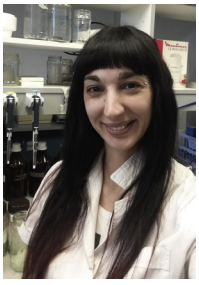
HIGHLIGHTS

- Cocaine disrupts mouse testicular epigenetic homeostasis, increasing DNA methylation in germ cells and sperm.
- Cocaine increased acetylated histone 3 and 4, decreased class I HDAC expression and altered DNMTs and TETs methylation markers.
- Addictive psychostimulants negatively impact male reproductive function and fertility.

RBMO

ARTICLE

Cocaine alters the mouse testicular epigenome with direct impact on histone acetylation and DNA methylation marks



BIOGRAPHY

Candela Gonzalez is a Researcher for the National Research Council in Argentina. Her areas of research cover issues of male infertility and reproduction. This forms part of translational medicine, bridging basic and clinical research and working in conjunction with medical institutions.

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KEY MESSAGE

Cocaine intake in mice disrupts testicular epigenetic homeostasis, increasing global methylated cytosine levels in spermatozoa, and alters global histone acetylation and the expression of DNA methylation markers. These results expand knowledge about the testicular effects of cocaine that negatively impact the reproductive male function.

ABSTRACT

Research question: Recent evidence suggests that cocaine administration in animal models can trigger non-genetic inheritance of addiction traits from father to offspring, affecting development and behaviour. Is chronic cocaine intake involved in alterations of epigenetic homeostasis in the testis?

Design: Epigenetic marks and mediators in testis and isolated germ cells of adult mice treated with cocaine (10 mg/kg) or vehicle (sterile saline solution) were evaluated in an intermittent binge protocol: three intraperitoneal injections, 1 h apart, one day on/off for 13 days, collecting tissue 24 h after the last binge administration (day 14).

Results: It was shown that chronic cocaine intake in mice disrupts testicular epigenetic homeostasis, increasing global methylated cytosine levels in DNA from germ cells and sperm. Cocaine also increased testicular and germ cell acetylated histone 3 and 4 and decreased expression of histone deacetylases HDAC1/2. Immunolocalization studies showed that HDAC1/2 and acetylated histone 3 and 4 proteins localize to meiotic germ cells. Analysis of mRNA expression in isolated germ cells shows decreased levels of Hdac1/2/8, Dnmt3b and Tet1 and increased levels of Dnmt3a gene expression after cocaine treatment.

Conclusions: Cocaine intake is associated with testicular toxicity and significant reproductive function impairment. The results presented here broaden the basic knowledge of the impact of addictive stimulants on testicular pathophysiology, fertility and male reproductive health and imply that altered epigenetic homeostasis by cocaine may have potential consequences on future generations.

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KEYWORDS

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HDAC
Histone acetylation

INTRODUCTION

Substance use disorders are a major health concern worldwide. An estimated 149–271 million people were reported to have used an illicit drug in 2009 (*Cadet et al., 2014*). Chronic cocaine use impacts brain structures and functions in neural pathways involved in reward, motivation and memory (*Cadet and Bisagno, 2014*). Addictive psychostimulant intake is also associated with toxic events and epigenetic changes in brain dopaminergic areas (*Robison and Nestler, 2011*). Emerging evidence shows that similar events take place in peripheral organs like the testis. In particular, cocaine intake has been associated with testicular toxicity and significant impairment of male reproductive function in humans (*Fronczak et al., 2012*).

Epigenetic mechanisms of gene regulation have been linked to both the acute effects and lasting changes to the brain caused by addictive drugs including cocaine (*Robison and Nestler, 2011*), but their impact on the testicular physiology has been poorly investigated. The epigenetic marks in male germ cells are mainly established in spermatogonia, spermatocytes and round spermatids, indicating that these stages would be especially susceptible to environment-induced changes in the germ cell epigenome (*Oakes et al., 2007; Rajender et al., 2011*). The time between chromatin compaction and the transit of mature sperm to the epididymis is at least 10 days, where epigenetic and transcriptional machineries are no longer active (*Bale, 2015*). Importantly, growing evidence indicates that cocaine can influence the physiology and behaviour of descendants through epigenetic mechanisms and male germ cell reprogramming (*Vassoler et al., 2013*; *Wei et al., 2011*). One such mechanism is the regulation of gene accessibility by histone tail acetylation, a highly regulated process of transcriptional activation elicited by the action of histone acetyltransferases (HAT) and counteracted by histone deacetylases (HDAC). In the testis it was also found that histone acetylation participates in the control of the spermatogenic process independently of gene transcription, via chromatin remodelling. For instance, an essential step in spermiogenesis is the hyperacetylation of histone 4, which allows the proper replacement of histones by protamines (*Hazzouri et al., 2000*). Also, gene silencing is regulated by marks placed on DNA itself, like methylation at the 5′ position of cytosines (5-mC), a process mediated by DNA methyltransferases (DNMT) and counteracted by dioxygenases, such as those in the ten-eleven translocation (TET) family (*Robinson and Nestler, 2011*). DNA methylation can modulate transcriptional outcome by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factors to DNA (*Moore et al., 2013*).

The mechanism by which cocaine alters the testicular physiology and epigenome has been poorly investigated. Toxic effects of cocaine on the testes include morphological changes, antioxidant depletion, apoptosis of germ cells and reduced sperm production both in humans and rodents (*Bracken et al., 1990; George et al., 1996; Li et al., 1999; Rodriguez et al., 1992*). It has been previously shown that cocaine administration in male mice induced testicular germ cell loss together with increased oxidative stress (*González et al., 2015*). Importantly, toxicity occurred in parallel with dysregulation of the testicular dopamine (DA) system: cocaine increased the expression of the rate-limiting enzyme of catecholamine synthesis, tyrosine hydroxylase, and induced down-regulation of DA receptors, DRD1 and DRD2, similarly to the mechanism described in the brain (*González et al., 2015*). These results highlighted a probable link between toxic events and testicular DA dysregulation that could influence the testicular epigenome.

The present study evaluated changes in the expression of epigenetic acetylation and methylation marks in adult mice after chronic cocaine treatment in both whole testicular tissue and isolated germ cells. Understanding that testicular cells are susceptible to exogenous environmental changes and identifying the type of epigenetic marks that could be altered is critical to detect underlying and transgenerational mechanisms of risk of diseases.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (2 months old) from the School of Exact and Natural Sciences of the University of Buenos Aires (UBA) were housed in a light- and temperature-controlled room. Principles of animal care were followed in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council, 2003) and approved on 8 July 2015 by the IACUC Committee of the Faculty of Pharmacy and Biochemistry (UBA) (Protocol Number: EXP_UBA_N°0040944/15).

Pharmacological treatment

Cocaine hydrochloride (10 mg/kg, Sigma-Aldrich, St Louis, MO, USA) or vehicle (saline) was administered in a binge-like regimen of three intraperitoneal injections 1 h apart, one day ON/one day OFF (FIGURE 1) (*González et al., 2015*). Animals were killed on day 14 and testes removed for isolation of germ cells, immediately frozen at –70°C for molecular analyses or fixed in 4% paraformaldehyde (PFA) for immunohistochemistry studies. The right and left epididymis were also removed and immediately used for sperm swim-up processing.

Isolation of germ cells

Germ cells were isolated from testes of vehicle- and cocaine-treated mice as previously described (*Goodyear and Brinster, 2017*). The right and left testes of each animal were decapsulated and digested with type I collagenase (0.23 mg/ml, Sigma) in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin for 10 min at 34°C in a shaking water bath. Collagenase activity was stopped by adding cold PBS and the seminiferous tubules were allowed to settle and washed three times with PBS. Then, the seminiferous tubules were mechanically dispersed and the supernatants were filtered (cell strainer, 41 μm) and centrifuged at 150*g* for 15 min. Finally, PBS was removed and the cells were kept at –80°C for molecular studies.

Sperm swim-up

Each cauda epididymis from vehicle- and cocaine-treated mice was cut five times for spermatozoa release and placed in 500 μl of HEPES-buffered HTF medium (Irvine Scientific, Santa Ana, CA, USA). Epididymides were incubated at 37°C for 20 min (to allow sperm to swim out). The sperm suspension from each cauda was pooled and kept at –80°C for molecular studies.

RNA isolation and real-time PCR

Total testicular and isolated germ cell RNA was extracted with TRIzol®

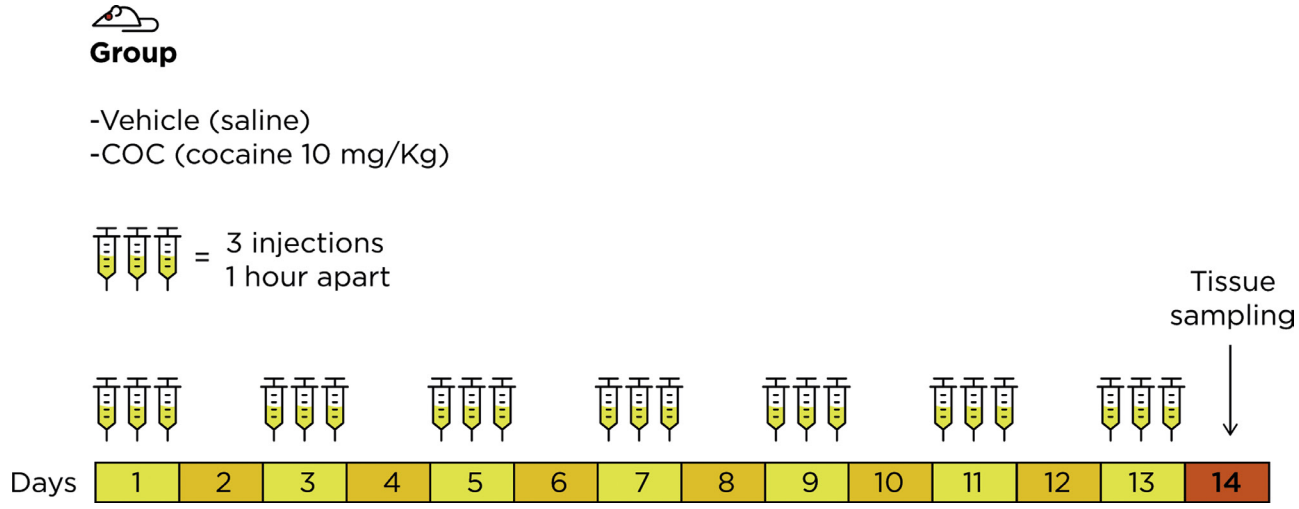

FIGURE 1 Schematic representation of the experimental cocaine treatment. Male C57BL/6 mice were subjected to an intermittent chronic treatment (one day ON/one day OFF for 13 days) with cocaine (COC) at 10 mg/kg or saline (vehicle) in a binge protocol: three intraperitoneal injections per day, 1 h apart. Tissue samples were taken 24 h after the last binge (day 14).

Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Testicular (1 μg) and germ cell (500 ng) RNA was treated with DNAse I (Invitrogen, Carlsbad, CA, USA) and used for reverse transcription in a 20 μl final volume containing M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) and random hexamer primers (Biodynamics, USA). Reverse transcribed cDNA was used for quantitative PCR reactions using SYBR Green PCR Master Mix (Foster City, CA, USA) and specific primers (Supplementary TABLE 1) in a Stratagene MPX500 cycler (Stratagene, USA). All samples were analysed in duplicate. Data from the reaction were collected and analysed by the complementary computer software (MxPro3005P v4.10 Build 389, Schema 85). Melting curves were run to confirm specificity of the signal. To assess quantitative differences in the cDNA target between samples, the mathematical model of Pfaffl was applied (*Pfaffl, 2001*). An expression ratio was determined for each sample by calculating $(E_{target})^{\Delta Ct(target)}/(E_{GAPDH})^{\Delta Ct(GAPDH)}$, where E is the efficiency of the primer set and Ct is threshold cycle with ΔCt = Ct (normalization cDNA) – Ct (experimental cDNA). The amplification efficiency of each primer set was calculated from the slope of a standard amplification curve of log(ng cDNA) per reaction versus Ct value [E = 10 – (1/slope)].

Immunohistochemistry

Mounted paraffin sections (5 μm) were dewaxed in xylene, rehydrated in graded alcohols and washed in tap water. Sections were subjected to antigen retrieval (30 min at 100°C in 10 mmol/l citrate buffer, pH 6.0). Then, endogenous peroxidase activity was inhibited in tissue sections using 0.5% v/v H_2O_2/methanol for 20 min at room temperature. Sections were blocked for 30 min with 1.5% normal goat (for HDAC2, H3ac and H4ac) or horse (for HDAC1) serum in PBS and incubated overnight at 4°C with primary antibody anti-HDAC1 (1:100, mouse monoclonal, 05-100-I Millipore), anti-HDAC2 (1:50, rabbit polyclonal, H54 sc-7899, Santa Cruz), anti-H3ac antibody (1:100, rabbit polyclonal, 06-599, Millipore), anti-H4ac antibody (1:100, rabbit polyclonal, 06-866, Millipore). Sections were incubated for 1 h at room temperature with appropriate 1:200 diluted biotinylated secondary antibodies (1:200, biotinylated goat anti-rabbit IgG, BA-1000 and biotinylated horse anti-mouse IgG, BA-2000; Vector Laboratories Ltd, Peterborough, UK). Then, sections were incubated for 30 min with 1:100 diluted streptavidin–peroxidase complexes (ABC Kit, Vector Labs). Development of peroxidase activity was achieved with 0.05% w/v 3,3′-diaminobenzidine and 0.1% v/v H_2O_2 in Tris-HCl. As negative controls, normal rabbit serum (for HDAC2, H3ac and H4ac) or normal mouse (for HDAC1) was used instead of primary antibodies. No specific immunoreactivity was detected in these sections (see Supplementary FIGURE 1).

Western blot

Nuclear extracts were prepared from testicular tissue. Briefly, tissue homogenates were prepared in a solution containing 10 μmol/l HEPES, 10 μmol/l KCl, 1.5 μmol/l, 1% IGEPAL (Sigma) with protease inhibitors (Roche). Samples were centrifuged at 14000*g* for 5 min and the cytosolic protein fraction was separated. After washing the nuclear protein fraction, samples were resuspended in buffer containing 20 μmol/l HEPES, 840 μmol/l NaCl, 1.5 μmol/l $MgCl_2$ and 0.4 μmol/l EDTA and then centrifuged at 15000*g* for 15 min. Protein samples from isolated germ cells were obtained from TRIzol (Invitrogen, Carlsbad, CA, USA), according to methods reported by *Kopec et al. (2017)*. Briefly, the phenolic phase obtained in mRNA extraction was washed with 0.3 mmol/l guanidine hydrochloride in ethanol, centrifuged at 7500*g* for 5 min and resuspended in a buffer containing 100 μmol/l Tris, 140 μmol/l NaCl, 20 μmol/l EDTA and 5% SDS for 2 h at 70°C. Protein concentration was determined by Lowry assay.

Western blot analyses were conducted as previously described (*González et al., 2015*). Protein samples (20–40 μg) were separated by 12.5% SDS-PAGE, and the separated proteins transferred to a PVDF membrane. Blots were incubated with primary anti-HDAC1 (1:1000, 05-100-I Millipore), anti-HDAC2 (1:500, H54 sc-7899, Santa Cruz), anti-H3ac antibody (1:3000, 06-599, Millipore) or H4ac (1:5000, 06-866, Millipore). Immune complexes were detected using anti-mouse and anti-rabbit secondary antibodies and chemiluminescence reagents (Amersham, NJ, USA).

Bands were visualized in a C-DiGit® Chemiluminescent Western Blot Scanner (LI-COR Biosciences). The resulting images were quantified with ImageJ (NIH) software. Then, membranes were stripped and re-probed with monoclonal antibody against α-tubulin (1:3000, Sigma, USA) to confirm equal loading and transfer of samples. Bands were quantified using ImageJ (NIH).

DNA extraction and ELISA-based global 5-methylcytosine (5-mC) determination

Genomic DNA from sperm samples was extracted with the Quick-DNA Kit (Zymo Research, USA) according to the manufacturer's instructions for biological fluids. Briefly, 200 µl of sample was digested with proteinase K (20 mg/ml) in a lysis buffer (included in kit). Samples were incubated at 55°C for 10 min, treated with a digestion buffer and loaded onto spin columns. After washing and elution steps, samples were resuspended in a final elution volume of 50 µl.

Genomic DNA of isolated germ cells was extracted using lysis buffer and incubated overnight with proteinase K, extracted with phenol/chloroform and precipitated overnight with EtOH 100% at –70°C. After suspension in 50 ml Tris-EDTA buffer, DNA was treated with RNAseA. In both cases, purified DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA). Global DNA methylation levels were obtained with an ELISA-based commercial kit (D5325, Zymo Research, USA) according to manufacturer's instructions. For each sample, methylation analysis was performed in duplicate (100 ng DNA each). The samples were incubated with capture and detection antibodies. HRP developer was added and the plate was allowed to develop at room temperature for 1 h. Absorbance was read at 405 nm.

Statistical analysis

Data were analysed with Student's *t*-test and expressed as the mean ± SEM. Data was checked for parametric test assumptions. Differences were considered statistically significant if $P < 0.05$.

RESULTS

Cocaine treatment decreased class I HDAC and increased histone acetylation in the testis

Chronic cocaine treatment was associated with a decrease in testicular *Hdac1*, *Hdac2*, *Hdac3* and *Hdac8* mRNA expression (FIGURE 2A). Among this family, HDAC1 and HDAC2 are part of the same regulatory complex and were found to be involved in proliferation, cell cycle and apoptosis (*Kelly and Cowley, 2013*). Therefore, these HDAC were selected for protein analysis. First, immunohistochemistry analysis of vehicle- and cocaine-treated animals revealed that HDAC1 and HDAC2 were mainly expressed in the nucleus of meiotic germ cells within the seminiferous tubule. HDAC1 was immunolocalized mainly in spermatocytes and persisted in the spermatid stage (FIGURE 2B). Interestingly, HDAC2 expression was strongly observed in primary spermatocytes, becoming weaker in the next cell stages of spermatogenesis (FIGURE 2B). Quantification of protein expression confirmed the mRNA profile, showing decreased expression of HDAC1 and HDAC2 after cocaine treatment (FIGURE 2C). The expression of pan-acetylated histone 3 (H3ac) protein was observed in the nucleus of spermatogonia and from primary spermatocytes to spermatid stage (FIGURE 2B). Moreover, H3ac protein levels increased in the testis of cocaine-treated mice compared with controls (FIGURE 2C). The expression pattern of pan-acetylated histone 4 (H4ac) proteins within the seminiferous tubules varied between vehicle- and cocaine-treated mice. H4ac was immunolocalized mainly in late meiotic stages in vehicle mice while it was detected in primary spermatocytes and in successive stages until round spermatid in cocaine-treated mice (FIGURE 2B). No differences were detected in H4ac protein levels between treatments (FIGURE 2C).

Cocaine treatment altered the expression of epigenetic methylation and demethylation enzymes in the testis

Changes in DNA methylation/demethylation enzymes were observed, specifically a cocaine-induced increase in the mRNA expression of *Dnmt3a* and *Tet2*, while *Tet1* and *Tet3* showed a decreased expression (FIGURE 3). No changes in the expression level of *Dnmt1* and *Dnmt3b* were detected.

Cocaine treatment decreased class I HDAC and increased histone acetylation in isolated germ cells

Chronic cocaine treatment was associated with decreased *Hdac1*, *Hdac2* and *Hdac8* mRNA expression in germ cells (FIGURE 4A). No changes in the expression levels of *Hdac3* were detected (FIGURE 4A). Quantification of protein expression showed decreased expression of HDAC1 and HDAC2 in germ cells after cocaine treatment (FIGURE 4B). Moreover, protein expression of H4ac significantly increased and H3ac showed a trend towards an increase ($P = 0.05$) in germ cells of cocaine-treated mice compared with controls (FIGURE 4B).

Cocaine altered DNA methylation/demethylation enzymes and increased global 5-mC levels in germ cells and sperm DNA

Cocaine treatment was associated with altered DNMT and TET expression by increasing *Dnmt3a* and decreasing *Dnmt3b* and *Tet1* mRNA levels in germ cells (FIGURE 5A). No changes were detected in the expression of *Dnmt1* and *Tet2/3* after cocaine treatment (FIGURE 5A). We evaluated global DNA methylation effects (% 5-mC) in isolated germ cells and cauda epididymal sperm from vehicle- and cocaine-treated mice. FIGURE 5B shows the percentage of 5-mC content, adapted for mouse CpG density/genome length. The 5-mC DNA content was ~50% higher in germ cells and ~35% higher in the sperm of cocaine-treated mice (germ cells: vehicle 13.15 ± 0.99 versus cocaine 20.113 ± 2.28; sperm: vehicle 15.81 ± 1.08 versus cocaine 21.35 ± 1.52).

DISCUSSION

The present study provides evidence to support cocaine having a disruptive effect on the epigenetic homeostasis of the testis, through alterations in the expression of different mediators of acetylation and methylation in mouse germ cells. Evidence is provided that chronic cocaine treatment decreases class I HDAC expression and increases whole testicular H3 and specific germ cell H4 acetylation. A description is also given for the first time that cocaine treatment alters *Dnmt3a/b*, *Tet1* and 5-mC levels in the germ line and increases DNA methylation levels in the sperm.

There is growing evidence indicating that environmental perturbations like chronic stress, dietary change and drug abuse can influence the physiology and behaviour of descendants through epigenetic inheritance (*Wei et al., 2015*).

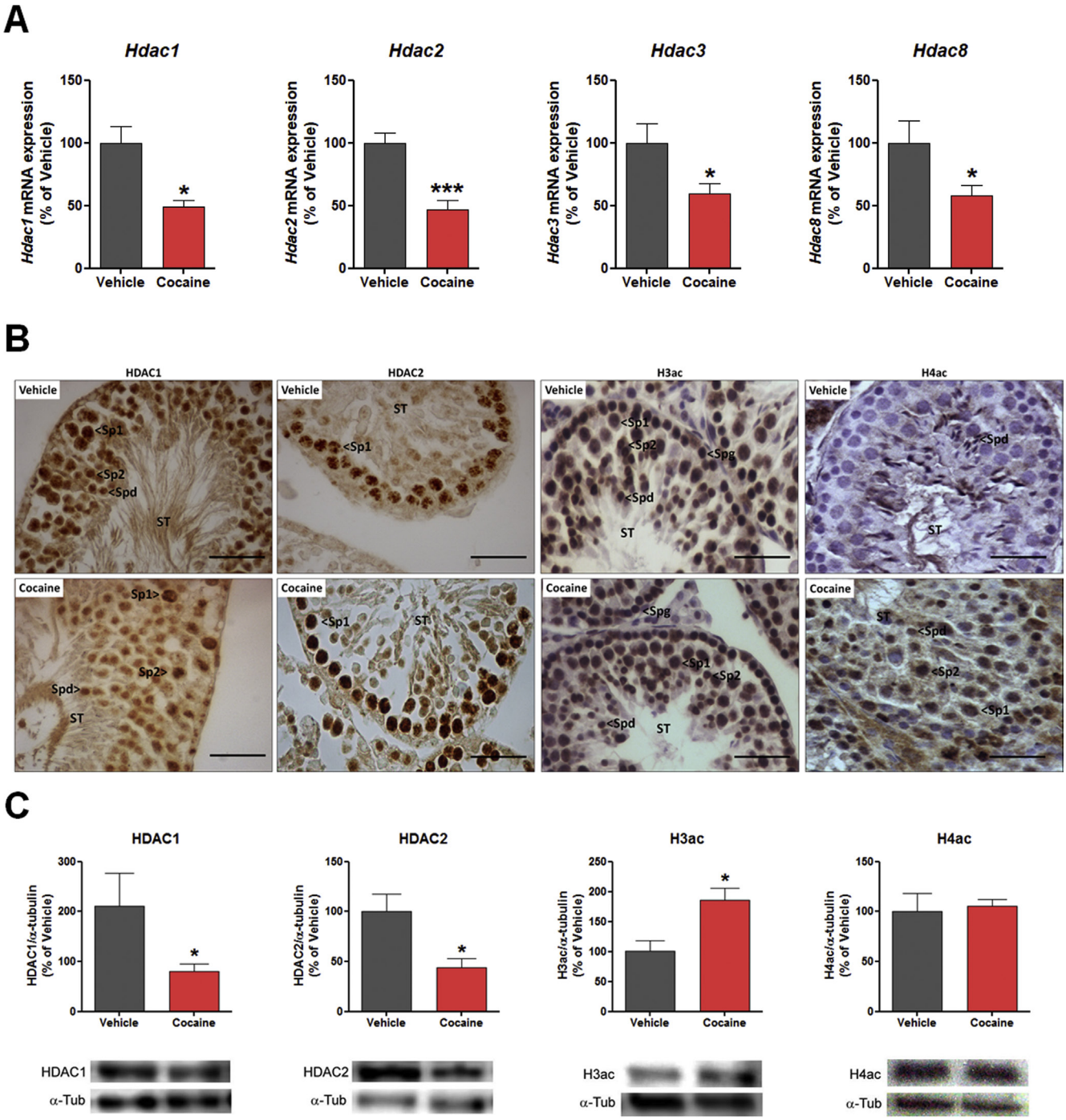

FIGURE 2 Effect of cocaine on acetylation marks and enzyme expression in the mouse testis. (A) Class I *Hdac1/2/3/8* mRNA expression measured by real-time PCR. (B) Testicular immunostaining of HDAC1/2, global pan-acetylated histone 3 (H3ac) and 4 (H4ac) and (C) HDAC1/2, H3ac and H4ac protein levels evaluated by Western blot in the testis of vehicle- and cocaine- treated mice. ST = seminiferous tubule; Spg = spermatogonia; Sp1 = primary spermatocyte; Sp2 = secondary spermatocyte; Spd = spermatid. Scale bar: 50 µm. Values indicate mean ± SEM ($N = 6$). Student's *t*-test, asterisks indicate statistical differences: $^{*}P < 0.05$; $^{***}P < 0.001$.

The mechanisms by which the paternal experience influences offspring are poorly understood, but a reprogramming of testicular germ cells seems to be essential to this process. Accumulating evidence indicates that epigenetic mechanisms, including DNA methylation, histone modification and the actions of small non-coding RNA such as microRNAs (miRNA) and PIWI-interacting RNAs (piRNA), have an important role in paternal germ cells reprogramming (*Wei et al., 2015*). For instance, it has been shown that specific miRNA and methylation patterns were altered in sperm of male rodents exposed to stressful and traumatic environments, or to a high-fat diet, and contribute to the inheritance of behavioural and metabolic changes by their offspring (*Diaz and Ressler, 2014; Fullston et al., 2013; Gapp et al., 2014; Rodgers et al., 2013*). *Vassoler et al. (2013)* found that male rats which self-administered cocaine produced offspring with behavioural alterations

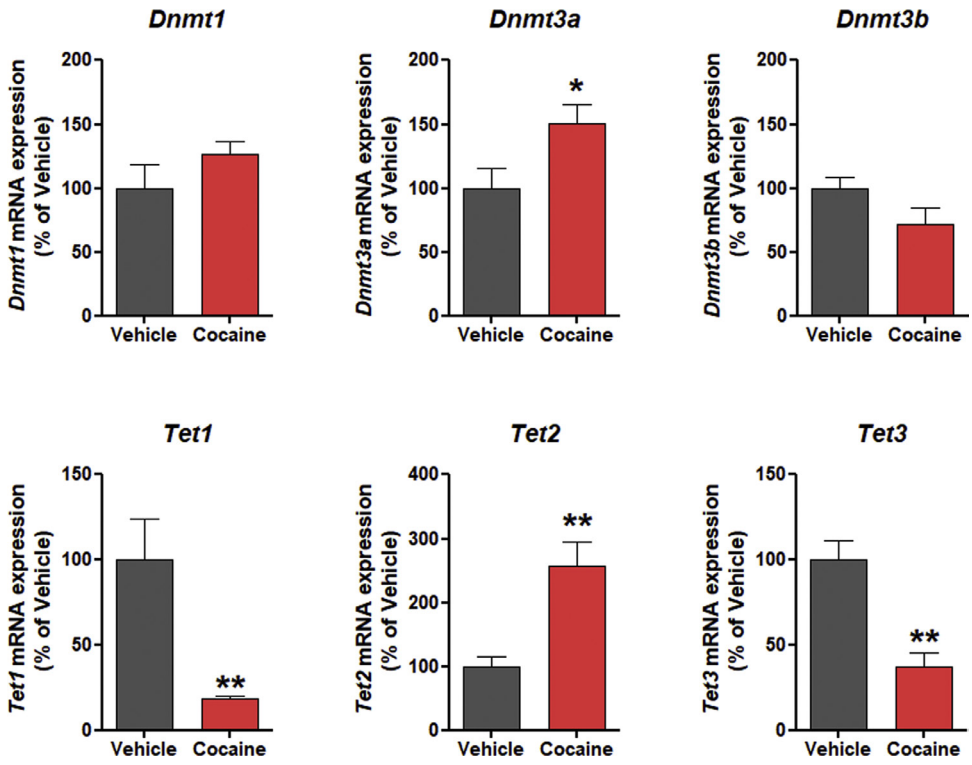

FIGURE 3 Effect of cocaine on methylation/demethylation enzyme expression in the mouse testis. *Tet1/2/3* and *Dnmt1/3a/3b* mRNA expression measured by real-time PCR. Values indicate mean ± SEM ($N = 6$). Student's *t*-test, asterisks indicate statistical differences: $*P < 0.05$; $**P < 0.01$.

transmitted through an increase in H3ac enrichment in germ cells. Although histones are replaced by protamines during sperm maturation, it has been established that a small fraction remains packaged into nucleosomes, which could explain paternal transmission effects through histone acetylation changes in the germline (*Hammoud et al., 2009*). An important open question is whether these epigenetic changes in male germ cells are reversible if the environmental conditions return to normal, and there is some evidence that these reprogramming effects in germ cells are potentially reversible (*Burggren, 2016; Gapp et al., 2016*).

Here, increased H3ac and H4ac protein expression was detected concomitantly with decreased expression of HDAC1/2 proteins and class I HDAC mRNA. Interestingly, it was reported that increased H3ac and H4ac, together with decreased HDAC activity, is part of the toxic effect of methoxiacetic acid, an agent that causes spermatocyte death (*Wade et al., 2008*). In the brain, class I HDAC and increased H3ac and H4ac levels were found to be central in mediating neural plasticity and behavioural responses to cocaine (*Kennedy et al., 2013; Rogge and Wood, 2013*). Moreover, local knockdown of HDAC1 in the nucleus accumbens led to a decrease of both *Hdac1* and *2* mRNA expression (*Kennedy et al., 2013*), and loss of *Hdac1* leads to an overall reduction in total class I HDAC activity in embryonic tissues, when tissue-specific gene expression patterns are being established (*Lagger et al., 2002*). In the testis, similar mechanisms elicited by cocaine could dampen HDAC expression and interfere with testicular acetylation patterns. In this context, histone hyperacetylation secondary to class I HDAC inhibitor trichostatin-A treatment has been shown to interfere with the spermatogenic process and produce infertility (*Fenic et al., 2004*). Interestingly, this study found a dramatic loss of pachytene-diplotene spermatocytes due to increased apoptosis, further supporting the notion that HDAC activity may be particularly important in this stage of meiosis. Therefore, HDAC dysregulation in germ cells may alter the spermatogenic process with a direct impact on male fertility.

It has been shown that cocaine can alter the DNA methylation patterns carried by male gametes and cause paternally inherited teratological effects in offspring (*He et al., 2006*). DNMT are expressed during the spermatogenic process, contributing to the establishment and/or maintenance of methylation patterns in male germ cells (*La Salle and Trasler, 2006*), and their disturbance can lead to hypomethylated spermatogonia, aberrant chromatin packaging and abnormal chromosome structure leading to meiotic arrest (*Webster et al., 2005*). In the mouse, it has been shown that *Dnmt3a* and *Dnmt3b* are expressed in spermatogonia and in meiotic germ cells until round spermatid stage (*La Salle and Trasler, 2006*). Here, it was found that cocaine increased *Dnmt3a*, as was previously reported in cocaine inhalation studies in mice (*He et al., 2006*), together with a decrease in *Dnmt3b*. In addition, decreased *Tet1* expression levels were found as another possible mediator of alterations in DNA methylation patterns. Expression of this enzyme was also found to be decreased after cocaine treatment in the brain (*Feng et al., 2015*). In adult human testis, *Tet1* was observed to be expressed through round spermatid stage and its levels were correlated with sperm concentration and pregnancy outcome (*Ni et al., 2016*). Although the precise nature of the changes that occur at the protein level remain to be fully elucidated, the alterations in *Dnmt3a/b* and *Tet1* gene expression found in this study may serve as markers of pathological mechanisms leading to the increased DNA methylation levels that were found in germ cells and

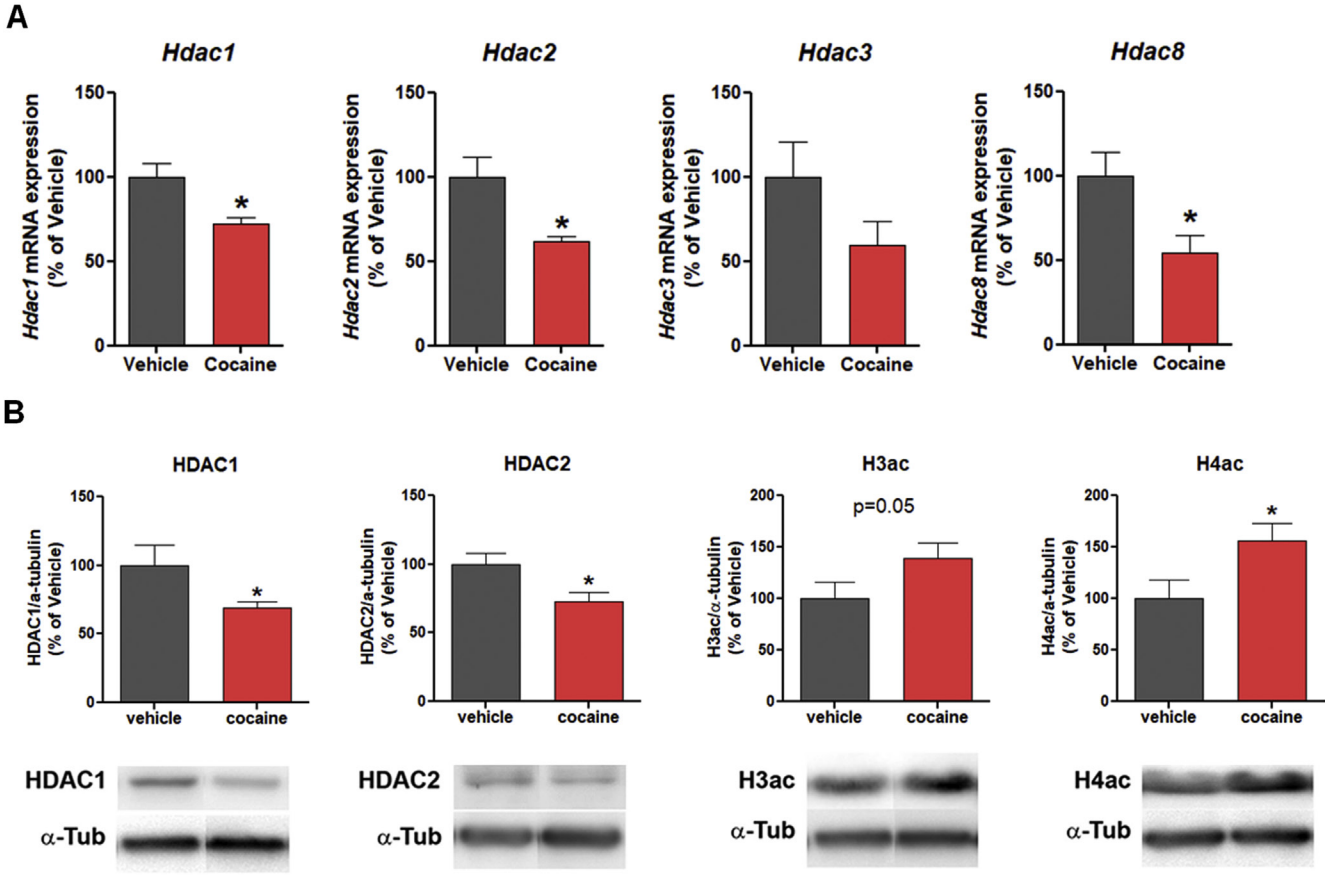

FIGURE 4 Effect of cocaine on acetylation marks and enzyme expression in isolated germ cells. (A) *HDAC1/2/3/8* mRNA expression measured by real-time PCR and (B) HDAC1/2 and global acetylated histone 3 (H3ac) and 4 (H4ac) protein levels evaluated by Western blot in germ cells of vehicle- and cocaine-treated mice. Values indicate mean ± SEM ($N = 7$). Student's *t*-test, asterisks indicate statistical differences: $^*P < 0.05$.

sperm after cocaine treatment. Supporting this, testicular biopsies of infertile patients with impaired spermatogenesis showed increased global DNA methylation that were correlated with *Dnmt1* mRNA levels (*Jaiswal et al., 2015*).

A key question concerns the precise intracellular signalling mechanism through which cocaine and other drugs of abuse induce changes in histone acetylation in the brain and other organs, and accumulating evidence suggests that these changes may be specific to DRD1 activation and involve regulation of growth factor-associated kinases (*Bertran-González et al., 2008; Schroeder et al., 2008*). The exact mechanism through which cocaine affects testicular physiology has not been elucidated, but the dual hydrophilic and lipophilic nature of this molecule may allow it to cross the blood–testis barrier, just as it crosses the blood–brain barrier (*Dietrich, 2009*). Interestingly, *Li et al. (1997)* found a testicular protein that binds cocaine in a saturable and specific manner, but with different sensitivity from the cocaine binding protein in the brain. There are not thought to be any reports showing DA transporter expression in the testis, which is the most studied binding site for cocaine in the brain (*dela Peña et al., 2015*). However, cocaine can also bind to neurotransmitter receptors, plasma proteins, voltage gated ion channels and metabolic enzymes (*Heard et al., 2008*). Therefore, in the testis cocaine may exert its effects through DAT-independent mechanisms that in the brain were also shown to increase DA tone (*O'Leary and Hancox, 2010; Steffensen et al., 2008*). As previously reported (*González et al., 2015*), DRD1 expression was found in the mouse spermatogonia, and testicular DRD1 was down-regulated after cocaine administration, suggesting that DRD1 may have a role in the deleterious action of cocaine on germ cell epigenome. These previous results, together with the data presented here, broaden the basic knowledge of the impact of addictive stimulants on testicular pathophysiology and fertility with implications for male reproductive health.

This is the first study showing that cocaine treatment increases global DNA 5-mC levels in motile sperm, which could have a direct impact on male fertility and next-generation phenotype (Stuppia *et al.*, 2015). In germ cells, epigenetic programmes are particularly dynamic, undergoing erasure, re-establishment and maintenance of patterns (*Meikar et al., 2013*). Parentally imprinted genes play an important role in regulating fetal and postnatal development of the offspring, and the nervous system is particularly vulnerable to alterations in this imprinting (*Davies et al., 2005; Wilkins and Úbeda, 2011*). Although strong barriers prevent the passage of abnormal epigenetic patterns between generations, recent high-resolution profiling studies have shown that not all epigenetic marks are erased during germ cell and embryonic reprogramming (*Ly et al., 2015*). This provides a potential explanation for the intergenerational inheritance of abnormal epigenetic marks that may affect offspring health.

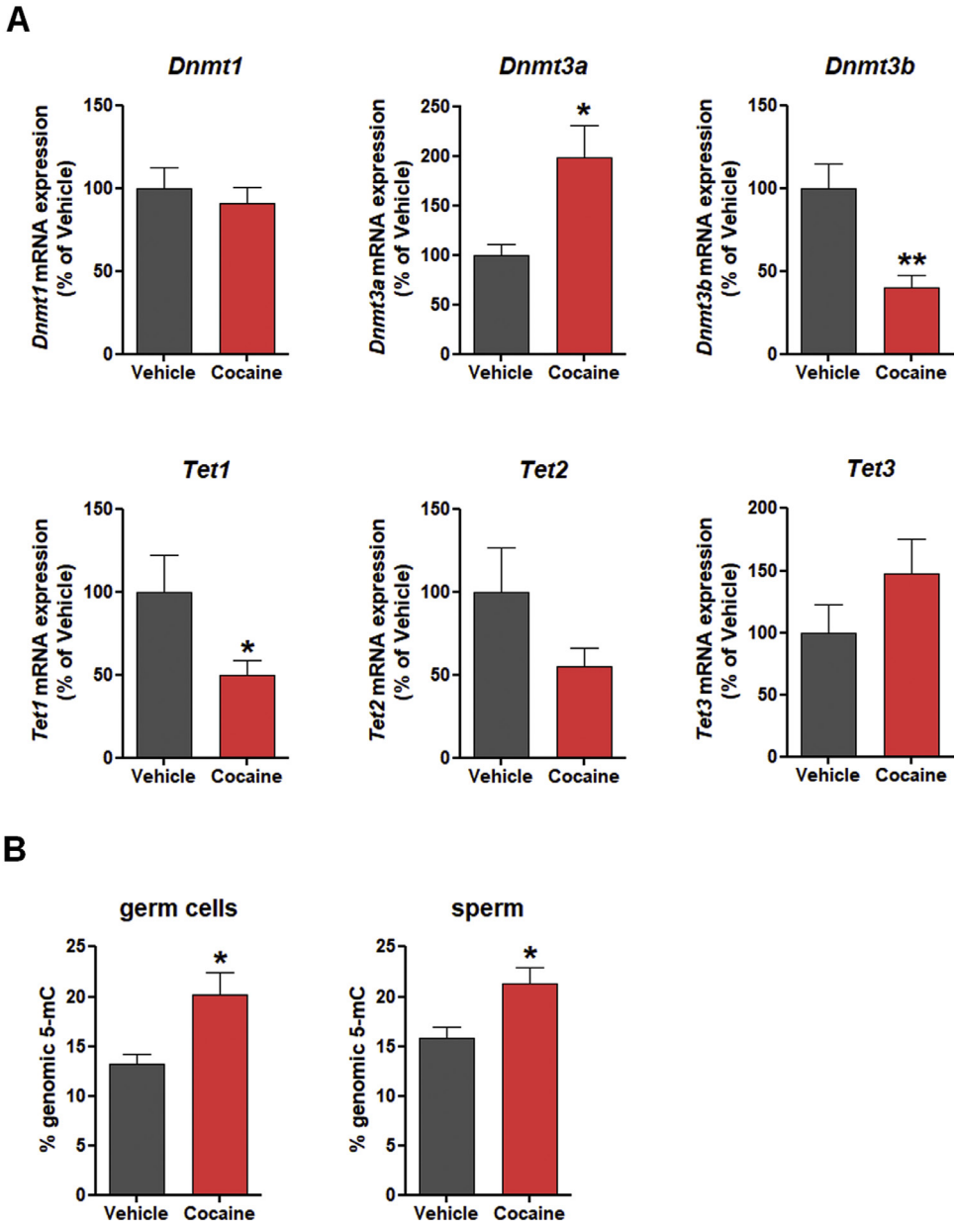

FIGURE 5 Effect of cocaine on methylation/demethylation enzyme expression in germ cells and percentage of 5-methylcytosine (5-mC) content in genomic DNA from isolated germ cells and sperm. (A) *Dnmt1/3a/3b* and *Tet1/2/3* mRNA expression measured by real-time PCR in germ cells and (B) 5-mC content in germ cells and sperm DNA of vehicle- and cocaine-treated mice. Values indicate mean ± SEM ($N = 7$). Student's *t*-test, asterisks indicate statistical differences: $^{*}P < 0.05$, $^{**}P < 0.01$.

The correlation between epigenetics and male reproduction represents a very interesting field of study, mainly due to the possible transgenerational effects related to epigenetic modifications in germ cells. Results presented here propose that cocaine abuse can induce epigenetic changes in testicular cells that could affect the spermatogenic process and even lead to germ cell reprogramming. Because different environmental agents have been shown to induce epigenetic modifications, the control of paternal lifestyle prior to conception could represent an upcoming hot topic in the management of male fertility and reproduction.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2018.05.014.

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