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XX

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The role of DNA methylation in the development of cocaine-induced behavioural sensitisation

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III **Anier K**, Malinovskaja K, Pruus K, Aonurm-Helm A, Zharkovsky A, Kalda A (2013) Maternal separation is associated with DNA methylation and behavioural changes in adult rats. *European Neuropsychopharmacology* [Epub ahead of print].

Contribution of author to original publications is following:

I The author was the main person in gene expression, DNA methylation, chromatin immunoprecipitation studies, analysed the data and participated in the study design and manuscript writing.

II The author was the main person in the gene expression, DNA methylation and cell culture studies, analysed the data, participated in the study design, manuscript writing and handled correspondence.

III The author performed all the molecular biological experiments, except Western immunoblotting, analysed the data, wrote the manuscript and handled the correspondence.

ABBREVIATIONS

ABC	avidin-biotin complex
AC	acute cocaine treatment
ACTH	adrenocorticotropic hormone
ADP	adenosine diphosphate
AFR	animal facility rearing
Ags3	activator of G-protein signaling 3
AID	activation-induced cytidine deaminase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	anaysis of variance
APOBEC	apolipoprotein B mRNA editing enzyme complex
AP-1	activator protein-1
Avp	arginine-vasopressin
$A_{2A}R$	adenosine A_{2A} receptor
Bdnf	brain-derived neurotrophic factor
bp	base pair
5-caC	5-carboxycytosine
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
Cck	cholecystokinin
Cdk5	cyclin-dependent kinase 5
cDNA	complementary DNA
c-Fos	FBJ murine osteosarcoma viral oncogene
ChIP	chromatin immunoprecipitation
Cnr1	cannabinoid receptor-1
CNS	central nervous system
CpG	cytosine-guanine dinucleotide
CPP	conditioned place preference
CRE	cAMP response element
CREB	cAMP response element binding protein
CRF	corticotrophin-releasing factor
Crhr2	corticotrophin-releasing factor receptor 2
CT	cycle threshold
DA	dopamine
DARPP-32	cAMP-regulated phosphoprotein 32 kDa
DAVID	database for annotation, visualization and integrated discovery
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
ERK	extracellular signal-regulated kinase
5-fc	5-formylcytosine
FC	fold change
fosB	FBJ osteosarcoma oncogene B
fra-1,2	fos-related antigen 1 and 2

GABA	γ-aminobutyric acid
GAD67	glutamate decarboxylase 67
GADD45	growth arrest and DNA damage-inducible protein 45
Gal	galanin
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
GC	glucocorticoid
GEO	gene expression omnibus
GluR1	glutamate receptor 1
GO	gene ontology
GR	glucocorticoid receptor
G9a	histone H3 lysine K9 methyltransferase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDM	histone demethylase
5-hmc	5-hydroxymethylcytosine
HMT	histone methyltransferase
HPA	hypothalamic-pituitary-adrenal axis
HP1	heterochromatin protein 1
5-HT	serotonin
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
JHDM1,2	Jumonji domain-containing histone demethylase 1 and 2
kb	kilobase
kDa	kilo Dalton, the unified atomic mass unit
LG	licking and grooming
LSD1	lysine specific demethylase 1
MAPK	mitogen activated protein kinase
MBD	methyl-CpG-binding domain protein
5-mc	5-methylcytosine
MeC	methylated cytosine
MeCP2	methyl-CpG-binding protein 2
MeDIP	methylated DNA immunoprecipitation
MET	L-methionine
mRNA	messenger ribonucleic acid
MS	maternal separation
MS15	15 min maternal separation
MS180	180 min maternal separation
MSK1	mitogen- and stress-activated protein kinase 1
MSP qPCR	methylation-specific quantitative polymerase chain reaction
NAc	nucleus accumbens
NGFI-A	nerve growth factor-inducible protein A
NH	non-handled
OD	optical density
PBS	phosphate buffered saline

PC12	pheochromocytoma cells
PFC	prefrontal cortex
PKA	protein kinase A
PND	postnatal day
PP1	protein phosphatase 1
PP1c	protein phosphatase 1 catalytic subunit
PVN	hypothalamic paraventricular nucleus
qPCR	quantitative polymerase chain reaction
Reln	reelin
RC	repeated cocaine treatment
RG-108	non-nucleoside DNA methyltransferase inhibitor
RNA	ribonucleic acid
RST	repeated SAM treatment
SAH	S-adenosylhomocysteine
SAHA	suberoylanilide hydroxamic acid
SAL	saline
SAM	S-adenosylmethionine
SDS	sodium dodecyl sulfate
SEM	standard error of mean
Ser133	serine 133
Sir2	silent information regulator 2
Sirt	sirtuin
Slc17a7	solute carrier family 17 member 7
SST	single SAM treatment
TBS	TRIS buffered saline
TET	ten-eleven translocation enzyme
Thal	thalamus
Tris-EDTA	trishydroxymethylaminomethane-Ethylenediaminetetraacetic acid
TSA	trichostatin A
VTA	ventral tegmental area
ZEB	zebularine
ΔcJun	truncated form of cJun, dominant negative of ΔFosB
ΔFosB	truncated form of FosB that is induced by chronic drug use

INTRODUCTION

Drug addiction is chronic relapsing disorder characterised by compulsive pattern of drug seeking and taking behaviour despite severe adverse consequences (Kalivas et al., 2005; Hyman et al., 2006; Koob and Kreek, 2007). Prolonged use of abused drugs, such as psychostimulants, may contribute to behavioural abnormalities that can last for months or even years after discontinuation of drug consumption. Drug addiction is a multi-factorial and polygenic disorder that does not conform to a simple Mendelian transmission pattern (Goldman, 1993; Enoch and Goldman, 1999; Goldman et al., 2005; Wong et al., 2011). Individuals are differentially vulnerable to substance abuse, not everyone who uses an addictive substance becomes addicted. Extensive epidemiological studies show that roughly half of an individual's risk for drug addiction is genetic, but the specific genes that confer risk for drug addiction are not well known (Nestler, 2001; Goldman et al., 2005; Hyman et al., 2006), although several possible candidates have been proposed (Wang et al., 2012).

Entrance into addicted state clearly results from the interplay between inherited predisposition (e.g. via genetic variants mediating the personality traits associated with drug-seeking behaviour and dependence) and the environment (e.g. actual exposure to drugs of abuse) (Nestler, 2001; Goldman et al., 2005; Wong et al., 2011). There is evidence that stressful and traumatic experiences in early life have also a long-lasting impact on individual's behaviour. However, the mechanisms which mediate the effects of the early environment on the behaviour are not yet fully understood. Recent findings suggest that epigenome, which consists of the machinery for programming long-term gene expression profiles and thus defines gene function and phenotype, can be modulated by a variety of environmental factors, including nutrients, chemicals and early-life environment (Weaver et al., 2004; Waterland et al., 2006; Roth et al., 2009; Szyf, 2009). Therefore, the epigenome provides an important interface between genes and environment and may be viewed as a potential mechanism underlying the rapid form of environmentally driven adaptation (Franklin and Mansuy, 2010).

Repeated administration of psychostimulants (such as cocaine) induces an enhanced behavioural response to subsequent drug exposure, a phenomenon known as psychomotor or behavioural sensitisation (Robinson and Berridge, 1993; Pierce and Kalivas, 1997). Psychostimulant-induced behavioral sensitisation in rodents provides a model of the addictive behaviours (such as those associated with craving and relapse) and psychotic complications of psychostimulant abuse (Robinson and Becker, 1986). Behavioural sensitisation is remarkable persistent phenomenon. In rodents, it can persist for month to years after drug treatment is discontinued. Persistent behavioural sensitisation indicates that drug-induced short- and long-term changes in gene expression may be involved. Accumulating data suggest that epigenetic mechanisms (such as DNA methylation, histone modifications and microRNA) - key cellular processes that interpret diverse environmental stimuli into long-lasting changes in gene expression via the regulation of chromatin structure - contribute to drug-induced transcriptional and behavioural changes (Kumar et al., 2005; Levine et al., 2005; Renthal et al., 2007; Renthal and Nestler, 2008; Wang et al., 2010).

The general aim of the present study was to investigate the role of DNA methylation in the development of cocaine-induced behavioural sensitisation in mice and rats. The more specific aims were: a) to determine the role of cocaine treatment on DNA methyltransferases (DNMT) and selected genes expression in the nucleus accumbens (NAc) of adult mice and to assess the effect of DNMT inhibitor zebularine on the development of behavioural sensitisation in mice; b) to investigate the role of methyl donor S-adenosylmethionine (SAM) on cocaine-induced gene expression changes and the development of behavioural sensitisation in mice; c) using maternal separation (MS) as an early life stress model, to evaluate whether the MS on rats could alter cocaine-induced behavioural sensitisation in adulthood via aberrant DNA methylation.

REVIEW OF THE LITERATURE

1. Drug addiction and behavioural sensitisation

Drug addiction can be defined as the loss of control over drug use, or the compulsive seeking and taking of drugs despite adverse consequences (Nestler, 2001). Once a person becomes addicted to drugs of abuse, only few effective therapies exist. Therefore, understanding of the neural mechanisms that underlie the transition from recreational drug use to a chronically addicted state, and the mechanisms which are responsible for the persistence of addictive behaviours even after prolonged drug abstinence, would provide clues into how block or reverse the addicted state and thereby diminish the rate of relapse (for a review see Renthal and Nestler, 2008).

Psychostimulants, such as cocaine and amphetamine, change a neuronal structure and function in the specific brain regions, resulting in persistent changes at the molecular, cellular systems and behavioural levels (Paulson et al., 1991; Koob and LeMoal, 2001; Nestler, 2001; McQuown and Wood, 2010). Repeated administration of psychostimulants induces an enhanced behavioural response to subsequent drug exposure, a phenomenon known as psychomotor or behavioural sensitisation that can persist for months (Robinson and Berridge, 1993; Pierce and Kalivas, 1997). Behavioural sensitisation can be separated into two components - induction and expression of sensitisation. Induction of sensitisation indicates to the progressive increase in locomotor activity during the repeated drug treatment. Expression of sensitisation is demonstrated following challenge with a low dose of psychostimulant after a drug-free period (McQuown and Wood, 2010). Psychostimulant-induced behavioural sensitisation in rodents provides a model for addictive behaviours such as those associated with craving and relapse, as well as for psychotic complications of psychostimulant abuse (Koob and Bloom, 1988; Robinson and Berridge, 1993; Chen et al., 2003).

Several neuropharmacological studies indicate that drugs of abuse activate the brain reward circuitry, which centres on dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain and their projections to the limbic system - in particular, the NAc, dorsal striatum, amygdala, hippocampus and regions of prefrontal cortex (Figure 1) (Kalivas and Volkow, 2005; Koob and LeMoal, 2005; Hyman et al., 2006; Robinson and Nestler, 2011). Under normal conditions, this reward circuitry controls an individual's responses to natural rewards, such as food, sex, play and social interactions. Compared to the natural rewards, drugs of abuse activate this reward circuitry far more strongly and persistently, and without association with productive behavioural outcomes. Chronic exposure to drugs modulates described brain reward regions in part through a homeostatic desensitisation that renders the individual unable to attain sufficient feelings of reward in the absence of drug (for a review see Robinson and Nestler, 2011).

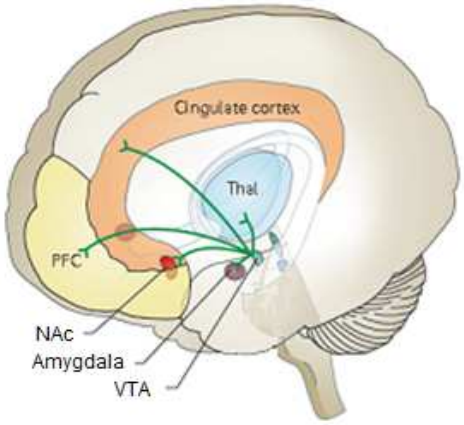

Figure 1. The brain describes dopaminergic afferents that originate in the ventral tegmental area (VTA) and release dopamine in the nucleus accumbens (NAc) and other limbic targets. Thalamus (Thal), prefrontal cortex (PFC) (modified from Robinson and Nestler, 2011).

The addictive phenotype may persists for the length of an individual's life with drug craving and relapse occurring after weeks, months or even years of abstinence. This persistence suggests that drugs of abuse induce long-lasting changes in the brain that underlie addiction behaviours (Robinson and Nestler, 2011). Therefore, it has been hypothesized that persistent alterations in gene expression could be responsible for the long-term behavioural and structural changes (Nestler and Aghajanian, 1997).

The classic mechanism for gene expression regulation is through the actions of transcription factors, which are proteins that in response to cell signaling pathways are able to bind to specific DNA sequences in the promoter regions of target genes, and increase or decrease gene expression by promoting or blocking the recruitment of the RNA polymerase-II transcriptional complex (Robinson and Nestler, 2011). It has been proposed that drugs of abuse activate certain transcription factors and thereby cause adaptive changes in neuronal structure and function (Kalivas et al., 2003). Despite the fact that several different transcription factors exist, two, the most and best characterised transcription factors (related with drug addiction) are cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) and ΔFosB (Nestler et al., 2001; McClung and Nestler, 2003).

Phosphorylation of CREB by protein kinase A (PKA) at serine133 (Ser133) is an important event in the activation of CREB and cAMP response element (CRE)-dependent gene expression. Furthermore, phosphorylation of CREB at Ser133 allows recruitment of transcriptional coactivator, CREB-binding protein (CBP), that in turn promotes transcription (Carlezon et al., 2005; Briand and Blendy, 2010; Robinson and Nestler, 2011). Psychostimulants and opiates upregulate the cAMP pathway and thereby increase CREB activity in multiple brain regions, including the NAc and dorsal striatum (Carlezon et al., 2005; Edwards et al., 2007; Briand and Blendy, 2010). Studies involving the inducible overexpression of CREB or a dominant negative mutant in bitransgenic mice or with viral vectors have demonstrated that CREB induction in the NAc decreases the rewarding effects of cocaine and opiates (Carlezon et al., 1998; Barrot et al., 2002; Robinson and Nestler, 2011). Data by Walters and Blendy (2001) have demonstrated that mice who had partially deficient in CREB ($CREB^{\alpha, \Delta}$ mutant mice lack the α and Δ isoforms of CREB) showed an enhanced

response to the reinforcing properties of cocaine compared with their wild-type controls in both conditioned place preference (CPP) and sensitisation behaviours (Walters and Blendy, (2001). These results suggest that drug-induced CREB activation/phosphorylation in the NAc comprises a negative feedback mechanism which dampens behavioural sensitivity to subsequent drug exposure (Carlezon et al., 2005; Chen et al., 2009). However, temporally CREB is induced rapidly after each drug treatment (effects are relatively short-lived) and returns to baseline after a few hours (Nestler, 2008).

Transcription factor ΔFosB (encoded by the *fosB* gene) is a member of the Fos family, which consists of *c-fos*, *fosb*, *fra-1* and *fra-2* genes (Nestler et al., 2001; Nestler, 2008). ΔFosB heterodimerizes with Jun family proteins (c-Jun, JunB, JunD) to form activator protein-1 (AP-1; known as transcription factor AP-1) complexes which bind to AP-1 sites in responsive genes to regulate transcription (Curran and Franza 1988; Jorissen et al., 2007; Nestler, 2008). It has been found that acute exposure to drug of abuse cause transient increase in members of the transcription factor Fos family (including *c-fos*, *fosB*) in the NAc and dorsal striatum (Nestler et al., 2001). During repeated drug of abuse exposure the expression of transcription factor ΔFosB is increased several fold and often persists long after drug exposure ceases. Thus, ΔFosB extraordinary stability in neurons has led to the theory that it plays an important role in the onset of drug addiction (Bowers et al., 2004; McClung et al., 2004; Kalivas and O'Brien, 2008). Indeed, several previous studies have demonstrated that ΔFosB is linked directly to addiction-related behaviours. It has been found that prolonged ΔFosB expression in the NAc increases the rewarding effects of cocaine. For example, mice overexpressing ΔFosB demonstrated increased CPP, self-administration and incentive motivation for cocaine (Kelz et al., 1999; Nestler, 2001; Colby et al., 2003; Peakman et al., 2003). However, mice that express a dominant-negative form of cJun (ΔcJun), which disrupts normal AP-1 function, demonstrated less preference for cocaine (Nestler, 2008). Thus, to summarize, it seems that gene expression induced by short-term ΔFosB and by CREB reduce the rewarding effects of cocaine, while prolonged ΔFosB expression increase drug reward. Furthermore, it has been demonstrated that gene expression after a short cocaine exposure was dependent on CREB, while gene expression after a longer cocaine treatment was ΔFosB dependent (McClung and Nestler, 2003; Nestler, 2008).

Moreover, altered expression of *Ags3* (activator of G protein signaling 3) (Bowers et al., 2004) and *Bdnf* (brain-derived neurotrophic factor) (Grimm et al., 2003) has been reported weeks after the last drug experience (Renthal and Nestler, 2008). Manipulation of these genes in rodents regulates drug relapse behaviour (Bowers et al., 2004; Lu et al., 2004; Graham et al., 2007; Renthal and Nestler, 2008). A multitude of microarray studies under different experimental conditions have identified several potential target genes for drugs of abuse in distinct brain reward regions that may promote to their long-lasting behavioural effects (Freeman et al., 2001; McClung and Nestler, 2003; Yuferov et al., 2003; Yao et al., 2004; McClung et al., 2005; Winstanley et al., 2007; LaPlant and Nestler, 2011). Several recent data suggest that epigenetic mechanisms - key cellular processes that interpret diverse environmental stimuli into long-lasting changes in gene expression via the regulation of chromatin structure - contribute to drug-induced transcriptional and behavioural changes (Kumar et al., 2005; Levine et al., 2005; Renthal et al., 2007; Renthal and Nestler, 2008; Wang et al., 2010).

2. Epigenetics and epigenetic mechanisms

The sequence of nucleotides comprising an individual's genome is identical, with the exception of a few rare somatic mutations, across all cells in the body. However, at a functional level the genome is anything but static and DNA is structurally much more complex than a string of nucleotides (Wong et al., 2011). Every cell in our bodies contains the same DNA sequence and each has its own unique phenotype characterised by a specific pattern of gene expression that is in a constant state of flux. In context of determining the phenotype of a cell it is important also the degree to which specific genes are functionally active at any particular time in development. Therefore, sequencing the genome was only the first step in our quest to understand how genes are expressed and regulated (for a review see Smith and Mill, 2011; Wong et al., 2011).

Accumulating evidences indicate that above the DNA sequence is a second layer of information - the epigenome - that regulates when and where genes are turned on or off. Historically, the term epigenetics (literally meaning "above genetics") was coined by Conrad Hal Waddington in 1942 to describe the examination of causal mechanisms whereby the genes of the genotype bring about phenotypic effects (Haig, 2004; McQuown and Wood, 2010). At present, epigenetics can be defined as long-lived and reversible modifications to nucleotides or chromosomes that do not change the sequence but can alter gene expression and phenotype (LaSalle et al., 2013). Epigenetic mechanisms are essential for normal cellular development and differentiation, and allow the long-term regulation of gene function through non-mutagenic mechanisms (Smith and Mill, 2011). Several data suggest that alterations of epigenetic mechanisms affect the vast majority of nuclear processes (including gene transcription and silencing), DNA replication and repair, cell cycle, telomere and centromere function and structure (Gonzalo, 2010). During the last decade the field of epigenetics has developed into one of the most influential areas of scientific research and has become an important topic in several neurobiology fields such as learning and memory, psychiatric and neurological disorders.

Epigenetics is used to refer to the extremely complex processes of organizing the genome in a manner that allows for regulated gene expression in the appropriate cell type upon appropriate cellular stimuli (LaPlant and Nestler, 2011). The fundamental unit that accomplishes this feat on a molecular level is chromatin, which is the complex of DNA, histones and non-histone proteins in the cell nucleus. The basic repeating structural unit of chromatin is the nucleosome (Figure 2), which consists of ~147 base pairs (bp) of DNA wrapped around a core nucleosome (Strahl and Allis, 2000). Nucleosomes are composed of octamers that contain four histone homodimers, one each of histones H2A, H2B, H3 and H4, with H1 binding to spans of non-nucleosomal DNA (Robinson and Nestler, 2011). The histone-DNA configuration is maintained by electrostatic bonds between positively charged histones and negatively charged DNA (Grunstein, 1997). This highly condensed histone proteins-DNA complex structure means that control over gene expression occurs partly by gating access of transcriptional activators to DNA (Felsenfeld and Groudine, 2003; Li et al., 2007; Renthal and Nestler, 2008).

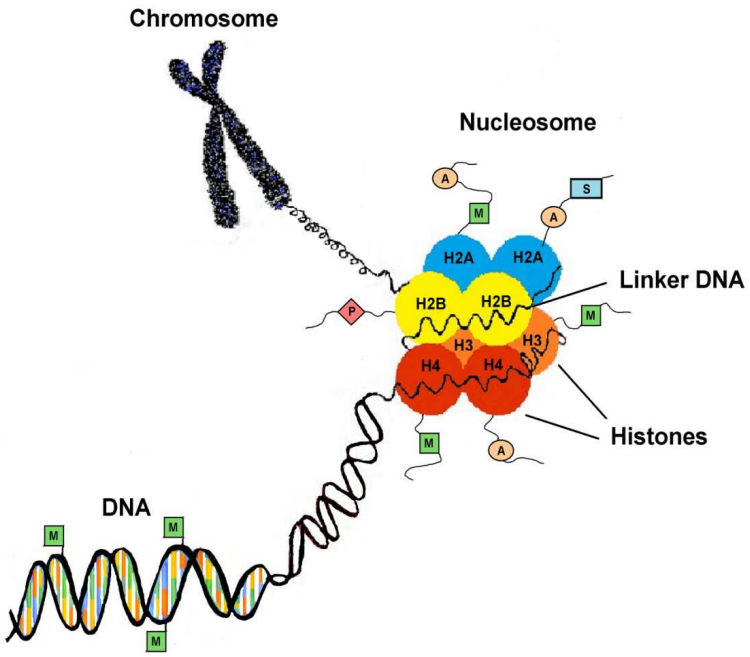

Figure 2. DNA is wrapped around a cluster of histone proteins to form nucleosomes. In histone modifications, a combination of different molecules can attach to the tails of histones, which consequently change the state of the chromatin around the DNA. When the chromatin becomes opened, the transcription of associated genes is activated and opposite when chromatin becomes closed. In DNA methylation, methyl marks added in CpG islands generally repress gene transcription (Wong et al., 2011). (A)-acetylation, (M)-methylation, (P)-phosphorylation, (S)-sumoylation (modified from Anier and Kalda, 2012).

The structure of chromatin and access to the DNA sequence wrapped around it is regulated by posttranslational modifications of histones and the DNA itself (Kouzarides, 2007). Numerous types of posttranslational modifications such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation of the amino (N)-terminal tails of histones alter chromatin compaction to create more open states (euchromatin, transcriptionally active) versus closed states (heterochromatin, transcriptionally inactive) (Cheung et al., 2000; Berger, 2007; Kouzarides, 2007; Robinson and Nestler, 2011). It has been found that histone modifications that weaken or disrupt histone-DNA contacts, such as histone acetylation, correlate with transcriptionally active states. In contrast, histone modifications that increase histone-DNA contacts, such as histone methylation at certain basic amino acid residues, promote transcriptional repression (Strahl and Allis, 2000; Maze and Nestler, 2011). Combinations of numerous posttranslational modifications occurring on amino (N)-terminal histone tails have shown to affect condensation of chromatin and to result in altered levels of gene expression in cells (Jenuwein and Allis, 2001; Maze and Nestler, 2011).

2.1. Histone modifications

Each histone protein is composed of a central globular domain and an amino (N)-terminal tail that contains multiple sites for posttranslational modifications, including acetylation, phosphorylation, methylation, ubiquitination, and ADP-ribosylation. Most histone

posttranslational modifications are dynamic (Allis et al., 2007; Berger, 2007; Kouzarides, 2007) and are regulated by large number of histone modifying enzymes like acetyltransferases, deacetylases, methyltransferases, demethylases, kinases, etc. (Gibney and Nolan, 2010).

2.1.1. Histone acetylation and deacetylation

The enzymes that regulate histone acetylation levels are histone acetyltransferases (HAT's). The primary function of HAT's is to neutralize the charges on histones to relax chromatin structure, allowing for greater access to the DNA by transcription factors and thereby increase transcription (Norton et al., 1989; Barrett and Wood, 2008). On the other hand, histone deacetylases (HDAC's) deacetylate histone tails and histone deacetylation has been linked to transcriptional repression (Kuo and Allis, 1998; Jenuwein and Allis, 2001; Narlikar et al., 2002). The HDAC family consists of a number of proteins that have a catalytic deacetylase domain and are divided into 4 classes. Class I HDACs (HDAC 1, 2, 3 and 8) are ubiquitously expressed and likely mediate the majority of deacetylase activity within cells. Class II HDACs (HDAC 4-7 and 9-10) are larger proteins containing deacetylase domain and an N-terminal regulatory domain that enables them to be shuttled in and out of the nucleus in a neural activity-dependent manner and enriched in specific tissues such as brain and heart (Chawla et al., 2003; Renthal and Nestler, 2008). Class III HDACs are homologs of Sir2 and sirtuins and HDAC11 is a class IV HDAC and shares homology to class I and II enzymes (Yang and Seto, 2008). The balance between the opposing activities of HAT's and HDAC's determines the gene expression levels.

2.1.2. Histone methylation

Another group of important enzymes are histone methyltransferases (HMT's, like SET, MLL, SUV39 etc.), which are methylated at lysine (K) or arginine (R) residues and removed by histone demethylases (HDM's) (Tachibana et al., 2001; Hake et al., 2004; Shi et al., 2004). Histone methylation has been associated with both transcriptional activation and repression, depending on the particular residue and the extent of methylation (Su and Tarakhovsky, 2006; Maze and Nestler, 2011). Lysine side chains may be mono-, di- or trimethylated, whereas the arginine side chain may be mono- or dimethylated. At present, there are 24 known sites of methylation on histones - 17 are lysine and 7 are arginine residues (Bannister and Kouzarides, 2005). It has been found that trimethylation of histone H3 lysines K4 (H3K4me3) and K36 (H3K36me3) are highly associated with transcriptional initiation and often correlated with increased levels of transcriptional activity, whereas di- and trimethylation on histone H3 lysines K9 (H3K9me2/3) and K27 (H3K27me2/3) are associated with transcriptional repression (Rice and Allis, 2001; Maze and Nestler, 2011). Histone methylation is dynamically regulated by HMT's and HDM's. The first discovered HDM was lysine specific demethylase 1 (LSD1). LSD1 is flavin-dependent monoamine oxidase which demethylate mono- and dimethylated lysines, specifically histone H3 lysines K4 and K9 (H3K4 and H3K9). Because lysines can be mono-, di-, and trimethylated and LSD1 only mediates mono- and didemethylation, the Jumonji domain-containing (JmjC) histone demethylases (like

JHDM1, JHDM2 - JmjC domain-containing histone demethylase 1 and 2, respectively) were discovered. They are able to demethylate mono-, di-, or trimethylated lysines that allow larger functional control of lysine methylation (Shi and Whetstine, 2007).

2.1.3. Histone phosphorylation

Histone phosphorylation has been shown to be involved in a variety of cellular processes, including transcriptional regulation, apoptosis, cell cycle progression, DNA repair and chromosome condensation (Banerjee and Chakravarti, 2011). The most described histone phosphorylation sites is serine 10 on histone H3 (H3S10). It has been found that this modification stabilizes the HAT (Gcn5) on gene promoters and antagonizes the methylation of lysine K9 on histone H3 (H3K9) and the recruitment of heterochromatin protein 1 (HP1) (Kouzarides, 2007; Renthal and Nestler, 2008). Phosphorylation of serine or threonine residues on histone tails can be accomplished by nuclear kinases such has mitogen- and stress-activated protein kinase (MSK-1) and can be dephosphorylated by protein phosphatases (such as protein phosphatase 1, PP1) (Brami-Cherrier et al., 2009; Koshibu et al., 2009; Day and Sweatt, 2011). The excat mechanism how phosphorylation contributes to transcriptional activation is not well understood, but it is hypothesized that the addition of negatively charged phosphate groups to histone tails neutralizes positive charge of histone tails and reduces their affinity for DNA (Grant, 2001; Tambaro et al., 2010).

2.2. DNA methylation and DNA methyltransferases

DNA methylation represents another important and unique epigenetic mechanism. The methylation of one of the four DNA bases, cytosine, is the relatively stable epigenetic modification, regulating the transcriptional plasticity of mammalian genomes. In DNA methylation, methyl group is added to the 5’ position on the cytosine pyrimidine ring and this occurs primarily where a cytosine (C) occurs next to guanine (G) in the DNA sequence (C-phosphate link-G, or cytosine-guanine dinucleotides, CpG) (Holliday and Pugh, 1975; Klose and Bird, 2006; Wong et al., 2011). The CpG sequences are not evenly dispersed throughout the genome, but are clustered in so-called CpG islands – short regions of 0.5 to 4 kb in length having a rich (60 – 70%) cytosine-guanine content. Over 50 - 60% of all dinucleotides in these islands are CpG, compared to the rest of the genome where the CpG content is ≤ 20% (Bird, 2002). Approximately 50% of CpG islands are located in the promoter regions and around the transcription start sites and are unmethylated in normal cells. Proper DNA methylation is required for normal development of an organism, genetic imprinting and X-chromosomal inactivation (Chahrour and Zoghbi, 2007; Suzuki and Bird, 2008).

DNA methylation is generally considered to suppress gene transcription through recruitment of co-repressor complexes (e.g., HDAC’s and HMT’s) that can modify nucleosome structure (Robinson and Nestler, 2011). Such complexes involve several DNA methyl-CpG-binding domain proteins (MBD, in mammals these are MeCP2, MBD1-4), which are necessary for normal cell growth and development (Robertson and Wolffe, 2000; Chahrour and Zoghbi, 2007; Kim et al., 2009). It should be noted that mechanistically MeCP2 may act as both an activator and repressor of gene transcription (Chahrour et al., 2008).

The methylation of cytosine is catalysed by DNA methyltransferases (DNMTs). In mammalian genomes, DNMT's are enzymes that have demonstrated to mediate the transfer of methyl group from S-adenosylmethionine (SAM or AdoMet) to cytosine (Figure 3) (Eden et al., 2003; Villar-Garea et al., 2003; Goll and Bestor, 2005).

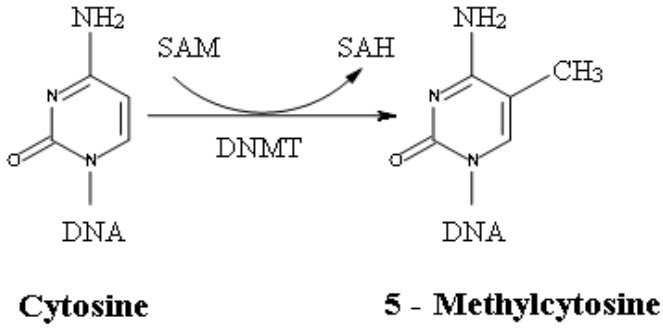

Figure 3. Methylation modification of DNA at the 5-carbon position of cytosine by DNA methyltransferases (DNMT's), where S-adenosylmethionine (SAM) is the methyl group donor ($-CH_3$) and converted to S-adenosylhomocysteine (SAH, modified from Wong et al., 2011).

There are two main enzyme groups: the DNMT1 and DNMT3 families. DNMT1, the first-identified eukaryotic DNMT, is essential for maintaining DNA methylation patterns in proliferating cells as it copies DNA methylation pattern from matrice chain to newly synthesized DNA chain. It is also involved in establishing new DNA methylation patterns (*de novo* methylation) (Bestor, 2000; Goll and Bestor, 2005; Siedlecki and Zielenkiewicz, 2006). The DNMT3 family includes two active *de novo* DNMT's - DNMT3A and DNMT3B, which are necessary for establishing new DNA methylation patterns (Okano et al., 1999), and one regulatory factor, DNMT3-Like protein (DNMT3L) (Bestor, 2000; Goll and Bestor, 2005). DNMT3L has not been shown to possess methyltransferase activity (Bourc'his et al., 2001), but regulates DNMT3A and DNMT3B by stimulating their catalytic activity (Cheng and Blumenthal, 2008). Organisms that contain members of the DNMT1 and DNMT3 families also have DNMT2, which displays weak DNMT activity (Okano et al., 1998; Yoder and Bestor, 1998; Siedlecki and Zielenkiewicz, 2006).

2.2.1. S-adenosylmethionine and DNA methylation

S-adenosylmethionine (SAM or AdoMet), first discovered in 1952, is formed from the essential amino acid methionine and adenosine triphosphate. SAM is the methyl group donor of multiple methylation reactions in all organisms (Chiang et al., 1996; Cheng and Roberts, 2001; Lu, 2000; Bottiglieri, 2002), whereas S-adenosylhomocysteine (SAH) is the product of transmethylation reactions and DNMT inhibitor (Chiang, 1998; Detich et al., 2003). The studies have revealed that exogenous administration of SAM increases the intracellular ratio of SAM to SAH (Garcea et al., 1989; Pascale et al., 2002; Detich et al., 2003). An increase in SAH concentrations, even without a concomitant reduction in SAM, results DNMT inhibition and DNA hypomethylation (Caudill et al., 2001). For that reason, the SAM/SAH ratio has been proposed as a "methylation index" to indicate the likelihood of hyper- or hypomethylation of DNA (Waterland, 2006).

Several studies suggest that exogenous SAM administration can increase the levels of intracellular SAM and trigger hypermethylation of DNA (Watson et al. 1999; Lu, 2000; Fuso et al. 2001), whereas methyl-deficient diets decrease intracellular SAM concentration, increase SAH concentrations, and trigger DNA hypomethylation (Pogribny et al., 1995; Steinmetz et al., 1998; Poirier, 2002). It has been found that dietary supplements, such as SAM, L-methionine (MET) and folic acid increases DNA methylation and thereby alter gene expression (Ross, 2003). SAM has been used as a dietary supplement in Italy (since 1979), Spain (since 1985), Germany (since 1989), in the United States (since 1999) and in several other countries (Bottiglieri, 2002).

2.2.2. DNA demethylation

In contrast to the large amount of information that has accumulated on DNA methylation, DNA demethylation is still a quite controversial and largely unresolved area of research (Kapoor et al., 2005; Wu et al., 2010). DNA demethylation occurs via a series of chemical reactions that modify 5-methylcytosine (5-mC) at two sites, the amino group and the methyl group. An alternative model for DNA demethylation involves the conversion of methylated cytosine (MeC) to thymine through deamination or loss of the amine group following conventional base and nucleotide excision repair processes, a nonmethylated cytosine is resynthesized (Figure 4) (Ma et al., 2009a; Day and Sweatt, 2010).

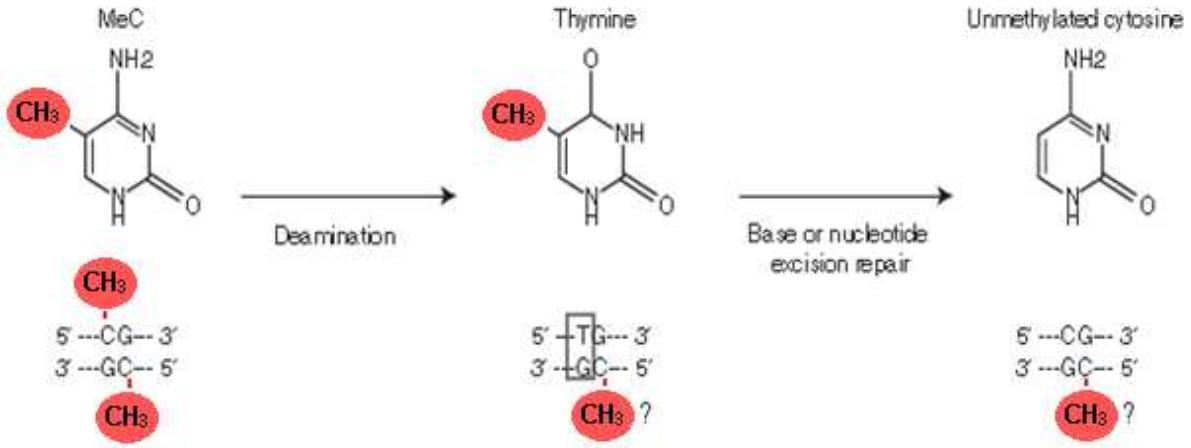

Figure 4. Methylated DNA is deaminated, converted to thymine and base or nucleotide excision repair processes are able to replace thymine with unmethylated cytosine. MeC- methylated cytosine (adapted from Day and Sweatt, 2010).

It is assumed that the Growth Arrest and DNA Damage-inducible protein 45 (GADD45) family of proteins (specifically GADD45β) could participate in each step of this process and thereby catalysing DNA demethylation (Ma et al., 2009b). Furthermore, it seems that DNMT's may also participate in deamination of methylated cytosine in a strand-specific manner (Métivier et al., 2008), giving them a role in both the DNA methylation and demethylation processes. Although, it is not clear how this model would affect methylation status on the complementary DNA strand, this mechanism would enable selective demethylation at specific sites in DNA allowing transience of methylation, active

demethylation and a route of entry for the nucleoside analog inhibitors of DNMT's into the DNA of non-dividing cells (Day and Sweatt, 2010). DNMT inhibitors, such as zebularine or 5-aza-2'-deoxycytidine, may operate by substituting for cytosine during base excision repair processes and this altered base is resistant to methylation and traps DNMT's, resulting in a decrease in DNMT activity and in the demethylation of the newly repaired strand (Szyf, 2009; Day and Sweatt, 2010).

Another active DNA demethylation mechanism is mediated by the ten-eleven translocation (TET) enzymes TET1-3, which add a hydroxyl group onto the methyl group of 5-methylcytosine (5-mC) to form 5-hydroxymethylcytosine (5-hmC) (Tahiliani et al., 2009; Ito et al., 2010). Once 5-hmC is formed, then 2 separate mechanisms can convert 5-hmC back into cytosine in mammals. First, iterative oxidation by TET enzymes continues to oxidize 5-hmC to 5-formylcytosine (5-fC) and then to 5-carboxycytosine (5-caC) (Ito et al., 2011) and the second, 5-hmC is deaminated by AID/APOBEC (activation-induced cytidine deaminase/apolipoprotein B mRNA editing enzyme complex) to form 5-hydroxymethyl-uracil (5-hmU) (Guo et al., 2011; Moore et al., 2013). These data indicate that 5-hmC may also play a role in the process of active DNA demethylation.

2.3. microRNA

Another level of epigenetic regulation by small non-coding RNAs (termed microRNAs) has been discovered (Bergmann and Lane, 2003). MicroRNAs, which generally are around 22 bp long, are posttranslational regulators that bind to complementary sequences on target mRNAs and regulate gene expression at different levels, i.e., the silencing of chromatin (affecting histone modifications), degradation of mRNA and blocking translation (Szyf et al., 2008; Taft et al., 2010; Li and van der Vaart, 2011). Thus, like histone modifications and DNA methylation, microRNAs are also important players in the epigenetic control of gene expression.

2.4. Epigenome

The pattern of epigenetic modifications in the genome, the epigenome, is the result of a complex interplay between enzymes that modify DNA and histones, proteins that could recognize these modifications and microRNAs (Bernstein et al., 2007; Szyf, 2009; Telese et al., 2013). Thus, epigenome consists of the machinery for programming long-term gene expression profiles, that defines gene function and phenotype, which is expressed in behaviour (Szyf, 2009). Unlike the underlying genome which is largely static within an individual, the epigenome can be dynamically altered by environmental factors such as nutrients, chemicals, early life environment (Weaver et al., 2004; Waterland et al., 2006; Szyf, 2009). Therefore, the epigenome provides an essential interface between genes and environment and may be viewed as a potential mechanism underlying the rapid form of environmentally driven adaptation (Franklin and Mansuy, 2010).

2.5. The role of epigenetic mechanisms in drug addiction

2.5.1. Histone modifications in drug addiction

Drugs of abuse (e.g. cocaine, amphetamine, ethanol) induce changes in histone modifications in the brain, and evidence has begun to accumulate that these modifications are related to some of the functional abnormalities found in addiction models (Kumar et al., 2005; Kim and Shukla, 2006; Kalda et al., 2007; Robinson and Nestler, 2011). For example, it has been found that acute and chronic cocaine exposure increased at global level histone H3 and H4 acetylation in the rodent NAc (Kumar et al., 2005). At the gene level, it has been shown that acute cocaine treatment was related to histone H4 hyperacetylation at the promoters of the immediate early genes, such as *c-fos* and *fosB*, while repeated cocaine treatment was associated with histone H3 hyperacetylation at the promoters of *Cdk5* and *Bdnf*.

Further studies have demonstrated that modifications of HDAC activity are substantial regulators of the rewarding properties of cocaine. Short-term systemic or intra-NAc administration of non-specific HDAC inhibitors prior to cocaine or morphine exposure enhances behavioural preferences for places associated with drug delivery (so-called CPP) (Kumar et al., 2005; Sanchis-Segura et al., 2009; Robinson and Nestler, 2011). It has been found that overexpression of HDAC4 or HDAC5 reduces behavioural responses to cocaine (Kumar et al., 2005; Renthal et al., 2007), whereas genetic deletion of HDAC5 hyper-sensitizes mice to the chronic effects (but not to the acute effects) of the drug (Renthal et al., 2007). It has been also demonstrated that mutant mice with decreased expression of CBP (a major HAT in brain), exhibit reduced sensitivity to chronic cocaine exposure (Levine et al., 2005; Robinson and Nestler, 2011). Moreover, chronic cocaine treatment increased expression of two sirtuins, Sirt1 and Sirt2, which are class III of HDAC's. Upregulation of sirtuins expression is associated with increased H3 acetylation and ΔFosB binding at Sirt1 and Sirt2 promoters, which indicates that sirtuins are downstream targets of ΔFosB (Robinson and Nestler, 2011). Pharmacological inhibition of sirtuins reduces CPP and cocaine self-administration, whereas activation increases rewarding responses to cocaine (Renthal et al., 2009). Despite the fact that single cocaine exposure does not alter sirtuin activity, upregulation after chronic cocaine exposure may mediate the stable neuroadaptive changes involved in maintaining addiction (McQuown and Wood, 2010).

There is evidence that histone methylation is also directly regulated by drugs of abuse (Robinson and Nestler, 2011). The experiments by Maze and colleagues (2010) have demonstrated that global levels of histone H3 lysine K9 dimethylation (H3K9me2) are decreased in the mouse NAc after chronic cocaine exposure (Maze et al., 2010). A genome-wide studies by Renthal et al. (2009) revealed alterations in H3K9me2 binding on the numerous genes promoter regions in the NAc. The global reduction in H3K9me2 in the NAc was mediated through the repression of G9a (known as HMT), which was regulated by the cocaine-induced transcription factor ΔFosB (Robinson and Nestler, 2011). It has been reported that acute cocaine exposure enhanced G9a binding in the *fosB* promoter region and therefore rapidly suppressing cocaine-induced increases in ΔFosB expression (Maze and Nestler, 2011). Following repeated cocaine exposure, ΔFosB accumulation in the NAc results in G9a repression and decreased global levels of H3K9me2, preventing G9a's ability to

maintain normal levels of gene expression and enhancing behavioural responses to the drug (Maze et al., 2010; Maze and Nestler, 2011).

Histone phosphorylation is also an important component of the epigenetic responses to drugs of abuse. Brami-Cherrier and colleagues (2009) reported that cocaine induces a robust phosphorylation of histone H3 within the NAc at the promoters of *c-Fos* and *c-Jun* (Brami-Cherrier et al., 2009) and histone H3 phosphorylation is positively regulated by MAPK/extracellular-signal regulated kinase (ERK) cascade, including phosphorylation of ERK and MSK-1-induced phosphorylation of histone H3 (Brami-Cherrier et al., 2005; Bertran-Gonzalez et al., 2008). Nuclear accumulation of 32 kDa dopamine and cAMP-regulated phosphoprotein (DARPP-32) acts to inhibit PP1, thereby preventing histone dephosphorylation (Stipanovich et al., 2008). Critically, these pathways control behavioural responses to cocaine through the inhibition of dopamine D1 receptors, ERK, DARPP-32 and MSK-1, all of which diminish drug-induced locomotor responses or drug CPP (Stipanovich et al., 2008; Brami-Cherrier et al., 2009). Thus, these data confirm that changes in histone modifications may influence the transcription of genes involved in mediating cocaine-induced behaviour.

2.5.2. DNA methylation and drug addiction

Although the majority of studies have focused on histone modifications, DNA methylation is also critical component of the epigenetic response to drug-related behaviours. Several recent studies have provided crucial evidence for the role of DNA methylation in cocaine-induced neuronal plasticity in the NAc and hippocampus (Im et al., 2010; LaPlant et al., 2010, Paper I). Reports demonstrated that acute cocaine treatment induces rapid changes in the expression of *Dnmt3a* and *Dnmt3b* genes in the NAc, suggesting dynamic control of DNA methylation by drugs of abuse. Cocaine treatment also resulted in increased methylation of *protein phosphatase-1 catalytic subunit (PP1c)* promoter region, binding of MeCP2 at promoter and these changes are associated with transcriptional downregulation of *PP1c* in the NAc. In contrast, acute and repeated cocaine administrations induced hypomethylation and decreased binding of MeCP2 at the *fosB* promoter, and these are associated with transcriptional upregulation of *fosB* in NAc (Paper I). Im and colleagues (2010) investigated a possible role for MeCP2 in the dorsal striatum in the escalating cocaine intake seen in rats with extended access to the drug, and found that MeCP2 knockdown prevents escalation of cocaine self-administration during extended access (Im et al., 2010). It has been shown that NAc-specific manipulations that block DNA methylation potentiate cocaine reward and NAc-specific *Dnmt3a* upregulation attenuated cocaine reward (LaPlant et al., 2010). Recent reports indicate that epigenetic changes in brain regions outside of the striatum are also important regulators of drug memories. It has been found that DNA methylation within the hippocampus and prelimbic cortex is necessary for the establishment and maintenance of cocaine CPP (Han et al., 2010; Day and Sweatt, 2011).

2.5.3. microRNA and drug-related behaviour

Several recent studies have demonstrated that microRNAs are important in addiction related behaviours. For example, Hollander and colleagues (2010) found that cocaine self-administration upregulated microRNA miR-212 expression in the rat striatum and enhanced miR-212 levels in this brain region decreased cocaine reward (Hollander et al., 2010). It seems that the actions of miR-212 depend on upregulation of CREB, which is known to decrease the rewarding effects of cocaine (Robinson and Nestler, 2011). Im et al. (2010) demonstrated that MeCP2 may interact homeostatically with miR-212 to control *Bdnf* expression and cocaine intake. Therefore, it has been suggested that CREB-miR212-MeCP2-Bdnf mechanism is partly responsible for cocaine tolerance and escalating intake (Robinson and Nestler, 2011). Moreover, recent studies have demonstrated that overexpression of miR-124 in the NAc of rats reduces cocaine place conditioning, whereas overexpression of miR-181 has the opposite effect. These data indicate that microRNA's may play essential role for drug tolerance and escalating intake (Chandrasekar and Dreyer, 2009; Robinson and Nestler, 2011).

3. The role of early life stress as a predictor for drug addiction

Data from various clinical and preclinical studies have presented that stressful experiences in early life represent one of the major risk factor for the development of a wide range psychopathology, including drug addiction. Early life stress can result in permanent hypothalamic-pituitary-adrenal (HPA) axis changes, morphological changes in the brain and gene expression changes in the mesolimbic dopamine reward pathway, which are implicated in the development of drug addiction (for a review see Enoch, 2011).

Stress, which refers to processes involving perception, appraisal and response to harmful, threatening or challenging events or stimuli (Levine, 2005; Sinha, 2008) activates the HPA axis (Figure 5). The magnitude of the HPA response to stress is a function of the neural stimulation of hypothalamic corticotropin-releasing factor (CRF) release, which stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland (Smith and Vale, 2006). ACTH in turn affects adrenal glands to release glucocorticoids (such as cortisol in humans and corticosterone in rodents). It is well established that glucocorticoids exert their effects via two ligand-dependent transcription factors: the glucocorticoid receptor and the mineralocorticoid receptor, which, in turn, regulate patterns of downstream gene expression during development and in adulthood (McEwen and Sapolsky, 1995; Seckl and Meaney, 2004; Akil, 2005; De Kloet et al., 2005). The rate of secretion of glucocorticoids is regulated by negative feedback at the level of hypothalamus and pituitary to suppress CRF and ACTH (Sapolsky, 1996; De Kloet et al., 1998; McEwen, 1998; Shea et al., 2004). The hippocampus is also important for glucocorticoid negative feedback regulation of the HPA axis. The hippocampus contains a high concentration of both glucocorticoid and mineralocorticoid receptors, and infusion of glucocorticoids into this structure reduces basal and stress induced glucocorticoid release (Diorio et al., 1993; McEwen, 2000; Smith and Vale, 2006)

The activation of the HPA axis in response to acute stress are essential for survival, whereas chronic activation results in increased risk for several physiological problems such as anxiety, depression and addiction to drugs (Sapolsky et al., 2000, Ambroggi et al., 2009; Enoch, 2011).

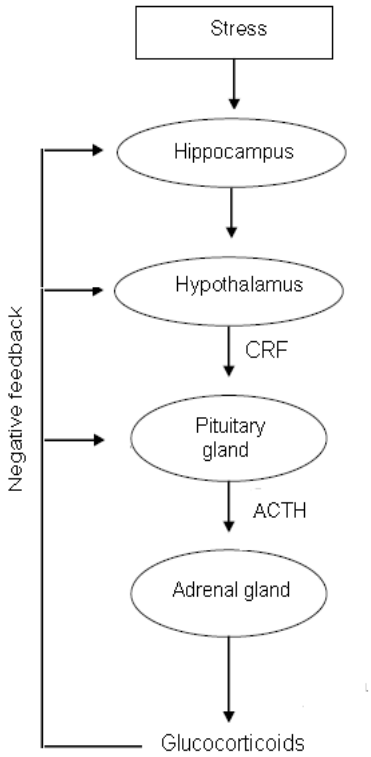

Figure 5. Schematic diagram of the hypothalamic–pituitary–adrenal (HPA) axis (modified from Shea et al., 2004).

Several preclinical studies have demonstrated that early life stress leads to heightened responsiveness to stress and alteration in the HPA system throughout the lifespan (Plotsky and Meaney, 1993; Heim and Nemeroff, 2001; Pryce et al., 2005). Disturbances in mother-infant interaction have been demonstrated as a natural stressor which may lead to maladaptive development (Daniels et al., 2004; Grace et al., 2009). Animal studies indicate that the quality of maternal care (arched back nursing, grooming, licking of pups) in the first two weeks of life influences the development of individual differences in behavioural and HPA responses to stress in offspring (Liu et al. 1997; Caldji et al. 2000; Meaney and Szyf, 2005). For example, it has been found thar poor maternal contact in early life increases plasma ACTH and corticosterone response to stress in adult rats, decreases hippocampal glucocorticoid receptor (GR) mRNA level and diminishes glucocorticoid feedback sensitivity (Liu et al. 1997; Weaver et al., 2001, 2004; Weaver, 2009; Enoch, 2011).

Rodent models of early life stress as maternal separation (MS) and neonatal isolation have been used to investigate the relationship between early life stress and susceptibility to drug addiction. Most of these studies suggest that early manipulations lead to increased drug taking behaviour in adulthood, however, the exact mechanism of how MS alters these behavioural changes is not yet understood.

3.1. Maternal separation and drug addiction

Maternal separation (MS) is an animal model that has been studied to characterize the long-term effects of early life experience on subsequent behaviour in adulthood (Plotsky and Meaney, 1993; Pryce and Feldon, 2003; Daniels et al., 2009). MS model involves the daily separation (15 min to 6 h) of litters from the dams during a critical period of development, usually from postnatal day (PND) 2 to 14 and these brief separations cause profound neurochemical and behavioural changes in the pups that are found in adulthood (Moffett et al., 2007). Rats that are separated for 15 minute per day (MS15) during the first 2 weeks of life (short MS) show less stress reactivity than animals separated for 180 min per day (MS180, long separation). MS15 and MS180 animals have been used in many studies and are usually referred to as “handled” and “maternally separated” animals, respectively. Commonly used control groups were a group reared under standard animal facility conditions (AFR) and a non-handled (NH) group (Meaney et al., 1988; Huot et al., 2002; Moffett et al., 2007).

Model of early life stress, MS and neonatal isolation (model, where pups are separated daily from the dams and also from the littermates) have been demonstrated to affect psychostimulants induced behaviour (Moffett et al., 2007). For example, it has been found that MS alters cocaine-induced locomotor activity in rats and mice (Brake et al., 2004; Kikusui et al., 2005) and behavioural sensitisation to cocaine (Li et al., 2003). Brake and colleagues (2004) have found that MS and NH rats displayed a dose-dependent higher sensitivity to cocaine-induced locomotor activity compared to the handled group (Brake et al., 2004). Studies by Marin and Planeta (2004) have demonstrated that male adolescent rats exposed to MS exhibited an enhanced locomotor response to cocaine, however, this response was not observed in adult rats (Marin and Planeta, 2004). Kikusui and colleagues (2005) have used mice in MS manipulation procedures and found that repeated MS increased the locomotor response to cocaine regardless of a mouse gender (Kikusui et al., 2005). It has been also found that handled rats (exposed to daily 15-min isolation periods as pups) showed an attenuated CPP for amphetamine compared to NH animals (Campbell and Spear, 1999). In addition, several previous studies have demonstrated that a daily neonatal isolation of 1 hour from PND 2-9 enhanced acquisition and maintenance of cocaine self-administration (Kosten et al., 2000; 2004; 2005; Zhang et al., 2005; Moffett et al., 2007) and separated rat pups drink more alcohol and self-administer more cocaine compared to the control animals (Matthews et al., 1996; Kosten et al., 2000; Huot et al., 2001; Flagel et al., 2003). Therefore, it is assumed that early life stress can lead to profound and lasting changes in the responsiveness of mesocorticolimbic dopamine (DA) neurons to stress and psychostimulants (Brake et al., 2004; Enoch, 2011). Studies by Piazza and colleagues have demonstrated that the liability of rats to self-administer drugs can be predicted by the response of mesolimbic DA-ergic neurons to stress - animals, that were more sensitive to the DA-releasing actions of stress were more likely to display addictive behaviour (Piazza et al., 1991; Piazza and LeMoal, 1996). Thus, it is highly likely that stress increases the activity of the dopaminergic brain systems which mediate drug-induced rewarding effects (Brady and Sonne, 1999).

Moreover, a growing body of evidence demonstrates that MS results in increased HPA responsivity to stress, and thus increased adrenal glucocorticoid (GC) release during stress and in turn, glucocorticoids seem to regulate mesolimbic DA systems (Meaney et al., 2002).

For example, it has been found that stress exposure and increased levels of GC enhance DA release in the NAc and suppression of GC by adrenalectomy reduces extracellular levels of DA under basal conditions (for a review see Sinha, 2008). Several animal studies have shown that depletion of GC by adrenalectomy reduces drug and alcohol consumption (Fahlke et al., 1994; Marinelli et al., 1997; Marinelli and Piazza, 2002). It has been also found that mice with deletion of the GR gene show a dose-dependent decrease in motivation to self-administer cocaine (Deroche-Gamonet at al. 2003; Sinha, 2008). Therefore, the HPA axis activation and subsequent release of GC might play also an important role in the acquisition of psychostimulant administration (Piazza and LeMoal, 1996; Mantsch et al., 1998; Goeders, 2002 and 2003; Meaney et al., 2002).

3.2. Maternal separation and epigenetic modifications

There is a growing body of evidence which demonstrate that early life adversity results in a change of epigenome. It has been found that epigenetic modifications, such as DNA methylation, alter gene expression programming in the brain in a way that lasts into adulthood. Using rodent models, several studies have demonstrated that the adult offspring of mothers that exhibit increased levels of pup licking/grooming (referred to as high-LG mothers) during the first week of life show increased hippocampal *GR* expression, decreased *CRF* expression, more modest HPA stress responses compared with low-LG mothers (Liu et al., 1997; Francis et al., 1999; Meaney and Szyf, 2005; Weaver et al., 2006; McGowan et al., 2008). Weaver and colleagues (2004) observed that poor maternal care in rats alters DNA methylation at a specific sequence motif upstream of the glucocorticoid receptor gene (*Nr3c1)* in the hippocampus of the offspring. They found significantly greater methylation of the exon 1_7 *GR* promoter sequence in the offspring of the low-LG mothers (Weaver et al., 2004). These data demonstrate that in low-LG offspring, higher methylation of the *GR* promoter suppress *GR* expression and therefore adult offspring of low-LG mothers have lower levels of GR (Liu et al., 1997; Champagne et al., 2003; Weaver et al., 2004). A study in humans of postmortem hippocampus shows that suicide victims exposed to childhood maltreatment have decreased *GR* (*Nr3c1)* mRNA level and increased cytosine methylation in *Nr3c1* promoter region (McGowan et al., 2009; Enoch, 2011).

The exact mechanism, how maternal behaviour alters epigenetic programming in the offspring brain, is unclear. According to one hypothesis, it is believed that maternal behaviour of the offspring increases hippocampal serotonin (5-HT) turnover and activation of a 5-HT_7 receptor increases cAMP activity. Increased cAMP activity results in activation of PKA and CREB and subsequent phosphorylated-CREB (pCREB) activity drives expression of the transcription factor NGFI-A (nerve growth factor-inducible protein A) (Figure 6). This transcription factor in turn recruits the histone acetyltransferase CBP and the MBD2 to the *GR* promoter (Weaver et al., 2004; Weaver, 2007). It is assumed that the increased histone acetylation triggered by CBP facilitates the demethylation of the gene by MBD2 and/or other DNA demethylases (Szyf, 2009).

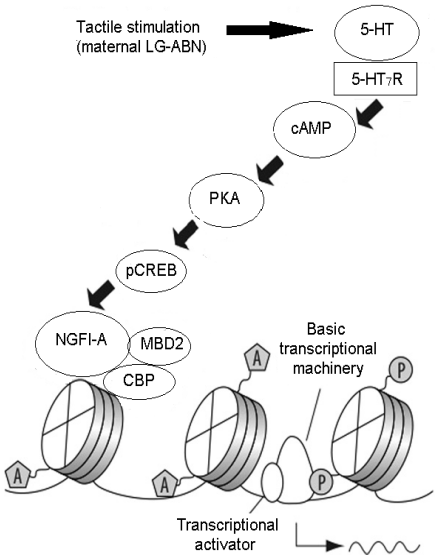

Figure 6. Model of epigenetic reprogramming of hippocampal glucocorticoid receptor gene expression and stress responses by maternal behaviour. (A)-acetylation; (P)-phosphorylation (modified from Bennett, 2011).

However, recent studies have revealed that patterns of epigenetic modification programmed early in life could be reversible using epigenetic modulators. For example, it has been found that injecting the HDAC inhibitor trichostatin A (TSA) into the left lateral ventricle of adult offspring of low-LG mothers reversed the epigenetic programming of the *GR* exon 1_7 promoter and re-established stress responsivity (Cervoni and Szyf, 2001; Cervoni et al., 2002; Weaver et al., 2004). Conversely, it has been found that injecting the amino acid methionine, which is the precursor of SAM into the ventricle of adult offspring of high-LG mother resulted in increased DNA methylation and downregulation of *GR* as well as heightened stress responsivity (Weaver et al., 2004 and 2005; Szyf, 2011).

Early life stress has been shown to bring about epigenetic changes of the other genes. For example, it has been found that early life stress in mice caused enduring hypersecretion of corticosterone and alterations in passive stress coping and this phenotype was accompanied by a persistent upregulation of arginine-vasopressin (*Avp*) expression and sustained DNA hypomethylation in the hypothalamic paraventricular nucleus (PVN) (Murgatroyd et al., 2009). The experiments by Roth and colleagues (2009) have demonstrated that early maltreatment produce persistent changes in methylation of *Bdnf* gene promoter and decreased *Bdnf* expression in the prefrontal cortex of adult rats (Roth et al., 2009). Later, Franklin et al. (2010) found that chronic and unpredictable MS alters the profile of DNA methylation in the promoter of several genes such as *Mecp2*, corticotrophin-releasing factor receptor 2 (*Crhr2*), cannabinoid receptor-1 (*Cnr1*) in the germline of the separated males and comparable changes in DNA methylation are present in the brain of the offspring and are associated with altered gene expression (Franklin et al., 2010).

Altogether, these data suggest that alterations in epigenomic programming have an impact on phenotype, which is expressed in behaviour, and that the early life stress leaves its impact on the genome through systematic readjustment of DNA methylation patterns (Szyf, 2009).

THE AIMS OF THE STUDY

The general aim of the present study was to investigate the role of DNA methylation in the development of cocaine-induced behavioural sensitisation in mice and rats.

The more specific aims for the current thesis were:

1. To determine: a) the role of cocaine treatment on DNMT and selected genes expression in the NAc of mice; and b) to assess the effect of DNMT inhibitor zebularine on cocaine-induced gene expression changes and the development of behavioural sensitisation in adult mice.

2. To investigate the role of methyl donor SAM on cocaine-induced gene expression changes and the development of behavioural sensitisation in adult mice.

3. To evaluate, whether the early life stress could alter DNMT and selected genes expression in the NAc of infant and adult rats, and whether these changes are associated with the development of cocaine-induced behavioural sensitisation in adulthood.

MATERIALS AND METHODS

1. Animals and housing conditions (Papers I-III)

All experiments were performed in accordance with EU guidelines (directive 86/609/EEC) on the ethical use of animals using the experimental protocol approved by the Ethics committee of the University of Tartu Medical Faculty. Male C57BL/6 mice, 4-5 months old, weight 25–30g were obtained from Scanbur BK, Sweden **(**Papers I, II) and 20-25 pairs of male and female Wistar rats were obtained from Harlan Laboratories, Netherlands **(**Paper III). All animals were housed in standard polypropylene cages under temperature and humidity-controlled rooms with 12 h light–dark cycle (light from 7:00 a.m.) and were allowed access to rodent chow and water *ad libitum*. Animals were allowed to acclimate to laboratory conditions and were handled at least 4-7 days before use in behavioural testing. All behavioural experiments were conducted during the light phase in an isolated experimental room

2. Maternal separation and handling procedures (Paper III)

As the pups were born (total 46, from 3-5 litters), female offspring were removed and male offspring were randomly assigned to one of three groups. During the experiment, every dam had 8 pups. The experimental groups are as follows: (1) animal-facility reared (AFR) where the dams and pups were handled only on PND10 for a cage change and were not separated; (2) handled group (MS15) where pups were handled and separated to a new cage on PND2-15 for 15 minutes and then returned to the home cage; (3) maternally-separated group (MS180) where pups were removed from the home cage on PND2-15 for 180 minutes and placed individually into a new cage. At the end of the separation pups were placed back into their home cage with the dam. The manipulation of pups in the MS15 and MS180 groups was initiated at 10:00 a.m., and after the manipulation, we monitored dam and pup behaviour in the home cage for 10 min. We performed seven separation procedures to collect pups from different dams for behavioural and neurochemical tests. We considered AFR as a control group for both handling (MS15) and separation (MS180) groups. A comparison of MS15 and MS180 groups enables collection of “time course” data on maternal separation. Cocaine-induced locomotor activity was evaluated on PND120-140. Animals were allowed to acclimate to laboratory conditions and were handled at least seven days prior to behavioural experiments. All behavioural experiments were conducted during the light phase in an isolated experimental room. For the evaluation of neurochemical changes, rats were euthanised on PND15 and PND120.

3. Drug administration (Papers I-III)

Cocaine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile saline (0.9% NaCl) and administered intraperitoneally (i.p.) at a dose of 15 mg/kg (Paper I) and 10 mg/kg (i.p.) (Papers II, III) immediately prior to locomotor activity training. Zebularine

(Tocris Bioscience, UK) was dissolved in sterile saline and administered intracerebroventricularly (i.c.v.) 300 ng/0.5 µl 20 min before cocaine or saline (0.1 ml/10 g body weight, i.p.) treatment (Paper I). SAM (Sigma-Aldrich Co, St. Louis, MO, USA) was dissolved in sterile saline and administered (i.p.) at a dose of 4520 mg/kg, 10 mmol/kg 20 minutes prior to cocaine treatment (Paper II).

3.1. Intracerebroventricular cannula implantation (Paper I)

For i.c.v. injection cannula implantation, mice were anesthetized with Fentanyl/fluanisone (trade name Hypnorm, VetaPharma, Leeds, UK) and midazolam (trade name Dormicum, Roche, Basel, Switzerland). During surgery, each mouse was implanted with a 26-gauge bilateral stainless steel guide cannula (Plastics One, Roanoke, USA) from which the injector extended 0.5 mm to end in the ventricle. Stereotaxic coordinates were follows: antero-posterior, -0.4 mm from bregma, -1.25 mm lateral from the midline, and -2.0 mm from bregma measured from the tip of the cannula guide (Paxinos and Franklin, 2001). Animals were habituated to dummy cannula removal and given 4 days of recovery and handling before the start of the experimental procedure. At the beginning of the study, target coordinates and proper cannula placement were verified by slowly injecting 1 µl of methylene blue dye into the cannulas of the first four mice, while they were under anesthesia. After waiting 5 min for the dye to circulate through the ventricles, the animals were sacrificed. Brains were removed, sliced at the point of cannula entry and target coordinates were verified.

4. Behavioural experiments

4.1. Locomotor activity measurement (Papers I-III)

Horizontal locomotor activity was monitored in standard polypropylene cages (36 x 20 x 15 cm) that were illuminated uniformly with dim light. A light-sensitive video camera, connected to a computer, was mounted about 1.5 m above the observation cage and locomotor activity of 8 animals at a time was monitored and analysed using VideoMot2 software (TSE Systems, Germany) (Papers I, II). In Paper I, mice (n=11) were randomly assigned to the following treatment groups: (1) Saline ("SAL"), mice were treated for 7 days with saline 0.1 ml/10 g body weigh (i.p.); (2) Acute cocaine ("AC"), mice were treated for 6 days with saline and on the 7th day with cocaine hydrochloride; (3) repeated cocaine ("RC"), mice were treated for 7 days with cocaine hydrochloride. On the 1st and 7th treatment days, locomotor activity was recorded for 60 min after the last injection. On the 2nd through to the 6th treatment days, mice were injected and placed in the test cages for 60 min without locomotion recording. To investigate the effect of zebularine, i.c.v. infusions of zebularine (300 ng/0.5 µl, injection speed 0.1 µl/min) or saline (0.5 µl) were performed 20 min before cocaine or saline (0.1 ml/10 g body weight, i.p.) treatment. Mice (n=7-12) were randomly assigned to the following treatment groups: (1) saline (i.c.v.) + saline (i.p.) "S+S"; (2) zebularine (i.c.v.) + saline (i.p.) "Z+S"; (3) saline (i.c.v.) + cocaine (i.p.) "S+C" and (4) zebularine (i.c.v.) + cocaine (i.p.) "Z+C". Locomotor activity was recorded for 60 min after i.p. injection, daily for 7 days.

In Paper II, mice were treated for 7 days i.p. with sterile saline (0.1 mL/10 g body weight) or SAM (4520 mg/kg, 10 mmol/kg, i.p.) 20 minutes prior to cocaine hydrochloride administration (10 mg/kg, i.p.). Animals (*n*=17-22) were randomly assigned into one of the following treatment groups: (1) Saline + Saline "S+S"; (2) SAM + Saline "M+S"; (3) Saline + Cocaine "S+C" and (4) SAM + Cocaine "M+C". Locomotor activity was recorded for 90 minutes after the second injection on days 1, 3, 5, and 7. On the days 14 and 28, all groups were tested for locomotor activity for 90 minutes after cocaine challenge (7 mg/kg, i.p., *n*=8). On the days 8-13 and 15-27 mice did not receive any treatment. Mice were sacrificed 24 h after the end of the repeated treatment.

In Paper III, cocaine hydrochloride was administered i.p. at a dose of 10 mg/kg immediately prior to locomotor activity training. Locomotor activity was monitored in the standard polypropylene cages (54 x 32 x 20 cm) of 4 animals at a time and analysed. For experiments of acute cocaine treatment, adult male rats (PND120-140) from each of the AFR, MS15 and MS180 groups (*n*=13; weight 485-520g) were treated for four days with saline (2.5 ml/kg, i.p.) and on the fifth day with cocaine hydrochloride (10 mg/kg, i.p.). For experiments assessing the effect of repeated cocaine treatment, all rats received cocaine (10 mg/kg, i.p.) once daily for five consecutive days. On the first, third and fifth treatment days, locomotor activity was recorded for 60 min after treatment. On the second and forth treatment days, rats were treated and placed in the test cages for 60 min without recording locomotion.

4.2. Exploration box test (Paper III)

At PND120, adult males from AFR, MS15 and MS180 groups (*n* = 8 animals in each group) were tested for exploratory behaviour. The test apparatus (modified from Matto et al., 1996 and Mällo et al., 2007) was made of metal and comprised an open area of 50 (width) × 100 (length) × 40 (height) cm with a small compartment (20×20×20 cm) attached to one of the shorter sides of the open area. The open area was divided into eight squares of equal size (25×25 cm) and four objects were situated in specific positions. Three objects were unfamiliar (a glass jar, a cardboard box, a wooden handle) and one was familiar (food pellet), and the objects remained the same throughout the experiment. The floor of the small compartment was covered with wood shavings and was directly linked to the open area via an opening (size 20 cm × 20 cm). The rat was placed into the small compartment, which was then covered with a lid, and during the 15 min test session, the following measures were taken by the observer: (a) latency to enter the open area with all four paws; (b) entries into the open area; (c) line crossings; (d) rearings; (e) exploration of the three unfamiliar objects in the open area and (f) the time spent exploring the open area. The exploration test box was cleaned after each animal. To provide an index of exploration (considering both the elements of inquisitive and inspective exploration), the scores for line crossings, rearings and object investigations were summed for each animal.

5. Tissue isolation (Papers I-III)

Mice and rats were sacrificed by decapitation. The NAc, hippocampus, cerebellum and prefrontal cortex (PFC) were rapidly dissected out on chilled ice-cold plate and frozen

immediately in liquid nitrogen and stored at -80 °C until RNA and DNA extracts and protein lysates were prepared. The NAc was dissected out using a round-shape puncher.

6. PC12 cells and DNMT activity measurement (Paper II)

Rat pheochromocytoma cells (PC12) purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) were cultured in a humidified 5% CO_2/95% air atmosphere at 37 °C. The PC12 cell line was maintained in RPMI 1640 medium (Gibco CO, USA) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (Gibco CO, USA). Cells were grown on polyethyleneimine (Sigma-Aldrich, St. Louis, MO, USA) coated plastic dishes (Nunc, Thermo Fisher Scientific, MA, USA) at a density of 10^6 cells/ml. The cells from the 4^{th} to 7^{th} passages were treated with 0.5 mM SAM and used for the DNMT activity, gene expression, and DNA methylation experiments. For single SAM treatment, 0.5 mM SAM solution (prepared in RPMI medium) was added to growing cells at time points 0, 2, 6, 12 and 24 hours. For repeated SAM experiment, 0.5 mM SAM was added to the cells once a day for 7 days and nuclear extract was prepared 0, 2, 6, 12 and 24 hours after last treatment. Control measurements were performed with vehicle (RPMI medium) treated cells. Nuclear extract was isolated from cells using EpiQuik Nuclear Extraction kit (Epigentek Group, Brooklyn, USA). Total DNMT activity was determined using an EpiQuik DNMT activity assay kit (Epigentek Group, Brooklyn, USA). DNMT activity (OD/mg/h) was calculated according to the formula:

$$X = \frac{OD(sample) - OD(blank)}{protein\ amount\ (mg) \times time\ (hour)} \times 1000$$

Two dishes combined as a sample, 4 samples per group were used. Experiments were repeated twice.

7. Gene expression analyses

7.1. Measuring mRNA levels by qPCR (Papers I-III)

Total RNA was extracted from mouse or rat NAc, hippocampus, cerebellum, PFC and from cells using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesised from 400 ng or 750 ng of total RNA using the First Strand cDNA Synthesis Kit (Fermentas Inc, Burlington, Canada). Quantitative PCR (qPCR) was performed using an ABI PRISM 7000 Sequence Detection System equipped with ABI Prism 7000 SDS Software (Applied Biosystems Inc, USA). Primers for mouse or rat *Dnmt1*, *Dnmt3a*, *Dnmt3b*, $A_{2A}R$, *fosB*, *Reln* were designed using Primer3 with BLAST sequence verification. Primers were synthesised by TAG Copenhagen AS (Copenhagen, Denmark) and were listed in table 1. Commercial assays were from SABiosciences (Qiagen, Hilden, Germany) for measuring mouse *PP1c* (Cat. No. PPM37272B), *Cck* (Cat. No. PPM24836G), *Gal* (Cat. No. PPM25148F), *Slc17a7* (Cat. No. PPM35361A) and rat *PP1c* (Cat. No. PPR42515B) mRNA expression. PCR amplification was performed in a total

reaction volume of 25 µl in three parallels. The reaction mixture consisted of 1 µl First Strand cDNA diluted template, 12.5 µl 2x Master SYBR Green qPCR Master Mix (Applied Biosystems Inc, USA), 10.5 µl H_20 and 1 µl gene-specific 10 µM PCR primer pair stock. The PCR amplification was performed as follows: denaturation step at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min, repeated for 40 cycles. SYBR Green fluorescence was measured after each extension step and amplification specificity was confirmed by melting curve analyses and gel electrophoresis of the PCR products. Serial dilutions (fivefold) of total RNA from one control sample were analysed for each target gene and used to construct linear standard curves from which the concentrations of the test sample and efficiency of the PCR reaction were calculated. Results were normalised to *β-actin* or *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase) using the ΔΔCT method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

7.2. Gene expression profiling (Paper II)

Total RNA was extracted from mouse NAc as described in the section 7.1. Tissues from two animals were combined to a sample, 4 samples per group used. RNA quantity and quality were assessed using the NanoDrop-1000 spectrophotometer and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Labeled cRNA was prepared using Illumina TotalPrep RNA amplification Kit according to the manufacturer's instructions using 750 ng of total RNA as a template (Ambion Inc, Austin, TX, USA). The Illumina BeadChip platform (Illumina, San Diego, USA) and the corresponding whole-genome Mouse Ref-8 v2.0 BeadChip (approximately 25,698 transcripts; over 19,100 genes) were used for the gene expression analysis. The raw data was analysed with Illumina BeadStudio Gene Expression Module v3.3.7 (Illumina, San Diego, USA). Further data analysis was performed with R version 2.13.0 (http://www.r-project.org)/Bioconductor software (www.bioconductor.com) using lumi (Du et al., 2008) and limma packages (Smyth, 2005). The 'fdr' method to adjust the *p* values for multiple testing was used to control the false discovery rate (Benjamini and Hochberg, 1995). With a statistical discrimination *p* value set at less than 0.05, limma software and B-statistics analyses were used to identify up- and downregulated genes and filtered for 1.5-fold or greater differences in expression in accordance with standards for microarray analysis (Allison et al., 2006). Gene ontology analysis was conducted using DAVID Bioinformatics Resources (Huang et al., 2009).

Microarray data have been deposited in the Gene Expression Omnibus (GEO) repository (Edgar et al., 2002) and are accessible through GEO Series accession number GSE48365 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48365).

8. DNA methylation assays

8.1. Methylated DNA immunoprecipitation (Papers I-III)

Methylated DNA immunoprecipitation (MeDIP) was performed using the EpiQuik Methylated DNA (Paper III) and Tissue Methylated DNA Immunoprecipitation kit (Epigentek Group Inc, USA) according to the manufacturer's protocol. Genomic DNA was

extracted from mouse or rat (PND15 and PND120) NAc, cerebellum, PFC, sonicated into fragments ranging from 200-1000 bp in size and divided into immunoprecipitated (IP) and input (IN) portions. IP DNA was incubated with anti-5-methylcytosine monoclonal antibody to bind methylated DNA. Normal mouse IgG from manufacturers of the EpiQuik MeDIP kit was used as a negative control. Methylated DNA (750 ng) was subjected to qPCR using commercial assays from SABiosciences (Qiagen, Hilden, Germany) for mouse *Dnmt3a* (Cat. No. EPMM102350-1A), *Dnmt3b* (Cat. No. EPMM106719-1A), *PP1c* (Cat. No. EPMM108835-1A), *fosB* (Cat. No. EPMM109802-1A), *Cck* (Cat. No. EPMM111951-1A), *Gal* (Cat. No. EPMM105518-1A), *Slc17a7* (Cat. No. EPMM110039-1A) (Papers I, II) and for rat *Dnmt3a* (Cat. No. EPRN107117-1A), *PP1c* (Cat. No. EPRN102434-1A), $A_{2A}R$ (Cat. No. EPMM100909-1A) and *Reln* (Cat. No. EPRN105980-1A) (Paper III). To evaluate the relative enrichment of target sequences after MeDIP, the ratios of the signals in the IP DNA vs. IN DNA was calculated. The resulting values were standardised against the unmethylated control sequence *Gapdh* and fold changes were calculated.

8.2. Methylation-specific qPCR analysis (Papers I, III)

DNA was isolated from mouse and rat NAc using QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany) and processed for bisulphite modification using Epitect Bisulfite Kit (QIAGEN, Hilden, Germany). qPCR was used to determine the DNA methylation status of mouse and rat *PP1c* and of mouse *fosB* genes. Methylation-specific qPCR primers were designed using Methprimer software (www.urogene.org/methprimer). Methylation-specific and unmethylated PCR primers were designed to target putative CpG islands detected *in silico* in the promoter or non-promoter regions of the *PP1c* and *fosB* genes and were synthesised by TAG Copenhagen AS (Copenhagen, Denmark). Primer sequences are listed in table 1. PCR reactions were performed as described in the section 7.1. The comparative Ct method was used to calculate differences in methylation between samples ((Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

8.3. Primer sequences (Papers I-III)

Table 1. The sequences of all primers (from TAG Copenhagen AS) used in the study.

Target gene	Primer sequences	Species
	mRNA qPCR primers	
Dnmt1 Forward:	CCCATGCATAGGTTCACTTCCTTC	Mouse
Dnmt1 Reverse:	TGGCTTCGTCGTAACTCTCTACCT	Mouse
Dnmt3a Forward:	GCCGAATTGTGTCTTGGTGGATGACA	Mouse
Dnmt3a Reverse:	CCTGGTGGAATGCACTGCAGAAGGA	Mouse
Dnmt3b Forward:	TTCAGTGACCAGTCCTCAGACACGAA	Mouse
Dnmt3b Reverse:	TCAGAAGGCTGGAGACCTCCCTCTT	Mouse
fosB Forward:	ACAGATCGACTTCAGGCGGA	Mouse
fosB Reverse:	GTTTGTGGGCCACCAGGAC	Mouse
$A_{2A}R$ Forward:	AACCTGCAGAACGTCAC	Mouse
$A_{2A}R$ Reverse:	GTCACCAAGCCATTGTACCG	Mouse
Gapdh Forward:	GTCATATTTCTCGTGGTTCACACC	Mouse
Gapdh Reverse:	CTGAGTATGTCGTGGAGTCTACTGG	Mouse
β-Actin Forward:	ATGGTGGGAATGGGTCAGAAG	Mouse
β-Actin Reverse:	TCTCCATGTCGTCCCAGTTG	Mouse
Dnmt1 Forward:	AACGGAACACTCTCTCTCACTCA	Rat
Dnmt1 Reverse:	TCACTGTCCGACTTGCTCCTC	Rat
Dnmt3a Forward:	CAGCGTCACACAGAAGCATATCC	Rat
Dnmt3a Reverse:	GGTCCTCACTTTGCTGAACTTGG	Rat
Dnmt3b Forward:	GAATTTGAGCAGCCCAGGTTG	Rat
Dnmt3b Reverse:	TGAAGAAGAGCCTTCCTGTGCC	Rat
$A_{2A}R$ Forward:	AGTCAGAAAGACGGGAAC	Rat
$A_{2A}R$ Reverse:	CAGTAACACGAACGCAA	Rat
Reln Forward:	CTGCTGGACTTCTCTACGGAT	Rat
Reln Reverse:	CAGTAGAGGTGGAAGGATGGG	Rat
Gapdh Forward:	TGCCATCACTGCCACTCAGA	Rat
Gapdh Reverse:	GTCAGATCCACAACGGATACATTG	Rat
β-Actin Forward:	GTAACCCGTTGAACCCCATT	Rat
β-Actin Reverse:	CCATCCAATCGGTAGTAGCG	Rat
	Methylation-specific qPCR primers	
fosB methylated Forward:	TGTTAATTTTAGTTTTCGGGATAGC	Mouse
fosB methylated Reverse:	TACGTCAAAAAAAATCCCTCG	Mouse
fosB unmethylated Forward	TTAATTTTAGTTTTTGGGATAGTGT	Mouse
fosB unmethylated Reverse	ATTACATCAAAAAAAATCCCTCACT	Mouse
PP1c methylated Forward:	TTTTATGGGTTCGTAAAGAAGTTTC	Mouse
PP1c methylated Reverse:	ACGAAAAAAACAAAATAACCGC	Mouse
PP1c unmethylated Forward:	TTTATGGGTTTGTAAAGAAGTTTTG	Mouse
PP1c unmethylated Reverse:	ACCACAAAAAAAACAAAATAACCAC	Mouse
PP1c methylated Forward:	TTTTATGGGTTTGTAAAGAAGTTTC	Rat
PP1c methylated Reverse:	CACGAAAAAAACAAAATAACCG	Rat
PP1c unmethylated Forward:	TTTATGGGTTTGTAAAGAAGTTTTG	Rat
PP1c unmethylated Reverse:	ACCACAAAAAAAACAAAATAACCAC	Rat

9. Global DNA methylation analysis (Paper III)

Total DNA was isolated from rat NAc and PFC using the QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany). Global DNA methylation analysis was performed using a MethylFlash Methylated DNA Quantification kit (Epigentek Group Inc., Farmingdale, NY, USA) according to the manufacturer's instructions. Briefly, sample DNA (100 ng) was bound to high-DNA-affinity strip wells. The methylated fraction of DNA was detected using capture and detection antibodies and then quantified colorimetrically by reading the absorbance at 450 nm using a Tecan Sunrise microplate reader (Tecan Group Ltd., Switzerland). The amount of methylated DNA is proportional to the measured OD intensity. The percentage of methylated DNA (5-mC) in total DNA was quantified according to the manufacturer's protocol and formula.

10. Chromatin immunoprecipitation assay (Paper I)

Chromatin immunoprecipitation (ChIP) of genomic DNA associated with MeCP2) was carried out according to the manufacturer protocol (Millipore Inc, USA). Mouse NAc was minced to small pieces and cross-linked in 1% formaldehyde (10 μl/mg) for 15 min at 37 °C. The minced, fixed tissue was homogenized in SDS lysis buffer, sonicated to produce 200-1000 bp genomic fragments, centrifuged for 15 min at 13,000 x g and the supernatant was used for ChIP assay. Immunoprecipitations were carried out at 4 °C overnight with 5 μg of rabbit polyclonal MeCP2 antibody (AbCam, Cambridge, UK), negative control was anti-Rat IgG (Vector Laboratories Inc, Burlingname, USA) and positive control was anti-RNA Polymerase II from manufacturers ChIP kit. A portion of the sonicated DNA was left untreated to serve as input control. Immune complexes were collected with protein A beads and, according to the manufacturers protocol, sequentially washed two times with low salt buffer, high salt buffer, LiCl immune complex buffer and TE (Tris-EDTA) buffer. Immunoprecipitated DNA was subjected to qPCR using ChIP commercial assays from SABiosciences for mouse *PP1c* (Cat. No. GPM1037458(-)01A) and *fosB* (Cat. No. GPM1052791(-)01A).

11. Western blotting (Papers I, III)

The NAc was lysed in 10 vol (w/vol) of RIP-A lyses buffer: 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X 100 and protease inhibitors, homogenized, incubated 1 h at 4 °C and centrifuged 13,000 x g for 10 min at 4 °C (Papers I, III). The supernatants were resolved by electrophoresis on 8% SDS-polyacrylamide gel. Proteins were transferred onto Hybond™-P PVDF Transfer Membranes (Amersham Biosciences, UK) in 0.1 M Tris-base, 0.192 M glycine and 20% (w/w) methanol using an electrophoretic transfer system. The membranes were blocked with 0.1% (w/w) Tween 20/TBS (T-TBS) containing 5% (w/w) non-fat dried milk at room temperature for 1 h. After blocking the membranes were incubated overnight with one of the following antibodies: the anti-PP1γ1 polyclonal antibody (1:1,000, Millipore, Temecula, CA, USA) followed by incubation with biotinylated anti-rabbit IgM secondary antibody (1:1,000) for 2 h and ABC solution for 30 min (Paper I); chicken anti-Dnmt3a

(1:1000; ab14291; AbCam, USA) followed by incubation with secondary antibody anti-chicken HRP conjugate (1:2000; Pierce, USA) (Paper III). The membranes were incubated with ECL detection reagent (ECL, Amersham, UK) for 5 min to visualise proteins, and then exposed to autoradiography X-ray film (Amersham hyperfilm ECL, UK). To normalise immunoreactivity of the proteins, β-actin was measured on the same blot using a mouse monoclonal anti-β-actin antibody (1:10000; Sigma, St. Louis, USA) followed by incubating with an anti-mouse HRP-conjugated secondary antibody (1:2000; Pierce, US). The ratio of proteins of interest to β-actin were calculated and expressed as the mean OD ratio in arbitrary units ± SEM.

12. Statistical analysis (Papers I-III)

Behavioural data of all experiments were analysed using one-way or two-way ANOVAs for repeated measures and followed with Bonferroni's post-hoc tests. Expression levels of genes and proteins and MeDIP and global DNA methylation and ChIP data were analysed using one-way or two-way ANOVAs or t-tests. Differences in methylation-specific qPCR and DNMT activity data were analysed using two-way ANOVAs with Bonferroni's post-hoc tests. GraphPad Prism software (GraphPad, San Diego, CA, USA) was used for statistical analyses, and all data are expressed as the mean ± SEM. Significance levels were set to $p<0.05$.

RESULTS

1. The effect of DNA methylation on cocaine-induced behavioural sensitisation in mice (Paper I)

1.1. Acute and repeated cocaine treatment increases locomotor activity of mice

The effects of acute (AC) and repeated cocaine (RC) treatment on adult mice were assessed. Our data showed that AC treatment significantly increased the locomotor activity of mice (Figure 7; two-way ANOVA repeated measures, treatment $F_{2,35}$=18.9, p<0.0001; days effect $F_{1,35}$=28.81, p<0.0001; interaction $F_{2,35}$=7.43, p=0.002). We also found that after a daily injection of cocaine for 7 days (RC), all of the mice displayed a significantly enhanced locomotion in response to the same dose of cocaine on day 7 compared with day 1 (p<0.001) indicating the behavioural sensitisation.

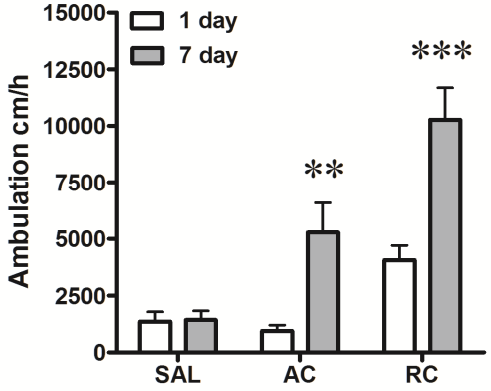

Figure 7. Cocaine-induced behavioural sensitisation in mice. Mice were treated daily for 7 days and ambulation was recorded for 1 h immediately after treatment. Treatment groups: saline (SAL), mice were treated with saline; acute cocaine (AC), mice were treated with saline for 6 days and with cocaine on the 7th day; repeated cocaine (RC), mice were treated daily for 7 days with cocaine. Two-way ANOVA, Bonferroni post-hoc test, **p<0.01 AC treatment 1st vs. 7th day, ***p<0.001 RC treatment 1st vs. 7th day, n=11. Error bars indicate SEM.

1.2. The effect of cocaine treatment on DNMT expression in the NAc and hippocampus

Earlier studies by Miller and Sweatt (2007) have demonstrated that mRNA levels of *Dnmt3a* and *Dnmt3b* were upregulated in the adult rat hippocampus following contextual fear conditioning. In our study, using qPCR, we found that AC administration displayed an increase of *Dnmt3a* mRNA levels in both time points (1.5 h and 24 h) and *Dnmt3b* mRNA level was increased at 24 h after AC treatment compared to saline control (Figure 8A-C). However, the RC treatment did not change significantly *Dnmt3a* and *Dnmt3b* mRNA levels compared to the saline control. There were also no significant changes of *Dnmt1* mRNA levels after AC and RC treatment.

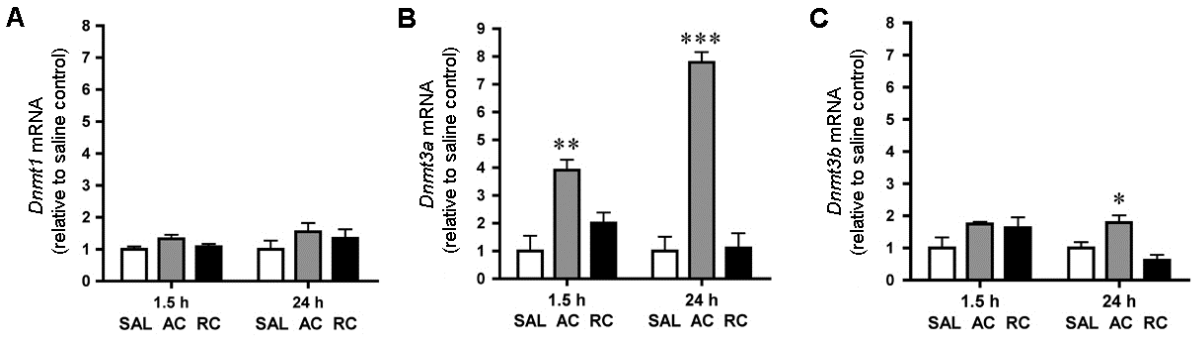

Figure 8. Acute (AC) and repeated (RC) cocaine treatment effects on (**A**) *Dnmt1*, (**B**) *Dnmt3a*, and (**C**) *Dnmt3b* mRNA levels at 1.5 and 24 h after treatment in the NAc. One-way ANOVA, Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001 compared with respective saline (SAL) group, n=11. Error bars indicate SEM.

We also assessed AC and RC treatment effects on *Dnmt's* mRNA levels in the hippocampus. Our data showed that AC and RC treatment increased *Dnmt3a* mRNA levels 1.5 h after treatment and *Dnmt3b* mRNA was enhanced only after AC treatment (Figure 9A-C). There were no changes in *Dnmt3a* and *Dnmt3b* mRNA levels 24 h after cocaine treatment and both AC and RC treatment did not alter *Dnmt1* mRNA level in the hippocampus of adult mice.

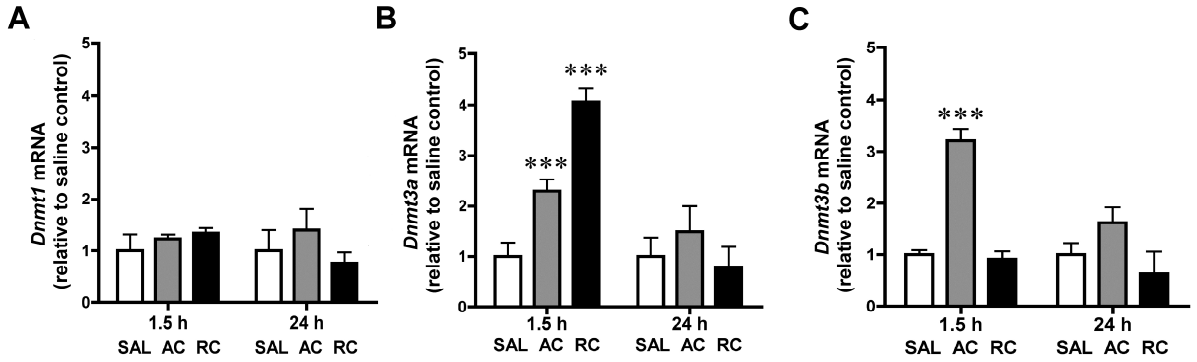

Figure 9. Acute (AC) cocaine treatment was associated with an upregulation of *Dnmt3a* and *Dnmt3b* mRNA levels in the hippocampus. (**A-C**) Cocaine effect on *Dnmt1*, *Dnmt3a* and *Dnmt3b* mRNA levels 1.5 h and 24 h after treatment in the hippocampus. One-way ANOVA, Bonferroni post-hoc test, ***p<0.001 compared with respective saline (SAL) group, n=8. Error bars indicate SEM.

1.3. Cocaine treatment alters selected genes expression in the NAc

Dnmt3a and *Dnmt3b* upregulation might cause hypermethylation of certain genes promoters and consequently downregulate the expression of these genes. Therefore, a search for genes that show a diminished expression following cocaine treatment was conducted. Several genes were tested, such as *Bdnf, PP1c, fosB,* and *adenosine A_{2A} receptor ($A_{2A}R$)* that participate in cocaine-induced neuroadaptations in the NAc and *in silico* analysis revealed CpG islands located within promoter regions. Our qPCR data showed that *PP1c* mRNA levels were not

altered 1.5 h after AC and RC treatment (Figure 10A). However, 24 h after AC and RC treatment, the *PP1c* mRNA levels were significantly decreased compared with the saline control. To further confirm *PP1c* mRNA downregulation in the NAc, western blot analysis was performed. We found that PP1C protein level was also significantly decreased 24 h after AC and RC treatment compared to the saline control (Figure 10B).

As expected, it was found that AC and RC treatment resulted in upregulation of *fosB* mRNA levels 1.5 h after treatment (Figure 10C), but there were no significant changes 24 h after treatment. We also found that both AC and RC treatment decreased $A_{2A}R$ mRNA level 1.5 h after treatment (Figure 10D). However, we could not found significant changes in *Bdnf* mRNA levels (data not shown). As our aim was to investigate cocaine-induced long-lasting effects, for future DNA methylation studies *PP1c* and *fosB* genes were selected.

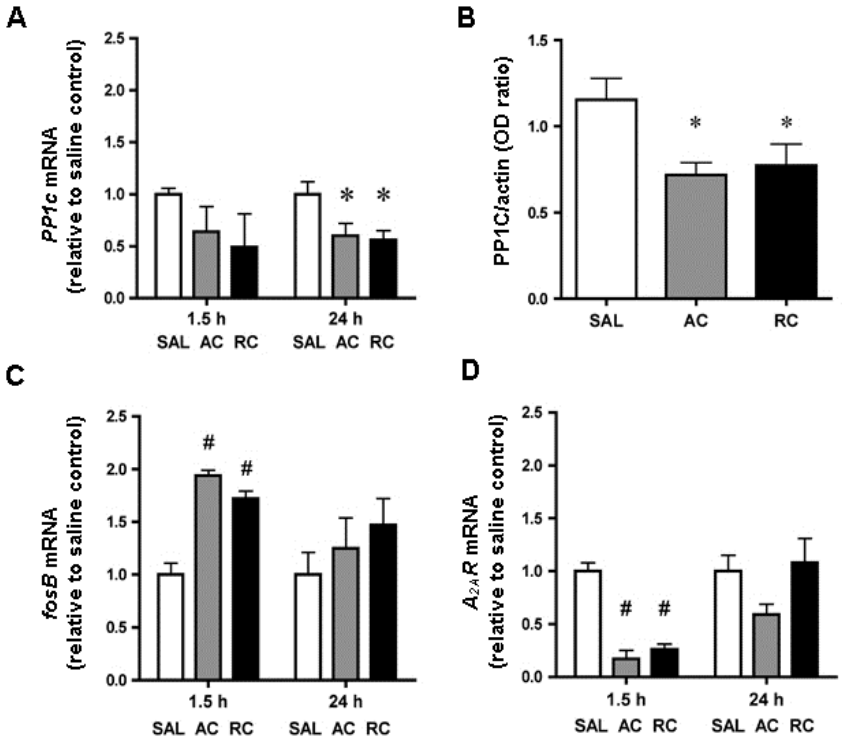

Figure 10. Altered patterns of *PP1c*, *fosB*, and $A_{2A}R$ genes expression after cocaine treatment in the NAc. (**A**) The effect of acute (AC) and repeated cocaine (RC) administration on *PP1c* mRNA levels at 1.5 and 24 h after treatment. (**B**) AC and RC treatment resulted in decrease of PP1C protein level 24 h after treatment in the NAc. (**C**) The effect of AC and RC administration on *fosB* and (**D**) on $A_{2A}R$ mRNA levels. One-way ANOVA, Bonferroni post-hoc test, $^{*}p<0.05$, $^{\#}p<0.001$ compared with saline (SAL) group, n=11. Error bars indicate SEM. OD, optical density.

1.4. Cocaine treatment alters DNA methylation in the *PP1c* and *fosB* promoters

To determine the role of DNA methylation on cocaine-induced behavioural sensitisation, cocaine-induced changes of DNA methylation patterns at the *PP1c* promoter region was evaluated. Using MeDIP assay, we found that both AC and RC treatment resulted in *PP1c* promoter associated CpG island hypermethylation 24 h after treatment (Figure 11B).

Methylation-specific qPCR (MSP qPCR) data confirmed DNA hypermethylation at the *PP1c* promoter region 24 h after AC and RC treatment (Figures 11A,C; two-way ANOVA, treatment $F_{2,66}$=2.77, p=0.0699; methylation effect $F_{1,66}$=31.24, p<0.0001; interaction $F_{2,66}$=8.34, p=0.0006).

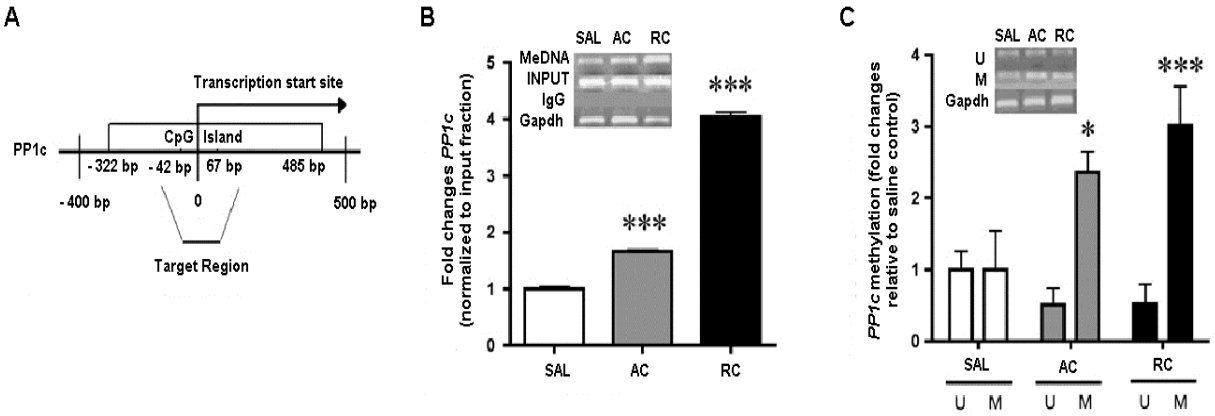

Figure 11. Cocaine treatment was associated with altered DNA methylation at *PP1c* promoters in the NAc at 24 h after treatment. (**A**) The position of the *PP1c* promoter-associated CpG island. The target region indicates the locations of methylation-specific qPCR (MSP qPCR) primer pairs. (**B**) Acute (AC) and repeated cocaine (RC) treatment elicited DNA hypermethylation associated with the *PP1c* promoter region, using methylaten DNA immunoprecipitation (MeDIP) assay. One-way ANOVA, Bonferroni post-hoc test, ***p<0.001 compared with saline (SAL) group, n=6. (**C**) AC and RC treatment elicited hypermethylation associated with the *PP1c* promoter region, using MSP qPCR. Two-way ANOVA, Bonferroni post-hoc test, *p<0.05, ***p<0.001 compared with methylated SAL group, n=11. Error bars indicate SEM. M, methylated; MeDNA, methylated DNA; U, unmethylated.

In correlation with upregulated *fosB* mRNA levels, our MeDIP data demonstrated hypomethylation at the *fosB* promoter 1.5 h after AC and RC treatment (Figures 12A,B).

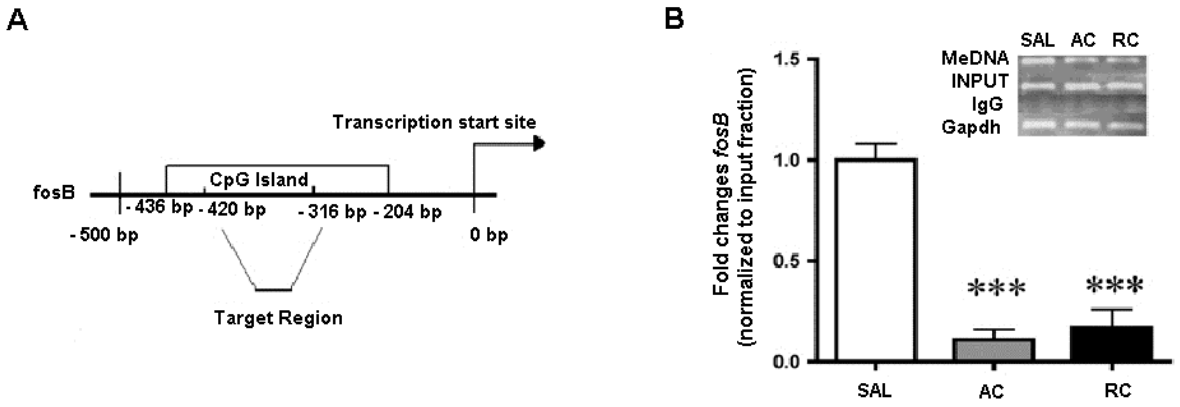

Figure 12. Cocaine treatment was associated with altered DNA methylation at *fosB* promoters in the NAc at 1.5 h after treatment. (**A**) The position of the *fosB* promoter-associated CpG island. The target region indicates the locations of the qPCR primer pairs. (**B**) Acute (AC) and repeated cocaine (RC) treatment elicited DNA hypomethylation associated with the *fosB* promoter region, using methylated DNA immunoprecipitation (MeDIP) assay. One-way ANOVA, Bonferroni post-hoc test, ***p<0.001 compared with saline (SAL) group, n=6. Error bars indicate SEM. MeDNA, methylated DNA

To further confirm that AC and RC administration altered DNA methylation at the promoter regions of *PP1c* and *fosB* genes, ChIP assay was performed. Consistent with the MeDIP and MSP qPCR results, ChIP analysis demonstrated that AC and RC cocaine treatment increased by 1.8- to 2-fold the *PP1c* promoter-associated MeCP2 binding 24 h after treatment in the NAc (Figure 13A). However, in the *fosB* promoter, AC and RC treatment was associated with a significant decrease in MeCP2 binding compared with the saline control at 1.5 h after treatment (Figure 13B).

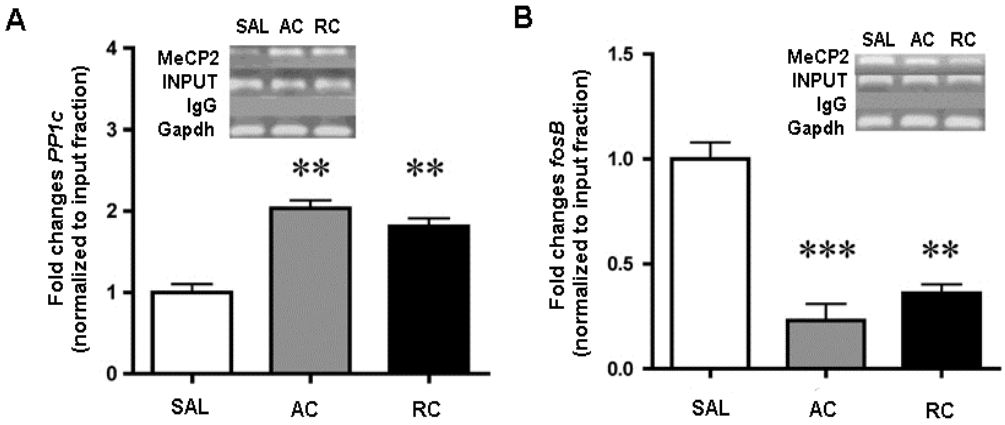

Figure 13. Altered patterns of MeCP2 binding at *PP1c* and *fosB* promoters in the NAc using chromatin immunoprecipitation (ChIP) assay. (**A**) Acute (AC) and repeated cocaine (RC) treatment increased MeCP2 binding at the *PP1c* promoter at 24 h after treatment and (**B**) decreased MeCP2 binding at the *fosB* promoter region at 1.5 h after treatment. One-way ANOVA, Bonferroni post-hoc test, **p<0.01, ***p<0.001 relative to SAL, n=6. Error bars indicate SEM.

1.5. The effect of DNMT inhibitor zebularine on cocaine-induced DNA methylation and the development of behavioural sensitisation in mice

As we found that cocaine treatment increased *Dnmt3a* and *Dnmt3b* mRNA levels and causes *PP1c* promoter region hypermethylation, the effect of DNMT inhibitor zebularine on cocaine-induced molecular changes and the development of behavioural sensitisation were assessed. To correlate the molecular changes with cocaine-induced behavioural sensitisation, a dose of zebularine (300 ng per 0.5 ml, i.c.v.) that did not affect basal locomotor activity and decreased DNA methylation at the *PP1c* promoter region at 1.5 h after acute treatment (Figure 14A) was selected from our pilot study.

For the co-treatment experiment, all mice received i.c.v. infusion of saline or zebularine followed after 20 min by i.p. saline or cocaine (15 mg/kg). Locomotor activity was recorded for 60 min immediately after the last i.p. injection. Repeated saline and cocaine (S+C) treatment for 7 days displayed a significantly enhanced locomotion on day 7 compared with that of day 1 (Figure 14B; two-way ANOVA with repeated measures, treatment $F_{3,39}$=15.98, p<0.0001; days effect $F_{1,39}$=10.26, p=0.0027; interaction $F_{3,39}$=5.69, p=0.0025). However, mice co-treated with zebularine and cocaine (Z+C) did not show any sensitisation on day 7 compared with day 1 (p<0.05). There was also no behavioural sensitisation after repeated zebularine and saline (Z+S) treatments (p<0.05). Thus, our results suggest that the inhibition of DNMT's with zebularine did not affect acute cocaine-induced locomotor activity, but instead delayed cocaine-induced behavioural sensitisation in mice.

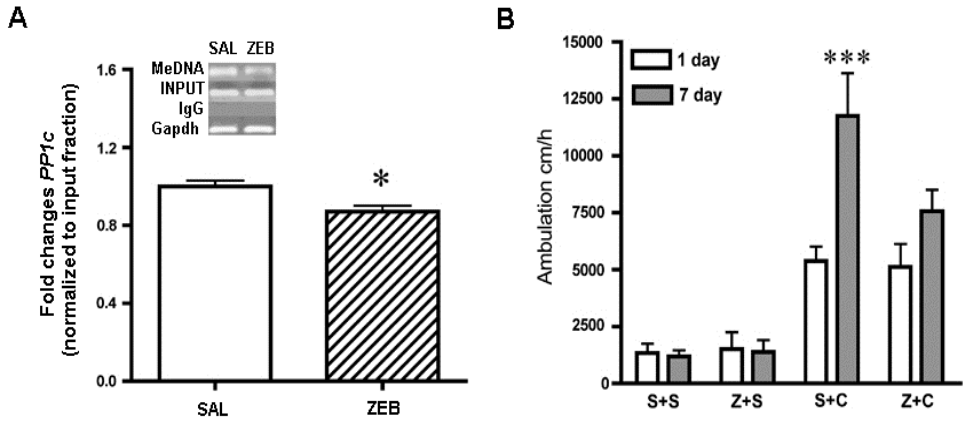

Figure 14. Inhibition of DNMT by zebularine delayed cocaine-induced behavioural sensitisation. (**A**) Acute zebularine (ZEB) treatment elicited DNA hypomethylation associated with the *PP1c* promoter region at 1.5 h after treatment in the NAc, using methylated DNA immunoprecipitation (MeDIP) assay. T-test, *p=0.034, n=4. (**B**) Mice were treated with cocaine (15.0 mg/kg, i.p.) alone or co-treated with zebularine (300 ng per 0.5 ml, i.c.v.) daily for 7 days, and their ambulation was recorded for 1 h immediately after treatment. Mice were treated with saline (0.5 ml, i.c.v.) + saline (0.1 ml per 10 g body weight, i.p.) (S+S); zebularine i.c.v. + saline i.p. (Z+S); saline i.c.v.+ cocaine i.p. (S+C); or zebularine i.c.v. + cocaine i.p. (Z+C). Two-way ANOVA with repeated measures, Bonferroni post-hoc test, ***p<0.001 S+C 1st vs. 7th day, n=7-12. Error bars indicate SEM. MeDNA, methylated DNA.

Finally, we studied for molecular evidence to confirm that zebularine infusions i.c.v. before cocaine treatment altered DNA methylation level at the *PP1c* promoter region in the NAc. Using MeDIP assay, we found that repeated cocaine treatment (S+C) induced at the *PP1c* promoter region DNA hypermethylation 24 h after treatment compared with the saline control group (S+S), and repeated zebularine and cocaine (Z+C) co-treatment avoided this effect (Figure 15A; one-way ANOVA, $p<0.001$ S+S vs. S+C, $p<0.001$ S+C vs. Z+C, $n=6$). Similarly, MSP qPCR data demonstrated that there was a significant decrease of DNA methylation associated with the *PP1c* promoter region in the Z+C group compared with the S+C group (Figure 15B; two-way ANOVA, treatment $F_{3,45}=39.0$, $p<0.0001$, methylation effect $F_{1,45}=42.8$, $p<0.0001$; interaction $F_{3,45}=80.8$, $p<0.0001$). There were also significant changes between the zebularine (Z+S) and the saline (S+S) treated groups ($p<0.05$).

In correlation with the *PP1c* promoter-associated CpG island methylation results, we observed that zebularine attenuated cocaine-induced downregulation of *PP1c* mRNA level (Figure 15C; one-way ANOVA, $p<0.001$ S+S vs. S+C, $p<0.05$ S+C vs. Z+C, $n=6$). These data indicate that the inhibition of DNMT's by zebularine reverses cocaine-induced *PP1c* gene hypermethylation and mRNA downregulation in the NAc.

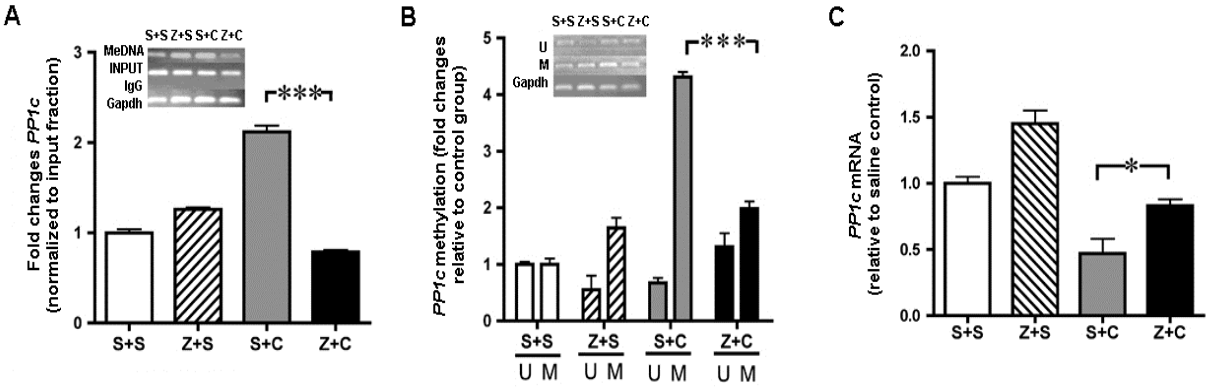

Figure 15. DNMT inhibitor zebularine altered DNA methylation at the *PP1c* promoter region 24 h after treatment in the NAc. Mice were treated with saline (0.5 ml, i.c.v.) + saline (0.1 ml per 10 g body weight, i.p.) (S+S); zebularine i.c.v. + saline i.p. (Z+S); saline i.c.v. + cocaine i.p. (S+C); or zebularine i.c.v. + cocaine i.p. (Z+C). (**A**) Repeated Z+C co-treatment decreased DNA methylation level associated with the *PP1c* promoter relative to the S+C group. (**B**) Repeated Z+C treatment decreased DNA methylation level associated with the *PP1c* promoter relative to the S+C group. Two-way ANOVA, Bonferroni post-hoc test, ***$p<0.001$ methylated S+C vs. methylated Z+C, $n=5$-7. (**C**) The blockade of *PP1c* methylation by zebularine was associated with an enhanced *PP1c* mRNA level. One-way ANOVA, Bonferroni post-hoc test *$p<0.05$ S+C vs. Z+C, $n=5$-7. Error bars indicate SEM. M, methylated; MeDNA, methylated DNA; U, unmethylated.

2. The effect of SAM treatment on cocaine-induced DNA methylation and behavioural sensitisation in mice (Paper II)

2.1. SAM pretreatment potentiated the development and expression of cocaine-induced behavioural sensitisation in mice

The effect of SAM (4520 mg/kg/day) on the behavioural sensitisation to cocaine (10 mg/kg/day) in adult mice was evaluated. Our data showed that repeated cocaine treatment (S+C) and repeated SAM and cocaine co-treatment (M+C) for 7 days displayed a significantly enhanced locomotion on day 7 compared with the day 1 (Figure 16A), indicating the development of the behavioural sensitisation. There were also significant differences (p<0.001) between S+C and M+C groups on day 7. However, we did not find difference in locomotor activity between SAM (M+S) and saline control (S+S) groups.

In cocaine (7 mg/kg i.p) challenge study (on days 14 and 28) S+C and M+C groups demonstrated a robust sensitisation exhibiting more locomotor activities than S+S group (Figure 16B). Importantly, M+C group had higher expression of sensitisation compared to the S+C group. Cocaine challenge also increased the locomotor activity in M+S group compared to the S+S group, but these changes were not substantial. These data demonstrated that exogenous SAM pretreatment did not affect acute cocaine-induced locomotor response, but instead potentiated the development and the expression of cocaine-induced behavioural sensitisation in mice.

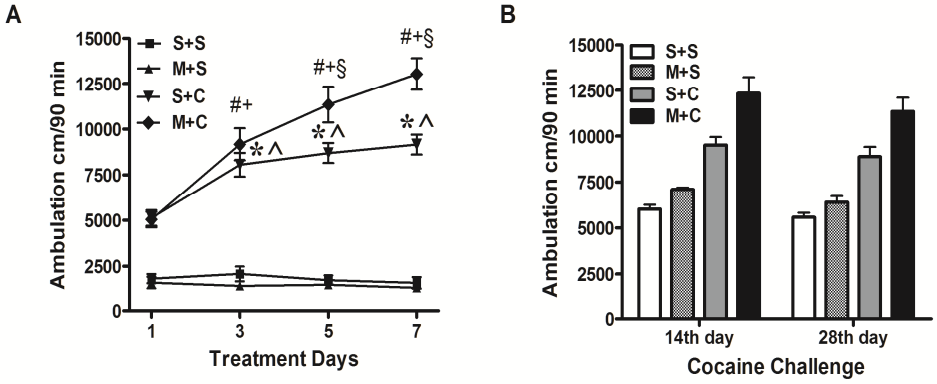

Figure 16. (**A**) The effect of S-adenosylmethionine (SAM) treatment on cocaine-induced behavioural sensitisation in mice. Two-way ANOVA repeated measurements, treatment $F_{3,198}$=103.47, p<0.0001; days effect $F_{9,198}$=47.07, p<0.0001; interaction $F_{9,198}$=21.86, p<0.0001; Bonferroni post-hoc test, *p<0.05, saline + saline (S+S) vs. S + cocaine (S+C); $^{\#}p$<0.05, S+S vs. S-adenosylmethionine + cocaine (M+C); $^{\wedge}p$<0.05, Saline + S-adenosylmethionine (M+S) vs. S+C; ^{+}p<0.05, M+S vs. M+C; $^{\S}p$<0.05, S+C vs. M+C on the indicated days; n=17 (S+S and M+C), n=18 (M+S and S+C). (**B**) Cocaine challenge, two-way ANOVA repeated measurements, treatment $F_{3,23}$=24.58, p<0.0001; time $F_{1,23}$=11.60, p=0.0024; interaction $F_{3,23}$=0.36, p=0.7831; n=8. Error bars indicate SEM.

2.2. SAM-modified cocaine-induced gene expression

An Illumina microarray was used to study persistent changes in gene expression in the NAc following repeated SAM and cocaine treatment. The samples for gene expression profiling

were collected 24 hours after the final treatment. Four different treatment groups - S+S, M+S, S+C and M+C (4 samples per group) were compared and differentially expressed genes were identified by a combination of statistical significance ($p<0.05$) and a fold change (FC) filter (FC>1.5). In total, 482 separate transcripts were expressed differently between the M+S, S+C and M+C groups, representing 1.88 % of the total number of transcripts analysed whereas 98.12 % of the transcripts remained unaltered.

To assess the direction of gene expression changes induced by the treatments, the M+S, S+C and M+C treatment groups were compared to the S+S group. Our data showed that in the M+S group a total of 18 transcripts (36%) were up- and 32 transcripts (64%) were downregulated (see Supplementary Tables S1, S2 in Paper II), representing 0.19 % (n=50) of the total number of transcripts analysed. In the S+C group, 93 transcripts (38.6%) were up- and 148 transcripts (61.4%) were downregulated (see Tables 2, 3 in Paper II), representing 0.94% (n=241) of the total number of transcripts analysed. In the M+C group, 54 transcripts (42.5%) were up- and 73 transcripts (57.5%) downregulated (see Supplementary Tables S3, S4 in Paper II), representing 0.49% (n=127) of the total number of transcripts analysed.

The comparisons between M+S, S+C, and M+C groups were also performed. Our analysis showed that 28 of 32 (87.5%) SAM-responsive transcripts were down- and 9 of 18 (50%) were upregulated in the S+C group (see Supplementary Figures S1A, S2A in Paper II). Comparisons between M+S vs. M+C groups demonstrated that 12 of 32 (37.5%) SAM-responsive transcripts were down- and 8 of 18 (44.4%) were upregulated in the M+C group (see Supplementary Figures S1B, S2B in Paper II). Interestingly, we found that 64 transcripts (43.2%) of the 148 cocaine-responsive genes were down- and 50 transcripts (53.8%) of the 93 cocaine-responsive genes were upregulated in the M+C group (see Supplementary Figures S1C, S2C in Paper II). These data suggest that SAM pretreatment reduced 56.8 and 46.2% (of genes down- and upregulated by cocaine, respectively) of cocaine-induced transcripts.

Gene ontology (GO) analysis was performed to group significantly regulated genes into similar biological or molecular functional categories. GO analysis showed an over-representation of downregulated genes in the M+S, S+C, and M+C groups encoding proteins involved in: (i) cell cycle, differentiation and proliferation, (ii) developmental process, and (iii) signal transduction (see Supplementary Table S5 in Paper II). The upregulated genes in all those groups were mainly aggregated into the categories of (i) multicellular organismal process, (ii) cell cycle, differentiation and proliferation, (iii) signal transduction, (iv) developmental process and/or ion transport (see Supplementary Table S6 in Paper II). Table S6 shows that genes, which are related to “cell-cell signaling” and “behaviour” were upregulated only in the S+C group.

2.3. SAM pretreatment altered cocaine-induced CpG island methylation and transcriptional activity in the NAc

Analysis of qPCR was performed to validate the subset of gene expression changes observed in the microarray analyses. Genes chosen for qPCR validation were selected based on their potential roles in cocaine-induced neuronal plasticity and on *in silico* analysis that revealed CpG islands located within their promoter regions. From the microarray data, 3 genes for validation were selected: a) solute carrier family 17 member 7 or vesicular glutamate

transporter 1 (*Slc17a7*) and cholecystokinin (*Cck*) as downregulated genes; b) galanin (*Gal*) as upregulated gene after repeated cocaine treatment.

Using the same RNA samples as in the gene expression profiling, transcription analysis of *Slc17a7* and *Cck* revealed a significant decrease in mRNA levels following repeated M+S, S+C, or M+C treatments in the NAc. There were significant differences (p<0.001) in both genes between the S+C and the M+C groups. Using mouse cerebellum as a reference brain region, we found that the selected genes mRNA were altered in the cerebellum as well, but these changes were not as extensive as in the NAc. *Slc17a7* and *Cck* mRNA levels comparisons in both brain tissues demonstrated that *Slc17a7* mRNA was significantly different (p<0.001) between the S+C group in the NAc vs. S+C group in the cerebellum and *Cck* mRNA level between the S+C and M+C groups in NAc vs. S+C and M+C groups in the cerebellum (Figures 17A,B).

Gal mRNA data in the NAc showed that repeated M+S, S+C and M+C treatments significantly increased (p<0.001) *Gal* expression compared to the S+S group. Furthermore, *Gal* mRNA was significantly different (p<0.001) between the S+C and M+C groups. In the cerebellum, we found that M+S and S+C treatments significantly (p<0.001) upregulated *Gal* expression compared to the saline control. There were also statistical differences (p<0.001) between S+C and M+C groups. *Gal* mRNA levels comparison in both brain regions demonstrated that there were significant differences (p<0.001) between the S+C and M+C groups in the NAc vs. S+C and M+C groups in the cerebellum (Figure 17C).

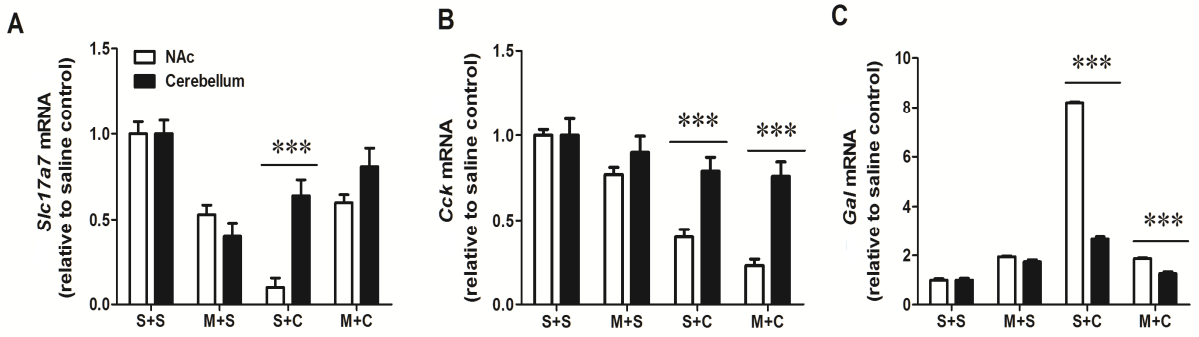

Figure 17. The effect of repeated S-adenosylmethionine (SAM) and cocaine administration on selected genes mRNA levels in the mouse NAc (n=8) and cerebellum (n=6). (**A**) *Slc17a7* mRNA, two-way ANOVA, treatment $F_{3,44}$=27.80, p<0.0001; tissue effect $F_{1,44}$=8.44, p=0.0057; interaction $F_{3,44}$=7.51, p=0.0004; Bonferroni post-hoc test, ***p<0.001 Saline + Cocaine (S+C) NAc vs. S+C cerebellum; (**B**) *Cck* mRNA, treatment $F_{3,44}$=27.67, p<0.0001; tissue effect $F_{1,44}$=36.35, p<0.0001; interaction $F_{3,44}$=7.66, p=0.0003; Bonferroni post-hoc test, ***p<0.001 S+C and S-adenosyl-methionine + Cocaine (M+C) NAc vs. S+C and M+C cerebellum; and (**C**) *Gal* mRNA, treatment $F_{3,44}$=2045.35, p<0.0001; tissue effect $F_{1,44}$=1269.06, p<0.0001; interaction $F_{3,44}$=885.82, p<0.0001; Bonferroni post-hoc test, ***p<0.001 S+C and M+C NAc vs. S+C and M+C cerebellum. Error bars indicate SEM.

Using MeDIP assay, *Slc17a7, Cck,* and *Gal* promoter-associated CpG island methylation analysis in the NAc was performed. For the *Slc17a7* promoter, MeDIP analysis revealed that both M+S and S+C treatment resulted in promoter hypermethylation compared

to the S+S group (Figure 18A). We also found that repeated SAM pretreatment significantly (p<0.001) decreased *Slc17a7* promoter hypermethylation compared to the S+C group. With regard to *Cck* promoter methylation, M+S and S+C treatments resulted in promoter hypermethylation (Figure 18B). However, there was an additive increase in *Cck* promoter methylation levels in the M+C group compared to the S+C group (p<0.001). *Gal* MeDIP analysis demonstrated that M+S and S+C treatments induced promoter-associated CpG island hypomethylation (Figure 18C). Remarkably, we found that repeated M+C treatment essentially reversed *Gal* promoter hypomethylation compared to the S+C treatment (p<0.001).

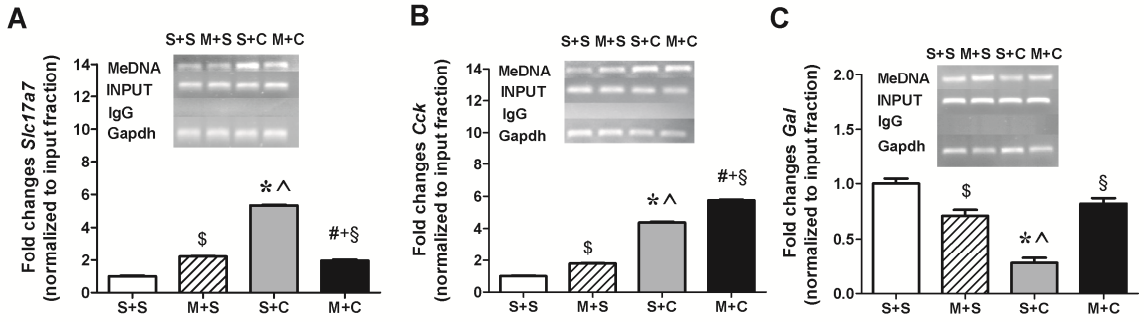

Figure 18. The effect of repeated S-adenosylmethionine (SAM) and cocaine treatment on selected genes promoter methylation levels in the mouse NAc. (**A**) *Slc17a7* promoter methylation, one-way ANOVA, $F_{3,28}$=1583, p<0.0001; Bonferroni post-hoc test; $^{\$}p$<0.05, Saline + Saline (S+S) vs. Saline + S-adenosylmethionine (M+S); *p<0.05, S+S vs. Saline + Cocaine (S+C); $^{\#}p$<0.05, S+S vs. S-adenosylmetionine + Cocaine (M+C); ^p<0.05, M+S vs. S+C; ^{+}p<0.05, M+S vs. M+C; $^{\S}p$<0.05, S+C vs. M+C, n=8; (**B**) *Cck* promoter methylation, $F_{3,28}$=2587, p<0.0001; Bonferroni post-hoc test; $^{\$}p$<0.05, S+S vs. M+S; *$p$<0.05, S+S vs. S+C; $^{\#}p$<0.05, S+S vs. M+C; ^p<0.05, M+S vs. S+C; ^{+}p<0.05, M+S vs. M+C; $^{\S}p$<0.05, S+C vs. M+C, n=8; and (**C**) *Gal* promoter methylation, $F_{3,28}$=39.58, p<0.0001; Bonferroni post-hoc test, $^{\$}p$<0.05, S+S vs. M+S; *$p$<0.05, S+S vs. S+C; ^$p$<0.05, M+S vs. S+C; $^{\S}p$<0.05, S+C vs. M+C, n=8. Error bars indicate SEM. MeDNA, methylated DNA.

MeDIP data in the mouse cerebellum showed that repeated M+S and S+C treatments significantly (p<0.001) enhanced (*Slc17a7, Cck*) promoter-associated CpG island methylation (see Supplementary Figures S3A,B in Paper II). Moreover, *Slc17a7* and *Cck* promoter methylation data comparison in both brain tissues demonstrated that there were significant differences (p<0.001) between the S+C and M+C groups in the NAc vs. S+C and M+C groups in the cerebellum. *Gal* MeDIP data comparison in both brain regions showed that there were significant changes (p<0.001) only between the S+C groups in the NAc vs. S+C groups in the cerebellum (see Supplementary Figure S3C in Paper II).

2.4. The effect of SAM treatment on methyltransferase activity and DNMT expression in PC12 cells

To evaluate the underlying mechanism of the SAM modifying effect at the gene and genome level, we studied the effects of a single and repeated dose (7 days) of 0.5 mM SAM on DNMT activity in PC12 cells. Time-course analyses showed that SAM altered methyltransferase activity in a biphasic manner: a single SAM treatment (SST) enhanced and a repeated SAM treatment (RST) decreased DNMT activity compared with vehicle controls

(Figures 19A,B). To link methyltransferase activity with *Dnmt3a* and *Dnmt3b* mRNA levels, we measured the SAM-altered DNMT activity on 1st, 3rd, 5th and 7th treatment days. On the 1st day, DNMT activity was similar to vehicle control, on the 3rd day its activity was reduced approximately by 50%, on the 5th by 65% and on the 7th day by 82% compared to controls (Figure 19C).

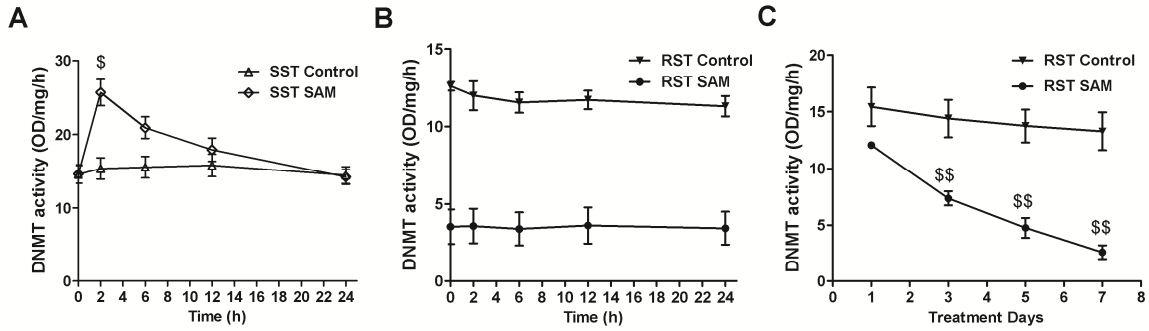

Figure 19. S-adenosylmethionine (SAM) altered DNMT activity in PC12 cells in a biphasic manner: (**A**) a single SAM treatment (SST) enhanced [(two-way ANOVA, treatment $F_{1,30}$=15.64, p=0.0004; time $F_{4,30}$=6.77, p=0.0005; interaction $F_{4,30}$=4.91, p=0.0037; Bonferroni post-hoc test, $^{\$}p$<0.001 SST SAM 2 h vs. SST control 2 h; n=4) whereas (**B**) repeated SAM treatment (RST) decreased DNMT activity compared to the control (two-way ANOVA, treatment $F_{1,30}$=206.74, p<0.0001; time $F_{4,30}$=0.18, p=0.9461; interaction $F_{4,30}$=0.13, p=0.9717; n=4)]. (**C**) The effect of RST SAM treatment on DNMT activity in cells, two-way ANOVA, treatment $F_{1,12}$=141.58, p<0.0001; time $F_{3,12}$=5.65, p=0.0447; interaction $F_{3,12}$=6.22, p=0.0086; Bonferroni post-hoc test, $^{\$\$}p$<0.001 RST SAM vs. RST control on the indicated days; n=4. Error bars indicate SEM. OD, optical density.

We found that the decrease in DNMT activity in PC12 cells correlated with downregulation of *Dnmt3a* mRNA (Figure 20A), but not remarkably with *Dnmt3b* mRNA level (Figure 20B) after repeated SAM treatment. *In silico* analysis of *Dnmt3a* revealed that CpG islands located within the promoter region, thus, we assessed *Dnmt3a* promoter methylation following repeated SAM treatment. The MeDIP results showed that repeated SAM treatment resulted in a significant increase in methylation of the *Dnmt3a* promoter (Figure 20C), thereby decreasing *Dnmt3a* gene transcription in PC12 cells.

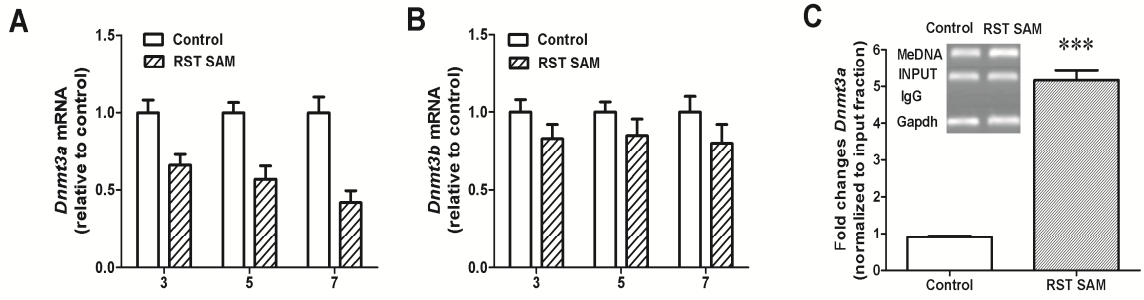

Figure 20. (**A**) Repeated S-adenosylmethione (RST SAM) treatment downregulated *Dnmt3a* mRNA levels compared to the control groups, two-way ANOVA, treatment $F_{1,18}$=47.49, p<0.0001; time $F_{2,18}$=1.15, p=0.3391; interaction $F_{2,18}$=1.15, p=0.3391; n=4. (**B**) The effect of RST SAM treatment on *Dnmt3b* expression, in each group n=4. (**C**) RST SAM treatment significantly hypermethylated *Dnmt3a* promoter region in PC12 cells. T-test, ***p=0.0003, n=4. Error bars indicate SEM. MeDNA, methylated DNA.

2.5. SAM treatment hypermethylated the *Dnmt3a* promoter and downregulated mRNA level in the NAc

To bridge PC12 cells and mice data, we assessed *Dnmt3a* and *Dnmt3b* promoter methylation patterns and transcriptional activity in the NAc. Using MeDIP, we discovered that M+S and M+C treatment increased *Dnmt3a* promoter methylation (Figure 21A), but there was no significant change in *Dnmt3b* promoter (Figure 21B). Using qPCR, our data demonstrated that *Dnmt3a* mRNA was significantly decreased following M+S and M+C treatments and increased following S+C treatment compared with the saline-treated group (Figure 21C). There were significant (p<0.001) differences in both *Dnmt3a* and *Dnmt3b* mRNA levels after the S+C and M+C treatment in NAc (Figures 21C,D). These data indicate that repeated SAM treatment may decrease methyltransferase activity *in vitro* and repeated SAM treatment is associated with hypermethylation of *Dnmt3a* promoter region both *in vitro* and *in vivo*.

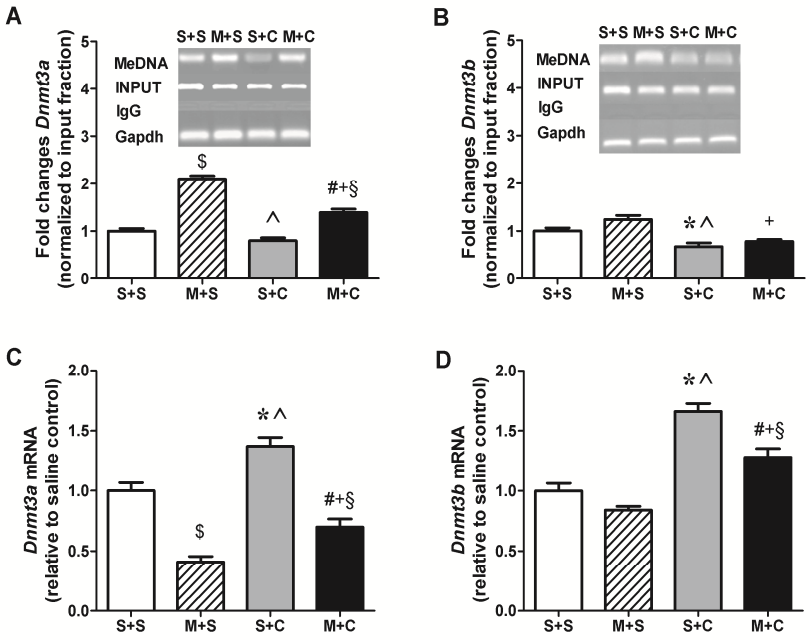

Figure 21. Repeated S-adenosylmethionine (SAM) and cocaine treatment altered DNA methylation (**A**) at the *Dnmt3a* promoter, one-way ANOVA, $F_{3,28}$=80.83, p<0.0001; Bonferroni post-hoc test, $^{\$}p$<0.05, Saline + Saline (S+S) vs. Saline + S-adenosylmethionine (M+S); $^{\#}p$<0.05, S+S vs. S-adenosylmethionine + Cocaine (M+C); ^p<0.05, M+S vs. Saline + Cocaine (S+C); ^{+}p<0.05, M+S vs. M+C; $^{\S}p$<0.05, S+C vs. M+C; n=8; and at the (**B**) *Dnmt3b* promoter in the NAc, $F_{3,28}$=13.04, p<0.0001; Bonferroni post-hoc test, *p<0.05, S+S vs. S+C; ^p<0.05, M+S vs. S+C; ^{+}p<0.05, M+S vs. M+C; n=8. The effect of SAM and cocaine treatment on (**C**) *Dnmt3a* mRNA level, one-way ANOVA, $F_{3,28}$=40.83, p<0.0001; Bonferroni post-hoc test, $^{\$}p$<0.05, S+S vs. M+S; *$p$<0.05, S+S vs. S+C; $^{\#}p$<0.05, S+S vs. M+C; ^p<0.05, M+S vs. S+C; ^{+}p<0.05, M+S vs. M+C; $^{\S}p$<0.05, S+C vs. M+C; n=8; and (**D**) on *Dnmt3b* mRNA level, $F_{3,28}$=35.27, p<0.0001; Bonferroni post-hoc test, *p<0.05, S+S vs. S+C; $^{\#}p$<0.05, S+S vs. M+C; ^p<0.05, M+S vs. S+C; ^{+}p<0.05, M+S vs. M+C; $^{\S}p$<0.05, S+C vs. M+C; n=8. Error bars indicate SEM. MeDNA, methylated DNA.

3. The role of MS on DNA methylation and behavioural changes in rats (Paper III)

3.1. The effect of MS on exploratory behaviour and cocaine-induced behaviour

Previous studies have demonstrated that MS alters exploratory behaviour in a novel environment (Kaneko et al., 1994; Marmendal et al., 2006). To validate our model of MS, we first used the exploratory box test to measure novelty-related behaviour in adult rats. The exploratory box test data demonstrated that MS for 180 minutes per day increased line crossing and the sum of exploratory activity in adult rats compared with rats from the AFR group (Figure 22; one-way ANOVA, $p<0.05$ AFR vs. MS180, $n=8$). No differences were found between the MS15 and MS180 groups. Based on these data, we considered that our MS model is behaviourally valid.

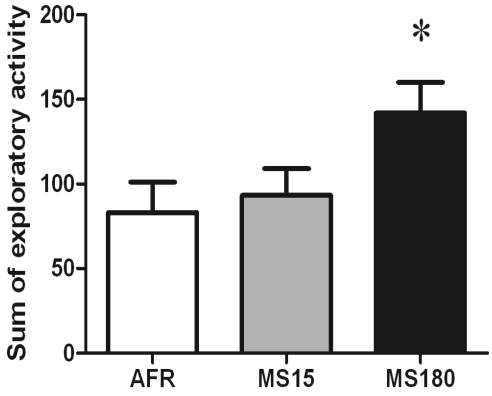

Figure 22. The effect of maternal separation (MS) on exploratory behaviour in adult rats. One-way ANOVA, followed by Bonferroni post-hoc test, *$p<0.05$ animal facility rearing (AFR) group vs. MS180 group, $n=8$. Error bars indicate SEM.

The acute cocaine (AC, 10 mg/kg, i.p.) treatment study was performed on PND120-140. Figure 23A shows that AC treatment considerably increased locomotor activity in both MS groups compared with the control group (two-way ANOVA repeated measures, MS effect $F_{2,64}=2.47$, $p=0.08$; days effect $F_{2,64}=48.87$, $p<0.0001$; interaction $F_{4,64}=4.40$, $p=0.0033$). There were also significant differences ($p<0.05$) between the MS15 and MS180 groups.

After a daily injection of cocaine over 5 consecutive days, both MS15 and MS180 rats developed behavioural sensitisation; however, there were no significant differences between groups (Figure 23B; two-way ANOVA repeated measures, MS effect $F_{2,66}=2.23$, $p=0.124$; days effect $F_{2,66}=7.93$, $p=0.008$; interaction $F_{4,66}=0.17$, $p=0.953$). These data indicate that MS for 180 minutes increased the response to acute cocaine treatment and MS enhanced the development of behavioural sensitisation in general when compared with AFR adult rats.

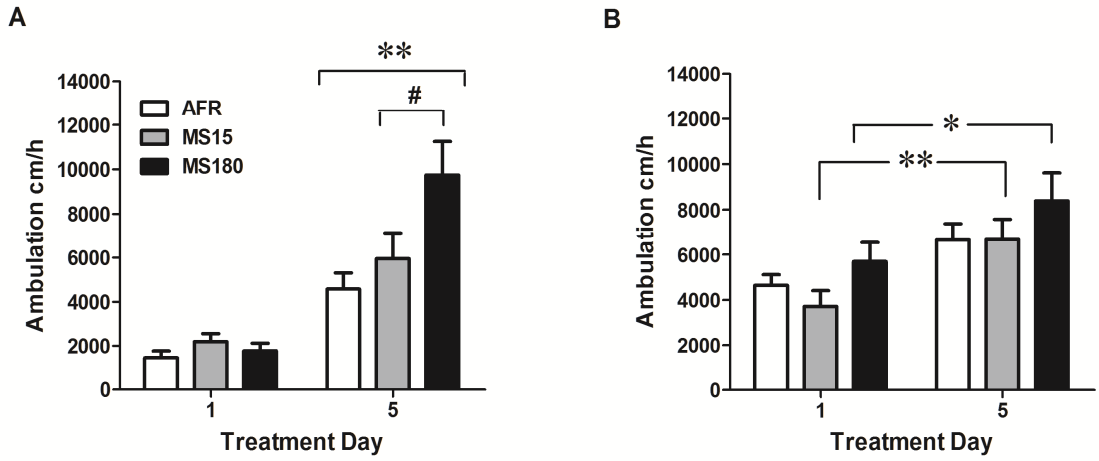

Figure 23. (**A**) Maternal separation (MS) was associated with an increased response to acute cocaine treatment. Two-way ANOVA repeated measures, Bonferroni post-hoc test, $^{**}p<0.01$ animal facility rearing (AFR) group vs. MS15 or MS180 groups; $^{\#}p<0.05$ MS15 vs. MS180, $n=13$. (**B**) The effects of repeated cocaine treatment on the development of behavioural sensitisation. Two-way ANOVA repeated measures, Bonferroni post-hoc test, $^{**}p<0.01$ MS15 1st day vs. MS15 5th day; $^{*}p<0.05$ MS180 1st day vs. MS180 5th day, $n=13$. Error bars indicate SEM.

3.2. MS-induced DNMT upregulation is maintained into adulthood

To evaluate the role of DNA methylation on MS-induced changes in gene expression, we first assessed the effect of MS on *Dnmt1*, *Dnmt3a* and *Dnmt3b* mRNA levels in the NAc of infant (PND15) rats. Using qPCR, we found that MS for 15 minutes per day during the first two weeks of life increased *Dnmt3b* mRNA levels significantly and that MS for 180 minutes enhanced all DNMT's expression levels in the NAc of PND15 rats (see Supplementary Figures S1A-C in Paper III).

Next, DNMT's expression levels in the NAc of adult rats (PND120) were assessed. We found that DNMT's were significantly upregulated both in MS15 and MS180 groups compared with AFR animals (Figures 24A-C). We also found that *Dnmt3a* and *Dnmt3b* mRNA levels were significantly different ($p<0.001$) between the MS15 and MS180 groups. To confirm our *Dnmt3a* mRNA results western blot analysis was performed. We found that NAc DNMT3A protein level was increased in MS15 and MS180 adult rats compared with AFR group (Figure 24D) and there was a significant difference ($p<0.01$) between the MS15 and MS180 groups. These findings indicate that early life stress (MS180) during the first two weeks of life significantly increased *Dnmt's* mRNA levels compared with MS15 and AFR groups and these changes persist into adulthood.

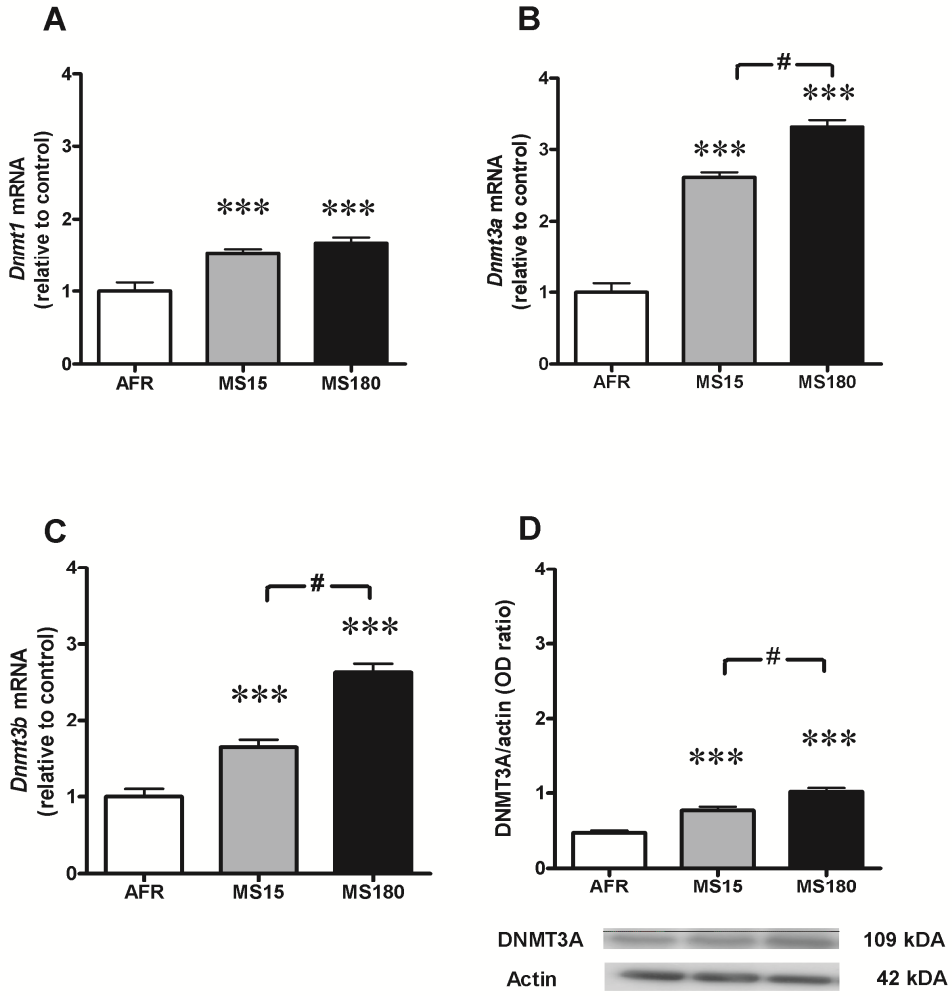

Figure 24. The effect of maternal separation (MS) on the levels of (**A**) *Dnmt1,* (**B**) *Dnmt3a* and (**C**) *Dnmt3b* mRNA in the NAc of adult rats. One-way ANOVA, Bonferroni post-hoc test, ***p<0.001 compared with the animal facility rearing (AFR) group; $^{\#}p$<0.001 MS15 vs. MS180, n=6. (**D**) The effect of MS on the protein levels of DNMT3A in the NAc of adult rats. One-way ANOVA, Bonferroni post-hoc test, ***p<0.001 compared with the AFR group; $^{\#}p$<0.01 MS15 vs. MS180, n=6. Error bars indicate SEM. OD, optical density.

3.3. The effect of MS on global DNA methylation levels in the NAc and PFC

In this part of study, we assessed whether MS could alter global DNA methylation levels in the NAc and in the PFC of adult rats. Global methylation analysis (the percentage of methylated DNA in total DNA) showed that DNA methylation was significantly decreased in the NAc of MS180 animals (Figure 25A; one-way ANOVA, Bonferroni post-hoc test, p<0.05 AFR vs. MS180, n=7). We also found that DNA methylation was decreased in the PFC of MS15 and MS180 animals but these changes were not statistically significant (Figure 25B). These data suggest that MS may alter global DNA methylation levels in the NAc of adult rats.

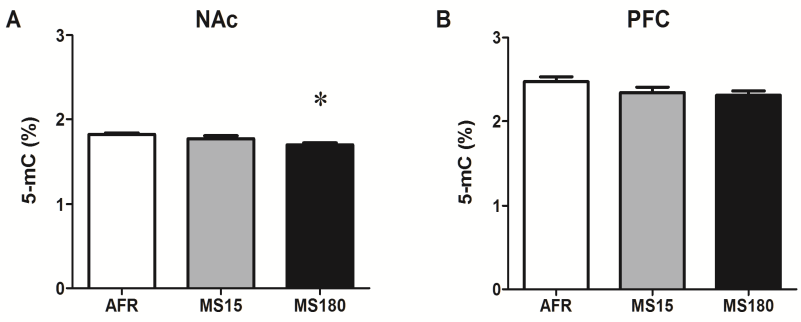

Figure 25. (**A**) The levels of global DNA methylation, shown as percentage methylated DNA (5-mC) of total DNA, in the NAc and (**B**) in the PFC of adult rats. One-way ANOVA, Bonferroni post-hoc test, **p*<0.05 animal facility rearing (AFR) group vs. MS180 group, *n*=7.

3.4. MS is associated with hypermethylation in the *PP1c* and $A_{2A}R$ promoter regions in the NAc

To investigate the role of MS at a gene level, *PP1c* and $A_{2A}R$ genes were chosen based on both their possible participation in cocaine-induced neuroplasticity mechanisms and on data from *in silico* analysis that revealed CpG islands within their promoter regions. We also examined the expression and promoter region methylation levels of *Reelin (Reln)*, which is not associated with cocaine-induced neuronal plasticity although its promoter is epigenetically regulated. Using MeDIP assay, we measured *PP1c* promoter-region methylation of PND15 rats. We found that MS15 and MS180 significantly upregulated *PP1c* promoter-region methylation levels (see Supplementary Figure S2A in Paper III). To validate the MeDIP results, we used a methylation-specific qPCR analysis (MSP qPCR). This analysis revealed that methylation of the *PP1c* promoter region was increased in both separated-animal groups on PND15 (see Supplementary Figure S2B in Paper III; two-way ANOVA repeated measures, treatment $F_{2,30}$=6.64, p=0.0041; methylation effect $F_{1,30}$=96.78, p<0.0001; interaction $F_{2,30}$=26.11, p<0.0001; Bonferroni post-hoc test, p<0.05 unmethylated AFR vs. unmethylated MS180; p<0.001 methylated AFR vs. methylated MS15 or MS180, n=6). The data from DNA methylation analysis correlated with qPCR results because we found that the *PP1c* mRNA level was decreased in the MS180 group compared with control animals (see Supplementary Figure S2C in Paper III).

To determine whether MS-induced *PP1c* gene promoter-region hypermethylation and transcriptional downregulation is persistent, we examined *PP1c* promoter methylation and transcriptional activity at PND120. Data generated using MeDIP and MSP qPCR assays demonstrated that both MS15 and MS180 elicited DNA hypermethylation at promoter region of *PP1c* (Figure 26A MeDIP; and Figure 26B; MSP qPCR assay, two-way ANOVA repeated measures, treatment $F_{2,26}$=15.37, p<0.0001; methylation effect $F_{1,26}$=94.92, p<0.0001; interaction $F_{2,26}$=36.90, p<0.0001; n=6). In correlation with this *PP1c* promoter-region hypermethylation, we found that *PP1c* mRNA was significantly decreased in the NAc of separated rats (Figure 26C).

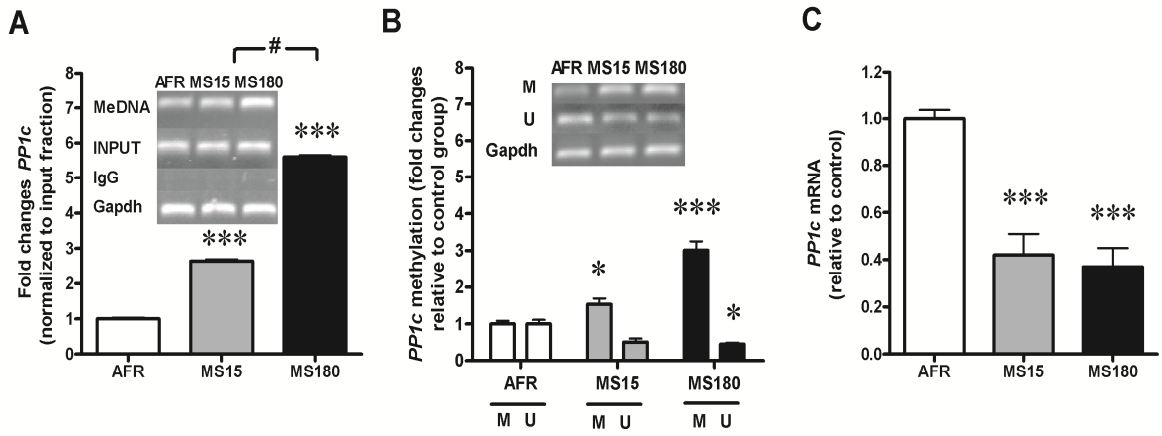

Figure 26. (**A**) The methylated DNA immunoprecipitation (MeDIP) assay showed that maternal separation (MS) elicited DNA hypermethylation of the *PP1c* promoter region, one-way ANOVA, Bonferroni post-hoc test, ***p<0.001 compared with the animal facility rearing (AFR) group; $^{\#}p$<0.001 MS15 vs. MS180, n=6. (**B**) Methylation-specific qPCR showed that MS elicited hypermethylation of the *PP1c* promoter region. Two-way ANOVA, Bonferroni post-hoc test, *p<0.05, ***p<0.001 compared with methylated or unmethylated DNA of the AFR group, n=6. (**C**) The effect of MS on the *PP1c* mRNA levels, quantified using qPCR. One-way ANOVA, Bonferroni post-hoc test, ***p<0.001 compared with the AFR group, n=6. Error bars indicate SEM. M, methylated; MeDNA, methylated DNA; U, unmethylated.

The second gene of interest was $A_{2A}R$. We observed that methylation of $A_{2A}R$ at PND15 was enhanced in the NAc of separated animals (see Supplementary Figure S3A in Paper III). Quantification of $A_{2A}R$ mRNA by qPCR demonstrated that hypermethylation of the $A_{2A}R$ promoter was associated with reduced mRNA levels in MS groups but these data did not achieve statistical significance (see Supplementary Figure S3B in Paper III).

To assess whether MS-induced $A_{2A}R$ hypermethylation is persistent, we evaluated the promoter methylation and mRNA at PND120. MeDIP results showed that changes in DNA methylation at the $A_{2A}R$ promoter is maintained into adulthood (Figure 27A). Furthermore, we found that $A_{2A}R$ hypermethylation was statistically different (p<0.001) between MS15 and MS180 animals. We also found that hypermethylation of the $A_{2A}R$ promoter leads to reduced mRNA in the MS180 group (Figure 27B). There were also significant differences (p<0.05) between the MS15 and MS180 groups. Thus, these data suggest that MS during the first two weeks of life results in persistent *PP1c* and $A_{2A}R$ promoter-region hypermethylation and transcriptional downregulation in the NAc. However, neither handling nor MS affected *Reln* promoter-region methylation or mRNA in the NAc (data not shown).

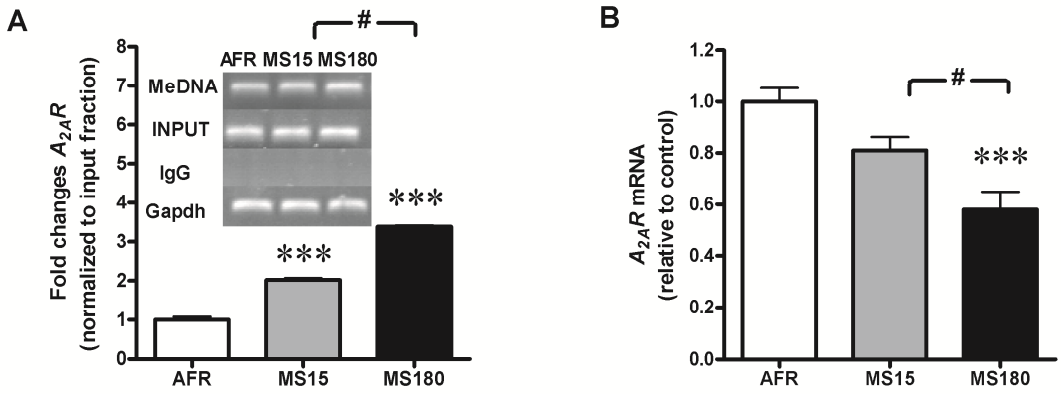

Figure 27. Maternal separation (MS) was associated with $A_{2A}R$ promoter methylation and transcriptional downregulation in the NAc of adult rats. (**A**) The methylated DNA immunoprecipitation (MeDIP) assay showed that MS elicited DNA hypermethylation of the $A_{2A}R$ promoter region. One-way ANOVA, Bonferroni post-hoc test, ***p<0.001 animal facility rearing (AFR) group vs. MS15 or MS180 groups; $^{\#}p$<0.001 MS15 vs. MS180, n=6. (**B**) The effect of MS on mRNA levels of the $A_{2A}R$ gene. One-way ANOVA, Bonferroni post-hoc test, ***p<0.001 AFR vs. MS180, $^{\#}p$<0.05 MS15 vs. MS180, n=6. Error bars indicate SEM. MeDNA, methylated DNA.

To determine whether the *PP1c* and $A_{2A}R$ promoter-region hypermethylation and transcriptional downregulation is NAc-specific, the PFC of adult rats was studied. Our data showed that neither handling nor MS affected the *PP1c* or $A_{2A}R$ mRNA and promoter methylation levels in the PFC (see Supplementary Figures S4A,B; S5A,B in Paper III). There were also no changes in *Reln* mRNA and promoter-methylation levels in the PFC of MS15 and MS180 animals (Fig. S4C; S5C). These data indicate that *PP1c* and $A_{2A}R$ mRNA and promoter methylation changes after MS are NAc-specific.

DISCUSSION

1. The role of DNA methylation on cocaine-induced behavioural sensitisation in mice (Paper I)

In study I, our aim was to investigate the role of cocaine treatment on DNMT's and selected genes (*PP1c, fosB)* expression in the NAc of adult mice; and to assess the effect of DNMT inhibitor on cocaine-induced gene expression changes and the development of behavioural sensitisation in mice.

1.1. The role of DNMT on cocaine-induced behavioural sensitisation

Previous findings have implied that the function of DNMT's in the central nervous system (CNS) might be involved in DNA repair and neurodegeneration (Brooks et al., 1996; Endres et al., 2000, 2001; Fan et al., 2001) and that misregulation of DNA methylation and DNMT's might be involved in cognitive disorders such as schizophrenia, Rett syndrome, and Fragile X mental retardation (Sutcliffe et al., 1992; Amir et al., 1999; Veldic et al., 2004).

Studies by Miller and Sweatt (2007) have demonstrated that DNA methylation levels were rapidly and dynamically regulated in the hippocampus following the associative training paradigm of contextual fear conditioning. They found that *Dnmt3a* and *Dnmt3b* mRNA levels were upregulated in area CA1 of the hippocampus following fear conditioning (Miller and Sweatt, 2007). Our data showed that acute cocaine treatment upregulated *Dnmt3a* and *Dnmt3b* mRNA levels in the NAc and hippocampus (Figures 8B,C and 9B,C; respectively). In addition, we found that *Dnmt3a* expression achieved the highest level after acute cocaine treatment and it diminished after repeated cocaine exposure. In relation to *Dnmt3a* and *Dnmt3b* expression levels in the NAc, cocaine-induced changes in the hippocampus were more dynamic as there were no significant changes 24 h after treatment. However, both acute and repeated cocaine treatment did not alter *Dnmt1* mRNA level in the NAc and hippocampus, thus, we hypothesize that cocaine induces rather *de novo* DNA methylation.

At present, signal transduction processes that might control cocaine-induced DNMT's expression in the adult CNS are unknown. Although DNA methylation is a part of the regulation of gene expression, it infers that there may be some overlap of intracellular signaling pathways with other chromatin modification mechanisms. In the adult CNS chromatin modification is governed by various intracellular signaling pathways, including the Ras-mitogen-activated protein kinase (MAPK/ERK) cascade (Renthal and Nestler, 2008; Brami-Cherrier et al., 2009). It has been found that targeting the Ras signaling pathway with drugs such as methotrexate and inhibitors of MAPK/ERK decreases DNA methylation in malignant hematological diseases and colon cancer cells, indicating a causal relationship between Ras signaling and DNA methylation (MacLeod et al., 1995; Philips, 2004; Morgan et al., 2007; Lu et al., 2007; Diaz-Flores and Shannon, 2007).

In recent years, the MAPK/ERK signaling cascade has been implicated in responses to most drugs of abuse (Berke and Hyman, 2000; Nestler, 2001; Fasano and Brambilla, 2002; Girault et al., 2007). It has been found that in response to cocaine, the MAPK/ERK and the

downstream MSK1 controls an early phase of histone H3 phosphorylation at the *c-fos* promoter in striatal neurons (Brami-Cherrier et al., 2005). MSK1 action may be potentiated by the concomitant inhibition of PP1 by nuclear translocation of dopamine and cAMP-regulated phosphoprotein DARPP-32 (Stipanovich et al., 2008; Brami-Cherrier et al., 2009). Cocaine also phosphorylates the transcription factor CREB via the MAPK/ERK–MSK1 cascade (Swank and Sweatt, 2001; Levine et al., 2005). As mentioned earlier, phosphorylation and activation of CREB recruits CBP, which regulates chromatin structure as part of CREB-dependent activation of nuclear gene transcription (Sweatt, 2009). Therefore, we speculate that cocaine-induced activation of the Ras signaling pathway might trigger both DNA methylation and histone modifications.

1.2. DNA methylation regulates *PP1c* and *fosB* transcription in the NAc after acute and repeated cocaine treatment

As we found that cocaine treatment significantly upregulated *Dnmt3a* and *Dnmt3b* expression in the NAc and thereby might cause hypermethylation of certain genes promoters, *PP1c* and *fosB* promoter region methylation and mRNA levels in the NAc of mice were evaluated. Our data demonstrated that DNMT activation results in hypermethylation of the *PP1c* promoter region after acute and repeated cocaine treatment (Figures 11B,C), increased MeCP2 binding in the *PP1c* promoter region (Figure 13A) and decreased *PP1c* expression of both mRNA and protein levels (Figures 10A,B). It has been shown that methylation of DNA brings about general deacetylation of histones H3 and H4, prevents methylation at histone H3 lysine K4, and induces methylation of histone H3 lysine K9 (Kouzarides, 2007; Li et al., 2007; Shahbazian and Grunstein, 2007; Patra and Szyf, 2008). Based on these data, we speculate that MeCP2 can recruit co-repressor complexes to methylated *PP1c* promoter regions, including histone-modifying enzymes such as HDAC's and HMT's. Repression of *PP1c* may result in several effects on the intracellular signal transduction level. For example, inhibition of *PP1* has been shown to increase phosphorylation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluR1 and Ca2+/calmodulin-dependent protein kinase II (Genoux et al., 2002). Reduced PP1 may have important effects for transcriptional regulation by CREB through the ability of PP1 to complex with HDAC. Canettieri and colleagues (2003) have demonstrated that a HDAC1-PP1 complex represses CREB activation under basal conditions and dephosphorylates CREB to return the system to baseline after a stimulus (Canettieri et al., 2003). This suggests that repression of PP1 is crucial in providing phosphorylated CREB with the capacity to recruit CBP to the promoter, at which time histones become acetylated and help to drive the transcription of particular genes. Thus, repression of the *PP1c* gene via methylation of promoter regions should allow aberrant phosphorylation of crucial receptors, protein kinases, and transcription factors during cocaine-induced neuroadaptation.

Previous studies have demonstrated that expression of Fos-family proteins (c-Fos, FosB, Fra-1, and Fra2) is rapidly induced in the NAc after acute exposure to drugs of abuse (Hope et al, 1994). This induction is transient, lasting only 4-12 h after drug exposure. In our study, we found that cocaine treatment hypomethylated *fosB* promoter (Figure 12B), decreased MeCP2 binding in the *fosB* promoter region (Figure 13B) and increased *fosB*

mRNA level (Figure 10C). These data suggest that cocaine may also induce DNA demethylation and thereby increase certain genes transcription. However, in contrast to the large amount of information that has accumulated on DNA methylation, DNA demethylation is still a quite controversial and largely unresolved area of research and there is no consensus about DNA demethylases in mammalian cells although several potential canditates have been proposed (Ooi and Bestor, 2008; Métivier et al., 2008).

1.3. DNMT inhibition by zebularine delay the development of cocaine-induced behavioural sensitisation

The effect of DNMT inhibitor zebularine on cocaine-induced molecular changes and the development of behavioural sensitisation were assessed. Our data showed that zebularine blocked hypermethylation in the *PP1c* promoter region and delayed the development of cocaine-induced behavioural sensitisation. The decrease of *PP1c* hypermethylation was triggered using i.c.v. infusion of zebularine and it was sufficient to re-establish *PP1c* gene expression in the NAc. It has been found that zebularine is a potent inhibitor affecting all DNMT subtypes (Weisenberger et al., 2004; Marquez et al., 2005). However, we were unable to determine which subtype of DNMT is important for the development of behavioural sensitisation. It is possible that both Dnmt3a and Dnmt3b work together to hypermethylate the promoter-associated CpG islands and to then transcriptionally repress these genes, aiding in the development of behavioural sensitisation.

In Figure 14A we show that a single dose of zebularine caused hypomethylation in the promoter of *PP1c* at 1.5 h after treatment. However, the effect of repeated zebularine (Z+S) administration resulted in *PP1c* promoter region hypermethylation 24 h after last treatment (Figures 15A,B). Based on zebularine pharmacokinetics, plasma zebularine concentrations in mice declined with terminal half-lives ($t_{1/2}$) of 40 minutes after 100 mg/kg intravenous (i.v.) injection (Holleran et al., 2005), we speculate that 24 h after the last administration of zebularine (Z+S) the DNMT inhibition effect is ended. Therefore, it is possible that a slight increase of methylation in *PP1c* promoter-associated CpG island in Z+S groups (Figures 15A,B) is caused by a compensatory increase of DNMT activity after inhibition by zebularine. Despite slight hypermethylation at the *PP1c* promoter region after Z+S treatment, we found that *PP1c* mRNA level was increased approximately 1.5-fold in the Z+S group compared with the saline control (S+S, Figure 15C) suggesting that smaller changes of DNA methylation may not alter gene transcription. Repeated Z+C co-treatment data demonstrated that zebularine treatment blocked *PP1c* promoter region hypermethylation (Figures 15A,B), which was associated with an enhanced *PP1c* mRNA level (Figure 15C). We speculate that in addition to *PP1c*, zebularine may also inhibit hypermethylation in promoter-associated CpG islands of other genes, re-establish these gene transcriptions, and therefore inhibit cocaine-induced neuroplasticity in the brain.

At behavioural level, we found that repeated Z+C co-treatment delayed the development of behavioural sensitisation, although, zebularine (Z+S) itself did not affect locomotor activity. Interestingly, zebularine did not modify acute cocaine effect, suggesting that the promoter region hypermethylation might be an important underlying mechanism for altered gene expression during the development of behavioural sensitisation. Therefore, we

hypothesize that zebularine via normalization of gene transcription at cellular level stabilized neuronal network function and therefore delayed the development of psychostimulant-induced behavioural sensitisation. As DNA methylation has an important role in learning and memory formation (Miller and Sweatt, 2007), zebularine may also delay cocaine-induced behavioural sensitisation by inhibiting DNA methylation in the hippocampus and altering cocaine-environment associated learning and memory formation.

2. The role of SAM on cocaine-induced DNA methylation and behavioural sensitisation in mice (Paper II)

In this study, we assessed the role of methyl donor SAM on cocaine-induced gene expression changes and the development of behavioural sensitisation in mice.

2.1. The effect of SAM treatment on cocaine-induced gene expression and CpG island methylation

Using whole-genome gene expression analysis, our data demonstrated that repeated SAM treatment significantly altered 50 transcripts. Of these transcripts, 36% were upregulated and 64% were downregulated suggesting that exogenous SAM treatment primarily silences gene expression, as expected from the typical silencing effect of DNA hypermethylation. Compared to the SAM, cocaine treatment has a broader impact upon gene expression. Repeated cocaine treatment changed 241 transcripts and predominantly downregulated these transcripts. 37 transcripts of the 50 SAM-responsive genes were also altered in the S+C group. Interestingly, the number of significantly affected genes in the M+C group was lower than that of the S+C group. These data demonstrate that 46% and 57% (respectively up- and downregulated) SAM and cocaine treatment-group transcripts were no longer significantly different compared to the respective transcripts from the S+C group, suggesting that SAM pretreatment modified the cocaine-induced gene expression pattern in the NAc. GO analysis revealed that SAM pretreatment decreased expression of several cocaine-induced genes in different functional groups, demonstrating that SAM's blunting effect is non-specific.

Other microarray studies have demonstrated that several genes are affected by cocaine (S+C) treatment (Freeman et al., 2010; Rodríguez-Borrero et al., 2010; Maze and Nestler, 2011). However, there have been only a few reports demonstrating an effect of SAM or MET (a precursor of SAM) at the genome level. In SK-N-BE neuroblastoma cells, SAM modulates 7 genes (of a total of 588 genes analysed) of which 3 were upregulated and 4 downregulated, showing low levels of modulation (Cavallaro et al., 2006). Weaver and colleagues (2006) demonstrated that repeated MET intraventricular treatment altered 337 transcripts, representing 1.08% of the population of genes on the DNA microarray, and of these altered transcripts, 217 (64%) were downregulated and 120 (35.6%) were upregulated in the rat hippocampus (Weaver et al., 2006). It is difficult to compare our results with these data but nevertheless, the ratio of up- and downregulated genes is similar between all of the studies. Both SAM and MET only affect the expression of a limited number of genes and did not affect the vast majority of the genome, suggesting that SAM or MET treatments, despite their

global nature, do not result in a general silencing of gene expression. The basis for the specificity of SAM or MET gene expression effects remains unknown. Thus, exogenous SAM treatment induces minor effects on whole-genome gene expression; however, SAM pretreatment significantly modified cocaine-induced gene expression by blunting non-specifically the cocaine response.

To determine the effect of SAM and cocaine at the gene level, we selected (from the gene expression profiling data) *Cck*, *Slc17a7*, and *Gal* genes for analysis based on their possible participation in cocaine-induced neuroadaptations in the NAc (Hökfelt et al., 1980; Josselyn et al., 1997; Fremeau et al., 2001; Narasimhaiah et al., 2009). We found that repeated SAM treatment caused both hypermethylation and hypomethylation in the promoter regions of the selected genes, and these changes associated with down- and upregulated mRNA expression, respectively (Figures 17A-C; 18A-C; see supplementary Figures S3A-C in Paper II). These results are comparable in part with the studies, which demonstrated that exogenous SAM treatment elicits gene silencing via promoter hypermethylation (Watson et al., 1999; Fuso et al., 2001; Pulukuri et al., 2007). Similarly, repeated treatment with MET is also associated with hypermethylation of *reelin* and *GAD67* promoter regions in mouse frontal cortex and striatum (Tremolizzo et al., 2002; Dong et al., 2008). We also found that SAM pretreatment inhibited cocaine-induced hyper- and hypomethylation (*Slc17a7* and *Gal*, respectively) in the NAc. Interestingly, SAM additively enhanced cocaine-induced hypermethylation at the *Cck* promoter, which was associated with *Cck* transcriptional downregulation in the NAc (Figures 17B and 18B). Thus, it seems that SAM pretreatment may both increase and decrease cocaine-induced DNA methylation on gene level.

To evaluate whether the effects of SAM are NAc-specific or not, we assessed the selected genes expression and promoter region methylation levels in the mouse cerebellum. Our data showed that repeated SAM treatment significantly changed *Slc17a7* and *Cck* promoter methylation levels in both brain regions (see supplementary Figures S3A,B in Paper II) indicating that SAM effects are rather non-specific. As expected, the cocaine effects on the gene expression were more prominent in the NAc than in the cerebellum. These data suggest that SAM treatment modifies cocaine (another epigenetic factor) specific effects in the NAc.

2.2. The effect of SAM on DNMT expression and methyltransferase activity

In this part of study, we analysed the consequences of acute and repeated SAM treatments on DNMT's expression and methyltransferase activity in PC12 cells. We found that exogenous SAM treatment induced a biphasic effect on methyltransferase activity (Figures 19A,B) since a single SAM treatment enhanced whereas repeated daily treatment significantly reduced methyltransferase activity. Both *in vitro* and *in vivo* data show that repeated SAM treatment increases *Dnmt3a* promoter-region hypermethylation, which was associated with downregulation of the *Dnmt3a* mRNA level (Figures 20A,C and 21A,C). SAM and cocaine co-treatment (M+C) also increased *Dnmt3a* promoter methylation compared with S+C group. As we found significantly downregulated *Dnmt3b* mRNA level in M+C group (Figure 21D), without methylation of *Dnmt3b* promoter, we speculate that SAM-induced a histone methylation might be involved in *Dnmt3b* expression. Moreover, based on accumulating data, we propose the following model for the regulation of DNA methylation/demethylation

activities. A single dose of SAM stimulates methyl-transferase activity resulting in increased DNA methylation. It is possible that SAM increases DNA methylation by also decreasing demethylation via inhibition of MBD2 (Detich et al., 2003). Repeated administration of exogenous SAM decreases methyltransferase activity via decreased *Dnmt3a* and *Dnmt3b* expression and via increased levels of intracellular SAH, which is the product of the transmethylation reactions and inhibits DNMT activity (Chiang, 1998). Therefore, we propose that the modifing effect on cocaine-induced gene expression in the NAc following repeated SAM treatment may result from an altered balance between methylation/demethylation activities. We also speculate that decrease of SAM-induced methyltransferase activity is reversible after discontinuing exogenous SAM treatment. While we found that SAM and cocaine treatment may also cause promoter-associated CpG-island hypermethylation (e.g., *Cck*), we speculate that other factor(s) may participate in fine-tuning the DNA methylation/demethylation balance.

2.3. The effect of SAM treatment on cocaine-induced behavioural sensitisation

At behavioural level, we found that repeated SAM treatment alone did not affect locomotor activity (Figure 16A); a related finding has been described following repeated MET treatment (Tremolizzo et al., 2002). Several recent studies indicate that injections of DNMT inhibitors into different brain regions may affect inversely the development of cocaine-induced behavioural sensitisation and CPP in mice. For example, continuous intra-NAc infusion over the 7 days of RG108 increased cocaine induced CPP and enhanced the induction of behavioural sensitisation to chronic cocaine (LaPlant et al., 2010). DNMT inhibitor 5-aza-2-deoxycytidine (5-aza) injections into hippocampus CA1 area restrained acquisition of cocaine-induced CPP, however 5-aza had no effect on acquisition after injection into prelimbic cortex (Han et al., 2010). We previously demonstrated that repeated zebularine intracerebroventricular injections decreased cocaine-induced behavioural sensitisation, suggesting that zebularine (due to diffusion to a brain tissue) affected DNMT activity in the hippocampus more than in the NAc (Paper I). In this study we found that SAM pretreatment significantly potentiated cocaine-induced ambulations during the development and expression of behavioural sensitisation in mice. Therefore, our behavioural data, coupled with the decreased *Dnmt3a* and *Dnmt3b* mRNA levels in the NAc, support the findings that decreased methyltransferase activity in the NAc positively regulates cocaine-induced behavioural sensitisation (LaPlant et al., 2010). Increased locomotor activity in M+C group on the 5-7 treatment days is also in line with decreased *Dnmt3a* mRNA level and methyltransferase activity in PC12 cells (Figures 19C and 20A). It is not known how exogenous SAM treatment affects *Dnmt3*a and *Dnmt3b* expression in other brain regions, but we speculate that reduced *Dnmt3a* and *Dnmt3b* expression in the NAc might had higher impact to cocaine-induced locomotor activity compared with other brain regions (e.g. CA1 area).

The cocaine challenge study suggests that an increased locomotor activity in the M+C group (compared to S+C group) is persistent indicating that the effects of SAM on cocaine are long-lasting even after withdrawal period of cocaine. Recently, it has been reported that chronic MET (0.78 g/kg, twice per day subcutaneously) diminished the rewarding effects of cocaine in the CPP procedure (LaPlant et al., 2010). There are a number of methodological

differences between the LaPlant's study and our study that could account for the disparate findings, including the drug (MET vs. SAM), dose of MET and SAM, and different pretreatment regimens. We think that an especially important factor is the pretreatment regimen as SAM or MET might affect different genes, depending on the interval between pretreatment and cocaine administration. However, our data indicate that methyl donor SAM modifies cocaine-induced gene expression in the NAc of mice and thereby contributes the development of behavioural sensitisation.

3. The role of MS on DNA methylation and behavioural changes (Paper III)

Using MS as an early life stress model, our aim was to investigate whether the MS on rats could alter DNMT's and selected genes (*PP1c*, $A_{2A}R$) expression in the NAc of infant and adult rats and whether these changes are associated with the development of cocaine-induced behavioural sensitisation in adulthood.

3.1. The effect of MS on exploratory behaviour and cocaine-induced behaviour in adult rats

To validate our model of MS, we used the exploratory box test to measure novelty-related behaviour in adult rats subjected to MS manipulation. Exploratory behaviour in novel environments is influenced by the conflicting motivators fear and curiosity. Mällo and colleagues (2007) found that animals with a high motivation to explore displayed reduced anxiety-like behaviour (Mällo et al., 2007). It has been also reported that chronic variable stress increased exploratory activity in exploratory box test and increased dopamine D_1 receptor-specific cAMP accumulation in the NAc of rats (Matrov et al., 2011). Our data demonstrated that in the adult MS180 group, rats showed increased exploratory activity compared with the AFR and MS15 groups. These data were consistent with the Marmendal et al. (2006) study, where early deprivation was found to increase exploratory behaviour in adult male Wistar offspring. The exploratory box test data are also consistent with our cocaine results, and several studies have demonstrated, that there is a significant correlation between a subject's locomotor response to a novel environment and its locomotor response to cocaine (Bardo et al., 1996; Hooks et al., 1991). There is a report demonstrating that exposure to novelty activates, at least in part, the same neural substrate that mediates the rewarding effects of drugs of abuse (Bardo et al., 1996), however, the exact mechanism underlying the effects of MS on exploratory behaviour, is unknown.

The acute cocaine treatment (10 mg/kg, i.p.) study demonstrated that adult rats from the MS180 group showed an increased response to acute cocaine treatment (Figure 23A), suggesting that the duration of MS effected this result. The repeated cocaine treatment study indicates that the early life stress (both handling and MS) increased susceptibility to cocaine-induced behavioural sensitisation in adult animals compared with the AFR group (Figure 23B). Few studies have investigated the influence of MS on the effects of cocaine. For example, Li and colleagues (2003) observed that in adulthood, AFR, MS15 and MS180 groups did not differ in their locomotor response to an acute intravenous cocaine treatment,

and that MS15 and MS180 groups showed significantly less robust effects of cocaine challenge than rats from an AFR group; however, they used only female rats (Li et al., 2003). Kikusui and colleagues (2005) found that repeated MS increased the hyperlocomotor response to 10.0 mg/kg cocaine regardless of mouse gender (Kikusui et al., 2005). Marin and Planeta (2004) found that male adolescent rats exposed to MS exhibited an increased locomotor response to cocaine; however, this response was not observed in adult rats (Marin and Planeta, 2004). Brake and colleagues (2004) demonstrated that MS and non-handled animals displayed a dose-dependent increased sensitivity to cocaine-induced locomotor activity compared with a handled group (Brake et al., 2004). These data suggest that the outcome of the model of early life stress may depend on various factors, such as environmental conditions, gender, specifics of the separation procedure, the duration of separation, etc.

3.2. The effect of MS on DNMT and selected genes expression

In present study, we showed that early life stress upregulated Dnmt's expression in the NAc of infant rats and that this upregulation persists into adulthood. We found that MS elevated expression levels of *Dnmt3a* primarily, suggesting that *de novo* methylation dominated. The signal transduction processes that control MS-induced DNMT's expression in the infant CNS are unknown. As we found upregulated *Dnmt3a* mRNA level in the NAc, we hypothesized that increased glucocorticoid receptor stimulation by corticosterone during MS could trigger a cascade of molecular events, which constantly increase DNMT's expression in the brain. These changes facilitate adaptation to acute environmental challenges, but may lead to behavioural pathologies in chronic stress conditions.

On a global level, we found that MS significantly decreased DNA methylation in the NAc, but not in the PFC of adult rats (Figure 25). There is a limited numbers of studies that describe MS-induced DNA methylation on a global level in the rat brain. Using mild and high prenatal stress, Mychasiuk et al. (2011) found that mild prenatal stress increased and high prenatal stress decreased global DNA methylation levels in the frontal cortex and hippocampus of rat offspring.

Studies have demonstrated that stressful experiences during early life may cause both DNA hypo- and hypermethylation of specific gene promoters (Weaver et al., 2004; Fuchikami et al., 2009; Murgatroyd et al., 2009; Roth et al. 2009; Franklin et al., 2010). We found that MS was associated with hypermethylation and transcriptional downregulation of the *PP1c* and $A_{2A}R$ promoter regions in the NAc, but not in the PFC. Neither handling nor MS affected *Reln* promoter-region methylation or transcription levels in the NAc and PFC. These data suggest that MS effects may be gene and brain region specific. Moreover, because we found that on global level, MS was associated with DNA hypomethylation and at gene level with DNA hypermethylation, our findings suggest that MS may regulate DNA methylation at different levels - on a global and a gene level. However, the exact mechanisms underlying these changes, are unknown. A similar phenomenon - global DNA hypomethylation and gene-specific hypermethylation - has been observed in aging-related conditions (Wilson et al., 1987; Oakes et al., 2003; Calvanese et al., 2009).

Furthermore, in many complex diseases including cancer, atherosclerosis and psychiatric disorders (Baylin and Herman, 2000; Pogribny and Beland, 2009), it is common to observe global DNA hypomethylation and gene-specific hypermethylation (Kile et al., 2010).

As mentioned above, gene expression analysis showed that MS induced persistent transcriptional downregulation of the neuronal plasticity-related genes *PP1c* and *$A_{2A}R$* in the NAc. The PP1 enzyme contains both a catalytic subunit and a regulatory subunit (Goldberg et al., 1995). Protein kinase A and PP1 regulate the phosphorylation state and activity of many physiological effectors, including neurotransmitter receptors, for example AMPA receptor, which regulate the excitability of medium spiny neurons (Yan et al., 1999). Repression of the *PP1c* gene via hypermethylation of its promoter region could enable aberrant phosphorylation of crucial receptors and transcription factors during and after MS. Our data suggest that both environmental factors, MS and cocaine treatment (cocaine resulted in *PP1c* promoter hypermethylation and transcriptional downregulation in the NAc of mice, Paper I) have a similar effect on *PP1c* promoter methylation and transcription activity in the NAc.

The activity of the $A_{2A}R$ determines the response to acute cocaine treatment. NAc contains a high density of $A_{2A}Rs$, which pre- and postsynaptically regulate glutamatergic and dopaminergic neurotransmission on GABAergic striatal efferent neurons, in part by acting on heteromers of adenosine receptors with dopamine and metabotropic glutamate receptors (Ferré et al., 2007). At the behavioural level, pharmacological blockade typically potentiates the acute behavioural effects of psychostimulants by acting on the NAc $A_{2A}Rs$ (Popoli et al., 1998; Filip et al., 2006). These finding are consistent with our results that demonstrate that reduced *$A_{2A}R$* mRNA levels in the NAc of MS180 rats is associated with an enhanced response to acute cocaine treatment.

Thus, we can conclude that stressful experiences in early life may create a background, via aberrant DNA methylation, which promotes the development of cocaine-induced behavioural sensitisation in adult rats.

CONCLUSIONS

1. Our data demonstrated that cocaine treatment causes a dynamic increase in *Dnmt3a* and *Dnmt3b* expression levels in the NAc and hippocampus suggesting that cocaine induces rather *de novo* DNA methylation. At the gene level, we identified that cocaine treatment induces both DNA methylation/demethylation and thereby affects certain genes transcription. DNMT inhibitor zebularine intracerebroventricular treatment normalised hypermethylated gene transcription and delayed the development of cocaine-induced behavioural sensitisation in adult mice indicating that DNA methylation/demethylation activities may be important processes during the development of behavioural sensitisation in adult mice.

2. The methyl donor SAM modifies cocaine-induced gene expression at the genome and gene level in the NAc and may thereby contribute the development of cocaine-induced behavioural sensitisation in adult mice.

3. Maternal separation may cause via aberrant DNA methylation persistent changes of gene expression and create a background that promotes the development of cocaine-induced behavioural sensitisation in adult rats.

In summary, our results suggest that DNA methylation plays important role in the development of cocaine-induced behavioural sensitisation. Furthermore, environmental factors such as SAM and early life stress may promote via DNA methylation the development of psychostimulant-induced drug addiction in mice and rats.

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SUMMARY IN ESTONIAN

DNA metüülimise roll kokaiini poolt põhjustatud käitumusliku sensitisatsiooni kujunemises

Ravimsõltuvust defineeritakse kui kroonilist psüühika- ja käitumishäiret, mille peamiseks iseloomustajaks on vastupandamatu tung tarbida psühhotroopseid aineid hoolimata sellega kaasuvatest tõsistest kõrvaltoimetest (Kalivas et al., 2005; Hyman et al., 2006; Koob and Kreek, 2007). Eksperimentaalsed uuringud on näidanud, et ravimsõltuvus kujuneb välja järk-järgult ja sõltuvusega seotud neurobioloogilised muutused võivad jääda püsima ka pärast psühhotroopsete ainete tarvitamise lõppu. Ulatuslikud epidemioloogilised uuringud on näidanud, et ravimsõltuvuse kujunemisel on oluline roll nii geneetilisel eelsoodumusel kui ka keskkonnateguritel (Nestler, 2001; Goldman et al., 2005; Hyman et al., 2006).

Geenid, mis võivad suurendada riski ravimsõltuvuse tekkeks, on käesoleval ajal teadmata. Uuringud on näidanud, et ravimsõltuvuse teket saab mõjutada erinevate keskkonnateguritega nagu stress, psühhotroopsete ainete kättesaadavus, varajase eluea keskkond (Nestler, 2001; Weaver et al., 2004; Goldman et al., 2005; Waterland et al., 2006; Szyf, 2009). Hüpoteesi kohaselt peavad püsivate käitumuslike muutuste aluseks olema püsivad muutused geeniekspressioonis. Seetõttu on viimasel ajal üha enam hakatud tähelepanu pöörama epigeneetikale, mis kirjeldab geeniekspressiooni regulatsioonis osalevaid molekulaarseid mehhanisme ning keskkonna ja geenide vahelisi seoseid.

Geeniekspressiooni reguleerimiseks on vajalik, et vastav geen oleks transkriptsiooni läbiviivatele valkudele kättesaadav. Rakutuumas olev DNA on kokkupakitud kromatiiniks, mis on DNA, histoonide ja teiste valkude kompleks. Kromatiini piirkonnas, kus ei toimu transkriptsiooni (heterokromatiin) on DNA tihedalt seotud histoonidega pärssides transkriptsiooni läbiviivate valkude (nt. RNA polümeraas II) seostumist DNA-ga (Fischle et al. 2003). Kromatiini osa, mis on vähem kokkupakitud ning kus toimub geenide transkriptsioon, nimetatakse eukromatiiniks. Epigeneetilised mehhanismid nagu histoonide modifitseerimine, DNA metüülimine ja mikroRNA-d, on olulised mehhanismid geeniekspressiooni regulatsioonis. Häired epigeneetilistes mehhanismides võivad põhjustada tõsiseid patoloogiaid nagu näiteks kasvajate teket, arengu- ja psühhiaatrilisi häireid (Rett sündroom, Fragiilse X-i sündroom, autism, depressioon, ärevus) jne.

Korduv psühhostimulaatori (nt. kokaiini või amfetamiini) manustamine põhjustab katseloomadel suurenenud käitumusliku vastuse. Sellist fenomeni nimetatakse psühhomotoorseks ehk käitumuslikuks sensitisatsiooniks ning see modelleerib adiktiivset käitumist ja psühhostimulaatorite psühhootilisi komplikatsioone inimesel (Robinson and Berridge, 1993; Pierce and Kalivas, 1997). Käitumusliku sensitisatsiooni tekke ja avaldumisega on seotud aju sarrustuse piirkonnad nagu naalduv tuum *(nucleus accumbens)* ja prefrontaalne korteks. Kuna käitumuslikule sensitisatsioonile on iseloomulikud püsivad muutused katseloomade käitumises, siis arvatakse, et osaliselt on selle põhjuseks lühi- ja pikaajalised geeniekspressiooni muutused, mis omakorda mõjutavad närviimpulsi ülekannet, sünapsite moodustamist ja närviringide funktsioneerimist.

Mitmed uuringud viitavad, et epigeneetilised mehhanismid (eriti geeni promootori piirkonna DNA metüülimine) on seotud pikaajaliste geeniekspressiooni muutustega. DNA metüülimise korral liidetakse DNA metüültransferaasi (DNMT) vahendusel metüülrühm DNA tsütosiin-guanosiin (CpG) dinukleotiidsele järjestusele (Holliday and Pugh, 1975; Klose and Bird, 2006). CpG järjestust esineb kogu genoomi ulatuses ja valdavalt on need metüülitud. CpG järjestused, mis asuvad geeni promootoris on metüülitud väiksemas ulatuses ja nende metüülimise aste korreleerub geeni repressiooniga ehk siis DNA hüpermetüülimine pärsib transkriptsiooni reguleerivate valkude seostumist DNA-ga. Muutused DNA metüülimise/demetüülimise protsesside tasakaalus võivad luua olukorra, kus ekspresseeruvad need geenid, mis peaksid olema vaigistatud või vastupidi ning seeläbi võivadki tekkida püsivad geeniekspressiooni muutused, mis omakorda soodustavad erinevate haiguste, sealhulgas ravimsõltuvuse, teket.

Töö eesmärgid

Antud töö peaeesmärgiks oli hinnata DNA metüülimise rolli kokaiini poolt põhjustatud käitumusliku sensitisatsiooni kujunemises hiirtel ja rottidel.

Täpsemad töö eesmärgid olid järgmised:

1. Uurida: a) kokaiini manustamise mõju DNMT-de ja valitud markergeenide ekspressioonile hiirte naalduvas tuumas; ja b) hinnata DNMT inhibiitori, zebulariini, toimet kokaiini poolt esilekutsutud geeniekspressiooni muutustes ning käitumusliku sensitisatsiooni kujunemises täiskasvanud hiirtel.

2. Hinnata metüülrühma doonor S-adenosüülmetioniini (SAM) mõju kokaiini poolt esilekutsutud geeniekspressiooni muutustes ja käitumusliku sensitisatsiooni kujunemises täiskasvanud hiirtel.

3. Selgitada, kas varajases elueas kogetud stress (maternaalse separatsiooni mudel, MS) võib mõjutada DNMT-de ja valitud markergeenide ekspressiooni noorte ja täiskasvanud rottide naalduvas tuumas ning kas need muutused on seotud kokaiini poolt põhjustatud käitumusliku sensitisatsiooni tekkega täiskasvanueas.

Töö tulemused ja järeldused

1. Käesoleva töö tulemused näitavad, et kokaiini manustamine suurendas dünaamiliselt *Dnmt3a* ja *Dnmt3b* ekspressiooni hiirte naalduvas tuumas ja hipokampuses, kuid ei mõjutanud oluliselt *Dnmt1* ekspressiooni. Kuna on leitud, et DNMT3 perekonda kuuluvad ensüümid DNMT3A ja DNMT3B vastutavad uute (*de novo)* metüülimismustrite loomise eest (Okano et al., 1999) ja DNMT1 olemasolevate metüülimismustrite säilitamise eest (Bestor, 2000; Goll and Bestor, 2005), siis võib järeldada, et kokaiini manustamine mõjutab pigem *de novo* DNA metüülimist. Kasutades metüülitud DNA immunosadestamist ja metüülspetsiifilist kvantitatiivset reaalaja PCR, leidsime, et nii

akuutne kui ka korduv kokaiini manustamine suurendasid ravimsõltuvusega seotud markergeeni proteiin fosfataas 1 katalüütilise alaühiku (*PP1c*) promootori piirkonna hüpermetüülimist hiirte naalduvas tuumas. *PP1c* promootori hüpermetüülimise tulemusena oli oluliselt vähenenud *PP1c* ekspressioon nii mRNA kui valgu tasemel. *PP1c* ekspressiooni langus võib omakorda põhjustada häireid rakusisestes signaaliülekande protsessides, kuna on leitud et *PP1c* osaleb transkriptsioonifaktorite (nt. CREB), kinaaside (nt. Ca^{2+}/kalmoduliin-sõltuv proteiin kinaas II) ja retseptorite (nt. AMPA retseptori alaühik GluR1) fosforüleerimise inhibeerimises (Genoux et al., 2002; Canettieri et al., 2003). Teiseks uuritavaks markergeeniks oli antud töös *fosB.* Erinevalt *PP1c*, oli *fosB* promootor kokaiini manustamise järgselt hüpometüülitud ning transkriptsioon suurenenud. Siit järeldub, et kokaiini manustamine võib põhjustada nii DNA metüülimist kui ka demetüülimist (metüülmärgiste eemaldamist geeni promootori piirkonnast).

DNMT inhibiitori, zebulariini, intraventrikulaarne manustamine enne kokaiini manustamist kõrvaldas *PP1c* promootori hüpermetüülimise hiirte naalduvas tuumas ja normaliseeris *PP1c* ekspressiooni. Käitumiskatsete tulemusena leidsime, et zebulariini manustamine pidurdas kokaiini poolt põhjustatud käitumusliku sensitisatsiooni väljakujunemist täiskasvanud hiirtel. Seega, kokaiini poolt põhjustatud muutused DNA metüülimise/demetüülimise protsessides võivad olla olulised mehhanismid käitumusliku sensitisatisooni kujunemises ning DNMT inhibiitori manustamine võib normaliseerida hüpermetüülitud geenide transkriptsiooni.

2. Kasutades Illumina platvormil põhinevat, kogu genoomi katvat geenieksрessiooni analüüsi, leidsime, et kuigi genoomi tasemel mõjutab SAM vaid väheste geenide ekspressiooni, avaldab ta modifitseerivat toimet kokaiini poolt esilekutsutud geenieksрessiooni muutustele täiskasvanud hiirte naalduvas tuumas. SAM-i modifitseeriv toime avaldus ka geeni tasemel, kuna avastasime, et korduv SAM-i manustamine põhjustas nii hüpo- kui ka hüpermetüülimist Illumina ekspressioonikiibi analüüsi andmete põhjal valitud markergeenide promootori piirkonnas. Lisaks leidsime, et korduv SAM-i manustamine vähendas metüültransferaasi aktiivsust ja langetas *Dnmt3a* ja *Dnmt3b* ekspressiooni nii *in vivo* kui ka *in vitro.* Siit järeldub, et SAM-i modifitseeriv toime kokaiini poolt esilekutsutud geenieksрessiooni muutustele võib olla seotud DNA metüülimise/demetüülimise protsesside tasakaalu muutustega hiirte naalduvas tuumas. Käitumiskatsete tulemused näitasid, et SAM ise ei suurenda hiirte lokomotoorset aktiivsust. SAM + kokaiini koosmanustamise katsetes (SAM-i manustati hiirtele 20 minutit enne kokaiini süstimist) aga leidsime, et SAM suurendab kokaiini poolt põhjustatud lokomotoorse aktiivsuse tõusu nii käitumusliku sensitisatisooni induktsiooni- kui ka ekspressioonifaasis. Seega, metüülrühma doonor SAM avaldab modifitseerivat toimet kokaiini poolt esilekutsutud geenieksрessiooni muutustele naalduvas tuumas ning soodustab käitumusliku sensitisatsiooni teket täiskasvanud hiirtel.

3. Varajases elueas kogetud stressi modelleerimiseks kasutasime MS mudelit, mille korral rotipojad eraldati emast esimesel kahel elunädalal (postnataalsed päevad PND1-14) iga päev 180-ks minutiks. Töö tulemused näitasid, et MS põhjustas püsivaid muutuseid Dnmt-de ja valitud markergeenide (*PP1c* ja $A_{2A}R$) ekspressioonis rottide naalduvas

tuumas. Kuna leidsime, et MS põhjustas globaalselt DNA hüpometüülimist ning *PP1c* ja $A_{2A}R$ promootorite hüpermetüülimist, siis oletame, et MS võib reguleerida DNA metüülimist nii globaalselt kui ka geeni spetsiifiliselt. Käitumiskatsete tulemused näitasid, et MS suurendas nii täiskasvanud rottide uudistamis- kui ka lokomotoorset aktiivsust. Antud töö tulemuste põhjal saab järeldad, et MS võib DNA metüülimise kaudu põhjustada püsivaid muutuseid geeniekspressioonis ning luua tausta, mis soodustab kokaiini poolt põhjustatud käitumusliku sensitisatsiooni teket täiskasvanueas.

Seega, käesoleva töö tulemused näitavad, et DNA metüülimine mängib olulist rolli kokaiini poolt põhjustatud käitumusliku sensitisatsiooni kujunemises. Veelgi enam, keskkonnategurid nagu SAM ja varajases elueas kogetud stress, võivad DNA metüülimise kaudu soodustada psühhostimulaatoritest tingitud ravimsõltuvuse teket nii hiirtel kui ka rottidel.

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PUBLICATIONS

I **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.

II **Anier K,** Zharkovsky A, Kalda A (2013) S-adenosylmethionine modifies cocaine-induced DNA methylation and increases locomotor sensitization in mice. *The International Journal of Neuropsychopharmacology* 16:2053-2066.

III **Anier K,** Malinovskaja K, Pruus K, Aonurm-Helm A, Zharkovsky A, Kalda A (2013) Maternal separation is associated with DNA methylation and behavioural changes in adult rats. *European Neuropsychopharmacology* [Epub ahead of print].

CURRICULUM VITAE

General data

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Education

2008 – ...	University of Tartu, Faculty of Medicine, PhD studies in neurosciences
2003 – 2006	University of Tartu, Faculty of Physics and Chemistry, Department of Chemistry, Chair of Organic Cemistry, MSc
1999 – 2003	University of Tartu, Faculty of Physics and Chemistry, Department of Chemistry, BSc
1987 – 1999	Tõrva Secondary School

Professional employment

2011 – ...	University of Tartu, Faculty of Medicine, Institute of Bio- and Translational Medicine, Department of Pharmacology, researcher
2008 – 2011	University of Tartu, Faculty of Medicine, Department of Pharmacology, researcher
2004 – 2008	University of Tartu, Faculty of Medicine, Department of Pharmacology, specialist

Special courses

2011	Medical bioinformatics, Tartu, Estonia
2009	Laboratory animal science: C-category competence course; Tartu, Estonia

Professional organizations

2005 – ...	Estonian Society of Pharmacology

Scientific work

The scientific work has focused on the role of DNA methylation on the development of cocaine-induced behavioural sensitisation and on the role of epigenetic modifiers to reverse cocaine-induced behavioural sensitisation.

List of publications

1. **Anier K,** Malinovskaja K, Pruus K, Aonurm-Helm A, Zharkovsky A, Kalda A (2013) Maternal separation is associated with DNA methylation and behavioural changes in adult rats. *European Neuropsychopharmacology* [Epub ahead of print].

2. **Anier K,** Zharkovsky A, Kalda A (2013) S-adenosylmethionine modifies cocaine-induced DNA methylation and increases locomotor sensitization in mice. *The International Journal of Neuropsychopharmacology* 16:2053-2066.

3. **Anier K,** Kalda A (2012) Epigenetics in the Central Nervous System. *Current Translational Geriatrics and Gerontology Reports* 1:190-198.

4. Jaako K, Aonurm-Helm A, Kalda A, **Anier K**, Zharkovsky T, Shastin A, Zharkovsky A (2011) Repeated citalopram administration counteracts kainic acid-induced spreading of PSA-NCAM-immunoreactive cells and loss of reelin in the adult mouse hippocampus. *European Journal of Pharmacology* 666:61-71.

5. **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.

6. Kipper K, **Anier K**, Leito I, Karjagin J, Oselin K, Herodes K (2009) Rapid Determination of Meropenem in Biological Fluids by LC: Comparison of Various Methods for Sample Preparation and Investigation of Meropenem Stability. *Chromatographia* 70:1423-1427.

7. Tamm R, Oselin K, Kallassalu K, Magi R, **Anier K**, Remm M, Metspalu A (2008) Thiopurine S-methyltransferase (TPMT) pharmacogenetics: three new mutations and haplotype analysis in the Estonian population. *Clinical Chemistry and Laboratory Medicine* 46:974-979.

8. Oselin K, **Anier K** (2007). Inhibition of human thiopurine S-methyltransferase by various nonsteroidal anti-inflammatory drugs in vitro: a mechanism for possible drug interactions. *Drug Metabolism and Disposition* 35:1452-1454.

9. Metsvaht T, Oselin K, Ilmoja M, **Anier K**, Lutsar I (2007) Pharmacokinetics of penicillin G in very-low-birth-weight neonates. *Antimicrobial Agents and Chemotherapy* 51:1995-2000.

10. Oselin K, **Anier K**, Tamm R, Kallassalu K, Mäeorg U (2006) Determination of thiopurine S-methyltransferase (TPMT) activity by comparing various normalization factors: Reference values for Estonian population using HPLC-UV assay. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 834:77-83.

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Haridus

2008 – ...	Tartu Ülikool, Arstiteaduskond, Neuroteaduste doktoriõpe, doktorant
2003 – 2006	Tartu Ülikool, Füüsika-keemiateaduskond, orgaanilise keemia õppetool, MSc
1999 – 2003	Tartu Ülikool, Füüsika-keemiateaduskond, keemia eriala, BSc
1987 – 1999	Tõrva Gümnaasium

Teenistuskäik

2011 – ...	Tartu Ülikool, Arstiteaduskond, Bio- ja siiremeditsiini instituut, Farmakoloogia osakond, teadur
2008 – 2011	Tartu Ülikool, Arstiteaduskond, Farmakoloogia Instituut, teadur
2004 – 2008	Tartu Ülikool, Arstiteaduskond, Farmakoloogia Instituut, spetsialist

Erialane enesetäiendus

2011	Meditsiiniline bioinformaatika, Tartu, Eesti
2009	Katseloomateadus: C-kategooria kursus; Tartu, Eesti

Erialaorganisatsioonid

2005 – ...	Eesti Farmakoloogia Selts

Teadustöö

Teadustöö põhisuunaks on uurida DNA metüülimise rolli kokaiini poolt põhjustatud käitumusliku sensitisatsiooni kujunemises ning hinnata epigeneetiliste modifitseerijate toimet kokaiini poolt esilekutsutud käitumusliku sensitisatsiooni pööratavusele.

Publikatsioonide loetelu

1. **Anier K,** Malinovskaja K, Pruus K, Aonurm-Helm A, Zharkovsky A, Kalda A (2013) Maternal separation is associated with DNA methylation and behavioural changes in adult rats. *European Neuropsychopharmacology* [Ilmumas].

2. **Anier K,** Zharkovsky A, Kalda A (2013) S-adenosylmethionine modifies cocaine-induced DNA methylation and increases locomotor sensitization in mice. *The International Journal of Neuropsychopharmacology* 16:2053-2066.

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8. Oselin K, **Anier K** (2007). Inhibition of human thiopurine S-methyltransferase by various nonsteroidal anti-inflammatory drugs in vitro: a mechanism for possible drug interactions. *Drug Metabolism and Disposition* 35:1452-1454.

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