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Sex dependent alteration of epigenetic marks after chronic morphine treatment in mice organs

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A B S T R A C T

Epigenetic marks may be also affected by several factors, such as age, lifestyle, early life experiences and exposure to chemicals or drugs, such as opioids. Previous studies have focused on how morphine epigenetically regulates different regions of the brain that are implicated in tolerance, dependence and other psychiatric disorders more related to the physio-pathological effects of opioids. Nevertheless, a significant knowledge gap remains regarding the effect of chronic treatment on other organs and biological systems. Therefore, the aim of this work is to increase our knowledge about the impact of chronic morphine exposure on DNA methylation and histone modification levels in each of the organs of male and female model mice *in vivo*. Our results reveal, for the first time, that chronic morphine treatment induced changes in DNA methylation/hydroxymethylation and histone modification *in-vivo* at the systemic level, revealing a potential physiological effect on the regulation of gene expression. Notably, morphine-induced epigenetic modification occurs in a sex-dependent manner, revealing the existence of different underlying mechanisms of epigenetic modification in male and female mice.

1. Introduction

Epigenetic modifications include environmentally induced changes in the genome that do not alter the DNA sequence. The main molecular mechanisms are DNA methylation at CpG-rich islands and histone modification (Bannister and Kouzarides, 2011; Russo et al., 1996). First, methylation modifies gene transcription by preventing the binding of transcription factors or by facilitating the binding of suppressive factors (Hsieh, 1994). Second, changes in histone modification can lead to chromatin dysregulation (Bannister and Kouzarides, 2011). These epigenetic modifications play major roles in gene expression, cellular differentiation, X-chromosome inactivation, genomic imprinting, transposable element repression and embryo development. Furthermore, both types of modification have been shown to be affected by several factors, including age (Christensen et al., 2009), lifestyle habits (smoking, alcohol consumption, physical activity, or nutrition) (Lim and Song, 2012), and early life experiences, such as maternal care (Weaver et al., 2004). Recently, it has been demonstrated that exposure to chemicals or drugs, such as opioids, also triggers both of these epigenetic modifications (Browne et al., 2020).

Morphine originates from opium and is its most active component. This analgesic drug is primarily used in medicine for pain relief (Subiran et al., 2011) despite its considerable number of adverse side effects. However, the mechanisms of epigenetic modification induced by chronic morphine exposure are not fully understood, although this topic is attracting increasing awareness (Rasoulpour et al., 2011).

DNA methylation is an epigenetic mark that aims to regulate gene expression. DNA cytosine methylation is catalysed by DNA methyltransferases (DNMTs), which act by transferring a methyl group from *S*-adenosyl methionine to the 5th carbon of a cytosine residue, ultimately forming 5-methylcytosine (5 mC) (Guo et al., 2014). The covalent C–C bond in 5 mC makes this mark stable and thus difficult to directly remove. While this modification is maintained through the action of DNMT1, de novo cytosine methylation patterns are mainly established by the methyltransferases DNMT3A and DNMT3B (Li et al., 1992). Although this modification is considered a long-lasting epigenetic mark, it can gradually and passively be lost if the maintenance of the methylation pattern is not ensured during replication throughout generations of cells. In addition, the ten-eleven translocation (TET) protein

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Abbreviation list

5 mC 5-methyllcytosine
CFP1 CXXC finger protein 1
CpG islands region of the genome with a high frequency of CpG sites
DNMT1/2/3A/3B DNA methyltransferases 1/2/3A/3B
EED embryonic ectoderm development
Ezh2 enhancer of zeste 2 polycomb repressive complex 2 subunit
G9a euchromatic histone lysine methyltransferase 1
GLP euchromatic histone lysine methyltranferase 2
H3K4me2/me3 di/trimethylation of lysine 4 on histone H3
H3K9me2 dimethylation of lysine 9 on histone H3
H3K27me3 trimethylation of lysine 27 on histone H3
hCG human chorionic gonadotropin
KMT histone lysine methyltransferase
MBD1/2/3 methyl-CpG binding domain 1/2/3
mESCs mouse embryonic stem cells
MLL1 lysine methyltransferase 2A
MOR μ opioid receptor
PMSG pregnant mare serum gonadotropin
PRC2 polycomb repressive complex 2
RBBP5 RB binding protein 5, histone lysine methyltransferase complex subunit
RTqPCR reverse transcription polymerase chain reaction
SETD1A/B SET domain containing 1A/B
SUZ12 polycomb repressive complex 2 subunit
SW swiss webstar
TET1/2/3 ten eleven translocation protein 1/2/3

family, which is composed of TET1, TET2, and TET3, is responsible for active DNA demethylation. These proteins work as oxygenases and can convert 5 mC to 5hmC, an intermediate of DNA methylation removal (Ito et al., 2011). Another important family is the methyl-CpG-binding domain (MBD) protein family (Hendrich and Tweedie, 2003); these proteins interact with multiple protein partners, providing a link between cytosine derivatives, functional chromatin states and their regulation (Martinowich et al., 2003).

Histone modification is other important mechanism of epigenetic modification that includes many types of posttranslational modifications, with histone methylation changes being the most frequently studied. For example, on histone H3, methylation of K27 and K9 is associated with repression, while methylation of K4 promotes transcriptional activation (Strahl and Allis, 2000; Barski et al., 2007; Liang et al., 2004). These posttranslational modifications of histone tails are catalysed by specific enzymatic machinery. For instance, polycomb repressive complex 2 (PRC2) mainly trimethylates H3K27 (H3K27me3), and it is composed of 4 core protein subunits. Notably, EZH2 is the enzymatic subunit, and EED and SUZ12 do not have catalytic functions but are crucial for the enzymatic activities of stabilization, nucleosome binding and target gene recruitment (Yuan et al., 2012). The two core subunits of the multimeric repressive histone lysine methyltransferase (KMT) complex, G9a and GLP, act primarily to catalyse the dimethylation of H3K9 (H3K9me2) throughout the genome (Fritsch et al., 2010). In contrast, the di- and trimethylation of H3K4 (H3K4me2/3) are catalysed by two different regulatory complexes, namely, the MLL1 complex and the SETD1 complex. In addition, SETD1A/B, which are components of the SETD1 complex, are recruited to sites near H3K4 (Shilatifard, 2012), and the catalytic subunit of the MLL1 complex, namely, MLL1, catalyses the di- or trimethylation of H3K4 (Hu et al., 2013). Furthermore, RBBP5 is one of the subunits of the WRAD complex, which acts by stabilizing and promoting the catalytic activities of MLL1 and the SETD1 complexes (Ernst and Vakoc, 2012), and CFP1 is the DNA-binding subunit of the SETD1 complex, which is required for the accumulation of H3K4 before the addition of histone modifications (Yu et al., 2017).

We have recently described that chronic morphine treatment has an important effect on mouse embryonic stem cells (mESCs) at the transcriptomic level (Munoa-Hoyos et al., 2020), which in turn could impact the developmental process of the embryo and the phenotype of the adult. In fact, most previous studies have focused on how morphine epigenetically regulates different regions of the brain that are implicated in tolerance, dependence and other psychiatric disorders (Browne et al., 2020; Maze and Nestler, 2011). However, a significant knowledge gap remains regarding the effect of chronic treatment on other organs and biological systems. Thus, the main objective of this work is to increase our knowledge about the impact of chronic morphine exposure on the levels of DNA methylation and histone modification in each of the organs of male and female model mice *in vivo*. To this aim and to search for a possible epigenetic mechanism, we investigated changes in DNA methylation/hydroxymethylation and histone modification associated with both transcriptional repression (H3K27me3 and H3K9me2) and activation (H3K4me3 and H3K4me2) as well as their regulatory complexes.

2. Material and methods

2.1. Animals and drug treatments

For all the *in vivo* experiments, 8- to 10-week-old Swiss Webstar (SW) mice (10 male and 10 female) were used. The mice were housed in groups (ten per cage) in a colony room maintained at constant temperature (+-23 °C) with a 12-h light/dark cycle (lights on from 8:00 a.m. to 8:00 p.m.) and were given ad libitum access to food and water. All the protocols involving mice were approved by the University of the Basque Country Animal Experimentation Ethical Committee (approval number CEEA/339/2013/SUBIRAN CIUDAD and M20/2016/142). The male and female Swiss mice were divided into two separate groups of 5 animals each before treatment, and the weights of all the mice were measured to calculate the specific doses of morphine to be administered. Five male and five female mice were chronically treated with morphine, as previously described (Crain and Shen, 1995). The mice were subcutaneously injected with morphine hydrochloride (purity 98.8%, Alcaliber S. L) twice daily at 12-h intervals (8:00 and 20:00) for 4 days, and the dose was increased each day (20, 30, 40, and 50 mg/kg). On day 5, all the animals received a single subcutaneous injection of morphine (10 mg/kg) at 8:00. Instead of morphine, normal saline (0.9% p/v NaCl) was administered to the control group, which was composed of five male and five female mice. The volume of the solution that was subcutaneously injected was maintained at 0.1 ml. After the treatment period, both the control and treated mice were weighed again to compare the measured values to those at measured the beginning of the treatment as an internal control of animal welfare (Supplementary Figure 1). Morphine treatment overlapped with ovarian hyperstimulation treatment in the female mice to synchronize ovulation and to control the hormonal status of the female mice. The female mice received hormone treatment composed of 5 IU PMSG (G4527, Sigma-Aldrich) and 5 IU hCG (C8554, Sigma Aldrich) at 48-h intervals. The chronic morphine treatment efficacy was confirmed by the "hot plate analgesia test". The treated male and female mice exhibited increased latency to pain reflex behaviour compared to the control mice when the animals were placed on a heat conductive (approximately 60 °C) surface (Ripoll et al., 2006). Finally, the animals were sacrificed by cervical dislocation, and organs from different biological systems (testis/ovaries, liver, pancreas, kidney, heart and lung) were collected for further experimentation (Fig. 1).

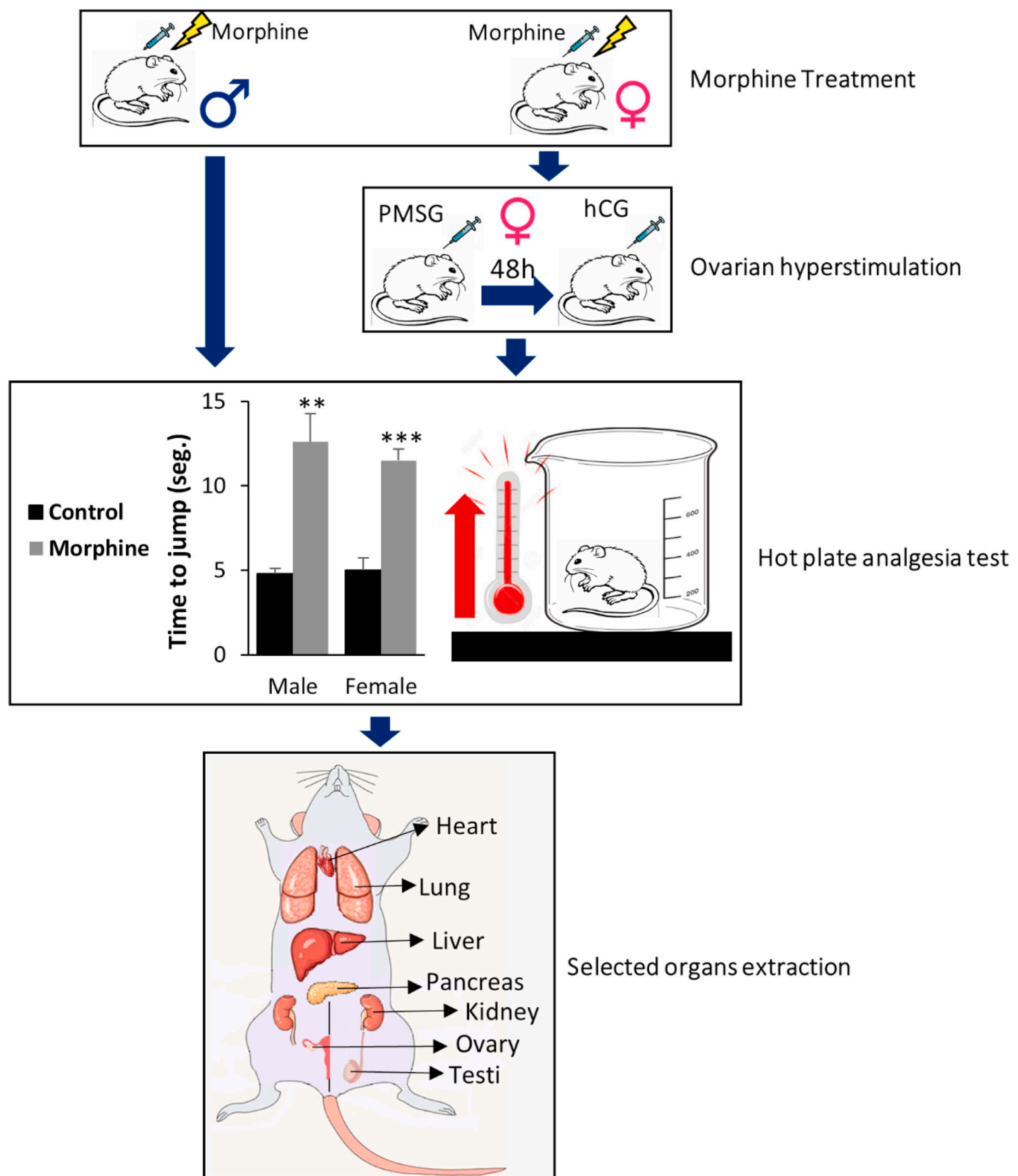

Fig. 1. ***In vivo*** **experimental design for the study of the epigenetic changes induced by morphine in male and female mice.** A hot plate analgesia test was carried out to measure the effect of morphine in male and female mice *in vivo*. Statistical significance was determined by Student's *t*-test (**$p < 0.01$; ***$p < 0.001$). Sample size n = 10 for each sex and 5 for each treatment group. *(Colour).* (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.2. Quantification of DNA methylation by mass spectroscopy

To obtain DNA from different mouse organswe took 50 mg of tissue from each organ that was representative of all the cell types composing the organ. For this purpose, each organ was homogenized in a frozen state (with liquid nitrogen), allowing mixing of all the cell types. Then, each sample was homogenized using DNA lysis buffer (100 mM Tris-HCl, 5 mM EDTA, 200 mM NaCl and 0.2% SDS) and a homogenizer (Heidolph GIRALT), producing a uniform mixture of different cells from the organ tissues. Then, each sample was treated with Proteinase K at 100 mg/ml (AM2546, Thermo Fisher Scientific) and incubated overnight with gentle shaking. Then, the samples were treated with 5 μl RNAse at 10 mg/ml (R5125, Sigma) for 1-h at 37 °C. DNA extraction from all the samples was performed using a classic phenol-chloroform/isoamyl methodology with phenol (P4557, Sigma), chloroform (CL01981000, Scharlau) and isoamyl alcohol (BP1150, Fisher BioReagents). After the extraction, the DNA concentration and purity were evaluated by measuring the 260/280 absorbance ratio in a Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific). The extracted DNA was enzymatically hydrolysed (DNA Degradase Plus; E2020, Zymo

Research), and aliquoted samples (10 μl containing 50 ng of digested DNA) were run in a reversed-phase UPLC column (Eclipse C18 2.1 × 50 mm, 1.8 μm particle size, Agilent), equilibrated, and eluted (100 μl/min) with water/methanol/formic acid (95/5/0.1, all by volume). The effluent from the column was added to an electrospray ion source (Agilent Jet Stream) connected to a triple quadrupole mass spectrometer (Agilent 6460/6400 QQQ). The machine was operated in positive ion multiple reaction monitoring mode using previously optimized conditions, and the intensity of specific MH+→fragment ion transitions was measured and recorded (5mCm/z 242.1 → 126.1, 5 hC 258.1 → 142.1 and dC m/z 228.1 → 112.1). The measured percentages of 5 mC and 5hmC in each experimental sample were calculated based on the MRM peak area divided by the combined peak areas for 5 mC plus 5hmC plus C (total cytosine pool).

2.3. Western blotting

Nuclear proteins were extracted from mouse organ tissues using a high-salt extraction protocol. After the organs were homogenized in liquid nitrogen, allowing the mixing of all the cell types, 50 mg of each organ tissue was homogenized in 100 mM Hepes (pH 7.8), 150 mM KCl, 10 mM EGTA, 10 mM EDTA, 3.2 M sucrose, 3% Triton X-100, 1 M DTT and a complete protease inhibitor cocktail (Complete tablets, Roche) with a homogenizer (Heidolph GIRALT). After 45–60 min of incubation on cold ice, the nuclear fraction was recovered from the samples. For nuclear protein extraction, a 45-min incubation in high-salt buffer (100 mM Hepes pH 7.8, 4 M KCl, 10 mM EGTA, 10 mM EDTA, 80% Glizerol, 1 M DTT and a complete protease inhibitor cocktail (Complete tablets, Roche)) was used. Finally, the nuclear proteins from each sample were quantified by the BCA method, mixed with 4x loading sample buffer containing DTT (10% v/v) and boiled for 10 min at 95 °C. The mouse organ tissue samples were loaded onto 12% resolving gels and separated by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to polyvinylidene fluoride membranes (PVDF) (Amersham Hybond, Sigma) using the Mini Trans-Blot electrophoretic transfer system (Bio-Rad Laboratories, Hercules, CA, USA). Then, the membranes were blocked with Blotto (20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% Triton X-100) containing 5% bovine serum albumin (BSA) for 1-h. Then, the membranes were incubated with a 1:1000 dilution of different histone modification-specific antibodies (polyclonal rabbit anti-trimethyl-histone H3K27 antibody (A2363, Abclonal); polyclonal rabbit anti-dimethyl-histone H3K9 antibody (A2359, AbClonal); polyclonal rabbit anti-dymethyl-histone H3K4 antibody (A2357, AbClonal) and polyclonal rabbit anti-trimethyl-histone H3K4 (A2356, AbClonal)) overnight at 4 °C. After washing (3 × 5 min) in Blotto buffer, the membranes were incubated for 1-h at RT with peroxidase-conjugated goat anti-rabbit antibodies (Blotto + 5% BSA 1:1000) (goat anti-rabbit IgG HRP, sc-2004; Santa Cruz Biotechnology). After washing (3 × 5 min), the peroxidase activity of the blots was visualized with an enhanced chemiluminescence system (ChemiDoc XRS detector, Bio-Rad). The monoclonal mouse anti-beta actin peroxidase antibody (A3854, Sigma, 1:25,000) was used for normalization. The results were analysed by semiquantitative Western blot densitometry analysis using ImageJ software (Image Processing and analysis in Java).

2.4. Real-time PCR (RT-qPCR)

Total RNA was extracted from mouse organ tissues with TRIzol reagent (Thermo Fisher) according to the manufacturer's instructions for each sample type. Briefly, after the organs were homogenized in liquid nitrogen, 50 mg of each organ tissue was homogenized with 1 ml of TRIzol reagent. After a 5-min incubation at RT, 200 μl of chloroform was added to the sample and mixed by vigorous hand shaking. The mix was incubated at RT for 5 min. The tube was then centrifuged at 12,000 g at 4 °C for 15 min. The aqueous phase containing the RNA was transferred to a clean 1.5-ml tube and mixed with 500 μl of isopropyl alcohol, and the sample was incubated for at least 1-h at −20 °C to precipitate the RNA. The sample was centrifuged at 12,000 g at 4 °C for 10 min. The supernatant was discarded, and the RNA pellet was washed twice with 75% ethanol and then centrifuged at 7500 g at 4 °C for 5 min. Then, each RNA sample was dried at RT for 5 min, and the pellet was redissolved in 10 μl of RNase-free water and incubated at 60 °C for 10–15 min. The concentration and purity of the RNA were determined by measuring the absorbance at 260 nm and the 260/280 ratio. The samples were reverse transcribed using an iScript cDNA synthesis kit (Invitrogen). To that end, 1 μg of RNA was mixed with 4 μl of 5x iScript Reaction Mix, 1 μl of iScript Reverse Transcriptase and nuclease-free water to achieve a total reaction volume of 20 μl per sample. Then, the mix was incubated in a thermal cycler (MyCycler) at 25 °C for 5 min (priming), 42 °C for 30 min (reverse transcription) and 85 °C for 5 min (reverse transcriptase inactivation) and then stored at −20 °C. Real-time PCR analysis was carried out using iTaq Universal SYBR Green SuperMix (Applied Biosystems, California USA) on an Abiprism 7000 Sequence Detection System. The PCR program included 39 cycles of 10 min at 95 °C (denaturation), 20 s at 95 °C (hybridization) and 1 min at 59 °C (annealing and extension). PCR amplification was repeated in more than three independent experiments conducted in triplicate. The relative fold induction of gene expression was quantified by the ddCT relative quantification method. The most stable reference genes were used as housekeeping genes for data normalization and were Pyruvate Carboxylase (*Pcx*) and Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) (primer sequences in Table 1).

2.5. Statistical data analysis

Statistical analysis of the hot plate results, methylation/hydroxymethylation results, RT-qPCR results and Western blot densitometry results was performed using Student's *t*-test. Statistical significance was considered at values of $^{*}p < 0.05$, $^{**}p < 0{,}01$ and $^{***}p < 0{,}001$.

3. Results

3.1. Chronic administration of morphine

To elucidate how morphine can induce epigenetic modifications we treated male and female mice with chronic morphine treatment. We performed a modified protocol for chronic morphine treatment in a mouse model (Crain and Shen, 1995), in which animals received injections of morphine (20, 30, 40, and 50 mg/kg) twice a day for 4 days and once (10 mg/kg) on day 5 (Fig. 1) to induce the development of morphine dependence. We considered that the choric morphine treatment is more likely to produce stable epigenetic changes rather than an acute morphine treatment. To determine whether the dose of morphine produced physical dependence we used the "hot plate analgesia test" (Ripoll et al., 2006; Yang and Pu, 2009). This methodology is the most sensitive and reliable index used to confirm the efficacy of treatment by assessing jumping. We observed a significant delay of jumps in the treated mice than in the control mice after the last dose of morphine (Fig. 1), confirming the efficacy of the treatment. This significant result confirmed the efficacy of chronic morphine treatment and was consistent with previous studies (Ripoll et al., 2006; Yang and Pu, 2009). In addition, the initial body weights of both groups were measured prior to drug administration and just after the last dose of the treatment. (Yang and Pu, 2009; Mucha and Kalant, 1016; Trang et al., 2003; Li et al., 2009). No change in body weight was observed in either the males or females (Supplementary figure 1). Thus, these findings showed that differences in epigenetic modification between the treated and control mice might be induced by chronic morphine treatment and excluded the possibility that variability in epigenetic modification is linked to changes in weight.

Table 1
Primer sequences used in the RT-qPCR experimentation.

GENE	Forward (F) -5' → 3′	Reverse (R) -5' → 3′
Gapdh, Glyceraldehyde 3-phosphate dehydrogenase	TATGACTCCACTCACGGCAAATT	TCGCTCCTGGAAGATGGTGAT
Pcx, Pyruvate Carboxylase	CAACACCTACGGCTTCCCTA	CCACAAACAACGCTCCAT
Ezh2, Enhancer Of zeste 2 polycomb repressive complex 2 subunit	AGAATGTGGAGTGGAGTGGTG	CAGTGGGAACAGGTGCTATG
Eed, Embryonic ectoderm development	CCACAAATACGCCAAATGC	CAAACACCAGAGGGTCTCCT
Suz12, Polycomb repressive complex 2 subunit	ACAGAAGCCAGAGACGACCT	GGAGCCATCATAACACTCATTG
G9a, Euchromatic histone lysine methyltransferase 1	TCGGAACAAAGAAGGAGACAC	ATTGACACAGGGGTGGGTA
Glp, Euchromatic histone lysine methyltransferase 2	GATGGATGGAGATTCAGAGGA	CTTTTCCGAGCAGGTTTGAT
Mll1, Lysine methyltransferase 2A	GCAGGCACTTTGAACATCCT	TTATGGGGACAGAGGTCAGG
Rbbp5, RB binding protein 5, histone lysine methyltransferase complex subunit	AGATTGCGACCAGAGGTTTC	ATCCGAGTCATCGTCTACCG
Setd1a, SET domain containing 1A	CAGACGGGCTTGTAGATTCC	GTGGGGGTAGGAGAGGGATA
Setd1b, SET domain containing 1b	CCAGCAGCACAGAGAGTGAG	GCCTCGGGTTGATTTTACTG
Cfp1, CXXC finger protein 1	ACAGCAACACCTGAGCCACT	AGGAACGGGGACTCTTCT
Dnmt1, DNA methyltransferase 1	GCCAGTTGTGTGACTTGGAA	GTCTGCCATTTCTGCTCTCC
Dnmt2, DNA methyltransferase 2	GAGGGCTGCTGATACAAACAA	GAAGGGGAAAGGCTCTGACT
Dnmt3A, DNA methyltransferase 3A	AAACTTCGGGGCTTCTCCT	ATGGGCTGCTTGTTGTAGGT-
Dnmt3B, DNA methyltransferase 3B	ACTTGGTGATTGGTGGAAGC	CCAGAAGAATGGACGGTTGT
Mbd1, Methyl-CpG binding domain protein 1	CACGACCTTCCTGACTTCCT	CCAACCCCTCCTATCTCCTC
Mbd2, Methyl-CpG binding domain protein 2	ACACATCTCAACCCCTCTGC	GTGCCTCCTCCAGTTTCTTG
Mbd3, Methyl-CpG binding domain protein 3	TTGAGTGCCTTTGACATTGC	AGGGTGCTGGTGTGTAGAGC
Mbd4, Methyl-CpG binding domain protein 4	GCAAAAACCATTATCAAGTTCTCA	GATTCTCCCAAAGCCAGTCA
Tet1, Tet methylcytosine dioxygenase 1	TGCTCCAAACTACCCCTTACA	CCCTCTTCATTTCCAAGTCG
Tet2, Tet methylcytosine dioxygenase 2	TGGCTACTGTCATTGCTCCA	TGTTCTGCTGGTCTCTGTGG
Tet3, Tet methylcytosine dioxygenase 3	CGCCGTGATTGTTATCTTGA	TGTTAGGGTCTTTGCCTTGG

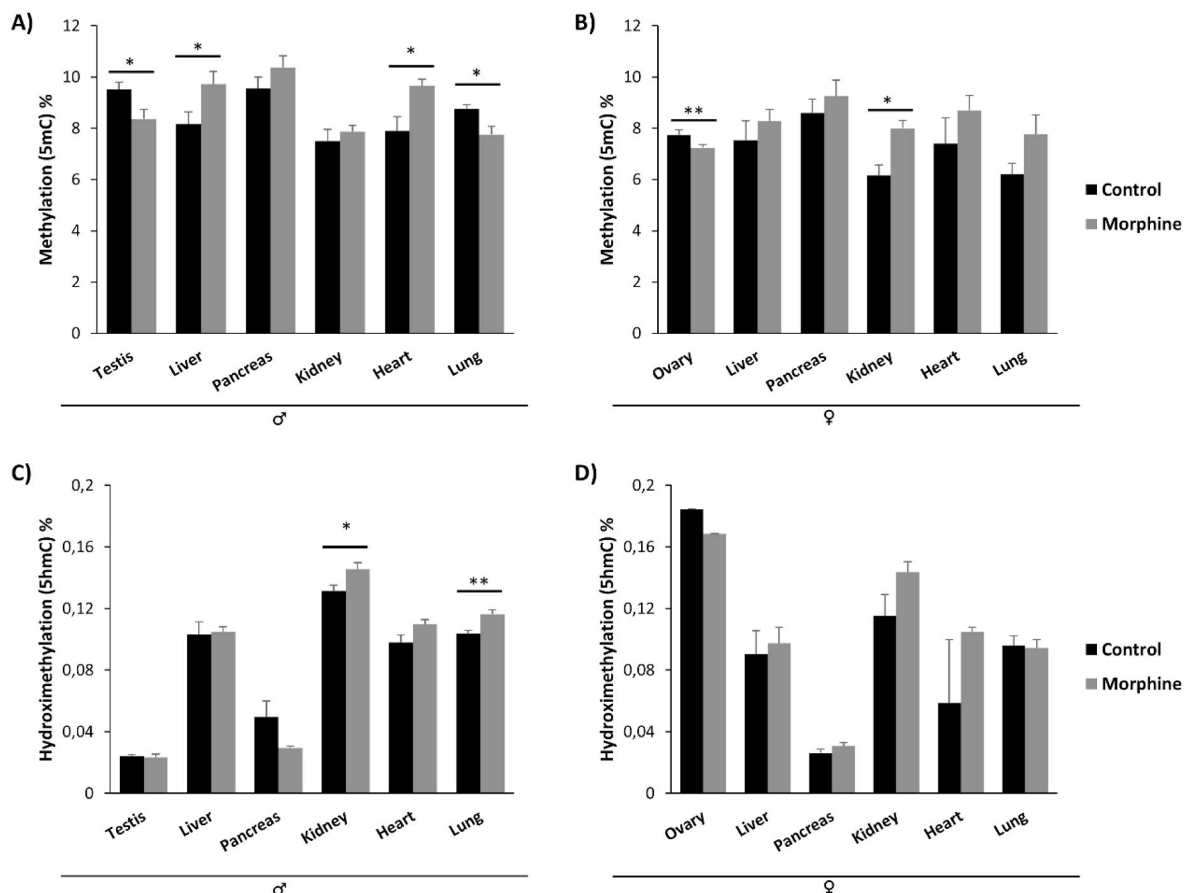

Fig. 2. Effect of chronic morphine treatment on DNA methylation and hydroxymethylation and their regulatory complexes in male and female mice *in vivo*. LC-MS/MS analysis measuring the total amount of methylation in (A) male and (B) female mouse organs and the total amount of hydroxymethylation in (C) male and (D) female mouse organs. The measured percentages of 5 mC and 5hmC in each experimental sample were calculated based on the MRM peak area divided by the combined peak areas for 5 mC plus 5hmC plus C (total cytosine pool). Statistical significance was determined by Student's *t*-test (*$p < 0.05$; **$p < 0.01$). Sample size n = 10.

3.2. Effect of chronic morphine treatment on DNA methylations/hydroxymethylations and regulatory complexes in male and female mice in-vivo

Our first objective was to study the effects of morphine on the DNA methylation and hydroxymethylation epigenetic marks (Fig. 2) and their regulatory complexes (Fig. 3) in a variety of organs from different biological systems *in-vivo*.

By analysing the methylation levels (Fig. 2A and B), we observed that the total amount of methylation was approximately 10%. Nevertheless, chronic morphine treatment had different effects on the methylation levels depending on the sex of the mouse and the analysed tissue. In the male mice (Fig. 2A), we identified a significant increase in the methylation levels in the liver and heart tissues and a significant decrease in the methylation levels in the testis and lung tissues. However, in the female mice (Fig. 2B), although we observed a general increasing trend in the methylation levels after morphine treatment, the only significant increase was observed in the kidney tissue. In contrast, the methylation

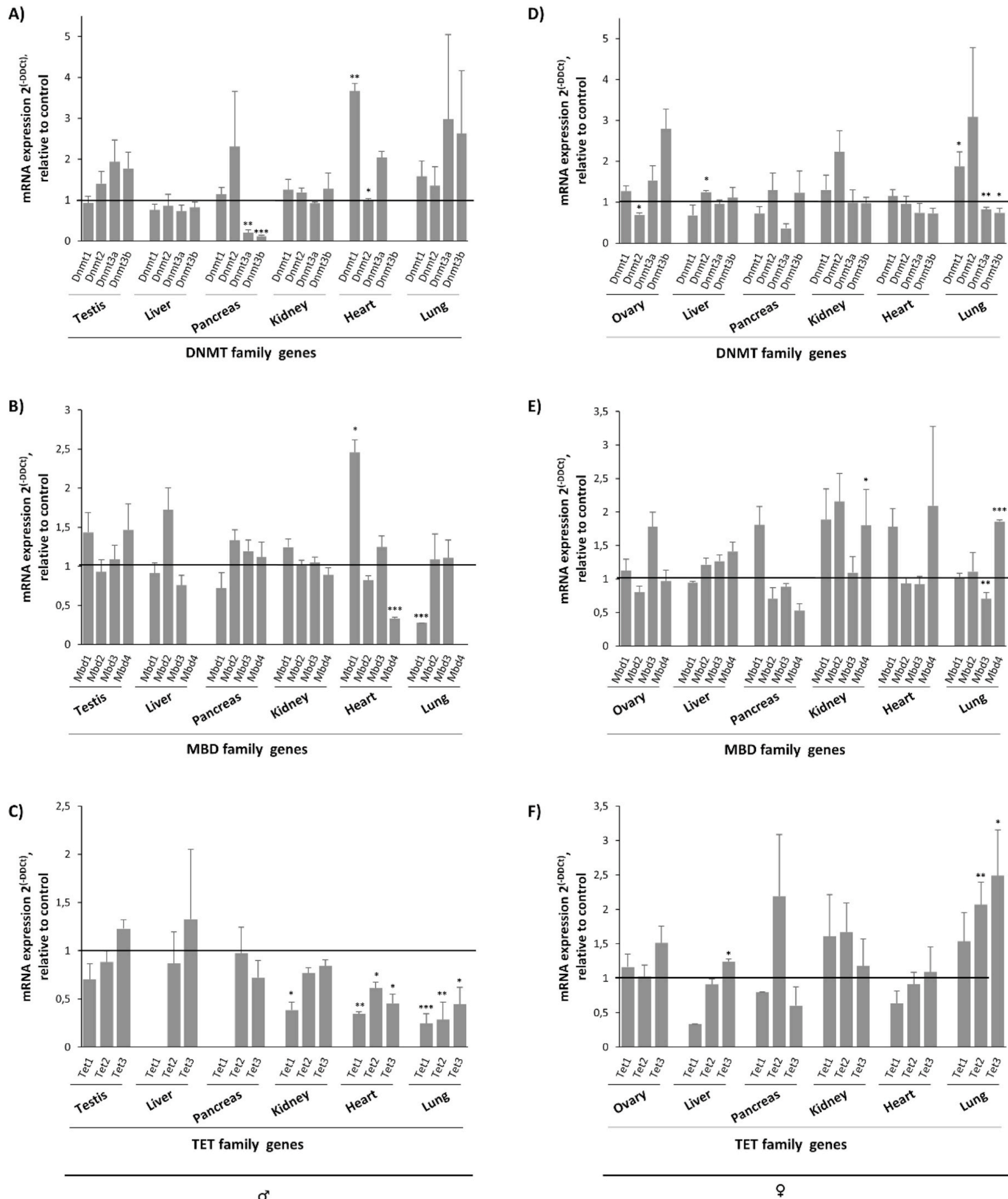

Fig. 3. Effect of chronic morphine treatment on the DNA methylation and hydroxymethylation regulatory complexes in male and female mice *in vivo*. RT-qPCR analysis of genes belonging to DNA methylation and hydroxymethylation regulatory complexes including A) Dnmt1 family genes, B) MBD family genes and C) TET family genes, three of which were analysed in male mouse organs, and D) Dnmt1 family genes, E) MBD family genes and F) TET family genes, three of which were analysed in female mouse organs. *Gapdh* and *Pcx* were used as the housekeeping genes. Acquired Ct values were normalized with respect to the control sample using the 2ddCT method. Statistical significance was determined by Student's *t*-test (*p < 0.05; **p < 0.01; ***p < 0.001). Sample size n = 10.

levels were significantly decreased in the ovarian tissue. Regarding the hydroxymethylation levels (Fig. 2C and D), the total amount of hydroxymethylation was at least ten times lower than the previously described methylation levels. In addition, the hydroxymethylation levels showed great variation among the studied organs. In the male mice (Fig. 2C), a significant increase in this modification was detected in the kidney and lung. However, we did not observe any significant change in hydroxymethylation in the female mice (Fig. 2D).

Additionally, by comparing the results from males and females (Fig. 2), we observed that morphine produced more remarkable changes in the males, while the changes in the female occurred to a lesser extent. These sex differences have been described to be dependent on many interacting variables, including some specific to the drug itself (dose, pharmacology, administration route and stress caused by the treatment) and others specific to the subject (species, genetic background, age and gonadal-hormonal status) (Craft et al., 2006; Kest et al., 2000). In our experiments, most of these variables, including the female mouse hormonal state, were monitored to ensure that morphine was the unique external factor producing the epigenetic changes. Nevertheless, because male and female hormonal cycles are different, these observations suggest that morphine-induced sex-dependent epigenetic differences could be influenced to some extent by gonadal hormone differences (Forman et al., 1989; Craft et al., 2004). On the other hand, it is worth mentioning that despite the sex-dependent differences in the methylation levels in most of the studied organs, both reproductive systems (testis and ovaries) showed the same patterns of methylation (increased after morphine treatment), but these systems showed no significant changes in the hydroxymethylation levels.

After the analysis of the effect of morphine on the methylation and hydroxymethylation levels in the different organs in the mouse model, our next goal was to study whether morphine induced alterations in the expression of genes related to the regulatory complexes of both types of epigenetic modification (Fig. 3). For that purpose, we used RT-qPCR to analyse three families that play a direct regulatory role: the DNMT and MBD families, which regulate methylation, and the TET family, which regulates hydroxymethylation. In the male mice (Fig. 3A, B, C) and regarding the DNMT family (Fig. 3A), chronic morphine treatment induced significantly upregulated gene expression of *Dnmt1*, *Dnmt3A* and *Dnmt3B* in the heart and downregulated gene expresion of *Dnmt3A* and *Dnmt3B* in the pancreas. In contrast, the expression of MBD family components (Fig. 3B) was altered only in the heart tissue, where the *Mbd1* gene was upregulated and the *Mbd4* gene was downregulated, and the lung, where only *Mbd1* expression was significantly decreased. These results were consistent only with the previously described increased the methylation levels in the heart and the decreased methylation levels in the lung. Concerning changes related to the TET family components (Fig. 3C), we observed a significant downregulation of the three members in the heart and lung and *Tet1* gene downregulation in the kidney. Nonetheless, these results did not correlate with the hydroxymethylation levels observed by the LC-MS/MS technique, showing opposite patterns.

On the other hand, in females (Fig. 3D, E, F), focusing on DNMT family components (Fig. 3D), we showed that *Dnmt2* expression was significantly downregulated in the ovary and upregulated in the liver. Furthermore, *Dnmt1* was increased while *Dnmt3A* and *Dnmt3B* were decreased in the lung after chronic treatment. In addition, related to MBD family components (Fig. 3E), we observed that *Mbd4* gene expression was downregulated in the pancreas. However, the *Mbd3* and *Mbd4* components exhibited opposite expression patterns in the lung, being decreased and increased, respectively. Uniquely, the downregulation of *Dnmt2* in the ovary was consistent with the observed methylation changes after chronic morphine treatment. However, the results of the MBD family components in the pancreas and lung did not correlate with the observed methylation levels. Finally, regarding the TET family members (Fig. 3F), we observed a significant downregulation of the expression of *Tet1*, an upregulation of the expression of *Tet3* in the liver tissue, and a significant upregulation of *Tet2* and *Tet3* in the lung. These results did not match the observed hydroxymethylation patterns after morphine treatment.

3.3. *Effects of chronic morphine treatment on histone modification and the regulatory complexes in male and female mice in vivo*

Our next objective was to study chromatin conformation by assessing histone modification and the regulatory complexes *in vivo* (Fig. 4); for this study, we used different biological systems from the previously mentioned mouse animal model subjected to chronic morphine treatment (Crain and Shen, 1995) (Fig. 1). Fig. 4A and B shows morphine-altered histone levels in both male and female mouse organs, respectively. In the male mice (Fig. 4A, C), morphine led to a global downregulation of the repressive histone mark H3K27me3 and a global upregulation of H3K9me2, H3K4me3 and H3K4me2 in the testis. In contrast, we observed a sharp increase in all the studied histone modificarion levels in the liver, pancreas and heart after morphine exposure, while a significant decrease was observed in the kidney. In the male lungs, however, morphine did not produce any effect on the histone modification levels. In the females (Fig. 4B, C), a global decrease was observed in H3K27me3 in the ovary, while H3K9me2, H3K4me3 and H3K4me2 were increased after morphine treatment. Moreover, in the female liver, we observed a global upregulation of the studied histone modifications, except for H3K4me3, whose histone modification level was decreased. In the pancreas, we observed an increase in the H3K27me3 and H3K4me2 levels; however, the H3K9me2 and H3K4me3 levels did not seem to be affected by morphine treatment. Furthermore, in the female kidney and heart, morphine induced an increase in all the histone levels, except for the H3K4me2 levels in the heart, which were not affected. Moreover, as we have described for the male mice, we did not observe any changes in the lungs of the female mice after chronic morphine exposure. In both the analyses of the male and female mice, protein expression was normalized by using β-actin, and negative controls were established by omitting the primary antibody before adding the secondary antibody (data not shown).

Additionally, by comparing the results from the male and female mice (Fig. 4), it was clear that morphine generated more striking changes in the tissues of male mice, while the changes observed in the tissues of the female mice were slight. In both sexes, the tissues of the reproductive system, namely, the testis and ovaries, showed the same levels of modification of the four histones studied. In contrast, in the other studied tissues, we observed several sex-dependent differences, except in the heart, where all the histone levels were increased in both sexes, and in the lung, where we did not find any significant changes.

We also studied the effect of chronic morphine treatment on the expression of genes related to histone modification regulatory complexes in the male and female organs (Fig. 5). On the one hand, in the samples from the male mice (Fig. 5A, B, C, D), we observed different expression patterns of the PRC2 subunits (Fig. 5A). In the testis, only *Eed* expression seemed to be significantly upregulated. In the liver, *Ezh2* was significantly downregulated, while *Eed* was substantially upregulated. In contrast, in the pancreas, kidney, lung and heart, only the catalytic subunit *Ezh2* was dysregulated, and it was downregulated in the first three cases and upregulated in the last case. Based on these results, *Eed* and *Ezh2* upregulation in the liver and heart, respectively, together with *Ezh2* downregulation in the kidney, were consistent with the H3K27me3 protein levels observed after chronic treatment. Regarding the G9a/GLP complex (Fig. 5B), both subunits were dysregulated only in the testis and heart, showing a completely opposite expression pattern (upregulation and downregulation, respectively). Furthermore, the *Glp* subunit was upregulated in the liver and downregulated in the lung. The observed changes in the *G9a* and *Glp* subunits confirmed the increase in H3K9me2 in the testis and liver, while the expression patterns were reversed when the histone protein levels in the heart and lung were analysed. With respect to the MLL1 complex (Fig. 5C), only the expression changes

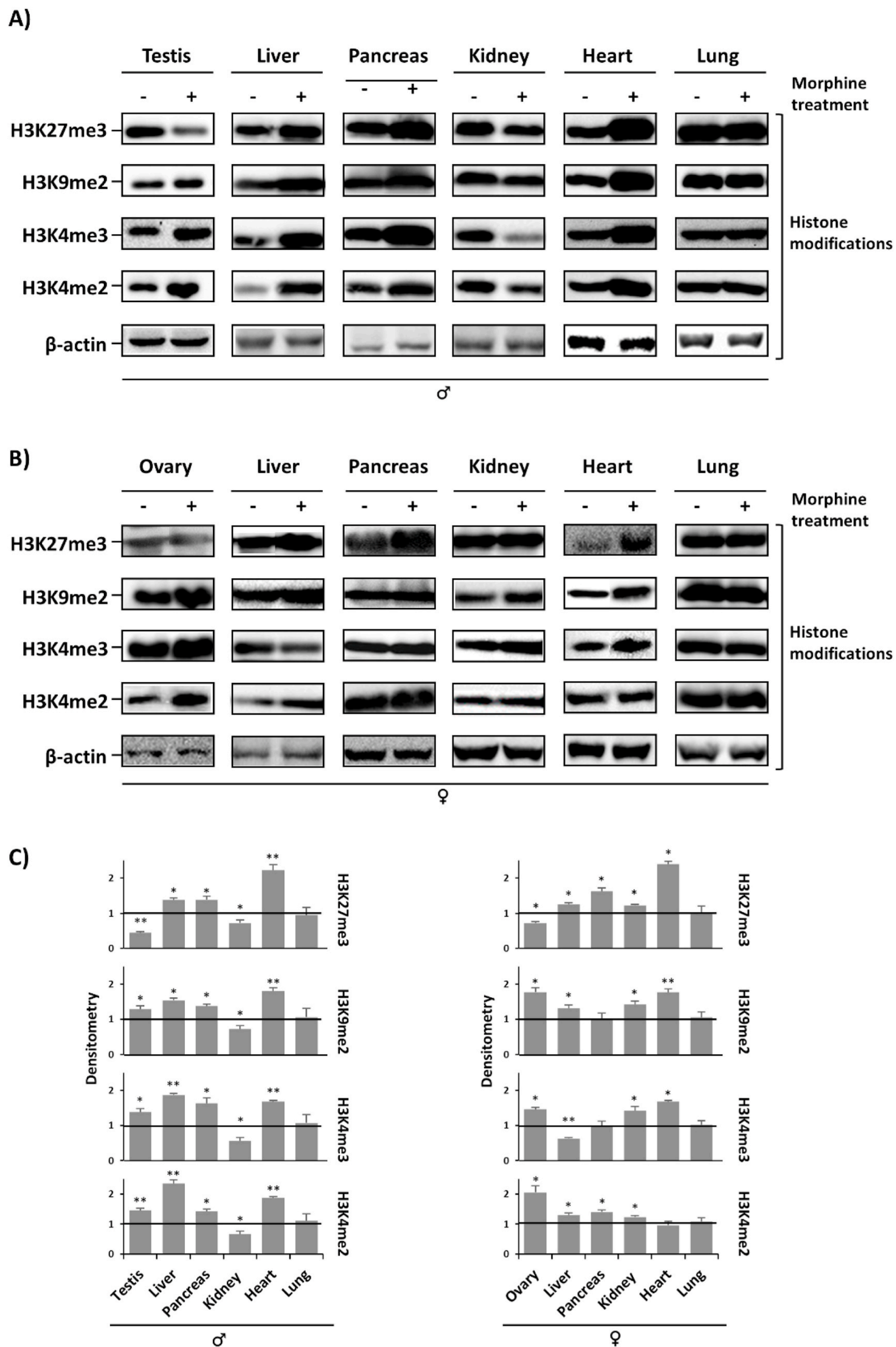

Fig. 4. Effect of chronic morphine treatment on histone modification and regulatory complexes in male and female mice *in vivo*. (A) Western blot analysis of H3K27me3, H3K9me2, H3K4me3, and H3K4me2 in male mouse organs (testis, liver, pancreas, kidney, heart and lung) after chronic morphine treatment. (B) Western blot analysis of H3K27me3, H3K9me2, H3K4me3, and H3K4me2 in female mouse organs (ovary, liver, pancreas, kidney, heart and lung) after chronic morphine treatment. C) Densitometries measured by ImageJ software. β-actin was used as the loading control. Sample size n = 10.

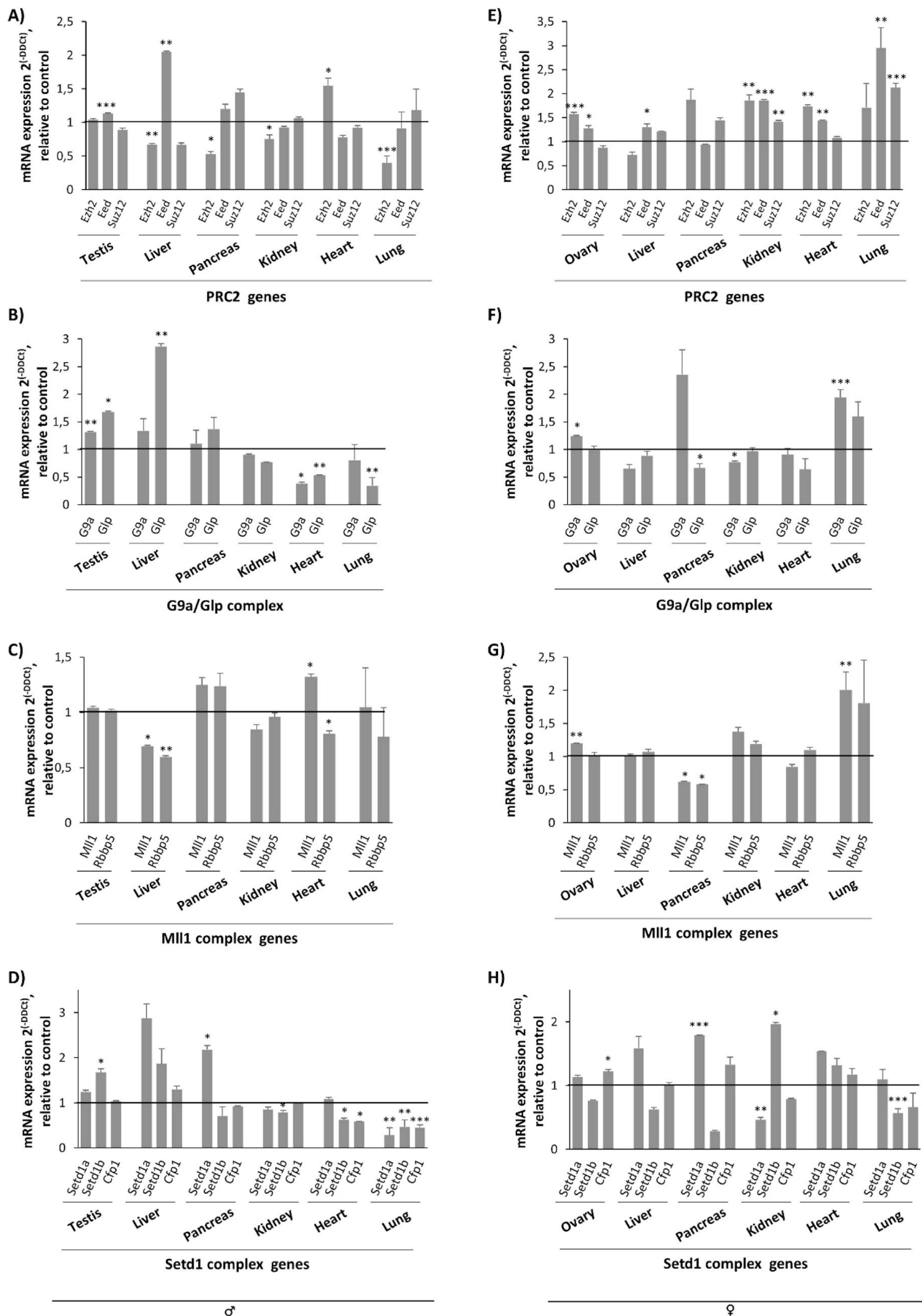

Fig. 5. Effect of chronic morphine treatment on histone modification regulatory complexes in male and female mice *in vivo*. RT-qPCR analysis of genes belonging to each histone modification methylation complex, including A) PRC2 genes, B) G9a/Glp complex genes, C) Mll1 complex genes and D) Setd1 complex genes, four of which were analysed in male mice organs, and E) PRC2 genes, F) G9a/Glp complex genes, G) Mll1 complex genes and H) Setd1 complex genes, four of which were analysed in female mice organs. *Gapdh* and *Pcx* were used as the housekeeping genes. Acquired Ct values were normalized with respect to the control sample using the 2ddCT method. Statistical significance was determined by Student's *t*-test (*p $<$ 0.05; **p $<$ 0.01; ***p $<$ 0.001). Sample size n = 10.

observed in the liver and heart appeared to be significant. Both subunits were downregulated in the liver, while the catalytic subunit *Mll1* was upregulated and the *Rbbp5* subunit was downregulated in the heart. In contrast, the H3K4me2/me3 levels were not consistent with the observed *Mll1* and *Rbbp5* expression levels in the liver, and only *Mll1* upregulation was consistent with the increased H3K4me2/me3 level. Finally, the SETD1 complex subunits were analysed at the gene expression level (Fig. 5D). Surprisingly, *Setd1a* and *Setd1b* were significantly upregulated in the pancreas and testis, respectively, while *Setd1b* was also downregulated in the kidney. Moreover, the heart tissue showed a significant downregulation of the *Setd1b* and *Cfp1* subunits, and the three subunits were significantly downregulated in the lung. Altogether, only the results observed in the testis, pancreas and kidney were consistent with the dysregulated protein levels observed in those tissues.

On the other hand, in the samples from the female mice (Fig. 5E, F, G, H), focusing on the PRC2 complex (Fig. 5E), the catalytic subunit *Ezh2* and the core component *Eed* were significantly upregulated in the ovary, heart and kidney, where interestingly, *Suz12* was also upregulated. Furthermore, *Eed* was upregulated in the liver, and the *Eed* and *Suz12* subunits were both upregulated in the lung. These results were uniquely consistent with the observed increase in H3K27me3 at the protein level in the liver, kidney and heart. Regarding the G9a/GLP complex (Fig. 5F), we observed a significant increase in *G9a* expression in the ovary and lung and a significant decrease in *G9a* expression in the kidney. In addition, the *Glp* subunit was only significantly altered in the pancreas, showing decreased gene expression. The observed dysregulation of the G9a/Glp regulatory complex at the gene expression level was only consistent with the increase in H3K9me2 in the ovary. With respect to the MLL1 complex (Fig. 5G), the catalytic subunit *Mll1* was significantly altered in three organs; it was upregulated in the ovary and lung and downregulated in the pancreas, where the *Rbbp5* component was also downregulated. These observed gene expression levels are perfectly consistent with the H3K4me3/me2 dynamics in the ovary after chronic morphine treatment. Finally, regarding the SETD1 complex subunits (Fig. 5H), *Setd1a* was significantly upregulated in the pancreas, *Setd1b* was downregulated in the lung, and *Setd1a and b* were significantly downregulated and upregulated, respectively, in the kidney. The DNA-binding subunit *Cfp1* was upregulated in the ovary after morphine treatment. These results were consistent with the changes in H3K4me3/me2 at the protein level, mainly in the ovary.

4. Discussion

Morphine is the most effective drug for the treatment of pain. The use of acute and/or chronic morphine treatment for the management of acute, severe or chronic pain related to advanced medical illness is considered the standard of care for inducing analgesia, despite the several side effects that morphine can cause (Nakatani, 2017). Although the main effects and properties of morphine have been linked to the nervous system and behaviour (Stein et al., 2003), wide-ranging side effects of morphine in different systems and organs have also been reported, as the mu-opioid receptor (the main target of morphine) is widely distributed throughout the body (Bodnar, 2018). Although morphine can cause several side effects, there is a critical gap in our understanding of how morphine leads to abnormal pain perception, behaviour and other physiological consequences. Morphine disrupts these processes, at least in part, by altering the gene expression, as it modifies the expression profile of certain mRNAs in many tissues and organs (Przewlocki, 2004). To date, a large number of studies have focused on identifying morphine-induced changes in gene expression (Loguinov et al., 2001; Ammon-Treiber and Hollt, 2005; Rhodes and Crabbe, 2005); nevertheless, none of these studies has identified a mechanism by which morphine induces these changes in expression. To provide additional data, we studied the main epigenetic marks related to morphine exposure in order to understand the effect of morphine on different organs in adults and the related mechanisms of epigenetic modification.

Mice are the most common species used in studies to mimic the antinociceptive effects of opioids in humans (Morgan and Christie, 2011; Holtz Nathan et al., 1007), as the affinity of the mu-opioid receptor is not critically different between humans and mice (Afsharimani et al., 2015). Furthermore, when the results of murine studies of opioid addiction were compared with the more limited data of human studies, the findings were observed to be similar and compatible (Angst and Clark, 2006). To identify specific changes in DNA methylation and histone modification in different systems and organs, male and female mice were chronically treated with morphine, as we consider that long-term morphine administration is more likely to produce stable epigenetic changes than acute morphine treatment (Crain and Shen, 1995; Yang and Pu, 2009; Yang et al., 2014). Our findings confirm that morphine induces systemic effects on males and females by changing the DNA methylation and histone modification levels in most of the studied organs in mice after morphine exposure. These changes may explain, at least in part, how morphine disrupts several physiological processes, such as sexual functions and hormones; gastrointestinal, renal and hepatic functions; kidney development; pancreatic secretions; cardiovascular responses; and metabolic and respiratory processes (Bodnar, 2018; Glanzmann et al., 2016; Ernest et al., 1998; Perneger et al., 2001).

A relevant aspect of our study is the use of both male and female mice to reveal the epigenetic changes induced by morphine at the systemic level. Until recent decades, most scientific studies have only studied male animals to understand the physiological and molecular effects of different drugs, and these results have been extrapolated to females, without testing whether the effects on the different sexes are similar or completely different (Hamberg, 2008). However, sex-related differences in opioid-induced analgesia and physical dependence and in opioid addiction have been reported (Pasternak, 1993; Cicero et al., 1996; Islam et al., 1993; Baamonde et al., 1989). Consistent with these results, our findings show sex-dependent effects on alterations in DNA methylation and histone modification induced by morphine in different organs; these findings can explain how mechanisms of epigenetic modification may also have a relevant physiopathological effect on the cardiovascular system or locomotor activity, among others (Back et al., 2011; McHugh et al., 2013). Sexual dimorphism in mu-opioid receptor splice variants, which occurs in specific tissues, has also been described as a mediator of sex-dependent adaptations in response to chronic morphine treatment (Chakrabarti et al., 2012; Verzillo et al., 2014). Furthermore, several studies have previously indicated that male rats and mice display greater opioid analgesia, with males being more sensitive than females to the antinociceptive properties (Kest et al., 2000; Cicero et al., 1996; Islam et al., 1993; Baamonde et al., 1989; Candido et al., 1992). All these findings may provide a basis for understanding why we observe more remarkable epigenetic changes in males than in females. Our results, therefore, highlight the need for sex parity in future studies focused on the effects of morphine, reinforcing the differences between males and females, which in fact exist.

The relation between DNA methylation and opioid addition has been studied mostly in the 5 mC context and with a focus on particular genes of interest after acute or chronic opioid exposure; in addition, previous studies have mainly focused on the functions of the brain or specific tissues, such as blood or sperm, in human and rodent models (Cecil et al., 2015; Chorbov et al., 2011). Our results show global hypomethylation in the testis, ovary and lung (in males), which is consistent with the same pattern observed in neuronal cells (Trivedi et al., 2014). In contrast, global DNA hypermethylation is observed in the livers and hearts of male mice and in the kidneys of female mice. Although there has not yet been a genome-wide mapping of such regulatory effects, as has been performed with other drugs such as cannabinoids (Watson et al., 2015) or other toxin and addiction models (Suzuki et al., 2013; Yoon et al., 2017), DNA hypermethylation has been reported in a variety of gene exons and gene bodies of human patients addicted to heroin (Kozlenkov

et al., 2017). This difference in the modulation of DNA methylation by opioids has also been identified in multiple brain regions in rats following chronic morphine exposure (Barrow et al., 2017). Therefore, in concordance with what has been previously reported, there is no global trends of methylation patterns, as these patterns change depending on sex and the analysed region or tissue (Barrow et al., 2017), probably because a variety of different cell types are present in each organ.

On the other hand, a limited number of studies have suggested a role of histone modification in opiate action (Maze and Nestler, 2011; Sanchis-Segura et al., 2009; Jing et al., 2011). In fact, very little is known about how opioids alter histone methylation compared to acetylation. Although most of the analysed histone modifications are globally increased, our results indicate that there is no uniform pattern after chronic morphine treatment, similar to DNA methylation. Notably, *in vivo* chronic morphine treatment led to a decrease in the H3K27me3 levels in reproductive organs, the testes and ovaries, and in the kidneys of male mice, which is consistent with our previous results of mouse embryonic stem cells after *in vitro* morphine exposure (Munoa-Hoyos et al., 2020). Histone modifications can result in a gain or loss of cytosine DNA methylation through the formation of compact or open chromatin in response to environmental factors (Tamaru and Selker, 2001; Lan et al., 2017). In this line, chronic morphine treatment causes a decrease in the levels of the repressive mark H3K27me3 and an increase in the levels of the active mark H3K4me2/3 in both the testes and ovaries after morphine exposure; these effects may be involved in the loss of methylation induced by morphine. In a similar way, the H3K27me3 and H3K9me2 repressive marks could form compact chromatin, facilitating DNA methylation in the livers and hearts of males and the kidneys of females. Our results, therefore, suggest the existence of cross-talk between different epigenetic marks induced by morphine in different organs.

Unfortunately, our results incompletely explain the roles of the DNA methylation and histone modification regulatory complexes in different organs after *in vivo* morphine exposure. Some researchers have previously described that the PRC2 and G9a/Glp regulatory complexes of H3K27me3 and H3K9me3, respectively, are sensitive to morphine exposure (Munoa-Hoyos et al., 2020; Maze et al., 2010; Sun et al., 2012; Koo et al., 2015). This is consistent with our findings regarding the PRC2 and G9a/Glp regulatory complex members in the testis, ovary and male kidney but not in other organs. In addition, although it has been recently described that the DNA methylation and hydroxymethylation machinery (composed of DNMTs and TETs) is also dysregulated after exposure to different drugs (Vaillancourt et al., 2017; Saad et al., 2019; Feng et al., 2015; Jiang et al., 2021), our results do not show changes in this machinery after *in vivo* morphine treatment. This difference in the results can probably be explained by the fact that we have only focused on the gene expression of the complex members. Therefore, further functional analyses will be required to elucidate the role of regulatory complexes in the chronic analgesia induced by morphine.

5. Conclusions

In conclusion, our overall results reveal, for the first time, that chronic morphine treatment induces changes in DNA methylation/hydroxymethylation and histone modification *in-vivo* at the systemic level in mice. We provide insights into how morphine leads to tissue- and sex-specific effects on the epigenome and the existence of cross-talk between the different epigenetic marks induced by morphine in different organs. This study reveals the need for detailed mapping of the changes in DNA methylation and histone modification in models of addiction to increase our understanding of the underlying mechanisms of morphine-induced epigenetic modification.

CRediT authorship contribution statement

Iraia Muñoa-Hoyos: Methodology, Investigation, Formal analysis, Data curation, and writing. **Manu Araolaza:** Methodology, Investigation, Formal analysis, Data curation, and writing. **Itziar Urizar-Arenaza:** Methodology. **Marta Gianzo:** Methodology. **Jon Irazusta:** Conceptualization, and, Resources. **Nerea Subiran:** Methodology, Investigation, Conceptualization, Resources, Supervision, and, Funding acquisition, The author(s) read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2021.112200.

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