Stochastic Loss of Silencing of the Imprinted \textit{Ndn/NDN} Allele, in a Mouse Model and Humans with Prader-Willi Syndrome, Has Functional Consequences

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Abstract

Genomic imprinting is a process that causes genes to be expressed from one allele only according to parental origin, the other allele being silent. Diseases can arise when the normally active alleles are not expressed. In this context, low level of expression of the normally silent alleles has been considered as genetic noise although such expression has never been studied further. Prader-Willi Syndrome (PWS) is a neurodevelopmental disease involving imprinted genes, including \textit{NDN}, which are only expressed from the paternally inherited allele, with the maternally inherited allele silent. We present the first in-depth study of the low expression of a normally silent imprinted allele, in pathological context. Using a variety of qualitative and quantitative approaches and comparing wild-type, heterozygous and homozygous mice deleted for \textit{Ndn}, we show that, in absence of the paternal \textit{Ndn} allele, the maternal \textit{Ndn} allele is expressed at an extremely low level with a high degree of non-genetic heterogeneity. The level of this expression is sex-dependent and shows transgenerational epigenetic inheritance. In about 50% of mutant mice, this expression reduces birth lethality and severity of the breathing deficiency, correlated with a reduction in the loss of serotonergic neurons. In wild-type brains, the maternal \textit{Ndn} allele is never expressed. However, using several mouse models, we reveal a competition between non-imprinted \textit{Ndn} promoters which results in monoallelic (paternal or maternal) \textit{Ndn} expression, suggesting that \textit{Ndn} allelic exclusion occurs in the absence of imprinting regulation. Importantly, specific expression of the maternal \textit{NDN} allele is also detected in post-mortem brain samples of PWS individuals. Our data reveal an unexpected epigenetic flexibility of PWS imprinted genes that could be exploited to reactivate the functional but dormant maternal alleles in PWS. Overall our results reveal high non-genetic heterogeneity between genetically identical individuals that might underlie the variability of the phenotype.

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Introduction

Imprinted genes are functionally mono-allelic in a parent-of-origin specific manner. Genomic imprinting is a non-Mendelian epigenetic form of gene regulation which is germline-inherited since the epigenetic marks are established in the parental gametes without altering the DNA sequence [1]. Compared to most other tissues the brain is enriched in genes showing an imprinted pattern of expression [2], vulnerable to environmental perturbation [3] and contributing to various neurodevelopmental diseases [4,5]. This vulnerability, linked to a plasticity of gene regulation, might also allow a positive adaptation of an organism to a new external environment. It is important to examine situations in which partial loss of imprinting (LOI) rescues a mutant phenotype. Understanding the mechanisms underlying this positive effect could lead to therapeutic avenues that manipulate this rheostat function.

\textit{Necdin (Ndn)} is an imprinted gene present in both human and mouse, and its maternally inherited allele is normally silenced [6–9]. The human \textit{NDN} gene is located in a large imprinted domain. All the paternally expressed genes from this domain are candidate genes for some of the symptoms of Prader-Willi Syndrome (PWS), an orphan neurodevelopmental genetic disease [10] (OMIM 176270). The essential clinical diagnostic criteria include neonatal hypotonia and abnormal feeding behavior with a poor suck
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Author Summary

Genomic imprinting is a process that causes genes to be expressed from only one of the two chromosomes, according to parental origin, the other copy of genes being silent. Prader-Willi Syndrome (PWS) is a neurodevelopmental disease involving imprinted genes, including NDN, which are only expressed from the paternally inherited chromosome, the maternally inherited copy of the gene normally being silent. Here we show that, in absence of the paternal Ndn copy only, the maternal Ndn allele is expressed at an extremely low level with a high degree of heterogeneity. The level of this expression is dependent on both the sex of the offspring and the genotype of the mother. In about 50% of mutant mice, this expression reduces birth mortality and severity of the breathing deficiency, showing a functional role of this low expression. Importantly, specific expression of the maternal NDN allele is also detected in post-mortem brain samples of PWS individuals. Our data reveal an unexpected epigenetic flexibility of PWS imprinted genes that could be exploited to reactivate the functional but dormant maternal alleles in PWS. Overall our results reveal high non-genetic heterogeneity between genetically identical individuals that might contribute to variability in the phenotype.

followed by a hyperphagia, resulting in severe obesity, and behavioral problems [11–14]. Breathing deficiency is a significant health concern for many patients and contributes to some cases of sudden death [15,16]. Notably, there is considerable variability in symptom severity among patients [11].

Mouse strains with targeted inactivation of single PWS genes have been created, and heterozygous mice with a paternally inherited deficiency (+m/−p) are generally considered to be functionally null. Four independent Ndn-deficient mouse lines have been created [9,17–19], three of which display PWS associated phenotypes [9,17–21] including partial early post-natal lethality due to respiratory distress [20,22].

In Muscatelli's Ndn-KO mouse model (named Ndn<sup>tm1.Mus</sup>), we observed a high level of phenotypic heterogeneity among the Ndn<sup>+m/+m</sup> mice within each litter; notably in the incidence and severity of apneas [22]. The stochastic nature of gene expression can generate pronounced phenotypic variations [23] and here we hypothesize that this inter-individual variability, among Ndn<sup>+m/+p</sup> mice, might result from a “stochastic” activation of the putatively silent maternal allele of Ndn.

In this study, we investigate this hypothesis by comparing homozygous mice deleted for both alleles of Ndn (Ndn<sup>−/−</sup>) with heterozygous Ndn (Ndn<sup>+m/+p</sup>) mice. We perform a comprehensive analysis of the in vivo expression and functional role of the Ndn maternal allele. We investigate the genetic context and the mechanism underlying this maternal expression. Finally, we show that the maternal allele of NDN is transcribed and that Necdin protein is present in human post-mortem Prader-Willi brains.

Results

**Ndn<sup>−/−</sup>** mutant mice present a more severe phenotype than Ndn<sup>+m/+p</sup> littermates

After 36 backcrosses on the C57Bl/6j genetic background, we measured the lethality of Ndn<sup>+m/+p</sup> mice versus Ndn<sup>+/+</sup> mice, both derived from crosses between a wild-type (WT) female and a heterozygous male deleted for the Ndn maternal allele (−m/4p). As expected [17], Ndn<sup>+m/+p</sup> mice were significantly under-represented at weaning (28% reduction, 125 +/- versus 91 Ndn<sup>−/+ −p</sup>, CHI² test, P<0.01). Furthermore, we confirmed that there was an equivalent number of Ndn<sup>+m/+p</sup> (118) versus Ndn<sup>+/+</sup> (116) pups at birth, and 21% lethality between postnatal day (P) P0 and P1 in both sexes. However, in a cohort of 73 Ndn<sup>−/−</sup> mutants, derived from crosses between a Ndn<sup>−/−</sup> female and a Ndn<sup>−/-</sup> male, we found a 43% lethality of the Ndn<sup>−/−</sup/+m pups (32/75), between P1 and P2, and these pups were visibly cyanotic. Altogether, these data suggest that, due presumably to respiratory deficiency, Ndn<sup>−/−</sup>/+m new-borns are twice as likely to die early compared to Ndn<sup>+/−</sup>m new-borns. This result is surprising because in theory there is no Ndn expression in either Ndn<sup>−/−</sup> or Ndn<sup>+/−</sup>m pups.

Next, we compared breathing pattern between Ndn<sup>−/−</sup> and Ndn<sup>+/−</sup>m mice. Previously, we demonstrated that newborn and young adult Ndn<sup>+/−</sup>m−p mice present an irregular respiratory rhythm with frequent apneas [22]. Importantly, such apneas were more than twice as frequent in Ndn<sup>−/−</sup> compared to Ndn<sup>+/−</sup>m−p mice (Table 1).

In summary, the respiratory phenotype of Ndn<sup>−/−</sup> homozygous mice is more severe than in Ndn<sup>+/−</sup>m heterozygotes, suggesting a role for the maternally inherited Ndn allele.

Quantitative expression of the Ndn maternal allele

Since we suspected a role of the maternal Ndn allele in the phenotype of Ndn<sup>+/−</sup>m−p mice, we further investigated the expression of this allele using a specific anti-Necdin antibody in immunoblot analyses of protein extracts from different P1 brains (Figure S1A) or from individual E12.5 embryos (Figure S1B). We detected a specific signal at the expected size for WT animals but also a fainter signal (10–20 fold less intense) in four of the eight Ndn<sup>−/−</sup>−p mutants, derived from crosses between a wild-type female and a Ndn<sup>−/-</sup> male.

Consistent with our previous study [17], we did not detect maternal Ndn allele expression by RT-PCR (Figure S1C). We therefore increased the experimental sensitivity using RT-qPCR. We focused on different developmental stages (E12.5, P1 and adult) [24], using total brain tissue as well as brain structures known to highly express Ndn (hypothalamus) or to be involved in

Table 1. Respiratory pattern and apnea in Ndn<sup>−/−</sup>−p young adult versus Ndn<sup>+/−</sup>m−p mice.

| Ndn<sup>−/−</sup>−p | Ndn<sup>+/−</sup>m−p |
|----------------|-----------------|
| **Animal weight (g)** | 16.7±2.3 | 16.4±2.7 |
| **Minute Ventilation (ml.min<sup>−1</sup> g<sup>−1</sup>)** | 2.0±0.6 | 2.6±1.0 |
| **Breathing Frequency (BPM)** | 256.7±56.6 | 298.3±81.4 |
| **Tidal Volume (ml.10<sup>−3</sup> g<sup>−1</sup>)** | 7.9±1.0 | 8.7±1.8 |
| **Expired Volume (ml.10<sup>−3</sup> g<sup>−1</sup>)** | 7.8±1.0 | 8.7±1.8 |
| **Expiratory Time (ms)** | 174.9±28.9 | 166.3±45.0 |
| **Apnea number, per hour** | 8.8±8.9 | 18.2±14.4 |
| **Apnea duration (ms)** | 900±109 | 929±107 |
| **% of animals with apnea >750 ms** | 85% | 84% |
| **Apnea scores (% time.hour<sup>−1</sup>)** | 0.2±0.2 | 0.5±0.4 |

While the mean apnea duration was similar between genotypes (~900 msec), evaluation of the total apnea duration expressed as the total recording time (i.e. apnea scores) revealed a two-fold increase in the percentage of total apnea duration in Ndn<sup>−/−</sup>−p compared to Ndn<sup>+/−</sup>m−p individuals.

Values are represented as Mean±SD; n = 20 for Ndn<sup>+/−</sup>m−p and 18 for Ndn<sup>−/−</sup>−p mice.

Mann Whitney t-test, two-tailed. P value = 0.02 (a) and 0.03 (b).

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In *Ndn*/*−/−* mutant mice, no *Ndn* transcripts were detected irrespective of the brain structures or stages analyzed (data not shown). In WT individuals (Figure 1A), as expected [17,24], we observed higher *Ndn* expression in P1 brains compared with expression in E12.5 embryos. In *Ndn/+−/−* individuals, maternal *Ndn* transcripts were detected, but the transcript level was reduced 800 (P1 brain) to 1500 (adult hypothalamus)-fold compared to WT individuals (comparing the medians, Figure 1B). Interestingly, there was a huge inter-individual variability (×100 to ×1000 between the extreme values) for all *Ndn/+−/−* mice, irrespective of the stages and tissues tested.

We searched for factors that influence the level of transcripts of the *Ndn* maternal allele. While our results show an absence of a significant effect of the paternal genotype (Figure 1D,E,F), the maternal genotype clearly influences maternal *Ndn* expression in the *Ndn/+−/−* offspring. Although in all cases the offspring (+m/−p) have inherited a wild-type (+m) allele from the mother, offspring from the *Ndn+/+ or Ndn+−/−* maternal genotype had a significant three-fold higher level of *Ndn* maternal expression compared to those from a *Ndn−/m+/p* maternal genotype (Figure 1D,E,F). Importantly, the extensive variability of *Ndn* maternal expression is also positively correlated with both those maternal genotypes. In contrast, litters issued from *Ndn−/m+/p* mothers showed both a lower level of *Ndn* expression and an absence of variability in the *Ndn/+−/−* offspring (Figure 1D,E,F).

In addition, there is a gender-specific effect on the *Ndn* maternal expression in *Ndn/+−/−* offspring, with females expressing two-fold more *Ndn* expression compared to males (Figure 1G).

Finally we compared this maternal expression in *Ndn/+−/−* offspring from a C57BL/6j or a 129Sv/Pas genetic background. In both mouse strains, a similar level of *Ndn* maternal expression was observed (Figure 1H).

We conclude that an extremely low but specific transcription of the maternally inherited *Ndn* allele in *Ndn/+−/−* individuals is found in at least two mouse strains (C57BL/6j, 129Sv/Pas). Transcript numbers are highly variable irrespective of the developmental stage or the brain structure analyzed, even among littermates. Finally, the quantity of maternal *Ndn* transcripts depends significantly on the maternal genotype and on the gender.

**Qualitative expression of the *Ndn* maternal allele at different developmental stages**

We asked whether maternal *Ndn* expression was due to: 1) low but homogeneous expression in all tissues and/or 2) reduced but focal expression in specific structures and cell types. At E12.5, using immunohistochemistry (IHC) and in situ hybridization (ISH) on frozen serial sections, Necdin protein and transcripts were detected in the same structures of four out of nine *Ndn/+−/−* embryos (Figure 2). Importantly, no protein or transcripts were detected in *Ndn−/−* individuals (Figure S2). Expression of the maternally inherited *Ndn* allele was detected in a restricted number of cells of specific nervous structures in which the paternally inherited *Ndn* allele is normally expressed in WT animals (Figure 2 A,B,C,D,E). Interestingly, the cerebral cortex, the tongue and the myotome, which show expression of the paternal allele in WT, do not express the maternal *Ndn* allele (data not shown). In contrast to WT E10.5 embryos, no maternal *Ndn* expression was detected in *Ndn/+−/−* E10.5 embryos (n=9) (Figure S3). At P1, a stage when expression normally peaks [24], we detected Necdin protein by IHC in the brain of *Ndn/+−/−* newborns (n=6) (Figure 3). Necdin presented a similar expression pattern in *Ndn+/−−* adults (Figure S4). At both developmental stages, this expression was restricted to a limited number of cells in several, but not all nuclei that express *Ndn* in WT animals, such as the hypothalamic (Figure 3B) and the raphe nuclei (Figure 3C,D).

Thus, at the anatomical level, using ISH and IHC, we conclude that the *Ndn* maternal allele is expressed in a subset of *Ndn+/−−* individuals and, compared to WT mice, is restricted to a limited population of cells in specific nervous system structures. Noticeably, there is considerable inter-individual variability, even between littermates (Figure 1 and data not shown).

We addressed the question of intra-individual variation by studying the raphe nuclei, a structure defined by 5HT-expressing neurons, all of which express Necdin in WT mice [22]. We double immunostained P1 brains using anti-Necdin and anti-5HT antibodies and determined the number of 5HT/Necdin positive neurons in the different raphe nuclei (B1 to B9) of WT, *Ndn+/−−* and *Ndn−/−* newborns (Figure 4A). We confirmed both inter-individual and intra-individual variation in the number of 5HT/Necdin double positive neurons in *Ndn+/−−* raphe nuclei. For instance, in the same individual, 78% of 5HT positive neurons in B1/B2 raphe nuclei were Necdin positive although in other raphe nuclei no Necdin expression was detected (Figure 4A). We conclude that there is also intra-individual variation in the expression of maternal *Ndn* allele in the raphe nuclei.

**Ndn maternal expression plays a functional role at the cellular level**

Previously, we observed alterations in the 5HT system [22] in *Ndn+/−−* mice. Here, we analyzed the cellular defects in *Ndn−/−* newborn mice (n=8) in comparison with *Ndn+/−−* (n=18) and WT newborns (n=9). Using 5HT immunolabelling, we counted the number of 5HT neurons in the B1/B2 raphe nuclei (Fig. 4B). We found a significant 28% reduction (WMW test, P<0.001) in the number of 5HT-expressing neurons between *Ndn−/−* (1306 (1204,1337); n=8) and WT newborns (1807 (1738,1882); n=9). Interestingly, in the B1/B2 raphe nuclei compared to WT mice, the *Ndn+/−−* individuals that expressed Necdin, with a mean of 46% of 5HT neurons Necdin-positive (*Ndn+/−−* (Nde+), Figure 4B), had only a 8% reduction (WMW test, P<0.001) in the number of 5HT-expressing neurons (1666 (1409,1733); n=9). In contrast, the *Ndn+/−−* individuals (*Ndn+/−−* (Nde−), Figure 4B) that do not show Necdin expression had a significant 20% reduction (WMW test, P<0.001) in the number of 5HT-expressing neurons (1313 (1250,1335); n=9) similar to the results observed in *Ndn−/−* P0 mice. Thus expression of the maternal *Ndn* allele in *Ndn+/−−* individuals correlates with an increased number of 5HT-expressing neurons.

The *Ndn* C57BL/6j maternal allele is never expressed in wild-type mice

We next asked whether the low level of maternal *Ndn* expression was also present in WT mice. In order to discriminate between paternal and maternal allele-specific *Ndn* expression in WT mice, we identified mouse strains carrying transcribed polymorphisms in the *Ndn* gene. Three such polymorphisms (two SNPs in the 3′-untranslated region (UTR) and one 5′bp indel in the 5′-UTR) were identified between *Mus musculus* (C57BL/6j) and *Mus spretus* strains.

First, to analyze the SNPs, we performed two quantifications of allele-specific expression by pyrosequencing (QUPAS) assays on RT-PCR products from F1 brains of six pups with a C57BL/6j mother and *Mus spretus* father, and did not detect expression of the
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maternal (C57Bl/6J) Ndn allele in these brain samples (data not shown). To further increase the sensitivity for detection of maternal Ndn transcripts, we designed specific TaqMan probes distinguishing between the presence and absence of the 5’ bp indel in the 5’-UTR and used RT-qPCR for allele-specific quantification. However, this assay also did not reveal any Ndn transcripts from the C57Bl/6j maternal allele in the C57Bl/6j maternal allele in C57Bl/6j × Mus spretus F1 brains from 32 pups (Figure S3). We conclude that in this wild-type mixed genetic context, we do not detect any expression of the maternal C57Bl/6j Ndn allele.

**Completion of the experiments of the Ndn alleles**

The Ndn+/− p heterozygous mice described by Gerard et al [9] (named Ndnm252Sw) present a more severe phenotype with particular a higher lethality at birth compared to Ndn−/− p offspring (n = 51) issued from a WT female crossed with a Ndn−/− male (C). Considering separately the effect of the maternal or paternal genotype, we showed that when the mother is WT and the father is (+/−), with a paternal Ndn mutant allele (+/− p) or a paternal Ndn mutant allele (−/− p), then there is no difference in the copy number of Ndn maternal transcripts between the Ndn+/− p individuals of the offspring of both types of crosses (n = 25 and n = 46, respectively, D,E,F). However, we observed an effect of the paternal Ndn genotype, with a significant difference in the level of Ndn maternal transcripts between the Ndn+/− p individuals (n = 22) issued from a (−/− p female X +/− male) compared with the Ndn+/− p individuals (n = 29) issued from a (+/− p female X −/− male) (D,E,F); the +/+ or Ndn+/− p paternal genotype is correlated with a significant three times higher level of Ndn maternal expression in the Ndn+/− p offspring. Values are represented as Median (Q1, Q3). WMW test, two-tailed.

* P value <0.05 and ** <0.01.

Figure 1. Ndn expression analyzed by RT-qPCR in Ndn+/− p mice. RT-qPCR analysis of Ndn transcripts. A) Ndn transcripts in E12 WT embryos and selected brain tissues from P1 (whole brain, hypothalamus, pons) or adult (hypothalamus) WT mice. B) Ndn transcripts in Ndn+/− p E12 embryos and brain tissues from P1 (whole brain, hypothalamus, pons) or adult (hypothalamus) Ndn+/− p mice. C–F) Quantification of Ndn transcripts in Ndn+/− p E12 embryos, P1 and adult Ndn+/− p brain tissues in male (M) and female (F) mice. H) Quantification of Ndn transcripts in F1 mice (E12, P1 brain, P1 pups; n = 71) issued from a cross between a WT female and a (−/− p male) × Ndn−/− p female. doi:10.1371/journal.pgen.1003752.g001

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Variation in DNA methylation at the DMRs of imprinted genes has been reported in different tissues, importantly in brain, and might be a source of gene expression and phenotypic variations [25]. We therefore studied DNA methylation in a secondary DMR (42 CpGs), previously shown to be correlated with imprinted regulation of Ndn expression [7,26] (Figure S9). We found no major changes in methylation on the Ndn maternal allele in PWS infant (9 months old with a deletion); age and sex matched (one with a deletion and one with a maternal disomy) and one PWS child (6 months old). Expression of NDN also occurred in the cortex of PWS patients (data not shown). The results contradict the widely accepted assumption that in PWS patients the maternal allele is totally silenced in the brain.

The maternally inherited allele of NDN is expressed in hypothalamus of PWS patients

We assessed whether the expression of the maternal Ndn allele observed in heterozygous Ndn+/− p mice also occurs in PWS patients. Using a specific human NDN RNA probe and an anti-Necdin antibody, we performed an ISH and IHC on hypothalamic sections obtained from brains of two adult PWS patients (one with a deletion and one with a maternal disomy) and one PWS infant (9 months old with a deletion); age and sex matched control individuals were included as positive controls (Table S1). In all patients, we found NDN transcripts and protein in the paraventricular and supra optic nuclei (Figure 6). We confirmed NDN mRNA expression in five more adult PWS patients (25–64 years of age) and one PWS child (6 months old). Expression of NDN also occurred in the cortex of PWS patients (data not shown).

The results contradict the widely accepted assumption that in PWS patients the maternal allele is totally silenced in the brain.
These findings are in full agreement with the results obtained in our heterozygous Ndn+/-p mice.

**Discussion**

In this article we report a stochastic expression of the maternally inherited allele of the Ndn gene in mice where the paternal gene has been inactivated. We showed an extremely low and very variable number of transcripts but nevertheless confirmed that these transcripts are translated into Necdin protein. Furthermore a comparison between Ndn-/- and Ndn+/-p pups showed that the lethality, due to respiratory distress, is decreased two-fold in Ndn+/-p compared to Ndn-/- . In agreement with this decreased lethality, surviving Ndn+/-p adult mice present two-
fold fewer apneas and more 5HT-expressing neurons, in a manner that is positively correlated with maternal $Ndn$ expression. This confirms the functional importance of the extremely weak expression of the $Ndn$ maternal allele. Finally $NDN$ transcripts and protein were also detected in brain tissue from human PWS patients.

Furthermore, our results strongly suggest that expression of the maternal allele of $Ndn$ only occurs in the absence of expression of the paternal $Ndn$ allele. In addition, they are consistent with a model where, without an imprinting regulation, competition between two $Ndn$ promoters results in a monoallelic expression. In this model, imprinting mechanisms create an allelic exclusion that dictates that the maternal allele is inactivated.

Loss of imprinting in mouse models for PWS and in PWS patients

Prior to this study, it was widely accepted that only the paternal alleles of PWS candidate genes are expressed, the maternal alleles being totally silenced. However, in brains of mice, with a deletion...
of the imprinting center, an incomplete silencing of paternally inherited PWS genes as well as a low level of expression of maternal alleles of PWS genes, was reported but not investigated [27]. LOI was also observed in lymphoblasts of two PWS patients with a deletion and two atypical PWS patients with a maternal disomy [28,29], but these studies were not extended to include expression profiles in the brain. LOI has been described in other contexts, particularly in some cancers [30]. Our study addresses for the first time the robustness of silencing of the maternal alleles of PWS candidate genes in brain. Our results show that in both mice and humans, in the absence of the paternally inherited Ndn gene, the maternal Ndn allele is expressed in the brain at very low level but sufficiently to allow Necdin protein production. A similar mechanism might be hypothesized for any of the PWS genes in PWS patients. For example, the imprinted Mage2/MAGEL2 PWS gene, showed a similar loss of imprinting in Mage2 deficiency in B1/B2 raphe nuclei.

The relevance of Ndn maternal allele expression

The two-fold reduction of post-natal mortality in Ndn+/−p mice compared to Ndn−/− mice, suggests that even the low level of maternal Necdin protein is sufficient to rescue 50% of the mice, in comparison with the Ndn−/− mice. Nevertheless 50% of Ndn−/− individuals survive suggesting that another compensatory system is activated when the level of Ndn expression is null or very low in Ndn+/−p mice.

A surprising degree of inter-individual variability was observed in the number of Ndn transcripts amongst Ndn+/−p mice. The degree of maternal Ndn expression is correlated with the severity of the phenotype, in that the number of apneas is significantly increased in Ndn−/− mice compared to Ndn+/−p mice. Previously, we published that those apneas might be correlated with an alteration of the 5HT system [22]. Here we showed that the number of 5HT-expressing neurons is reduced by 20% in Ndn−/− compared to WT mice while Ndn+/−p mice are divided in two distinct populations with 30% and 10% few 5HT-neurons respectively. The lowest reduction (10%) of 5HT-expressing neurons is observed in those Ndn+/−p individuals co-expressing Necdin in 5HT neurons. These data support a link between the expression of the Ndn maternal allele and the degree of survival, the severity of apneas and the number of 5HT neurons in the B1/B2 raphe nuclei.

Variability of Ndn maternal expression

Ndn maternal expression presents a high inter-individual variability (1 to 3 orders of magnitude), even among Ndn+/−p individuals from the same litter, irrespective of the age and brain structure analyzed. Intra-individual variability of Ndn expression was also detected in the brain structures. This expression is limited to some, but not all, of the brain regions that normally express Ndn with no evidence of ectopic expression. In those brain regions the number of neurons expressing Ndn is clearly less than in wild-type animals and variability of Ndn expression amongst the Ndn+/−p offspring was linked to both maternal genotype and gender, being additive factors. A Ndn+/−p mouse with a +/- or Ndn+/−p mother (a mouse who has inherited a wild-type Ndn allele from her grandmother) is predisposed to the highest level of expression and to a greater inter-individual variability, a phenomenon referred to as transgenerational epigenetic inheritance [31]. In contrast, paternal genotype has no impact. Furthermore maternal Ndn allele expression was two-fold higher and more variable in female mice compared to male mice. This may reflect the increased genetic variability in females: some genes escaping X inactivation, such as Jarid1c, which codes for a histone demethylase [32], showing higher expression in females. This could explain our observations concerning maternal allele Ndn expression. Alternatively or additionally, female-specific hormones could be involved.

An interesting observation resulting from transcriptome profiling is the very high variability between individuals in steady state levels of a range of mRNAs, often reaching an order of magnitude [33]. This might explain why the penetrance of a given genotype is often incomplete [23,31,33]. This type of epigenetic phenomenon might also be involved in the variable expression of the maternal Ndn gene and consequently might lead to survival of some Ndn+/−p mice. Nevertheless, even in Ndn−/− mice the penetrance of the phenotype (postnatal lethality and apneas) is not complete, suggesting that another mechanism involving a “compensatory pathway” takes place. This compensatory pathway might result from an increase of a gene expression linked to the lack of Ndn expression or might also result from the stochastic variability in gene expression described above [33] that occurs independent of the state of Ndn expression.

Mechanism underlying the maternal expression

The lack of detection of expression of the C57B1/6J paternal allele in WT mice (with a paternal M. Spretus allele) suggests that expression of the Ndn maternal allele is associated with the absence of an active paternal Ndn promoter, as confirmed by our study of the Ndn+/−p Ndn+/−trans mice. However, the paternal allele expression was two-fold higher and more variable in female mice compared to male mice. This may reflect the increased genetic variability in females: some genes escaping X inactivation, such as Jarid1c, which codes for a histone demethylase [32], showing higher expression in females. This could explain our observations concerning maternal allele Ndn expression. Alternatively or additionally, female-specific hormones could be involved.

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detectable by immunohistochemistry and by Western blot. Importantly, the absence of antibody staining on samples from \(-/-\) mice ruled out the possibility of cross-reactivity with proteins sharing epitopes with Necdin. Until relatively recently, it has been assumed that transcript abundance is the main, although not the only, determinant of protein abundance. Experiments aimed at addressing this question have lead to an emerging body of evidence changing this view and, in every organism that has been examined to date at a global level, steady-state transcript abundance only partially predicted protein abundance [38]. This lack of correlation suggests a strong regulatory role for all processes downstream of transcription. Furthermore, it has been shown that in many situations, transcription, translation and degradation are often extensively coupled and regulate each other through feedback loops. This coupling might enhance responsiveness to the environment and might help reduce inter-cellular variability in gene expression, which is by nature a stochastic event [39].

Collectively, these results suggest that very low level expression of PWS maternally silenced genes might be sufficient to alleviate specific PWS symptoms [23]. Importantly, we show that the quantity of Ndn transcripts is not, at least in neurons, a good indicator of its protein level and hence its functional importance [38].

An understanding of the context in which the Ndn maternal allele might be transcribed is an important step towards the development of a pharmacological therapy to trigger and/or increase the expression of this maternal allele in PWS patients. Furthermore, our results provide a further indication of the high non-genetic heterogeneity between genetically identical individuals that might, in this case, underlie LOI and contribute to variability in the phenotype [40].

Materials and Methods

Breeding of mice

Mice were handled and cared in accordance with the Guide for the Care and Use of Laboratory Animals (N.R.C., 1996) and the European Communities Council Directive of September 22th 2010 (2010/63/EU, 74). Experimental protocols were approved by the institutional Ethical Committee guidelines for animal research with the accreditation no. B13-055-19 from the French Ministry of Agriculture.

Ndn deficient mice were maintained on the C57BL/6J background and the paternal mutation was transmitted by crossing Ndn\(-/-\) males with C57BL/6J WT females (from Janvier Company). In parallel, since the Ndn-KO allele was created using a 129/SvPas ES cell line, we maintained the mutation via a paternal transmission on the 129/SvPas genetic background using Charles River male mice. All Ndn mice were genotyped by PCR as previously described [21]. Genotype of Ndn\(-/-\) mice was confirmed by a secondary intra-deletional PCR whose primers were: 5’-GATCGGAAAGGCGGAGACATG-3’ and 5’-CTGCCCATGACCTTTTTCG-3’ generating a 420 bp fragment indicating the presence of Ndn WT allele.

The Ndn ++ over-expressing mouse line is the Magel2 KO (+/m/−p and −/−) mouse line created previously in our team. Consequently, it is an over-expression of the endogenous Ndn gene rather than being a transgenic mouse. Magel2 being imprinted, closed to Ndn and belonging to the MAGE family gene, as Ndn. We observed this overexpression at the transcript and protein level and we estimated, by western blot quantification, the level of overexpression [a factor of 1.7 fold]. They were also maintained onto C57BL/6J background.

Importantly, all the mouse lines used in this study, excepted the Ndn\(tm2Stw\)-m/−p mouse line, have been created in our laboratory and maintained on pure genetic background. The Ndn\(tm2Stw\)-m/−p mouse have also been bred onto C57B/6 for over 30 generations in Wervick’s laboratory.3

Western blotting

P1 brain and E12 whole embryos were rapidly dissected and crushed in lysis buffer as previously [41]. For each sample, proteins (30 mg) were separated on a 12% SDS-PAGE and transferred onto nitrocellulose membranes (Protran Whatman, Dutscher). Membranes were incubated overnight with a rabbit polyclonal antibody against Necdin (Upstate; 1:1000) or with a rabbit polyclonal antibody against GFP (Sigma, G1544) and subsequently with an anti-rabbit horseradish peroxidase antibody (GE Healthcare, Buckinghamshire,UK; 1:3000). In both experiments, membranes were reprobed using a mouse anti-α-Tubulin antibody (Sigma, T6074). For Necdin immunolabeling was visualized by enhanced chemiluminescence. For GFP immunolabeling was visualized by Gbox (Syngen). Quantification was performed using ImageJ.

Plethysmography

We performed plethysmography in weight-matched littermate mice that were 6 weeks old, unrestrained and unanesthetized. Spontaneous breathing activities were recorded in normoxic conditions using whole-body plethysmograph (EMKA Technologies, Paris, France). After a 30 min period of stabilization in the apparatus, respiratory parameters were calculated breath-by-breath during a 30 min period of measurement. The mean of each parameter was automatically calculated from this 30 min period of measurement using EMKA technologies Datanalyst software. Apneas have been defined here as an absence of a respiratory signal during at least three respiratory cycles in resting conditions.

Reverse transcription and real-time quantitative PCR

Classical RT-PCR was performed as previously described [17]. For RT-qPCR, mice were sacrificed at E12.5, P1 or as adults. Whole embryos, whole P1 brains, P1 pons and P1 or adult hypothalamus tissues were rapidly collected and frozen in liquid nitrogen prior to RNA isolation using standard conditions. Subsequently, total RNA samples were incubated with DNase (TURBO DNA-free; Ambion). Messenger RNAs from 1 μg of total RNAs were reverse-transcribed in a total volume of 20 μL using the M-MLV, reverse transcriptase RNase H minus, point mutant (Promega) and oligod(T)15 in the presence of a synthetic external, heterologous and noncompetitive poly(A) Standard RNA (SmRNA) used to calibrate the reverse transcription [42] (patent WO2004.092414). At the end of the RT, total volume was brought up to 100 μL and real-time PCR was performed using the Rotorgene System (Qiagen) to determine the number of SmRNA and Ndn cDNA molecules in 5 μL of the RT product. The specific

Figure 6. NDN expression in PWS patients. Detection of NDN transcripts revealed by ISH, and using a NDN anti-sense probe, on PVN brain sections from control individuals (A,C,E) and PWS patients (B,D,F). A NDN-sense probe was used as a negative control. IHC on SON brain sections, using a NEDCIN specific antibody, was performed on the same control and PWS patients. The expression was studied in the 94-118 adult control male (A) and the 95104 adult PWS patient with a maternal uniparental disomy (B), in the 88-017 adult control male (C) and the 00-028 PWS adult patient with a deletion (D), in the 97-153 control infant (E) and the 99-079 PWS infant with a deletion (F). Scale bar: 20 μm.
forward and reverse primers were designed using “Universal Probe Library” software (Roche Diagnostics) in the region deleted in the Ndn KO-allele. The sequences of the primer pair used were: Necdin-Forward 5'-AAACAACGTTGACCCATGA-3', Necdin-Reverse 5'-CTTCCATGATAGGCTGCTG-3' (60 bp). The primer sequences and the quantification conditions of calibrator cDNAs (Standard cDNAs) are protected by the patent WO2004/092414. To discriminate specific from non-specific cDNA products, a melting curve was obtained at the end of each run, by a slow temperature elevation up to 98°C (0.1°C/s). Before RT, absence of traces of genomic DNA in the purified total RNA samples was ruled out by real-time PCR of the non-deleted Ndn sequence. Quantification cycles were converted into the number of cDNA copies using the quantification curve specific for each primer pair that had been previously established from serial dilutions of purified PCR products. The equation of the calibration curve for NDN cDNA was performed in four replicates for each dilution ranging from 10 to 1 × 10^8 copies: \( C_t = -3.3417 \log \text{[cDNA]} + 39.049, r^2 = 0.9988 \). No amplification was obtained in Ndn-/-/- individuals only. In the other mice, the lowest and highest copies number quantified were 92 and 4,638,062, respectively. For each sample, the number of Ndn cDNA copies was normalized according to relative efficiency of RT determined by the standard cDNA quantification. Finally, gene expression was expressed as the cDNA copy number quantified in 5 μl aliquot of RT product.

**Immunohistochemistry**

Specificity of Necdin protein detection was controlled on tissues from Ndn-/-/- animals. Fixed brains was dissected, cryopreserved and sectioned (14 μm) using a cryostat (Leica CM3050S). Embryos and post-natal mice (P1) were sacrificed and treated as previously [21]. Antibodies used were: rabbit polyclonal anti-Necdin (07-565; Millipore, Bedford, MA, USA; 1:500), mouse monoclonal anti-GFP (Interchim, NB600-597; 1:500), goat anti-Necdin (07-565; Millipore, Bedford, MA, USA; 1:500), mouse Alexa Fluor 488 (Molecular Probes, Invitrogen; 1/500), Alexa Fluor 555 (Molecular Probes, Invitrogen; 1/500), goat anti-mouse Alexa Fluor 488 (Molecular Probes, Invitrogen; 1/500), donkey anti-goat Cy3 (Chemicon, AP180C; 1/1000) diluted in the blocking buffer without BSA. Sections were examined on a Zeiss Axioplan 2 microscope with an Apotome module. Quantification of labeled cells was performed using ImageJ.

For quantification of immunofluorescence, images were acquired using a confocal microscope (SP5-X, Leica), z stacks of 70 μm were performed for each image, and analyzed using ImageJ.

**In Situ hybridization**

All Ndn in situ hybridization experiments for the study of Ndn gene expression were performed on serial slices of those used for immunohistochemistry and performed as previously [43]. Specificity of Ndn mRNA detection was controlled on tissues from Ndn-/-/- animals and with the sense control riboprobes. A peroxidase-conjugated anti-digoxigenin-POD (1:1250) antibody (Roche) was used to detect the Ndn hybridized riboprobe, visualized using a tyramide signal amplification (TSA-plus Biotin Kit, Perkin Elmer).

**Quantification of allele-specific expression**

We could identify two transcribed Single Nucleotide Polymorphisms (sSNPs) in the 3' UTR of Ndn to discriminate between the C57BL/6J and Mus spretus alleles.

To determine allele-specific transcriptional level, we performed QUASEP and RT-qPCR with allele-specific TaqMan probes on cDNA of C57BL/6J × Mus spretus F1 brains from P10–P14 pups. All RNA samples were treated with DNaseI (Agilent) to minimize any risk of contamination with genomic DNA. Subsequently, 2 μg of high-quality total RNA were reverse transcribed into cDNA (SuperScript III First Strand Synthesis System, Invitrogen) and oligo(dT)-priming according to manufacturer's instructions.

The QUASEP assays were designed using the PyroMark Assay Design Software 2.0 (Qiagen). PCR was performed with the FastStart High Fidelity PCR System (Roche) according to manufacturer’s recommendations using the cDNA of C57BL/6J × Mus spretus F1 brains from 6 P10–P14 mice. Pyrosequencing was done on a PSQ 96MA Pyrosequencing System (Qiagen) with a sequencing primer (Table S2) and PyroGold SQA reagents (Qiagen). Data were analyzed with the PSQ 96MA 2.1.1 software (Qiagen) as previously described [44].

For allele-specific RT-qPCR, we used the 5 bp indel in the 5' UTR of Ndn to design TaqMan probes specific for C57BL/6J and Mus spretus, respectively [45,46]. Quantitative PCR was performed on an ABI 7500 Fast Real time PCR System using the cDNA of C57BL/6J × Mus spretus F1 brains from 32 P10–P14 mice. Briefly, the 20 μl reaction contained 10 μl TaqMan Fast Universal PCR Master Mix (2×), 3.6 μl 5 μM combined forward (C57BL/6J): 5'-CTTCTCTCTGTCCTGCTC-3', Mus spretus: 5'-CTTCTCTCTGTCCTGCTC-3' and reverse (C57BL/6J): 5'-GGTGTCCTCAGTCCTTC-3', Mus spretus: 5'-GGTGTCCTCAGTCCTTC-3') primers (0.9 μM); 2 μl of each 2 μM TaqMan probe (C57BL/6J: FAM-CTCAGAGCCGG- CATCCGTTCTGCTG- BHQ1, Mus spretus: ATTO550- CTCACAGGGCATCGAGTCC- BHQ2; 0.2 μM) and 2.4 μl cDNA. The qPCR thermal profile consisted of 95°C for 10 min, followed by 48 cycles of 95°C for 30 s and 60°C for 30 s. Real-time PCR data were analyzed with ABI SDS 2.0.6 software.

**Methylation study**

Unfertilized oocytes and blastocysts were collected from C57BL/6 superovulated females and directly embedded in agarose beads for bisulphite treatment as previously described [47]. Sperm was recovered from the epididymis. Adult brain and kidney were dissected from interspecific M. spretus X M. musculus F1 mice or from Ndn+/- mice and DNA extracted according to standard techniques.

Bisulphite treatment of HindIII-digested adult brain, kidney and sperm genomic DNAs was carried out as described [48]. Oocyte and blastocyst DNAs were treated as described [47]. A semi-nested PCR was used to amplify regions A (CpG sites 1 to 20) and B (CpG sites 21 to 42) from bisulphite treated DNA samples. Primers used to amplify region A were: 5'-ATTGTTGATATGAAGATGAGTAGG-3' (outside forward; first and second rounds), 5'-AACCTACCTTAATACCTTC-3' (inside reverse) primers (0.9 μM); 2 μl of each 2 μM TaqMan probe (F1 brains from 32 P10–P14 mice. Briefly, the 20 μl reaction contained 10 μl TaqMan Fast Universal PCR Master Mix (2×), 3.6 μl 5 μM combined forward (C57BL/6J): 5'-CTTCTCTCTGTCCTGCTC-3', Mus spretus: 5'-CTTCTCTCTGTCCTGCTC-3' and reverse (C57BL/6J): 5'-GGTGTCCTCAGTCCTTC-3', Mus spretus: 5'-GGTGTCCTCAGTCCTTC-3') primers (0.9 μM); 2 μl of each 2 μM TaqMan probe (C57BL/6J: FAM-CTCAGAGCCGG- CATCCGTTCTGCTG- BHQ1, Mus spretus: ATTO550- CTCACAGGGCATCGAGTCC- BHQ2; 0.2 μM) and 2.4 μl cDNA. The qPCR thermal profile consisted of 95°C for 10 min, followed by 48 cycles of 95°C for 30 s and 60°C for 30 s. Real-time PCR data were analyzed with ABI SDS 2.0.6 software.

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Quantification of allele-specific expression**

We could identify two transcribed Single Nucleotide Polymorphisms (sSNPs) in the 3'-UTR of Ndn to discriminate between the C57BL/6J and Mus spretus alleles.
were cloned into the pGEM-T Easy TA Vector (Promega) and sequenced using standard methods. For oocytes and blastocysts, PCRs were performed on samples prepared from at least three different batches of oocytes and blastocysts. Identical clones derived from oocytes and blastocysts secondary PCRs were considered as derived from one single allele and represented only once.

Generation of transgenic mice with Ndn-eGFP modified BAC

The BAC603M20 (Research Genetics; referred as BAC109 [43]) contains a 104 kb Nol insert including the Ndn gene. BAC109 was modified by homologous recombination in E.coli as described [49] in order to replace the Ndn open reading frame (ORF) by the eGFP ORF.

Cesium chloride gradient purified BAC DNA was microinjected in the pronucleus of C57BL/6 x CBA mouse zygotes. Founders containing the BAC transgene were identified by amplifying an eGFP fragment by PCR. Transgenic founders were maintained on C57BL6 genetic background. Transgene copy numbers were determined by Southern blot of BglII digested genomic DNA hybridized with PCR probes.

Human material and studies

Hypothalamic material from 3 PWS patients and from controls, matched for age, sex, postmortem delay and fixation time were obtained through The Netherlands Brain Bank (NBB, Director Dr. I. Huitinga). Clinicopathological details are given in Supplementary Table S1.

Sections throughout the hypothalamus were collected at 1200 μm intervals and mounted and pretreated as previously [50], with 2 μg/ml of proteinase K.

For the detection of NDA mRNA we hybridized the sections with a 2000 ng/ml DIG-labeled RNA probe, complementary to bpl258-1578 of the human NDA mRNA (NM_002487.2). Hybridization and stringency washes were performed as previously described [51] at 60°C. Anti-DIG-Alkaline phosphatase-fab fragments [Roche], diluted 1:3000 in buffer 1 (100 mM Tris, 150 mM NaCl pH 7.5) were used to detect DIG labeled RNA hybrids [50]. Specificity of the hybridization signal was verified by comparison with sections processed with sense probe under identical conditions.

For IHC, 6 μm sections of hypothalamic tissue, containing SON, PVN, and INF were collected, mounted and microwaved in Citrate Buffer as previously [50]. Necdin was detected with rabbit IgG, anti-Necdin (07-565; Millipore, Bedford, MA, USA) diluted 1:500 in Supermix (SUMI: 0.25% gelatin [Merek] (w/v), 0.5% Triton X-100 in TBS, pH 7.6) for 1 hour at RT, followed by an overnight incubation at 4°C. Detection of Necdin immunoreactivity was performed according to the ABC method described before [52]. Antibody specificity was confirmed by the absence of ICC staining in the human hypothalamus after omission of the first antibody from the staining protocol.

Statistical analyses

Nonparametric statistical tools (Sigmastat software) or exact statistical tools (StatXact software) were used depending on the size of the sample (n). All tests are two-tailed tests. In the results, values are indicated as following: Mean±SD or (Q2 (Q1, Q3), n, P value) where Q2 is the median, Q1 is the first quartile and Q3 is the third quartile. Mann Whitney t-test or Wilcoxon-Mann-Whitney test (WMW in the text) are used. The level of significance was set at a P-value less than 0.05.

Supporting Information

Figure S1 Ndn expression analyzed by RT-PCR and Western blot. (A and B) Western-blots analysis on homogenates from P1 whole-brain (A) or from E12.5 embryos (B) using an anti-Necdin antibody and an anti-Tubulin antibody as positive control. Necdin-specific immunoreactivity (at 37 Kd) was visible in all WT and in four Ndn+/− p mice, while four Ndn+/− p and Ndn−/− mutants showed no Necdin expression (A,B). (C) RT-PCR analysis to detect a 562 bp fragment of Ndn transcripts in whole brain of WT, Ndn+/− p and Ndn−/− neonates. Note the complete absence of Ndn expression from the maternal allele in the neonatal brain using this approach. A positive control PCR was performed using Hprt primers in order to amplify a 429 bp fragment of Hprt transcripts (C, Hprt). (TIF)

Figure S2 Ndn expression in Ndn+/− E12.5 embryos. Expression of Ndn in the nervous system of WT and Ndn+/− embryos at E12.5 revealed by IHC or ISH on frozen sections using an anti-Necdin antibody (red) or Ndn RNA probe (green). Tissue sections are visualized using a Hoechst labeling (blue). Although expression is detected in WT embryos at the protein and transcript levels in the preoptic area (A), supraoptic area (B), thalamus (C), pons (D) and in the dorsal root ganglia (E), no transcripts or protein are detected in Ndn−/− embryos. Scale bar: 50 μm. (TIF)

Figure S3 Ndn expression in Ndn+/− E10.5 embryos. Expression of Necdin in the nervous system of WT and Ndn+/− Ndn embryos at E10.5 revealed by IHC or ISH on frozen sections using an anti-Necdin antibody (red) or a Ndn RNA probe (green). Tissue sections are visualized using a Hoechst labeling (blue). Expression is detected in WT embryos at the protein and transcript levels in the preoptic area (A), supraoptic area (B), thalamus (C), pons (D) and in the dorsal root ganglia (E). No expression is found in Ndn+/− p embryos (n = 9). Scale bar: 50 μm. (TIF)

Figure S4 Necdin expression in Ndn+/− p adult brains. Expression of Necdin in WT, Ndn+/− p and Ndn−/− adult brains revealed by IHC using an anti-Necdin antibody (in red) on coronal sections at the lateral septum level (A), nucleus of thalamus (B), accrete nucleus of hypothalamus (C), paraventricular hypothalamic nucleus (D) and suprachiasmatic nucleus (D). Scale bar: 500 μm (A), 250 μm (B), 200 μm (C and D). (TIF)

Figure S5 RT-qPCR shows an absence of Ndn maternal allele expression in wild-type mice. TaqMan probe-based RT-qPCR analysis for allele-specific quantification of the 5 bp indel polymorphism on brain cDNA samples of Mus musculus (C57Bl/6j) (A), Mus spretus (B) and two F1 hybrid offspring from a female Mus musculus X male Mus spretus cross (C and D). The blue curve and the horizontal blue line represent transcripts from the Mus musculus allele and the corresponding threshold C, value, the red curve and the horizontal red line transcripts from the Mus spretus allele and the corresponding threshold C, value. The results obtained for the brain cDNA samples of the other 30 F1 hybrid mice were identical to those shown in C and D. (TIF)

Figure S6 Expression of Ndn in the nervous system of wild-type and Ndn+/− p Ndn+2Stw embryos at E12.5. We performed IHC or ISH on frozen sections using an anti-Necdin antibody (in red) or Ndn RNA probe (in green). Tissue sections are visualized using a
Hoechst labeling (blue). An expression is detected at the protein and transcript levels in WT embryos only, in the septal area (A), preoptic area (B), thalamus (C), pons (D) and in the dorsal root ganglia (E). Note that there is no expression in Ndn<sup>tm1/m</sup>/+ embryos. Scale bar: 50 µm.

**Figure S7** BAC Ndn-eGFP transgene construction and analyses. Structure and copy number of the 603M20-deltaNdn-eGFP transgene in line TG45 (referred in the text to TG) and TG57 (not studied here but shown for comparison to TG45). (A) The Ndn genomic region and (B) 603M20-deltaNdn-eGFP transgene are represented on the upper diagonal. (C) Genomic DNA was isolated from WT or transgenic mice (lines 45 and 57), digested by BglII, separated by gel electrophoresis, blotted and hybridized to the probe 1. In line 45, the 4.4 kb transgenic and the 4.9 kb endogenous fragments detected after hybridization were of same intensity, indicating that the transgene was present in 1 or 2 copies although several copies are detected in line 57. Only the TG45 mouse line expresses the eGFP transgene and was used in our experiments.

**Figure S8** Quantitative analyses of eGFP expression and Necdin expression in the hypothalamus of WT TG+ mice. (A) Western blot analysis to quantify the eGFP expression relative to α-Tubulin in WT, Ndn<sup>tm1/m</sup>−/+ and, Ndn<sup>−/−</sup> hypothalamus of TG+ mice. The ratio of eGFP/α Tubulin (R) was calculated for each Ndn<sup>tm1/m</sup>−/+ individual and a mean (n = 5) is given for the WT and Ndn<sup>−/−</sup> genotypes. R is an indicator of the hypothalamic quantity of eGFP per genotype. (B,C) Immunofluorescence (green) of the Ndn-eGFP transgene and immunolabeling of the Necdin-positive cells (red) in the PVN (b) or the NSC (C). (D,E) Graph of the quantification of green and red fluorescence performed for each individual cell from the PVN (D) and NSC (E) brain structures.

**Figure S9** Methylation profile at the Ndn DMR. (A) Localization of CpG dinucleotides in relation to the Ndn transcriptional start site, translational start site and promoter is represented. The transcriptional start site is between CpG sites 10 and 11, and the translational start site (ATG) between CpG sites 16 and 17. (B) and (C) CpG dinucleotides are very sparsely methylated or completely unmethylated in regions A and B of the paternal alleles in brain (B) and kidney (C). In regions A and B, the maternal alleles display a much higher level of methylation, the average percentage of methylated CpG being 44% in region B in brain (D) and kidney (E) of WT mice as in brain of Ndn<sup>tm1/m</sup>−/+ mice (F). It should be noted that no particular bias of amplification in either region A or B of paternal versus maternal alleles was observed and that the maternal methylation profiles described were identical in interspecific M. spreitus × M. musculus F1 mice. The proportion of methylation at each CpG dinucleotide in a WT mouse clearly demonstrates that the level of methylation increases from the 5′ to the 3′ region of the CpG island (D,E). Analysis of the blastocyst DNA (G) showed an almost complete lack of methylation in region A and in region B. Although parental identity of sequenced alleles for blastocyst DNA could not be determined, the sequenced alleles are most likely derived from both parents since no bias of amplification was observed in adult tissues. In sperm DNA (H) and in ovulated oocytes (I), none of the 42 CpG dinucleotides were ever methylated, consistent with the complete absence of methylation in blastocysts and on the adult brain and kidney paternal alleles. The number of analyzed alleles is indicated (n).

**Table S1** Cinicopathological details of Prader-Willi Syndrome and control subjects. BMI, body mass index; M, male; NBB no, Netherlands Brain bank number; ND, not determined; PMID, post-mortem delay; SIDS, Sudden Infant Death Syndrome.

**Table S2** Sequence of the QUASEP primers.

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**Author Contributions**
Conceived and designed the experiments: VM UU RW UZ DS FM. Performed the experiments: AR FS UU VM FW ML BG JB FG SC FJM MB. Analyzed the data: VM UU RW UZ DS LB FM. Contributed reagents/materials/analysis tools: AR FS UU VM FW ML BG JB FG SC FJM. Wrote the paper: KD FM. Provided critical comments for the manuscript: VM UU RW UZ DS LB.

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