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Enrichment of genomic pathways based on differential DNA methylation associated with chronic postsurgical pain and anxiety in children – a prospective, pilot study

Vidya Chidambaran[1,5], **Xue Zhang**[2], **Kristie Geisler**[1], **Bobbie L Stubbeman**[1], **Xiaoting Chen**[3], **Matthew T. Weirauch**[3,4,5], **Jarek Meller**[4], and **Hong Ji**[2,6,7]

[1]Department of Anesthesiology, Cincinnati Children's Hospital, Cincinnati, OH, USA

[2]Pyrosequencing core for genomic and epigenomic research, Cincinnati Children's Hospital, Cincinnati, OH, USA

[3]Center for Autoimmune Genomics and Etiology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

[4]Divisions of Biomedical Informatics and Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

[5]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA

[6]Department of Environmental Health, University of Cincinnati

[7]Division of Asthma Research, Cincinnati Children's Hospital, Cincinnati, OH, USA

Abstract

We have reported child anxiety sensitivity (CASI) predicts chronic post-surgical pain (CPSP). Here, we evaluated DNA methylation profiles to understand gene-environmental interactions underlying CPSP and CASI, in order to identify shared, enriched, genomic pathways. In 73 prospectively recruited adolescents undergoing spine fusion, preoperative CASI, and pain data over 12 months post-surgery were collected. DNA from peripheral blood of evaluable subjects with (n=16) and without CPSP (n=40) were analyzed using MethylationEPIC arrays. We identified 637 and 2,445 differentially methylated positions (DMPs) associated with CPSP and CASI respectively ($p \leq 0.05$). Ingenuity pathway analysis of 39 genes with DMPs for both CPSP and CASI revealed enrichment of several canonical pathways, including GABA receptor ($p=0.00016$ (CPSP); 0.0008 (CASI)) and Dopamine-DARPP32 Feedback in cAMP ($p=0.004$

Corresponding Author: Vidya Chidambaran, MD, 3333 Burnet Ave, MLC 2001, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, Ph: 5136361786; vidya.chidambaran@cchmc.org.

Declaration of Author Contributions: VC conceived, conducted, coordinated data collection and analysis, and wrote the initial draft of the manuscript. Research coordinators KG and BLS, who consented and recruited subjects, and collected data. XZ and HJ contributed to statistical plan, sample size, analysis of the samples in the pyrosequencing laboratory, and analysis of epigenetic data and IPA. XC worked with MTW to conduct the histone and TF bioinformatics analyses while JM conducted the Enrichr, Pinet and LINCS bioinformatics analyses.

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(CPSP) and 0.00003 (CASI)) Signaling. Gene–gene interaction network enrichment analysis revealed participation of pathways in cell signaling, molecular transport, metabolism and neurological diseases (p-value <10–8). Bioinformatic approaches to identify histone marks and transcription factor (TF) binding events underlying DMPs, showed their location in active regulatory regions in pain pathway relevant brain cells. Using Enrichr/Pinet enrichment and Library of Integrated Network-Based Cellular Signatures (LINCS) knockdown signatures, we identified TFs regulating genes with DMPs in association with CPSP and CASI. In conclusion, we identified epigenetically enriched pathways associated with CPSP and anxiety sensitivity in children undergoing surgery. Our findings support GABA hypofunction and Dopamine-DARPP32 pathway's roles in emotion/reward and pain. This pilot study provides new epigenetic insights into the pathophysiology of CPSP, and a basis for future studies in biomarker development and targetable interventions.

Perspective: Differential DNA methylation in regulatory genomic regions enriching shared neural pathways were associated with chronic post-surgical pain and anxiety sensitivity in adolescents undergoing spine surgery. Our findings support GABA hypofunction and Dopamine-DARPP32 pathway's roles in emotion/reward contributing to behavioral maintenance of pain 10–12 months after surgery.

Keywords

Bioinformatics; epigenetics; anxiety; chronic postsurgical pain; DNA methylation; functional genomics

Introduction

Chronic postsurgical pain (CPSP) is often defined as pain that lasts beyond 3–6 months post-surgery, in the absence of other preexisting problems or postoperative complications.[46; 77] In children, the median prevalence of CPSP is 20%,[57] however the incidence ranges from 11–54% after spine fusion,[9; 40; 66] a painful surgery that adolescents undergo. CPSP involves multiple peripheral and central signaling and modulatory pathways regulated by genes.[32] While chronic pain conditions have a heritable risk of 45%,[78] and genetic factors explain some of the individual differences in pain perception,[1; 53] a genetic basis for CPSP has been elusive,[35] attributed partly to lack of replicability[38] and inconsistent findings[61] in genetic association studies,[4; 74] and lack of consideration of gene-environmental interactions. In addition, especially in children, caregiving environment and psychological factors like anxiety, prime children's pain responses influence his or her response to further surgical stress.[15; 30] Twin studies have shown that environmental factors are involved in the inter-personal differences in pain sensitivity.[1] Since epigenetic mechanisms such as DNA methylation (addition of a methyl group to the 5' position of a cytosine - guanine residue (CpG dinucleotide))[70] are known to mediate the influence of environmental factors on genetic expression,[60] and have been known to influence pain processing and the transition of acute to chronic pain, [6] it leads us to hypothesize that *elucidating gene-environmental influences through epigenetics will explain critical gaps in predisposition and mechanisms involved in CPSP*.[13; 17]

We recently identified psychological and perioperative factors associated with CPSP in adolescents undergoing spine fusion surgery. Among psychosocial factors studied, we reported that child anxiety sensitivity index (CASI) was significantly associated with CPSP in the spine cohort. μ-In addition, we identified opioid receptor gene (*OPRM1)* DNA methylation markers as predictors of acute and chronic postsurgical pain. However, effect sizes of single CpG sites are small, and sometimes identify associations that cannot be replicated. Hence, in this pilot study, we compare DNA methylation profiles and use a global bioinformatics-based approach to identify pathways, gene-gene interactions, histone marks, and protein-DNA binding events enriched in DNA methylation differences associated with CPSP and CASI. This approach integrates epigenetic-level data with biologic processes, pathways, and networks, and *overcomes pitfalls of hypothesis-driven candidate marker association studies, which overlook unknown possible causal variants*.[80] Such approaches have been used previously to study epigenetics of chronic pain conditions (such as fibromyalgia)[11] and psychological conditions (such as panic disorder),[65] but not CPSP or anxiety. We will test the hypothesis that biological processes enriched in gene sets with differentially DNA methylated positions (DMPs) will be shared by CPSP and anxiety, thus suggesting new avenues for preventing and treating CPSP.

Methods

An observational prospective cohort study was conducted in 73 adolescents with idiopathic scoliosis undergoing posterior spine fusion. The surgical, anesthetic and pain plans were standardized (see Supplement). The studies are registered with ClinicalTrials.gov (Identifier: NCT01839461, NCT01731873), as part of a larger pharmacogenomics study. The study was approved by the institutional review board. Written informed consent was obtained from parents and assent was obtained from children before enrollment.

Participants:

Healthy non-obese children aged 10–18 years were recruited for the study if they fulfilled following criteria: American Society of Anesthesiologists (ASA) physical status less than or equal to two (mild systemic disease), a diagnosis of idiopathic scoliosis and/or kyphosis, scheduled to undergo elective spinal fusion. We excluded females who were pregnant or breastfeeding, diagnosis of chronic pain or opioid use in the past six months, had hepatic or renal disease or developmental delays.

Data Collection

Prior to surgery, following data were collected: demographics (sex, age, race), weight, pain scores (numerical rating scale/0–10 NRS)[72] and home medications. We assessed anxiety in both child and a parent using the 0–10 visual analog scale (VAS), a simple scale validated for this use.[5] Questionnaires to assess pain catastrophizing and anxiety sensitivity were administered (Table 1). Surgical and anesthetic data collected included propofol and remifentanil doses, duration of surgery, and number of vertebral levels fused. On postoperative days (POD) one and two, we recorded pain scores (every four hours), and doses of morphine equivalents and diazepam administered. After hospital discharge,

research coordinators administered questionnaires over phone in a standard fashion, at 10–12 months after surgery (Table 1) to obtain pain measures.

Outcomes

Outcomes evaluated were a) CPSP, defined as NRS>3/10 at 10–12 months post-surgery.[47; 77] NRS cut-offs of 3/10 were used because they depict moderate/severe pain, are associated with functional disability, and have been described as a predictor for persistence of pain.[24] b) Child Anxiety Sensitivity Index (CASI), an 18-item self-report validated measure of symptoms of anxiety in children and adolescents (range 18–54), was chosen as the anxiety measure, because CASI has previously been shown to be strongly correlated with state and trait anxiety.[58] It measures how anxiety-related symptoms are interpreted as being physically, psychologically or socially harmful. [67] The CASI has been shown to have good test-retest reliability, and has been validated with high internal consistency in clinical and nonclinical pediatric samples (aged 8– 15.8 years).[67] Our own studies have shown that the odds of pain persistence at 1 year after spine surgery was 1.24 times higher per unit increase in CASI score (95% CI 1.09–1.42, p=0.002),[10] and is supported by studies in other pediatric cohorts.[54] Higher anxiety sensitivity is associated with fear of pain, avoidance behavior and maladaptive coping styles, leading to increased pain persistence and disability.

Measurement of DNA methylation

Blood samples were collected before surgery in EDTA. Genomic DNA was isolated and frozen at −20 °C. To study DNA methylation, 500ng of genomic DNA (measured by Thermo Scientific NanoDrop spectrophotometer), with quality controls maintaining a purity of 260/280 ratio from 1.6 – 2.0, was extracted, and treated with bisulfite using Zymo EZ DNA Methylation Gold kit (Zymo Research, Orange, CA, USA), according to manufacturer's instructions. Bisulfite-converted samples were hybridized in the Human Infinium MethylationEPIC BeadChip microarrays (©Illumina Inc., San Diego, CA). This array provides unparalleled coverage of CpG islands, genes, and enhancers.

Data analysis

The clinical characteristics and demographics of the cohort were described using mean (with standard deviation), median (IQR) and frequency (percentage) depending on data distribution. Prior to the DNA methylation analysis, the quality of the methylation arrays was assessed using sample-independent and dependent internal control probes included on the array for staining, extension, hybridization, specificity and bisulfite conversion. The number of probes with detection P value ≤0.05 was examined for each sample. Only samples that passed the quality control with >95% probes detected were included in the analysis. If CpG sites were not detected in all samples at p=0.01 level, or if they were located on the X and Y chromosomes, they were excluded. Of the 73 samples, one was excluded from analysis as it did not pass all quality control steps. The remaining samples all had more than 99% of the probes detected. The signal intensities were background-adjusted using out-of-band probes (noob) and normalized using subset-quantile within array normalization (swan in R 3.4.4'minfi_1.22.1'). [2] Beta values, calculated as

$beta = \frac{signal_{methylation}}{signal_{methylation} + signal_{unmethylation}}$, and M values, the logit transformation of the beta values,[19] were used. Surrogate variable analysis (SVA) was used to control batch effect and unknown confounders such as *cell composition*. The method "irw" in SVA_3.24.4 was used[42].[41] Age and race were included in the full model when generating the SVs. Models on beta and M values with different clinical measures generated different numbers of SVs, ranging from 6 to 7. To test whether the surrogate variables are associated with the outcome (CPSP and CASI), we performed Pearson and Spearman correlation.

For each of the CpG sites, the association of DNAm with CPSP and CASI was tested with linear regression. The linear models were adjusted to include age, sex, race and significant surrogate variables. CpG sites whose DNAm (both beta and M values) were associated with CPSP or CASI at $p \leq 0.05$ level were selected for further evaluation. The selected DMPs should also have differences ≥ 0.05 in beta between CPSP yes and no groups. (Figure 1) As impact of non-genetic covariates were previously found on CPSP and CASI,[9] to ensure the robustness of the association identified from the above analyses in which the DNAm was used as the dependent variable, we conducted logistic and linear regression for CPSP and CASI, respectively, in which CPSP and CASI were used as dependent variables and beta value as primary independent variable. Models were adjusted to include non-genetic co-variables. Significant non-genetic co-variables were identified by univariate analysis for CPSP (factors tested: age, sex, race, morphine dose in mg/kg POD1 and 2, preoperative anxiety score (VAS) for child and parent, duration of surgery, vertebral levels fused, PCS-P and CASI) and CASI (factors tested: age, sex, race, PCS-P, diazepam doses and parent anxiety score), and selection of co-variables associated at $p < 0.10$. Analyses were performed using Statistical Analysis System (SAS), version 9.4 (SAS Institute Inc., Cary, NC) and R 3.4.4. Only CpG sites showing significant association with beta values in these models ($p<0.05$) were extracted from Methylation EPIC array annotation files and imported into *Ingenuity Pathway Analysis* (IPA, Ingenuity Systems, Redwood City, CA) for pathway mapping, gene network detection, and upstream regulator identification. Gene networks mapped by overlapping genes (with p-score>8; p-score = −log10(p-value)) and hence, p-values $< 10^{-8}$ were identified and constructed in IPA.

To identify potential regulatory mechanisms altered by CpG methylation differences, we evaluated CpG sites that were significant in the previous step ($p<0.05$) against a control set of CpG sites ($p>0.4$) using a compiled large collection of functional genomics datasets from various sources, including ENCODE[12], Roadmap Epigenomics[3], Cistrome[43], and ReMap-ChIP[26]. In total, this database contains 4,045 datasets performed in 1,069 different cell types and conditions. 1,544 datasets monitor binding interactions of proteins such as transcription factors with the human genome using ChIP-seq; 1,213 measure the presence of histone marks using ChIP-seq; 277 measure open chromatin through DNase-seq; 55 measure expression quantitative loci (eQTLs); and 558 predict "ActiveChromatin" states using combinations of histone marks[20]. Overall, 240 experiments were performed in cell lines and cell types related to brain regions relevant to pain/anxiety outcomes.

We next used the RELI algorithm to estimate the statistical enrichment of histone marks and protein binding events at the genomic loci displaying altered DNA methylation.[28] As

input, the method took a set of genomic loci (in this case, regions with differential methylation marks). The coordinates of each locus were padded by 100 bases in either direction (to account for experimental resolution). The resulting loci were then systematically intersected with the ChIP-seq and epigenetic data set libraries described above, and the number of input regions overlapping each dataset by at least one base was counted. Next, a P-value describing the significance of this overlap was estimated using a simulation-based procedure. To this end, the control set of CpG sites that do not change ($p>0.4$) was used as a negative, background set. A distribution of expected overlap values was then created from 2,000 iterations of randomly sampling from the negative set, each time choosing a set of negative examples that match the input set in terms of the total number of genomic loci and the length of each locus. The distribution of the expected overlap values from the randomized data resembles a normal distribution and can thus be used to generate a Z-score and corresponding P-value estimating the significance of the observed number of input regions that overlap each data set. Collectively, this procedure controlled for the count and sizes of the input loci, and the count and sizes of each individual dataset in the library. The final output of the method is a p-value based ranking of all the functional genomics datasets, in terms of their overlap with the input set.

With the goal of further elucidating pathways and potential regulatory mechanisms underlying the observed epigenetic changes, we performed enrichment analysis using a comprehensive, curated library of transcription factor targets that combines results from ENCODE and literature-based CHEA ChIP-seq experiments, available through Enrichr (http://amp.pharm.mssm.edu/Enrichr/). Next, we used the Library of Integrated Network-based Cellular Signatures (LINCS) of genetic perturbations (gene knockdowns of the 39 genes common to both outcomes with DNA methylation changes) and connectivity analysis, with the focus on kinase signaling pathways, available through Pinet (http://pinet-server.org) and Enrichr.[39] One of the goals of LINCS library is to enable analysis of connectivity between genetic [36] and chemical perturbations by measuring correlations between their transcriptional echo (correlation between landmark gene expression vectors). Here, we use signatures of genetic knock-downs of gene encoding protein kinases, which consist of genes whose mRNA expression is downregulated in response to the loss of function for each kinase.

Results

The mean age of participants was 14.4 years (SD 1.6); they were mostly white (82%) and female (84%). Demographics and description of variables evaluated, are presented in Table 2. Median preoperative pain score was 0.0 (IQR 0.0–1.0) and mean (SD) for AUC on POD1 and 2 was 202.6 (84.3). As expected, there was a significant difference in NRS pain scores at 10–12 months between the non-CPSP (0.0 (0.0–1.0)) and CPSP (5.0 (4.0–6.0)) groups ($p<0.001$). Of 73 subjects recruited, follow-up for CPSP outcomes was successful for 56 subjects. Incidence of CPSP in this cohort was 15/56 (29%).

Non-genetic covariates for DNAm-outcome analyses

At a significance threshold of p<0.1, univariate analyses identified age and CASI as significant determinants of CPSP (p= 0.070 and 0.090 respectively) (Table 2); and PCS-P (p=0.015) and diazepam dose (p=0.090) for CASI. Preoperative pain, AUC and pain at 10–12 months were all significantly higher in the CPSP group compared to the non-CPSP group (p=0.006, 0.002 and <0.001 respectively). Since preoperative pain, AUC and CPSP are correlated pain outcomes, with possible overlap of DNA methylation associations, we did not include them as co-variables in the multivariate DNAm model for CPSP.

DMPs associated with CPSP/CASI

Based on SVA analyses, no surrogate variables were associated with CPSP or CASI at nominal level (p<0.05). Based on the workflow for analyses of DMPs associated with CPSP/CASI, adjusted for non-genetic covariates (Figure 1), we identified 637 DMPs for CPSP and 2,445 DMPs for CASI. The distribution of differentially methylated regions in association with CPSP and CASI (p-value<0.05 and delta-beta>5) with regard to genomic location, are presented in Table 3. The entire list of CpG sites (DMPs) including location, effect size and p-values, selected for IPA analysis for CPSP and CASI shared pathway analyses, will be provided as supplementary viewing files.

Pathway enrichment and gene network analyses

Annotation information on the DMPs was used for the analysis; in total, 310 genes (CPSP) and 1526 genes (CASI) were annotated to the DMPs. The genes associated with DMPs were over-represented in several canonical pathways (p-value<0.05) for CPSP and CASI. Pathways for CPSP included GABA receptor signaling, Protein kinase C signaling, dopamine receptor and cAMP mediated signaling among others. Pathways for CASI included beta-adrenergic, CDK5, protein kinase A, dopamine receptor and G-protein coupled receptor signaling among others.

Shared, enriched genomic pathways – CPSP and CASI

At gene level, 39 genes had DMPs associated with both outcomes, and they enriched 14 pathways (Table 4), among which the key ones were GABA receptor signaling and Dopamine-DARPP32 Feedback in cAMP Signaling. Gene network analyses of genes with DMPs common to CASI and CPSP revealed three networks associated with these phenotypes (p-value<10^{-8}). Of the three, two play a role in cell signaling, molecular transport, cancer, vitamin and mineral metabolism (p-scores of 33 and 19), while the third is involved in neurological disease and connective tissue function (p-score of 12). Two representative networks are illustrated in Fig 2.

Functional genomics analyses

DMPs associated with CPSP are located in active regulatory regions with open chromatin marked by H3K27ac, H3K4me1 and H3K4me3 in brain cells from the hippocampus, frontal lobe, temporal lobe, anterior cingulate cortex, etc. (Table 5). Also depicted in Table 5 are the significant (p<0.05, after correction for multiple testing) protein (e.g., transcription factor) binding events identified to overlap significantly at the CpG sites, significant for CASI. Of

note, many involve the RNA polymerase subunit POLR2A, suggesting that many differential methylation events might result in altered gene expression. The results of the Enrichr experiments are shown in Figure 3, where for each transcription factor, e.g., REST, its (here ENCODE mapped) targets among genes identified in our study are indicated by red squares (and include in this case RIMS2, CDH13, SPTBN4 etc.). Note that statistical significance of the enrichment is indicated by red vertical bars associated with each transcription factor. The results of the LINCS analysis are summarized in Table 6.

Discussion

We have previously shown that psychological variables (CASI),[9] clinical variables and *OPRM1* promoter DNA methylation[10] are associated with CPSP. In this study, we evaluated DNA methylation profiles for association with CPSP and CASI (anxiety sensitivity) in children undergoing spine surgery and followed up on our findings with an integrative computational analysis to identify common, targetable pathways enriched by the genes with differentially methylated CpG sites associated with these outcomes.

Epigenetic research into acute to chronic pain transitions[6] is still in its infancy. To our knowledge, there is only a handful of clinical epigenetic studies in postsurgical patients. DNA methylation of the Secreted Protein, Acidic, Rich in Cysteine (*SPARC*) promoter was shown to play a role in chronic low back pain related to degenerated intervertebral discs. [69] CpG methylation within the Tumor Necrosis Factor (*TNF*) gene promoter has also been identified as a mechanism by which *TNF* alters the risk for mild persistent breast pain in subjects with breast cancer undergoing surgery.[68] We previously reported on two CpG sites in an active regulatory region of the *OPRM1* gene that binds multiple transcription factors, to be predictive of CPSP in another subset of the spine surgery cohort.[10] Hence, although the study sample size is small, we believe this study provides novel insight and evidence for the role of epigenetics in CPSP. Moreover, similar analyses have been reported with smaller sample sizes (n=47; case + controls) in prior epigenetic-chronic pain studies. [11] Another epigenome based pathway analyses used whole blood DNA for DNAm in a large cohort of adults, with chronic widespread musculoskeletal pain.[45] They found that 6% of variance for the pain phenotype was explained by epigenetic factors, and showed enrichment for neurological pathways, including synaptic long-term depression, axonal guidance signaling, CREB, neuropathic pain signaling and melatonin signaling.[45] While some of the pathways are similar to what we have identified for CPSP, the differences may be reflective of differences in the nature of pain and cohorts evaluated.

We will focus our discussion on shared enriched pathways common to CPSP and CASI. Of great interest is that the top canonical pathways enriched by genes with DMPs common to both these outcomes were the GABA receptor signaling and Dopamine-DARPP32 pathways. This is aligned with previous literature citing hypofunction of GABAergic inhibitory tone in the dorsal horn of the spinal cord as a key factor in central neuropathic pain after spinal cord injury. [18] Mechanisms proposed for GABAergic hypofunction include decreased number of GABA receptors (through apoptosis),[62] downregulation of GABA synthesizing enzyme (GAD), [48] and decreased GABA concentrations.[27] Several studies, both *in vitro* and *in vivo,* support the role of DNA methyltransferases in the

regulation of GABAergic gene expression in brain regions relevant for pain and anxiety (cortex, striatum and hippocampus).[33] DNA epigenetic modifications of GABAergic interneurons in the basolateral amygdala were shown to be involved in the anxiety-like phenotypes in prenatal stress mice, and importantly, this was shown to be reversible with a demethylating agent, 5-Aza deoxycytidine.[79] Further, our functional genomics analysis show that many of the CpG sites identified are located in regions of the brain marked by lysine 27 tri-methylation (H3K27me3), which is known to negatively regulate gene expression. Our study thus provides new evidence for DNA methylation as a mechanism for possibly reduced function of the GABA receptor pathway genes and its role in CPSP and anxiety pathogenesis.

Our findings are also aligned with postulated roles for the DARPP-32 dopamine pathway (Supplementary figure 1) in the actions of drugs of abuse,[49; 76] inflammatory states,[75] and psychiatric conditions like schizophrenia and bipolar disorder.[31] DARPP-32 is a substrate of cAMP-dependent protein kinase (PKA) highly concentrated in dopamine-innervated brain areas, which functions as a PKA-regulated inhibitors of protein phosphatase-1 (PP1). The identification of epigenetic enrichment of this pathway is exciting as animal studies suggest a role for this phosphoprotein as intracellular detector of convergent dopamine-1 receptor and N-methyl-D-aspartate (NMDA) receptor activation,[7] which are target receptors for pain medications (opioids) and antipsychotic medications (for example haloperidol), and may suggest therapeutic interventions for CPSP, based on epigenetic profile.[23] Cyclin-dependent kinase5 (Cdk5) inhibitor (roscovitine) has been shown to decrease DARPP-32 phosphorylation,[75] and present exciting opportunities for future research, as its intrathecal use was found to decrease formalin-induced nociceptive response in rats[75] and remifentanil-induced hyperalgesia.[44] Since dopamine is involved in reward-mechanisms[64] and motivation to engage in pain self-management behaviors is an important predictor of adaptation/coping with acute pain, anxiety induced avoidance or lack of motivation[50] is the plausible mechanism by which dopamine signaling might be a player in development of CPSP in the presence of anxiety.

Genes with common DMPs associated with CPSP and CASI were also over-represented in nitric oxide signaling (NOS), which deserves mention. Nitric oxide is formed by N-methyl-d-aspartate (NMDA)-receptor activation. It has been shown to be an analgesic [55] and algesic [29] mediator at spinal, supraspinal and systemic sites in experimental animals. Pu et. al. postulated a dual control mechanism, wherein, excitatory NMDA is counteracted by inhibitory μ-opioid receptor signaling to modulate cyclic GMP/nitric oxide release, [56] thus influencing neuronal plasticity. Moreover, NOS also plays a role in morphine dependence and tolerance, which has been shown to be prevented using NOS inhibitors. [59]

There is evidence from prior studies for the role of pathways we have identified to be enriched by genes with DMPs associated with CASI. Bioinformatics analysis of microRNAs with differential expression in association with anxiety disorder used gene ontology and KEGG pathway analysis to predict target genes and functions. Epigenetically enriched pathways were elucidated which involved those related to neuronal brain functions, similar to our findings (GnRH signaling pathway).[21] Protein Kinase A signaling pathway, closely related to the DARPP-32 pathway described above, is involved in neuronal plasticity in the

amygdala, is responsible for amplification of anxiety behaviors in response to stressful stimuli. Several clinical studies have shown that alterations in PKA are associated with anxiety, depression, and other psychiatric disorders,[37] which supports our findings.

The limitations of this study are the use of blood samples for DNA methylation measurement. While measurement in the primary target tissue (brain) would be ideal, these are inaccessible in clinical human studies. Some evidence for use of blood samples as a correlate for brain samples comes from a previous study compared methylation profiles derived from 12 tissues and found high correlation of DNAm between somatic tissues.[22] Davies *et. al.* concluded that peripheral tissues may be useful in studies of complex neurobiological phenotypes,[14] based on their findings that inter-individual variation in DNA methylation was reflected across brain and blood. However, the translational relevance of findings in easily available tissue like blood cannot be overemphasized. In addition, we have used bioinformatics approaches to provide evidence for functional relevance of our findings in the brain. ChIP assay findings show that the DMPs identified are located in active chromatin areas in pain pathway relevant brain cells, which is a strong indicator of DNAm in these CpG sites possibly affecting gene expression and function in neural tissue. Moreover, Enrichr and Pinet analyses revealed that the genes with DMPs (common to CPSP and CASI) are regulated by several TFs previously associated with neuronal phenotypes. These TFs include REST, TRIM28, POU5F1, NFE2L2, GATA2 and NANOG (Figure 3). NANOG is also associated with POU5F1, KLF4 etc. Further evidence for the GABA pathway involvement in CPSP and CASI comes from the analysis of overlaps with LINCS knockdowns. This reveals several GABA receptor subunits (including GPRC5C), among other potential positive upstream regulators of genes with shared DMPs (Table 6). GPRC5C has been previously shown to be an activator of NANOG,[25] which seems to be consistent with NANOG's (and related TFs) targets being among (in this case predicted to be) positively regulated genes. Also, LINCS knockdowns of NANOG are strongly positively correlated (in multiple cell lines) with SMAD1/2/3, POLR2A (and other units of PolIIa), EP300 and other putative TFs targeting the overlap genes, which adds supporting evidence for them working together (not shown in the figures). The results of pathways analyses are limited due to being highly dependent on the input list of CpG loci – hence, validation/ replication of the sites will be needed for translational impact. Nevertheless, the findings of this pilot study provide a significant basis for future research in predictive biomarkers and development of DNA methylation integrated prediction models for CPSP. In addition, it demonstrates that epigenetic markers could be used to identify downregulated and upregulated pathways which can then be manipulated using drugs, another step towards individualized medicine. Cross sectional studies are potentially affected by reverse causation bias and genetic variation confounders[51] and do not provide causation inferences. Hence, future longitudinal studies will be necessary to identify epigenetic changes over time resulting from surgical insult and pain/opioid exposures.

In conclusion, our findings provide a better understanding for the shared role of epigenetic regulation of CPSP and anxiety. While future studies are required in larger prospective cohorts with longitudinal evaluation of DNAm with replication of our findings, these pilot results are promising, and open new avenues of epigenetics-based pain research, since DNA methylation mechanisms can be modified by factors like diet, exercise, stress and

meditation.[34; 73] Our findings provide a basis for biopsychosocial profiles involved in CPSP and suggest consideration of behavioral and other pathway-targeted strategies, based on the individual's methylation profile.[52] There is promise from animal models for epigenetic modification to prevent the progression to chronic postsurgical pain,[15; 16] and use of demethylating drugs in other diseases,[63; 71] for such therapies to be useful for the treatment of chronic pain. Recent advent of targeted epigenetic modification[8] also provides hope for decreasing nonspecific effects and poor delivery of epigenetic modulating to target cells and tissues, a major impediment to the development and clinical application of such analgesics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- First pilot epigenome wide study in pediatric chronic postsurgical pain (CPSP).
- DNA methylation in shared pathways associated with CPSP and anxiety sensitivity.
- Differential DNA methylation enrich GABA and Dopamine-DARPP32 pathways.
- GABA hypofunction and dopamine risk-reward pathways may be involved in CPSP.
- Functional bioinformatics supports results and transcription factor targets proposed.

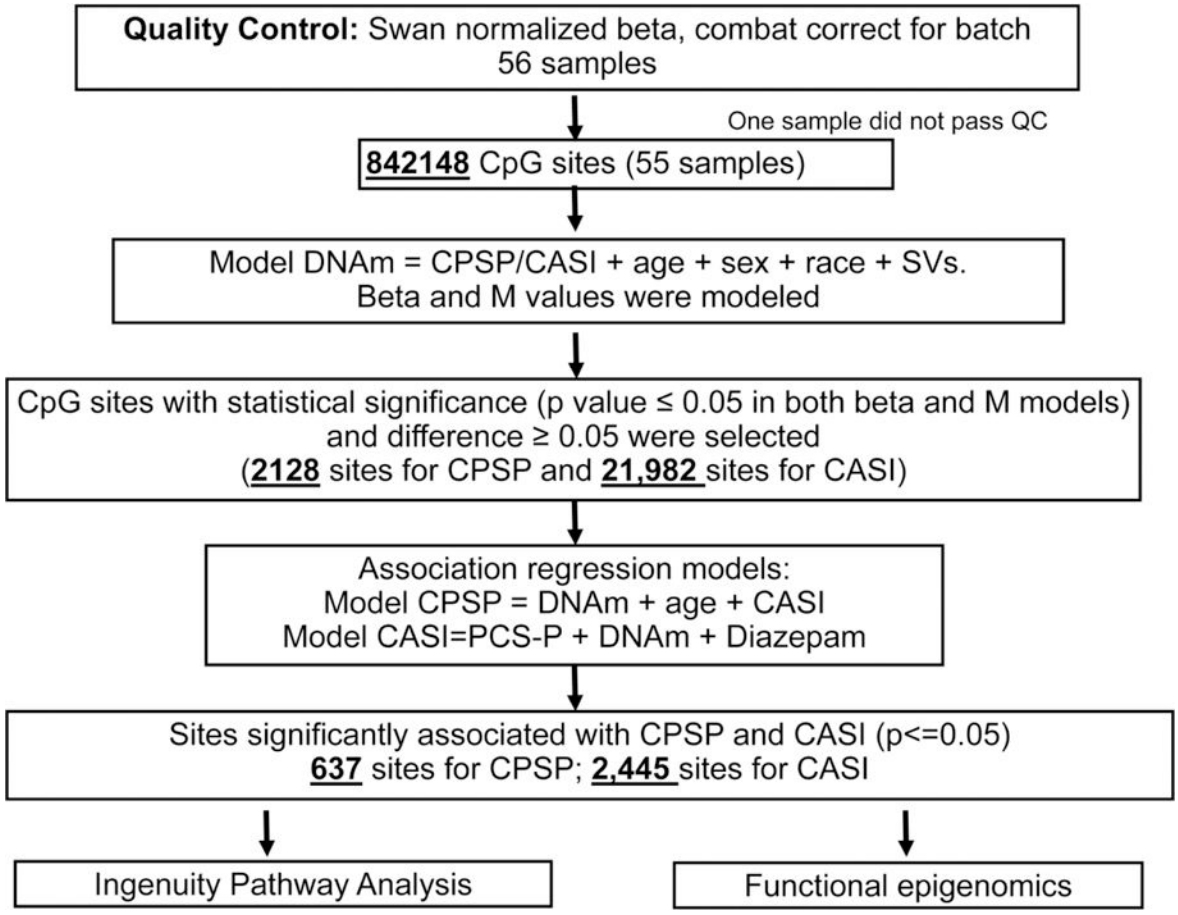

Figure 1:

Workflow for the statistical analysis of MethylationEPIC array data for chronic postsurgical pain (CPSP) and anxiety sensitivity (CASI) outcomes. One sample did not pass quality control. DNA methylation beta and M values were modeled and CpG sites with methylation satisfying certain criteria were included in logistic (for CPSP) and linear (CASI) regression models adjusted for other covariates. Methylation at sites significantly associated with outcomes were then included in the pathway and functional analyses.

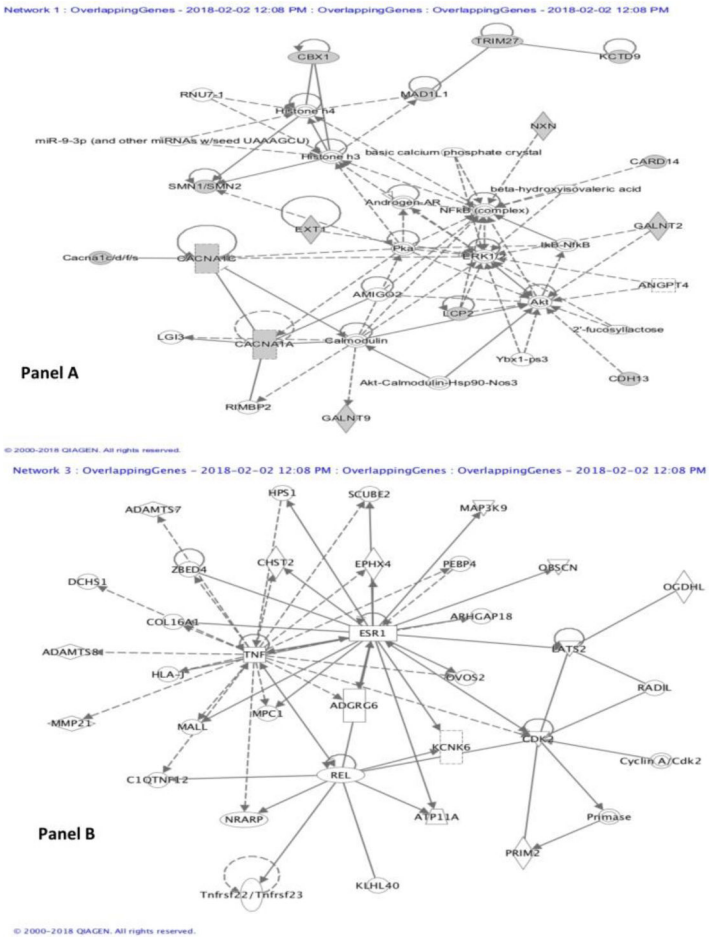

Figure 2:

Gene-gene interaction networks. Genes associated with the differentially methylated sites were uploaded to Ingenuity pathway analysis. Based on p-value cutoffs of 10^{-8}, three networks were identified. Two of them were similar in function with several overlapping molecules. Hence, two of the different networks are presented here. The network in panel A is associated with Cell Signaling, Molecular Transport, Vitamin and Mineral Metabolism. It had a p-score of 33 and 14 focus molecules (including CACNA1A, CACNA1C, Calmodulin, ERK1/2, Histone h3, Histone h4, IkB-NfkB, NFkB (complex), miR-9–3p). The network in Panel B is associated with Neurological Disease, Organismal Injury and Abnormalities, Connective Tissue Development and Function; with a p-score of 12, and 6 focus molecules (including ESR1, KCNK6, PRIM2, TNF).

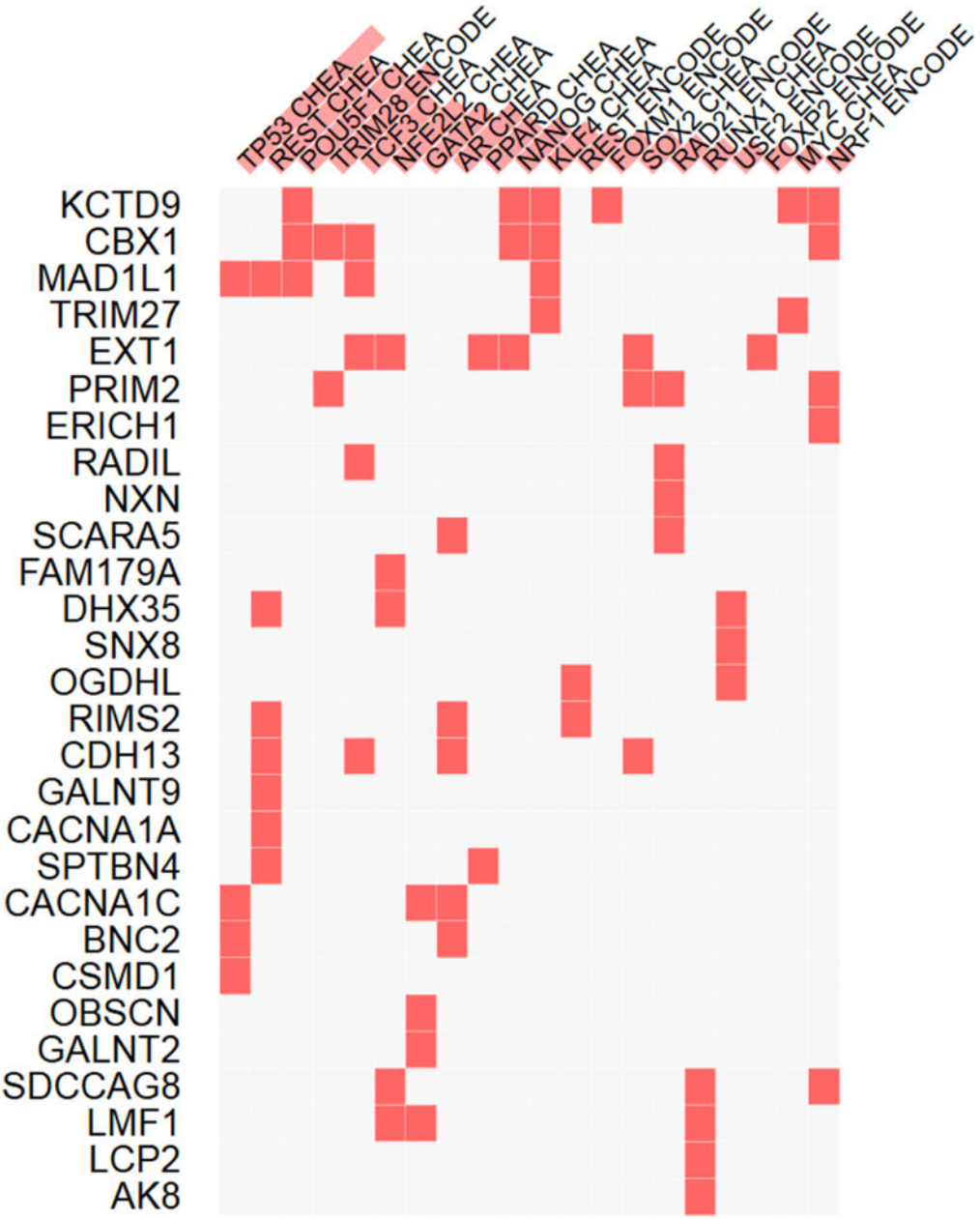

Figure 3:

The results of enrichment analysis using Enrichr are shown here, where for each transcription factor, e.g., REST, its (here ENCODE mapped) targets among genes identified in our study are indicated by red squares (and include in this case RIMS2, CDH13, SPTBN4 etc.). Note that statistical significance of the enrichment is indicated by red vertical bars associated with each transcription factor.

Table 1:

Data collection schema

Data variables	Pre-operative	Intra-operative	Over 48 hours after surgery	10–12 months after surgery
Demographics VAS Anxiety scores (parent and child) Pain score (child)	x			
Surgical duration Vertebral levels fused Propofol dose Remifentanil dose		x		
Pain assessment Opioid consumption Diazepam use Analgesic adjuncts	x		x	x
Child Questionnaires CASI	x			
Parent Questionnaires PCS-P	x			

* time calculated from end of surgery

Abbreviations: VAS: Visual analog scale; CASI = Childhood Anxiety Sensitivity Index; PCS-P = Pain Catastrophizing Scale (Parent version)

Table 2:

Demographics and other variables considered in univariate analysis for outcomes

	All	Chronic Post-surgical pain (CPSP)*			CASI (N=56)	
	N=73	No (N=40)	Yes (N=15)	p value	[d]Correlation coefficient	p value
[a]Age (years)	14.4 ± 1.6	14.3 ± 1.8	15.2 ± 1.3	0.07	0.01	0.96
[b]Sex (Male)	9 (16%)	8 (20%)	1 (7%)	0.42	0.06	0.68
[b]Race (White)	45 (82%)	33 (83%)	12 (80%)	1.00	0.04	0.76
[c]Weight (Kg)	53.7 (50.4–58.0)	53.5 (50.4–59.8)	53.9 (51.0–58.0)	0.84	−0.19	0.25
[c]Preoperative pain score	0.0 (0.0–0.1)	0.0 (0.0–0.0)	1.0 (0.0–2.0)	0.006	-	-
[c]VAS Anxiety (Child)	4.4 (3.0–6.9)	4.4 (3.0–6.8)	3.3 (3.0–8.5)	0.75	0.26	0.14
[c]VAS Anxiety (Parent)	6.7 (4.7–8.1)	5.4 (4.4–8.0)	7.6 (5.0–8.8)	0.37	0.25	0.15
[c]Number of vertebral levels fused	12.0 (10.0–13.0)	12.0 (11.0–13.0)	12.0 (10.0–12.0)	0.40	−0.01	0.95
[a]Surgical duration (hours)	4.2 ± 1.1	4.3 ± 1.0	3.9 ± 1.4	0.38	−0.12	0.47
[a]Pain AUC POD1&2	201.8 ± 85.8	181.0 ± 76.6	261.1 ± 85.4	0.002	-	-
[c]Morphine dose POD1&2 mg/kg	1.2 (0.9–1.7)	1.2 (1.0–1.8)	1.7 (0.9–2.9)	0.13	0.15	0.27
[c]CASI	28.4 (24.0–32.5)	27.0 (24.0–31.0)	29.8 (26.0–34.3)	0.09	-	-
[c]Pain scores at 6–12 months	1.0 (0.0–4.0)	0.0 (0.0–1.0)	5.0 (4.0–6.0)	<0.001	0.15	0.38
[a]PCS-P	21.7 ± 11.6	20.4 ± 12.5	24.1 ± 9.7	0.48	0.40	0.015
[c]Diazepam use mg/kg	0.1 (0.1–0.2)	0.1 (0.1–0.2)	0.2 (0.1–0.2)	0.12	0.26	0.09

Note:

a: data exhibited normal distribution; shown as mean ± SD and compared using t tests for PP.

b: shown as frequency (proportion) and compared using Fisher's exact tests for PP.

c: data did not exhibit a normal distribution; shown as median (IQR) and compared using Wilcoxon rank sum tests for PP.

d: Spearman correlation coefficient.

* Data from 55 subjects who had CPSP outcomes and evaluable Methylation EPIC array data are presented here

Abbreviations: VAS: Visual Analog Scale; POD: Postoperative Day; CASI: Childhood anxiety sensitivity index; PCS-P: Pain catastrophizing scale -parents; AUC: Area under curve; POD: postoperative day

Table 3:

Distribution of differentially methylated regions associated with chronic post-surgical pain (CPSP) and anxiety sensitivity (CASI) based on genomic location

Genomic location	CPSP – differentially methylated sites		CASI – differentially methylated sites	
	Number	%	Number	%
TSS1500	211	11.01	103	11.77
TSS200	93	4.85	35	4.00
5'UTR	40	2.09	64	7.31
1st exon	17	0.89	2	0.23
Gene body	729	38.03	318	36.34
3'UTR	3	0.16	22	2.51
N_Shelf	31	1.62	14	1.60
N_Shore	57	2.97	18	2.06
S_Shelf	24	1.25	12	1.37
S_Shore	42	2.19	19	2.17
CpG island	117	6.10	29	3.31
Open sea	553	28.85	239	27.31

CpG: 5'-C-phosphate-G-3': cytosine and guanine separated by only 1 phosphate.

Table 4:

Shared overlapping pathways identified by gene overlap for CpG sites associated with both chronic post surgical pain and child anxiety sensitivity index.

Shared Ingenuity Canonical Pathways for CPSP and CASI*	P-value CPSP	Ratio	Molecules	P-value CASI	Ratio
GABA Receptor Signaling	0.0001 6	0.07 4	*ABAT,ADCY5,CACNA1H,CACNA1C,GABBR1, KCNH2, CACNA1A*	0.0008	0.15 8
Dopamine-DARPP32 Feedback in Camp Signaling	0.004	0.04 3	*PPP1R1B, ADCY5, PLCG2, CAMKK1, CACNA1C, DRD4, CACNA1A*	0.00003	0.15 2
Cellular Effects of Sildenafil (Viagra)	0.005	0.04 6	*ADCY5, PLCG2, CACNA1C, NPPA, KCNH2, CACNA1A*	0.0002	0.15 3
GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells	0.012	0.04 5	*ADCY5,PLCG2,CACNA1H,CACNA1C,CACNA1A*	0.0002	0.16 1
Calcium Signaling	0.013	0.03 4	*CAMKK1, TRDN, CACNA1H, CACNA1C, CACNA1A, ATP2B2, CAMK2B*	0.0026	0.11 7
nNOS Signaling in Skeletal Muscle Cells	0.013	0.07 3	*CACNA1H, CACNA1C, CACNA1A*	0.0008	0.22 0
Dopamine Receptor Signaling	0.015	0.05 2	*PPP1R1B, ADCY5, DRD4, SLC18A2*	0.00007	0.19 5
Synaptic Long Term Depression	0.020	0.03 5	*PLBD1,PLCG2,PLA2G4C,CACNA1H,CACNA1C, CACNA1A*	0.02570	0.10 3
cAMP-mediated signaling	0.022	0.03 1	*PDE9A, ADCY5, VIPR2, PTH1R, GABBR1, DRD4, CAMK2B*	0.00000 2	0.15 0
Corticotropin Releasing Hormone Signaling	0.028	0.03 6	*ADCY5,PLCG2,CACNA1H,CACNA1C,CACNA1A*	0.0064	0.12 2
Netrin Signaling	0.045	0.04 6	*CACNA1H, CACNA1C, CACNA1A*	0.0006	0.18 5
CREB Signaling in Neurons	0.046	0.02 8	*ADCY5,PLCG2,CACNA1H,CACNA1C,CACNA1A, CAMK2B*	0.0008	0.12 3

*Two pathways not included in the table above include Gustatory pathway and sperm motility which are not relevant to the outcomes being studied

Table 5:

Overlap between CpG sites associated with chronic postsurgical pain (CPSP) and childhood anxiety sensitivity index (CASI) with functional genomics datasets in cells derived from brain tissue

CpG sites associated with Chronic postsurgical pain (Histone markers)				
Dataset	**Cell type**	**Epigenetic mark**	**Ratio**	**Corrected P-value**
Roadmap Epigenomics (Histone narrow)	Brain (Hippocampus, Middle)	H3K27me3	0.226	1.37E-14
Roadmap Epigenomics (Histone narrow)	Brain (Mid Frontal Lobe)	H3K27me3	0.190	3.56E-13
Roadmap Epigenomics (Histone narrow)	Fetal (Brain, Male)	H3K27me3	0.187	1.96E-09
Roadmap Epigenomics (Histone narrow)	Brain (Inferior Temporal Lobe)	H3K27me3	0.154	1.11E-07
Roadmap Epigenomics (Histone narrow)	Brain (Cingulate Gyrus)	H3K27me3	0.146	5.68E-07
Roadmap Epigenomics (Histone narrow)	Fetal (Brain, Male)	H3K4me1	0.349	6.03E-07
Roadmap Epigenomics (Histone narrow)	Fetal (Brain, Female)	H3K27me3	0.185	8.03E-06
Roadmap Epigenomics (Active Chromatin)	Brain (Germinal Matrix)	Bivalent enhancer	0.046	9.36E-05
Roadmap Epigenomics (Histone narrow)	Brain (Substantia Nigra)	H3K27me3	0.119	0.00014
Roadmap Epigenomics (Histone narrow)	Brain (Angular Gyrus)	H3K27me3	0.127	0.0015
Roadmap Epigenomics (Active Chromatin)	Fetal (Brain, Male)	Bivalent enhancer	0.096	0.0055
Roadmap Epigenomics (Active Chromatin)	Fetal (Brain, Male)	Bivalent TSS	0.019	0.0109
eQTLs (GTEx V6)	Brain (Anterior cingulate cortex BA24)	eQTL	0.025	0.015
CpG sites associated with Childhood anxiety Sensitivity Index (Histone markers)				
Roadmap Epigenomics (Histone narrow)	Brain (Cingulate Gyrus)	H3K4me3	0.321	6.76E-10
Roadmap Epigenomics (Histone narrow)	Brain (Mid Frontal Lobe)	H3K4me3	0.321	8.98E-09
Roadmap Epigenomics (Dnase narrow)	H1 Derived Neuronal Progenitor Cells	Dnase	0.381	1.37E-08
Roadmap Epigenomics (Histone narrow)	Brain (Inferior Temporal Lobe)	H3K4me3	0.321	2.26E-07
Roadmap E pigenomics (Histone narrow)	H9_Derived_Neuron_Cultured_Cells	H2A.Z	0.251	4.26E-07
Roadmap Epigenomics (Histone narrow)	H9 Derived Neuronal Progenitor Cells	H2A.Z	0.334	5.73E-07
Roadmap Epigenomics (Histone narrow)	Brain (Hippocampus Middle)	H3K4me3	0.333	7.51E-07
Roadmap Epigenomics (Histone narrow)	Brain (Anterior Caudate)	H3K4me3	0.330	1.09E-06
Roadmap Epigenomics (Histone narrow)	Brain (Angular Gyrus)	H3K4me3	0.299	1.49E-06
Roadmap Epigenomics (Histone narrow)	Brain (Substantia Nigra)	H3K4me3	0.294	2.00E-06
Roadmap Epigenomics (Histone narrow)	H9_Derived_Neuron_Cultured_Cells	H3K4me3	0.256	1.11E-05
Roadmap Epigenomics (Histone narrow)	H9 Derived Neuronal Progenitor Cells	H3K4me3	0.250	3.48E-05
Roadmap Epigenomics (Active Chromatin)	Brain (Hippocampus Middle)	2_TssAFlnk	0.103	0.0001
Roadmap Epigenomics (Histone narrow)	H1 Derived Neuronal Progenitor Cells	H3K4me2	0.283	0.0002
Roadmap Epigenomics (Active Chromatin)	Brain (Anterior Caudate)	1_TssA	0.232	0.0006
DNaseI Duke	Cerebellum	Dnase	0.314	0.0007
Roadmap Epigenomics (Active Chromatin)	Brain (Cingulate Gyrus)	ActiveChromatin	0.381	0.0007
Roadmap Epigenomics (Active Chromatin)	Brain (Inferior Temporal Lobe)	ActiveChromatin	0.383	0.0009
Roadmap Epigenomics (Active Chromatin)	Brain (Inferior Temporal Lobe)	2_TssAFlnk	0.091	0.0010
Roadmap Epigenomics (Active Chromatin)	H9_Derived_Neuron	ActiveChromat in	0.356	0.0014
Roadmap Epigenomics (Active Chromatin)	Brain (Substantia Nigra)	1_TssA	0.211	0.0019

CpG sites associated with Chronic postsurgical pain (Histone markers)				
Dataset	**Cell type**	**Epigenetic mark**	**Ratio**	**Corrected P-value**
Roadmap Epigenomics (Active Chromatin)	Brain (Anterior Caudate)	ActiveChromatin	0.391	0.0022
Roadmap Epigenomics (Active Chromatin)	H1_Derived_Neuronal_Progenitor	ActiveChromatin	0.317	0.0024
Roadmap Epigenomics (Active Chromatin)	Brain (Angular Gyrus)	1_TssA	0.220	0.0028
Roadmap Epigenomics (Histone narrow)	Brain (Substantia Nigra)	H3K9ac	0.264	0.0052
Roadmap Epigenomics (Active Chromatin)	Brain (Hippocampus Middle)	1_TssA	0.201	0.0078
Roadmap Epigenomics (Active Chromatin)	Brain (Substantia Nigra)	ActiveChromatin	0.363	0.0085
Roadmap Epigenomics (Active Chromatin)	Brain (Cingulate Gyrus)	2_TssAFlnk	0.088	0.0112
Roadmap Epigenomics (Active Chromatin)	Brain (Cingulate Gyrus)	1_TssA	0.203	0.0116
Roadmap Epigenomics (Histone narrow)	Brain (Anterior Caudate)	H3K9ac	0.302	0.0227
DNaseI Duke	Frontal cortex	Dnase	0.386	0.0232
Roadmap Epigenomics (Histone narrow)	Brain (Mid Frontal Lobe)	H3K9ac	0.267	0.0246
Roadmap Epigenomics (Histone narrow)	Brain (Inferior Temporal Lobe)	H3K9ac	0.310	0.0279
Roadmap Epigenomics (Histone narrow)	Brain (Cingulate Gyrus)	H3K9ac	0.294	0.0314
Roadmap Epigenomics (Active Chromatin)	H9_Derived_Neuronal_Progenitor	ActiveChromatin	0.340	0.0329
Roadmap Epigenomics (Active Chromatin)	Brain (Hippocampus Middle)	ActiveChromatin	0.402	0.0395
Roadmap Epigenomics (Active Chromatin)	Brain (Angular Gyrus)	2_TssAFlnk	0.069	0.0478
CpG sites associated with Childhood anxiety Sensitivity Index (Transcription factors)				
Dataset	**Cell type**	**Protein**	**Ratio**	**Corrected P-value**
ENCODE	HepG2+forskolin	POLR2A	0.175	0.00032
ENCODE	MCF10A-Er-Src+4OHTAM_1uM_36hr	POLR2A	0.169	0.00053
ENCODE	MCF10A-Er-Src+EtOH_0.01pct	POLR2A	0.171	0.00058
Cistrome	HEK293	BRD2	0.209	0.0012
ENCODE	MCF-7	POLR2A	0.155	0.0013
ENCODE	MCF-7+serum_stimulated_media	POLR2A	0.172	0.0017
Cistrome	CUTLL1	ETS1	0.169	0.0028
ENCODE	ProgFib	POLR2A	0.160	0.0044
ENCODE	HCT-116	POLR2A	0.190	0.0047
ENCODE	HeLa-S3	POLR2A	0.186	0.0068
ENCODE	Gliobla	POLR2A	0.165	0.0078
ENCODE	A549+DEX_100nM	POLR2A	0.191	0.0078
ENCODE	A549+EtOH_0.02pct	POLR2A	0.191	0.0082
ENCODE	ECC-1+DMSO_0.02pct	POLR2A	0.174	0.0093
ENCODE	MCF-7+serum_starved_media	POLR2A	0.167	0.0093
ENCODE	NB4	POLR2A	0.156	0.011
ENCODE	H1-hESC	POLR2A	0.173	0.013
ReMap	hek293	BRD3	0.077	0.014
ENCODE	A549	POLR2A	0.169	0.014
ENCODE	H1-hESC	RBBP5	0.112	0.014

CpG sites associated with Chronic postsurgical pain (Histone markers)				
Dataset	**Cell type**	**Epigenetic mark**	**Ratio**	**Corrected P-value**
ENCODE	HUVEC	POLR2A	0.198	0.016
Misc (GEO)	LoVo	ASCL2	0.124	0.020
ENCODE	SK-N-MC	POLR2A	0.137	0.020
ENCODE	GM19099	POLR2A	0.170	0.023
ENCODE	GM15510	POLR2A	0.165	0.025
ENCODE	MCF-7+serum_starved_media	CTCF	0.113	0.026
ENCODE	GM12878	POLR2A	0.188	0.034
Misc (GEO)	LoVo	GMEB2	0.166	0.035
Pazar	CD4+	HMGN1	0.198	0.039
Misc (GEO)	LoVo	MEF2C	0.039	0.039
Misc (GEO)	LoVo	RAD21	0.261	0.044

‘Ratio’ indicates the fraction of CPSP or CASI differentially methylated CpGs whose genomic coordinates intersect the indicated dataset. P-value is based on the significance of the ratio, and is adjusted for multiple testing, based on simulations (see Methods). No significant transcription factors in brain cells were identified for CpG sites associated with CPSP

Table 6:

Signatures of gene knockdowns for protein kinases and potential downstream targets among genes with DNA methylation associated with Chronic postsurgical pain and Childhood anxiety sensitivity index

Pathway	P Value	Gene List
ABL1_knockdown_96h_HEPG2	0.0003	TNXB, NXN, OGDHL, TRIM27, ADAMTS8,
DAPK3_knockdown_96h_HA1E	0.0003	SPTBN4, GALNT2, NXN, OGDHL, ADAMTS8,
RARB_knockdown_96h_HA1E	0.003	EXT1, PRIM2, ZNF814, LMF1,
GPR31_knockdown_96h_PC3	0.003	RIMS2, ZNF814, CACNA1C, ADAMTS8,
GABRG1_knockdown_96h_HCC515	0.003	BNC2, ERICH1, ZNF814, CARD14,
RIPK2_knockdown_96h_HCC515	0.003	EXT1, OBSCN, TNXB, TRIM27,
RIOK3_knockdown_96h_HA1E	0.003	SPTBN4, OBSCN, TNXB, ADAMTS8,
GABBR1_knockdown_96h_HA1E	0.003	OBSCN, NXN, ZNF814, TRIM27,
GPRC5C_knockdown_96h_PC3	0.003	RIMS2, PRIM2, SPTBN4, CACNA1C,
GPR151_knockdown_96h_A375	0.003	EXT1, SPTBN4, ERICH1, CARD14,
ADRA2A_knockdown_96h_A375	0.003	ADRA2A_knockdown_96h_A375
CHRM3_knockdown_96h_PC3	0.003	SPTBN4, OBSCN, ERICH1, CACNA1C,
FLT3_knockdown_96h_HA1E	0.003	PRIM2, SPTBN4, NXN, ADAMTS8,
CSNK1G2_knockdown_96h_HA1E	0.003	PRIM2, NXN, OGDHL, ADAMTS8,
TNIK_knockdown_96h_HEPG2	0.003	SPTBN4, GALNT2, NXN, OGDHL,

LINCS L1000 Kinase Perturbations show genes identified in this study as undergoing epigenetic regulation (right column) being significantly downregulated by kinase knock-down signatures in the left column, thus representing potential downstream targets of the respective kinase signaling cascade. Note that for each kinase, the corresponding cell line is also indicated, e.g., GABRG1 knock-down in HCC515 cells in the 5th row