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Juliette Salles, Sanaa Eddiry, Saber Amri, Mélissa Galindo, Emmanuelle Lacassagne, et al.. Differential DNA methylation in iPSC-derived dopaminergic neurons: a step forward on the role of SNORD116 microdeletion in the pathophysiology of addictive behavior in Prader-Willi syndrome. *Molecular Psychiatry*, 2024, 29 (9), pp.2742-2752. <10.1038/s41380-024-02542-4>. <hal-04560462>

HAL Id: hal-04560462

<https://hal.science/hal-04560462v1>

Submitted on 9 Oct 2024

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Full title:

Differential DNA methylation in iPSC-derived dopaminergic neurons: a step forward on the role of *SNORD116* microdeletion in the pathophysiology of addictive behavior in Prader-Willi syndrome

Running Title: SNORD116 microdeletion in iPSC-derived dopaminergic neurons

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Differential DNA methylation in iPSC-derived dopaminergic neurons: a step forward on the role of *SNORD116* microdeletion in the pathophysiology of addictive behavior in Prader-Willi syndrome

Abstract

Introduction: A microdeletion including the *SNORD116* gene (*SNORD116* MD) has been shown to drive the Prader-Willi syndrome (PWS) features. PWS is a neurodevelopmental disorder clinically characterized by endocrine impairment, intellectual disability and psychiatric symptoms such as a lack of emotional regulation, impulsivity, and intense temper tantrums with outbursts. In addition, this syndrome is associated with a nutritional trajectory characterized by addiction-like behavior around food in adulthood. PWS is related to the genetic loss of expression of a minimal region that plays a potential role in epigenetic regulation. Nevertheless, the role of the *SNORD116* MD in DNA methylation, as well as the impact of the oxytocin (OXT) on it, have never been investigated in human neurons.

Methods: We studied the methylation marks in induced pluripotent stem-derived dopaminergic neurons carrying a *SNORD116* MD in comparison with those from an age-matched adult healthy control. We also performed identical neuron differentiation in the presence of OXT. We performed a genome-wide DNA methylation analysis from the iPSC-derived dopaminergic neurons by reduced-representation bisulfite sequencing. In addition, we performed RNA sequencing analysis in these iPSC-derived dopaminergic neurons differentiated with or without OXT.

Results: The analysis revealed that 153,826 cytosines were differentially methylated between *SNORD116* MD neurons and control neurons. Among the differentially methylated genes, we determined a list of genes also differentially expressed. Enrichment analysis of this list encompassed the dopaminergic system with *COMT* and *SLC6A3*. *COMT* displayed hypermethylation and under-expression in *SNORD116* MD, and *SLC6A3* displayed hypomethylation and over-expression in *SNORD116* MD. RT-qPCR confirmed significant

over-expression of *SLC6A3* in *SNORD116 MD* neurons. Moreover, the expression of this gene was significantly decreased in the case of OXT adjunction during the differentiation.

Conclusion: *SNORD116 MD* dopaminergic neurons displayed differential methylation and expression in the *COMT* and *SLC6A3* genes, which are related to dopaminergic clearance.

Key words: *SNORD116 MD*, iPSC-derived dopaminergic neurons, oxytocin, *SLC6A3*, *COMT*, dopamine clearance

Introduction

Prader-Willi syndrome (PWS) is a rare neurodevelopmental genetic imprinting disorder caused by the lack of expression of the paternal alleles in the q11-q13 locus of chromosome 15. PWS is characterized by a nutritional trajectory from anorexia at birth to hyperphagia in children and adults, frequently leading to severe obesity. The eating behavior encompasses compulsory food searching, storing, and hoarding, and a constant drive for food that is a major source of stress and anxiety for patients, families, and caregivers. In addition, these behaviors prevent socialization. For these reasons, the hyperphagia in patients with PWS could be described as an addiction-like behavior for food (1). Patients with PWS also present other compulsive behaviors—including tobacco use in adolescence to early adulthood—that have prompted health supervision guidelines (2). However, few data have linked addictive behaviors with PWS. A genetic hereditary influence (“nature”) has been identified in the development of addiction, as in twin studies, with genetic heritability found in 33-71% for nicotine dependence (3–6), 48-66% for alcohol dependence (7–9), and 49% for gambling (10). Polymorphisms in the dopamine system, such as the genes for receptor transporters and enzymes for dopamine, have been linked with addiction and obesity and have been discussed in other review articles (11–13). Nevertheless, genetic factors are not the exclusive prerequisites for the development of addiction, as the environment (“nurture”) also contributes. These factors include exposure to high-risk environments (typically, socially stressful environments with poor familial and traumatic early-childhood experiences) (14,15). Therefore, individual factors of resilience and vulnerability may contribute to the addiction framework and treatment (16), the environment being considered salient in influencing the phenotype acting at different levels, including gene expression via epigenetics. In humans, technological advances in epigenetics have now

provided novel descriptive information about the involvement of DNA methylation in addiction, including addiction-like eating behaviors (17,18).

With regard to the high-risk environments, which are typically related to caregiver-infant interactions, attachment theory postulates that early interactions give rise to caregiver-infant attachment, especially through the action of oxytocin (OXT), a neuropeptide produced by the hypothalamic paraventricular nucleus (PVN) neurons, whose terminated axons within the dopamine-rich brain regions may activate the dopaminergic reward pathways in response to social cues (19). OXT influences physiological and psychological processes by modulating brain sensitivity, playing a role in maternal and offspring bonding (20) and sociality and emotion regulation (21). In humans, recent studies for addiction treatment have suggested that intranasal OXT reduces the negative emotional consequences of abstinence and diminishes vulnerability to anxiety, stressors, and cue-induced relapse (22). Quite interestingly, PWS is also characterized by behavioral issues (23) including social deficits related to attachment impairment (24) and a decrease in OXT-producing neurons (25). Intranasal treatment for patients with PWS has been studied with contradictory results. Nevertheless, it seems that OXT treatment can be beneficial for patients with PWS in certain situations and potentially during a critical period of development (26). Our team especially demonstrated that intranasal OXT treatment administered to infants provided a positive effect on feeding and social skills, and administration to adults resulted in increased trust in others, decreased sadness tendencies and less disruptive behaviors (27,28).

We recently proposed that within the PWS locus, the *SNORD116* MD, which is considered the minimal critical region responsible for PWS, may serve as a model for the propensity toward addiction (29). This microdeletion was described by our team in a patient with PWS features and encompassed 3 genes: *SNORD 116*, *SNORD 109A* and *IPW* (30). Strikingly, the *SNORD116* gene encodes a small nucleolar RNA (snoRNA) and a long non-coding RNA (LncRNA); LncRNAs have been implicated in epigenetic regulation, including DNA methylation (31,32), whereas no data currently document such a role for snoRNAs. Therefore, in line with our understanding of the nature-nurture dichotomy associated with addictive behavior, we hypothesized that *SNORD116* MD could be involved in DNA methylation epigenetic regulation. The hypothesis of DNA methylation changes associated with the genes of the 15q11-q13 locus was reinforced by the results of our previous study, which we conducted in blood samples from a cohort of patients with PWS (including a patient with *SNORD116* MD) compared with controls. Indeed, we found that the PWS patients

displayed differential methylation in the genes involved in the neurodevelopmental and nutritional trajectories (33).

We now aim to further specify the relationship between genes of the 15q11-q13 locus and methylation epigenetic regulation, as well as its impact on RNA expression, in a cell model related to dopaminergic functioning in line with addiction pathophysiology. We therefore investigated DNA methylation and gene expression in the *SNORD116* MD, in iPSC-derived dopaminergic neurons. In addition, as explained above, OXT being potentially associated with an impact on neurogenesis and neural differentiation (34,35), we performed neuron differentiation with OXT added during the process in order to investigate the impact of this treatment on DNA methylation and RNA expression.

Methods

Cell line selection

As noted, we previously conducted a study from blood samples that included 7 patients with PWS displaying different mutations of 15q11-q13 (including *SNORD116* MD) and 2 controls, with the objective of identifying differentially methylated regions (DMRs) related to PWS (33). We also investigated the clustering of methylation patterns between the PWS patients and controls and found consistent clustering according to control or PWS phenotype (33). This result confirmed the significant effect of the absence of expression of the genes of the 15q11-q13 locus (all-encompassing the *SNORD116* MD) in PWS, which appeared to be independent of the individual genetic backgrounds of the patients. Therefore, in the present study we focused on the microdeletion, *SNORD116* MD, obtained from a patient whose clinical features regarding addiction patterns are detailed in supplementary material S1.

Fibroblast reprogramming to iPSC and differentiation into neurons

Subjects were recruited from a clinical research program (Clinical trial NCT01298180; n°EudraCT 2008-004612-12) approved by the CPP Toulouse 1 Ethics Committee, informed consent was obtained from all subjects. Primary human fibroblasts from an unaffected control (adult matched for age and gender adjusted from Waldhorn et al. 2022 (36)) and a patient with *SNORD116* MD were differentiated into neurons using the method previously described (37). In brief, iPSC cultures were routinely retained on mouse embryonic fibroblasts (MEFs) (Globalstem) and cultured in human embryonic stem neuron (ES) media. iPSCs were differentiated into neurons using a modified SMAD inhibition protocol from fibroblasts from

PWS patients and control patients (38). Neurons were harvested between days 28 and 32 for DNA and RNA extraction. *TH*, *GIRK2*, *DRD2*, *MAP2* and *B3TUB*, which are markers for dopaminergic neurons from midbrain (39–41), were tested to validate the neural differentiation. Four neuron lines were differentiated, half in standard conditions, and in other differentiations we added OXT 1 μ M to the culture medium every day from day 14 to day 28-32.

DNA methylation analysis

The DNA was extracted from the iPSC-derived neurons using the Quick-DNATM Miniprep Plus Kit (D4068 & D4069). We prepared libraries using the RRBS sample preparation kit from Diagenode (Cat. No. C02030032 or C02030033) according to the manufacturer's instructions. Briefly, 100 ng of genomic DNA were digested using MspI for 12 hours at 37°C. DNA fragments were end-repaired, and Diagenode indexed adapters were ligated. DNA was quantified using the KAPA Library Quantification Kit (Roche, Basel, Switzerland) prior to pooling 6 samples. Each pool was incubated with sodium bisulfite and purified using the EZ DNA Methylation Lightning Kit (Zymo Research; Cat. No. D5030). The optimal PCR cycle number was determined by qPCR (KAPA Library Quantification Kit, Roche, Basel, Switzerland) before the final enrichment PCR. PCR products were purified using AMPure XP Beads (Beckman Coulter Genomics, Brea, CA, USA). The size distribution of the resulting libraries was monitored using a Fragment Analyzer (Agilent Technologies, Santa Clara, CA, USA) and the libraries were quantified using the KAPA Library Quantification Kit (Roche, Basel, Switzerland). The libraries were denatured with NaOH, neutralized with Tris-HCl, and diluted to 100 pM. Clustering and sequencing were performed on a NovaSeq 6000 (Illumina, San Diego, CA, USA) using the single-end 100 nt protocol on one lane of a flow cell S1. Image analyses and base calling were performed using NovaSeq Control Software and the Real-Time analysis component (Illumina). Demultiplexing was performed using Illumina's conversion software (bcl2fastq 2.20). The quality of the raw data was assessed using FastQC (v0.11.8) from the Babraham Institute and Illumina software SAV (Sequencing Analysis Viewer). FastqScreen (v0.14.0) was used to identify potential contamination. The rate of non-conversion ranged between 0.4% and 1.24%, indicating that the conversion stage was efficient. The percentage of pass-filter (PF) sequences compared to raw clusters was greater than 79%.

RNA expression analysis

Total RNA was extracted using TrIZol® reagent (Invitrogen), according to the manufacturer's instructions. The quality and integrity of the obtained RNA were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies) after a denaturing step at 70 °C for 2 minutes.

RNA sequencing: Sample quality controls and library preparations were performed at the GeT-Santé facility (Inserm, Toulouse, France, get.genotoul.fr). RNA concentration and purity were determined using a ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was checked with a Fragment Analyzer (Agilent Technologies, Santa Clara, CA, USA), using the RNA Standard Sensitivity Kit. 260/280 purity ratios were all > 1.8, and integrity indices revealed good values (8.6-10 RIN and > 1.6 28S/18S ratios). RNA-seq paired-end libraries were prepared according to Illumina's protocol with some adjustments, using the TruSeq Stranded Total RNA Library Prep Gold Kit (Illumina, San Diego, CA, USA). Briefly, between 230 and 600 ng of total RNA were first ribo-zero-depleted using Illumina Ribo-Zero probes. Library quality was assessed using the HS NGS kit on the Fragment Analyzer (Agilent Technologies, Santa Clara, CA, USA). Library quantification and sequencing were performed at the GeT-PlaGe core facility (INRAE, Toulouse, France). Libraries were quantified by qPCR using the KAPA Library Quantification Kit (Roche, Basel, Switzerland) to obtain accurate quantification. Libraries were equimolarly pooled and RNA sequencing was then performed on one S4 lane of the Illumina NovaSeq 6000 instrument (Illumina, San Diego, CA, USA), using the NovaSeq 6000 S4 v1.5 Reagent Kit (300 cycles) and a paired-end 2 x 150 pb strategy. This resulted in 2 billion raw paired-end reads, for an average of 2 x 127 million reads per sample.

qRT-PCR: RNA was reverse transcribed into cDNA with the Super Array RT2 First Strand Kit (SABiosciences, Qiagen) according to the manufacturer's instructions. Amplification of the cDNA was conducted in a Light Cycler 480 detection system (LC480, Roche Applied Science) using an RT2 profiler PCR array (SABiosciences, Qiagen) according to the manufacturer's instructions. The qRT-PCR results were analyzed with sequence detection software from Light Cycler 480 (Roche Applied Science). Primers were validated with a 5-point standard curve.

Primer sequences:

- COMT: Fr ACGCTTCTCTTGGAGGAATGTGG/ Rv TCACGTTGTCAGCCAGTAGCAC
- SLC6A3: Fr: TCTATGCGGCCTACAAGTTCTGC/ Rv: ATGGCGTAGGCCAGTTTCTCTC

GAPDH was used as a housekeeping gene. The data shown represent the mean $\Delta\Delta Ct$ with the respective standard deviation when $\Delta\Delta Ct = Ct \text{ gene of interest} - Ct \text{ GAPDH}$. The data represent results from several independent experiments as indicated in “n” mention.

Bioinformatics and statistical analysis

For the RRBS data analysis, Trim Galore v0.6.0 (Babraham Bioinformatics), which uses Cutadapt v2.7 (42), was used to perform adapter trimming and quality control of raw reads (--rrbs option). Bismark v0.22.1 (Babraham Bioinformatics) (43) was used to align the trimmed RRBS reads to the human hg38 genome, and the methylation call for every single cytosine was retrieved with the bismark_methylation_extractor module. Differential methylation analysis was performed using the methylSig (v1.1.0) (44) Bioconductor R package (R v3.6.1) on CpG island cytosines. A site-specific analysis on CpG sites and a tiled analysis on 200 bp windows were performed. A cytosine or region is called differentially methylated if the adjusted p-value (according to the Benjamini-Hochberg multiple testing correction) is below 5% and the difference of methylation above 25%. Differentially methylated CpGs were annotated by the closest gene using bedtools closest tool (v2.29.2). The gene annotation was retrieved from UCSC on 2020-02-25 and NCBI gene IDs from gene2refseq.gz file (NCBI; 2020-02-25). The refgene_getlocationsummary from Cisgenome (v1.2) was used to annotate the CpGs with the genome region types (exon, intron, promoter region, etc.). The Gene Ontology (GO) analysis was conducted on the Kyoto Encyclopedia of Genes and Genomes (KEGG) (45) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) (46) .

For RNA sequencing data, normalization was made by DESeq2, which relies on the hypothesis that most features are not differentially expressed. This normalization was performed with locfunc="median". For the fold change template, the design was counts ~ batch + group to estimate the models' coefficients, which can be interpreted as $\log_2(FC)$. A generalized linear model (GLM)-based method (fitType="parametric") was applied for the statistical procedure. RRBS and RNA sequencing cross-match analyses were made with genes from RRBS that displayed more than 2 differentially methylated cytosines (DMCs), a significant FDR, and a mean methylation rate $\geq 25\%$ or $\leq -25\%$, and with genes from RNA sequencing that displayed a positive p-value and an RNA fold change ≥ 2 or ≤ 0.5 .

Results

Genome-wide methylation analysis (GWMA) of differentially methylated cytosines (DMCs) in iPSC-derived dopaminergic neurons differentiated without OXT

Without OXT, we found 153,826 cytosines differentially methylated between control neurons and *SNORD116* MD neurons, with 61% in intragenic regions. The complete description is presented in S2a.

The enrichment analysis performed from the gene list revealed that 21 pathways were modified between *SNORD116* MD neurons and control neurons. Among them, 11 (52%) were relevant to PWS features, including pathways related to endocrine processes, neurodevelopmental processes, addictive pathways and the OXT signaling pathway. As an illustration, the heatmap of genes related to OXT and dopaminergic pathways is provided in S3 and the complete list of pathways is detailed in S4.

Genome-wide methylation analysis (GWMA) of differentially methylated cytosines (DMCs) in iPSCs-derived dopaminergic neurons differentiated with OXT

We found that after OXT exposure, 239,411 cytosines were differentially methylated between control neurons and *SNORD116* MD neurons, with 62% of the DMCs in intragenic regions. A complete description is presented in S2b.

When comparing *SNORD116* MD dopaminergic neurons differentiated without or with OXT, we found that 290,009 cytosines were differentially methylated, with 63% in intragenic regions. When comparing control dopaminergic neurons differentiated without or with OXT, we found that 222,438 cytosines were differentially methylated, with 61% in intragenic regions. We performed an enrichment analysis with the genes associated with DMCs within the *SNORD116* MD group (iPSC-derived neurons differentiated with OXT compared with those differentiated without OXT). This analysis showed that a large number of pathways (72 pathways) were modified, depending on the OXT adjunction. We performed the same analysis in the control (iPSC-derived neurons differentiated with OXT compared with those differentiated without OXT). We found that fewer pathways were modified (47 pathways). When we compared the pathways modified in *SNORD116* MD and control, we noted that 45 pathways were common to both, 29 were exclusively found in *SNORD116* MD and 3 were exclusively found in control.

The enriched pathways are detailed in S5. The dopaminergic synapse was one of the pathways exclusively found in *SNORD116* MD, suggesting a specific interaction of OXT with *SNORD116* MD for the gene related to dopamine synapse functioning. Figure 1 presents the changes in methylation levels observed in the genes related to this pathway in *SNORD116* MD iPSC-derived neurons and the changes observed for the same genes in the control iPSC-derived neurons.

The list of the 200 genes with the highest methylation differences is provided in S6 for differentiations performed without OXT and in S7 for differentiations performed with OXT.

Differences in methylation and RNA expression

We selected genes from the RRBS and RNA sequencing databases by following the process described in the methods section. We then crossed the databases to determine the overlap between RRBS and RNA sequencing selected genes.

In the differentiation without OXT, we found that 162 genes were both differentially methylated and differentially expressed (the list of genes is provided in S8). The Venn diagram describes the overlaps between the RNA expression and DNA methylation (Figure 2A). The scatterplot of RNA expression and DNA methylation found significant negative correlations between the DNA methylation level and the RNA gene expression ($r=-0.96$; p value= $4.1e-10$) (Figure 2B). We performed enrichment analysis based on the 162 genes overlapping DNA methylation analysis and RNA sequencing in the differentiations performed without OXT, which revealed that dopamine clearance from the synaptic cleft and neuronal system were part of the 7 significant pathways found in enrichment. These pathways namely contained the solute carrier family 6-member 3 (*SLC6A3*) gene and the catechol-O-methyltransferase (*COMT*) gene.

In the differentiations performed with OXT, 232 genes were both differentially methylated and differentially expressed (the list is provided in S9). The Venn diagram describes the overlap between the expression and methylation (Figure 2C). The scatterplot of RNA expression and DNA methylation found significant negative correlations between the DNA methylation level and the RNA gene expression ($r=-0.41$; p value= 0.001) (Figure 2D). We performed an enrichment analysis based on the 252 genes overlapping DNA methylation analysis and RNA sequencing in the differentiations performed with OXT. This analysis identified the signal transduction pathways displaying the proopiomelanocortin (*POMC*) gene, whereas the melanocortin pathway is highly related to appetite regulation in eating disorders including in PWS (47).

Genes potentially involved in addiction-related PWS features

SLC6A3 gene

In the differentiation without OXT, 14 CpGs were found in the *SLC6A3* gene. Three CpGs in exons were hypermethylated in *SNORD116* MD. The others were positioned in the gene promoter and hypomethylated in *SNORD116* MD (Table 1). In the differentiation with OXT, 6 CpGs were found to be hypermethylated in *SNORD116* MD and located in exons or introns of *SLC6A3* (Table 2).

In the absence of OXT during differentiation, RNA expression was 3.6 times higher in iPSC-derived neurons with *SNORD116* MD (81 versus 22; p-value=0.03). In the presence of OXT during differentiation, RNA expression remained 3-fold higher in *SNORD116* MD neurons (18 versus 6; p-value=0.1). OXT significantly decreased the expression in iPSC-derived neurons in both *SNORD116* MD (p-value=0.04) and control (p-value<0.01) (Figure 3A).

RT-qPCR analysis found significantly higher expression in *SNORD116* MD versus control in differentiation without OXT (1.6 versus 0.27, p=0.03). The expression level was significantly lower in differentiation performed with OXT in *SNORD116* MD iPSC-derived neuron lines (2.3 versus 0.29, p=0.04). In control iPSC-derived neuron lines, *SLC6A3* expression was not significantly different between differentiations performed with or without OXT (Figure 3B).

COMT gene

In the differentiation performed without OXT, we found 11 CpGs, all hypermethylated in *SNORD116* MD. The CpGs were positioned in the gene promoter (Table 1). In the differentiation performed with OXT, 15 CpGs were found; one CpG was hypermethylated in *SNORD116* MD and another in the control line. The CpGs were positioned in the gene promoter (Table 2).

In the absence of OXT during differentiation, RNA expression was 15.1 times lower in neurons with *SNORD116* MD (180 versus 2728; p-value=9.9E-25). In the presence of OXT during differentiation, RNA expression remained 16 times lower in *SNORD116* MD neurons (142 versus 2305; p-value=2.8E-08) (Figure 3A).

RT-qPCR analysis found significantly lower expression of *COMT* (relative quantification strategy) in *SNORD116* MD versus control in differentiation without OXT (0.08 versus 0.18, p=0.09). The level of expression was not different in differentiation with OXT (Figure 3B).

POMC gene

In the differentiation performed without OXT, 4 CpGs were in the *POMC* gene. Half the CpGs were hypermethylated in *SNORD116* MD. The CpGs were positioned in the gene exons (Table 1). In the differentiation with OXT, 9 CpGs were found in *POMC*. Seven CpGs were hypermethylated in *SNORD116* MD and located in the promoter region. The others were in introns and half were hypermethylated. For this gene, we found no relevance between the modifications with OXT treatment observed in each neuron line (*SNORD116* MD and control) and the modifications observed by comparing *SNORD116* MD and control (Table 2). In the absence of OXT during differentiation, *POMC* RNA expression was not significantly different. In the presence of OXT during differentiation, *POMC* RNA expression was 10.5 times higher in *SNORD116* MD neurons (42 versus 4; p-value=0.02). We found a significant difference in RNA expression between *SNORD116* MD neuron lines differentiated without OXT and those differentiated with OXT. RNA expression of the *POMC* gene was 3.3 times higher in the lines differentiated with OXT (Figure 3A). As the level of RNA expression was not different in the differentiation without OXT, we did not perform complementary RT-qPCR analysis.

For the 3 genes, S10 details the position of methylated CpG and the level of methylation. S10a presents the analysis conducted in control and *SNORD116* MD iPSC-differentiated dopaminergic neurons for differentiations performed without OXT and S10b for differentiations performed with OXT.

Discussion

Our study investigated the correlations between *SNORD116* MD, DNA methylation and RNA expression in iPSC-derived neurons compared with control, in differentiation performed with and without OXT.

The GWMA analysis revealed that *SNORD116* MD was associated with methylation modifications in 153,826 cytosines. We performed enrichment analysis with genes associated with these DMCs and found consistent results with the enrichment analysis performed in our PWS blood cohort (33). Indeed, we found that 4 (66%) pathways were common to the 6 KEGGs identified in blood. We then selected a list of genes associated with these cytosines that overlapped both the differential DNA methylation and the differential RNA expression and conducted an enrichment analysis from this gene list. This analysis attested to the dopaminergic

clearance pathway among the results, including *SLC6A3* and *COMT*. We thus focused on these genes, and doing so was in line with our pathophysiology hypothesis (29).

SLC6A3 was hypomethylated in the promoter region and over-expressed in *SNORD116* MD iPSC-derived neurons. RNA sequencing results were confirmed by RT-qPCR analysis, which found significant differences in gene expression in *SNORD116* MD versus control. In addition, RT-qPCR also confirmed the lower gene expression level in *SLC6A3* in differentiation performed with OXT versus that performed with OXT in *SNORD116* MD—a result that was confirmed by RT-qPCR. This suggests that *SLC6A3* expression could be modulated by OXT. The *SLC6A3* gene codes for a dopaminergic transporter (DAT) that has been related to addiction (48) and attention deficit/hyperactivity disorder (ADHD) (49,50), and it is the target of psychoactive drugs such as methylphenidate and amphetamines, which are used in the treatment of ADHD symptoms, as well as being drugs of abuse (51,52). A study identified hypermethylation of the *SLC6A3* gene promoter and an upregulation of the DAT expression in patients with eating disorders, especially anorexia nervosa (53). The human *SLC6A3* gene is highly sensitive to epigenetic regulation, in line with the enrichment in GC nucleotides in the promoter-proximal region and the presence of multiple CpG sites in the entire gene. However, the epigenetic regulation of the *SLC6A3* gene is still not well understood (54). Recent developments in transcription mechanisms have uncovered the formation of liquid-phased condensate containing various proteins including RNAs for gene transcription. In line with this, known transcription factors of the *SLC6A3* gene may contain intrinsically disordered regions, along with lncRNA, and are well positioned to contribute to such a condensate. This *SLC6A3* condensate engages both enhancers and silencers (55).

COMT was hypermethylated in the promoter region and under-expressed in *SNORD116* MD iPSC-derived neurons. This result was observed in differentiation with and without OXT. The same tendency as noted in the RNA sequencing results was observed in RT-qPCR analysis, which found differences in gene expression in *SNORD116* MD versus control, although not reaching a significant p-value. The *COMT* gene codes for an enzyme involved in dopamine degradation, and the expected consequences of the under-expression of *COMT* are therefore an increase in the concentration of dopamine (or its metabolites) to neurotoxic levels. This gene predisposes to self-medicating via any substance or behavior—such as alcohol, nicotine, psychostimulants, opiates, sex, excessive eating, gambling, and gaming—that will activate dopamine release (56). It has also been suggested that it might play a role in psychiatric disorders such as ADHD (57,58) and borderline personality disorder (59,60), 2 disorders associated with emotional dysregulation and impulsivity and expected to develop addictive

behaviors (61,62). In addition, it was reported that the administration of inflammatory stimulant led to decreased COMT protein expression (63).

GWMA analysis revealed that the OXT adjunction also had an influence on the methylation marks. The modifications induced by OXT adjunction in biological pathways were substantial in both *SNORD116* MD and control iPSC-derived neurons. However, we noted that some modifications were specific to *SNORD116* MD, especially for the dopaminergic synapse pathway. Moreover, in the list of genes overlapping both the DNA methylation and RNA sequencing databases, we noted the mention of *POMC*, which appeared especially relevant to PWS features in *SNORD116* MD compared with control. *POMC* encodes a preproprotein that undergoes extensive, tissue-specific, and post-translational processing via cleavage by subtilisin-like enzymes known as prohormone convertases. In the hypothalamus, POMC neurons suppress appetite, but this observation seemed irrelevant to the increase in expression observed in our study, given that PWS patients display hyperphagia. Nevertheless, our observation of the increased expression of *POMC* concerned iPSC-derived dopaminergic neurons, and the effect of an over-expression of *POMC* in dopaminergic neurons is still almost unknown. A previous study in rat found POMC RNA in the ventral tegmental area (VTA), but this RNA was in low concentration, ranging from 21% to 31% with respect to the hypothalamus (64). In addition, there is evidence for a direct function of the melanocortin system in the VTA to modulate body weight; indeed, the introduction of α -MSH into the VTA induced an increase in dopamine in the NAc (65). However, it is possible that other cleavage products of the POMC peptide, such as ACTH, β -endorphin or γ -MSH, may mediate the observed physiological effect either directly in the VTA or through projections to other brain regions (66).

Despite interesting results, we should note the limitations of our work. First, there are still questions concerning the validity of the iPSC model to study epigenetic marks; indeed, an epigenetic memory has been suggested for some neuron lines (67). Nevertheless, in our study we chose to compare iPSCs coming from the same cell type (fibroblast). Moreover, other studies have underlined that the variations in the methylome could be related to individual genetic factors and recommended multiplying the number of subjects more than the number of clones (68). As to *SNORD116* MD, we unfortunately had only one individual as this is an extremely rare mutation. This point could raise questions about a possible role of the genetic background in our results. We nevertheless conducted this work following a previous study that focused on DNA methylation in a cohort of patients with PWS. The results in this cohort showed that the patient with *SNORD116* MD clearly clustered with the other PWS patients presenting other mutations, different deletions, or uniparental disomy (33). To complete this

published data, we performed additional analysis on this blood dataset including principal component analysis (PCA). PCA on the entire cohort demonstrated that all PWS patients clustered distinctly apart from the control subjects (S11a). The same analysis applied to PWS patients alone showed that all PWS patients (including the patient with the *SNORD116 MD*) clustered, except one (S11b). In addition, DMCs were also found in this blood database for *COMT*, *SLC6A3* and *POMC* (S11c). This suggests that the absence of expression of genes in the 15q11-q13 locus (including the *SNORD116 MD*) in all these patients with PWS is mainly responsible for the effect of differential DNA methylation observed in the whole genome analysis. Finally, we examined the expression of *COMT* and *SLC6A3* using the dataset provided by Eddiry et al. 2021 (69), which included primary fibroblasts from three patients with PWS, including those with the *SNORD116 MD* and two other deletions, in comparison to four control subjects. Our analysis revealed a significant increase in the expression of *SLC6A3* within the PWS group, further reinforcing the assertion that the deregulation of *SLC6A3* is linked to the absence of gene expression in the 15q11-q13 locus. Regarding *COMT*, our analysis showed a decreased expression in the PWS group; however, these findings did not reach statistical significance. This makes it challenging to entirely rule out the possibility that *COMT* deregulation may be influenced by the presence of distinct allelic variants in the two cell lines (S11d). It is therefore inferred that *SNORD116 MD* may constitute an accurate model to investigate the epigenetic abnormalities observed in PWS patients. In addition, the *SNORD116 MD* iPSC cell line that we used had already been described in previous published studies demonstrating the stability of the imprinting region after reprogramming (37). Moreover, in our data we found that DNA methylation marks (including those in the promoting region) emerged during the differentiation. For the control line, we also studied neurons from a single subject and, although this choice was made to ensure gender- and age-matching, it is a limitation of our work. In addition, for the same individual, iPSC random stochastic methylation reprogramming has been described on specific regions (70), and we cannot exclude the possibility that the iPSC reprogramming induced artifacts as described for GABA genes (70). Nevertheless, for the genes presented in this study, we observed that differential methylation compared with control appeared during the differentiation. We chose the model of dopaminergic neurons to be in line with the model of addiction and reward pathways. Yet, this choice might have limited our results and their interpretation. Last, by using the EpiFactors database (71) we found that epigenetic regulators are present in differentially expressed genes related to *SNORD116 MD*, and we therefore cannot exclude the possibility that differential methylation was related to indirect mechanisms.

The strength of this work is the proposal of a translational approach using the iPSC model to test a pathophysiological hypothesis. We found that the correlation between DNA methylation and RNA expression was relevant for some genes. Among them, we identified the *SLC6A3* and *COMT* genes, which previously showed interactions to control a complex phenotype (activation of the reward system) (72) and influence neural activation during response inhibition (73), as well as significant interactions related to eating psychopathology and binge eating (74), which thus may be related to our initial hypothesis (29). Figure 4 displays an incrementation of the current results to the postulated hypothesis. Moreover, these genes could be related to psychiatric disorders, including neurodevelopmental disorders like ADHD or addictive disorders, which are also relevant with our model. These observations raise perspectives for future research to better characterize the role of the *SNORD116* MD, especially in psychiatric cohorts. This is all the more likely given that recent data indicated the role of snoRNA in psychiatric disorders, including for treatment response (75).

In conclusion, these results suggest that the *SNORD116* MD is associated with epigenetic methylation regulation in reward systems, in line with the hypothesis of a role of sensitization for this gene regarding addictive behaviors in neurodevelopment. The more striking data concern the methylation and expression of the *COMT* and *SLCA3* genes, which seem promising and should encourage further research on the *SNORD116* MD, especially the *SNORD116* gene.

Data availability

The RNA sequencing data reported in this article were deposited in the NCBI's Gene Expression Omnibus (GEO) database: [GSE249891](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE249891)

Acknowledgments

This work was supported by a grant from the French Association for Prader-Willi Syndrome (grant R15062BB).

MGX acknowledges financial support from the France Génomique National infrastructure, funded as part of “Investissement d’Avenir” program managed by the Agence Nationale pour la Recherche (contract ANR-10-INBS-09).

Conflict of Interest

The authors declare they have no conflict of interest.

Author Contributions

JS, MT, and JPS developed the initial study design. NF, IG, and FJ provided feedback to improve the design. JS, SA, EL, MG and SE performed the cell culture and RT-qPCR. XA and GS performed DNA methylation and related bioinformatics analysis. EL performed RNA sequencing and related bioinformatic analysis. JS and MT performed the combined statistical analysis. JS, MT, JPS and SE wrote the initial manuscript. All authors contributed to improving the manuscript and responding to the reviewers.

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Figure and table legends

Figure 1. A heatmap representation of the changes in methylation levels observed in genes related to the dopaminergic synapse pathway observed in SNORD116 MD iPSC-derived neurons differentiated with and without OXT and the changes observed for the same genes in the control iPSC-derived neurons differentiated with and without OXT. The colors correspond to the mean level of methylation of all cytosines related to the mentioned gene.

Figure 2A. Venn diagram of the differentially methylated genes and the differentially expressed genes in SNORD116 MD neuron lines differentiated without OXT. 2B. Venn diagram of the differentially methylated genes and the differentially expressed genes in SNORD116 MD neuron lines differentiated with OXT. 2C. A scatterplot in genes with both differential methylation and expression (SNORD116MD vs control) in differentiation without OXT. The log-fold changes in methylation levels (x-axis) vs the log-fold changes in gene expression (y-axis). 2D. A scatterplot in genes with both differential methylation and expression (SNORD116MD vs control) in differentiation with OXT. The log-fold changes in methylation levels (x-axis) vs the log-fold changes in gene expression (y-axis).

*Figure 3A. Comparison of RNA expression between SNORD116 and control iPSC-derived dopaminergic neurons differentiated with and without oxytocin. Data are presented as mean \pm standard deviation. * Indicates significant difference (p -value $<$ 0.05). 3B. RT-qPCR on SLC6A3 gene showed higher gene expression in SNORD116 MD versus control in differentiation without OXT and a decrease*

in RNA expression in differentiation performed with OXT. **3B.** RT-qPCR on COMT gene showed a lower gene expression in SNORD116 MD versus control in differentiation without OXT. No significant changes were found in differentiation with OXT ($n=3$ for SNORD116 and $n=3$ for control). * p -value <0.05 , + p -value <0.1 . Center values are mean and error bars are s.e.m.

Figure 4. Effect of the SNORD116 deletion on gene expression in dopaminergic neurons differentiated from iPSC. The left part of the figure is adapted from the perspective article of Salles et al. (32) with implemented results of the present study. In the right part of the schema, we reported the genes modified and their suggested effects on the system. Abbreviations: Reward sens.: reward sensitivity, COMT: catechol-O-methyltransferase, DOPA: dopamine, SLC6A3: solute carrier family 6 member 3, ~OXT: effect of OXT treatment

Table 1. Position of methylated CpG and level of methylation when we compared control and SNORD116 MD iPSC-differentiated dopaminergic neurons without OXT. The values consist in the mean of methylation on the CpG position for total number of differentiations as specified in Methods. Significant threefold was $p<0.05$.

Table 2. Position of methylated CpG and level of methylation when we compared control and SNORD116 MD iPSC-differentiated dopaminergic neurons with OXT. The values consist in the mean of methylation on the CpG position for total number of differentiations, as specified in Methods.

Tables

Table 1

Gene	CpG position	Position	Methylation level SNORD116 MD	Methylation level Control	Methylation difference	p-value
COMT	19941880	Promoter	100	6.15	93.85	1.28E-41
COMT	19941885	Promoter	86.32	6.15	80.17	7.84E-27
COMT	19941888	Promoter	95.79	4.62	91.17	1.53E-36
COMT	19941890	Promoter	100	6.15	93.85	2.08E-41
COMT	19941905	Promoter	100	6.15	93.85	1,28E-41
COMT	19941939	Promoter	100	0	100	2,49E-95
COMT	19941944	Promoter	100	0	100	2,49E-95
COMT	19941994	Promoter	89.84	11.54	78.3	6,83E-49

COMT	19941999	Promoter	92.51	39.23	53.28	9,7E-26
COMT	19942017	Promoter	90.37	16.15	74.22	5,76E-44
COMT	19942074	Promoter	100	16.88	83.12	2,82E-56
POMC	25161334	Exon	65	100	-35	1,48E-11
POMC	25161381	Exon	98.73	70.37	28.36	5,55E-08
POMC	25161395	Exon	40	79.27	-39.27	0,000000222
POMC	25161587	Exon	100	72.5	27.5	0,0000506
SLC6A3	1393762	Exon	100	50	50	1,58E-08
SLC6A3	1420648	Exon	100	70.71	29.29	7,92E-10
SLC6A3	1420673	Exon	83.12	57.58	25.54	0,00021
SLC6A3	1445374	Promoter	4.17	47.83	-43.66	0,000045
SLC6A3	1445376	Promoter	0	40	-40	0,0000158
SLC6A3	1445386	Promoter	0	47.83	-47.83	0,00000127
SLC6A3	1445389	Promoter	0	47.83	-47.83	0,00000127
SLC6A3	1445395	Promoter	0	47.83	-47.83	0,00000189
SLC6A3	1445400	Promoter	0	47.83	-47.83	0,00000189
SLC6A3	1445402	Promoter	0	47.83	-47.83	0,00000189
SLC6A3	1445417	Promoter	0	69.57	-69.57	4,8E-10
SLC6A3	1445422	Promoter	0	47.83	-47.83	0,00000189
SLC6A3	1445427	Promoter	0	47.83	-47.83	0,00000189
SLC6A3	1445434	Promoter	0	47.83	-47.83	0,00000189

Table 2

Gene	CpG position	Position	Methylation level SNORD116 MD	Methylation level Control	Methylation difference	p-value
COMT	19962713	Exon	9.38	38.46	-29.08	0,000129
COMT	19962727	Promoter	100	74.36	25.64	2,15E-07
COMT	19962740	Promoter	38.89	75.31	-36.42	0,00361
COMT	19962749	Promoter	61.11	100	-38.89	0,00000026
COMT	19962800	Promoter	38.89	70.37	-31.48	0,0134

COMT	19941939	Promoter	100	19.88	80.12	9,02E-88
COMT	19941940	Promoter	83.45	18.82	64.63	9,34E-23
COMT	19941944	Promoter	100	19.88	80.12	9,02E-88
COMT	19941945	Promoter	100	3.53	96.47	2,66E-60
COMT	19941994	Promoter	98.03	19.39	78.64	7,83E-78
COMT	19941995	Promoter	92.09	3.53	88.56	1,15E-45
COMT	19941999	Promoter	100	19.88	80.12	9,02E-88
COMT	19942000	Promoter	87.05	3.53	83.52	6,46E-40
COMT	19942017	Promoter	90.13	19.88	70.25	2,81E-55
COMT	19942018	Promoter	100	3.53	96.47	2,66E-60
POMC	25168555	Promoter/ Enhancer	27.27	0	27.27	7,16E-09
POMC	25168575	Promoter/ Enhancer	25.42	0	25.42	1,74E-08
POMC	25168412	Promoter/ Enhancer	56.67	0	56.67	1,71E-16
POMC	25168381	Promoter/ Enhancer	30.88	0	30.88	3,91E-08
POMC	25168379	Promoter/ Enhancer	63.24	0	63.24	5,28E-18
POMC	25168343	Promoter/ Enhancer	28.33	0	28.33	1,75E-07
POMC	25168303	Promoter/ Enhancer	30.88	0	30.88	3,25E-08
POMC	25168142	Intron	17.65	45.65	-28	0,00264
POMC	25168141	Intron	52.02	25.12	26.9	5,96E-17
SLC6A3	1394489	Exon	68.09	100	-31.91	0,0000359
SLC6A3	1394501	Exon	68.09	100	-31.91	0,0000359
SLC6A3	1394542	Exon	61.7	100	-38.3	0,00000417
SLC6A3	1411252	Exon	50	100	-50	1,41E-15
SLC6A3	1411318	Exon	50	93.04	-43.04	9,68E-09
SLC6A3	1442944	Exon	69.57	100	-30.43	0,0000323

hsa04728 Pathway: Dopaminergic synapse







