Research Article

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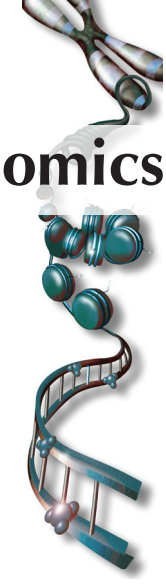
Epigenomics



Methadone induces hypermethylation of human DNA

Background: Increased global DNA methylation in the blood of patients chronically exposed to opioids had been interpreted as an indication of an epigenetic action of this drug class. **Materials & methods**: To strengthen the causality, human MCF7 cells were cultured in media with the addition of several known or potential modulators of DNA methylation including methadone. **Results**: Following 3 days of incubation with several different known or potential epigenetic modulators, global DNA methylation, quantified at *LINE-1* CpG islands, showed a large variability across all treatments ranging from 27.8 to 63%. Based on distribution analysis of the global methylation of human DNA exposed to various potential modulators, present *in vitro* experiments showed that treatment with the opioid methadone was associated with an increased probability of hypermethylation. **Conclusion**: This strengthens the evidence that opioids interfere with mechanisms of classical epigenetics.

Keywords: epigenetic drug effects • Gaussian mixture modeling • human DNA • opioids • pharmacoepigenomics

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Epigenetic effects are exerted by many factors such as early social experiences [1–3], physical training [4], age [5] as well as nutritional or chemical factors including Royal jelly [6], benzene [7], asbestos or smoking [5]. Accumulating evidence shows that common drugs may also induce alterations in DNA methylation patterns or histone conformations [8]. This is not restricted to novel classes of epigenetic therapeutics, which have been especially developed to interfere with the patient's epigenome [9–11], but includes common drugs as well [12]. These epigenetic effects may be clinically relevant by possibly contributing to wanted and unwanted drug effects.

Epigenetic effects have also been proposed to be produced by opioids [8]. Specifically, morphine decreased the expression of histone methyltransferase G9a which caused a lower methylation degree at H3K9me2 found in the nucleus accumbens of mice [13]. Besides interfering with histone modification, opioids have been suggested also to interfere with a second classical epigenetic mechanism, in other words, with DNA methylation. Specifically, the global DNA methylation, quantified by means of *LINE-1* CpG island methylation, was increased in blood cells of patients chronically treated with opioids [14], in other words, methadone substituted former heroin addicts and pain patients [14].

However, while the interference of opioids with mouse histone modification has been directly shown [13], the interference with human DNA methylation was only interpreted from an association found in patients chronically exposed to opioids, without a direct proof of causality in that cross-sectional study [14] where various further unaccounted factors might have influenced the patients' epigenome. Present experiments therefore addressed this question by exposing a human cell line to various known modulators of DNA methylation and to the opioid methadone to which the largest patient group in the previous association had been exposed [14]. As a positive result, it was expected that among several controlled factors influencing DNA

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methylation, the opioid indeed induced a significant shift toward DNA hypermethylation.

Materials & methods

Study design

In this *in vitro* study, MCF7 cells were cultured in media with addition of several substances, alone or in combinations, that reportedly modulate the DNA methylation via various mechanisms (Table 1) to mimic a large variability of DNA methylation observed in humans [14]. The main conditions; however, comprised controls that were cultured either untreated or with the addition of the solvent DMSO, methadone as the main focus of the present analysis, being the opioid that was most consistently involved in the previous observation of DNA hypermethylation in methadone substituted former heroin addicts or pain patients, respectively [14] and 5-aza-2′-deoxycytidine (5-Aza-CdR) as a substance acting as a cytidine nucleoside analog which is incorporated solely into DNA [15] and causes depletion of DNA methyltransferases [15,16] and in consequence a hypomethylation of DNA [17,18]. By including several further potential or known modulators of DNA methylation (Table 1), a large variability in DNA methylation was obtained to reflect the variability observed in humans [14]. Thus it could be analyzed by means of data structure analysis whether as hypothesized, opioids represented by methadone indeed induce a comparatively pronounced shift toward hypermethylation. The analysis focused on the methylation of CpG islands within the *LINE-1* retrotransposon as a widely used marker for global DNA methylation [7,19–22] as those showed the most pronounced effect in the previous observation [14] that had triggered the present experiments.

Cell culture & drug exposure

Drugs and culture conditions are summarized in Table 1. MCF7 cells (courtesy of S Grösch, Goethe – University, Frankfurt am Main, Germany) were cultured and incubated in Dulbecco's modified Eagle's Medium (DMEM) + GlutaMax™ (Gibco, Darmstadt, Germany) supplemented with 10% (v /v) fetal calf serum (FCS) and 1% penicillin/streptomycin (PAA, Cölbe, Germany) at 37°C in humidified atmosphere containing 5% CO_2. Before treatment application, cells were seeded at a density of 3×10^5/10 cm^2 and allowed to settle for 24 h in complete media. Subsequently, cells were incubated for 72 h with addition of the known or potential modulators of DNA methylation at various concentrations (Table 1). Specifically, 5-Aza-CdR is a cytidine analog that functions as a suicide inhibitor of DNA methyltransferases (DNMTs) and covalently traps DNMTs, leading to global DNA hypomethylation [17–18,23–24]; RG108 is a specific DNMT inhibitor that directly blocks the active site of the enzyme and is therefore expected to work as a demethylating agent [17,24]; S-adenosyl methionine (SAM) is a methyl donor that is catalyzed by DNMTs to form 5-methyl cytosine at CpG sites [25], which is expected to increase DNA methylation or at least inhibit global hypomethylation induced by 5-Aza-CdR [26]; 2,4-dichlorophenol

Table 1. Conditions, concentrations and numbers of MCF7 cell experiments, and summary statistics of the observed degree of global DNA methylation†.

Condition	Concentrations	Experiments (n)	Methylation, median (range); %
Untreated	–	7	50 (48.25–56.75)
DMSO	0.1 (%)	8	
5-Aza-CdR	0.1/0.3/1 (μM)	12	30.62 (27.75–48.25)
SAM	10/50/100 (μM)	8	51.12 (50–58)
DCP	0.1/1/10 (mg/l)	6	50.62 (49.25–54)
Methadone	1/10/25/50/75/100 (μM)	11	56.25 (49.5–57.75)
RG108	10/30/50/70/100 (μM)	9	49 (47.5–51.75)
5-Aza-CdR + SAM	0.3 + 50/100 (μM)	6	43.25 (32.25–46.25)
5-Aza-CdR + DCP	0.3 (μM) + 10 (mg/l)	4	31.62 (28.25–46.25)
5-Aza-CdR + methadone	0.3 + 10/25/50/75/100 (μM)	9	40 (33.25–51.5)
5-Aza-CdR + SAM + DCP	0.3 (μM) + 100 (μM) + 10 (mg/l)	3	49.25 (43–63)
RG108 + DCP	100 (μM) + 10 (mg/l)	2	49.12 (47.75–50.5)
SAM + DCP	100 (μM) + 10 (mg/l)	3	50.25 (49.5–56.75)

†For single data, see Figure 3.
5-Aza-CdR: 5-aza-2 -deoxycytidine; DCP: 2,4-dichlorophenol; SAM: S-(5′-Adenosyl)-l-methionine chloride.

(DCP) is an environmental pollutant reported to increase global methylation [27]. Finally, methadone was chosen as an opioid because it had been involved in the largest group of patients (heroin addicts) in whom the clinical association of opioid-induced hypermethylation had been observed [14]. On each day, media was replenished and treatment was refreshed. Methadone hydrochloride (Fagron, Barsbüttel, Germany) was dissolved in DPBS without calcium and magnesium (Dulbecco's phosphate buffered saline without $CaCl_2$ and $MgCl_2$; Gibco, Germany, Darmstadt; 14190–094). 5-Aza-CdR, SAM, DCP (Sigma-Aldrich, Taufkirchen, Germany) and RG108 (Biomol, Hamburg, Germany) were dissolved in DMSO and solvent was added to obtain a final concentration of 0.1% DMSO (0.25% for RG108) to the cell media in all incubations. Cells incubated with 0.1% solvent alone or without any substance addition (i.e., control condition) served as controls.

Quantification of global DNA methylation

The analysis of *LINE-1* DNA methylation was performed identically to the quantification in blood of methadone treated former heroin addicts and opioid treated chronic pain patients and has been described previously in full detail [14]. In brief, genomic DNA was extracted from MCF7 cells with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Bisulfite treatment was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Freiburg, Germany) as instructed by the manufacturer. That is, 10 µl genomic DNA (~1000 ng) and 10 µl water were incubated with 130 µl CT conversion reagent at 98°C for 10 min and at 64°C for 2.5 h and were subsequently stored at 4°C for up to 20 h. Finally, the bisulfite treated DNA was purified and eluted in 30 µl M-Elution buffer followed by storage at -20°C until assay.

LINE-1 retrotransposons are dispersed in more than 500,000 copies across the human genome [28] and are widely exploited for the analysis of global DNA methylation [29] agreeing with the 'gold standard' of measuring DNA methylation by means of HPLC [20,29], which has the major drawback of requiring large quantities of DNA. The present pyrosquencing assay is based on the PyroMark *LINE-1* assay proposed by the manufacturer (Qiagen), which includes four CpG sites located at positions 329–305 of the *LINE-1* sequence (Genbank X58075.1) and has been shown to be suitable as global DNA methylation marker [30]. The analyzed region of a CpG island located in the promoter region (L1Hs) DNA (PubMed GenBank X58075.1; lower strand) has the bisulfite-converted sequence 5′-TTTT-*GAGTTAGGTGTGGGATATAGT*TT**YG**TGGT-G**YG**T**YG**TTTTTTAAGT**YG**GTTTGAAAAGC-TAATATTCGGGTGGGAGTGATTCGATTTTT-TAGGTGCGTTCGTTATTTTTTTTTTTT-GATTCG*GAAAGGGAATTTTTTGATTTT*-3′ where the 146 bp PCR product was shown with the position of the analyzed CpG methylation sites (bold) and the localization of the PCR primers (underlined) and the sequencing primer (italics), respectively [7,19]. Relevant *LINE-1* DNA segments were amplified using forward primers with sequence 5′-TTTTGAGTTAGGTGTGGGATATA-3′ and reverse primer with sequence 5′-biotin-AAAAT-CAAAAAATTCCCTTTC-3′. PCR reactions were run on a Mastercycler nexus gradient flexlid instrument (Eppendorf, Hamburg, Germany) using a 50 µl reaction volume with 5 µl bisulfite treated DNA, mixed with 0.5 µl MyTaq™ HS DNA Polymerase (5 U/µl) (Bioline, Luckenwalde, Germany), 10 µl 5× MyTaq Reaction Buffer, 0.2 µl of each PCR primer (100 µM) and 34.1 µl HPLC-purified water. The following PCR program was used: 95°C for 1 min, 40 amplification cycles at 95°C for 15 s, 56°C for 15 s, 72°C for 15 s and a final elongation step at 72°C for 5 min.

Analysis of the global methylation marker *LINE-1* was done by means of Pyrosquencing™ (Qiagen) as described previously [7,14,19]. In brief, 50 µl of the PCR template was pipetted into a well containing 55 µl binding buffer (3 µl Streptavidin Sepharose High Performance; GE Healthcare Bio-Sciences AB, Uppsala, Sweden, 37 µl binding buffer; Qiagen and 15 µl HPLC-purified water). After sample purification with the PyroMark Vacuum Prep Worktable (Biotage, Uppsala Sweden) complexes were transferred onto a PSQ 96 Plate Low (Biotage, Uppsala, Sweden) prefilled with 0.16 µl of 100 µM sequencing primer (5′-AGT-TAGGTGTGGGATATAGT-3′) and 39.84 µl annealing buffer (Qiagen). The sequencing primer was let to anneal to the template at 80°C for 2 min on a PSQ 96 Sample Prep Thermoplate Low (Biotage, Uppsala, Sweden) and then cooled down to room temperature.

Sequence analysis took place on a PSQ 96 MA System using the PyroMark Gold Q96 Reagents (Qiagen) with the following sequence to analyze: TTYGTGGTGYGTYGTTTTTTAAGTYGGTTT. Pyro Q-CpG methylation software (version 1.0.9) had been used to determine the nucleotide dispensation order (ATCAGTGTGTCAGTCAGTC-TAGTCTG). Non-CpG cytosine residues were used as built-in controls to verify the bisulfite conversion (yellow shaded in Figure 1). The acceptable percentages for passed and checked quality were 4.5 and 7%, respectively. *LINE-1* methylation values rep-

resent the mean percentage methylation across all four CpG sites, which were measured in duplicate samples within one run. In addition, each sample was measured in two independent runs, which were subsequently averaged. Samples not meeting the criteria for complete bisulfite conversion or Pyrosequencing™ quality control checks were excluded. To verify the accuracy of the analysis, each run included control DNA from the EpiTect PCR Control DNA Set (Qiagen) that contains both, bisulfite converted 100% methylated and completely unmethylated DNA as positive controls and unconverted unmethylated DNA as negative control. The bisulfite converted methylated control DNA reached on average 74.36% ± 1.286% methylation while the bisulfite converted unmethylated control DNA reached only 2.71% ± 0.42% methylation, which well agrees with published values [30], and the PCR negative control did not show specific spikes for any injected nucleotide demonstrating assay specificity (Figure 1).

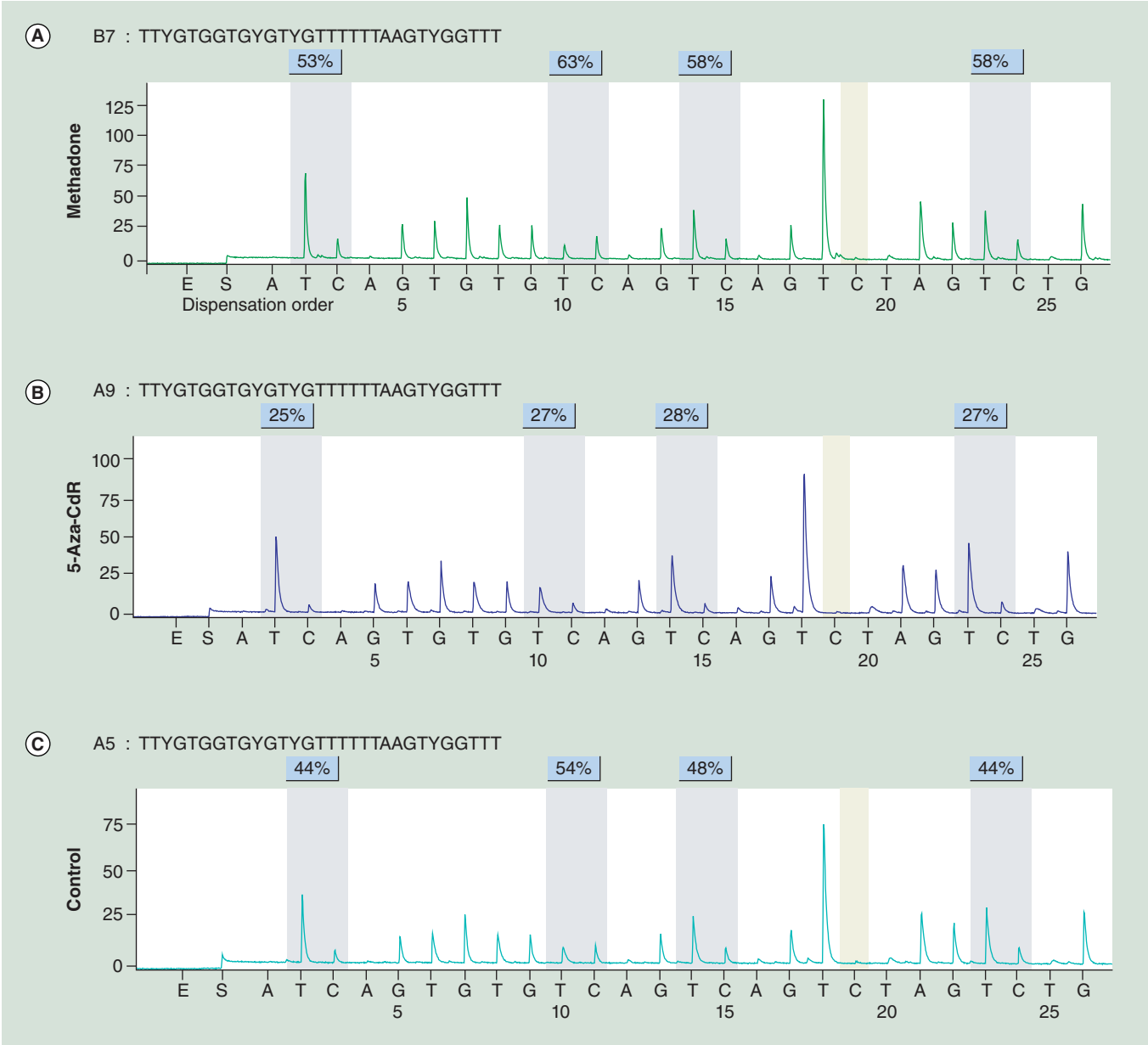

Figure 1. Exemplary pyrogram of a human *LINE-1* methylation analysis using pyrosequencing including four CpG sites (gray shaded). The percentages show the methylation degrees at each locus. **(A)** MCF7 cells treated with methadone (50 μM) for 72 h. **(B)** MCF7 cells treated with 5-Aza-CdR (0.3 μM) for 72 h. **(C)** MCF7 cells under control conditions. The yellow shaded position shows the bisulfite control, included in each sample to ensure complete bisulfite conversion of each unmethylated cytosine.
5-Aza-CdR: 5-aza-2′-deoxycytidine.

Data analysis

The principal focus of the analysis was the verification of the hypothesis that following methadone administration the degree of DNA methylation was higher than in untreated cells whereas following 5-Aza-CdR administration, the DNA became comparatively less methylated. This was expected to be reflected in the position of the methadone and 5-Aza-CdR conditions at the right or the left from the untreated condition, respectively, along the whole data range sorted from left to right for increasing DNA methylation, expressed as percent methylation of CpG islands analyzed in the *LINE-1* retrotransposon. Therefore, the distribution of the methylation degree observed in the various experimental conditions was analyzed using the Pareto Density Estimation (PDE [31]), which served to estimate the probability density function (PDF) of the DNA methylation and comprises a kernel density estimator representing the relative likelihood of a given continuous random variable taking on specific values that has been shown to be particularly suitable for the discovery of structures in continuous data hinting at the presence of distinct groups of data and particularly suitable for the discovery of mixtures of Gaussians [31]. Indeed, the PDE analysis indicated a multimodal distribution of DNA methylation within these experimental settings. Therefore, the data were subsequently modeled as a mixture of Gaussian distributions, which is an established probabilistic model for representing the presence of subpopulations within an overall population [32] (for further details, also see [33], and for a further example of the use of this model in biomedical research, see [34]). Specifically, a Gaussian mixture model (GMM) is a weighted sum of M component Gaussian densities as given by the equation:

$$p(x) = \sum_{i=0}^{M} w_i N(x \mid m^i, s^i) = \sum_{i=1}^{M} w_i \cdot \frac{1}{\sqrt{2\pi s^i}} \cdot e^{\frac{(x-m_i)^2}{2s_1^2}}$$

where $N(x|m_i,s_i)$ denotes Gaussian probability densities (components) with means m_i and standard deviations, s_i. The parameters w_i are the mixture of weights indicating the relative contribution of each component Gaussian to the overall distribution, which add up to a value of 1, and M denotes the number of components in the mixture. The parameters of the GMM were optimized using the expectation maximization (EM) algorithm [35]. Subsequently, the Bayes' theorem was used to obtain the limits in the DNA methylation degree at which the Gaussian modes were separated into M classes, c_i, $I = 1,\ldots,M$, of the complete DNA methylation pattern. This enabled the assignment of the DNA methylation degrees observed during the 5-Aza-CdR, the control or the methadone conditions, to the different Gaussian modes. As control condition served observations from both the DMSO and untreated conditions, which had identical methylation degrees (Wilcoxon test: W = 34.5; p = 0.48) and could therefore be pooled to obtain a sufficiently powered control sample. Subsequently, the hypothesis was tested whether the methylation observed under the three different conditions were unequally distributed among the obtained Gaussians, using Fisher's exact tests [36]. The α level was set at 0.05. Statistical analyses were performed using the software packages R (version 3.0.2 for Linux [37]).

Results

A total of 88 MCF7 cell experiments could be analyzed for the degree of methylation in the DNA samples extracted following 3-day treatments. Following cell culture under the various experimental conditions (Table 1), *LINE-1* CpG island methylation (Figure 1) was modulated across a wide range of methylation degrees from 27.75 to 63% (Figure 2). This provided a first prerequisite for the intended Gaussian mixture modeling analysis. As a second prerequisite, the clear multimodality in the PDE of the data hinted at the existence of subgroups of DNA methylation degrees (black line in Figure 3).

The analysis of the multimodal distribution of the DNA methylation in more detail identified four Gaussian modes (blue dotted lines in Figure 3) as fitting the envelope curve of the probability density function (red line in Figure 3). Parameters describing the four Gaussian modes are given in Table 2. The Bayesian decision limits among the four Gaussians were calculated to be located at DNA methylations of curves 35.68, 46.83 and 53.82% between Gaussians 1 and 2, 2 and 3 and 3 and 4, respectively (magenta perpendicular lines in Figure 3). The distribution of the DNA methylation observed during the various cell culture conditions is shown in Figure 4.

Following identification of the detailed distribution structure of the DNA methylation degrees, observations made with the cells submitted to the 5-Aza-CdR, the control or the methadone conditions could be assigned to Gaussian modes based on the Bayesian limits. This identified that DNA methylations observed during the three conditions were unequally distributed among the Gaussians (Fisher's exact test: $p = 1.619 \cdot 10^{-7}$). Specifically, while methadone treated cells were more often found in the fourth Gaussian mode comprising cells with comparatively high DNA methylation, 5-AZA-CdR treated cells were over-represented in the first Gaussian mode comprising samples with a low DNA methylation, The distribution of samples from both treatments differed statically significantly from that of control samples, which were mainly found in the third Gaussian, in other

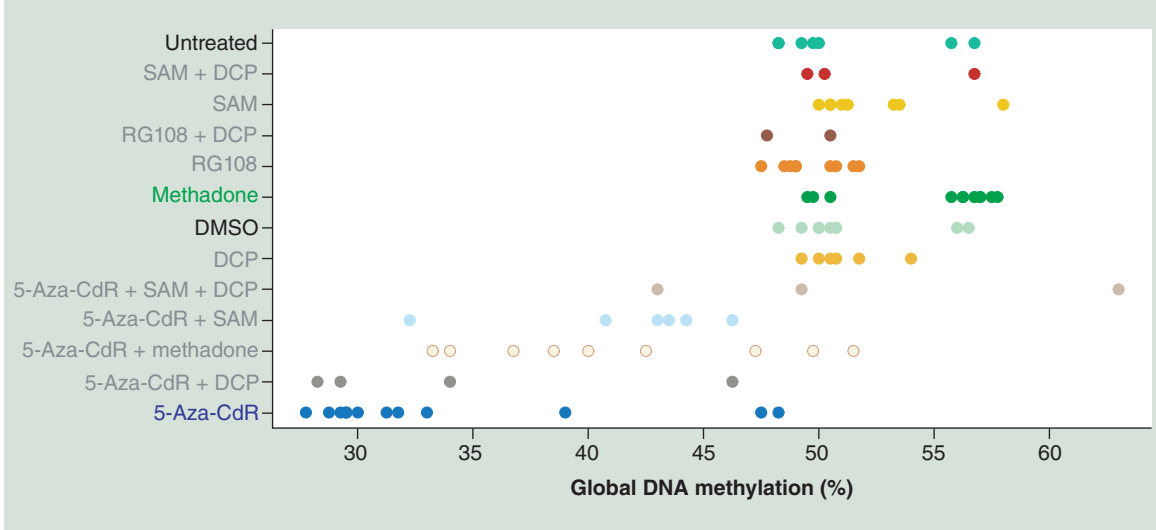

Figure 2. Dotplot of *LINE-1* DNA methylation of different treatment conditions of MCF7 cells. The different concentrations of inserted substances are summarized to a single category of conditions. The color code represents the different treatment conditions.
DMSO: Dimethyl sulfoxide; SAM: S-adenosyl methionine; DCP: 2,4-dichlorophenol; 5-Aza-CdR: 5-aza-2′-deoxycytidine.

words, as indicated by significant Fisher's exact tests ($p = 2.243 \cdot 10^{-5}$ and $p = 0.04474$, respectively). This substantiated, at the statistical level, the observation that most DNA methylation observations during 5-Aza-CdR condition were assigned to Gaussian 1 whereas most DNA methylation observations during methadone condition were assigned to Gaussian 4. By contrast, observations for the control conditions were mostly assigned to Gaussian 3 (Figure 4).

Discussion

The hypothesis that human cells treated with opioids displays a comparatively high degree of global methylation could be verified by the present results, which indicated that methadone-treated cells were mainly assigned to a subgroup within the DNA methylation pattern that was distinct to that to which untreated cells belonged, and that the distribution of methadone-treated versus untreated cells to different subgroups of DNA methylation was statistically significant. This supports the previous interpretation of *LINE-1* hypermethylation, observed in patients in a cross-sectional study, as an indication that opioid use is associated with increased global DNA methylation [14]. The present experiments were designed to mimic the clinical situation in which DNA is exposed to many different epigenetic factors that may modulate its methylation toward both directions to different degrees. The conclusion of a hypermethylation effect of opioids, in particular of methadone, could be drawn by the clear evidence that among several factors, methadone treatment produced the most pronounced increase in the probability that the exposed DNA was among those samples that displayed hypermethylation. The validity of the results is supported by the clear hypomethylation induced by treatment with 5-Aza-CdR as a known inhibitor of DNA methylation currently used for cancer treatment [38,39].

The present results provide further evidence that opioids are among common primary nonepigenetic drugs that nevertheless produce epigenetic effects in humans [8]. In particular, further evidence is presented that opioids may not only modulate the methylation of histones [13] but may also induce DNA hypermethylation. Opioid effects on the human epigenome are an active research topic that has; however, led to occasionally conflicting findings. For example, in SHSY5Y cells treated with morphine for either 4 or 24 h [40], methylation binding protein sequencing showed a hypomethylation after long-term morphine treatment (24 h) while hypermethylation was observed after short-term treatment (4-h). Moreover, DNA methylation assessed by pyrosequencing of *LINE-1* depended on the observed CpG site and the overall changes in the *LINE-1* promoter region tended toward hypermethylation after 4-h treatment and a hypomethylation after 24-h treatment. The latter is inconsistent with the present results, which; however, were found using an experimental design that had the previously observed opioid-induced hypermethylation after long-term treatment in mind and therefore the incubation time was 72 h and shorter exposures were not assessed.

Present experiments were designed as a step toward proof of evidence about opioid-induced DNA hyper-

methylation that we have reported previously [14], without addressing the molecular pathways yet, which still needs to be elucidated. Downregulation of histone methylation (H3K9me2), on the other hand, seems to comprise a decrease in G9a expression as a core subunit of the histone lysine methyltransferase [13]. Perhaps, a mechanistic hypothesis can be derived from an analogy to the DNA hypermethylation observed in differentiated keratinocytes after treatment with endocannabinoids [41]. The observed global DNA hypermethylation in the keratinocytes was shown to result from an increase in DNA methyltransferase (DNMT) activity. The activity of this enzyme increased as a result of the activation of the p38 and p42/44 MAPK dependent pathway after binding of the agonistic endocannabinoids to the CB_1 receptors [41]. Cannabinoid and opioid receptors share as G_i-coupled receptors some of the same downstream pathways. From this raises a hypothesis that MAPK pathways may induce DNA hypermethylation also in the context of opioids.

In addition to not showing the molecular mechanism of opioids signaling toward DNMTs, additional limitations of the present study emerge from the included cell line. First, loss of DNA methylation in repetitive elements of the DNA has been proposed to account for most of the global hypomethylation that characterizes a large percentage of human cancers [20]; however, tumor suppressor genes may be hypermethylated [26]. Cancer-related changes in the methylation status could have affected the magnitude of the effects

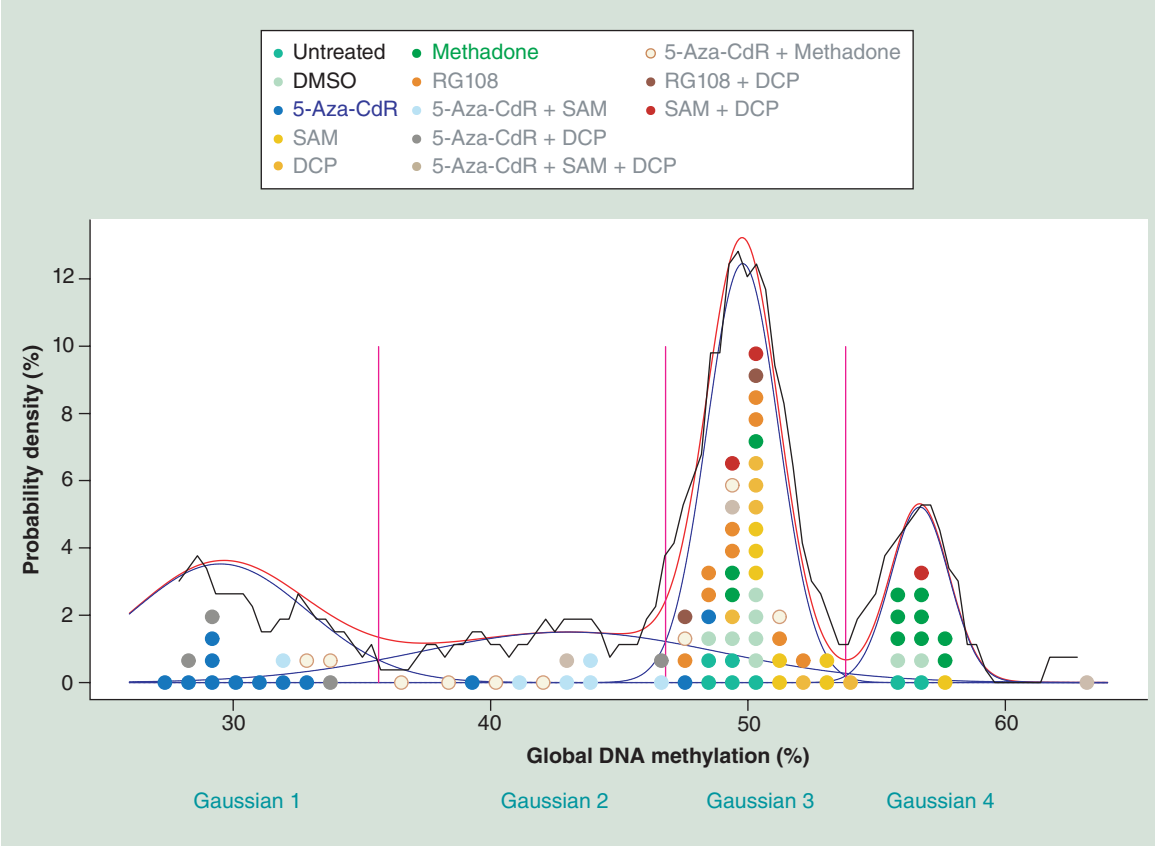

Figure 3. Distribution of the DNA methylation degrees from n = 88 MCF7 cell experiments subjected to different substances known or expected to modify DNA methylation (Table 1). DNA methylation degrees are expressed as percent methylation of *LINE-1* CpG islands. The density distribution is presented as probability density function, estimated by means of the Pareto Density Estimation ([31]; black line). A Gaussian mixture model (Equation 1; GMM given as $sp(x) = \sum_{i=0}^{M} w_i N(x \mid m_i, s_i)$), was fit (red line) to the data, for which the number of mixes was found to be $M = 4$ (blue dotted lines). DNA methylation distribution among the obtained four Gaussians was n = 15, n = 12 and n = 45 and n = 16 for Gaussian 1–4, respectively, starting from the left. The Bayesian boundaries (magenta perpendicular lines) between the four Gaussians were observed at DNA methylation degrees of 35.68, 46.83 and 53.82%. The overlaid dotplot shows how the single observations of *LINE-1* DNA methylation observed following cells grown under addition of different substances are distributed among the four Gaussians. This shows that the forth Gaussian is mainly populated by observations made under methadone condition.
5-Aza-CdR: 5-aza-2′-deoxycytidine; DCP: 2,4-dichlorophenol; DMSO: Dimethyl sulfoxide; SAM: S-adenosyl methionine.

Table 2. Parameter values obtained following modeling of the distribution of the DNA methylation degrees†.

Gaussian #	m_i	s_i	w_i
i = 1 (1st Gaussian)	29.53	3.38	0.3
i = 2 (2nd Gaussian)	43.07	5.84	0.22
i = 3 (3rd Gaussian)	49.83	1.39	0.44
i = 4 (4th Gaussian)	56.71	1.13	0.15

†Expressed as percent methylation of *LINE-1* CpG islands, by means of a Gaussian mixture model (GMM given as:

$$p(x) = \sum_{k=0}^{M} w_i N(x \mid m_i, s_i)$$

for which the number of mixes was found to be $M = 4$ (Figure 3) where m_i, s_i and w_i are the parameters mean, standard deviation and relative weight of each of the Gaussian, respectively.

on DNA methylation observed in the MCF7 breast cancer cell line and the results may quantitatively differ from those that could be observed in noncancer cells including blood cells [14]. Second, while the use of a non-neuronal cell line to assess opioid-induced DNA hypermethylation was not in contradiction to the previous report of opioid-induced DNA hypermethylation in *LINE-1* from peripheral white blood cells [14], the observed consequences with respect to the correlated increased pain [14] strongly suggest an effect within the nervous system, which should be substantiated in a neuronal cell line. Epigenetic mechanisms may be tissue-specific [42–44] although chromosome-wide analyses suggest that only a small proportion of genes are differentially methylated in different tissues [45]. Hence, opioid-induced effects on DNA methylation need to be assessed in multiple cell lines including normal or cancer cells and neuronal and non-neuronal cells, until the epigenetic consequences of opioid exposure can be regarded as being understood.

A further potential limitation of the present laboratory analysis is attributable to the quantification of global DNA methylation in contrast to its quantification at the single gene level. However, quantification of global DNA methylation analysis is widely established in research [29] and has been shown to be linked with altered global gene expression, in particular hypermethylation is often associated with gene silencing [46]. Global DNA methylation, including for *LINE-1*, qualifies as epigenetic biomarker, for example, in metabolic diseases [47] or in glioma where a correlation with the methylation of a disease-relevant gene (*MGMT* promoter) has been shown [48]. Indeed, a re-analysis of data available from [14] (cohorts 'methadone substituted opiate addicts' and 'controls') resulted in a weak but statistically significant correlation between the methylation of the μ-opioid receptor gene (mean across 22 CpG positions) and *LINE-1* (Pearson's r = 0.242; p = 0.002) supporting a relevance of the present global methylation data.

With the correlation of *OPRM1* and *LINE-1* methylation in mind (see above), three possible scenarios of possible biological implications emerge. First, the methadone-induced hypermethylation of *LINE-1* is a marker of a hypermethylation of *OPRM1* and the previously observed increased pain in long-term opioid-treated pain patients [11] owes to a reduced μ-opioid receptor expression. Second, *LINE-1* hypermethylation could be a marker of hypermethylation of still unspecified genes involved in the nocifensive system. Third, the hypermethylation of *LINE-1* could also be causally involved in the observed biological effects without reducing it to a biomarker. Specifically, *LINE-1* is epigenetically silenced by hypermethylation of promoter CpG islands or histones [49]. Transcription is presumed to occur during embryogenesis in germ cells [50,51] or in neural progenitor cells [52]. However, *LINE-1* can be released from epigenetic suppression [53] by chemical noxes such as cocaine, which reduced the histone methylation at *LINE-1* repeats in the nucleus accumbens [54], or voluntary exercise, which increased *LINE-1* insertion in hippocampal neuronal progenitor cells [55]. Its role in neuronal reshaping [56] qualifies *LINE-1* also for a role in pain. Release from epigenetic suppression [53] may change the genome of individual neurons [57] by rendering protein-coding genes active, which may lead to somatic mosaicism in the nervous system [58]. Retrotransposition may occur in conditions associated with DNA hypomethylation [59], which has been also described for neuropathic pain [60]. All these mechanisms potentially qualify as providing a novel epigenetic mechanism of opioid-induced hyperalgesia, adding to the hypotheses involving a sensitization of peripheral nerve endings or second-order neurons, enhanced descendent facilitation of nociceptive pathways, increased production, release and decreased reuptake of neurotransmitters involved in nociception [61–63] or alterations in chemokine regulation or the MAPK pathway [63]. The elevation of the amount or response to excitatory neurotransmitters like glutamate and glycine via neural plasticity received most attention [62,64]. Finally, further biological consequences of the epigenetic effects of nonepigenetic drugs including opioids are possible as outlined previously [8].

The present results were obtained employing an experimental design that aimed at mimicking the exposure of human DNA to various other known active epigenetic modulators of DNA methylation. This approach was chosen to reflect the structure often met in data obtained from clinical observations where relevant epigenetic effects are being observed while exploiting the possibility of controlled conditions as an advantage of cell culture experiments over

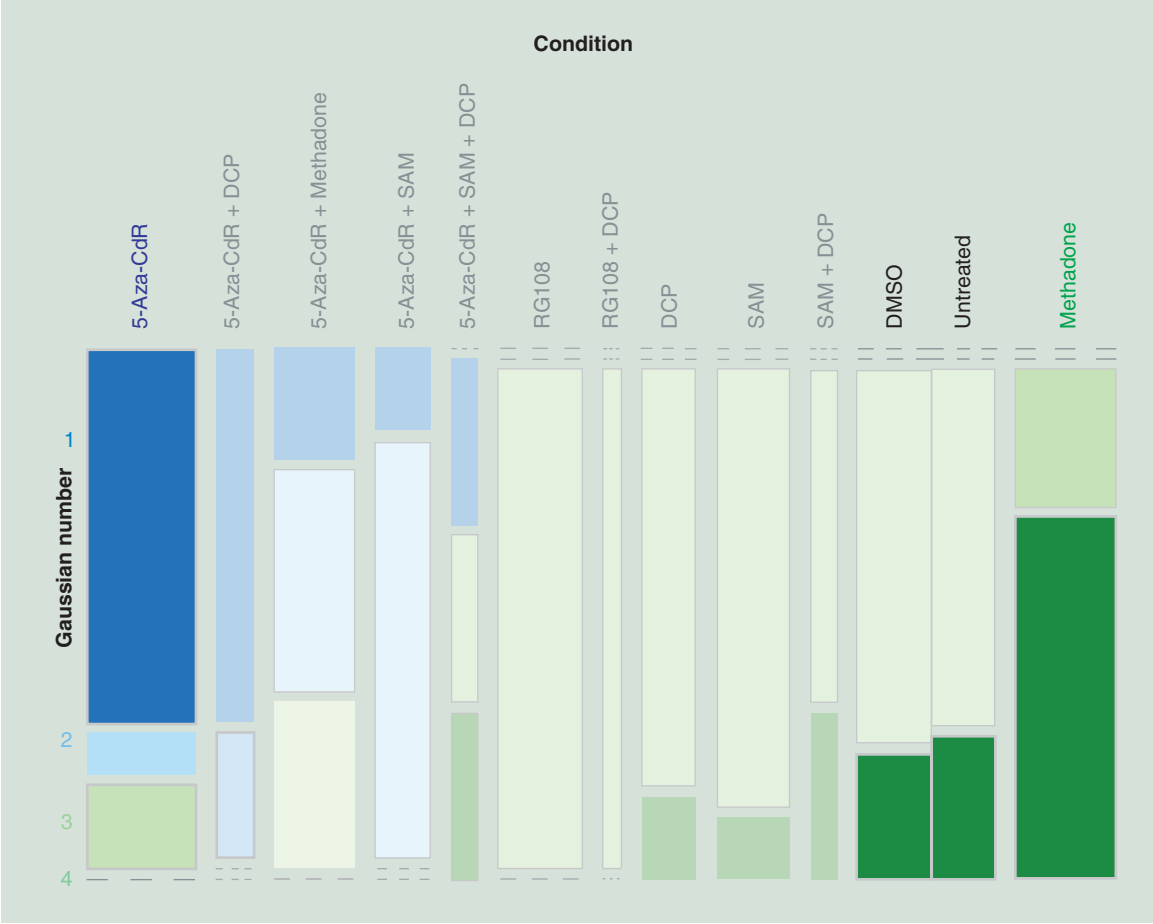

Figure 4. Mosaicplot of the assignment of global DNA methylation expressed as percent of *LINE-1* CpG island methylation among the four Gaussian mode determined groups (ordinate color coded from top to bottom: dark blue = Gaussian 1, in other words, lowest methylation, light blue = Gaussian 2, in other words, methylated below the control or untreated conditions, light green = Gaussian 3, which includes the control and untreated conditions, and dark green = Gaussian 4, in other words, increased methylation from untreated or control conditions) versus the conditions applied to the human MCF7 cell line (abscissa). The latter are grouped in bars with widths proportional to the number of cell experiments analyzed per condition (Table 1). The conditions on which the principal analytical focus was aid are shown in bright colors whereas further conditions used to establish a large variability of DNA methylation degrees as a prerequisite for Gaussian mixture modeling (Figure 3) are dimmed.
5-Aza-CdR: 5-aza-2′-deoxycytidine; DCP: 2,4-dichlorophenol; DMSO: Dimethyl sulfoxide; SAM: S-adenosyl methionine.

cross-sectional clinical observations [34]. The results are mainly based on data distribution analysis as previously applied to complex human data [34]. The analysis pooled the conditions as it focused on identifying subgroups while using several conditions and drug concentrations merely to create the large variability of DNA methylation which is a prerequisite of the Gaussian mixture model analysis. Following establishment of such subgroups, we show that the conditions/treatments in the focus of this work were assigned to different Gaussians respective subgroups of DNA methylation. The assignment to the groups differed statistically significantly as indicated by the results of the Fisher's exact test. This novel approach was successful in showing the hypermethylating effect of methadone, yet disregard of the concentration effects, which would have to rely on very small sample sizes, seems acceptable. The possible effect of this procedure is occasional misplaying of single samples among Gaussians, which obviously did not deter the statistical significances. The validity of this approach is supported by the agreement of the observed epigenetic effects of the tested modulators with their expected effects from prior knowledge of their activities (Table 1). Specifically, 5-Aza-CdR exerted its DNA hypomethylating effect as expected from its use as a cancer therapeutic in order to demethylate and induce the expression of tumor suppressor genes [26,38–39],

including a reversal of the methadone effects when combined with it (Figures 2 & 3). Similarly, the previously observed effects of SAM [26] could be reproduced, in other words, while treatment with SAM alone did not affect DNA methylation compared with controls (Gaussian 3), SAM inhibited the global hypomethylation induced by 5-Aza-CdR (Gaussian 2), which corresponded to the expectation from [26]. The poor demethylating effects of the DNMT inhibitor RG108 can be explained by its activity directed against the bacterial rather than the human enzyme [65–67]. Only the environmental pollutant DCP, reported to slightly increase DNA methylation in *Carassius auratus* livers [27], was unable to induce a right-shift of DNA methylation, for which species differences known to be relevant in epigenetics [68,69], concentration response related causes or a comparatively smaller effect of that of methadone may be contemplated.

Conclusion

Based on distribution analysis of the global methylation of human DNA exposed to various potential modulators, the present *in vitro* experiments showed that treatment with the opioid methadone was associated with an increased probability of hypermethylation. This supports a previous interpretation of a similarly directed association in clinical data [14] and strengthens the evidence that opioids interfere with both main mechanisms of classical epigenetics comprising alteration of histone modification [13] and DNA methylation. The results encourage initiating experiments that assess the molecular pathways between the opioid receptor as the common target of opioids and the human DNA methyltransferases.

Future perspective

Present results strengthen the evidence that opioids play a role as modulators of DNA methylation. During pain therapy, they therefore interplay with other epigenetic factors including strong pain [70]. When considering that the degree of *LINE-1* methylation had been reported to positively correlate with the pain intensity [14], the possibility of an epigenetic mechanism of opioid-induced hyperalgesia becomes more likely. This adds to other hypotheses of opioid-induced hyperalgesia such as sensitization of peripheral nerve endings or second-order neurons, enhanced descendent facilitation of nociceptive pathways or increased production, release and decreased re-uptake of neurotransmitters involved in nociception [61–63]. Further strengthening the epigenetic role of opioids; however, will require the inclusion of additional cell lines, neuronal and non-neuronal, cancer and non-cancer and the analysis of CpG islands in unique structural genes.

Disclaimer

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Executive summary

- Epigenetic effects are exerted by many factors including common drugs.
- An interference with human DNA methylation was interpreted from a cross-sectional study, and this was correlated with increased pain.
- The so far lacking causality of this observation was presently addressed in *in vitro* experiments.
- In a human cell line exposed to several modulators of DNA methylation, method induced the most pronounced shift toward hypermethylation.
- This strengthens the evidence that opioids interfere with the main mechanisms of classical epigenetics including DNA methylation.

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