Pharmacological Treatment for Neonatal Abstinence Syndrome is Associated with Altered DNA Methylation and Neurobehavior

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List of Abbreviations:

CpG: cytosine-phosphate-guanine

DNAm: DNA methylation

NAS: Neonatal abstinence syndrome

NNNS: NICU Network Neurobehavioral Scales

Abstract

Objective: To determine whether pharmacological treatment for neonatal abstinence syndrome (NAS) is associated with changes in DNA methylation (DNAm) of the mu-opioid receptor gene (*OPRM1*) and improvements in neonatal neurobehavior.

Study Design: Buccal swabs were collected from 37 neonates before and after morphine treatment for NAS. Genomic DNA was extracted and DNAm was examined at four CpG sites within the *OPRM1* gene. The NICU Network Neurobehavioral Scales (NNNS) was also performed before and after NAS treatment. Changes in DNAm ($\text{DNAm}_{\text{post-tx}} - \text{DNAm}_{\text{pre-tx}}$) and NNNS summary scores ($\text{NNNS}_{\text{post-tx}} - \text{NNNS}_{\text{pre-tx}}$) were then calculated. Path analysis was used to examine associations among pharmacologic treatment (length of treatment and total dose of morphine), changes in DNAm, and changes in NNNS summary scores.

Results: DNAm significantly decreased from pre- to post-treatment at 1 of 4 CpG sites within the *OPRM1* gene. Neonates also demonstrated decreased excitability, hypertonia, lethargy, signs of stress and abstinence, and increased quality of movement and regulation from pre- to post-treatment. Increased length of treatment and higher morphine doses were associated with greater decreases in DNAm; greater decreases in DNAm were associated with greater decreases in excitability and hypertonia on the NNNS.

Conclusions: Pharmacological treatment of NAS is associated with decreased DNAm of the *OPRM1* gene and improved neonatal neurobehavior. Epigenetic changes may play a role in these changes in neonatal neurobehavior.

Introduction

The prevalence of prenatal opioid exposure increased by 333% from 1999 to 2014 in the United States [1]. This has been accompanied by a steep rise in the incidence of neonatal abstinence syndrome (NAS), from 1.2 to 8.8 per 1,000 hospital births between 2000 and 2016 [2–6]. NAS describes a constellation of signs in the neonate including irritability, inconsolable, high-pitched crying, hypertonia, feeding difficulty, tremors, and gastrointestinal dysfunction (e.g., vomiting, loose stools) [2].

The most common pharmacological treatment for NAS is morphine, with a minority of neonates treated with other opioid replacements [7]. Pharmacological treatment for NAS is associated with improved neurobehavior as assessed by the NICU Network Neurobehavioral Scales (NNNS)[8,9]. Specifically, neonates show improvements in orientation, quality of movement, excitability, hypertonia, and signs of stress (e.g., high pitched cry) over the course of pharmacological treatment. NNNS summary scores have also been linked to maternal buprenorphine dose and have been found to differentiate between neonates who require pharmacological treatment for NAS and those that do not [10,11].

The average length of stay (LOS) for NAS is approximately 12 days [7]. However, there is significant variability in the severity of NAS, including the amount (e.g., total morphine dose) and type(s) of pharmacologic treatment (e.g., adjunctive medication) needed. Genetic and epigenetic factors likely account for some of this variability [12]. Specifically, epigenetic variation in the mu opioid receptor (*OPRM1*) gene has been linked to NAS severity [13,14]. It is unknown whether pharmacological treatment for NAS is associated with changes in DNA methylation (DNAm) of *OPRM1* and whether these changes are associated with improvements in neurobehavior commonly observed following pharmacologic treatment for NAS. Given that

increased methylation of *OPRM1* is associated with more severe NAS, we hypothesized that pharmacologic treatment for NAS would result in decreased DNAm and improvements in neonatal neurobehavior.

Methods

Participants

This study included 37 mothers treated with methadone (43%) or buprenorphine (57%) for an opioid use disorder. Neonates were treated with morphine for NAS at Women and Infants Hospital (Providence, RI). Neonates who exceeded a predetermined opioid dose also received phenobarbital. Exclusion criteria included gestational age < 37 weeks and evidence of sepsis, major congenital anomalies, or genetic disorders. The mean gestational age of the infants was 38.9 ± 1.1 weeks and mean birth weight was 3011 ± 382 g. The study was approved by the Institutional Review Board at each site and study investigators obtained informed consent.

Measures

Neonatal Neurobehavior was assessed shortly after birth and at hospital discharge by trained examiners using the NNNS, a standardized neurobehavioral exam that includes neurological integrity, motor and behavioral function, and signs of stress and abstinence [15]. It has been used previously in studies of prenatal drug exposure [8,16–19]. The NNNS results in 12 summary scores (e.g., quality of movement, hypertonia, excitability) including stress/abstinence, and has been used previously in studies of prenatal drug exposure [8,16–19].

DNA was sampled from buccal swabs collected immediately following the NNNS exam shortly after birth and at hospital discharge. Quantitative DNA methylation was measured as previously described [20]. Briefly, genomic DNA was extracted from pooled saliva samples using QIAmp DNA Mini Kits (Qiagen, Inc.) following manufacturer's protocols and purified

DNA was used for quantitative methylation analysis by pyrosequencing. DNA samples (1µg) were bisulfite-modified using the EZ DNA methylation Kit (Zymo Research, Irvine, CA) following the manufacturer's protocol. Pyrosequencing was performed on a Qiagen Q48 pyrosequencing platform on PCR product amplified from bisulfite-modified DNA using assay reagents from EpigeneDx (Hopkinton, MA) for 4 CpG sites in the *OPRM1* promoter at positions (-93, -90, -80, -71). The sequence interrogated was CGGCGAAAGGAAGCGGCTGAGGCG. Dilution series of fully methylated referent DNA into fully unmethylated referent DNA were run with each assay to assess the performance characteristics of the assay.

Statistical Analyses

Data analysis proceeded in three steps. First, paired samples t-tests determined whether there was significant change in DNAm at the 4 CpG sites within *OPRM1* before and after pharmacologic treatment. Next, paired samples t-tests were used to test whether there was significant improvement in NNNS summary scores before and after pharmacologic treatment. Finally, path analysis was used to test the full mediated pathway from NAS treatment (i.e., length of treatment [LOT], and total dose of morphine) to change in DNAm of *OPRM1*, to change in neurobehavior. All path models were run using full-information maximum likelihood to account for missingness in any outcome variables. We adjusted t-tests for multiple testing by using the Benjamini-Hochberg procedure. Results remained significant after imposing a false discovery rate (FDR) of 10%.

Results

Descriptive statistics for mothers and neonates are shown in Table 1. The sample was split in terms of prenatal exposure to methadone (43%) or buprenorphine (57%). A majority of neonates (75%) had concurrent exposure to maternal tobacco use. Information about NAS

treatment is shown in Table 2. Neonates were treated for an average of 14.2 days (95% CI = 12.7 – 15.8 days) and the average total dose of morphine administered was 13.2 mg (95% CI = 10.5 – 15.9 mg). About half (56%) of neonates were also treated with phenobarbital.

DNAm before and after NAS treatment

DNAm significantly decreased from pre- ($M = 1.37\%$) to post-treatment ($M = 0.92\%$) at one CpG site (-71), $t(36) = -2.09$, $p = .04$ (Table 3).

Neurobehavior before and after NAS treatment

Neonates exhibited significant decreases in excitability, hypertonia, lethargy, and signs of stress/abstinence and significant increases in quality of movement and regulation from pre- to post-treatment (all $p < .05$; Table 3).

Relationship between changes in DNAm and neurobehavior

We investigated whether changes in DNAm at the -71 CpG site were related to changes in NNNS summary scores. Neonates with larger decreases in DNAm at the -71 CpG site had larger decreases in excitability ($r = .40$, $p = .02$) and hypertonia ($r = .36$, $p = .05$) and greater increases in regulation ($r = -.40$, $p = .05$).

NAS Treatment, DNAm, and NNNS outcomes

A series of path models were tested linking NAS treatment to changes in NNNS scores (Δ NNNS) via changes in DNAm (Δ DNAm). Because there was significant change in 6 NNNS subscales (excitability, hypertonia, lethargy, quality of movement, regulation, and stress/abstinence), only these measures were examined. Similarly, we only included DNAm at 1 CpG site (-71) that significantly changed pre- to post-treatment.

Greater LOT ($\beta = -.29$, $p = .04$) and total morphine dose ($\beta = -.31$, $p = .04$) were associated with greater decreases in DNAm at the -71 CpG site in all models (Table 4). In the

LOT model for hypertonia, there was a significant association between LOT and Δ DNAm ($\beta = -.29, p = .04$) and between Δ DNAm and Δ NNNS ($\beta = .37, p = .001$). These results indicate that greater LOT was associated with greater decreases in DNAm at the -71 CpG site and greater decreases in DNAm were in turn associated with greater decreases in hypertonia. The indirect path from LOT to Δ DNAm to Δ NNNS was not significant ($\beta = -.11, p = .09$).

A similar pattern emerged in the morphine models. For excitability, greater morphine dose was associated with greater decreases in DNAm ($\beta = -.31, p = .04$) and greater decreases in DNAm were associated with greater decreases in excitability ($\beta = .40, p = .005$). The indirect path from morphine dose to Δ DNAm to Δ NNNS was significant ($\beta = -.13, p = .04$). For hypertonia, results were similar to the LOT model. Greater morphine doses were associated with greater decreases in DNAm ($\beta = -.31, p = .04$). Greater decreases in DNAm were associated with greater decreases in hypertonia ($\beta = .45, p < .001$). The indirect path from morphine dose to Δ DNAm to Δ NNNS was significant ($\beta = -.14, p = .05$).

Discussion

The overall goal of this study was to examine whether pharmacologic treatment for NAS was associated with changes in DNAm of *OPRM1* and whether changes in DNAm were associated with improvements in neonatal neurobehavior. DNAm significantly decreased from pre- to post-treatment in one CpG site within *OPRM1*. Neonates also demonstrated significant improvements in multiple aspects of neurobehavior (e.g., hypertonia, regulation, signs of stress/abstinence) following pharmacologic treatment for NAS. Path models provide support for DNAm as a pathway that may be associated with treatment-related improvements in neonatal neurobehavior. Our findings are novel in that they provide evidence for changes in DNAm

following pharmacologic treatment for NAS and highlights the potential mechanistic role of DNAm in treatment-related improvements in neurobehavior.

NAS is a highly variable condition whose severity is influenced by a host of factors, including type of antenatal maternal opioid use as well as neonatal genetic and epigenetic factors [12,19]. DNAm of the *OPRM1* gene has been shown to be higher in opioid-exposed versus opioid-naïve neonates [21]. Other studies have demonstrated that greater DNAm of *OPRM1* in both mothers and neonates is associated with greater need for pharmacologic treatment for NAS and with a more severe phenotype [13,14]. These studies also demonstrated that hypermethylation (e.g., gene silencing) was associated with the most severe NAS phenotypes. These findings suggest that DNAm of opioid receptor genes is potentially altered by exposure to opioids *in utero*, with increased DNAm playing a role in the severity of NAS.

Our findings support the hypothesis that pharmacologic treatment for NAS may be beneficial by decreasing DNAm of *OPRM1*, albeit only at one CpG site. The CpG site identified in this study (-71) is different from the CpG sites that have been identified in prior studies as being related to NAS severity (e.g., -18, -14, -10, +23, +84) [13,14]. Most of these previously-identified CpGs are located within putative transcription factor binding sites, meaning that increased DNAm of these sites may be associated with decreased gene expression [22,23]. Increased DNAm of the *OPRM1* gene has been shown to decrease protein expression of mu-opioid receptors which could increase the need for pharmacologic treatment of NAS symptoms [13,24]. Despite being located outside of a transcription factor binding site, the -71 CpG site is the most proximal of all investigated CpG sites to the *OPRM1* promoter region. This suggests that differences in DNAm at this site may have increased biological relevance compared to other sites we investigated.

Since prenatal opioid exposure is associated with greater DNAm of *OPRM1*, the decrease in DNAm of the -71 CpG site suggests that pharmacologic treatment may ‘correct/reverse’ altered patterns of DNAm [21]. Just as greater DNAm is associated with increased NAS severity via reduced gene expression, decreases in DNAm following pharmacologic treatment may be associated with increased gene expression and the amelioration of NAS. The exact mechanism by which changes in DNAm of *OPRM1* impact gene expression and the severity of NAS remains to be determined, but these findings provide additional support that epigenetic mechanisms are involved (directly or indirectly) in explaining the efficacy of pharmacologic treatment. The extent to which treatment-related changes in DNAm persist and whether they differ as a function of the specific drug treatment (e.g., morphine, buprenorphine) requires further study.

Neonatal neurobehavior has been extensively studied in relation to prenatal opioid exposure, with studies documenting differences in NNNS summary scores based on opioid exposure and type and dose of maternal opioid (e.g., methadone versus buprenorphine) [10,11,16,17]. More recently, NNNS scores have been shown to differentiate neonates with high- and low-severity of NAS [25]. Neonates with severe NAS were more likely to have a neurobehavioral profile characterized by poor regulation, high arousal, hypertonicity, and physiological signs of stress and abstinence. Pharmacologic treatment for NAS has been associated with improvements in neonatal neurobehavior [8,9] and lower rates of infant mortality [26]. Indeed, the current study confirmed that there were improvements in neonatal neurobehavior following pharmacologic treatment for NAS. Given that atypical NNNS profiles have been linked to abnormal long-term developmental outcomes, our findings suggest that neonates who show positive changes in NNNS post-treatment may also have better long-term outcomes [25,27].

Path analysis models suggested that changes in DNAm may underlie the association between pharmacologic treatment for NAS and improvements in neonatal neurobehavior. Neonates who exhibited greater treatment related decreases in DNAm also showed the most significant improvements in excitability and hypertonia. These findings suggest that pharmacologic treatment for NAS is associated with changes in *both* DNAm and neurobehavior which provides preliminary support for epigenetics as a putative mechanism underlying the efficacy of pharmacologic treatment for NAS. Additionally, a growing number of studies have shown that DNAm may be one predictor of individual differences in neonatal neurodevelopment [28,29]. By linking changes in DNAm to neonatal neurobehavior, this study provides stronger support for the putative role of DNAm as a determinant of neurobehavior.

Several limitations of this study should be noted. First, our small sample size may have limited our power to detect small changes in DNAm and neurobehavior, or to detect relatively small associations between DNAm and neurobehavior. However, the significant associations reported are of medium to large effect sizes meaning that they may have increased practical significance [30]. Nonetheless, study findings should be confirmed in a larger cohort of neonates. Second, we were unable to compare changes in DNAm to an untreated comparison sample and cannot definitively conclude that changes in DNAm are solely due to pharmacologic treatment. It is possible that changes in NNNS and potentially in DNAm may be due to maturation, rather than pharmacologic treatment. Since neonates had DNAm and NNNS measured before and after treatment and because neonates treated longer were more likely to receive greater doses of morphine, both LOT and morphine dose are confounded by chronological age. Teasing apart the impact of treatment versus time would require selecting a comparison group(s) such as opioid exposed and untreated or unexposed neonates. Neonates exposed to opioids *in utero* who are

treated for NAS likely differ in many ways from unexposed neonates and from those exposed to opioids who do not develop NAS. These difficulties may therefore not be easily addressed in future studies.

In conclusion, pharmacologic treatment for NAS is associated with decreased DNAm of *OPRM1* and improved neonatal neurobehavior. These findings suggest that changes in DNAm may be one mechanism explaining how pharmacologic treatment for NAS improves neonatal neurobehavioral outcomes.

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Table 1

Maternal and Neonatal Characteristics

Variable	N	M (95% CI) or N (%)
Maternal age (years)	37	29.4 (27.8 – 31.0)
Med Assisted Treatment		
Methadone	37	16 (43%)
Buprenorphine	37	21 (57%)
Concurrent Exposures		
Cigarette Smoking	36	27 (75%)
Alcohol	37	1 (3%)
Benzodiazepines	35	4 (11%)
SSRIs	35	6 (7%)
Male sex	37	20 (54%)
Caucasian	36	31 (86%)
Gestational age (weeks)	37	38.9 (38.5 – 39.2)
Birth weight (grams)	37	3,011 (2,884 – 3,138)
Head circumference (cm)	37	34.0 (33.6 – 34.4)
Feeding		
Breastfed (any)	37	21 (57%)
Formula only	37	16 (43%)

Table 2

NAS treatment

Variable	N	M (95% CI) or N (%)
Length of treatment (days)	37	14.2 (12.7 – 15.8)
Length of stay due to NAS (days)	37	17.2 (15.7 – 18.8)
Maximum Finnegan score	37	11.6 (11.0 – 12.2)
Morphine, total dose (mg)	37	13.2 (10.5 – 15.9)
Phenobarbital use	37	21 (57%)

Table 3

DNA methylation and neurobehavior before and after pharmacological treatment

Variable	Pre-tx	Post-tx					
	M(SD)	M(SD)	df	t	Raw p	Adj p	Cohen's D
<i>DNA methylation (OPRM1)</i>							
-93	1.54 (1.12)	1.51 (1.37)	36	-0.10	0.92	0.92	0.02
-90	1.57 (0.90)	1.42 (1.40)	36	-0.57	0.57	0.66	0.09
-80	1.27 (0.95)	1.57 (1.81)	36	0.96	0.34	0.46	0.16
-71	1.37 (1.08)	0.92 (1.07)	36	-2.09	0.04*	0.09*	0.34
<i>Neurobehavior</i>							
Arousal	3.76 (0.82)	4.09 (0.92)	28	1.88	0.07	0.13	0.34
Asymmetric Reflexes	0.10 (0.40)	0.03 (0.18)	28	-0.81	0.42	0.53	0.15
Attention	3.51 (1.23)	4.37 (1.37)	12	1.30	0.22	0.33	0.36
Excitability	4.27 (2.46)	2.78 (2.52)	29	-2.42	0.02*	0.05*	0.44
Handling	0.62 (0.20)	0.61 (0.19)	26	0.21	0.84	0.90	0.04
Hypertonia	1.50 (1.22)	0.34 (0.55)	29	-5.46	< 0.01*	< 0.01*	0.99
Hypotonia	0.17 (0.46)	0.03 (0.18)	29	-1.44	0.16	0.27	0.25
Lethargy	6.33 (2.47)	4.22 (2.20)	29	-3.55	< 0.01*	< 0.01*	0.66
Quality of Movement	3.97 (0.56)	4.72 (0.62)	27	5.23	< 0.01*	< 0.01*	0.99
Regulation	4.60 (0.82)	5.44 (0.86)	24	3.61	< 0.01*	< 0.01*	0.72
Stress/Abstinence	0.15 (0.06)	0.11 (0.08)	29	-2.37	0.02*	0.06*	0.50

Note. *denotes p-values that are significant with a 10% false discovery rate (FDR).

Table 4

Summary of Path Coefficients

Path	β(SE)	β(SE)	β(SE)	β(SE)
Length of Treatment (LOT)	LOT $\rightarrow$ DNAm	DNAm $\rightarrow$ NNNS	LOT $\rightarrow$ NNNS	LOT $\rightarrow$ DNAm $\rightarrow$ NNNS
Excitability	-.29*	.24+	-.34*	-.07
Hypertonia	-.29*	.37***	.09	-.11+
Lethargy	-.29*	-.31*	-.19	.09
Quality of Movement	-.29*	-.20	.10	.06
Regulation	-.29*	-.26	.24	.07
Stress/Abstinence	-.29*	.08	-.43*	-.02
Morphine, total dose (MOR)	MOR $\rightarrow$ DNAm	DNAm $\rightarrow$ NNNS	MOR $\rightarrow$ NNNS	MOR $\rightarrow$ DNAm $\rightarrow$ NNNS
Excitability	-.31*	.40**	.06	-.13*
Hypertonia	-.31*	.45***	.27	-.14+
Lethargy	-.31*	-.37*	-.36**	.12+
Quality of Movement	-.31*	-.32+	-.19	.10
Regulation	-.31*	-.35	.03	.11
Stress/Abstinence	-.31*	.22	-.10	-.07

Note. * $p < .05$, ** $p < .01$, *** $p < .001$