profiling method.

The hypothalamus is a highly sexually dimorphic brain region that is influenced by steroid hormones in development (Tobet & Fox, 1992; McCarthy, 2008; Tobet et al., 2009). The downstream effectors of steroid hormones include γ -aminobutyric acid (GABA), nitric oxide (NO), and brain-derived neurotrophic factor (BDNF) among many others (Tobet et al., 2009). As snoRNA editing may influence neurotransmitter receptor function there may be interesting sex dependent interactions in conditions where transmitter signaling is altered. For example, disruption of GABA_B receptor signaling by deleting the R1 subunit of the GABA_B receptor affected the organization of cell populations in the PVN and surrounding area in a sex dependent manner when no sex difference was evident in wild-type mice (McClellan et al., 2010). In the current study, results from ISH experiments comparing female and male hypothalami were suggestive of greater levels of *Snord116* in females, but inconclusive. Quantitative analyses revealed that *Snord116* levels were similar levels in tissue between males and females. It remains to be determined whether changes in transmitter function (e.g., GABA_B receptor disruption; McClellan et al., 2010) might reveal changes in *Snord116* function.

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Highlights

- The expression of snoRNA *Snord116* was examined in perinatal, weaning age and young adult mouse brain by *in situ hybridization* and qPCR.
- *Snord116* expression in weaning and adult mice is prominent in the medial hypothalamus, particularly within nuclei that are part of feeding circuitry.
- This prominent expression pattern of *Snord116* was observed in adult but not in newborn, indicating it was developmentally regulated.
- The result provides a link between *Snord116* expression and PWS phenotypes at a cellular level.

Figure 1.

Digital images show digoxigenin-based *in situ* hybridization (ISH) illustrating the expression of *Snord116* in the brain including hypothalamus and surrounding area in neonatal (P0) mice. Panels A and B show the representative images of *Snord116* in mice at birth (P0). Panel C is the magnified image from the boxed area in panel B. Scale bars are located in the lower left of each panel.

Figure 2.

Representative images from a female mouse at weaning age to illustrate digoxigenin-based *Snord116 in situ* hybridization (ISH) signal in multiple brain regions. Panels A (caudal diencephalon) and B (rostral mesencephalon) illustrate coronal sections hundreds of microns apart containing *Snord116* ISH labeling. The numbered brain regions correspond to images shown at higher magnifications in panels C through I (1=C, 2=D, 3=E, 4=F, 5=G, 6=H, 7=I). Representative positive cells are highlighted by arrows shown in panels C, D, G, H and I. The *Snord116* ISH signal was most readily noticeable in hypothalamic nuclear groups (panel C). In most other brain regions, the *Snord116* ISH signal was lower or not detectable compared to the hypothalamus, as shown in the representative regions of cortex (panel D), striatum (panel E) and brain stem (panel F). Other brain regions in the same coronal plane of hypothalamus that also have high *Snord116* ISH signal include cells in the dentate gyrus of the hippocampus (panel G), medial amygdala (panel H) and pyriform cortex (panel I). Scale bars are located in the lower left of each panel.

Figure 3.

Representative images from a female mouse at weaning age show digoxigenin-based *Snord116* *in situ* hybridization (ISH) signals in the hypothalamus. The ISH signal was detected in the paraventricular nucleus (PVN; panel A), ventromedial nucleus of the hypothalamus (VMH; panel B) and arcuate nucleus (ARC; panel B). Panel C shows a representative higher magnification image of *Snord116* from the boxed area in the ARC in panel B. The arrows point to representative cells that show nucleoli labeling of *Snord116*. Scale bars are located in the lower left of each panel.

Figure 4.

Representative digoxigenin-based *in situ* hybridization (ISH) images of *Snord116* from the rostral to caudal hypothalamus in a female mouse at weaning age. The images from panels A to L are hypothalamic sections taken from rostral to caudal. The *Snord116* ISH signals are prevalent in the nuclear groups of the anteroventral periventricular nucleus (AvPV; panel A), rostral periventricular area of the third ventricle (RP3V; panel B), paraventricular nucleus (PVN; panels E,F), ventromedial nucleus of the hypothalamus (VMH; panels G, H), arcuate nucleus (ARC; panels G, H), and posterior periventricular nucleus of the hypothalamus (PVp; Panels K, L). Letter V in the images indicates the third ventricle of the brain. Scale bar is located in the lower left of panel A and is applied for the rest of panels in this figure.

Figure 5.

Relative levels of *Snord116* determined by quantitative reverse-transcription PCR (qRT-PCR). *Snord116* levels were detectable by RT-PCR in the hypothalamus (HYP; panel A) and cortex (COR; panel A) in the brain but not in the liver (LIV; panel A). qRT-PCR revealed the relative level of *Snord116* in the HYP and COR in neonatal pups (panel B). Relative levels of *Snord116* were determined at weaning age in the COR, dorsal hypothalami (D-HYP) including the paraventricular nucleus, and ventral hypothalami (V-HYP) including the arcuate nucleus (panel C). The mean value of *Snord116* relative levels in the COR was normalized to 1. The values of other groups are the fold differences of the mean value in the COR. The number of animals per group is given within each of the bars, which represent mean values $\pm$ SEM.

Figure 6.

Relative level of *Snord116* in female and male mouse brains determined by qRT-PCR. At weaning, the relative level of *Snord116* was less in COR of males and females than in dorsal hypothalami (D-HYP) or ventral hypothalami (V-HYP), but similar by sex. The mean value of *Snord116* relative levels in the male COR was normalized to 1. The values of other groups are the fold differences of the mean value in the male COR. The number of animals per group is given within each of the bars, which represent mean values $\pm$ SEM.