Published in final edited form as:

Eur J Neurosci. 2019 May ; 49(9): 1115–1126. doi:10.1111/ejn.14310.

Impaired cocaine-induced behavioral plasticity in the male offspring of cocaine-experienced sires

Dr. Mathieu E. Wimmer¹, Fair M. Vassoler², Samantha L. White³, Heath D. Schmidt^{3,4}, Simone Sidoli⁵, Yumiao Han⁵, Benjamin A Garica⁵, and R. Christopher Pierce, Ph.D.³

¹Department of Psychology and Neuroscience Program, College of Liberal Arts, Temple University

²Department of Biomedical Sciences, Cummings School of Veterinary Medicine, Tufts University, Peabody Pavilion, 200 Westborough Road Grafton, MA 01536

³Center for Neurobiology and Behavior, Department of Psychiatry, Perelman School of Medicine at the University of Pennsylvania

⁴Department of Biobehavioral Health Sciences, School of Nursing, University of Pennsylvania

⁵Epigenetics Institute, Department of Biochemistry and Biophysics, Perelman School of Medicine at the University of Pennsylvania

Abstract

Our previous work indicated that male, but not female, offspring of cocaine experienced sires display blunted cocaine self-administration. We extended this line of investigation to examine behavioral sensitization, a commonly used model of cocaine-induced behavioral and neuronal plasticity. Results indicated that male, but not female, offspring of cocaine-taking sires showed deficits in the ability of repeated systemic cocaine injections to induce augmented locomotor activity. The reduced cocaine sensitization phenotype in male progeny was associated with changes in histone post-translational modifications, epigenetic processes that regulate gene expression by controlling the accessibility of genes to transcriptional machinery, in the nucleus accumbens of first-generation male progeny. Thus, five histone PTMs were significantly altered in the male progeny of cocaine-exposed sires. Moreover, self-administration of nicotine was unaltered in male and female offspring suggesting that the intergenerational effects of paternal cocaine taking may be drug-specific. Interestingly, the reduced sensitivity to cocaine previously observed in the male offspring of cocaine-taking sires dissipated in the grand-offspring. Both male and female grand-progeny of cocaine-exposed sires showed unaltered cocaine-induced behavioral sensitization and cocaine self-administration. Taken together, these findings indicate that paternal cocaine taking produces changes in multiple cocaine addiction-related behaviors in male progeny,

Corresponding author: Chris Pierce, Ph.D., Center for Neurobiology and Behavior, Perelman School of Medicine at the University of Pennsylvania, 125 South 31st Street, Philadelphia, PA 19104, USA, Telephone: 215-746-8915, rcpierce@mail.med.upenn.edu.

Authors Contributions: MEW, FMV, HDS, BAG and RCP Designed Research; MEW, FMV, SLW, YH Performed Research, MEW, SS and HDS Analyzed Data, MEW and RCP Wrote the paper.

Conflict of Interest: Authors report no conflict of interest.

Data Accessibility Statement: All of this article's supporting data and materials can be readily accessed by contacting the corresponding author.

which do not persist beyond the first generation of offspring. Moreover, the altered sensitivity to cocaine in first generation male progeny of cocaine-sired male offspring was associated with epigenetic modifications in the nucleus accumbens, a nucleus that plays a critical role in cocaine-associated behavioral plasticity.

Graphical Abstract

Male first generation progeny of sires that chronically self-administered cocaine show lower cocaine sensitivity and changes in histone posttranslational modifications in the nucleus accumbens. In contrast, second generation offspring of cocaine-taking sires show unaltered cocaine self-administration and cocaine behavioral sensitization. These data indicate that paternal cocaine exposure confers altered cocaine sensitivity in male offspring but not in subsequent generations.

Keywords

epigenetics; multigenerational; transgenerational; behavioral sensitization; cocaine self-administration

Introduction

A growing body of evidence indicates that ancestral exposure to all of the major classes of abused drugs influences the physiology and behavior of descendants (Vassoler *et al.*, 2013; Szutorisz *et al.*, 2014; Finegersh *et al.*, 2015a; Finegersh *et al.*, 2015b; White *et al.*, 2015; Yohn *et al.*, 2015; Vassoler *et al.*, 2016; Vassoler *et al.*, 2017). Several reports have consistently shown that sire exposure to cocaine influences cocaine-associated behaviors in the next generation. For example, the progeny of sires that self-administered or received non-contingent cocaine displayed reduced cocaine self-administration in male offspring (Vassoler *et al.*, 2013; Le *et al.*, 2017). Blunted cocaine conditioned reward in female offspring of sires that received non-contingent cocaine also has been observed (Fischer *et al.*, 2017). Studies of this sort have not consistently examined the effects of paternal cocaine beyond the first generation of offspring and the mechanisms underlying these cross-generational phenotypes remain an active area of research. The current study examined

addiction-related behaviors in male and female progeny and grand-progeny of cocaine-exposed sires as well as associated neuroepigenetic processes.

The N-terminal tails of histone proteins that package DNA are sites for several post-translational modifications (PTMs), including acetylation, phosphorylation and methylation (Strahl & Allis, 2000; Maze *et al.*, 2013). These histone marks change the conformation of chromatin, which impacts the accessibility of genes by the transcriptional machinery and regulates gene expression. Chromatin remodeling is associated with stable and persistent changes to the transcriptome that may contribute to the development of addiction and other psychiatric diseases (Renthall & Nestler, 2008; Maze *et al.*, 2011). Paternal drug exposure produces broad changes in gene expression in the brain reward pathway of the next generation (Vassoler *et al.*, 2013; Szutorisz *et al.*, 2016; Rompala *et al.*, 2017), which may be driven by remodeling of histone modifications (Vassoler *et al.*, 2013). Therefore, we also examined histone PTMs in the nucleus accumbens, a region important for regulating forms of cocaine-induced behavioral plasticity including cocaine self-administration and behavioral sensitization.

Materials and Methods

Animals and housing:

For the F0 generation, male and female Sprague-Dawley rats (*Rattus norvegicus*) weighing 250–300 g were obtained from Taconic Laboratories (Germantown, NY). Animals were individually housed except during the mating period at which point they were pair housed. Food and water were available ad libitum. A 12/12 hr light/dark cycle was used with the lights on at 7:00 a.m. All experimental procedures were performed during the light cycle. All animal care and experiments were approved by the Institutional Animal Care and Use Committee of the Perelman School of Medicine at the University of Pennsylvania and conducted in accordance with the National Institute of Health guidelines.

Materials:

All self-administration experiments used Med-Associates (East Fairfield, VT) instrumentation enclosed within ventilated, sound attenuating chambers. The apparatus was equipped with response levers, stimulus lights, food pellet dispensers, and injection pumps for injecting drugs intravenously. The sensitization apparatus consisted of plexiglass arenas ((17'' l x 17'' w x 15'' h), San Diego Instruments, San Diego, CA) equipped with infrared beam sensors spaced 1 inch apart.

Surgery:

Prior to surgery, the rats were anesthetized with 80 mg/kg ketamine and 12 mg/kg xylazine. An indwelling silastic catheter was placed into the right jugular vein (side opposite the heart) and sutured in place. The catheter was then threaded subcutaneously over the shoulder blade and was routed to a mesh backmount platform (CamCaths, Cambridge, UK/ Strategic Applications Inc., Libertyville, IL) that was sutured below the skin between the shoulder blades. Animals were allowed to recover for a minimum of 7 days following surgery prior to behavioral testing and drug self-administration. Catheters were flushed daily with 0.3 ml of

an antibiotic (Timentin, 0.93 mg/ml) dissolved in heparinized saline. The catheters were sealed with plastic obturators when not in use.

F0 Cocaine Self-Administration:

For all experiments, cocaine was obtained from the National Institute on Drug Abuse (Rockville, MD) and dissolved in bacteriostatic 0.9% saline. Following a 7-day recovery period from surgery, the male rats were placed in operant chambers and allowed to lever press for intravenous cocaine infusions (0.25 mg cocaine/56 µl saline/infusion over 5 sec). Rats were trained using an FR1 schedule of reinforcement. A 20-second timeout period during which responses had no scheduled consequences followed each cocaine infusion. The rats were limited to a maximum of 75 cocaine infusions per daily 2-hour self-administration session. Self-administration continued for a total of 60 days.

Breeding:

Twenty-four hours after the last day of cocaine self-administration, male rats were placed into a cage with naïve female rats. The animals remained co-housed for 7 days. This breeding resulted in 13 litters from cocaine-experienced sires and 13 litters from saline control sires. 1–3 animals from each litter was used for each behavior or molecular biological assay, such that no litter was over-represented in any of the experiments. A subset of the male F1 saline- and cocaine-sired progeny were mated to drug naïve females to generate the F2 generation of animals.

Behavioral Sensitization:

When they reached 60 days of age, 1–3 male and female offspring from each litter were placed in plexiglass locomotor activity monitoring chambers for 2.5 hours. Following this habituation day, all animals were habituated to the chambers daily for 30 minutes prior to each injection. Following this 30 minute habituation period, male offspring received 15mg/kg cocaine intraperitoneal (i.p.) injections daily for seven days and remained in the activity chambers for 2 hours after each injection. Female offspring received 5mg/kg cocaine injections on days 1 and 7 of the sensitization protocol. Female rats are more sensitive to both acute and chronic behavioral effects of cocaine than are male rats (Sircar & Kim, 1999). The lower dose (5mg/kg) on days 1 and 7 was chosen to limit stereotypies (Walker *et al.*, 2001), which are difficult to detect using a beam-break based activity monitoring system. On days 2 through 6, female offspring received 15mg/kg cocaine injections and remained in the activity monitoring chambers 2 hours after each daily injection.

Nicotine Self-administration:

Following recovery from surgery (1 week), separate drug-naïve offspring were placed in the operant chambers and allowed to lever press for intravenous nicotine (0.03mg/kg nicotine/59µl saline, infused over 5 seconds) on a fixed-ratio 1 schedule of reinforcement. Each nicotine infusion was paired with a tone and light cue. Each infusion was followed by a 20-second timeout, during which the drug was not available and responses were recorded. Responses on the inactive lever had no consequences and were recorded throughout each 2-

hour session. All nicotine self-administration experiments were conducted during the dark phase.

Quantitative high-throughput characterization of histone post translational modifications

Nucleus accumbens punches were quickly dissected from naïve male offspring following decapitation and histones were acid-extracted. Nuclei were isolated by suspending cells into nuclei isolation buffer (15 mM Tris-HCl (pH 7.5), 15 mM NaCl, 60 mM KCl, 5 mM MgCl₂, 1 mM CaCl₂, 250 mM sucrose, 0.2% NP-40) including the following inhibitors: 1 mM DTT, 0.5 mM AEBSF, and 10 mM sodium butyrate. Nuclei were separated by centrifugation (1000 g for 10 min). Two mL of cold 0.4 N H₂SO₄ was added on the nuclei pellet and sample was incubated at 4°C with shaking for 2 h. The nuclei were pelleted at 3400 g for 5 min. The supernatant was collected, trichloroacetic acid (TCA) was added at a final concentration of 33% TCA (w/v), and the sample was incubated overnight at 4°C. Afterwards, precipitated histones were pelleted by centrifugation (3400 g) for 10 min. Pellet was washed from leftover acid using ice cold acetone. Purified histones were processed with two cycles of chemical derivatization (propionic anhydride), trypsin digestion, and desalting. Sample was dissolved in 30 µl of 50 mM NH₄HCO₃, pH 8.0. Derivatization reagent was prepared by mixing propionic anhydride with acetonitrile in the ratio 1:3 (v/v) and added to the histone sample in the ratio of 1:2 (v/v) for 20 min at room temperature. The reaction was performed twice to obtain complete labeling (Lin & Garcia, 2012). Histones were then digested with trypsin with an enzyme:sample ratio of 1:20 (w:w) overnight at room temperature in 50 mM NH₄HCO₃, pH 8.0. After digestion, the derivatization reaction was performed again twice to derivatize peptide N-termini. Samples were analyzed by using a nanoLC-MS/MS setup. nanoLC was configured with a 75 µm ID x 17 cm Reprosil-Pur C18-AQ (3 µm; Dr. Maisch GmbH, Germany) nano-column using an EASY-nLC nanoHPLC (Thermo Scientific, San Jose, CA, USA). The HPLC gradient was 2–35% solvent B (A = 0.1% formic acid; B = 95% MeCN, 0.1% formic acid) over 30 min and from 34% to 100% solvent B in 30 minutes at a flow-rate of 300 nL/min. LC was coupled with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA). Full scan MS spectrum (m/z 290–1400) was performed in the Orbitrap with a resolution of 60,000 (at 400 m/z) with an AGC target of 1×10⁶. The acquisition method contained both data-dependent and targeted scans. The targeted signals were the histone H3 and H4 peptides in isobaric forms, if any. MS/MS was performed with collision induced dissociation (CID) with normalized collision energy of 35, an AGC target of 10⁴ and a maximum injection time of 100 ms. MS/MS data were collected in centroid mode. Precursor ion charge state screening was enabled and all unassigned charge states as well as singly charged species were rejected. The selected modified peptides were quantified using label-free based extracted ion chromatography. EpiProfile was used for the purpose with a peak extraction mass tolerance set to 10 ppm. The peptide relative ratio was calculated by considering the peak area of all peptides that share the same amino acid sequence as total peptide abundance, and estimating the percentage of each individual species by dividing the peak area by the total peptide abundance.

F2 Cocaine Self-Administration:

F2 offspring were trained using an FR1 schedule of reinforcement for 10 consecutive days. Animals were switched to and FR3 reinforcement schedule for 2 days and lastly to a progressive ratio (PR) schedule. Under the PR schedule, the response requirement for each subsequent infusion increased until the animal failed to meet the requirement. The response requirement for i^{th} reinforcement was $R(i)=[5e^{0.2i}-5]$ and the session expired when an animal took longer than 30 minutes to earn a cocaine infusion. The breakpoint was defined as the total number of responses or the final completed ratio.

Statistics:

Behavioral sensitization and drug self-administration data were analyzed using a two-way repeated measures analysis of variance (ANOVA), using “day” as the within subject factor and “sire” as the between subject factor. Post-hoc tests using Bonferroni correction were used when an interaction effect was found to be significant. For measurement of histone PTMs, Tukey Studentized range tests were used to compare the abundance of each modified residue between the two groups.

Results

Unaltered nicotine self-administration in male and female progeny of cocaine-exposed sires.

We previously demonstrated that male, but not female progeny of cocaine-exposed sires show reduced cocaine self-administration due to a reduction in the reinforcing efficacy of cocaine (Vassoler *et al.*, 2013). We also showed that sucrose self-administration was not changed in the male offspring of cocaine-treated sires, suggesting that changes in cocaine self-administration were not caused by impaired operant learning (Vassoler *et al.*, 2013). Here, we tested the reinforcer specificity of these effects by assessing whether paternal cocaine exposure influenced self-administration of nicotine, another stimulant drug of abuse. First generation male and female offspring had daily 2-hour access to nicotine (0.03mg/kg/infusion) on a fixed ratio 1 reinforcement schedule for 10 consecutive days. Each 5 second nicotine infusion was accompanied by light and tone cues. Cocaine-sired male offspring showed similar levels of nicotine self-administration compared to control rats (Figure 1A, Sire: $F(1,32)=0.3519$, $p=0.5572$). Paternal cocaine history did not affect nicotine self-administration in female F1 offspring (Figure 1B; Sire: $F(1,17)=1.948$, $p=0.1807$). Overall, these results indicated that paternal cocaine history did not influence nicotine self-administration in male or female first-generation progeny.

Male and female grand-progeny of cocaine-exposed sires showed unaltered cocaine self-administration.

Cocaine self-administration and the reinforcing efficacy of cocaine were evaluated in male and female grand-offspring of saline- and cocaine-exposed sires. Animals had daily access to cocaine self-administration (1 mg/kg/infusion, 2 hours/day) for 10 consecutive days. Male grand-offspring increased their intake over the course of the 10 days (Figure 2A, Day: $F(9,288)=23.09$, $p<0.0001$), regardless of sire (Interaction Sire x Day: $F(9,288)=0.6313$,

$p=0.7701$). Consistent with these results, when animals were switched to a progressive ratio of reinforcement, F2 animals did not show any difference in breakpoint ($p=0.8366$, Figure 2B). Female grand-progeny of both saline- and cocaine-treated sires increased their cocaine intake over the course of 10 days (Figure 2C, Day: $F(9,153)=29.69$, $p<0.0001$) irrespective of sire treatment group (Interaction Sire x Day: ($F(9,153)=0.7436$, $p=0.6685$). There was no difference in F2 female progeny breakpoint when animals were switched to a progressive ratio reinforcement schedule (Figure 2D, $p=0.6113$). Taken together, these findings demonstrate that the grand-progeny of cocaine-exposed sire show normal levels of cocaine self-administration and the reinforcing efficacy of cocaine is not altered by paternal cocaine history.

Lack of behavioral sensitization to cocaine in male offspring of cocaine-experienced rats.

Next we extended these multigenerational findings to the cocaine behavioral sensitization paradigm, a popular measure of cocaine-induced behavioral plasticity. Daily non-contingent administration of cocaine results in progressive increases in cocaine-induced behavioral hyperactivity. This phenomenon, termed behavioral sensitization, is associated with increases in the reinforcing effectiveness of cocaine (Vezina, 2004). We assessed this form of behavioral plasticity in naïve male cocaine-sired and saline-sired male rats, which received seven consecutive daily injections of 15 mg/kg i.p. cocaine. After 30 minutes of habituation to an open field chamber, cocaine-induced locomotor activity was assessed for 30 minutes following each daily injection. Male saline-sired animals demonstrated an increase in locomotor activity on day 7 relative to day 1 of cocaine administration (Figure 3A, Day x Sire interaction: $F(1,13)=11.96$, $p=0.0042$; Bonferroni posttest $p=0.0072$); in contrast, cocaine-induced behavioral sensitization was not observed in male cocaine-sired animals (Bonferroni post test $p=0.1028$). Female F1 progeny received a 5 mg/kg injection on day 1 and day 7 and 15 mg/kg i.p. cocaine injections on days 2–6 to minimize stereotypies during behavioral testing (Walker *et al.*, 2001). Female offspring of saline- and cocaine-exposed sires showed increases in locomotor activity on day 7 relative to day 1 (Figure 3B; Day: $F(1,18)=14.67$, $p=0.0012$; day x sire interaction $F(1,18)=0.8076$, $p=0.3807$). Thus, male but not female offspring of cocaine-treated sires had deficits in cocaine-induced behavioral plasticity as manifested by blunted behavioral sensitization following repeated non-contingent cocaine injections.

Epigenetic remodeling of histone post-translational modifications in the nucleus accumbens of male progeny of cocaine-experienced sires.

Epigenetic marks such as the chemical changes on histone proteins that package DNA are highly dynamic and play an important role in regulating gene expression and synaptic plasticity (Abel & Zukin, 2008; Maze *et al.*, 2013). Behavioral sensitization to cocaine is mediated, in part, by plasticity in the nucleus accumbens (Pierce & Kalivas, 1997; Vanderschuren & Kalivas, 2000). Here, a global assessment of the relative abundance of histone PTMs was assessed by using mass spectrometry in the nucleus accumbens of drug-naïve F1 progeny. Out of 58 modifications assessed on histone H3 and histone H4, 5 marks were altered in male progeny of cocaine-experienced sires (Table 1). Principal component analysis (PCA) revealed that cocaine sire exposure did not alter the overall composition of histone proteins or their overall pattern of modifications (Figure 4A). The changes elicited

by paternal cocaine taking were specific to particular residues. One modification, H3K14ac, was enriched in cocaine-sired male offspring. The other 4 altered modifications were less abundant in the male progeny of cocaine-exposed sires (H3K18ac, H3K27me2, H3K4me2 and H4K20me2, Figure 4B).

Male and female grand-offspring of cocaine-exposed sires show intact cocaine sensitization.

We assessed whether impairments in cocaine-induced behavioral plasticity in F1 male progeny were transmitted to the next (F2, grand-offspring) generation. Naïve F1 males from each group were bred with drug naïve female rats to produce the cohort of F2 progeny. Cocaine behavioral sensitization was measured in naïve adult male and female grand-offspring of saline- and cocaine-treated sires. Male F2 rats received seven consecutive daily injections of 15 mg/kg i.p. cocaine. After 30 minutes of habituation to an open field chamber, cocaine-induced locomotor activity was assessed for 30 minutes following each daily injection. Male grand-offspring of saline- and cocaine-exposed sires showed an increase in locomotor activity on day 7 relative to day 1 of cocaine administration (Figure 5A, Day: $F(1,27)=22.92$, $p<0.0001$ Day x Sire interaction: $F(1,27)=0.4991$, $p=0.4860$). Female F2 progeny received a 5 mg/kg injection on day 1 and day 7 and 15mg/kg i.p. cocaine injections on days 2–6 as described for the F1 female progeny. Female grand-offspring of saline- and cocaine-treated sires had increases in locomotor activity on day 7 relative to day 1 (Figure 5B; Day: $F(1,8)=20.47$, $p=0.0019$; day x sire interaction $F(1,8)=0.7244$, $p=0.4194$). Thus, male and female grand-offspring of cocaine-exposed sires have normal behavioral plasticity following chronic experimenter-delivered cocaine treatment.

Discussion

The current results demonstrate that male, but not female offspring of cocaine-exposed sires have blunted cocaine-induced behavioral plasticity. These effects appear to be drug-specific in that nicotine self-administration was unaltered by cocaine sire history. Changes in cocaine-induced behavioral plasticity in male progeny were accompanied by remodeling of histone modifications in the nucleus accumbens, a brain region that plays a critical role in both the acquisition of cocaine self-administration as well as cocaine-induced behavioral sensitization. Interestingly, the behavioral phenotype observed in male progeny of cocaine-exposed sires was not found in the next generation. Both male and female grand-offspring of cocaine-taking sires showed unaltered cocaine sensitization and comparable levels of cocaine self-administration as control grand-offspring rats. Taken together, these findings indicate that paternal cocaine taking blunts cocaine-induced behavioral plasticity in male offspring and that this phenotype may be mediated by chromatin remodeling in the nucleus accumbens.

This report adds to a growing body of evidence that paternal cocaine exposure produces altered cocaine reward processing and cocaine addiction-related behavior in first generation offspring. Two recent publications indicate that the male offspring of sires exposed to an extensive cocaine self-administration regimen have altered cocaine self-administration

(Vassoler *et al.*, 2013; Le *et al.*, 2017). The current results are consistent with these studies, showing that male, but not female offspring of cocaine-taking sires have blunted cocaine sensitization. In contrast, a multigenerational mouse model recently demonstrated that female offspring of sires that received experimenter delivered cocaine showed attenuated conditioned place preference for cocaine while both male and female offspring displayed higher sensitivity to acute psychostimulant challenge (Fischer *et al.*, 2017). These observations further underscore the need to include both male and female subjects in investigations of multigenerational effects and underlines the complexity of the mechanisms underlying altered behaviors in adult progeny elicited by paternal environmental insults. The discrepancies in sex-specificity of paternal cocaine-derived phenotypes may be tied to the mode of cocaine delivery in the sires (self-administration vs. experimenter-delivered cocaine) and the species used (mice vs. rats). The idea that methodological considerations could impact behavioral outcomes is not surprising, particularly in light of a recent report indicating that the motivational state of cocaine-taking sires has profound implications for the epigenetic transmission of phenotypic traits to the next generation of animals (Le *et al.*, 2017).

The epigenetic inheritance of cocaine exposure is likely conferred by a combination of complex mechanisms that warrant further study. Evidence that direct interactions between sperm and cocaine are possible (Sweet & Murdock, 1987) suggests that the experimenter-delivered and yoked-cocaine mediated effects in progeny may be caused by direct reprogramming of the germline epigenome, independent of the nervous system. Consistent with this possibility, experimenter-delivered cocaine impacts both progeny behavior (Fischer *et al.*, 2017) as well as sperm morphology and reproductive endocrine levels (Abel *et al.*, 1989). On the other hand, the volitional component of sire cocaine consumption seems to play an important role in influencing behavior and biology in the next generation (Le *et al.*, 2017). These results suggest that epigenetic inheritance of cocaine exposure is mediated by an interaction between cocaine-induced effects on brain physiology and spermatogenesis. Additional research using cocaine analogs that do not cross the blood brain barrier, such as cocaine methiodide (Schindler *et al.*, 1992; Hill *et al.*, 2009) combined with rigorous assessments of the germline epigenome could help unravel which cocaine-induced epigenetic reprogramming events in the germline are functionally important for influencing neurodevelopment of descendants.

Defining the mechanisms by which environmental perturbations alter development via epigenetic reprogramming of the germline has been a highly debated topic. Even within well controlled animal models, establishing causal links between paternal environmental insults and behavioral abnormalities in adult progeny is challenging. This is partly due to the fact that many studies have only examined first generation offspring and phenotypic characterizations have rarely been carried out in subsequent generations. An epigenetic mark must be erased and then re-established in germ cells in the absence or re-exposure to the insult in order to be considered a transgenerational epigenetic mechanism (Dunn *et al.*, 2010). In the case of paternal drug exposure, phenotypes would have to be observed in both the first and second generation offspring for the transmission to be deemed transgenerational. We assessed both cocaine-induced behavioral sensitization in the F1 generation as well as cocaine self-administration in male and female grand-progeny of

cocaine-taking sires. Interestingly, the second generation of offspring produced by cocaine-treated sires showed normal cocaine self-administration and behavioral sensitization, suggesting that any remodeling events in F0 sperm are rectified in the germline of first generation progeny.

A handful of articles have examined addiction-related behaviors following paternal cocaine exposure (Vassoler *et al.*, 2013; Fischer *et al.*, 2017; Le *et al.*, 2017) and only one of them characterized these effects beyond the first generation (Le *et al.*, 2017). Our findings are at odds with this recent report, which demonstrated that some of the effects derived from paternal cocaine exposure are carried in to the second generation progeny. There is a major methodological difference that may account for the divergence in phenotypes between the two studies. Le and colleagues selected sires that showed the highest motivation (top 25 percentile) to earn cocaine infusions as their experimental treatment for sires and compared them to “low” cocaine-taking animals (bottom 40 percentile). Our experiments did not attempt to segregate sires into “high-” or “low-” taking groups as there was little variability in the amount of cocaine infusions earned in the F0 generation in the current study. Taken together, these results suggest that the volitional component of cocaine exposure is important for mediating the reprogramming of the germline and may impact the stability of these effects. Another potential interpretation is that there is a threshold, below which paternal cocaine exposure does not impact subsequent generations. Expression of DNA methyl transferase enzymes, methylation of DNA and histone modifications are altered in sperm of sires following chronic cocaine exposure (He *et al.*, 2006; Vassoler *et al.*, 2013; Le *et al.*, 2017). We postulate that the germline epigenetic signature of cocaine-exposed sires is not maintained in germ cells of F1 male progeny in our experiments. As novel tools to manipulate the epigenome during early developmental stages become widely available, defining epigenetic marks that are functionally relevant to shape the next generation will soon be within reach.

Our results demonstrated that the alterations in cocaine sensitivity in the offspring of cocaine-exposed sires were accompanied by chromatin remodeling in the nucleus accumbens, one of the brain regions critical for reward processing and addiction. We found that the abundance of 5 histone marks were altered by paternal history of cocaine in adult, drug-naïve male progeny. Abundance of dimethylated lysine 4 of histone H3 (H3K4me2) was decreased in male offspring of cocaine-experienced sires compared to controls. This was the largest change in abundance out of all of the histone modifications assessed. Interestingly, H3K4me2 is one of the few epigenetic marks that remains highly enriched in mature sperm at regulatory regions of genes with developmental functions (Hammoud *et al.*, 2009; Brykczynska *et al.*, 2010). Furthermore, these sperm-inherited modified nucleosomes are retained during embryo development (van der Heijden *et al.*, 2005; Torres-Padilla *et al.*, 2006), suggesting that H3K4me2 may be a critical player in the transfer of cocaine-related information from sires to male offspring via sperm. Furthermore, H3K4me2, in the nucleus accumbens has been associated with methamphetamine-induced conditioned reward (Aguilar-Valles *et al.*, 2014). Our findings suggest H3K4me2 in the nucleus accumbens may contribute to altered cocaine reward in first generation male progeny of cocaine-taking sires.

We also found that H3K14ac was enriched in the accumbens of cocaine-sired F1 male progeny. This histone modification is increased in the accumbens of depressed patients and in animals that were chronically stressed by social defeat (Covington *et al.*, 2009). Furthermore, chronic exposure to 9-THC, the psychoactive compound in marijuana, also elicits enrichment of H3K14ac in the nucleus accumbens (Prini *et al.*, 2017). Paternal cocaine reduced accumbal H3K18ac, which is typically associated with active transcriptional start sites (Wang *et al.*, 2008; Ernst *et al.*, 2011) and is also affected by chronic alcohol treatment (Finegersh *et al.*, 2015b). We also found a decrease in the presence of H3K27me2 in F1 progeny following paternal cocaine exposure. Interestingly, reducing H3K27me2 abundance by manipulating the activity of the lysine dimethyl transferase G9a was recently shown to prevent the neurodegeneration elicited by neonatal ethanol exposure in mice (Subbanna *et al.*, 2013). Overall, the epigenetic histone marks identified by our unbiased high-throughput screen of F1 cocaine-sired nucleus accumbens have been associated with models of psychiatric disease and constitute important molecular correlates of cocaine addiction-related behaviors. Our results demonstrate that these five histone modifications are associated with resistance to cocaine addiction-like behavior.

Future studies are required to determine if these five PTMs in the accumbens may serve as potential biomarkers and/or therapeutic targets for cocaine addiction. The accumbens is composed of two key subregions: the core and the shell, which have known distinct projections and functions for addiction-related behaviors (Schmidt *et al.*, 2005; Pierce & Wolf, 2013). It is notable that the changes in histone PTM abundance were detected in combined core and shell lysates, which suggest one of the following possibilities. The identified marks are changed in either the core, the shell or both subregions in our multigenerational model. A major challenge, besides teasing out the nature of the chromatin remodeling events in each subregion, will be to define the loci where the abundance of these marks is altered by paternal cocaine exposure. Future work should also focus on testing the functional relevance of these histone modifications on reward processing and drug-taking behavior.

In summary, our findings have potentially profound implications for the progeny of cocaine-abusing fathers. We employed an animal model of cocaine exposure to systematically study the behavioral and biological consequences of paternal cocaine abuse on their descendants. We have delineated five histone modifications in the nucleus accumbens that may be predictive of cocaine addiction resistance phenotypes, which may lead to the discovery of novel targets for the treatment of cocaine abuse.

Acknowledgements:

The authors thank Rachel Schassburger, Thomas Hopkins, Blake Kimmey and John Maurer for technical assistance, Mark Alter for help with the behavioral sensitization experiment, and Lisa Briand for advice on experimental design. This work was supported by the following grants from the National Institutes of Health: R01 DA033641 (R.C.P.), K02 DA18678 (R.C.P.), R21 DA040837 (R.C.P.), K01 DA039308 (M.E.W.) and R21 DA039393 and R01037897 (H.D.S.), GM110104 (B.A.G.), AI118891 (B.A.G.)

List of abbreviations:

9-THC 9-tetrahydrocannabinol

ANOVA	Analysis of variance
F1	first generation/ offspring
F2	second generation/grand-offspring
F0	parent generation
FR	fixed ratio
HPLC	High pressure liquid chromatography
i.p.	intraperitoneal
kg	kilogram
mg	milligram
MS	Mass spectrometry
PCA	principal component analysis
PR	progressive ratio
PTMs	post-translational modifications

References

- Abel EL, Moore C, Waselewsky D, Zajac C & Russell LD (1989) Effects of cocaine hydrochloride on reproductive function and sexual behavior of male rats and on the behavior of their offspring. *J Androl*, 10, 17–27. [PubMed: 2708121]
- Abel T & Zukin RS (2008) Epigenetic targets of HDAC inhibition in neurodegenerative and psychiatric disorders. *Curr Opin Pharmacol*, 8, 57–64. [PubMed: 18206423]
- Aguilar-Valles A, Vaissiere T, Griggs EM, Mikaelsson MA, Takacs IF, Young EJ, Rumbaugh G & Miller CA (2014) Methamphetamine-associated memory is regulated by a writer and an eraser of permissive histone methylation. *Biol Psychiatry*, 76, 57–65. [PubMed: 24183790]
- Brykczynska U, Hisano M, Erkek S, Ramos L, Oakeley EJ, Roloff TC, Beisel C, Schubeler D, Stadler MB & Peters AH (2010) Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. *Nat Struct Mol Biol*, 17, 679–687. [PubMed: 20473313]
- Covington HE 3rd, Maze I, LaPlant QC, Vialou VF, Ohnishi YN, Berton O, Fass DM, Renthal W, Rush AJ 3rd, Wu EY, Ghose S, Krishnan V, Russo SJ, Tamminga C, Haggarty SJ & Nestler EJ (2009) Antidepressant actions of histone deacetylase inhibitors. *J Neurosci*, 29, 11451–11460. [PubMed: 19759294]
- Dunn GA, Morgan CP & Bale TL (2010) Sex-specificity in transgenerational epigenetic programming. *Horm Behav*
- Ernst J, Kheradpour P, Mikkelsen TS, Shores N, Ward LD, Epstein CB, Zhang X, Wang L, Issner R, Coyne M, Ku M, Durham T, Kellis M & Bernstein BE (2011) Mapping and analysis of chromatin state dynamics in nine human cell types. *Nature*, 473, 43–49. [PubMed: 21441907]
- Finegersh A, Ferguson C, Maxwell S, Mazariegos D, Farrell D & Homanics GE (2015a) Repeated vapor ethanol exposure induces transient histone modifications in the brain that are modified by genotype and brain region. *Front Mol Neurosci*, 8, 39. [PubMed: 26300722]
- Finegersh A, Rompala GR, Martin DI & Homanics GE (2015b) Drinking beyond a lifetime: New and emerging insights into paternal alcohol exposure on subsequent generations. *Alcohol*, 49, 461–470. [PubMed: 25887183]

- Fischer DK, Rice RC, Martinez Rivera A, Donohoe M & Rajadhyaksha AM (2017) Altered reward sensitivity in female offspring of cocaine-exposed fathers. *Behav Brain Res*, 332, 23–31. [PubMed: 28552600]
- Hammoud S, Liu L & Carrell DT (2009) Protamine ratio and the level of histone retention in sperm selected from a density gradient preparation. *Andrologia*, 41, 88–94. [PubMed: 19260844]
- He F, Lidow IA & Lidow MS (2006) Consequences of paternal cocaine exposure in mice. *Neurotoxicol Teratol*, 28, 198–209. [PubMed: 16458479]
- Hill ER, Tian J, Tilley MR, Zhu MX & Gu HH (2009) Potencies of cocaine methiodide on major cocaine targets in mice. *PLoS One*, 4, e7578. [PubMed: 19855831]
- Le Q, Yan B, Yu X, Li Y, Song H, Zhu H, Hou W, Ma D, Wu F, Zhou Y & Ma L (2017) Drug-seeking motivation level in male rats determines offspring susceptibility or resistance to cocaine-seeking behaviour. *Nat Commun*, 8, 15527. [PubMed: 28556835]
- Lin S & Garcia BA (2012) Examining histone posttranslational modification patterns by high-resolution mass spectrometry. *Methods Enzymol*, 512, 3–28. [PubMed: 22910200]
- Maze I, Feng J, Wilkinson MB, Sun H, Shen L & Nestler EJ (2011) Cocaine dynamically regulates heterochromatin and repetitive element unsilencing in nucleus accumbens. *Proc Natl Acad Sci U S A*, 108, 3035–3040. [PubMed: 21300862]
- Maze I, Noh KM & Allis CD (2013) Histone regulation in the CNS: basic principles of epigenetic plasticity. *Neuropsychopharmacology*, 38, 3–22. [PubMed: 22828751]
- Pierce RC & Kalivas PW (1997) A circuitry model of the expression of behavioral sensitization to amphetamine-like psychostimulants. *Brain Res Brain Res Rev*, 25, 192–216. [PubMed: 9403138]
- Prini P, Penna F, Sciuccati E, Alberio T & Rubino T (2017) Chronic Delta(8)-THC Exposure Differently Affects Histone Modifications in the Adolescent and Adult Rat Brain. *Int J Mol Sci*, 18.
- Renthal W & Nestler EJ (2008) Epigenetic mechanisms in drug addiction. *Trends Mol Med*, 14, 341–350. [PubMed: 18635399]
- Rompala GR, Finegersh A, Slater M & Homanics GE (2017) Paternal preconception alcohol exposure imparts intergenerational alcohol-related behaviors to male offspring on a pure C57BL/6J background. *Alcohol*, 60, 169–177. [PubMed: 27876231]
- Schindler CW, Tella SR, Katz JL & Goldberg SR (1992) Effects of cocaine and its quaternary derivative cocaine methiodide on cardiovascular function in squirrel monkeys. *Eur J Pharmacol*, 213, 99–105. [PubMed: 1499661]
- Sircar R & Kim D (1999) Female gonadal hormones differentially modulate cocaine-induced behavioral sensitization in Fischer, Lewis, and Sprague-Dawley rats. *J Pharmacol Exp Ther*, 289, 54–65. [PubMed: 10086987]
- Strahl BD & Allis CD (2000) The language of covalent histone modifications. *Nature*, 403, 41–45. [PubMed: 10638745]
- Subbanna S, Shivakumar M, Umapathy NS, Saito M, Mohan PS, Kumar A, Nixon RA, Verin AD, Psychoyos D & Basavarajappa BS (2013) G9a-mediated histone methylation regulates ethanol-induced neurodegeneration in the neonatal mouse brain. *Neurobiol Dis*, 54, 475–485. [PubMed: 23396011]
- Sweet F & Murdock GL (1987) Affinity labeling of hormone-specific proteins. *Endocr Rev*, 8, 154–184. [PubMed: 3038507]
- Szutorisz H, DiNieri JA, Sweet E, Egervari G, Michaelides M, Carter JM, Ren Y, Miller ML, Blitzer RD & Hurd YL (2014) Parental THC exposure leads to compulsive heroin-seeking and altered striatal synaptic plasticity in the subsequent generation. *Neuropsychopharmacology*, 39, 1315–1323. [PubMed: 24385132]
- Szutorisz H, Egervari G, Sperry J, Carter JM & Hurd YL (2016) Cross-generational THC exposure alters the developmental sensitivity of ventral and dorsal striatal gene expression in male and female offspring. *Neurotoxicol Teratol*, 58, 107–114. [PubMed: 27221226]
- Torres-Padilla ME, Bannister AJ, Hurd PJ, Kouzarides T & Zernicka-Goetz M (2006) Dynamic distribution of the replacement histone variant H3.3 in the mouse oocyte and preimplantation embryos. *Int J Dev Biol*, 50, 455–461. [PubMed: 16586346]

- van der Heijden GW, Dieker JW, Derijck AA, Muller S, Berden JH, Braat DD, van der Vlag J & de Boer P (2005) Asymmetry in histone H3 variants and lysine methylation between paternal and maternal chromatin of the early mouse zygote. *Mech Dev*, 122, 1008–1022. [PubMed: 15922569]
- Vanderschuren LJ & Kalivas PW (2000) Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. *Psychopharmacology (Berl)*, 151, 99–120. [PubMed: 10972458]
- Vassoler FM, Oliver DJ, Wyse C, Blau A, Shtutman M, Turner JR & Byrnes EM (2017) Transgenerational attenuation of opioid self-administration as a consequence of adolescent morphine exposure. *Neuropharmacology*, 113, 271–280. [PubMed: 27729240]
- Vassoler FM, White SL, Schmidt HD, Sadri-Vakili G & Pierce RC (2013) Epigenetic inheritance of a cocaine-resistance phenotype. *Nature Neuroscience*, 16, 42–47. [PubMed: 23242310]
- Vassoler FM, Wright SJ & Byrnes EM (2016) Exposure to opiates in female adolescents alters mu opiate receptor expression and increases the rewarding effects of morphine in future offspring. *Neuropharmacology*, 103, 112–121. [PubMed: 26700246]
- Vezina P (2004) Sensitization of midbrain dopamine neuron reactivity and the self-administration of psychomotor stimulant drugs. *Neurosci Biobehav Rev*, 27, 827–839. [PubMed: 15019432]
- Walker QD, Cabassa J, Kaplan KA, Li ST, Haroon J, Spohr HA & Kuhn CM (2001) Sex differences in cocaine-stimulated motor behavior: disparate effects of gonadectomy. *Neuropsychopharmacology*, 25, 118–130. [PubMed: 11377925]
- Wang Z, Zang C, Rosenfeld JA, Schones DE, Barski A, Cuddapah S, Cui K, Roh TY, Peng W, Zhang MQ & Zhao K (2008) Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet*, 40, 897–903. [PubMed: 18552846]
- White SL, Vassoler FM, Schmidt HD, Pierce RC & Wimmer ME (2015) Enhanced anxiety in the male offspring of sires that self-administered cocaine. *Addict Biol*
- Yohn NL, Bartolomei MS & Blendy JA (2015) Multigenerational and transgenerational inheritance of drug exposure: The effects of alcohol, opiates, cocaine, marijuana, and nicotine. *Prog Biophys Mol Biol*, 118, 21–33. [PubMed: 25839742]

Figure 1. Male progeny of cocaine-experienced sires show unaltered nicotine self-administration
A. Progeny of saline- and cocaine-exposed sires were given daily 2-hour access to nicotine (0.03mg/kg/infusion) for 10 consecutive days under an FR1 reinforcement schedule. Acquisition and maintenance of nicotine self-administration were unchanged by paternal cocaine taking and the total number of infusions earned over the 10 days of nicotine self-administration was similar in saline- compared to cocaine-sired male progeny ($p=0.5572$; $n=12$ per group). **B.** Female offspring of cocaine-experienced sires show similar nicotine self-administration compared to saline-sired control progeny. The total number of nicotine

infusions earned over 10 days is comparable between the two groups of F1 offspring ($p=0.1807$; $n=9-10$ per group).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2. Grand-progeny of cocaine-exposed sires have normal cocaine self-administration.

A. There was no difference in cocaine self-administration (1mg/kg/inf) between saline- and cocaine-sired grand-progeny ($p=0.7701$; $n=17$ per group). **B.** When cocaine was self-administered under a progressive ratio reinforcement schedule, saline- and cocaine-sired grand-progeny showed similar breakpoints ($p=0.8366$; $n=17$ per group). **C.** Female grand-progeny of saline- and cocaine-experienced sires have similar levels of cocaine self-administration under a fixed ratio schedule ($p=0.6685$) and have comparable breakpoints (**D**) on a progressive ratio reinforcement schedule ($p=0.6113$; $n=8-11$ per group). Means $\pm$ s.e.m. are shown in every panel.

Figure 3. Male offspring of cocaine-experienced sires have blunted cocaine-induced locomotor sensitization.

A. Total locomotor activity counts post cocaine (15mg/kg) injection on the first (day 1) and last day (day 7) of the cocaine sensitization protocol for male offspring. Male saline-sired progeny demonstrate significant cocaine-induced locomotor sensitization ($p=0.0072$). In sharp contrast, cocaine-sired progeny show similar ambulation following cocaine injections on day 1 compared to day 7 ($p=0.1028$; $n=7-8$). **B.** Total locomotor activity counts post cocaine (5mg/kg) injection on day 1 and day 7 of the cocaine sensitization protocol for female offspring. Both saline- and cocaine-sired female progeny show cocaine-induced locomotor sensitization ($p=0.0012$; $n=10$). Data are expressed as the mean ($\pm$ SEM), * $p<0.05$ compared to day 1 (Bonferroni correction).

Figure 4. Paternal cocaine taking elicits epigenetic remodeling of histone PTMs in the nucleus accumbens of male progeny.

A. A principal component analysis representing the overall distance between samples for the relative abundances of all histone peptides. There are no global differences between conditions, suggesting that any differences between the two groups of F1 offspring would be limited to small individual changes in the pattern of histone modifications. **B.** Volcano plot representing the distribution of the histone peptides in relation to their difference between condition (ratio, x-axis) and the reproducibility of such observation (t-test p-value, y-axis). Statistically different peptides (p-value < 0.05, two tails t-test) are above the dashed line and shown in black (n=6).

Figure 5. Grand-offspring of cocaine-exposed sires show intact cocaine-induced locomotor sensitization

A. Total locomotor activity counts post cocaine injection on the first (day 1) and last day (day 7) of the cocaine sensitization protocol for male grand-offspring. Male saline- and cocaine-sired grand-progeny demonstrate significant cocaine-induced locomotor sensitization ($p < 0.0001$; $n = 13-15$ per group). **B.** Total locomotor activity counts post cocaine injection on day 1 and day 7 of the cocaine sensitization protocol for female grand-offspring. Both saline- and cocaine-sired female grand-progeny show cocaine-induced locomotor sensitization. ($p = 0.0019$; $n = 5$ per group). Data are expressed as the mean ($\pm$ SEM), * $p < 0.05$ compared to day 1 (Bonferroni post-hoc tests).

Table 1.
Histone modifications in NAC.

The abundance of each post-translational modification was measured for the following peptides. The unmodified portion is reported in the first row for each peptide.

Peptide	Modification	Saline-sired abundance (%)	Cocaine-sired abundance (%)	p-value
TKQTAR(H3 3–8)	unmodified	81.9%	79.5%	0.1631
	H3K4me1	18.0%	20.5%	0.1634
	H3K4me2	4 × 10^{−3} %	2 × 10^{−3} %	0.0411
	H3K4me3	3.4 × 10 ^{−3} %	6.0 × 10 ^{−3} %	0.6584
	H3K4ac	7.7 × 10 ^{−3} %	16.0 × 10 ^{−3} %	0.3606
KSTGGKAPR(H3 9–17)	unmodified	11.5%	11.0%	0.7115
	H3K9me1	9.6%	8.9%	0.6507
	H3K9me2	21.9%	19.5%	0.0618
	H3K9me3	7.9%	7.3%	0.3228
	H3K9ac	0.2%	0.1%	0.4258
	H3K14ac	8.8%	9.7%	0.0087
	H3K9me1K14ac	10.5%	12.2%	0.1119
	H3K9me2K14ac	22.2%	23.4%	0.6873
	H3K9me3K14ac	6.4%	6.6%	0.8101
	H3K9acK14ac	1.1%	1.3%	0.1476
KQLATKAAR(H3 18–26)	unmodified	61.4%	58.6%	0.4457
	H3K23me1	0.1%	0.2%	0.4392
	H3K18me1	0.4%	0.3%	0.7024
	H3K18ac	2.7%	2.4%	0.0123
	H3K23ac	31.7%	34.5%	0.3575
	H3K18acK23ac	3.6%	4.0%	0.6212
KSAPATGGVKKPHR(H3 27–40)	unmodified	2.0%	3.1%	0.4078
	H3K36me1	0.6%	0.4%	0.6791
	H3K27me1	1.8%	1.3%	0.6607
	H3K27me2	12.7%	10.4%	0.0283
	H3K36me2	2.1%	1.9%	0.6029
	H3K27me3	4.2%	3.7%	0.1217
	H3K27ac	0.1%	0.1%	0.7569
	H3K27me1K36me1	3.3%	3.8%	0.6791
	H3K27me2K36me1	33.2%	32.2%	0.5956
	H3K27me1K36me2	10.6%	10.9%	0.6919
	H3K27me2K36me2	11.1%	11.9%	0.6378
	H3K27me3K36me1	13.5%	13.2%	0.6746
	H3K27me1K36me3	1.6%	2.6%	0.0547
	H3K27me3K36me2	3.2%	4.5%	0.2930

Peptide	Modification	Saline-sired abundance (%)	Cocaine-sired abundance (%)	p-value
YQKSTELLIR(H3 54–63)	unmodified	86.3%	95.0%	0.2599
	H3K56ac	13.7%	5.0%	0.2599
EIAQDFKTDLR(H3 73–83)	unmodified	48.8%	45.4%	0.6006
	H3K79me1	19.3%	16.7%	0.5596
	H3K79me2	31.6%	37.5%	0.3295
	H3K79ac	0.3%	0.2%	0.3619
GKGGKGLGKGGAKR(H4 4–17)	unmodified	55.1%	52.4%	0.3275
	H3K5ac	0.5%	0.7%	0.2600
	H3K8ac	0.5%	0.4%	0.2973
	H3K12ac	0.2%	0.3%	0.4932
	H3K16ac	32.1%	33.0%	0.3329
	H3K5acK16ac	0.5%	0.8%	0.0551
	H3K8acK16ac	1.8%	2.5%	0.1916
	H3K12acK16ac	5.8%	5.8%	0.9740
	H3K5acK8acK12ac	1.5%	1.7%	0.4927
	H3K5acK8acK16ac	$4.8 \times 10^{-3} \%$	$3.1 \times 10^{-3} \%$	0.5512
	H3K5acK12acK16ac	$5.3 \times 10^{-3} \%$	$2.7 \times 10^{-3} \%$	0.4616
	H3K8acK12acK16ac	1.0%	1.3%	0.1971
	H3K5acK8acK12acK16ac	0.8%	1.1%	0.3135
KVLIR(H4 20–23)	unmodified	0.5%	3.9%	0.2999
	H3K20me1	14.3%	17.2%	0.0961
	H3K20me2	72.4%	65.6%	0.0442
	H3K20me3	12.8%	13.3%	0.6525