Effect of short-term prescription opioids on DNA methylation of the *OPRM1* promoter

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Abstract

Background. Long-term opioid use has been associated with hypermethylation of the opioid receptor mu 1 (*OPRM1*) promoter. Very little is currently known about the early epigenetic response to therapeutic opioids. Here we examine whether we can detect a similar increase in DNA methylation in the days following the use of prescribed opioids. Longitudinal changes in DNA methylation was assayed using the Illumina Infinium Human MethylationEPIC array in a cohort of 33 opioid-naïve participants who underwent standard dental surgery followed by opioid medication self-administration. Saliva samples were collected before surgery (visit 1), and at two postsurgery visits at 2.7 ± 1.5 days (visit 2), and 39 ± 10 days (visit 3) after the discontinuation of opioid analgesics.

Results. The perioperative methylome underwent significant changes over the three visits. This was unrelated to opioids, and primarily due to postoperative inflammatory response and alteration in cellular composition. Deconvolution of cell heterogeneity indicated an increase in granulocytes at visit 2 and compensatory decline by visit 3. To specifically examine the effect of opioids, we applied a candidate gene approach and evaluated 10 CpGs located in the *OPRM1* promoter. There was significant cross-sectional variability in opioid use, and for participants who self-administered the prescribed drugs, the total dosage ranged from 5–210 morphine milligram equivalent (MME). Participants were categorized by cumulative dosage into three groups: <25 MME, 25–90 MME, ≥90 MME. Using mixed effects modeling, 4 CpGs had significant positive associations with opioid dose at 2-talied p-value < 0.05, and overall, 9 of the 10 CpGs showed the predicted higher methylation in the higher dose groups relative to the lowest dose group. After adjustment for age and cellular heterogeneity, the promoter mean methylation

also had positive associations with cumulative MME (regression coefficient = 0.0002, 1-tailed p-value = 0.02), and duration of opioid use (regression coefficient = 0.003, 1-tailed p-value = 0.001), but this effect was significant only for visit 3.

Conclusion. The present study provides evidence that the hypermethylation of the *OPRM1* promoter is in response to opioid use, and such epigenetic restructuring can be induced even by short-term use of therapeutic opioids.

Key words: prescription opioids, DNA methylation, addiction, opioid use disorder

Background

Prescription opioids were once considered as a relatively benign treatment for pain management [1, 2]. However, over the past decade, prescribed analgesics have emerged as a major socio-environmental factor that has contributed to the opioid epidemic [3, 4]. For many individuals who develop opioid use disorder (OUD), the initiation phase may begin with treatment for acute pain or minor surgery, with primary care physicians and dentists accounting for a large fraction of prescribed opioids [5-11]. Even short-term use (e.g., up to three days) is a risk factor for some individuals, and the risk for addiction increases proportionally with dosage and duration of use [8, 9, 12-15].

Drug addiction is a chronic disease that is triggered by an exposure to an environmental agent. Following the initial exposure, the addictive substance continues to have a persistent effect, and this suggests a form of cellular memory. There is strong evidence that epigenetic processes, including DNA methylation, play a key role in maintaining the long-term effects of the additive substance [16, 17]. Studies particularly in model organisms have shown that drugs of abuse trigger intracellular signaling cascades that alter gene transcription; the repeated exposure to the drug then results in remodeling of the epigenome that persists over time; and these epigenetic processes maintain the long-term changes in steady-state gene expression that underlie addiction [16, 18-20]. Work in humans generally relies on postmortem tissue from long-term drug users, and studies have found significant epigenetic differences in the brain of former addicts compared to non-addicts [21, 22]. While the brain is the most relevant tissue in terms of neuroadaptation and drug seeking behavior, epigenetic marks of addiction have also been detected in peripheral tissues such as blood and sperm [23-28]. Easily accessible

peripheral tissues are clearly the practical choice when it comes to defining biomarkers of drug use and/or predictors of individual risk for addiction.

The μ-opioid receptor gene (*OPRM1*) encodes the primary target for both endogenous and exogeneous opioids and plays a central role in mediating the rewarding and therapeutic effects. The CpG island located in the promoter of this gene is a potential sensor for drug use, and multiple studies in leukocytes and sperm have found higher DNA methylation among long-term opioid users compared to control samples [23, 29-33]. Hypermethylation of the promoter region has also been found among people with alcohol dependence [34]. However, as all these studies are cross-sectional comparisons between opioid exposed individuals and controls, there is no definite way to discern whether the epigenetic differences are the cause, or effect, of drug use. Since genetic variants within and near the *OPRM1* gene have also be associated with susceptibility to addiction and drug sensitivity[35-37], it is plausible that such epigenetic marks represent genetic effects that preceded drug use. Another lingering question is, if the epigenetic marks are induced by drug use, does the hypermethylation of the promoter CpGs occur only after repeated and sustained exposure, or are these indicators of the early epigenomic, and potentially transcriptomic, responses to drugs? In the case of potent drugs such as opioids, the initial exposure is a crucial phase in the pathway to drug dependence and addiction, and it is reasonable to expect some of the modification to the epigenome to occur within the first few exposures.

To address these questions, we applied a longitudinal design and collected saliva samples and self-reports of opioid use from a group of opioid naïve dental patients before oral surgery, and at two follow-up visits after surgery. We assayed genome-wide DNA methylation and explored

(1) the methylome during the perioperative period, (2) how demographic variables such as age and race/ethnicity relate to the methylome changes and immune response, and (3) whether we can discern opioid associated CpGs from the highly heterogeneous methylome data. As the site of surgery and postsurgery inflammatory response, saliva is a particularly challenging specimen. To overcome this, we applied *in-silico* approaches to deconvolute the underlying cellular heterogeneity and demonstrate the utility of the methylome-based cell estimates as proxies for the immune changes induced by surgery. For the effect of opioids, we specifically focused on the *OPRM1* promoter CpGs and evaluated whether we can replicate the CpG hypermethylation. Overall our results show a dosage-dependent increase in methylation that can be discerned despite extensive heterogeneity, indicating that the epigenetic response to opioids occurs within the first few days to weeks following exposure to the drug.

Results

The number of enrolled participants (N = 41) and timeline of sample collection are shown in Fig. 1. Only 33 patients (19 females) received prescription opioids after an oral procedure. The baseline characteristics and opioid use data are reported only for these 33 participants (Table 1). Following the pre-surgery visit (visit 1 or v1), the second visit (visit 2 or v2) occurred after surgery and within a week of the last opioid dose (average number of days between last opioid dose and visit 2 was 2.7 ± 1.5 days). The last sample collection (visit 3 or v3) occurred between 32–88 days from surgery, and the number of days between the last opioid dose and visit 3 was 39 ± 10 days. In total, 26 participants provided saliva samples at all three visits, 6 participants provided saliva at two visits, and one provided saliva only at v1 (Table 1). The mean age was 33.61 ± 13.84 years and ranged from 19 to 61 years (Table 1). Based on self-reported

race/ethnicity, there were 13 Caucasians (mean age = 31.69 ± 14.11 years), 13 African Americans (mean age = 39.92 ± 13.91), and the remaining 7 were of "other" racial/ethnic group (mostly Hispanic/Latino; mean age = 25.43 ± 8.02). Individual level information is provided as Additional file 1: Table S1. The African American group was slightly older but there was no statistically significant difference in age between the groups (p-value = 0.06). Sex distribution was not significantly different between the race/ethnic groups.

Postoperative opioid dosing data was based on self-reported pill counts, converted to morphine milligram equivalent (MME). With the exception of one individual who used no opioids (and we consider this individual to represent a dose of 0 MME with 0 days of use), all the patients started opioid treatment generally within 24 hours of surgery, and continued use for an average of 6 ± 4 days for up to 17 days (Additional file 1: Table S1). As expected, the cumulative dosage correlated with length of use (r = 0.67, p-value < 0.0001). For the 32 participants that self-administered opioids, the total cumulative dosage over the course of treatment ranged from 5–210 MME. Based on the quantile distribution of the cumulative MME, participants were classified into three groups: <25 MME (those below the 25th percentile or quartile 1 for opioid dosage) , 25–90 MME (those within the interquartile range), and ≥90 MME (those above the 75th percentile or quartile 3) (Table 1). Opioid dosage showed no significant association with age, sex, and self-reported race/ethnicity.

130 **Table 1. Participant characteristics and postoperative opioid use**

Variables[a]	
Sex	
Female	19
Male	14
Age (years)	33.61 ± 13.84
Self-reported race/ethnicity	
African-American	13
Caucasian	13
Other[b]	7
Prescribed opioid medication	
Hydrocodone 5mg	19
Oxycodone 5mg	12
Oxycodone 10mg	1
Oxycodone 5mg and Codeine 30mg	1
Length of opioid use in days (mean ± sd)	6 ± 4
MME[c] (mean ± sd)	64.91 ± 48.88
<25 MME (<Q1)	10
25–90 MME (Q1–Q3)	13
≥90 MME (≥Q3)	10
Surgery to visit 2 in days (mean ± sd)	8.0 ± 4.1
Surgery to visit 3 in days (mean ± sd)	43.9 ± 10.9
Number of completed visits	
Three visits (v1, v2, and v3)	26 participants
Two visits (v1 and v2)	5 participants [d]
Two visits (v2 and v3)	1 participant
Only v1	1 participant

131 [a] Mean and standard deviation (sd) for continuous variables and counts for categorical variables
132 [b] Other= Hispanic/Latino, Asian, Middle-eastern, and Native American
133 [c] Opioid dose converted to morphine milligram equivalent (MME) according to medication type; Q1 is the first
134 quartile (25%) and Q3 is the third quartile (75%)
135 [d] Methylome data for one participant with v1 and v2 samples were excluded during the methylome data check
136 (see methods)

137

138 Global shift in postoperative methylome

139 For an overview of the methylome and the variance structure, we started with a principal

140 component analysis (PCA) using the full set of high-quality probes (736,432 probes passed QC

141 criteria). The top PC (PC1) captured a vast portion of the variance at 63.5%, and following that,

PC2 and PC3 captured only 2.5% and 1.6% of variance, respectively (PCs for each methylome data in Additional file 1: Table S2). PC1 was not significantly associated with the demographic variables (sex, age, self-reported race/ethnicity). Instead, visit was the most significant explanatory variable for PC1 ($F_{2,86}$= 5.94, p-value = 0.004), and the pattern indicated a significant change in the methylome with the strongest contrast between v3 and v2 (Tukey-Kramer *post hoc* p-value = 0.003) (Fig. 2a). To deduce whether the longitudinal variance capture by PC1 could be explained by the length of time from surgery or opioid self-administration, we performed bivariate analyses between PC1 and the following variables: opioid dose, days from surgery to sample collection, and days from last opioid self-administration to sample collection. This analysis was done for the three visits separately, and at v2, PC1 had a modest but significant correlation with days from surgery to v2 (r = 0.40, p-value = 0.03, n = 31 participants with methylome data at v2; Fig. 2b). Similarly, at v3, PC1 was correlated with days from surgery to v3 (r = 0.41, p-value = 0.04, n = 27 participants with methylome data at v3). PC1 was not correlated with opioid dose or the number of days from the last opioid use. From this, we can infer that the longitudinal shift in the methylome is primarily due to surgery.

To profile the CpGs that changed longitudinally over the three visits we performed a mixed effects ANOVA with visit as a fixed variable and the person ID as random effect (Fig. 2c). The p-values for visit showed a significant deviation from the null hypothesis (Fig. 2d histogram). However, only 2 intergenic CpGs (cg05639411 and cg24904009) were above the genome-wide significant threshold of 5.0e-8 (Fig. 2c) and overall, the pattern indicated a modest shift in the methylome across several CpGs. At a genome-wide suggestive threshold of p-value = 1.5e-5, there were 1701 CpGs that underwent change over the visits (Additional file 1: Table S3). The

164 majority of these CpGs (>65%) decreased in methylation between v1 and v2, and regained
165 methylation by v3 such that these sites showed significantly higher levels of methylation at v3
166 compared to both v1 and v2 (Fig. 2e). Similarly, for the ~35% of CpGs that gained methylation
167 between v1 and v2, these sites generally declined in methylation by v3 such that these sites
168 were significantly lower in methylation compared to both v1 and v2 (Fig. 2e). Gene set
169 enrichment analysis (GSEA) of the 1133 annotated genes represented by the CpGs conveyed
170 mostly an innate immune inflammatory response (Additional file 1: Table S4). The most
171 overrepresented pathway was natural killer cell mediated cytotoxicity (KEGG ID hsa04650;
172 normalized enrichment score = -1.93, FDR = 0.03), and the most overrepresented function was
173 genes involved in cellular defense response (GO ID 0006968; normalized enrichment score = -
174 1.83 p = 0.001, FDR = 0.3), and these immunity related categories were enriched among the
175 CpGs that decreased in methylation at v2. The opioid receptors were not represented in the list
176 of visit associated CpGs. Based on these observations, a possible explanation for the shift in the
177 methylome is that it is the result of surgery induced immune response and changes in the oral
178 cell composition. The opioid use, if it had an impact, is likely to exert a weaker signal, and given
179 the limited sample size, more suitable for a focused candidate gene study.

180 Deconvolution of cellular heterogeneity

181 To decompose cell types from the composite DNA methylation signal, we applied a reference-
182 free approach [38]. The bootstrapping method described in Houseman et al. [38] determined K
183 = 4 cell types (Additional file 1: Table S2). Cell 1, which represented the most abundant cell
184 type, showed an increase at v2 right after surgery, and a decline by v3 (Table 2). Aside from cell
185 1, no other cell showed significant change over the visits (Table 2). To deduce what cell types

186 are represented by the 4 groups, we also estimated blood leukocyte proportions (mainly
187 lymphocytes and granulocytes/neutrophils) using a reference-based approach [39], and
188 compared correlations between the 4 cell types to the reference-based cell estimates (Table 2;
189 Additional file 1: Table S2). Cell 1 had a strong positive correlation with granulocytes, and cell 4
190 had a strong positive correlation with lymphocytes indicating that cells 1 and 4 are chiefly
191 representative of the leukocyte population in saliva and serves as a proxy for the increase in
192 granulocyte proportions after surgery. Cells 2 and 3 had only modest correlations with the
193 leukocyte estimates (|r| of 0.4–0.5) and may be more representative of the epithelial cells.

194 The cell estimates were not associated with opioid dose. To evaluate if the baseline
195 characteristics were related with the cellular composition, we tested association with age, sex
196 and race/ethnicity and the three visits. Cell 1 had a significant negative correlation with age (r =
197 -0.48, p-value = 0.006) only at v2 that suggests an age-dependent immune response in the days
198 immediately after surgery (Fig. 3a). Cell 3 had the strongest association with age at all three
199 visits (Fig. 3b). Cells 2 and 3 showed extensive cross-sectional variability without longitudinal
200 change, and both were significantly associated with race/ethnicity at all three visits that
201 indicates that these could serve as proxies for the cellular composition differences between
202 populations (Fig. 3c, 3d). Cell 4 was not associated with any of the baseline variables, and sex
203 was not a factor for any of the cell types.
204
205
206
207

Table 2. Reference-free and reference-based estimates of cellular proportions

Cell types	Cell proportions by visit (mean ± SD) Visit 1	Visit 2	Visit 3	Visit p-val	Pearson r with reference-based estimates Lymphocytes	Granulocytes
	Reference-free estimates					
Cell 1	0.60 ± 0.28	0.74 ± 0.23	0.47 ± 0.32	$F_{2,86}$ = 6.6, 0.002	-0.96	0.95
Cell 2	0.13 ± 0.13	0.10 ± 0.10	0.17 ± 0.15	ns	0.51	-0.47
Cell 3	0.21 ± 0.20	0.12 ± 0.18	0.22 ± 0.19	ns	0.43	-0.40
Cell 4	0.07 ± 0.18	0.03 ± 0.08	0.14 ± 0.23	$F_{2,86}$ = 2.7, 0.07	0.77	-0.81
	Reference-based estimates					
Granulocytes	0.71 ± 0.14	0.77 ± 0.10	0.64 ± 0.18	$F_{2,86}$ = 6.1, 0.003		
Lymphocytes	0.27 ± 0.12	0.21 ± 0.09	0.33 ± 0.15	$F_{2,86}$ = 6.6, 0.002		

Effect of opioid dose on *OPRM1* promoter methylation

To examine if higher opioid dose is related to higher promoter methylation, we used a candidate gene approach and focused on the CpGs located in the *OPRM1* promoter. In total, 10 promoter CpGs were targeted by the Illumina probes and these encompassed the CpG island described by Nielsen et al. and replicated by Chorbov et al. [29, 30] (Fig. 4a; Table 3; individual level β-values in Additional file 1: Table S2). We first applied a mixed regression model with opioid dosage group and visit as fixed categorical variables, and each participant ID as random intercept. With the exception of the last CpG, the regression estimates for all the *OPRM1* promoter CpGs were positive with higher methylation levels for the two higher dosage groups (i.e., 25-90 MME and ≥ 90 MME) relative to the lowest dosage group (<25 MME) (Table 3). At a nominal p-value of 0.05, 4 CpG were significantly associated with opioid dosage groups. The ANOVA plots for these CpGs showed that the difference between the dosage groups was pronounced at v3 (for CpG1, CpG2, CpG6) and v2 (for CpG7) but not at v1 (Fig. 4a).

To check whether the association with opioid dosage can be robustly detected, we summarized the overall methylation pattern in the promoter by taking the mean DNA methylation β-values for the nine CpGs that were positively associated with opioid dosage (CpG1 to CpG9). We applied a linear regression model and tested whether higher mean methylation was associated with either higher MME or longer length of opioid use. This analysis was done for the three visits separately and adjusted for age and cellular heterogeneity. Both MME and length of opioid use were associated with higher mean methylation, but this effect was significant only at v3, further indicating that the hypermethylation of the *OPRM1* receptor is more likely a response rather than a predisposing factor (Fig. 4b; Table 4). Our results are consistent with the opioid associated hypermethylation and indicates that even a relatively short-term use may induce increase in methylation that is proportional to dosage at the *OPRM1* promoter.

Table 3. Dose dependent methylation of individual *OPRM1* promoter CpGs

		<25 MME vs. 25–90 MME[1]		<25 MME vs. ≥90 MME[1]		Dosage anova[2]	
CpG	ProbeID	Coef	t-val	Coef	t-val	$F_{2,29}$	*p*
CpG1	cg22370006	0.041	2.66	0.023	1.39	3.53	**0.04**
CpG2	cg14262937	0.051	2.77	0.014	0.69	4.20	**0.02**
CpG3	cg06649410	0.047	1.65	0.010	0.31	1.56	0.23
CpG4	cg23143142	0.018	1.56	0.000	-0.04	1.71	0.20
CpG5	cg23706388	0.010	0.76	0.006	0.38	0.29	0.75
CpG6	cg05215925	0.019	2.73	0.012	1.59	3.74	**0.04**
CpG7	cg14348757	0.042	2.78	0.019	1.16	3.92	**0.03**
CpG8	cg12838303	0.026	2.08	0.022	1.64	2.38	0.11
CpG9	cg22719623	0.006	0.52	0.004	0.33	0.14	0.87
CpG10	cg15085086	-0.029	-0.94	-0.040	-1.19	0.78	0.47

[1]Regression estimates for higher dose groups (25–90 MME and ≥90 MME) relative to lowest dose group (<25 MME) based on linear mixed effects model: lmer(CpG ~ dose + visit + (1|ID))

[2]Two-tailed p-values for the main effect of dosage groups

Table 4. Mean methylation in the *OPRM1* promoter and association with opioid dose and days of use

	MME dosage effect Visit 3[2]			Days of opioid use effect Visit 3[2]		
	Coef	t-val	*p* (1-tailed)	Coef	t-val	*p* (1-tailed)
Promoter methylation [1]	0.0002	2.16	0.02	0.003	3.47	0.001

[1] *OPRM1* promoter methylation summarize by averaging the β-values for CpG1 to CpG9

[2] Linear regression at visit 3, one-tailed p-value to test hypermethylation with higher cumulative MME or longer duration of use

Discussion

Here we carried out DNA methylation assay in longitudinally collected saliva samples from a cohort of opioid naïve dental patients who received prescription opioids following oral surgery. Due to the small sample size and the heterogeneity in the methylome data, we used a candidate gene approach rather than an epigenome-wide study to evaluate the effect of therapeutic opioids. To summarize the main result, we found increased methylation at the *OPRM1* promoter associated with higher cumulative opioid dose. This replicates the hypermethylated profile among long-term opioid users and alcohol dependent individuals [23, 29-34]. The pattern of methylation we observed indicates that the increase in methylation is more likely the response to, rather than the cause of, opioid use [20]. The present study provides evidence that such epigenetic modifications are induced within the early days of drug use and may represent the early epigenomic responses to an addictive substance.

A peculiar challenge we faced was that the site of sample collection was also the site of surgery. Saliva has a highly heterogeneous cellular makeup and is estimated to constitute about ~45% epithelial cells, and about ~55% leukocytes from circulating blood [40]. The main goal of the

study was to detect the effect of short-term and comparatively low-dose opioids, while accounting for the larger perturbation caused by surgery. The oral surgery was a minor and relatively non-invasive procedure; nonetheless, the patients would experience injury induced inflammatory response that can induce changes in numbers of circulating immune cells [41], and consequently, changes in the oral cell composition. As DNA methylation is highly cell-type specific, the heterogeneity in cells will be a major source of "noise" in the methylome data [39, 42-44]. The longitudinal variability in DNA methylation that was captured by the top PC can therefore be attributed to cell composition rather than opioid use. We could deduce this by the significant correlation between PC1 and the number of days from surgery to the follow-up visits. We were able to partly resolve the cell heterogeneity by applying reference-free and reference-based estimates of cell proportions. The reference-free method estimated four major cell types. Although the saliva certainly has more than just 4 types of cells [40], the classification into 4 broad groups likely reflects the limitation in the *in-silico* approach to resolve finer differences between cellular subtypes. Cell 1 most likely represented the granulocyte population (chiefly neutrophils), which constitutes the most abundant leukocyte subtype in circulating blood, and responsible for innate immunity and acute inflammatory response. Consistent with the known increase in granulocyte-to-lymphocyte ratio in the few days following surgery [45], we also found an increase in cell 1 and relative abundance of granulocytes compared to lymphocytes at visit 2. This was followed by a compensatory decrease in granulocyte proportions by visit 3. Cell 2 and cell 3 are presumed to represent a portion of the epithelial cell population, and these showed no significant within-individual changes over the visits. However, these cells exhibited significant association with age and self-

283 reported race/ethnicity. Although cell type decomposition was not the primary objective of the
284 study, our analyses demonstrate that the saliva methylome can be highly informative of
285 individual differences in perioperative immune profile.

286 For the effect of postsurgical opioid use, we focused on the *OPRM1* promoter region as an
287 epigenetic sensor of opioid dose. The CpG-rich promoter harbors a CpG island and several
288 studies in different populations have demonstrated higher DNA methylation at this site among
289 opioid users and methadone-maintained heroin addicts [23, 29-33]. The increased methylation
290 of the *OPRM1* promoter is not only limited to OUD but has also been detected among
291 individuals with alcohol dependence, suggesting that the hypermethylation is generally
292 associated with substance use disorder and addiction [34]. A question has been whether such
293 epigenetic differences are the result of drug use or the cause of increased vulnerability to
294 addiction [20]. To address this, we interrogated 10 CpGs in a 550 bp region that encompassed
295 the promoter CpG island investigated by Nielson et al. and Chorbov et al. (the CpG island is
296 depicted in Fig. 4) [29, 30]. With the exception of the last CpG, the remaining 9 CpGs showed
297 higher methylation in the two higher-dose groups relative to the low-dose group, and four of
298 these CpGs were significantly associated with dosage at nominal alpha of 0.05. Comparison of
299 mean methylation differences between the dosage groups across the three visits indicated that
300 the higher methylation in the higher dose groups is more apparent at the postsurgery visits,
301 particularly visit 3. The positive association between the mean promoter methylation and
302 cumulative MME, and mean promoter methylation and days of opioid use, were also significant
303 only at visit 3. The heightened inflammatory state at visit 2, which occurred within a few days

304 from surgery, may have been the reason why the more subtle effect of opioids was not
305 significant at visit 2, and the positive association emerged only at visit 3.

306 The *OPRM1* locus presents a prime site for gene x environment interaction, a critical aspect of
307 addiction since the addictive substance is an environmental agent that has a long-lasting
308 biological effect. The *OPRM1* gene has been the subject of several candidate gene studies for
309 addiction. Much attention has been paid to the missense SNP that alters the *OPRM1* protein
310 function, although its impact on addiction trait and OUD is somewhat ambiguous [46, 47].
311 Several studies have also identified non-coding variants in the *OPRM1* locus that alters DNA
312 methylation and gene expression [33, 35, 48]. At least one genome-wide association study has
313 also identified a genome-wide significant association between a SNP upstream of *OPRM1* and
314 methadone-maintenance dosing [37]. These studies collectively provide evidence that common
315 genetic variants in the proximal region of *OPRM1* affect DNA methylation and gene expression
316 and could have a downstream impact on opioid response that could potentially influence
317 vulnerability to addiction. Our present work was carried out in a small sample size and our
318 primary goal was to track the within-individual trajectory across the visits. If there were
319 genetically modulated small cross-sectional differences at baseline, the present sampling would
320 be underpowered to detect the differences, and the significant association with opioid dose we
321 found may have been the result of opioid-induced augmentation of differential methylation at
322 the postoperative visits.

323 The small sample size and the heterogeneity in methylome signal, partly due to cell
324 composition and partly due to the heterogenous population group, were limitations that
325 prevented a more comprehensive epigenome-wide exploration. The *OPRM1* promoter CpGs

may only be part of a network of genes that represent the acute cellular response to drug exposure. The present study also does not address if these epigenetic changes will linger in the absence of continued use. A more comprehensive longitudinal study of the early effects of prescriptions opioids with a longer follow-up period would be the next phase of study.

Regarding the potential for epigenetic persistence, we must point out that any peripheral tissue serves only as a proxy for the possible epigenetic changes in the brain. A distinction is that blood and epithelium are mitotically active tissues and cells are renewed within few days to few weeks, with the exception of the long-lived memory T-cells. For methylation signals to persist, it will require either continued presence of the perturbation (i.e., continued exposure to opioids), or methylation changes in mitotically active stem cells that can be faithfully transmitted to daughter cells. The brain, on the other hand, is mitotically inactive and consists of mostly terminally differentiated cells that last a lifetime. If the relatively modest dose and short-term use of prescription opioids has a similar impact in brain cells, the effects may not readily decay and may be long-lasting in the central nervous system.

Conclusion

In conclusion, our study replicates the hypermethylation of the *OPRM1* promoter with opioid use. Previous studies reported on the effects of chronic opioid use; here we provide evidence that the epigenetic restructuring begins within the initial stage of opioid exposure. The present findings on the acute effects of prescription opioids require further replication with a well-powered and more comprehensive study in a larger cohort.

Methods

Participants

Eligible participants were scheduled for tooth extractions at an oral and maxillofacial surgery clinic that were typically followed by postoperative prescriptions of hydrocodone/acetaminophen (7.5/325 mg q4-6h prn pain) or oxycodone/acetaminophen (5mg/325mg q6h prn pain). For inclusion in the study, individuals were required to be 18 years of age or older, opioid naïve, able to consent, able to understand and speak English, and willing to provide saliva samples. Individuals were excluded if they reported previous use of opioids, had current substance use dependence, were pregnant, were incarcerated, had other causes of pain, were unable to consent, or had a developmental disability that prevented participation. The study received approval by the university Institutional Review Board. Eligible participants were provided a summary of the consent form by the study coordinator and allowed to read and ask questions before enrollment. All participants provided written informed consent.

Forty-one individuals consented to the study and provided contact information and responded to a demographic questionnaire. The clinical staff provided routine opioid medication and recovery instructions for all participants right after surgery. The opioids prescribed to participants were Hydrocodone, Oxycodone, and Codeine in doses that varied between 5mg to 30mg (Table 1). The study coordinator also provided opioid medication logs to record self-administration including the date, time, individual dose per opioid pill, and the number of opioid pills taken. For the 33 participants who received opioid medication, only one participant (person ID 142) reported no opioid usage.

Sample processing and DNA methylation assay

Saliva was collected using the Oragene DNA sample collection kit (OGR 500) by DNA Genotek (http://www.dnagenotek.com). The first set of samples was collected before surgery. The second saliva sample was collected a few days after opioid discontinuation, and the third was collected on a follow-up visit (Fig. 1; Table 1). DNA was purified using the DNA Genotek PrepIT L2P kit according to manufacturer's instruction. Genome-wide DNA methylation was assayed on the Illumina Infinium Human MethylationEPIC BeadChips following the manufacturer's standard protocol at the HudsonAlpha Genomic Services Lab (https://gsl.hudsonalpha.org).

Data processing

Raw intensity IDAT files were loaded to R and all quality checks, data preprocessing, and normalization were carried out using the R package, minfi (v.1.31) [49]. Methylation levels were estimated as β-values (ratio of methylated by unmethylated probes) and quantile normalized. The initial QC involved comparison between the log median intensities of methylated and unmethylated channels, and the density plots for β-values (Additional file 2: Fig. S1a). All samples passed these checks. Sex estimated from the DNA methylation data also matched the self-reported sex. To retain only high-quality data, probes with detection p-value > 0.01 (14,676 probes) were excluded. Probes that target CpGs on the sex chromosomes were also removed (18,605 probes). Finally, a total of 96,146 probes that overlapped annotated SNPs and/or were flagged for poor mapping quality (MASK.general list from [50]) were also filtered out. A total of 736,432 high quality probes were retained and used for downstream analysis.

As further QC, we performed unsupervised hierarchical clustering using the full set of high-quality probes (Additional file 2: Fig. S1b). While samples longitudinally collected from the same

individual tended to cluster together, there were also several samples that did not cluster with self. To check for possible errors in sample labeling, we repeated the cluster analysis using a subset of 30,435 probes that had been filtered out due to overlap with common SNPs. While these are deemed as poor-quality probes and unfit for differential methylation analysis, in terms of sample identity check, these probes can serve as proxy genotype markers that can help verify if samples came from the same person. Using this set, almost all samples collected from the same participant clustered with self, and for the most part, the clusters also aligned with self-reported race/ethnicity groups (Additional file 2: Fig. S1c). Only one of the 33 participant who received prescription opioids (person ID 108) did not pair with self and data from this person was excluded from all downstream analysis.

Estimation of cellular proportions

To infer the relative proportions of the major cell types, we first implemented a reference-free deconvolution of the methylome data using the R package RefFreeEWAS (v2.2) [51]. The RefFreeEWAS algorithm applies a non-negative matrix factorization to decompose a matrix Y = MΩ, where M represents an *m x K* matrix with *m* as CpG specific methylation for an unknown number of *K* cell types, and Ω as the cell-type proportion constrained to sum to a value ≤ 1. For computational efficiency, the *K* cell types has to be first specified, and as described in Houseman et al. [51], we set the *K* to vary from 2 to 10 cell types and decomposed the Y = MΩ. Following this, we applied bootstrapping to estimate the optimal *K* value. For this estimation, we applied 10 interactions with replacement every 1000 times. The optimal *K* = 4 was selected based on the minimum value of the average of bootstrapped deviances for each putative cell type. While this method provides the relative proportions of the cell types, the identity of the

four cells are unknown. Since a significant proportion of saliva consists of leukocytes, we also applied a reference-based approach to estimate the relative proportions of lymphocytes and neutrophils [39, 42, 43]. To infer the putative identities of cells, we performed Pearson correlations between the *K* cells and the proportions of leukocyte types.

Statistical analyses

For the global analysis, PCA was done on the full set of 736,432 probes using the prcomp function in R. To evaluate which variable had the most significant association with PC1, we examined the association between PC1 and the following variables: sex, age, self-reported race/ethnicity, opioid dose, days from surgery to sample collection, and days from last opioid dose to sample collection. We used ANOVA for categorial variables, and Pearson correlation for continuous variables, and these tests were done for the three visits separately. We also performed similar analyses for the estimated cell proportions to examine whether the variables were significantly related to the estimated cell proportions. PC1 and the cell proportions was also related to visit using ANOVA. Since visit was the most significant explanatory variable for PC1, we identified the CpGs that showed longitudinal change over the three visits by applying a mixed-effects ANOVA: β-value ~ visit + Error(ID/visit). This epigenome-wide analysis was done in the 26 participants with data from all 3 visits. For the set of genome-wide suggestive CpGs that changed over the visits (uncorrected p-value $\leq 10^{-5}$), GSEA was implemented on the WebGestalt platform (http://www.webgestalt.org) with each CpG ranked by the mean β-value difference between v2 and v1.

For candidate gene analysis, we surveyed the promoter region of *OPRM1*. The CpG island that was interrogated by Nielson et al., and Chorbov et al. is located at 154360587–154360922 bp of chromosome 6 (GRCh37/hg19) [29, 30]. Within that exact coordinate, our data only had 4 CpG probes. We therefore considered a slightly wider region (550 bp) and in total, and the array data contained 10 probes that targeted promoter CpGs at chr6:154360344-154360894 bp. To evaluate methylation at individual CpGs, we applied a linear mixed effects model with the dosage group and visit as fixed categorical variables, and person ID as random intercept: lmer(β-value ~ dosage + visit + (1|ID)). This was done using the "lmertest" R package, and to get the p-values for the main effect of dosage groups, the degrees of freedom were computed by the Satterthwaite's method [52, 53]. Following the CpG level analysis, we estimated the general methylation trend for the promoter by averaging the β-values for the 9 CpGs that had a positive regression coefficient with the dosage groups. We then tested the association between the promoter mean methylation score, and two opioid related continuous variables: length of opioid use in days, and cumulative MME. This analysis was done for the three visits separately, and adjusted for age and cellular heterogeneity using the equations lm(mean-β ~ MME + age + cell1 + cell2 + cell3), and lm(mean-β ~ days-of-use + age + cell1 + cell2 + cell3).

List of Abbreviations

FDR: False discovery rate

GO: Gene ontology

GSEA: Gene Set Enrichment Analysis

KEGG: Kyoto encyclopedia of genes and genomes

MME: Morphine milligram equivalent

OPRM1: Opioid receptor mu 1

OUD: Opioid use disorder

PC: Principal component

PCA: Principal component analysis

QC: Quality control

SNP: Single nucleotide polymorphism

Declarations

Ethics approval and consent to participate: All participants provided written informed consent and study received IRB approval.

Data availability. The full de-identified raw DNA methylation data will be made available from the NCBI NIH Gene Expression Omnibus repository upon official publication.

Competing interests: We have no financial or non-financial conflicts of interest.

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Author contributions: JVSS: performed lab work and data analysis and contributed to manuscript; FISG: contributed to data analysis; JHB: identified suitable patients and facilitated participant recruitment at the dental clinic; KJD: contributed to study conception and design; KM: contributed to study conception, design and data analysis, and wrote the manuscript. All authors contributed to and approved the final version of the manuscript.

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Figure legends

Fig 1. Timeline of sample collection. Saliva samples were collected before surgery and the two follow-up visits after surgery and end of opioid self-administration. The notations above the arrows show the range of days between events.

Figure 2. Global patterns in DNA methylation across visits

(a) The top principal component (PC1) extracted from the methylome-wide data explained 63.5% of the variance, and the ANOVA plot shows significant differences between the three visits ($F_{2,86}$= 5.94, p-value = 0.004). **(b)** At visit 2, PC1 is correlated with number of days from surgery to the second visit (r = 0.40, p-value = 0.03, n = 31). **(c)** The epigenome-wide association plot depicts the location of each CpG (autosomal chromosomes 1 to 22 on the x-axis) and the $-\log_{10}$(p-value) for the effect of visit (y-axis). Genome-wide significant threshold is set at p-value = 5×10^{-8} (upper red horizontal line); suggestive threshold is set at p-value = 10^{-5} (lower blue horizontal line). **(d)** Distribution of p-values for the effect of visit shows a significant deviation from the null hypothesis. **(e)** For the CpGs above the suggestive threshold, comparison of mean differences between visit 1 and visit 2 (x-axis), and visit 3 and visit 2 (y-axis) indicates a reversal in methylation patterns from visit 2 to visit 3, with majority of sites showing lower methylation at visit 2, and then increasing in methylation by visit 3.

Figure 3. Estimated cell type proportions and associated variables

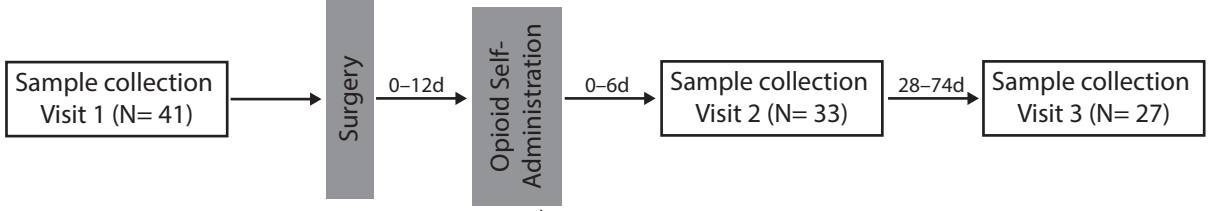
644 **(a)** Cell 1 shows both longitudinal and cross-sectional variability. Proportion of cell 1 is
645 negatively correlated with age at visit 2 (r = -0.48, p-value = 0.006, n = 31; black squares and
646 dashed line), but not at visit 1 (r = -0.13, p-value = 0.49, n = 31; red x markers and dotted line),
647 and only slightly at visit 3 (r = -0.32, p-value = 0.10, n = 27; grey circles and solid line). **(b)** Cell 3
648 is associated with cross-sectional variability but no significant longitudinal change. The
649 estimated proportion has a strong positive correlation with age at all three visits. At visit 1, r =
650 0.36 (p-value = 0.05); visit 2, r = 0.57 (p-value = 0.0009); visit 3, r = 0.48 (p-value = 0.01). **(c)** Cell
651 3 also shows a significantly higher proportion in African Americans at all three visits ($F_{2,28}$ =
652 15.66, p-value < 0.0001 at visit 1). **(d)** Cell 2 is also ethnicity specific and associated with lower
653 proportion in African Americans at all three visits ($F_{2,28}$ = 4.77, p-value < 0.02 at visit 1).

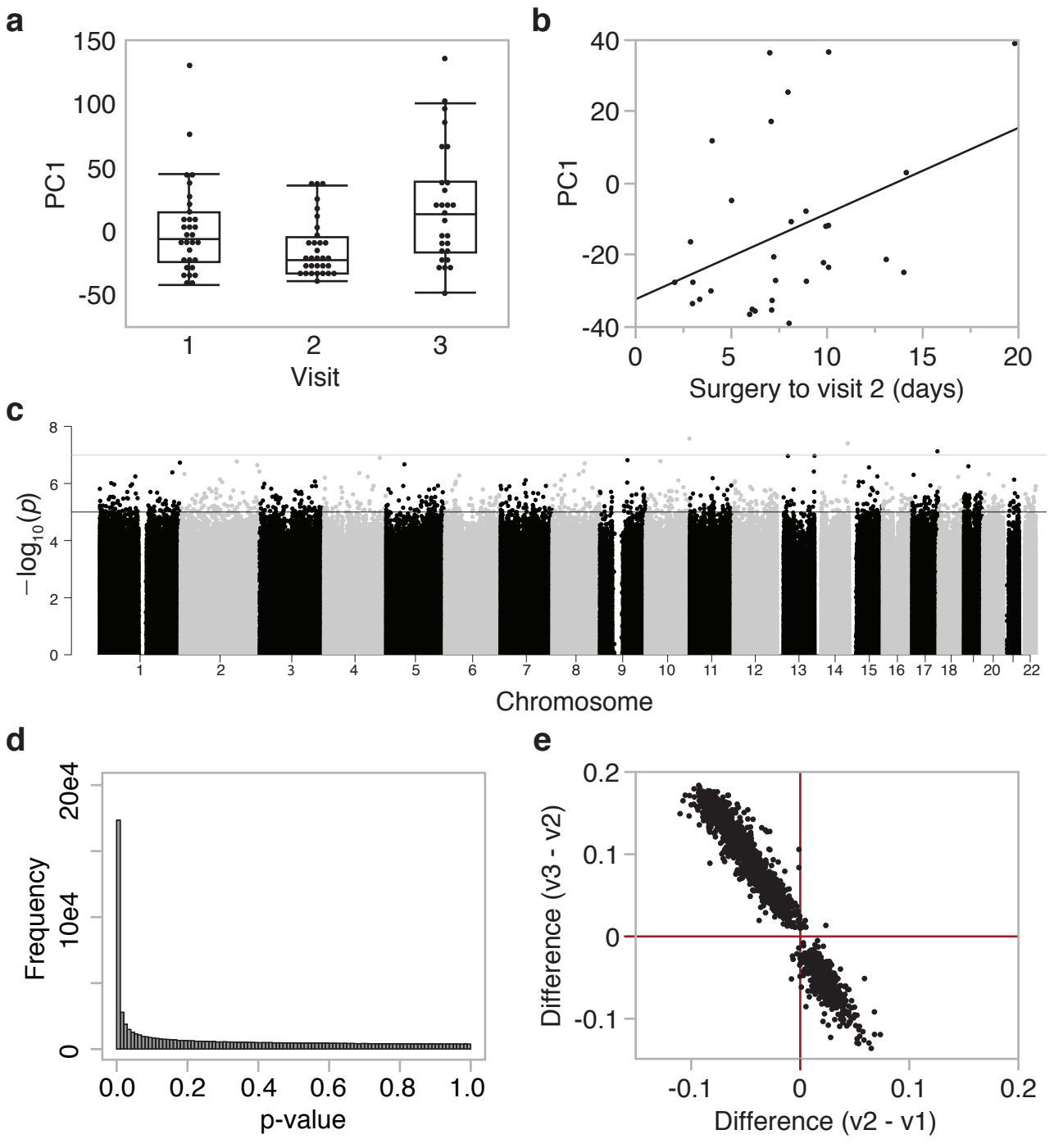
654

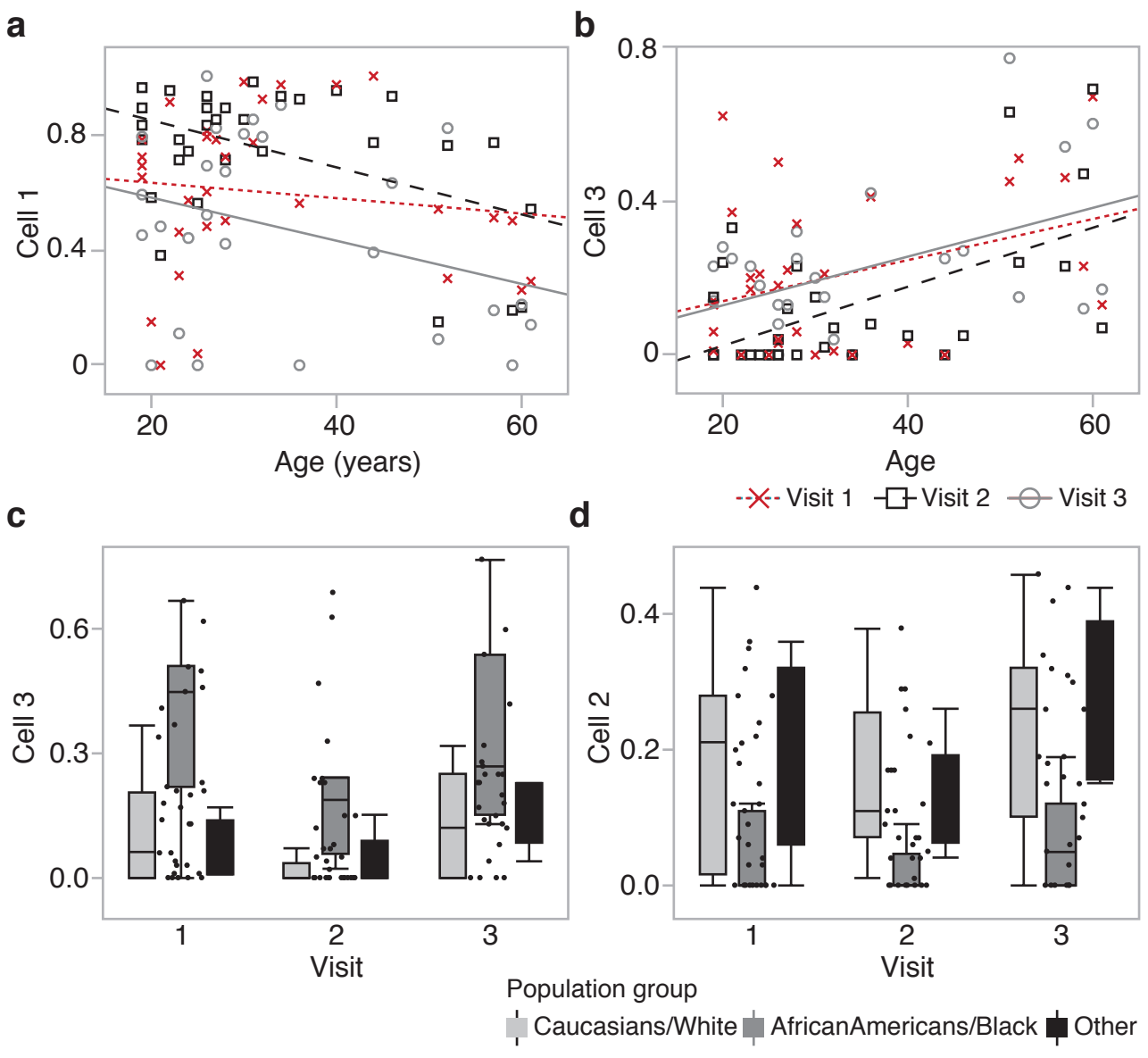
655 **Figure 4. *OPRM1* promoter CpG methylation**

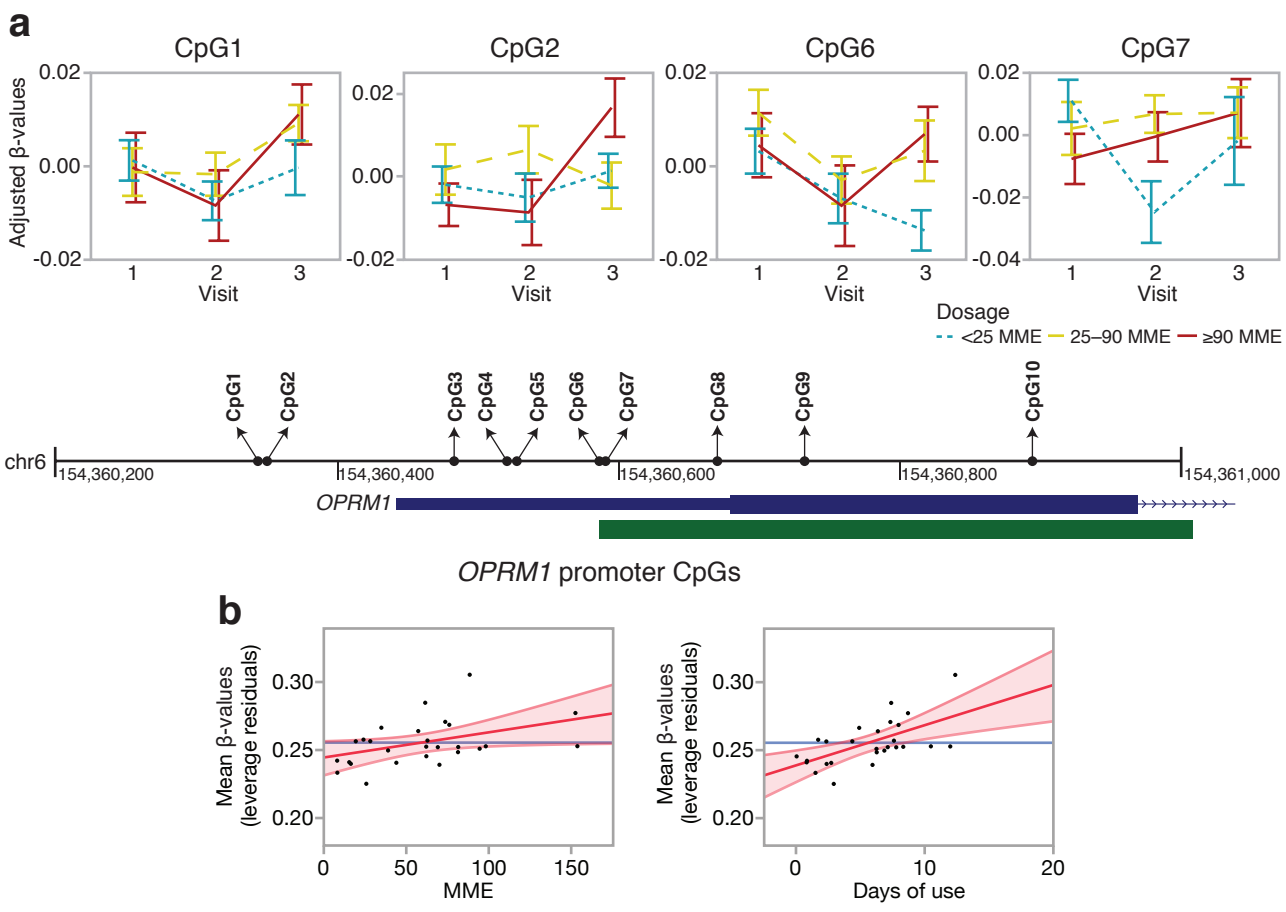
656 **(a)** The *OPRM1* promoter and the CpG island (green block) are depicted along with base pair
657 coordinates (black line; GRCh37/hg19), and location of the 10 CpGs (filled circles). Residual β-
658 values were extracted after fitting participant ID as random intercept, and the plots show the
659 methylation patterns across the three visits for CpG1, CpG2, CpG6, and CpG7 (panels with
660 ANOVA line plots; error bars are standard error). The difference between the dosage groups
661 appear at visit 3 (for CpG1, CpG2, CpG6) and visit 2 (for CpG7). The lowest cumulative dose
662 group (<25 MME: blue dotted line) has lower average methylation compared to the two higher
663 cumulative dose groups (25–90 MME: yellow dashed line; ≥90 MME: red solid line). **(b)** The
664 promoter mean methylation was taken as the average β-values for CpG1 to CpG9. After fitting a

665 regression model with adjustment for age and cell proportions, the leverage plots show a
666 significantly higher average promoter methylation (y-axes) associated with higher MME (x-axis,
667 left panel), and longer duration of use (x-axis; right panel). MME is morphine milligram
668 equivalent.


Sample collection
Visit 1 (N= 41)
Surgery
0–12d
Opioid Self-
Administration
1–17d
0–6d
Sample collection
Visit 2 (N= 33)
28–74d
Sample collection
Visit 3 (N= 27)


a
PC1
Visit
b
PC1
Surgery to visit 2 (days)
c
$-\log_{10}(p)$
Chromosome
d
Frequency
p-value
e
Difference (v3 - v2)
Difference (v2 - v1)


a
b
c
d
Cell 1
Cell 3
Cell 2
Age (years)
Age
Visit
Visit 1
Visit 2
Visit 3
Population group
Caucasians/White
AfricanAmericans/Black
Other


a
CpG1
CpG2
CpG6
CpG7
Adjusted β-values
0.02
0.00
-0.02
-0.04
1
2
3
Visit
Dosage
<25 MME
25–90 MME
≥90 MME
CpG1
CpG2
CpG3
CpG4
CpG5
CpG6
CpG7
CpG8
CpG9
CpG10
chr6
154,360,200
154,360,400
154,360,600
154,360,800
154,361,000
OPRM1
OPRM1 promoter CpGs
b
Mean β-values
(leverage residuals)
0.30
0.25
0.20
0
50
100
150
MME
0
10
20
Days of use