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Prenatal opioid exposure alters pain perception and increases long-term health risks in infants with neonatal opioid withdrawal syndrome

Uppala Radhakrishna[1,2]*, Rupa Radhakrishnan[3], Lavanya V. Uppala[4], Tithi S. Trivedi[5], Jignesh Prajapati[6], Rakesh M. Rawal[7], Srinivas B. Muvvala[8], Ray O. Bahado-Singh[2] and Senthilkumar Sadhasivam[1]

[1]Department of Anesthesiology and Perioperative Medicine, University of Pittsburgh, Pittsburgh, PA, United States, [2]Department of Obstetrics and Gynecology, Corewell Health William Beaumont University Hospital, Royal Oak, MI, United States, [3]Department of Radiology and Imaging Sciences, Indiana University School of Medicine, Indianapolis, IN, United States, [4]Department of Pharmacology & Neuroscience, School of Medicine, Creighton University, Omaha, NE, United States, [5]Department of Botany, Bioinformatics and Climate Change Impacts Management, School of Sciences, Gujarat University, Ahmedabad, Gujarat, India, [6]Department of Biochemistry & Forensic Sciences, Gujarat University, Ahmedabad, India, [7]Department of Medical Biotechnology, Gujarat Biotechnology University, Gandhinagar, Gujarat, India, [8]Department of Psychiatry, Yale School of Medicine, New Haven, CT, United States

Background: Opioids are often prescribed for pain relief, yet they pose risks such as addiction, dependence, and overdose. Pregnant women have unique vulnerabilities to opioids and infants born to opioid-exposed mothers could develop neonatal opioid withdrawal syndrome (NOWS). The study of opioid-induced epigenetic changes in chronic pain is in its early stages. This study aimed to identify epigenetic changes in genes associated with chronic pain resulting from maternal opioid exposure during pregnancy.

Methods: We analyzed DNA methylation of chronic pain-related genes in 96 placental tissues using Illumina Infinium Methylation EPIC BeadChips. These samples comprised 32 from mothers with infants prenatally exposed to opioids who needed pharmacologic NOWS management (+Opioids/+NOWS), 32 from mothers with prenatally opioid-exposed infants not needing NOWS pharmacologic treatment (+Opioids/-NOWS), and 32 from unexposed control subjects (-Opioids/-NOWS).

Results: The study identified significant methylation changes at 111 CpG sites in pain-related genes among opioid-exposed infants, with 54 CpGs hypomethylated and 57 hypermethylated. These genes play a crucial role in various biological processes, including telomere length regulation (*NOS3, ESR1, ESR2, MAPK3*); inflammation (*TNF, MAPK3, IL1B, IL23R*); glucose metabolism (*EIF2AK3, CACNA1H, NOTCH3, GJA1*); ion channel function (*CACNA1C, CACNA1H, CLIC4, KCNQ5*); autophagy (*CTSS, ULK1, ULK4, ATG5*); oxidative stress (*NGF, NRG1, OPRM1, ATP1A2*); aging (*GRIA1, NGFR, PRLR, EIF4E*); cytokine activity (*TRPV4, RUNX1, CXCL8, IL18R1*); and the risk of suicide (*ADORA2A, ANKK1, GABRG2, IGSF9B*). These epigenetic changes may influence 48 signaling pathways—including cAMP, MAPK, GnRH secretion, estrogen signaling, morphine addiction, circadian rhythms, and insulin secretion—profoundly affecting pain and inflammation-related processes.

Conclusion: The identified methylation alterations may shed light on pain, neurodevelopmental changes, and other biological mechanisms in opioid-exposed infants and mothers with OUD, offering insights into NOWS and maternal–infant health. These findings may also pave the way for targeted interventions and improved pain management, highlighting the potential for integrated care strategies to address the interconnected health of mothers and infants.

Introduction

Chronic pain, also known as persistent pain, is a complex condition influenced by physical injuries, underlying medical conditions, and neurological issues (1). It can result from injuries, post-surgery complications, cancer, and pregnancy-related discomfort (2). Pain is categorized into nociceptive, neuropathic, and hyperalgesia types. Nociceptive pain stems from tissue damage like injuries or inflammation, felt as sharp or aching, that persists longer than the normal healing time (3). Neuropathic pain, frequently persistent, emerges from nerve system damage, characterized by enduring burning sensations observed in conditions like diabetic neuropathy lasting beyond 3 months. Hyperalgesia, marked by heightened pain sensitivity to even mild stimuli, can occur locally or globally due to factors like injury, inflammation, or certain neurological conditions (4–6). Pain management uses diverse medications and therapies, both prescription and nonprescription, to ease severe pain (7). Opioids are recognized for their pain-relieving properties and euphoric effects, making them susceptible to misuse (8).

Prolonged opioid use during pregnancy can induce genetic and epigenetic alterations in both mother and fetus, increasing addiction vulnerability and impacting neurodevelopment (9–13). These changes may contribute to birth defects, miscarriage, premature birth, low birth weight, respiratory and feeding issues, maternal health risks, NOWS, and future behavioral challenges (14–16).

The relationship between epigenetic dysregulation and persistent pain is an evolving research domain, with ongoing studies enhancing our understanding of its impact on pain tolerance (17). Recent studies have highlighted the role of epigenetic changes in chronic pain (18), particularly through DNA methylation, which influences genes regulating pain signaling, inflammation, and stress responses (19). The relationship between chronic pain and DNA methylation is bidirectional. Pain can induce DNA methylation changes that affect pain sensitivity, creating a complex feedback loop that perpetuates chronic pain states (20). Opioids alleviate pain by acting on the central nervous system, rather than directly influencing pain-related genes (21). Chronic pain is influenced by a complex interplay of factors, including inflammation, nerve sensitization, changes in the central nervous system, genetics, epigenetics, environmental influences, and gender differences

(22, 23). These factors can significantly impact pain perception, tolerance, and the effectiveness of pain management strategies, as well as the side effects experienced by individuals.

This study aims to identify key gene networks and epigenetic changes associated with opioid-associated pain dysregulation through a genome-wide analysis of methylation and transcriptomic data. The findings may provide a foundation for advancing pain management strategies.

Materials and methods

Patients diagnosed with OUD were identified based on the criteria outlined in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) (24). Demographic and clinical-pathological data, such as age, sex, ethnicity, gestational age, and history of drug exposure, were gathered and this information has been previously published (25, 26). The study received approval from the Institutional Review Board of Beaumont Health System, Royal Oak, MI, USA (HIC#: 2019-086). Pregnant women were identified retrospectively via chart review from William Beaumont Hospital, Royal Oak, MI. Informed consent was waived since the study solely involved collecting discarded placental tissues and obtaining limited de-identified demographic data from hospital records.

The diagnosis of NOWS, coded as P96.1, was established by neonatologists following clinical criteria. Newborns born to mothers with a history of opioid or illicit drug use underwent monitoring in the inpatient unit for 4–5 days to detect signs of NOWS. Assessment of the infant's condition was conducted using the Finnegan Neonatal Abstinence Scoring Tool (FNAST), administered by postpartum and/or NICU nurses. If the FNAST scores indicated the need for pharmacologic intervention, the infant was transferred to the NICU for continued monitoring, scoring, and treatment. Throughout the treatment process, parental involvement was encouraged to optimize non-pharmacologic approaches, which remained the primary focus before and during pharmacologic treatment, regardless of the need for medication. The FNAST scoring system guided decisions regarding the initiation of pharmacologic management with morphine.

A total of 96 formalin-fixed, paraffin-embedded (FFPE) placental tissue biopsies were processed for DNA extraction.

These samples were categorized into three distinct groups for analysis. Group 1 comprised 32 placental tissue samples from newborns exposed prenatally to opioids and requiring pharmacologic treatment for NOWS (+Opioids/+NOWS). Group 2 consisted of 32 placental tissue samples from newborns prenatally exposed to opioids but not requiring pharmacologic treatment for NOWS (+Opioids/-NOWS). Lastly, Group 3 served as the control, comprising placental tissue samples from newborns with no prenatal opioid exposure and no NOWS (-Opioids/-NOWS, control).

Illumina infinium methylationepic BeadChip

We performed genome-wide DNA methylation analysis on bisulfite-treated genomic DNA using the Illumina Infinium EPIC BeadChip (850 K) according to the manufacturer's protocol at the Beaumont Health Genomics Core Facility. The detailed methodology has been previously described (25, 26). To ensure the integrity of our microarray experiments, sample placement on arrays was randomized for both cases and controls age and gender (27). The EPIC array encompasses over 850,000 individual CpG sites across the genome at a single-nucleotide resolution, encompassing multiple genes linked to pain. Following scanning with Illumina iScan scanners, intensity data (iDAT) files were imported into Illumina's Genome Studio methylation analysis package program.

Statistical and bioinformatic analysis

The IDAT files containing data were normalized using Genome Studio software's functional normalization method to ascertain Cytosine methylation levels (represented as ß-values) for each CpG site. Before analysis, CpG-probes with missing ß-values were excluded. Differential methylation was evaluated by comparing the ß-values of cytosines at individual CpG loci between NOWS and control groups. To minimize confounding variables, probes targeting sex chromosomes, non-specific regions, and CpG sites within 10 base pairs of SNPs were eliminated (28–30). Additionally, SNPs with a minor allele frequency of ≤0.05 were exclusively considered for subsequent analysis.

Distinguishing methylated CpG sites between individuals with NOWS and controls was determined using predetermined cutoff criteria with a false discovery rate (FDR) threshold of $p < 0.05$. When multiple CpG sites were found within a gene, the one with the highest area under the curve (AUC) receiver operating characteristic (ROC) ranking and the lowest p-value was selected. The calculation of p-values for methylation disparities between the case and control groups at each locus followed previously established methods (25, 31). Both raw p-values and FDR-adjusted p-values, corrected for multiple testing using the Benjamini-Hochberg method, were computed. Additionally, the AUC for combinations of loci was computed using the "ROCR" package (version 3.5.0) in the 'R' programming environment.

Chronic pain-associated gene selections

Pain-associated genes were identified through a comprehensive analysis using an integrated genetic database. This database combines data from genome-wide association studies (GWAS), gene expression profiles, and curated literature on pain phenotypes (32, 33). The focus was on genes with robust GWAS evidence linking them to pain perception or chronic pain, genes documented in known pain pathways, and those associated with opioid use, particularly in the context of chronic pain management or opioid-induced hyperalgesia. Detailed information on these genes, including their functions, pathways, and relevant SNPs or mutations, was carefully extracted and cross-referenced with multiple databases to ensure accuracy and relevance, particularly in the context of opioid use, addiction, and chronic pain. This comprehensive approach highlights the genetic underpinnings that link opioid use disorder with pain-related pathways and addiction susceptibility. Additionally, to identify potential genes implicated in pain, relevant review articles were consulted and reviewed (34–38). These articles offered valuable insights by synthesizing existing research, with a particular focus on genes involved in pain perception, chronic pain, and opioid use. A total of 897 pain-associated genes were retrieved including genes from the Human Pain Genetics Database (https://diatchenko.lab.mcgill.ca/hpgdb/), a hand-curated resource of genetic associations with human pain phenotypes (Supplementary Table S1). Genes overlapping between this database and published literature were included only once.

Supplementary Table S2 presents details on several key genes and their biological roles associated with pain. The table categorizes these genes based on their involvement in various physiological and pathological processes, including diabetes, telomere maintenance, glucose metabolism, aging, and suicide, among others. Each entry provides information on the gene's function and its relevance to the context of pain.

Heatmap

The heatmap was created with the Complex Heatmap module (v1.6.0) in the R package (v3.2.2). It displays the distribution of methylated CpG sites among pain-associated CpGs, where each site represents a single data point. This analysis aims to visually represent methylation patterns across these regions. Hierarchical clustering of the samples was performed using Ward's method (39). Significantly different methylated CpG sites between NOWS and control groups were determined using FDR P-values ≤0.05. The area under the receiver operating characteristic (AUC-ROC) was calculated based on methylation levels at the most significant CpG loci.

Protein-protein interaction (PPI) network analysis

We performed a PPI network analysis on the 111 differently methylated genes implicated in pain perception in NOWS using

the STRING database (version 12.0). Parameters set for the analysis included a maximum false discovery rate (FDR) of 0.05 to focus on statistically significant interactions, a minimum interaction strength of 0.01 to include relevant but not necessarily strong interactions, and a minimum count of 2 to be displayed in the network, ensuring that only genes with at least one connection were included. Notably, the *MALAT1* gene (lncRNA), identified as a noncoding gene, was excluded from this analysis due to its inability to encode protein and hence participate in PPIs.

Gene ontology (Go) and KEGG pathway analysis

The functional implications of these genes were further investigated through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses via the DAVID v6.8 tool. Enrichment analysis was conducted, focusing on biological processes (BP), cellular components (CC), molecular functions (MF), and metabolic pathways. Significant terms and pathways (p-value < 0.05, Benjamini-Hochberg corrected) were identified to elucidate the biological basis of NOWS pain perception. Results from the GO and KEGG analyses were visualized using bubble plots in R using the ggplot2 package. Each bubble represents a GO term or KEGG pathway, with size indicating gene count and color showing significance, providing an intuitive view of the enriched biological themes.

Venn diagram analysis

A Venn diagram analysis was performed to identify unique and shared methylation patterns among genes associated with NOWS-associated pain. Gene lists from four independent analyses were compiled and visualized using InteractiVenn (40). This approach enabled the identification of genes unique to each analysis as well as those common across multiple analyses.

Results

The study's four analyses identified differentially methylated CpGs linked to pain-related genes, opioid exposure, and NOWS. The first analysis compared the (+Opioids/+NOWS) group with the (+Opioids/-NOWS), identifying 50 significant genes (Supplementary Table S3). The second analysis, which compared the combined (+Opioids/+NOWS) and (+Opioids/-NOWS) groups with the (-Opioids/-NOWS, control), yielded 42 significant genes (Supplementary Table S4). The third analysis compared the (+Opioids/+NOWS) group with the (-Opioids/-NOWS, control), identifying 40 dysregulated genes (Supplementary Table S5). Finally, the fourth analysis compared the (+Opioids/-NOWS) group with the (-Opioids/-NOWS, control) group, revealing 49 significant markers (Supplementary Table S6).

After combining and overlapping the results from the four analyses, a total of 111 CpG sites (FDR p-values ≤0.05), associated with 111 pain-related genes in individuals with NOWS, were identified. When multiple genes overlapped in the analyses, each was included only once. Of these, 54 CpG sites exhibited hypomethylation, while 57 showed hypermethylation, with each site included only once. (Table 1).

Heatmap assessment

The heatmap reveals two distinct clusters of CpGs, (i.e., NOWS and control) utilizing CpG methylation markers associated with pain sensation. One cluster is characteristic of NOWS patients, while the other corresponds to the control group. This strong observation provides significant backing to the idea that these methylation markers are dependable indicators for discriminating between individuals with NOWS and those without. These results validate the precision and effectiveness of the methylation markers in distinguishing between the two patient groups. Supplementary Figures S1-S4 illustrate four distinct analysis combinations.

PPI network characteristics

The Protein-Protein Interaction (PPI) network analysis, which incorporated 110 nodes after excluding the noncoding *MALAT1* gene, revealed significant connectivity among proteins implicated in pain perception in NOWS. The network consisted of 350 edges, which is notably higher than the expected number of edges (154), suggesting a non-random pattern of protein interactions. The average node degree was calculated to be 6.36, indicating a moderate level of interaction per protein, while the average local clustering coefficient was 0.402, reflecting the network's tendency to form clusters. Remarkably, the PPI enrichment p-value was less than 1.0e−16, indicating highly significant enrichment beyond chance. This suggests that the differentially methylated genes in NOWS are interconnected and likely play a substantial role in pain perception. Visual analysis of the PPI network revealed several highly connected nodes that may be key regulators in NOWS pathophysiology (Figure 1).

Gene ontology (Go) analysis

The GO analysis identified significant terms across Biological Process (BP) (Figure 2), Cellular Component (CC) (Figure 3), and Molecular Function (MF) (Figure 4), that provide insights into the molecular basis of pain perception in NOWS. Key Biological Processes highlighted include the positive regulation of cytosolic calcium ion concentration, calcium ion import across the plasma membrane, and vasodilation, which suggest alterations in ion regulation and vascular function may play a critical role in NOWS-associated pain. Protein phosphorylation and signal transduction were also prominent, indicative of

TABLE 1 A comprehensive list of 111 CpG targets exhibiting significant methylation differences in NOWS is provided.

TargetID	Gene	Location	p-Val	FDR p-Val	% Methylation			AUC	CI	
					Cases	Control	Difference		Lower	Upper
cg26635219	CFTR	7q31.2	9.67×10^{-39}	8.36×10^{-33}	24.59	14.19	10.40	0.76	0.64	0.88
cg13999099	IL6ST	5q11.2	2.41×10^{-38}	2.08×10^{-32}	26.41	16.95	9.46	0.92	0.85	0.99
cg22018329	GNA11	19p13.3	1.03×10^{-37}	8.91×10^{-32}	85.92	78.79	7.13	0.77	0.65	0.88
cg03659519	GALR1	18q23	5.45×10^{-37}	4.72×10^{-31}	17.00	10.84	6.16	0.68	0.56	0.80
cg15677797	KLF11	2p25.1	7.74×10^{-21}	6.70×10^{-15}	6.37	13.28	−6.91	0.57	0.45	0.69
cg02904605	NOTCH3	19p13.12	6.01×10^{-17}	5.20×10^{-11}	64.42	74.86	−10.43	0.78	0.67	0.89
cg21517792	MTA1	14q32.33	5.80×10^{-15}	5.01×10^{-9}	78.15	85.62	−7.46	0.82	0.71	0.92
cg14117934	IL1B	2q14.1	1.07×10^{-14}	9.26×10^{-9}	77.75	69.36	8.39	0.81	0.70	0.91
cg14929554	N4BP1	16q12.1	2.57×10^{-14}	2.22×10^{-8}	60.20	70.37	−10.17	0.76	0.65	0.88
cg13377102	KCNAB3	17p13.1	6.63×10^{-14}	5.73×10^{-8}	70.71	79.30	−8.59	0.80	0.69	0.91
cg04708753	CASP9	1p36.21	1.07×10^{-13}	9.24×10^{-8}	5.83	11.12	−5.28	0.61	0.49	0.74
cg15929698	NPY	7p15.3	1.25×10^{-13}	1.08×10^{-7}	50.16	40.25	9.90	0.75	0.63	0.87
cg08929188	CALCA	11p15.2	2.00×10^{-13}	1.73×10^{-7}	25.95	17.92	8.02	0.75	0.63	0.87
cg16839955	ARNTL	11p15.3	4.03×10^{-13}	3.48×10^{-7}	28.33	20.13	8.20	0.81	0.71	0.92
cg15442907	CACNA1C	12p13.33	5.63×10^{-13}	4.87×10^{-7}	45.17	55.79	−10.62	0.78	0.67	0.90
cg06976250	ANKK1	11q23.2	5.99×10^{-12}	5.18×10^{-6}	16.26	10.96	5.30	0.59	0.46	0.71
cg22623236	PDE10A	6q27	9.31×10^{-12}	8.06×10^{-6}	62.35	71.33	−8.98	0.72	0.60	0.85
cg09711113	NLGN2	17p13.1	1.11×10^{-11}	9.60×10^{-6}	53.95	63.63	−9.69	0.75	0.62	0.87
cg25885356	RUNX1	21q22.12	1.19×10^{-11}	1.03×10^{-5}	44.32	35.28	9.04	0.72	0.59	0.85
cg27331241	PRKAR1B	7p22.3	1.77×10^{-11}	1.54×10^{-5}	52.56	62.30	−9.74	0.71	0.59	0.84
cg22197205	CLIC4	1p36.11	1.83×10^{-11}	1.58×10^{-5}	75.08	82.18	−7.10	0.75	0.64	0.87
cg08253824	SCN8A	12q13.13	1.85×10^{-11}	1.60×10^{-5}	60.21	69.29	−9.09	0.71	0.59	0.84
cg03037030	TNF	6p21.33	3.01×10^{-11}	2.61×10^{-5}	17.77	12.39	5.38	0.73	0.62	0.85
cg16243402	OPRM1	6q25.2	3.25×10^{-11}	2.81×10^{-5}	22.90	30.46	−7.56	0.62	0.50	0.74
cg17546721	TGFBR2	3p24.1	5.00×10^{-11}	4.32×10^{-5}	53.38	44.44	8.95	0.75	0.63	0.87
cg03652989	ULK1	12q24.33	5.15×10^{-11}	4.45×10^{-5}	80.91	74.25	6.66	0.77	0.65	0.89
cg11978118	ADORA2A	22q11.23	6.95×10^{-11}	6.01×10^{-5}	71.99	79.40	−7.40	0.77	0.66	0.89
cg00248439	GRK5	10q26.11	8.53×10^{-11}	7.38×10^{-5}	89.72	84.80	4.93	0.70	0.57	0.83
cg16293347	TAOK3	12q24.23	1.15×10^{-10}	9.95×10^{-5}	80.32	73.63	6.68	0.77	0.65	0.88
cg17782167	PLCE1	10q23.33	1.20×10^{-10}	1.03×10^{-4}	63.77	55.31	8.46	0.75	0.63	0.87
cg15118537	PRKG1	10q11.23-q21.1	1.31×10^{-10}	1.13×10^{-4}	72.65	64.95	7.69	0.73	0.60	0.85
cg09778136	ATG5	6q21	1.32×10^{-10}	1.14×10^{-4}	49.51	58.94	−9.42	0.75	0.63	0.87
cg11211173	ATP1A2	1q23.2	1.47×10^{-10}	1.27×10^{-4}	75.90	68.59	7.31	0.74	0.61	0.86
cg24062706	OSM	22q12.2	1.60×10^{-10}	1.38×10^{-4}	36.26	28.16	8.10	0.71	0.59	0.84
cg20629735	IL18R1	2q12.1	1.72×10^{-10}	1.49×10^{-4}	73.99	80.95	−6.96	0.76	0.65	0.88
cg24368031	MRC2	17q23.2	1.75×10^{-10}	1.52×10^{-4}	28.42	20.97	7.45	0.75	0.63	0.87
cg12339476	MAPK10	4q21.3	2.34×10^{-10}	2.02×10^{-4}	63.21	71.53	−8.32	0.73	0.60	0.85
cg25387779	GNAO1	16q13	4.10×10^{-10}	3.55×10^{-4}	18.94	26.52	−7.58	0.65	0.51	0.78
cg01015652	ESR2	14q23.2-q23.3	4.44×10^{-10}	3.84×10^{-4}	16.52	10.78	5.74	0.78	0.66	0.89
cg09031352	PTN	7q33	5.47×10^{-10}	4.73×10^{-4}	38.19	47.35	−9.16	0.75	0.63	0.87
cg14299235	ABCA1	9q31.1	5.51×10^{-10}	4.77×10^{-4}	67.75	59.84	7.91	0.68	0.55	0.81
cg05313261	MAPK3	16p11.2	5.72×10^{-10}	4.95×10^{-4}	6.60	11.74	−5.14	0.88	0.80	0.97
cg03402505	GRIA1	5q33.2	5.84×10^{-10}	5.05×10^{-4}	57.48	49.10	8.38	0.78	0.67	0.90
cg10274696	C7orf10	7p14.1	5.86×10^{-10}	5.07×10^{-4}	64.73	72.72	−7.99	0.78	0.67	0.90
cg06942027	KCNJ2	17q24.3	6.04×10^{-10}	5.23×10^{-4}	20.18	14.00	6.19	0.70	0.57	0.83
cg19690051	CAPN1	11q13.1	8.10×10^{-10}	7.00×10^{-4}	15.48	9.98	5.51	0.89	0.80	0.97
cg12578250	PRDM16	1p36.32	1.10×10^{-9}	9.50×10^{-4}	58.24	66.72	−8.48	0.73	0.60	0.85
cg18302652	CXCL8	4q13.3	1.24×10^{-9}	1.07×10^{-3}	15.36	21.35	−5.99	0.65	0.53	0.77
cg02081889	PCSK5	9q21.13	1.32×10^{-9}	1.14×10^{-3}	14.37	9.15	5.22	0.79	0.68	0.90
cg07616332	SHMT1	17p11.2	1.35×10^{-9}	1.17×10^{-3}	63.11	55.05	8.06	0.71	0.59	0.84
cg12602112	EDNRB	13q22.3	1.41×10^{-9}	1.22×10^{-3}	11.09	17.06	−5.97	0.70	0.58	0.83
cg15787454	CPQ	8q22.1	1.53×10^{-9}	1.33×10^{-3}	76.04	82.37	−6.32	0.74	0.62	0.86
cg16910896	LPAR1	9q31.3	1.55×10^{-9}	1.34×10^{-3}	64.85	72.65	−7.80	0.72	0.59	0.84
cg12056044	IL23R	1p31.3	1.78×10^{-9}	1.54×10^{-3}	60.52	68.71	−8.20	0.75	0.63	0.87
cg20583095	ESR1	6q25.1-q25.2	1.84×10^{-9}	1.59×10^{-3}	34.11	26.51	7.61	0.77	0.65	0.88
cg13480658	AJAP1	1p36.32	2.05×10^{-9}	1.77×10^{-3}	89.37	93.23	−3.87	0.72	0.59	0.84
cg04329125	CRIP2	14q32.33	2.05×10^{-9}	1.77×10^{-3}	47.33	39.10	8.23	0.70	0.58	0.83

(Continued)

TABLE 1 Continued

TargetID	Gene	Location	p-Val	FDR p-Val	% Methylation			AUC	CI	
					Cases	Control	Difference		Lower	Upper
cg21176263	LMX1B	9q33.3	2.65×10^{-9}	2.29×10^{-3}	64.92	72.69	−7.77	0.75	0.62	0.87
cg09021274	DLG2	11q14.1	2.98×10^{-9}	2.58×10^{-3}	84.55	79.06	5.50	0.86	0.77	0.95
cg17500968	TRPV4	12q24.11	3.37×10^{-9}	2.91×10^{-3}	84.86	80.02	4.83	0.68	0.56	0.80
cg09964361	CAMK4	5q22.1	3.42×10^{-9}	2.96×10^{-3}	63.94	56.11	7.83	0.71	0.58	0.84
cg07164211	CACNA2D1	7q21.11	4.58×10^{-9}	3.96×10^{-3}	79.12	84.79	−5.67	0.75	0.63	0.87
cg14972143	EIF4E	4q23	4.70×10^{-9}	4.07×10^{-3}	16.56	11.10	5.46	0.84	0.74	0.94
cg02695252	PRLR	5p13.2	4.71×10^{-9}	4.07×10^{-3}	72.17	78.88	−6.71	0.81	0.70	0.92
cg17369032	NGFR	17q21.33	5.51×10^{-9}	4.76×10^{-3}	75.13	81.46	−6.33	0.77	0.65	0.89
cg25067242	NGF	1p13.2	5.88×10^{-9}	5.09×10^{-3}	48.57	57.28	−8.72	0.77	0.65	0.88
cg25944168	EIF2AK3	2p11.2	6.28×10^{-9}	5.43×10^{-3}	63.88	56.16	7.72	0.71	0.58	0.84
cg21685789	GABRG2	5q34	6.54×10^{-9}	5.65×10^{-3}	57.62	49.72	7.90	0.73	0.60	0.85
cg06444178	ANKH	5p15.2	7.39×10^{-9}	6.39×10^{-3}	15.10	9.97	5.13	0.78	0.66	0.89
cg17349736	NR3C1	5q31.3	7.40×10^{-9}	6.40×10^{-3}	58.87	66.91	−8.04	0.76	0.64	0.88
cg21486834	RHBDF2	17q25.1	8.81×10^{-9}	7.62×10^{-3}	81.54	86.77	−5.23	0.76	0.64	0.87
cg02111786	NRXN3	14q24.3-q31.1	9.60×10^{-9}	8.31×10^{-3}	85.00	79.74	5.26	0.73	0.60	0.85
cg14129053	MYT1l	2p25.3	1.02×10^{-8}	8.86×10^{-3}	72.28	66.10	6.18	0.65	0.53	0.77
cg11590170	GJA1	6q22.31	1.03×10^{-8}	8.87×10^{-3}	79.56	73.47	6.09	0.74	0.61	0.86
cg21963925	CACNA1H	16p13.3	1.06×10^{-8}	9.16×10^{-3}	90.85	87.19	3.66	0.60	0.48	0.73
cg24397382	STX1A	7q11.23	1.10×10^{-8}	9.53×10^{-3}	71.48	64.43	7.05	0.70	0.57	0.83
cg15002761	IGSF9B	11q25	1.14×10^{-8}	9.87×10^{-3}	87.39	91.52	−4.14	0.76	0.64	0.88
cg22849544	THRB	3p24.2	1.16×10^{-8}	1.00×10^{-2}	18.73	24.80	−6.07	0.57	0.45	0.69
cg12078872	DDO	6q21	1.33×10^{-8}	1.15×10^{-2}	80.37	85.75	−5.38	0.80	0.70	0.91
ch.12.28033R	WNK1	12p13.33	1.48×10^{-8}	1.28×10^{-2}	11.78	17.49	−5.71	0.61	0.48	0.75
cg23817893	CCDC81	11q14.2	1.50×10^{-8}	1.30×10^{-2}	42.44	50.91	−8.46	0.75	0.63	0.87
cg08408433	PTGIR	19q13.32	1.54×10^{-8}	1.34×10^{-2}	71.09	77.74	−6.65	0.72	0.59	0.84
cg09713515	DOCK4	7q31.1	1.56×10^{-8}	1.35×10^{-2}	81.31	75.54	5.77	0.76	0.65	0.88
cg00781169	PTGER3	1p31.1	1.76×10^{-8}	1.52×10^{-2}	60.07	67.91	−7.85	0.70	0.57	0.83
cg09070522	REST	4q12	1.91×10^{-8}	1.66×10^{-2}	14.44	9.48	4.96	0.88	0.79	0.97
cg09397542	PHACTR1	6p24.1	1.97×10^{-8}	1.70×10^{-2}	14.23	9.36	4.87	0.77	0.65	0.89
cg23947039	BDNF	11p14.1	2.04×10^{-8}	1.76×10^{-2}	7.56	3.79	3.78	0.87	0.78	0.96
cg07539983	SPARC	5q33.1	2.17×10^{-8}	1.88×10^{-2}	79.24	73.23	6.01	0.74	0.61	0.86
cg02726883	NF1	17q11.2	2.37×10^{-8}	2.05×10^{-2}	14.59	9.63	4.96	0.91	0.84	0.99
cg05931684	EHMT2	6p21.33	2.48×10^{-8}	2.15×10^{-2}	17.20	11.93	5.27	0.86	0.76	0.95
cg10439765	SLC12A5	20q13.12	2.61×10^{-8}	2.26×10^{-2}	15.53	21.81	−6.27	0.70	0.57	0.82
cg19621317	ASIC1	12q13.12	2.63×10^{-8}	2.27×10^{-2}	71.62	78.17	−6.56	0.80	0.70	0.91
cg19753937	NRG1	8p12	2.63×10^{-8}	2.28×10^{-2}	74.82	80.88	−6.06	0.73	0.61	0.85
cg08644772	IKBKAP	9q31.3,	2.82×10^{-8}	2.44×10^{-2}	83.19	87.94	−4.75	0.76	0.64	0.88
cg22952017	CTSS	1q21.3	2.99×10^{-8}	2.59×10^{-2}	31.95	25.90	6.05	0.76	0.65	0.87
cg18793036	MME	3q25.2	3.02×10^{-8}	2.61×10^{-2}	14.89	21.02	−6.12	0.65	0.52	0.79
cg09045305	ADARB2	10p15.3	3.20×10^{-8}	2.77×10^{-2}	69.88	76.54	−6.66	0.74	0.62	0.86
cg21295398	BECN1	17q21.31	3.33×10^{-8}	2.88×10^{-2}	74.35	67.82	6.53	0.83	0.73	0.94
cg08914905	PIK3C3	18q12.3	3.43×10^{-8}	2.97×10^{-2}	75.40	68.99	6.41	0.71	0.58	0.84
cg01183713	ULK4	3p22.1	3.50×10^{-8}	3.03×10^{-2}	67.27	74.21	−6.94	0.71	0.59	0.84
cg19137569	KCNN3	1q21.3	3.52×10^{-8}	3.04×10^{-2}	32.61	40.65	−8.04	0.78	0.66	0.89
cg22762326	OXR1	8q23.1	3.59×10^{-8}	3.10×10^{-2}	82.45	76.99	5.46	0.75	0.62	0.87
cg16298405	RUNX2	6p21.1	3.85×10^{-8}	3.33×10^{-2}	9.95	14.56	−4.61	0.76	0.66	0.87
cg06069187	SARM1	17q11.2	4.15×10^{-8}	3.59×10^{-2}	21.62	28.62	−7.00	0.67	0.54	0.80
cg18501142	MALAT1	11q13.1	4.46×10^{-8}	3.86×10^{-2}	18.71	13.32	5.39	0.79	0.68	0.90
cg17457918	SCN1A	2q24.3	4.48×10^{-8}	3.87×10^{-2}	41.52	49.70	−8.19	0.73	0.60	0.85
cg05337454	NOS3	7q36.1	4.58×10^{-8}	3.97×10^{-2}	88.15	83.70	4.45	0.70	0.57	0.83
cg03786924	KCNQ5	6q13	4.94×10^{-8}	4.27×10^{-2}	29.22	22.61	6.61	0.74	0.62	0.86
cg17403731	HCN2	19p13.3	5.12×10^{-8}	4.43×10^{-2}	67.65	60.59	7.05	0.70	0.57	0.83
cg26701226	WSCD1	17p13.2	5.62×10^{-8}	4.86×10^{-2}	4.69	8.29	−3.60	0.57	0.44	0.69
cg06422471	SHANK3	22q13.33	5.69×10^{-8}	4.93×10^{-2}	21.70	15.91	5.80	0.71	0.58	0.83

This includes CpG sites with target ID, pain-associated gene IDs, chromosome locations, p-values, FDR p-values, and the percentage of methylation difference.
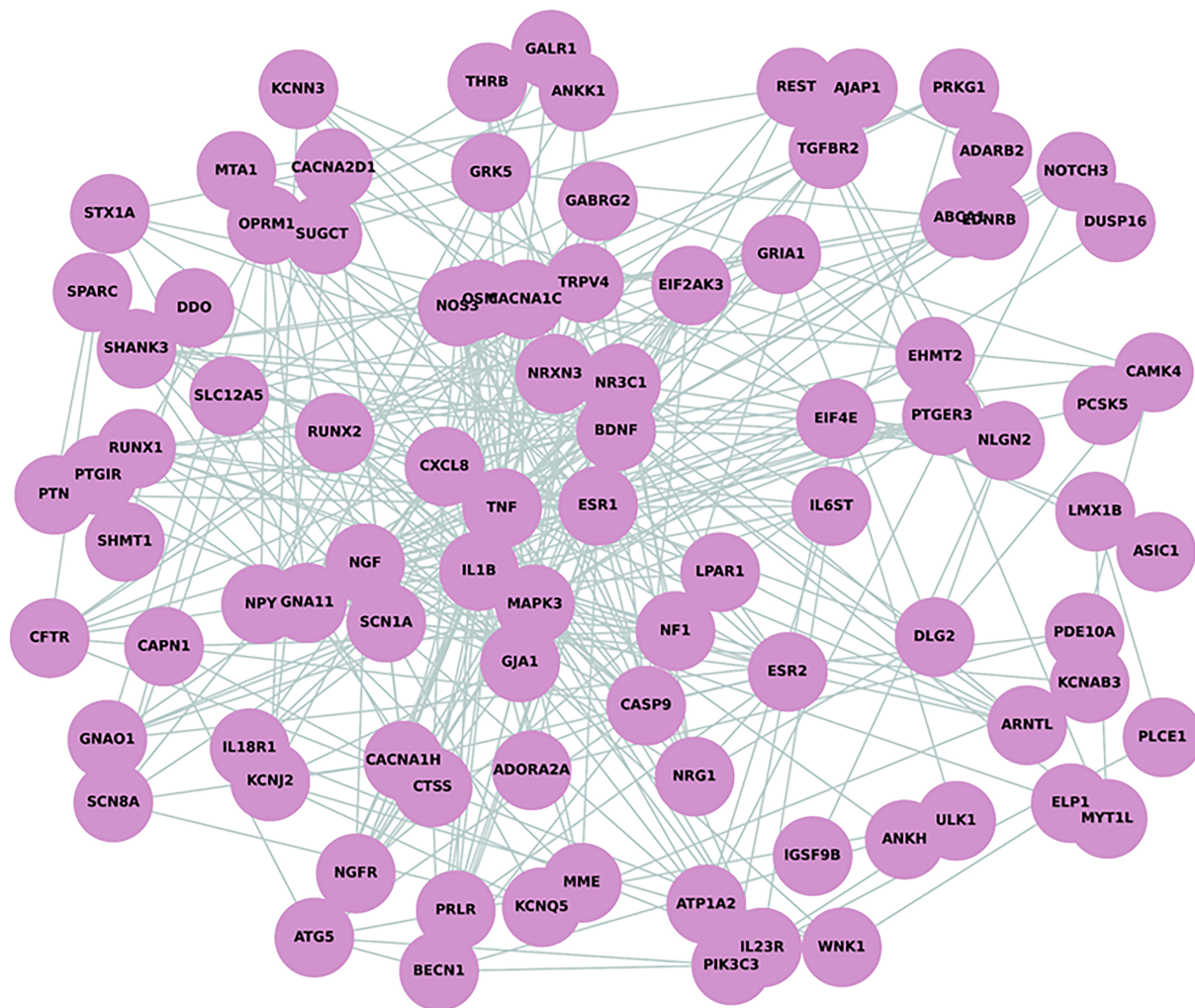
FIGURE 1
Protein-Protein interaction (PPI) network analysis depicting 110 nodes and 350 edges. The network demonstrates a higher-than-expected level of connectivity with an average node degree of 6.36 and an average local clustering coefficient of 0.402, indicative of a tightly knit network. This is supported by a PPI enrichment p-value of less than 1.0e−16.

complex signaling pathways involved in pain response. Terms related to cardiac muscle function and nerve development were notably enriched, reflecting the potential influence of these genes on the neurophysiological adaptations in NOWS.

In Cellular Components a significant enrichment in genes associated with the plasma membrane points to the importance of membrane-bound proteins and receptors in nociception. Other notable components included axons, neuronal cell bodies, and various synapse structures, such as glutamatergic synapses, suggesting a focus on neuron-specific structures and interactions. Significant Molecular Functions implicated in the data include scaffold protein binding and calmodulin binding, which may influence intracellular signaling pathways related to pain. The activity of voltage-gated calcium channels and protein kinase activities were also highlighted, suggesting potential targets for modulating neuronal excitability and signal transduction in pain pathways specific to NOWS.

The GO terms identified suggest that the differently methylated genes in NOWS are significantly involved in neural signaling and development, membrane dynamics, and intracellular signaling mechanisms These biological themes provide a molecular framework that may explain the altered perception of pain in NOWS and could help in guiding further research into targeted therapies for pain management in affected neonates (Supplementary Tables S7–S9).

Pathway analysis

KEGG pathway analysis of the 111 differently methylated genes identified their involvement in 48 pathways (FDR p-value <0.05), with a primary focus on neuronal function and substance use—both highly relevant to pain (Supplementary Table S10). Notably, the cAMP and MAPK signaling pathways, which
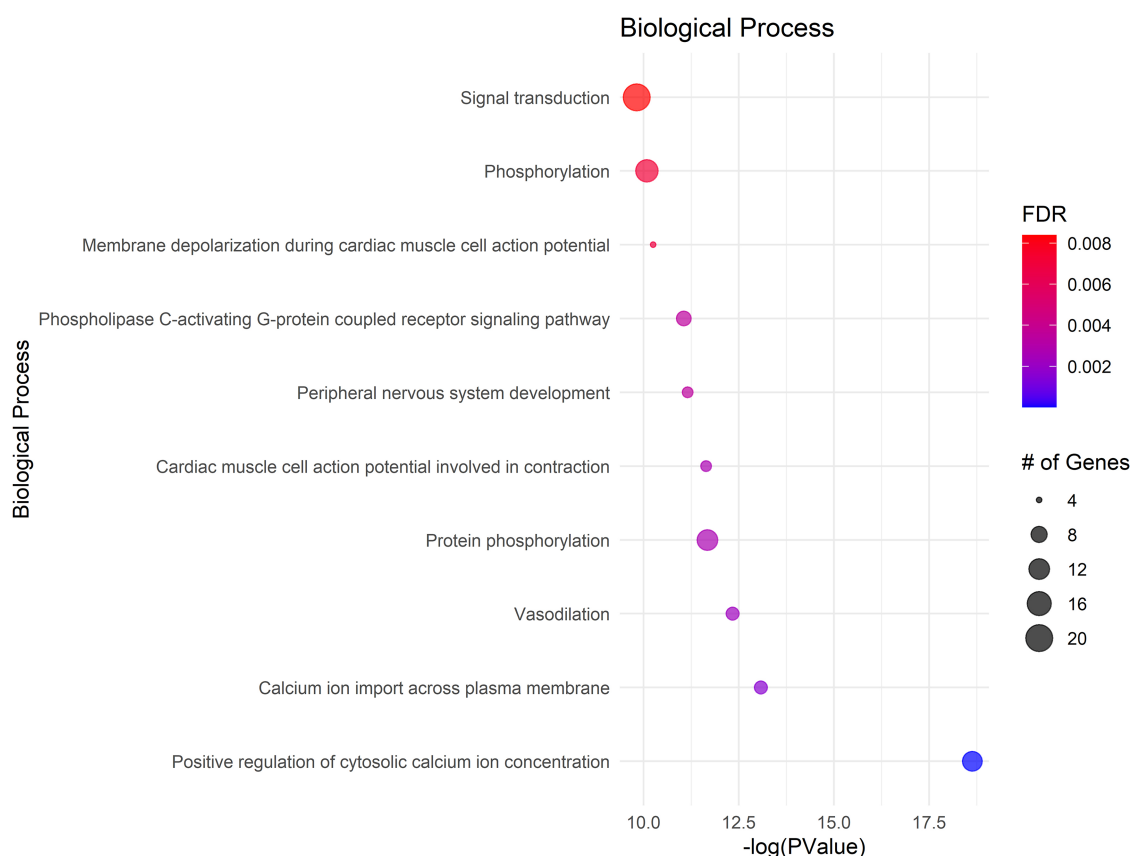
FIGURE 2
Displays the enrichment analysis for biological processes (BP). The size of each bubble corresponds to the number of genes associated with that term, and the color gradient represents the FDR value, with darker shades indicating greater significance.

exhibited high gene counts, play crucial roles in cellular responses to external stimuli and pain modulation. The neuroactive ligand-receptor interaction pathway, also enriched with a substantial number of genes, suggests alterations in neurotransmitter dynamics that may influence pain perception. Additionally, the enrichment of multiple disease-related pathways, including cancer and various infections, underscores the broader impact of epigenetic modifications on cellular stress and immune responses, which may have implications for pain regulation and opioid withdrawal (Figure 5).

Venn diagram

The Venn diagram analysis revealed distinct and overlapping methylation patterns, illustrating the complex epigenetic landscape of NOWS-associated pain (Figure 6). Each analysis identified a unique set of differentially methylated genes, while several genes exhibited overlapping methylation changes, suggesting their involvement in shared biological pathways. Notably, the *PLCE1* gene was consistently methylated across all analyses, highlighting it as a core epigenetic marker for NOWS-related pain. These findings reveal key regulatory

networks with therapeutic potential. See Supplementary Table S11 for the full gene list.
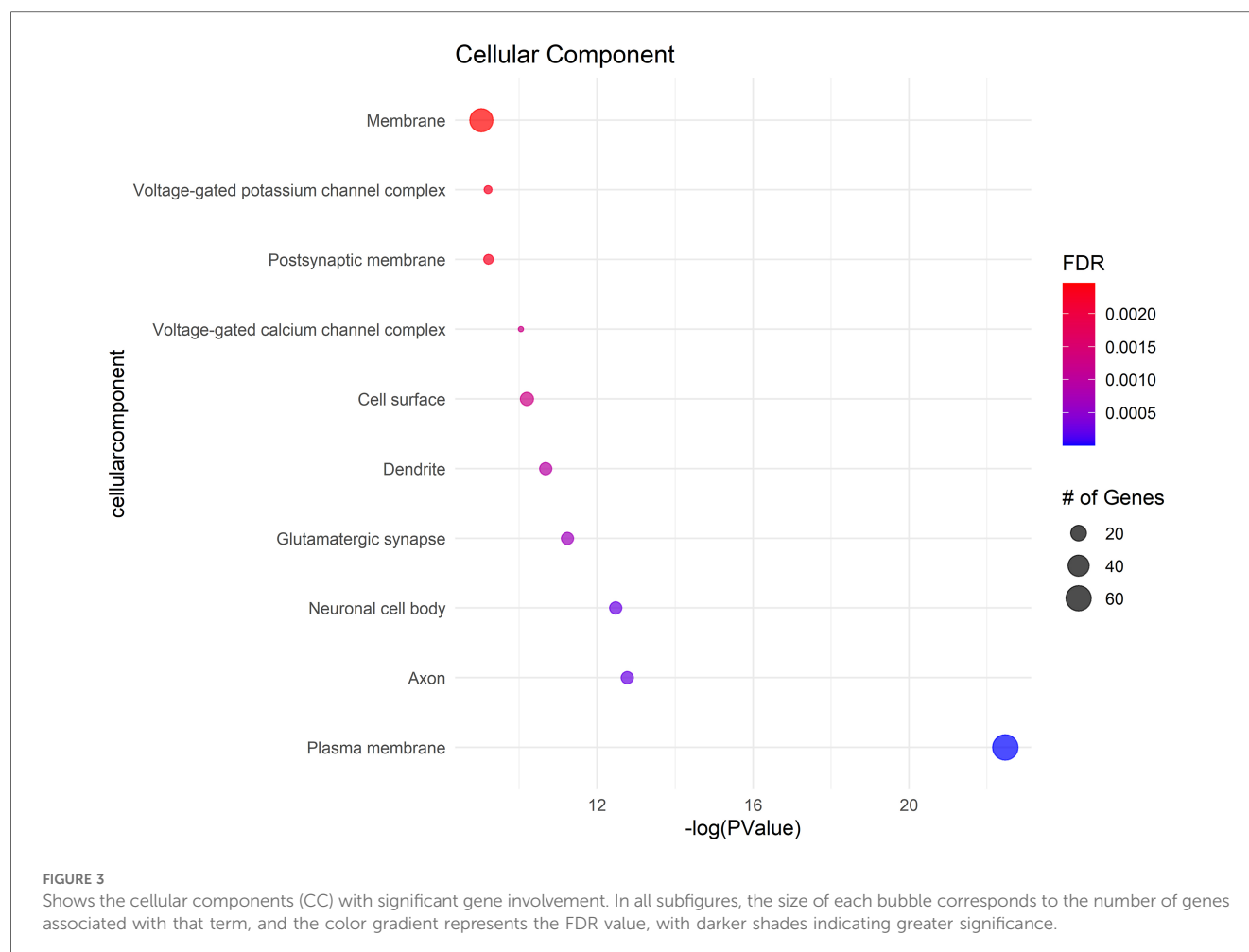
Discussion

Pregnant women may use opioids for pain relief, addiction, or treatment, but misuse can lead to epigenetic changes that impact fetal development, affecting genes related to pain sensitivity and neurological functions (41, 42). Epigenetic changes during critical periods of fetal development can shape pain perception and increase the likelihood of severe health issues that may arise in adulthood, often rooted in early infancy (33). Yet, how prenatal opioid exposure disrupts epigenetic regulation and its lasting effects on pain remains unknown.

Autophagy genes contribute to chronic pain

We identified ten dysregulated autophagy-related genes—*BDNF, LMX1B, ESR2, ULK4, ATG5, BECN1, MAPK3, PIK3C3, CTSS,* and *ULK1*—that affect neuronal development, pain

FIGURE 3
Shows the cellular components (CC) with significant gene involvement. In all subfigures, the size of each bubble corresponds to the number of genes associated with that term, and the color gradient represents the FDR value, with darker shades indicating greater significance.

sensitivity, and long-term health. *BDNF* is essential for neuronal survival and plasticity (43). *LMX1B* and *ESR2* support serotonergic neuron development and pain modulation. Dysregulation of *LMX1B* increases pain sensitivity and mood disorder risk, while *ESR2* affects pain thresholds and mood via estrogen signaling, underscoring their role in the complex interplay of pain and mood regulation (44). *ULK4* and *ULK1* are critical enzymes that initiate autophagy, dysregulation of *ULK1* or *ULK4* impairs autophagy, causing damaged proteins to accumulate in neurons. This can lead to neurodegeneration, increased cellular stress, inflammation, and heightened pain sensitivity, raising the risk of chronic pain (45). *ATG5* is vital for autophagosome formation; abnormal expression can impair autophagy, causing neuroinflammation and chronic pain (46). *BECN1* regulates autophagy initiation; dysregulation may result in neuron damage and an increased risk of neurodegenerative diseases (47). *MAPK3,* part of the *MAPK/ERK* pathway, affects pain perception and neural plasticity, impacting cognitive function (48). *PIK3C3* is involved in autophagy and endocytic trafficking; dysfunction may lead to neuroinflammation and chronic pain (49). *CTSS,* linked to neuropathic pain, may increase neuroinflammation and affect immune function (50).

Cytokines may affect pain perception

Dysregulation of cytokine genes, such as *IL23R*, can alter pain perception through immune modulation (51); while *NR3C1*, encoding the glucocorticoid receptor, affects pain pathways and inflammation, with its dysregulation potentially leading to inflammatory pain (52). Reduced *NR3C1* expression is associated with chronic cocaine use and linked to anxiety disorders and depression (53); *MAPK3* (*ERK1*) is key in the MAPK pathway, facilitating pain signal transmission and modulating the nociceptive pathway, crucial for pain perception regulation (48); *TNF* and *IL-1*β, key pro-inflammatory cytokines, play crucial roles in glial cell function, aging, obesity, depression, and pain modulation by heightening oxidative stress, exacerbating neuroinflammation, and increasing pain perception (54); *NGF* promotes neuropathic pain through nerve sensitization and sprouting (55); *IL-18* and its receptor *IL18R1* may contribute to pain signaling, diverging from their traditional roles in the immune system (56); *CXCL8* promotes inflammatory pain through neutrophil recruitment (57); *BDNF*, a neurotrophin, is crucial in numerous pain conditions, especially neuropathic pain (58), and is linked to obesity, type 2 diabetes mellitus, and other facets of metabolic syndrome (59); *TRPV4*, a cation channel,

FIGURE 4
Bubble plots of molecular functions (MF) enrichment analysis.
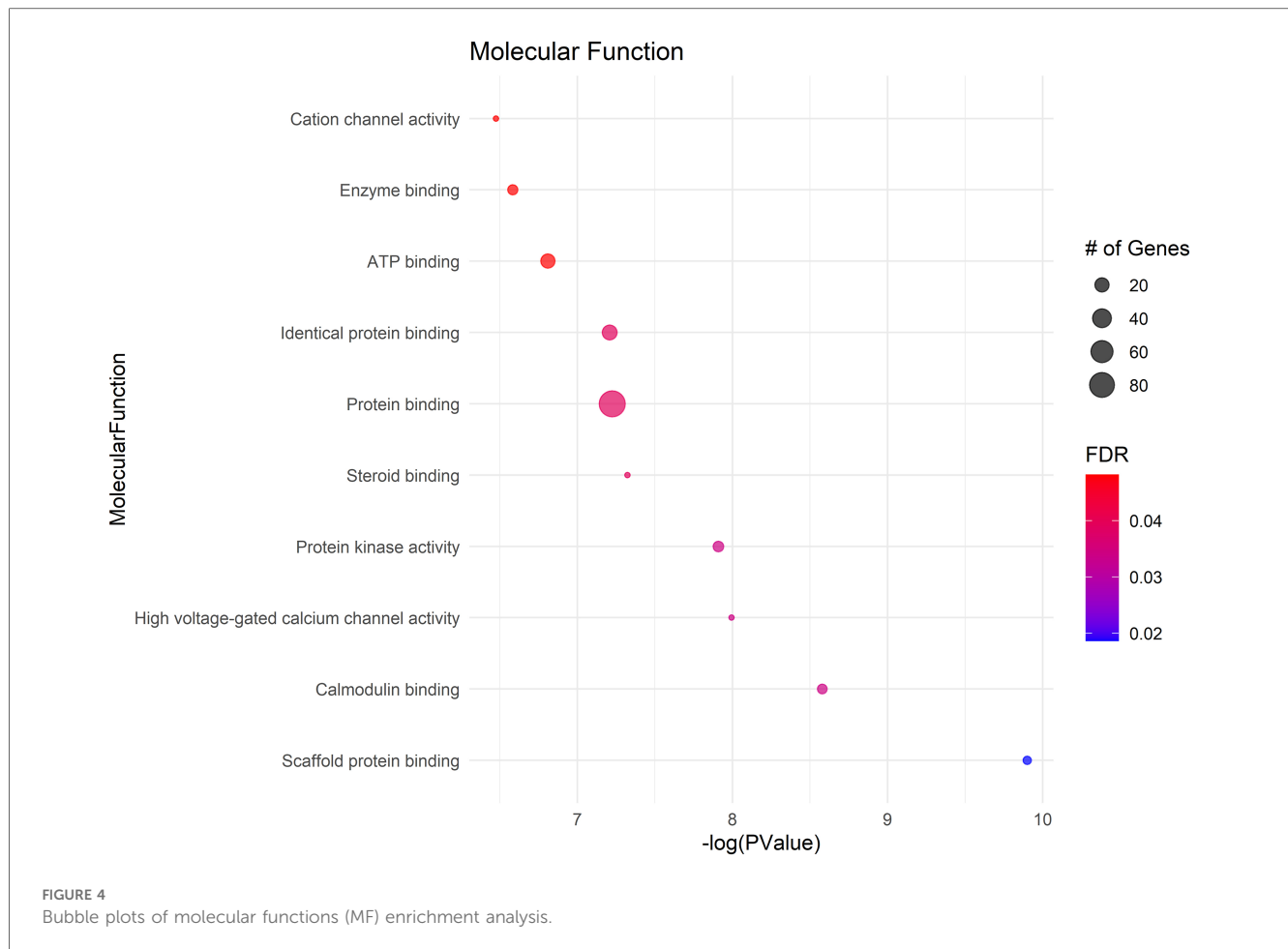
plays a role in nociception and pain sensation (60); The lncRNA *MALAT1* is significantly upregulated in the spinal cord and microglia, making it a key player in pain regulation (61), Its modulation of miR-129-5p and *HMGB1* further highlights its role in neuropathic pain mechanisms (62). Estrogen receptors *ERα* and *ERβ*, encoded by *ESR1* and *ESR2*, are essential for pain modulation and metabolic functions, including glucose metabolism and insulin sensitivity with their dysregulation affecting both pain sensitivity and overall metabolic processes (63). Variants in *ESR1* and *ESR2* genes affect menopause timing and symptom severity, with *ESR1* influencing the onset and *ESR2* affecting symptoms like hot flashes and bone density (64). In women with opioid addiction, these variants are linked to irregular menstruation, early menopause, worsened symptoms, and increased anxiety, depression, and chronic pain (65, 66); *CAMK4*, a kinase in calcium signaling, is linked to pain processing and synaptic plasticity (67); *OSM*, a cytokine, contributes to inflammatory and neuropathic pain (68); while *TGFBR2*, a receptor for *TGF-β*, is involved in pain-related cellular processes (69). Also, *RUNX1*, a transcription factor, affects sensory neuron development and pain processing (70).

Chronic pain can trigger addiction

Chronic pain frequently leads to opioid and alcohol use, disrupting brain chemistry and causing dependence (71). We identified 22 dysregulated pain-related genes in infants exposed to opioids *in utero*, affecting neurodevelopment, stress response, and reward pathways, thereby increasing the likelihood of addiction and opioid use later in life. *SCN8A* affects neuronal excitability, increasing addiction risk (72), while *BDNF* influences dopamine signaling, with lower levels linked to vulnerability (73). Changes in *OPRM1* may alter opioid sensitivity (74), and variants in *ANKK1/DRD2* disrupt dopamine signaling, increasing susceptibility to addiction (75). *NPY* impacts stress responses (76), and *MAPK* dysregulation affects synaptic plasticity (77). Variants in *NR3C1*, calcium channels *(CACNA1C, CACNA1H, CACNA2D1)*, and GABAergic genes *(ABRG2, GRIA1)* further influence addiction pathways. *PRKAR1B, NRXN3, SHANK3*, and immune genes *(TGFBR2, IL1B, TNF)* contribute to addiction risk, with autophagy-related genes *(BECN1, ULK1, ULK4)* may contribute to neurodegeneration, as previously detailed (78–80).
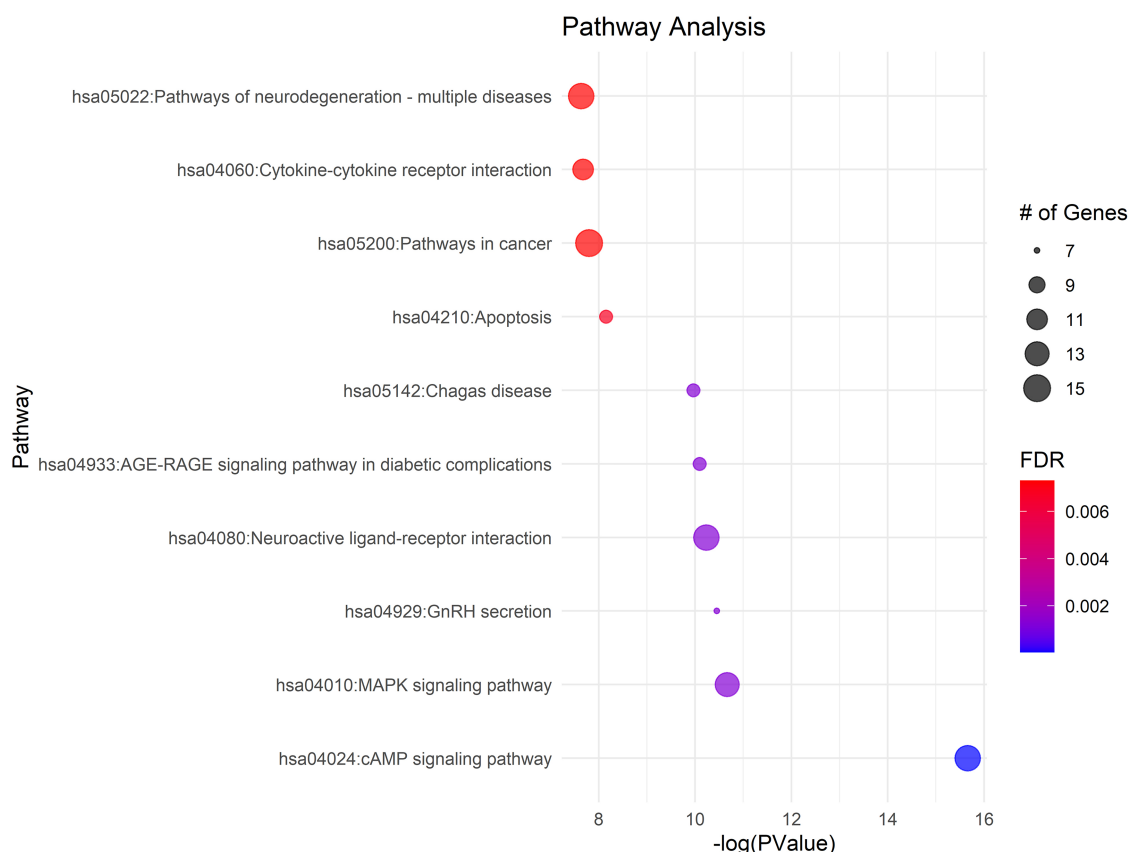
FIGURE 5
Gene ontology enrichment and KEGG pathway analysis of differentially methylated genes in NOWS cases, compared to normal controls, highlight significant involvement in pain-related pathways. The size of the bubble corresponds to the number of enriched genes within each pathway.

Chronic pain can lead to depression and suicidality

Chronic pain can lead to depression, a major risk factor for suicidality (81). We found multiple deregulated genes involved in pain, such as *TNF, IL1B,* and *IL6ST* (82–84), were identified as being associated with both depression and suicide. Gene variations affecting inflammation play a key role in depression, linking chronic pain, mood disorders, and suicide risk. Suicide is also reported in conditions like major depressive disorder, Alzheimer's disease, and Autism Spectrum Disorder. In our study, we identified several key genes associated with suicidal behavior, such as *BDNF, KCNJ2, NOS3, CACNA1C, PCSK5, GRIA1, ESR1, ANKK1, DLG2, GABRG2, IGSF9B, PRDM16, and NR3C1.* Variations in these genes may contribute to the complex interaction of biological, psychological, and environmental factors that may increase suicide risk in individuals in later life (83, 85–88).

Telomere genes in pain

Telomere maintenance genes, essential for preserving telomere length and integrity, have emerged as potential modulators of pain

sensitivity (89). As telomeres shorten with age and stress, they are linked to cellular aging, and increased inflammation, and can influence pain perception (90). The dysregulated genes identified in this study, including *NOS3, CFTR, ESR1, MAPK3, MTA1, ESR2, PRDM16, IKBKAP, RUNX2, REST, DLG2, PIK3C3, SHMT1,* and *PLCE1,* may impact pain perception by affecting inflammation, oxidative stress, and neuronal excitability pathways.

Pain can promote impulsivity

"Pain can lead to impulsive behavior by affecting decision-making and self-control (91). Those experiencing pain may engage in risky activities, such as overusing opioids, which can result in addiction (1). Pain also amplifies emotional distress and weakens self-regulation, further increasing impulsivity (92). We identified five dysregulated genes associated with impulsivity: *BDNF, MAPK10, NRG1, NRXN3,* and *PRKG1. BDNF,* a key biomarker for impulsivity, regulates impulse control by shaping neural circuits for decision-making (93). *MAPK10* influences impulsivity through stress pathways and brain regions controlling behavior. *NRG1* affects synaptic networks tied to impulse control (94), while *NRXN3* variants alter synaptic efficacy, contributing
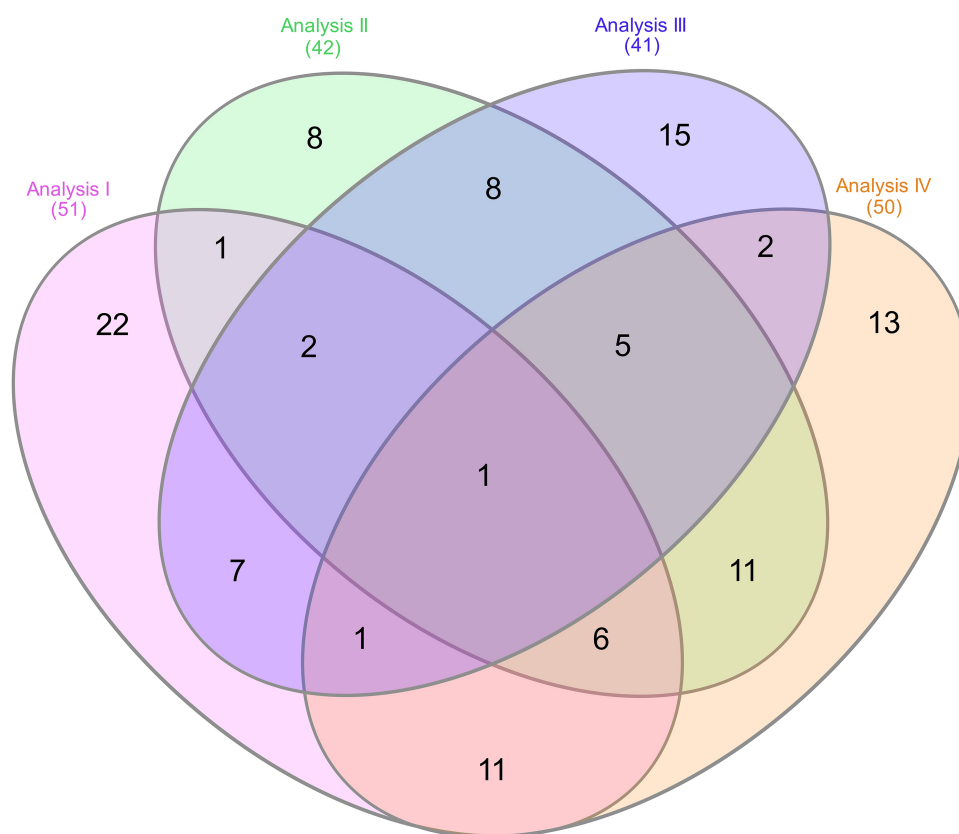
FIGURE 6
Venn diagram showing the overlap of differentially methylated genes across four analyses, each comparing methylation patterns related to NOWS-associated pain. • Analysis I: Compares (+Opioids/+NOWS) vs. (+Opioids/-NOWS) to identify methylation differences linked to NOWS-associated pain. • Analysis II: Compares (+Opioids/+NOWS) and (+Opioids/-NOWS) vs. (-Opioids/-NOWS, control) to identify CpG targets associated with pain.• Analysis III: Compares (+Opioids/+NOWS) vs. (-Opioids/-NOWS, control) to pinpoint methylation changes in NOWS-related pain genes. • Analysis IV: Compares (+Opioids/-NOWS) vs. (-Opioids/-NOWS, control) to identify CpG targets with differential methylation in pain-related genes. Analysis 1. Description of opioid use in opioid-exposed infants with and without NOWS: Comparison of infants requiring pharmacologic management for NOWS + Opioids/+NOWS) vs. those who did not require treatment (+Opioids/−NOWS). This analysis identifies epigenetic and molecular differences between opioid-exposed newborns with NOWS requiring treatment and those without withdrawal, pinpointing factors that trigger withdrawal. Analysis 2. Comparison of Opioid-Exposed Infants vs. Unexposed Controls: This analysis compares opioid-exposed infants—both those requiring pharmacologic management for NOWS (+Opioids/+NOWS) and those not requiring treatment (+Opioids/-NOWS)—with unexposed controls (-Opioids/-NOWS). Understanding these variations helps uncover prenatal opioid exposure and its influence on the newborn's molecular and epigenetic makeup. Analysis 3. NOWS vs. unexposed controls. (opioid-exposed infants who required pharmacologic management for NOWS vs. unexposed controls (+Opioids/+NOWS vs. -opioids/-NOWS, control), revealing key markers underlying withdrawal symptoms. Analysis 4. Distinguishing opioid-induced epigenetic changes: Comparison between NOWS infants who do not require pharmacologic management (+Opioids/-NOWS) vs. (-Opioids/-NOWS, control) revealing key opioid-induced alterations that illuminate early molecular mechanisms.

to impulsivity (95). *PRKG1* disrupts synaptic plasticity and neurotransmitter release, impacting impulse control (96).

Glucose metabolism

Glucose metabolism is crucial for physiological functions, including pain perception, possibly due to glucose level fluctuations (97, 98). Glucose metabolism and pain are bidirectional; elevated glucose levels can reduce pain tolerance, as seen when glucose infusion lowers pain thresholds (98). Diabetic patients often experience heightened pain sensitivity (hyperalgesia), linking altered glucose metabolism to pain perception (98). Conversely, acute severe pain decreases insulin sensitivity by affecting nonoxidative glucose metabolism, indicating that pain influences glucose metabolism (99). The hormonal response to pain, involving stress hormones, underscores the importance of pain management in maintaining metabolic balance (99). The study identified genes *like NR3C1, CACNA1C, CACNA1H, ESR1, CFTR, KLF11, EIF2AK3, NOTCH3, GJA1,* and *STX1A* as linked to glucose metabolism and pain sensitivity. *NR3C1* mediates glucocorticoids' anti-inflammatory and analgesic effects, affecting glucose metabolism and pain (100). *CACNA1C* and *CACNA1H*, which encode voltage-gated calcium channels, influence neurotransmitter release and pain sensitivity through glucose balance disruption (101). CFTR, linked to cystic fibrosis, causes metabolic irregularities that impact pain (102). *KLF11* regulates glucose

metabolism and insulin, with variations affecting pain sensitivity and diabetes (103). *EIF2AK3*, involved in ER stress and glucose metabolism, is associated with chronic pain (104). *NOTCH3*, a cell signaling receptor, may modulate pain through interactions with metabolic regulators (105). *GJA1*, a gap junction protein, influences glucose metabolism and pain signaling. *STX1A*, important for neurotransmitter release, affects pain sensitivity and glucose metabolism (106).

Obesity

Obesity worsens chronic pain by increasing inflammation and aggravating conditions like rheumatoid arthritis and fibromyalgia, while also raising the risk of metabolic syndrome, heart issues, diabetes, and cancer (107). This bidirectional relationship fuels a cycle where obesity heightens pain and reduces physical function, while chronic pain limits activity, contributing to weight gain and further exacerbating both conditions (108). Our study identifies that dysregulation in genes like *NOS3, TNF, BDNF, ESR1, ESR2, LMX1B, ATP1A2, OPRM1, NPY, THRB, NRXN3, SPARC,* and *MYT1l* significantly impacts the interplay between obesity and chronic pain. Earlier discussions covered *NOS3, TNF, BDNF, ESR1,* and *ESR2. LMX1B* plays a key role in obesity by regulating adipogenesis and energy metabolism (109). *LMX1B* mutations linked to nail-patella syndrome (NPS) may also contribute to chronic pain, suggesting heightened pain sensitivity (110); *ATP1A2* helps regulate metabolism by maintaining ion gradients across cell membranes and is linked to pain disorders such as neuropathic pain and fibromyalgia. The *OPRM1* gene, linked to the mu-opioid receptor, affects obesity and pain, especially in NOWS infants, with prolonged opioid use disrupting appetite and metabolism, leading to weight gain (111); *NPY* regulates appetite and energy balance, promoting feeding behavior and fat storage, thereby contributing to obesity development; *THRB* regulates metabolism and energy expenditure through thyroid hormones, affecting metabolic rate and potentially causing obesity if dysregulated. It also influences pain sensitivity and inflammation; *NRXN3* affects obesity traits like BMI and adiposity through appetite-regulating neural circuits and is linked to pain sensitivity (112). *SPARC* regulates tissue remodeling, impacting obesity-related processes and pain modulation. *MYT1l*, a transcription factor important for neural development, is associated with intellectual disability, autism, and obesity (113).

Circadian rhythm

Circadian rhythms, which regulate body processes over a 24-hour cycle, significantly affect pain perception and management (114). Disrupted rhythms can worsen pain and alter opioid processing, while pain and opioids can also impact circadian rhythms (115, 116). Disruptions affect factors like endogenous opioid levels, sleep, gene expression, and hormonal regulation. We identified 12 genes linking circadian rhythms to

pain regulation: *ESR1, CACNA1C, KLF11, IL1B, ABCA1, CAMK4, OSM, SHMT1, ARNTL, DOCK4, GNA11,* and *CRIP2. ESR1* affects females' circadian systems via estrogen levels. *CACNA1C* modulates calcium influx, potentially disrupting rhythms. *KLF11* influences glucose metabolism, impacting circadian patterns. *IL1B*'s proinflammatory effects affect the *SCN*, disrupting rhythms. *ABCA1* affects lipid signaling, possibly disrupting rhythms (117). *CAMK4* synchronizes peripheral clocks. *OSM* modulates rhythms through inflammatory pathways (68). *SHMT1* impacts metabolic processes. *ARNTL*, the core clock gene, regulates other clock genes, and mutations cause disorders. *DOCK4*, associated with neuronal development, may regulate rhythms. *GNA11* influences light signaling pathways. *CRIP2* affects rhythmic gene expression in the heart, potentially disrupting circadian rhythms (118).

Essential minerals and electrolytes in pain management

Minerals and electrolytes are essential for nerve function and pain regulation, governing nerve signaling and muscle contractions, while dysregulated metal-associated genes can disrupt DNA methylation and exacerbate chronic pain conditions (119). Calcium ions, crucial for neurotransmitter release and signaling, are disrupted by dysregulated genes such as *CACNA1C* and *CACNA1H*, heightening pain sensitivity and altering pain thresholds (120). Potassium channels (e.g., *KCNJ2, KCNQ5*) regulate neuronal excitability by maintaining membrane potential (121), while sodium channels (e.g., *SCN1A, SCN8A*) are vital for action potential transmission and pain signaling (122). Disruptions in these channels due to genetic variations or prenatal opioid exposure can lead to abnormal pain signaling. Selenium (*PCSK5, BDNF*) aids in antioxidant defense (123) and neurotransmitter regulation, potentially reducing chronic pain risk and neurodevelopmental issues from opioid withdrawal. Lithium (*BDNF*) influences neurogenesis and pain regulation by affecting BDNF levels, impacting the nervous system's adaptation to pain in opioid-exposed infants (124). *ATP1A2* encodes a Na +/K + ATPase subunit crucial for ion balance, and disruptions can impact pain processing and heighten the risk of pain or neurological issues such as migraine later in life (125).

Transporters can influence pain perception

Dysregulated transporters like *ABCA1, SLC12A5*, and *ABCC7 (CFTR)*, critically affect infants exposed to opioids *in utero* by altering neurotransmitter and ion movement across cell membranes (9). *ABCA1* affects lipid metabolism and cholesterol transport. Altered *ABCA1* can disrupt lipid balance, increasing inflammation and pain sensitivity, and potentially impacting nervous system development and long-term pain processing (126). *SLC12A5* encodes the K + -Cl- cotransporter KCC2, essential for maintaining chloride homeostasis in neurons, which is critical for proper synaptic inhibition and neuronal excitability.

Impaired *SLC12A5* function can lead to altered pain perception and increased sensitivity, effects that may be aggravated in individuals with a history of opioid exposure (127). *ABCC7 (CFTR)* regulates chloride ion transport across epithelial membranes, impacting tissue homeostasis and inflammation. While primarily associated with cystic fibrosis, *CFTR* abnormalities can indirectly influence pain pathways by promoting chronic inflammation and epithelial dysfunction, leading to altered pain sensitivity and management (128).

Pathways

Our study identified 48 significant canonical pathways (FDR < 0.05). We highlight key findings relevant to pain and infants born after prenatal opioid exposure.

cAMP signaling pathway

The cAMP signaling pathway is crucial for pain perception and transmission in the nervous system. Activating the cAMP pathway in the central nervous system induces hyperalgesia, whereas inhibiting this pathway alleviates hyperalgesia in inflammatory, non-inflammatory, and neuropathic pain models (129). There were 14 genes identified in this pathway.

MAPK signaling pathway

The MAPK pathway regulates diverse cellular functions and is crucial for transmitting pain signals from the injury site to the brain, where they are interpreted as pain (48). Thirteen differentially methylated genes have been identified in this pathway.

AGE-RAGE signaling pathway in diabetic complications

The AGE-RAGE pathway is critical in diabetic complications, including pain (130). Advanced Glycation End-products (AGEs) accumulate in tissues due to hyperglycemia, activating RAGE and initiating signaling cascades (131). In diabetic pain, this pathway contributes to neuroinflammation, oxidative stress, and nerve damage, which are key factors in diabetic neuropathy. RAGE activation leads to the production of pro-inflammatory cytokines, chemokines, and reactive oxygen species (ROS), exacerbating neuroinflammation and contributing to nerve dysfunction and pain hypersensitivity (132). Eight genes are involved in this pathway. Neurodegeneration pathways in NOWS, like oxidative stress, inflammation, and apoptosis, may alter long-term pain sensitivity and modulation (133). Infants with NOWS might experience increased pain sensitivity or altered pain responses, potentially persisting into childhood and adulthood. Calcium signaling and Alzheimer's disease-related pathways, essential for

neural function, are disrupted in NOWS (134, 135). These disruptions, combined with epigenetic changes, may contribute to heightened pain sensitivity and neurological dysfunction in affected infants. The PI3K-Akt pathway, vital for cell survival and growth, significantly influences pain processes. It can drive both adaptive responses that manage pain and maladaptive processes that worsen chronic pain (136). The enrichment of oxytocin signaling and circadian entrainment pathways in NOWS affects both pain perception and broader physiological systems in neonates, necessitating comprehensive care strategies (137, 138).

Limitations

Current human placental studies are inadequate for identifying biomarkers, and the underlying epigenetic mechanisms are not well understood. While these studies offer preliminary evidence linking therapeutic opioid use to epigenetic changes, further research is essential to investigate the temporal dynamics of these modifications in response to both prescription opioids and pain. Establishing causality will require additional factors to be considered. This study provides an intriguing potential proof-of concept, but larger cohort studies are necessary to validate these findings. Moreover, the differentially methylated genes identified have not been subjected to further validation.

In conclusion, our cohort study revealed that opioid use for pain management during pregnancy leads to disruptions in epigenetic factors. These alterations could serve as biomarkers, offering potential therapeutic targets and insights into the molecular mechanisms underlying prescription opioid use and pain. Notably, further investigation is needed to understand how epigenetic changes in genes related to chronic pain, due to maternal opioid use during pregnancy, affect NOWS. Looking ahead, our future research will focus on validating key epigenetic markers through functional studies, exploring their therapeutic potential in preclinical models, and assessing the long-term impact of these markers on individuals exposed to opioids.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The study received approval from the Institutional Review Board of Beaumont Health System, Royal Oak, MI, USA (HIC#:2019-086). Informed consent was waived since the study solely involved collecting discarded placental tissues and obtaining limited de-identified demographic data from hospital records.

Author contributions

UR: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. RR: Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing. LU: Validation, Writing – review & editing, Data curation, Formal Analysis, Investigation, Methodology, Software. TT: Data curation, Formal Analysis, Methodology, Software, Validation, Writing – review & editing. JP: Data curation, Formal Analysis, Methodology, Software, Validation, Writing – review & editing. RR: Conceptualization, Data curation, Formal Analysis, Methodology, Software, Supervision, Validation, Writing – review & editing. SM: Conceptualization, Data curation, Formal Analysis, Investigation, Supervision, Validation, Visualization, Writing – review & editing. RB-S: Software, Supervision, Validation, Visualization, Writing – review & editing, Formal Analysis, Investigation, Methodology. SS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – review & editing.

Funding

Conflict of interest

Drs. SS and RR are supported by their collaborative NIH grants (R01HD096800 PI: Sadhasivam) and R01DA059321 PI: Radhakrishnan). Sadhasivam's efforts are also supported by R44DA055407, R44DA056280, R41DA053877 (MPI: Sadhasivam), R01DA054513 (MPI: Chelly Sadhasivam) and U01TR003719 (PI: Sadhasivam).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpain.2025.1497801/full#supplementary-material

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