Effect of chronic heroin and cocaine administration on global DNA methylation in brain and liver.

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¹Abbreviations: CpG, cytosine-phosphate-guanine; DNMT, DNA methyltransferase; DDM, DNA demethylase; OPRM1, Opioid receptor mu 1; SP1, Specificity protein 1; AP1, Activator protein 1; PKCε, Protein kinase C epsilon; CDKL5, Cyclin-dependent kinase-like 5; MOPr, mu opioid peptide receptor; 5-MedC, 5-methyl-2'-deoxycytidine; dG, 2'-deoxyguanosine; G, guanosine; dA, 2'-deoxyadenosine; T, thymidine; dC, 2'-deoxycytidine; OD, optical density; NAc, nucleus accumbens; PFC, prefrontal cortex; PP1c, protein phosphatase-1 catalytic subunit; fosB, FBJ murine osteosarcoma viral oncogene homolog B; LC-MS, liquid chromatography-mass spectrometry; HPLC-UV, high performance liquid chromatography-ultra violet.

Abstract

Drug abuse is associated with epigenetic changes, such as histone modifications and DNA methylation. The purpose of the present study was to examine the effect of chronic cocaine and heroin administration on global DNA methylation in brain and liver. Male, 8 week old, C57BL/6J mice received heroin in a chronic 'intermittent' escalating dose paradigm, or cocaine in a chronic escalating dose 'binge' paradigm, which mimic the human pattern of opioid or cocaine abuse respectively. Following sacrifice, livers and brains were removed and DNA was extracted from them. The extracted DNA was hydrolyzed and 2'-deoxycytidine and 5-methyl-2'-deoxycytidine were determined by HPLC-UV. The % 5-methyl-2'-deoxycytidine content of DNA was significantly higher in the brain compared to the liver.

There were no differences between the control animals and the cocaine or heroin treated animals in neither of the tissues examined, which is surprising since cocaine administration induced gross morphological changes in the liver. Moreover, there was no difference in the % 5-methyl-2'-deoxycytidine content of DNA between the cocaine and the heroin treated animals. The global DNA methylation status in the brain and liver of mice chronically treated with cocaine or heroin remains unaffected, but this finding cannot exclude the existence of anatomical region or gene-specific methylation differences. This is the first time that global DNA methylation in the liver and whole brain has been studied following chronic cocaine or heroin treatment.

Keywords: DNA methylation; global; liver; brain; cocaine; heroin

1. Introduction

The field of epigenetics has grown fast during the last decade. There is now substantial evidence that supports the involvement of epigenetic mechanisms in gene expression alterations related to psychiatric disorders, including drug addiction (Haycock, 2009; Kalsi et al., 2009; Kovatsi et al., 2011; Lutz, 2008; Malvaez et al., 2009; McQuown and Wood, 2010; Nestler, 2008; Renthall and Nestler, 2008, 2009; Tsankova et al., 2007). Drug abuse, as an environmental stimulus, can trigger epigenetic changes which result in altered gene expression. These changes are heritable and target the epigenome, while the DNA sequence remains unaffected (Kovatsi et al., 2011). Epigenetic changes include histone modifications (histone methylation, acetylation and phosphorylation) and DNA methylation. DNA methylation occurs at the carbon 5 position of the cytosine residue, preferably in the so-called CpG islands, which are regions rich in CpG dinucleotides within the DNA molecule. The enzymes responsible for the addition of the methyl group are called DNA methyltransferases (DNMT1, 2, 3a and 3b) (Kovatsi et al., 2011). For the reverse process of demethylation, the formation of 5-hydroxymethylcytosine from 5-methylcytosine is required, which is carried out by the ten-eleven translocation proteins (Guo et al., 2011, Dahl et al., 2011). Deaminases convert 5-hydroxymethylcytosine to 5-hydroxymethyluracil which is then repaired to cytosine by the base excision repair pathway (Guo et al., 2011). Methylation of the DNA has been associated with gene silencing, whereas active transcription occurs as a result of demethylation (Franco et al., 2008; Kovatsi et al., 2011; Tang and Ho, 2007).

Drug abuse and addiction have been associated with epigenetic alterations, and specifically changes in DNA methylation. For instance, increased DNA methylation was observed in the OPRM1 gene in lymphocytes of former heroin users currently on methadone maintenance (Nielsen et al., 2009). Maternal exposure to cocaine was found to cause methylation of the AP-1 and SP1 binding sites and hyper-methylation of the promoter region of PKC ϵ in the fetal rat heart (Meyer et al., 2009; Zhang et al., 2007, 2009), as well as changes in the global methylation status of hippocampal pyramidal neurons (Novikova et al., 2008). Direct exposure to cocaine was found to cause hyper-methylation of the CDKL5 gene in the striatum (Carouge et al., 2010).

Drug abuse has been associated with alterations in gene expression in the brain (Yoo et al., 2012). For instance, it was shown that cocaine administration elevates MOPr mRNA levels in various brain regions (Yuferov et al., 1999; Yoo et al., 2012). Although it is not yet clear to what extent these alterations in gene regulation are controlled by epigenetic

mechanisms, the fact that these mechanisms are reversible could possibly lead to a powerful therapeutic tool (Fragou et al., 2011; Kouidou et al., 2010; Kovatsi et al., 2011).

We have currently studied the effect of chronic heroin or cocaine administration on global DNA methylation in liver and brain. We used chronic “intermittent” escalating dose heroin and chronic “binge” escalating dose cocaine administration paradigms in mice, which are common *in vivo* models used to mimic the human pattern of either opioid or cocaine use and abuse (Kreek et al., 2002; Muller and Unterwald, 2004; Spanagel, 1995).

This is the first study to investigate global DNA methylation in this model. The animals were culled, the tissues of interest were removed, the DNA was extracted from the tissues and global DNA methylation, in terms of % 5-methyl-2'-deoxycytidine content, was determined by an HPLC-UV method previously published (Kovatsi et al., 2012).

2. Materials and methods

2.1 Animals

Eight-week old, male C57BL/6J mice (B & K Universal, Hull, UK), weighing 20-25 g, were individually housed in a temperature- and humidity-controlled room with a 12 h light /dark schedule for 1 week before experiments commenced. Food and water were available *ad libitum*. All animals were weighed daily throughout the study. All studies were performed in accordance with protocols approved by the Home Office (Animals Act 1986) UK and the European Community Council Directive of the 24th Nov 1986 (86/609/EEC).

2.2 Chronic heroin administration

Following acclimatization, intraperitoneal (i.p.) injections of either saline (10 mL/kg) or heroin were administered to the mice in a chronic ‘intermittent’ escalating dose paradigm to mimic a common pattern of self-administration in human heroin abusers (Kreek et al., 2002). Animals were randomized into two groups (n=9). Each group received either saline or heroin injections for 7 days. Two intraperitoneal injections of saline or heroin were administered daily (17.00 and 09.00 h), in accordance with a protocol that has been used as an animal model to investigate heroin- and morphine-addictive processes (Bailey et al., 2010; Muller and Unterwald, 2004; Spanagel, 1995). The heroin-treated animals received 2 x 1 mg/kg/injection on day 1, 2 x 2 mg/kg/injection on days 2 and 3, 2 x 4 mg/kg/injection on days 4 and 5, and 2 x 8 mg/kg/injection on days 6 and 7. One hour after the last treatment injection,

animals were killed by decapitation after a 30 s exposure to CO₂. Brains and livers were rapidly removed, frozen by immersion in liquid nitrogen and stored at -80 °C.

2.3 Chronic cocaine administration

Following acclimatization, intraperitoneal (i.p.) injections of either saline (4 mL/kg) or cocaine were administered to the mice in an escalating dose 'binge' paradigm as described by Bailey et al. (Bailey et al., 2005a, 2005b, 2007, 2008; Schlussman et al., 2005), to mimic a common pattern of self administration in human cocaine abusers (Tsukada et al., 1996). Animals were randomized into two groups (n=9). Each group received either saline or cocaine injections for 14 days. Three intraperitoneal injections of saline or cocaine (1 h apart) were administered daily, with the first injection 60 min after the start of the light cycle. The cocaine-treated animals received 3 x 15 mg/kg/day on days 1-4, 3 x 20 mg/kg/day on days 5-8, 3 x 25 mg/kg/day on days 9-12, and 3 x 30 mg/kg/day on days 13 and 14. One hour after the last injection, animals were killed by decapitation after a 30 s exposure to CO₂. Brains and livers were rapidly removed, frozen by immersion in liquid nitrogen and stored at -80 °C.

2.4 Chemicals and reagents

Cocaine HCl and heroin were purchased from Sigma-Aldrich (Poole, UK). 5-methyl-2'-deoxycytidine (5-MedC) was purchased from US Biological (Massachusetts, U.S.A.). Guanosine (G), 2'-deoxyguanosine (dG), thymidine (T), 2'-deoxycytidine (dC) and 2'-deoxyadenosine (dA) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Sodium acetate and ammonium formate were supplied by Fluka (Taufkirchen, Germany). Calcium chloride, sodium succinate, micrococcal nuclease, calf thymus DNA, nuclease P1 and zinc chloride were purchased from Sigma-Aldrich (Taufkirchen, Germany). Calf spleen phosphodiesterase was supplied by Calbiochem-Merck (Darmstadt, Germany) and Slide-A-Lyser cassette by Pierce-Thermo Scientific (Illinois, U.S.A.). Proteinase K and RNase T1 were obtained from Roche (Basel, Switzerland) and RNase A from Novagen-Merck (Darmstadt, Germany). The Genomic-tip and the Genomic DNA buffer set were manufactured by Qiagen (Düsseldorf, Germany). HPLC grade water, ethanol, methanol and hydrochloric acid of analytical grade were obtained from Panreac (Barcelona, Spain). Isopropanol was purchased from Riedel-de-Haën, Sigma-Aldrich (Taufkirchen, Germany).

2.5 Preparation of standards

5-MedC and dC stock standard solutions were prepared by diluting the appropriate amount of purchased powder in HPLC grade water. The absorbance of the stock solutions was measured at 277 nm for 5-MedC and at 271 nm for dC. The measured absorbances were used to calculate the precise concentration of the solutions using the molar absorption coefficient, ϵ ($\epsilon = 8500 \text{ M}^{-1} \text{ cm}^{-1}$ for 5-MedC and $\epsilon = 9000 \text{ M}^{-1} \text{ cm}^{-1}$ for dC) (Dawson et al., 1986). The stock standard solutions were diluted with HPLC grade water to prepare working standards with the following concentrations: 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 mM for 5-MedC and 0.05, 0.1, 0.25, 0.5 and 1 mM for dC. All standard solutions were stored at -20°C and were stable for up to 1 month.

The appropriate amount of purchased calf thymus DNA was diluted in HPLC grade water to prepare a calf thymus DNA solution. The precise concentration of the solution was calculated by measuring the absorbance of the solution at 260 nm and assuming that 1 OD=50 $\mu\text{g/mL}$ double stranded (ds) DNA. Aliquots of 50 μg ds DNA were prepared and evaporated to dryness in a speedvac. Aliquots were stored at -20°C .

2.6 DNA Extraction

Extraction of the DNA was carried out using the Qiagen DNA Buffer set and the Qiagen genomic tip. All tissues were weighted (100-500 mg used) and homogenized in 19 mL of Buffer G2 (800 mM guanidine hydrochloride, 30 mM Tris-Cl, pH 8.0, 30 mM EDTA, pH 8.0, 5% Tween 20, 0.5% Triton X-100). 250 μL of RNase A (10 mg/mL) and RNase T1 (100 U) were added and the homogenate was incubated at 37°C for 30 min. 500 μL of Proteinase K (25 mg/mL) were then added and the incubation was continued for an additional 2.5 h. The tip was equilibrated with 10 mL of Buffer QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% v/v isopropanol, 0.15% v/v Triton x-100) and the homogenate was then loaded onto it. The tip was subsequently washed with 2 x 15 mL Buffer QC (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% v/v isopropanol) and the DNA was eluted with 15 mL of Buffer QF (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5, 15% v/v isopropanol). Cold isopropanol (10.5 mL) was added and the sample was centrifuged twice at 4000 rpm and at 4°C for 25 min, always keeping the pellet. The pellet (DNA) was washed initially with ethanol and then with 70% ethanol/30% HPLC grade water. The DNA was dried and then reconstituted with 100 μL HPLC grade water for liver samples and 200 μL for brain samples. The reconstituted DNA was left on a rotating table overnight at room temperature to dissolve and its concentration and purity were determined the next day on an Ampliquant AQ-07 photometer at 260 and 280 nm. Appropriate amounts of DNA were aliquoted into Eppendorf tubes to give 50, 100

and 200 µg of DNA. The tubes were subjected to speedvac evaporation till dryness and stored at -80 °C.

2.7 DNA hydrolysis

Calf spleen phosphodiesterase (0.001 U/µL, prepared by dialysis using the Slide-A-Lyser cassette), micrococcal nuclease (0.4 U/µL) and digestion buffer (100 mM sodium succinate, 50 mM CaCl₂, pH 6.0) were added to the dried DNA aliquots (containing 50 µg DNA) and were incubated at 37°C overnight. Nuclease P1 (2 U/µL) was added to the samples and the incubation was continued for an additional 4 h. The hydrolyzed DNA was then centrifuged at 14,000 rpm for 20 min and the supernatant was evaporated to dryness in a speedvac. 25 µL HPLC grade water were added to the samples, to yield a final DNA concentration of 2 µg/µL and the samples were stored at -20 °C for less than one week, until they underwent HPLC-UV analysis.

2.8 Instrumentation and chromatography

HPLC analysis was performed on a liquid chromatograph (Shimadzu Prominence) coupled with a Diode Array UV detector (Shimadzu SPD-M20A, Kyoto, Japan). Separation was achieved on a C18 Phenomenex Synergi Fusion-RP 80A (4 µm, 250 mm × 2.0 mm) column, which was attached to a guard column (C18 Synergi Fusion-RP 80A, 4 µm, 4.0 mm × 2.0 mm) and a Krudkatcher filter (0.5 µm) (California, U.S.A.).

The mobile phase consisted of Solvent A (50 mM ammonium formate, pH 5.4) and Solvent B (HPLC grade methanol). The gradient time program used was: solvent B 2% at 0 min, solvent B 3% at 18 min, solvent B 27% at 25 min, solvent B 35% at 40 min, solvent B 2% at 45 min. Total analysis time was 50 min. The flow rate was set at 0.2 mL/min and the column oven temperature was maintained at 25°C. UV detection was carried out at 277 nm.

2.9 Calculation of global DNA methylation

Global DNA methylation was expressed as the percentage of 5-MedC against the total cytosine present in the DNA and was calculated using the following equation:

$$\%5 - MedC = \frac{5 - MedC(nmol)}{dC(nmol) + 5 - MedC(nmol)} \times 100$$

2.10 Statistical Analysis

All statistical analyses were carried out using the SPSS software v. 18. The Mann-Whitney U test was used, with a confidence interval of 95 %.

3. Results

Four groups of animals (n=9) were used in this study. Both for the cocaine and the heroin treated animals, an equal number of control animals receiving saline were used.

For DNA extraction, only a part (100-500 mg) of liver was used. On the other hand, in the case of brain, the whole organ was used for DNA extraction. The amount of DNA extracted was always sufficient, with a good purity index.

The hydrolysis and HPLC-UV analysis were carried out in batches of five samples, accompanied by a control calf thymus DNA each time. Commercially available calf thymus DNA was used as a positive control to ensure that both the hydrolysis and the HPLC-UV procedure were effective for every batch (Mean % 5-MedC 6.7%, SD 0.2%, RSD 3.2 %). The hydrolysis was a two day process and the samples underwent HPLC-UV analysis on the third day, with the exception of samples prepared on Friday, which were analyzed on the fifth day. In this way, hydrolyzed DNA remained at -20 °C, prior to HPLC analysis, for a limited period of time (less than one week). This special care was necessary and was taken because we have previously shown that hydrolyzed DNA is less stable compared to non-hydrolyzed DNA when stored at -20 °C (Kovatsi et al., 2012).

Representative chromatograms of DNA extracted from liver and brain tissues of cocaine and heroin treated animals, as well as a representative chromatogram of calf thymus DNA are shown in Fig. 1. Zoomed in areas on each chromatogram (A, B, C, D, E) in Figure 1 depict the chromatographic separation between 5-MedC and guanosine.

The mean % 5-MedC content of the DNA isolated from the studied tissues, as well as the p values corresponding to the statistical comparison between different groups of animals are shown in Table 1.

In cocaine treated animals, the mean % 5-MedC content of liver DNA ($4.6\% \pm 0.3$) was identical to that of control animals receiving saline ($4.6\% \pm 0.2$). Results were similar in the brain, where both in the cocaine treated and the control animals the DNA had a mean % 5-MedC content of $4.9\% \pm 0.1$ (Table 1). Statistical analysis revealed no significant difference in the % 5-MedC content of liver ($p = 0.583$) and brain ($p = 0.887$) DNA between the cocaine treated and the control animals.

Similarly, in heroin treated animals, the mean % 5-MedC content of liver DNA ($4.5\% \pm 0.0$) was identical to that of control animals receiving saline ($4.5\% \pm 0.1$) and the mean % 5-

MedC content of brain DNA ($4.9\% \pm 0.1$) was identical to that of control animals receiving saline ($4.9\% \pm 0.1$). As expected, statistical analysis revealed no significant difference in the % 5-MedC content of liver ($p = 0.903$) and brain ($p = 0.837$) DNA between the heroin treated and the control animals.

When different tissues were compared, a statistically significant difference in the % 5-MedC content of DNA between liver and brain was found (Table 2). In all cases, the % 5-MedC content of DNA was significantly higher in the brain, compared to the liver.

4. Discussion

Although animals treated with an identical cocaine administration protocol are known to exhibit profound alterations in locomotor behavior and stereotypy and profound changes in gene expression in several brain regions (Bailey et al., 2005a, 2005b, 2007, 2008, 2010; Schlussman et al., 2005; Yoo et al., 2012), we found no changes in the global DNA methylation status in either brain or liver, in cocaine treated animals.

The lack of global DNA methylation alterations induced in the liver by cocaine is surprising considering the severe toxic effect that cocaine has on the liver. Indeed, the livers of cocaine treated animals were significantly larger (in terms of % weight of liver relative to body weight) compared to the livers of saline treated animals ($6.12 \pm 0.50\%$ vs $4.27 \pm 0.26\%$, $p < 0.001$). Furthermore, gross pathology of the livers of cocaine treated animals revealed regions of necrosis and intense eosinophilia (data not shown). Based on the above, we believe that the lack of overall DNA methylation changes in the liver observed in our study can be attributed to the cancelling out of hyper- and hypo- methylation in different anatomical regions of the liver, or in different genomic sites.

This is the first study to investigate the epigenetic effects of cocaine on liver. On the other hand, there is a large body of literature demonstrating profound DNA methylation alterations in liver pathology. For instance, global DNA hypo-methylation has been reported in liver injury induced by Wilson's disease (Medici et al., 2012), as well as in liver cells following treatment with triclosan, which is thought to cause liver tumors (Ma et al., 2013). Furthermore, it has been shown that hypo-methylation plays a crucial role in the onset of liver fibrosis and the progression of the disease to cancer (Komatsu et al., 2012). Genomic DNA hypo-methylation is also thought to provide a molecular mechanism for the role of hypoxia in hepatocellular carcinoma (Liu et al., 2011). Hypo-methylation has also been reported as a result of exposure to furan and has been linked to tumors induced by this carcinogen (Chen et al., 2012). On the other hand, cadmium has been found to increase global gene methylation in

the liver of rats and this effect has been linked to increased apoptosis and the onset of neoplastic lesions (Wang et al., 2012). Another environmental pollutant, perfluorooctanoic acid, has been found to cause dose-dependent hyper-methylation of the glutathione-S-transferase Pi promoter in human liver cells (Tian et al., 2012a). Global DNA hyper-methylation has been also linked to fibroblast activation during liver fibrogenesis (Tao et al., 2011a), while hyper-methylated genes have also been found in hepatocellular carcinoma (Tao et al., 2011b).

In contrast to the liver, there is a large body of literature on the effects of cocaine on DNA methylation in the brain. However, these studies have focused on DNA methylation either on particular brain regions, such as the nucleus accumbens (NAc) (Anier et al., 2010; LaPlant et al., 2010), the hippocampal pyramidal neurons (Novikova et al., 2008), the striatum (Carouge et al., 2010) and the prefrontal cortex (PFC) (Tian et al., 2012b), or on specific genes, such as the protein phosphatase-1 catalytic subunit (PP1c) promoter, the fosB promoter (Anier et al., 2010), the cyclin-dependent kinase-like 5 (CDKL5) gene (Carouge et al., 2010) and not the whole genome (global methylation). For instance, it was shown that the PFC exhibits hypo-methylation, while the striatum of the brain exhibits hyper-methylation in response to chronic cocaine exposure in mice and rats (Carouge et al., 2010; Tian et al., 2012b). Moreover, it was shown that the methylation status of different genome sites may differ within the same anatomical brain region. For instance, in the NAc of mice exposed to cocaine, DNA hyper-methylation was observed at the PP1c promoter, while hypo-methylation was observed at the fosB promoter (Anier et al., 2010). In the offspring of rats which were treated with cocaine during gestation, global hypo-methylation in the hippocampal pyramidal neurons was observed, whereas, when individual CpG islands were examined, 34 % were found to be hyper-methylated and 66 % were found to be hypo-methylated (Novikova et al., 2008).

The overall unaffected DNA methylation status observed in our study could be attributed to the fact that the whole brain, rather than specific anatomical regions, was studied. Hyper- or hypo-methylation in some regions could be counter balanced by hypo- or hyper-methylation respectively in other regions, yielding an overall unchanged methylation status. Moreover, as global methylation was studied, it is possible that hyper-methylation of specific genome sites might have cancelled out the hypo-methylation of other genome sites, resulting in lack of global DNA methylation changes overall.

The protocol of chronic heroin administration currently used is known to induce profound alterations in locomotor behavior and gene expression in brains of mice (Bailey et

al., 2010). Nevertheless, according to our results, in the liver and brain of mice treated with this protocol, global DNA methylation remained unaffected. Only one study has been published so far on the effect of heroin on DNA methylation, showing hyper-methylation of the OPRM1 gene promoter in lymphocytes of former heroin addicts currently on methadone maintenance (Nielsen et al., 2009). The present study is the first to investigate the effect of chronic heroin treatment on global DNA methylation, in either brain or liver. Our findings suggest that there is no observable change in the global DNA methylation status in these tissues following chronic heroin treatment in mice. It is possible however, as with the case of cocaine, that hyper- and hypo-methylation cancel out in different anatomical regions or in different genome sites, therefore resulting in no observable change in the overall DNA methylation status.

When the global DNA methylation status was compared between different tissues, in all cases the % 5-MedC content of DNA was statistically higher in the brain, compared to the liver. Our findings are in agreement with a previous report which showed that the methyl cytosine content of DNA was higher in the brain compared to the liver, both in control as well as in haloperidol treated rats (Shimabukuro et al., 2006). Furthermore, another report showed that in fetal baboons global DNA methylation was higher in the brain (frontal cortex) compared to the liver (Unterberger et al., 2009).

5. Conclusions

This study aimed at evaluating the effect of chronic heroin and cocaine administration on global DNA methylation on the tissue level. Although tissue-specific differences were observed, the global DNA methylation status in the brain and liver of mice treated chronically with cocaine or heroin remained unaffected. It is possible that hyper- and hypo-methylation cancel out in different anatomical regions or in different genome sites, resulting in an overall unchanged global DNA methylation status, but this is an assumption that remains to be elucidated in the future. Further research on different anatomical regions and different genomic sites, as well as on key enzymes such as DNMTs will aid towards this direction.

References

- Anier, K., Malinovskaja, K., Aonurm-Helm, A., Zharkovsky, A., Kalda, A., 2010. DNA methylation regulates cocaine-induced behavioral sensitization in mice. *Neuropsychopharmacology* 35, 2450-2461.
- Bailey, A., Gianotti, R., Ho, A., Kreek, M.J., 2007. Downregulation of kappa-opioid receptors in basolateral amygdala and septum of rats withdrawn for 14 days from an escalating dose "binge" cocaine administration paradigm. *Synapse* 61, 820-826.
- Bailey, A., Gianotti, R., Ho, A., Kreek, M.J., 2005a. Persistent upregulation of mu-opioid, but not adenosine, receptors in brains of long-term withdrawn escalating dose "binge" cocaine-treated rats. *Synapse* 57, 160-166.
- Bailey, A., Metaxas, A., Al-Hasani, R., Keyworth, H.L., Forster, D.M., Kitchen, I., 2010. Mouse strain differences in locomotor, sensitisation and rewarding effect of heroin; association with alterations in MOP-r activation and dopamine transporter binding. *Eur. J. Neurosci.* 31, 742-753.
- Bailey, A., Metaxas, A., Yoo, J.H., McGee, T., Kitchen, I., 2008. Decrease of D2 receptor binding but increase in D2-stimulated G-protein activation, dopamine transporter binding and behavioural sensitization in brains of mice treated with a chronic escalating dose 'binge' cocaine administration paradigm. *Eur. J. Neurosci.* 28, 759-770.
- Bailey, A., Yuferov, V., Bendor, J., Schlussman, S.D., Zhou, Y., Ho, A., Kreek, M.J., 2005b. Immediate withdrawal from chronic "binge" cocaine administration increases mu-opioid receptor mRNA levels in rat frontal cortex. *Brain Res. Mol. Brain Res.* 137, 258-262.
- Carouge, D., Host, L., Aunis, D., Zwiller, J., Anglard, P., 2010. CDKL5 is a brain MeCP2 target gene regulated by DNA methylation. *Neurobiol. Dis.* 38, 414-424.
- Chen, T., Williams, T.D., Mally, A., Hamberger, C., Mirbahai, L., Hickling, K., Chipman, J.K., 2012. Gene expression and epigenetic changes by furan in rat liver. *Toxicology* 292, 63-70.
- Dahl, C., Grønbæk, K., Guldberg, P., 2011. Advances in DNA methylation: 5-hydroxymethylcytosine revisited. *Clin Chim Acta* 412, 831-836.
- Dawson, R.M.C., Elliott, D.C., Elliott, W.H., Jones, K.M., 1986. *Data for Biochemical Research*, third ed. Oxford University Press, New York.
- Fragou, D., Fragou, A., Kouidou, S., Njau, S., Kovatsi, L., 2011. Epigenetic mechanisms in metal toxicity. *Toxicol. Mech. Methods* 21, 343-352.
- Franco, R., Schoneveld, O., Georgakilas, A.G., Panayiotidis, M.I., 2008. Oxidative stress, DNA methylation and carcinogenesis. *Cancer Lett.* 266, 6-11.

- Guo, J.U., Su, Y., Zhong, C., Ming, G.L., Song, H., 2011. Emerging roles of TET proteins and 5-hydroxymethylcytosines in active DNA demethylation and beyond. *Cell Cycle* 10, 2662-2668.
- Haycock, P.C., 2009. Fetal alcohol spectrum disorders: the epigenetic perspective. *Biol. Reprod.* 81, 607-617.
- Kalsi, G., Prescott, C.A., Kendler, K.S., Riley, B.P., 2009. Unraveling the molecular mechanisms of alcohol dependence. *Trends Genet.* 25, 49-55.
- Komatsu, Y., Waku, T., Iwasaki, N., Ono, W., Yamaguchi, C., Yanagisawa, J., 2012. Global analysis of DNA methylation in early-stage liver fibrosis. *BMC Med. Genomics* 5, 5.
- Kouidou, S., Kovatsi, L., Ioannou, A., 2010. Seeking the 5th Base of DNA using chromatographic methods of analysis. *Curr. Org. Chem.* 14, 2268-2281.
- Kovatsi, L., Fragou, D., Samanidou, V., Njau, S., Kouidou, S., 2011. Drugs of abuse: epigenetic mechanisms in toxicity and addiction. *Curr. Med. Chem.* 18, 1765-1774.
- Kovatsi, L., Fragou, D., Samanidou, V., Njau, S., Kouidou, S., Bailey, A., 2012. Evaluation of 5-methyl-2'-deoxycytidine stability in hydrolyzed and nonhydrolyzed DNA by HPLC-UV. *Bioanalysis* 4, 367-372.
- Kreek, M.J., LaForge, K.S., Butelman, E., 2002. Pharmacotherapy of addictions. *Nat. Rev. Drug Discov.* 1, 710-726.
- LaPlant, Q., Vialou, V., Covington, H.E., 3rd, Dumitriu, D., Feng, J., Warren, B.L., Maze, I., Dietz, D.M., Watts, E.L., Iniguez, S.D., Koo, J.W., Mouzon, E., Renthal, W., Hollis, F., Wang, H., Noonan, M.A., Ren, Y., Eisch, A.J., Bolanos, C.A., Kabbaj, M., Xiao, G., Neve, R.L., Hurd, Y.L., Oosting, R.S., Fan, G., Morrison, J.H., Nestler, E.J., 2010. Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens. *Nat. Neurosci.* 13, 1137-1143.
- Liu, Q., Liu, L., Zhao, Y., Zhang, J., Wang, D., Chen, J., He, Y., Wu, J., Zhang, Z., Liu, Z., 2011. Hypoxia induces genomic DNA demethylation through the activation of HIF-1 α and transcriptional upregulation of MAT2A in hepatoma cells. *Mol. Cancer. Ther.* 10, 1113-1123.
- Lutz, U.C., 2008. Alterations in homocysteine metabolism among alcohol dependent patients--clinical, pathobiochemical and genetic aspects. *Curr. Drug Abuse Rev.* 1, 47-55.
- Ma, H., Zheng, L., Li, Y., Pan, S., Hu, J., Yu, Z., Zhang, G., Sheng, G., Fu, J., 2013. Triclosan reduces the levels of global DNA methylation in HepG2 cells. *Chemosphere* 90, 1023-1029.

- Malvaez, M., Barrett, R.M., Wood, M.A., Sanchis-Segura, C., 2009. Epigenetic mechanisms underlying extinction of memory and drug-seeking behavior. *Mamm. Genome* 20, 612-623.
- McQuown, S.C., Wood, M.A., 2010. Epigenetic regulation in substance use disorders. *Curr. Psychiatry Rep.* 12, 145-153.
- Medici, V., Shibata, N.M., Kharbanda, K.K., Lasalle, J.M., Woods, R., Liu, S., Engelberg, J.A., Devaraj, S., Torok, N.J., Jiang, J.X., Havel, P.J., Lonnerdal, B., Kim, K., Halsted, C.H., 2012. Wilson disease: Changes in methionine metabolism and inflammation affect global DNA methylation in early liver disease. *Hepatology* Sep 4. doi: 10.1002/hep.26047. [Epub ahead of print].
- Meyer, K., Zhang, H., Zhang, L., 2009. Direct effect of cocaine on epigenetic regulation of PKCepsilon gene repression in the fetal rat heart. *J. Mol. Cell. Cardiol.* 47, 504-511.
- Muller, D.L., Unterwald, E.M., 2004. In vivo regulation of extracellular signal-regulated protein kinase (ERK) and protein kinase B (Akt) phosphorylation by acute and chronic morphine. *J. Pharmacol. Exp. Ther.* 310, 774-782.
- Nestler, E.J., 2008. Review. Transcriptional mechanisms of addiction: role of DeltaFosB. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 363, 3245-3255.
- Nielsen, D.A., Yuferov, V., Hamon, S., Jackson, C., Ho, A., Ott, J., Kreek, M.J., 2009. Increased OPRM1 DNA methylation in lymphocytes of methadone-maintained former heroin addicts. *Neuropsychopharmacology* 34, 867-873.
- Novikova, S.I., He, F., Bai, J., Cutrufello, N.J., Lidow, M.S., Undieh, A.S., 2008. Maternal cocaine administration in mice alters DNA methylation and gene expression in hippocampal neurons of neonatal and prepubertal offspring. *PLoS One* 3, e1919.
- Renthal, W., Nestler, E.J., 2008. Epigenetic mechanisms in drug addiction. *Trends Mol. Med.* 14, 341-350.
- Renthal, W., Nestler, E.J., 2009. Chromatin regulation in drug addiction and depression. *Dialogues Clin. Neurosci.* 11, 257-268.
- Schlussman, S.D., Zhou, Y., Bailey, A., Ho, A., Kreek, M.J., 2005. Steady-dose and escalating-dose "binge" administration of cocaine alter expression of behavioral stereotypy and striatal preprodynorphin mRNA levels in rats. *Brain Res. Bull.* 67, 169-175.
- Shimabukuro, M., Jinno, Y., Fuke, C., Okazaki, Y., 2006. Haloperidol treatment induces tissue- and sex-specific changes in DNA methylation: a control study using rats. *Behav. Brain Funct.* 2, 37.

- Spanagel, R., 1995. Modulation of drug-induced sensitization processes by endogenous opioid systems. *Behav. Brain Res.* 70, 37-49.
- Tang, W.Y., Ho, S.M., 2007. Epigenetic reprogramming and imprinting in origins of disease. *Rev. Endocr. Metab. Disord.* 8, 173-182.
- Tao, H., Huang, C., Yang, J.J., Ma, T.T., Bian, E.B., Zhang, L., Lv, X.W., Jin, Y., Li, J., 2011a. MeCP2 controls the expression of RASAL1 in the hepatic fibrosis in rats. *Toxicology* 290, 327-333.
- Tao, R., Li, J., Xin, J., Wu, J., Guo, J., Zhang, L., Jiang, L., Zhang, W., Yang, Z., Li, L., 2011b. Methylation profile of single hepatocytes derived from hepatitis B virus-related hepatocellular carcinoma. *PLoS One* 6, e19862.
- Tian, M., Peng, S., Martin, F.L., Zhang, J., Liu, L., Wang, Z., Dong, S., Shen, H., 2012a. Perfluorooctanoic acid induces gene promoter hypermethylation of glutathione-S-transferase Pi in human liver L02 cells. *Toxicology* 296, 48-55.
- Tian, W., Zhao, M., Li, M., Song, T., Zhang, M., Quan, L., Li, S. Sun, Z.S., 2012b. Reversal of cocaine-conditioned place preference through methyl supplementation in mice: altering global DNA methylation in the prefrontal cortex. *PLoS One* 7, e33435.
- Tsankova, N., Renthal, W., Kumar, A., Nestler, E.J., 2007. Epigenetic regulation in psychiatric disorders. *Nat. Rev. Neurosci.* 8, 355-367.
- Tsukada, H., Kreuter, J., Maggos, C.E., Unterwald, E.M., Kakiuchi, T., Nishiyama, S., Futatsubashi, M., Kreek, M.J., 1996. Effects of binge pattern cocaine administration on dopamine D1 and D2 receptors in the rat brain: an in vivo study using positron emission tomography. *J. Neurosci.* 16, 7670-7677.
- Unterberger, A., Szyf, M., Nathanielsz, P.W., Cox, L.A., 2009. Organ and gestational age effects of maternal nutrient restriction on global methylation in fetal baboons. *J. Med. Primatol.* 38, 219-227.
- Wang, B., Li, Y., Tan, Y., Miao, X., Liu, X.D., Shao, C., Yang, X.H., Turdi, S., Ma, L.J., Ren, J., Cai, L., 2012. Low-dose Cd induces hepatic gene hypermethylation, along with the persistent reduction of cell death and increase of cell proliferation in rats and mice. *PLoS One* 7, e33853.
- Yoo, J.H., Kitchen, I., Bailey, A., 2012. The endogenous opioid system in cocaine addiction: what lessons have opioid peptide and receptor knockout mice taught us? *Br. J. Pharmacol.* 166, 1993-2014.

- Yuferov, V., Zhou, Y., Spangler, R., Maggos, C.E., Ho, A., Kreek, M.J., 1999. Acute "binge" cocaine increases mu-opioid receptor mRNA levels in areas of the rat mesolimbic mesocortical dopamine system. *Brain Res. Bull.* 48, 109-112.
- Zhang, H., Darwanto, A., Linkhart, T.A., Sowers, L.C., Zhang, L., 2007. Maternal cocaine administration causes an epigenetic modification of protein kinase Cepsilon gene expression in fetal rat heart. *Mol. Pharmacol.* 71, 1319-1328.
- Zhang, H., Meyer, K.D., Zhang, L., 2009. Fetal exposure to cocaine causes programming of Prkce gene repression in the left ventricle of adult rat offspring. *Biol. Reprod.* 80, 440-448.

Figure Legends

Fig. 1. Chromatograms

- A. Hydrolyzed DNA extracted from the liver of a cocaine-treated mouse.
- B. Hydrolyzed DNA extracted from the brain of a cocaine-treated mouse.
- C. Hydrolyzed DNA extracted from the liver of a heroin-treated mouse.
- D. Hydrolyzed DNA extracted from the brain of a heroin-treated mouse.
- E. Hydrolyzed C.T. DNA used as positive control.

5-MedC, 5-methyl-2'-deoxycytidine; dG, 2'-deoxyguanosine; G, guanosine; dA, 2'-deoxyadenosine; T, thymidine; dC, 2'-deoxycytidine

Table 1: Comparison between different groups of animals

Mean % 5-MedC content of the DNA isolated from the studied tissues, standard deviation (SD), relative standard deviation (RSD) and p values corresponding to the statistical comparison between different groups of animals.

Tissue - Experiment	Liver - Cocaine		Brain - Cocaine		Liver - Heroin		Brain - Heroin	
Drug Exposure	Cocaine	Control	Cocaine	Control	Heroin	Control	Heroin	Control
Mean % 5-MedC	4.6	4.6	4.9	4.9	4.5	4.5	4.9	4.9
SD (%)	0.3	0.2	0.1	0.1	0.0	0.1	0.1	0.1
RSD (%)	5.7	4.2	1.6	3.0	1.0	2.0	1.7	2.2
p value (95%)	0.583		0.887		0.903		0.837	

Table 2: Comparison between different tissues

Mean % 5-MedC content of the DNA isolated from the studied tissues, standard deviation (SD), relative standard deviation (RSD) and p values corresponding to the statistical comparison between different tissues.

Tissue - Experiment	Liver	Brain	Liver	Brain	Liver	Brain	Liver	Brain
Drug Exposure	Cocaine Control		Cocaine Treated		Heroin Control		Heroin Treated	
Mean % 5-MedC	4.6	4.9	4.6	4.9	4.5	4.9	4.5	4.9
SD (%)	0.2	0.1	0.3	0.1	0.1	0.1	0.0	0.1
RSD (%)	4.2	3.0	5.7	1.6	2.0	2.2	1.0	1.7
p value (95%)	0.009		0.005		< 0.005		< 0.005	