DISSERTATIONES
NEUROSCIENTIAE
UNIVERSITATIS
TARTUENSIS
33

MARI URB

DNA methylation in the predisposition, expression and abstinence of cocaine addiction

DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS
33

DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS
33

MARI URB

DNA methylation in the predisposition, expression and abstinence of cocaine addiction

UNIVERSITY OF TARTU
Press

Department of Pharmacology, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia. Department of Chemistry and Biotechnology, Faculty of Science, Tallinn University of Technology.

Dissertation was accepted for the commencement of the degree of Doctor of Philosophy in Neurosciences on 24.04.2020, by the Joint Council for the Curriculum of Neurosciences

Supervisors:	Anti Kalda, MD, PhD, Professor, Department of Pharmacology, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia
	Tõnis Timmusk, PhD, Professor, Department of Chemistry and Biotechnology, Faculty of Science, Tallinn University of Technology, Estonia
Reviewers:	Kristina Mäemets-Allas, PhD, Researcher, Institute of Cell and Molecular Biology, University of Tartu, Estonia
	Urmas Arumäe, PhD, Docent, Department of Chemistry and Biotechnology, Faculty of Science, Tallinn University of Technology, Estonia
Opponent:	Petteri Piepponen, PhD, University Lecturer, Division of Pharmacology and Pharmacotherapy, Faculty of Pharmacy, University of Helsinki, Finland
Commencement:	25.09.2020

This research was supported by Estonian Research Council grant PUT1686, institutional research funding IUT2-3 and IUT19-18 and European Union through the European Regional Development Fund (Project No. 2014-2020.4.01.15-0012).

ISSN 1736-2792
ISBN 978-9949-03-367-6 (print)
ISBN 978-9949-03-368-3 (pdf)

University of Tartu Press
www.tyk.ee

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I. **Urb** M, Anier K, Matsalu T, Aonurm-Helm A, Tasa G, Koppel I, Zharkovsky A, Timmusk T, Kalda A. Glucocorticoid Receptor Stimulation Resulting from Early Life Stress Affects Expression of DNA Methyltransferases in Rat Prefrontal Cortex. J Mol Neurosci. 2019 May; 68(1): 99–110. http://doi.org/10.1007/s12031-019-01286-z.

II. Anier K, **Urb** M, Kipper K, Herodes K, Timmusk T, Zharkovsky A, Kalda A. Cocaine-induced epigenetic DNA modification in mouse addiction-specific and non-specific tissues. Neuropharmacology. 2018 Sep 1; 139:13–25. http://doi.org/10.1016/j.neuropharm.2018.06.036.

III. **Urb** M, Niinep K, Matsalu T, Kipper K, Herodes K, Zharkovsky A, Timmusk T, Anier K, Kalda A. The role of DNA methyltransferase activity in cocaine treatment and withdrawal in the nucleus accumbens of mice. Addict Biol. 2019 Feb 7. http://doi.org/10.1111/adb.12720.

Author's contribution to the publications listed above is as follows:

I. The author contributed to the design of the study, performed most experiments, conducted data analysis and wrote the manuscript.
II. The author performed gene expression, behavioral and specific loci 5-mC (5-methylcytosine) quantification experiments, conducted data analysis and co-wrote the manuscript.
III. The author performed molecular cloning, primary culture, immunohistochemistry and gene expression experiments, participated in behavioral and in vivo AAV injection experiments, analysed the data and wrote the manuscript.

ABBREVIATIONS

5-HT	5-hydroxytryptamine
5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine
AAV	Adeno-associated virus
AC	Acute cocaine
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AZN	azanucleoside
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CACNA1D	calcium voltage-gated channel subunit alpha1 D
Cer	cerebellum
ChIP	Chromatin immunoprecipitation
CHRNA5	cholinergic receptor nicotinic alpha 5 subunit
CNR1	cannabinoid receptor 1
CNS	central nervous system
CORT	corticosterone
CpG	Cytosine-phosphate-guanine
CPP	Conditioned place preference
CREB	cAMP response element-binding protein
CRH	Corticotrophin releasing hormone
DA	Dopamine
DAT	Dopamine transporter
DNMT	DNA methyltransferase
Esr1	estrogen receptor-1
FKBP5	FKBP prolyl isomerase 5 (human), FK506 binding protein 5 (house mouse)
GABA	γ-aminobutyric acid
GR	Glucocorticoid receptor
GRE	Glucocorticoid receptor element
Grin1	glutamate receptor subunit zeta-1
HDAC	Histone deacetylase
HPA	Hypothalamus-pituitary-adrenal
IEG	Immediate early genes
lncRNA	Long non-coding RNA
MeCP2	N-methyl-D-aspartate methyl-CpG binding protein 2
MS	Maternal separation
NA	noradrenaline
NAc	*Nucleus accumbens*
NF-κB	Nuclear factor κB
NMDA	N-methyl-D-aspartate
PBC	Peripheral blood cells

PFC	Prefrontal cortex
PND	Postnatal day
PKA	Protein kinase A
PVN	(hypothalamic) paraventricular nucleus
RG108	N-phthalyl-L-tryptophan, DNMT1 inhibitor
RC	Repeated cocaine
RS	Repeated saline
SAL	Saline
SAM	S-adenosyl methionine
SEM	Standard error of the mean
shRNA	Short hairpin RNA
SUD	Substance use disorder
SGI-110	quadecitabine
TDG	Thymine DNA glycosylase
TET	Ten eleven translocation
TUJ1	Tubulin beta 3 class III (*Tubb3*)
VTA	Ventral tegmental area

INTRODUCTION

Substance use disorder (SUD) is one of the major unresolved health problems affecting contemporary societies. According to the updated Diagnostic and Statistical Manual of Mental Disorders SUD is a brain disease in which psychoactive drug(s) evoke clinically significant distress or social or occupation impairments. In the current classification of both substance use and dependence the following criteria are included: craving, abuse, tolerance, withdrawal, continued used despite health problems. Craving is defined as a strong urge or desire to use the substance. Withdrawal is exhibited by either (1) a person having characteristic withdrawal symptoms for the specific substance or (2) a person using a substance or one closely related to it to avoid the substance-specific withdrawal symptoms. The substance classes are based on their mechanism of action: alcohol, cannabis, caffeine, hallucinogens, inhalants, opioids, sedatives-hypnotics-anxiolytics, stimulants, tobacco and others (Administration, 2016).

The use of biomarkers would aid in making SUD diagnoses, unfortunately none are used due to lack of suitability. Drugs or their metabolites in body fluids, hair or breath are present only during a short time period after an acute use, the replicated genetic variants are rare in many populations and the specificity of brain imaging techniques is still under development, thus, biomarkers are not yet appropriate for use in diagnosis making and need further research (Hasin et al., 2013).

Currently there is no causal treatment for SUD. Addictive drugs change various brain systems that are not yet well understood. They have a reinforcing effect by activating the mesolimbic dopamine pathway where drugs usurp synaptic plasticity processes. Moreover, addictive drugs also induce neuroadaptations between other neurotransmitter and hormone systems and changes in gene transcription and function that are partly mediated by epigenetic mechanisms. These adaptations also affect stress responsivity. Therefore, a better understanding of the contribution of various basic mechanisms to the transition from drug use to abuse furthers the development of effective treatments for SUD.

LITERATURE OVERVIEW

1. Reward learning and incentive motivation

Numerous philosophical and psychological studies have been performed to answer the question "what motivates us to act? ". Generally, it is understood that a reward will prompt us to obtain an incentive, whereas a punishment will motivate us to avoid it. Reward is defined as "an event that increases the probability of a response with a positive hedonic component" (Koob and Volkow, 2016). A neutral stimulus (conditioned stimulus) can be paired with a natural reward and a drug reward (unconditioned stimulus) to motivate behavior (Siegel, 1979). The behavioral response can be directed towards the outcome or towards the conditioned stimulus. Some have thought that the goal-oriented learning can explain all motivated behaviors (Donahoe et al., 1997), however, other studies have shown evidence of another form of learning, instrumental or operant learning (Konorski, 1948). Instrumental actions are either (1) performed to acquire a reward through an association created with a stimulus that predicts the reward or (2) since there is no understanding or the causal relationship between the action and the outcome, action evaluation becomes irrelevant and instrumental actions are only dependent on the adjoining of the action and the reward. Therefore, instrumental behavior under certain circumstances can shift from goal-oriented behavior to stimulus-reward oriented behavior, in which environmental stimuli that have been paired with an action that obtains a reward, trigger the instrumental action. This performance is said to be under habitual control and in this case, the outcome does not motivate behavior directly, but the stimuli-reward actions are strengthened by the outcome. Numerous studies have shown that animals need learn about the increased value of an instrumental outcome and with increased amount of training, the action selection shifts from goal-directed to habitual behavior. Thus, the drug-seeking behavior increases due to the enhanced incentive value of the drug (Hutcheson et al., 2001).

2. Theories of drug addiction

The concept of addiction can be explained as a three-stage periodically repeating circle that worsens over time and involves neuroplastic modifications in the brain reward, stress and executive function systems (Koob and Volkow, 2016). The three stages comprise of binge/intoxication, withdrawal/negative effect, and preoccupation/anticipation (craving). Different reinforcements affect and drive the recurring circle, positive reinforcement of the binge/intoxication stage, negative reinforcement to avoid adverse emotional state (dysphoria, anxiety, irritability) when access to the drug is prevented and the positive and negative conditioned reinforcement associated with the preoccupation/anti-

cipation stage (Koob and Moal, 2001). Impulsivity is defined as "a predisposition toward rapid, unplanned reactions to internal and external stimuli without regard for the negative consequences of these reactions to themselves or others" and is accompanied by feelings of pleasure, gratification (Moeller et al., 2001). Compulsivity manifests as "perseverative, repetitive actions that are excessive and inappropriate" and in obsessive-compulsive disorder are carried out to reduce anxiety and tension from obsessive thoughts (Berlin and Hollander, 2014). Impulsivity and compulsivity drive drug-taking behavior at different stages of the addiction cycle and in the context of addiction, individuals shift from impulsivity to compulsivity (Koob and Volkow, 2016).

The drug seeking and self-administration behaviors can be understood with these two underlying concepts – conditioned reinforcement and incentive salience. Drugs of abuse have a thorough effect on previously neutral stimuli and can be coupled with them. The phenomenon, called conditioned reinforcement, is defined as when a previously neutral stimulus strengthens certain behaviors through its association with drug of abuse and becomes a reinforcer itself (Koob and Volkow, 2016). The motivation for rewards coming from both one's physiological state and previously learned associations about a reward cue is defined as incentive salience and is mediated by the mesocorticolimbic dopamine system (Koob and Volkow, 2016). The ability of conditioned cues to recruit reward circuits and increase the progression through the addiction cycle helps to explain the intensity of the desire for the drug (craving) and the compulsive use even when the pharmacological effects weaken (Koob and Volkow, 2016).

Several theories have been developed to explain how recreational drug use transitions into compulsive drug seeking, in which the individual continues to use it, because of the feeling of need, despite serious negative consequences and reduced pleasure. According to the incentive motivation theory, chronic drug use results in persistent alterations in the neural circuitry associated with stimulating motivation that allows drugs and their cues to enhance drug-seeking behavior (Robinson and Berridge, 1993).

3. Brain reward system

Many studies have linked dopamine (DA) with reward behavior. The brain reward system was first described by Olds and Milner in 1954, where they showed that rats learned to work inexhaustibly to receive or avoid an intracranial electrical stimulation of specific septal nuclei, described as rewarding or aversive (Olds and Milner, 1954). DA was identified to maintain brain stimulation in medial prefrontal cortex (PFC), *nucleus accumbens* (NAc, a part of ventral striatum), ventral tegmental area (VTA) (Phillips and Fibiger, 1978). In agreement with these studies, DA receptor antagonists (Franklin and McCoy, 1979) and lesions in dopaminergic regions reduce self-stimulation behavior (Strecker et al., 1982). Overactivation of the DA system, however downregulates

the DA receptors and repeated drug intake decreases the importance of alternative stimuli (Volkow et al., 2017)

Natural rewards and addictive drugs alike are able to increase synaptic DA in the NAc via dopaminergic neurons in the VTA. The NAc is mainly composed (>90%) of medium spiny neurons classified by their expression of different DA receptors (Mews et al., 2018). Fast and steep increases of DA is associated with the subjective sensation of the so-called "high" (Hyman et al., 2006; Volkow et al., 2003). The fast and steep augmentation of DA is mediated by low-affinity D_1 receptors (via increased cAMP signaling), which are necessary for reward and cue-induced reponses (Caine et al., 2007). D_3 receptors that co-localize with D_1 receptors in the NAc are also associated with drug-seeking behavior (Vorel et al., 2002). Slower DA signaling is associated with motivated behaviors and incentive motivation (Berridge, 2007; Schultz, 2002). D_2 receptors, in contrast, are not necessary for drug reward (Caine et al., 2002).

The neurocircuitry that is associated with drug reward has been broadened to include projections that interact with the basal forebrain and other neurotransmitters and neuromodulators (Koob and Volkow, 2016, 2010). The neurons in the PFC are regulated by DA activity through D_1 and D_2 receptors and send a glutamatergic projection to the NAc. The aforementioned glutamatergic projection to the NAc among others is associated with drug reinstatement. Blockade of DA receptors in the PFC reduced cocaine-induced reinstatement; while conversely, DA injections to the PFC elicit a reinstatement of cocaine self-administrative behavior (McFarland and Kalivas, 2001). Furthermore, drug administration can trigger synaptic changes in glutamatergic projections from the prefrontal cortex and amygdala to the VTA and NAc (Egervari, 2016). Glutamate receptors include NMDA ionotropic receptors that modulate cell excitability. *Grin1* encodes a critical subunit for proper NMDA channel function. Thus, repeated drug use impairs the capacity of PFC to regulate cocaine-seeking habits due to dysregulation in glutamate signaling.

4. Neurobiology of drug addiction

4.1. Primary neurobiological targets of psychostimulants and brain adaptations to long-term psychostimulant use

Addiction is a state in human behavior manifested by compulsive drug use despite serious negative consequences (Hyman et al., 2006). Drug addiction can be defined as a chronic relapsing disorder of compulsive drug use and loss of control over drug intake despite serious negative consequences (Hyman et al., 2006; Koob and Volkow, 2016). An important goal of current neurobiological research is to understand the changes at the molecular, cellular and neurocircuitry levels of the transition from occasional and controlled drug taking to loss of control in drug use, dependence and the way how these changes persist in the vulnerability to relapse (Koob and Moal, 2001).

Psychostimulants increase DA neurotransmission in the NAc, their primary mechanism of action is either blocking the DA transporter (cocaine) or directly increasing DA efflux (amphetamine) (McCreary et al., 2015). Psychostimulants have several primary targets including DA, noradrenaline (NA) and serotonin (5-HT) transporters. There can be several mechanisms of action among different psychostimulants. For example, amphetamine is a monoamine transporter inhibitor (via trace amine-associated receptor 1 agonism) and releases DA, NA, 5-HT from synaptic vesicles via vesicular monoamine transporter 2 and dysfunctions DA metabolism by inhibiting monoamine oxidase (McCreary et al., 2015). However, the reinforcement properties of cocaine-like drugs correlate with their affinity to DA but not to NA or 5-HT receptors (Ritz et al., 1987).

Cocaine and other psychostimulants cause long-term drug-induced changes on molecular, cellular and behavioral level (Nestler, 2013; Paulson et al., 1991). For transcriptional and epigenetic changes to affect the behavior in addiction, it must change some functional output, such as neuronal excitability (intrinsic membrane properties) or connectivity (synapse number or strength). Psychostimulants also affect neuronal connectivity; the effect is most clearly seen on the changes in number, shape and size on medium spiny neurons in NAc (Russo et al., 2010).

Altered signal transduction and gene expression are thought to mediate the cellular and behavioral adaptations that drugs induce and sustain (Nestler, 2013; Nestler and Aghajanian, 1997). The causal role of cAMP (cyclic adenosine monophosphate) pathway-CREB (cAMP response element binding protein) upregulation in tolerance and dependence has gathered substantial support in recent decades (Nestler, 2016). Chronic administration of opiates and stimulants upregulates cAMP formation, cAMP-dependent protein kinase A (PKA) activation and PKA-dependent protein phosphorylation in the nucleus accumbens (NAc) (Nestler, 2016; Terwilliger et al., 1991). Drug-seeking behavior refers to patient's manipulative and demanding behavior with the aim of obtaining the drug. Self-administrating animals display drug-seeking behavior by persistant learned response (lever pressing) when drug is unavailable. Various interventions of cAMP/PKA signaling pathway have shown to increase drug self-administration or enhance drug-seeking behavior (Edwards and Koob, 2010; Self et al., 1998). Thus, the upregulation of cAMP/PKA pathway in NAc may be an important neuroadaptation for the establishment and maintenance of the addicted state.

The best studied transcription factors that are activated by drugs of abuse are *CREB*, Δ*FosB*, *NF-κB* and *Nr3c1* alias *GR* (the latter will be discussed in the following chapters), which in turn activate target genes and specific behavioral aspects of addiction. CREB is a major cAMP-regulated transcription factor. CREB serves to reduce an animal's sensitivity to the rewarding effects of the drug (tolerance) in NAc and mediates the negative emotional state during withdrawal (Barrot et al., 2002; DiNieri et al., 2009). These manifestations drive increased drug self-administration (Larson et al., 2011). The effect of CREB overexpression in NAc medium spiny neurons helped to limit behavioral

sensitivity to cocaine and thus by decreasing the reinforcing value of natural and drug rewards, cAMP-CREB upregulation contributes to the decrease in reward pathway and to the negative emotional state in withdrawal (Dong et al., 2006; Koob and Volkow, 2016). CREB-mediated transcription increases in NAc response to chronic stress, while knockdown of CREB leads to opposite phenotype, thus CREB is also involved in stress susceptibility and depression-like behavior (Barrot et al., 2002; Covington et al., 2011).

Repeated substance use increases transcription factor ΔFosB levels persistently that has been demonstrated with many drugs of abuse (Nestler, 2008). It has also been shown in human addicts (Robison et al., 2013). The behavioral phenotype of ΔFosB overexpressing animals resembles rodents after chronic drug administration. For example, in response to acute and chronic cocaine administration mice show increased locomotor activity (Kelz et al., 1999). The mice also show enhanced sensitivity to rewarding effects in place-conditioning assays and ΔFosB increases self-administration of the very low doses of cocaine, apparently through a process of positive reinforcement (Colby et al., 2003; Kelz et al., 1999).

Nuclear factor-κB (NF-κB) is a transcription factor activated by various intra- and extracellular stimuli, previously characterized by its functions in the immune system. NF-κB has been recently associated with synaptic plasticity and memory by being activated by basal synaptic input through a pathway requiring Ca^{2+}/ calmodulin-dependent kinase and local Ca^{2+} elevation (Meffert et al., 2003).

NF-κB expression is induced after chronic cocaine administration in the NAc and this is necessary but not sufficient for sensitization to the drug's rewarding effects (Russo et al., 2009). NF-κB has a role in medium spiny neuron spinogenesis in stress models which is particularly interesting considering the phenomenon of stress induced relapse to addiction (Christoffel et al., 2011).

4.2. Animal models in psychostimulant research

Understanding the neurobiology of addiction has been acquired through animal models and brain imaging studies of individuals with addiction. Animal models permit investigations of specific phenomena associated with the human pathological state such as models of genetic, epigenetic, cellular, transcription and network systems; symptoms like craving, relapse, depressive-like state and others (Koob and Volkow, 2016).

The modern neuropsychopharmacological studies of drug addiction began in 1962, when the rat intravenous drug self-administration procedure was developed (Weeks, 1962). Animal models aim to resemble the human condition (face validity) and to possess explanatory power of the pathology (construct validity). Additionally, animal models should predict the pharmacological potential of a compound in humans or a variable dependent of drug addiction. Drugs of abuse and the drug-associated stimuli have a powerful reinforcing effect. Animal

models of drug abuse include non-contingent (experimenter-administered) and contingent (self-administered) drug delivery. Behavioral sensitization is measured after repeated non-contingent drug treatments, whereas the recent contingent studies mostly focus on the reinstatement of drug-use or drug-seeking behavior. Both model types assess the effect of repeated drug use to neural function, but differ by drug administration.

Sensitization is defined as an enhanced response to a stimulus after a repeated exposure to that stimulus (Steketee and Kalivas, 2011). Behavioral sensitization is manifested as an increased psychomotor response that occurs with repeated and intermittent drug exposure by inducing dopaminergic pathways. Psychostimulant-induced behavioral sensitization models addictive behaviors, namely those associated with craving, relapse and psychotic complications of psychostimulant abuse in rodents (J.-F. Chen et al., 2003; Robinson and Berridge, 1993). Motor activity is commonly monitored and repeated exposure increases the motor-stimulant response. However, sensitization with higher doses of amphetamine can escalate to stereotypic behavior that interferes with locomotion (Leith and Kuczenski, 1982). The environment of drug administration can be paired with drug acquisition and thus influence sensitization. Many studies have shown that repeated psychostimulant administration in the same environment as previous drug exposure can enhance behavioral sensitization (Steketee and Kalivas, 2011). Behavioral sensitization can also be measured by conditioned place preference (CPP) or drug self-administration. Sensitization in the CPP paradigm is exhibited as an increased time spent in psychostimulant-associated environment.

Repeated administration of psychostimulants to rodents causes an enhanced locomotor response to subsequent drug injection, a phenomenon known as behavioral sensitization that can be divided into induction and expression phase of sensitization (Pierce and Kalivas, 1997; Robinson and Berridge, 1993). Initiation phase shows the immediate neural effects induced by drug exposure, while expression is the long-term consequences of initial events. Initiation is commonly associated with the ventral tegmental area and expression is linked to NAc.

In the self-administration studies, rodents acquire the drug by performing a behavioral response (pressing a lever) that is a sign of the drug's reinforcing properties. In the most commonly used fixed ratio schedules, the number of responses required for a dose infusion is set as a constant number and a certain minimum dose is required for stable behavioral responding. Drug infusion is signaled by an environmental stimulus that becomes a conditioned stimulus for the drug. Drug self-administration models show stable responding during various sessions and the response can be changed predictably by neurotransmitter antagonists.

In the human condition, abstinence occurs often voluntarily and due to adverse consequences of drug use and relapse is triggered by the reinforcing properties of drug-associated stimuli (reviewed in Kõks, 2015). Newer preclinical self-administration models are emerging that can also distinguish various psycho-

logical constructs of the disorder that facilitate compulsive relapse after voluntary abstinence and thus are focused on assessing the motivational and reinforcing value of drug-associated stimuli (Belin-Rauscent et al., 2016). In this "incubation of craving" method, longer abstinence periods result in increased drug-seeking behavior when reintroduced to the drug-associated environment that resembles the human phenomenon of increased craving after prolonged abstinence. To date, this version of the self-administration model in rodents is regarded as the most translational to the human condition (Lax and Szyf, 2018).

Current animal models have face validity (resembles the human condition) and some construct validity (has explanatory capacity) in three different stages of addiction. Psychopathologies and stress may influence human drug-seeking behavior, but animal studies showed that these pre-existing conditions are not necessary for drugs to be rewarding (Kõks, 2015). Moreover, self-administration of addictive drugs by laboratory animals supports understanding that drugs are universal reinforcers and human-specific factors are not necessary for reinforcement to take place (Kõks, 2015). Thus, both contingent and non-contingent models have construct validity for drug intoxication.

Animal models have greatly contributed to the knowledge of the molecular targets and neuroadaptations occurring after acute and chornic drug exposure place (Kõks, 2015). Both the behavioral sensitization and the self-administration have construct validity at the drug reinstatement phase in that they seem to correctly predict the neurocircuitry of substance abuse (Steketee and Kalivas, 2011). The "incubation of craving" model has provided a new possibility to study an additional human aspect of increased craving behavior after prolonged abstinence. The disharmony of animal and human studies likely indicates that the latter are influenced by additional factors not duplicated in animal studies or from subject/experimenter biases.

5. Activity-dependent drug induced neuroplasticity

The term neural plasticity has been in use in brain sciences for more than a century to refer to the changes in neural organization that would explain the modifiability of behavior, including short- or long lasting behavior, forms of learning and adaptability in response to injury. The fundamental mechanism of behavioral modifiability is attributed to synaptic transmission between neurons, whereby existing pathways are strengthened or new ones formed. The outcome of this process leads to structural (anatomical/morphological), functional (physiological) and biochemical changes in the brain (Berlucchi and Buchtel, 2009). The modifiability of synapses to store information on a long-term basis involves synapse-to-nucleus signaling pathways. New mRNA and protein synthesis to provide substrates for alterations in the synapse is seen as a requirement to store information / stabilize experiences. This synaptic activity-dependent transcription is distinguished from the basal gene expression. Thus, animal studies

have revealed that the basis of cognitive functions such as learning is synaptic activity-driven transcriptional mechanism (Yap and Greenberg, 2018).

Neurotransmitter-induced genomic response requires an influx of cytoplasmic calcium by stimulation of ligand-gated ion channels, such as the NMDA glutamate receptors and voltage-sensitive calcium channels or by the release of calcium from intracellular stores. The increase in cytoplasmic calcium leads to the activation of Ras-mitogen-associated protein kinase, calcium/calmodulin-dependent protein kinases, and calcineurin-mediated signaling pathways that mediate local alterations in synapses and also induce the activity-controlled transcription factors. These include CREB, myocyte enhancer factor 2 (MEF2), and the serum response factor (SRF) and induce the expression of immediate early genes (IEG) that are defined as "a class of genes that are rapidly and transiently induced by extracellular stimuli, without a requirement of new protein synthesis". IEGs, such as *Fos,* regulate a delayed response of gene expression of many genes related to cellular processes, such as dendritic growth, synapse formation, maturation and elimination, excitatory-inhibitory balance of circuits, as well as neuronal metabolic homeostasis and survival (Yap and Greenberg, 2018).

Activity-regulated DNA methylation-based priming mechanisms have been proposed to allow a permissive genomic state through recurring activity for a healthy neuronal transcriptome (Pruunsild and Bading, 2019). An alternative isoform of DNA methyltransferase 3A (*Dnmt3a*), that is *Dnmt3a*, is upregulated by synaptic activity and may be a central regulator for maintaining the transcriptomic profile (Oliveira et al., 2012). *DNMT3A* enzyme activity is associated with cocaine seeking, and its expression is altered during withdrawal (LaPlant et al., 2010; Massart et al., 2015). Besides the role of DNMT3A2 in experience-dependent behavior, the role of DNMT3A2 cocaine-related behavior was recently elucidated (Cannella et al., 2018).

Drug addiction can be viewed as a dysfunctional neural plasticity in individuals induced by repeated drug use. Roughly 50% of the risk to drug addiction can be attributed to genetic contribution. Genome-wide studies have identified numerous genes with changed expression in rodent and primate models of addiction and human addicts (Albertson et al., 2006a; Freeman et al., 2001; Zhou et al., 2011). For example, in the human NAc, opioid peptide prodynorphin increases in cocaine addicts (Albertson et al., 2006b) that has been established on animal models previously and dynorphin may counteract the resposes of overstimulated dopamine system. Candidate-gene driven approaches have revealed a number of single nucleotide polymorphisms that were associated with cocaine addiction. These polymorphisms were located near L-type calcium channel *CACNA1D*, cannabinoid receptor 1, delta opioid receptor (Pierce et al., 2018). Some results have been replicated, but not all and perhaps the abovementioned can be considered as targets for novel drug development.

Other genome-wide studies have identified first risk genes for cocaine dependence and only few have been replicated, such as rs16969968 in the *CHRNA5* gene and rs806368 in *CNR1* (Bühler et al., 2015). The association

between both *CHRNA5* and *CNR1* and cocaine addiction was protective (Bühler et al., 2015). A missense polymorphism rs16969968:G > A in nicotinic acetylcholine receptor α-5 subunit is strongly associated as a nicotine risk gene (Bühler et al., 2015) and because both cocaine and nicotine and frequently co-abused, the polymorphism was studied also in cocaine addiction. However, the association between the polymorphism in nicotinic α-5 subunit and cocaine abuse was found to be protective for cocaine addiction (Grucza et al., 2008). The physiological basis of this finding is unknown, but contributes to the idea that common genetic factors underlie drug abuse. The risk factors that contribute the other half include adverse early life experiences (discussed below) and other environmental stimuli that render the individual more vulnerable to drug exposure (Peña et al., 2013). Neurons activated during drug-associated learning require distinct profiles of gene expression in key brain areas involved in the reward circuit. Epigenetic mechanisms that underlie normal cellular and synaptic functioning are viewed as mediators both in addiction vulnerability and in drug-induced maladaptations in the brain.

Some genome-wide studies have been performed to examine DNA methylation changes with expression analysis after neuronal activity (Guo et al., 2011a; Halder et al., 2016). Differentially methylated and expressed genes in the hippocampus were functionally categorized under “ion channels” and “transcriptional regulation” (Halder et al., 2016). Thus, DNA methylation is thought to regulate synaptic transmission and gene transcription critical for memory maintenance (Oliveira, 2016) and that could include drug-associated memories. DNA methylation seems to induce stable changes in drug-associated memory genes (Bayraktar and Kreutz, 2018). Neurons activated during learning require distinct profiles of gene expression. Epigenome is seen as a signal integration platform through which environment interacts with an individual’s genome to integrate new information in the service of the stable neuronal functioning.

6. Epigenetic mechanisms

6.1. Basic mechanisms of epigenetic modifications

While the “central dogma” of the transcription of genetic information from DNA to RNA and subsequent translation into proteins remains a guiding principle in gene expression, the field of epigenetics was developed to explain the interaction of genome to external stimuli to stabilize cell phenotype (Crick, 1970). Epigenetics can be defined as “the study of any potentially stable and, ideally, heritable change in gene expression or cellular phenotype that occurs without changes in Watson-Crick base-pairing of DNA” (Goldberg et al., 2007). DNA is packed into chromosomes through highly regulated organizations to allow the selective accessibility of transcription machinery to specific genomic elements (including promoters, enhancers, silencers, open reading frames, insulators etc). Epigenetic processes comprise of chemical (modifications on DNA and histone

proteins) and structural alterations (chromatin remodeling and inter/intra-chromosomal DNA-DNA interactions) that enable a long-term regulation of gene function and hence influence cell lineage, function and fate (Chen et al., 2017a).

The human genome of more than 3 billion base pairs of DNA is compacted 400,000-fold to fit into the nuclear volume of ~1000 μm^3 (Schneider and Grosschedl, 2007). Histones are the main proteins used to coil the DNA to form nucleosomes, the basic repeating structural unit. Nucleosomes contain about 146 bp of DNA and an octamer of histones composed of two of each histone monomer (H2A, H2B, H3, and H4). Histones are positively charged in the N terminus (enriched with arginine and lysine residues) that facilitates the binding to the negatively charged DNA molecule. The nucleosomes are further packaged into higher-order structures of chromatin fibers and chromosomes (Chen et al., 2017a). Yet, the compacted genetic information remains accessible to transcriptional and repair mechanisms also due to numerous modifications on the N-terminal tail of histones and on DNA that increase histone-DNA contacts to promote transcriptional repression and vice versa. It is well known that chromatin structure and nuclear organization have major roles in regulating gene expression during development and abnormal functioning of the cell (Cremer et al., 2006; Schneider and Grosschedl, 2007).

6.2. Histone tail modification and noncoding RNAs

One form of epigenetic information includes more than 130 posttranslational modifications of histones, including acetylation, methylation, phosphorylation, ubiquitylation and sumoylation that correlated with promoters and gene bodies. Each modification of histones can change the accessibility of DNA and recruit transcription factors and coactivators/corepressors to various genomic regions (e.g. promoters, genme bodies, enhancers) to activate or silence transcription. Posttranslational modifications also interact with DNA methylation. The information can be actively erased (e.g. lysine demethylases and deacetylases), but passive copying during cell division is currently less clear (Chen et al., 2017a).

Another layer of information is provided by noncoding RNAs (ncRNAs) that are not translated into proteins and can be distinguished by their size (e.g. small ncRNAs, microRNAs), for example long ncRNAs (lncRNAs) are more than 200 bases in length. lncRNAs are classified according to their genomic loci or associated DNA strands into sense, antisense, intronic, intergenic, enhancer, or circular RNAs. lncRNAs are characterized by the lack of an open reading frame, shorter encoded RNA sequences, relatively low expression levels in comparison with mRNAs. lncRNAs are localized mostly in the nucleus and they are produced in a cell type, tissue and developmental stage specific manner (Chen et al., 2017a). An example of lncRNA-mediated silencing is the X chromosome inactivation in female mammals. The hallmarks of X chromosome silencing are reorganization of the chromosomal architecture, recruitment of repressor

complexes, modification of histones, methylation of CpG islands and others that lead to remarkable folding on an entire chromosome in each cell, exclusion of the transcription machinery and silencing of most of its genes (Galupa and Heard, 2018).

6.3. DNA methylation and demethylation

DNA methylation is an epigenetic mechanism that allows for a long-term adaptability of gene expression in response to developmental or environmental signals. DNA methylation comprises of a covalent addition of a methyl group at the 5th carbon of cytosine and predominantly occurs at cytosine-phosphate-guanine (CpG) dinucleotides (Chen et al., 2017b). 5-methylcytosine (5-mC) in the mammalian genome is involved in numerous biological functions (e.g. embryonic development, monoallelic expression of imprinted genes, X-chromosome-inactivation in female cells, repression of transposable elements, cancer, regulation of transcription) (Bird, 2002). Methylation signals are interpreted by transcription factors or by methylated-DNA-binding proteins (also known as readers, e.g. methyl-CpG binding protein 2, MeCP2) that affect further biological processes. DNA methylation of CpGs in promoter regions usually results in suppression of downstream gene expression by recruiting co-repressor complexes that repress transcription (Lin et al., 2007). However, the methylation of 5'-upstream, gene body, and 3'-downstream CpGs has been associated with transcriptional activation in various cell types, including neurons (Mo et al., 2015a). It is speculated that transcription factors could recruit other factors to change the status of heterochromatin to euchromatin and readers may affect splicing regulation during transcription (Meehan et al., 1989; Zhu et al., 2016). Cytosine methylation at non-CpG sites has also been reported, including in mature neurons, however, the function and mechanisms of this type of methylation are not yet elucidated (Jang et al., 2017; Lister et al., 2013). Therefore, we are beginning to understand how the transcriptional outcome of DNA methylation is affected by the genomic location of the methyl mark.

DNA methylation can be mediated by different DNA methyltransferases (DNMT), including complexes and some of them are associated with non-specific DNA sequences, while others are targeted to specific loci (Hervouet et al., 2018). DNA methylation is processed by the DNMT family of enzymes, subdivided into three classes: DNMT1, DNMT2 and the DNMT3A/3B/3L/3C. DNMT1 recognizes hemimethylated CpG sites on newly synthesized DNA during replication and methylates the daughter-strand cytosine at the complementary CpG, thus providing a way for passing epigenetic information through cell generations (Bostick et al., 2007; Sharif et al., 2007). New sites of DNA methylation are inserted by *de novo* DNA methyltransferases DNMT3A and DNMT3B and it occurs on both strands independently of DNA replication (Chen et al., 2017a). Recently discovered DNMT3C is a catalytically active enzyme and was previously annotated as a pseudogene (Barau et al., 2016).

DNMT3L is a structural protein and lacks the methyltransferase domain, but is required for gene imprinting and the regulation of DNMT3A/3B. DNMT3L and DNMT3C are linked to reproduction: DNMT3L stimulates germ line genome-wide methylation and DNMT3C methylates evolutionally young retrotransposons during spermatogenesis (Barau et al., 2016; Bourc'his and Bestor, 2004).

The methylation of both strands can be achieved more effectively through the cooperation of DNMT3 enzymes with DNMT1 in post-mitotic neurons (Feng et al., 2010). The interaction between DNMT1 and DNMT3A/B has been shown to be necessary in HEK-293 cells, thus there is evident crosstalk between maintenance and *de novo* methylation machineries (Kim et al., 2002). The functions of DNMT2 (renamed as tRNA aspartic acid methyltransferase 1 by the HUGO Gene Nomenclature Committee) have been poorly studied, it is mainly involved in the methylation of specific tRNAs that links its role to post-transcriptional gene regulation and protein translation (Goll et al., 2006; Lyko, 2018; Okano et al., 1998). This expands our knowledge of the regulatory capacities of the DNMT family.

The modification can be removed, either passively during cell division or by enzyme-mediated reactions coupling with base excision repair that result in unmethylated cytosine. Ten-eleven translocation (TET) methylcytosine dioxygenases together with thymine DNA glucosylase (TDG) form the capabilities for oxidation and removal of 5-mC. TET enzymes catalyze the oxidation of 5-mC to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC), and *in vitro* studies suggest that TET enzymes can use various combinations of substrates, including 5-hmC, 5-fC, 5-caC paired with 5-mC on the complementary strand (Chen et al., 2017a). Subsequently, a DNA repair protein, thymine DNA glycosylase (TDG) by hydrolyzing the carbon-nitrogen bond between the sugar-phosphate backbone of DNA and the mispaired thymine prepares the substrate for removal and replacement with an unmethylated cytosine by the base excision repair system (Maiti and Drohat, 2011; Weber et al., 2016). TET-mediated demethylation is involved in development, embryonic stem cell maintenance and differentiation, neuronal functions and cancer (Wu and Zhang, 2017).

6.4. Neuronal functions of DNA methylation and TET-mediated demethylation

DNMT1, DNMT3A/3B/3L are mutated in diverse human syndromes, those affecting neuronal functions will be discussed further. Dominant, heterozygous mutations of *DNMT3A* in the germ line causes Tatton-Brown-Rahman syndrome (OMIM 602769), characterized by tall stature, a distinctive facial appearance and intellectual disability and although there is no methylation data yet available, it is thought that a disruption of normal expression of imprinted genes is involved in this syndrome. *DNMT3A de novo* mutations have been identified in some individuals with autism spectrum disorder (Sanders et al., 2015). Germ

line origin, dominant mutations of *DNMT1* cause a heterogeneous group of adult-onset neurological disorders that include cerebellar ataxia, sensorineural deafness, narcolepsy, dementia, and other neurological and psychiatric abnormalities (OMIM 126375 and 605712). Methylation abnormalities are likely to be involved in the cause of all *DNMT*-affected conditions and the specific loci involved remain to be defined in the future (Edwards et al., 2017).

Relatively few functions of DNMT have been elucidated in the central nervous system (CNS). A constitutive *Dnmt1* and *Dnmt3b* knockout in the CNS is lethal and the knockout of *Dnmt3a* results in early postnatal death in mice (Fan et al., 2001; Li et al., 1992; Okano et al., 1999). *Dnmt1* regulates the survival of retinal and cortical interneurons and the impairments in γ-aminobutyric acid (GABA)-ergic neurotransmission have also been reported in the patho-physiology of different CNS diseases like schizophrenia and autism suggesting a role of *Dnmt1* in the regulation of cell death (Symmank and Zimmer, 2017).

Several reports have studied the neuronal phenotype on *Dnmt* knockdown mice. Feng and colleagues induced a conditional deletion of *Dnmt1* and *Dnmt3a* in excitatory postnatal neurons (specifically in neurons expressing calcium/ calmodulin-dependent protein kinase IIα) in the mouse forebrain (Feng et al., 2010). In their previous studies, single gene deletions of *Dnmt1* or *Dnmt3a* in the mouse brain did not cause any gross abnormalities in brain structures or DNA methylation levels in repetitive elements, therefore they hypothesized that *Dnmt1* and *Dnmt3a* may compensate for each other. The deficiency of DNMT1 and DNMT3A in forebrain neurons impairs the long-term potentiation and long-term depression in the hippocampus and causes spatial learning and memory deficits and no such alterations were seen in single gene knockout mice (Feng et al., 2010). However, a more recent report indicated that *Dnmt1* and *Dnmt3a* cannot replace each other, as spatial and novel object recognition and conditioned taste aversion learning impairments as well as long-term potentiation alterations were seen in forebrain-specific single knockout of *Dnmt3a*, but not of *Dnmt1* (Morris et al., 2014). Thus, specific deficits in learning were associated with the loss of *Dnmt3a*, but not *Dnmt1,* suggesting that DNMT3A is involved in certain types of learning and memory that are distinct from DNMT1. The reason for discrepancies between two aforementioned reports is unclear, but differences in the sex and age of subjects may have contributed to these findings.

Both DNMT1 and DNMT3A were associated with affective disorders. *Dnmt3a* overexpression induced a depressive-like behavior in the NAc, but in the PFC *Dnmt3a* overexpression decreased anxiety, while *Dnmt3a* knockdown in the PFC caused anxiogenic effects (E. Elliott et al., 2016; LaPlant et al., 2010). Thus, it seems that *Dnmt3a* knockdown has opposing effects in the PFC vs. NAc on anxiety and depressive-like behavior. Moreover, deletion of *Dnmt1* in the postnatal forebrain neurons resulted in anxiolytic effects in contrast with *Dnmt3a* deletion in the PFC (E. Elliott et al., 2016; Morris et al., 2016). Thus, DNMT1 and DNMT3A may have contrasting effects in some behavioral paradigms and future studies can clarify the region-specific roles on anxiety- and depressive-like behavior.

TET proteins have been shown to have various roles in the nervous system. 5-hmC (5-hydroxymethylcytosine) is highly abundant in various subtypes of neurons, suggesting that stem cells and neurons may be particularly susceptible to changes in DNA methylation state (Kriaucionis and Heintz, 2009; Münzel et al., 2010). Many studies have shown that neuronal activity induces DNA demethylation (Chen et al., 2003; Guo et al., 2011b, 2011a; Lubin et al., 2008; Ma et al., 2009; Martinowich et al., 2003; Miller and Sweatt, 2007). Repeated cocaine administration downregulates TET1 in NAc, alters 5-hmC distribution and the expression of differentially spliced isoforms (Feng et al., 2015). *Tet1* knockout mice have impaired hippocampal neurogenesis, abnormalities in learning and memory, and synaptic plasticity, along with the downregulation of several neuronal-activity-induced genes in the hippocampus (Rudenko et al., 2013; Zhang et al., 2013). TET1 overexpression results in transcriptional and behavioral changes that may be independent of its catalytic activity (Kaas et al., 2013). TET proteins may function independently of their catalytic functions by recruiting or repelling other factors (Wu and Zhang, 2017). TET2 functions are relatively unexplored, but seems to be involved in adult neurogenesis in the hippocampus (Gontier et al., 2018). *Tet3* knockout mice are not viable (Gu et al., 2011).

7. DNA methylation in psychostimulant-induced neuroplasticity

DNMT and TET enzymes both play a role in cocaine abuse in reward-related brain regions, such as the NAc, prefrontal cortex, amygdala, and the VTA (Kalda and Zharkovsky, 2015; Lax and Szyf, 2018). Chronic cocaine treatment altered the expression of *Dnmt3a,* but not other DNMT enzymes (LaPlant et al., 2010). Furthermore, DNMT3A had a negative regulation effect on cocaine reward in the NAc, authors showed that inhibition of DNMT3A increased and overexpression decreased reward-like behavior in rats (LaPlant et al., 2010). Further studies have elucidated the drug-seeking behaviors and neural adaptations in drug addiction with chronic cocaine treatment. Methionine is a methyl group donor that increases general DNA methylation levels, treatment with methionine during chronic cocaine exposure attenuated behavioral response to cocaine, including self-administration and *Dnmt3a* and *Dnmt3b* specifically increased in the NAc independently of methionine treatment (Wright et al., 2015). Methionine may have a specific effect on psychostimulant reward behavior, as no behavioral differences were observed with natural rewards (Wright et al., 2015). Cue-induced cocaine seeking behavior decreased with RG108 treatment after short abstinence from cocaine and the behavioral effect endured up to 1 month (Massart et al., 2015). Conversely, SAM (S-adenosyl methionine) treatment had an increasing effect on cocaine seeking lasting up to 1 month (Anier et al., 2013; Massart et al., 2015). These results imply that DNA

methylation is essential to cue-induced cocaine seeking after an abstinence period. Cocaine also increases MeCP2 expression in the *dorsal striatum* and the protein knockdown in this region inhibits cocaine self-administration (Im et al., 2010), indicating that DNA methylation-mediated repression of gene expression generally blunts the drug abuse-like behavior.

DNA methylation is involved in long-term changes in gene expression induced by psychostimulants (Anier et al., 2010; Han et al., 2010; LaPlant et al., 2010). Further evidence of DNA methylation in psychostimulant abuse comes from studies on specific gene promoters. First studies have begun to elucidate also the genome-wide changes in methylation patterns (Feng et al., 2015; Massart et al., 2015). Massart and colleagues studied the status of methylation marks over prolonged withdrawal period and their analysis showed that some marks remain stable and others are enhanced, while abstinence progressed (Massart et al., 2015). Increased 5-hmC after cocaine exposure at intron-exon boundaries was correlated with alterations in global gene expression. The results indicated that cocaine induced changes in exonic 5-hmC enrichment could regulate alternative splice site usage (Feng et al., 2015).

8. Drugs affecting DNA methylation

Azanucleosides (AZN) are pyrimidine analogues that are considered the first epigenetic drugs. 5-azacytidine (azacitidine) and 5-aza-2'-deoxycytidine (decitabine) are used for the treatment of acute myeloid leukemia and myelodysplastic syndromes. Both drugs are known to have two main mechanisms of antitumor activity. First, they cause cytotoxicity due to incorporation into DNA (azacitidine is incorporated into DNA as well as into RNA) and lead to DNA damage response. Secondly, AZN lead to DNA hypomethylation by irreversible inhibition of DNMT, mostly DNMT1. DNMT1 recognizes the incorporated cytidine analogue as a natural substrate and binds to it irreversibly inhibiting DNA methyltransferase function during replication and is therefore degradated. Consequently, methylation marks are lost and hypomethylation in silenced tumor suppressor genes leads to their reactivation and restoration of normal cell growth and differentiation in tumor cells. AZN act as DNA demethylating drugs at lower doses and are cytotoxic at higher doses, the latter limiting also dose regimens at the clinic due to unacceptable toxicity against normal cells (Diesch et al., 2016). Molecular targets of AZN include DNA demethylation of the promoter of silenced tumor suppressor genes, e.g. a key gene in cell cycle regulation p15, and downregulation of overexpressed metabolic genes. The ratio of cytidine deaminase (CDA) to deoxycytidine kinase (DCK) was higher in decitabine responders compared to non-responders (Qin et al., 2011) suggesting that increased deamination and decreased phosphorylation of the drug may be the mechanism of primary resistence. However, not all studies identify demethylation in a tumor suppressor gene and the correlation between the levels of DNA demethylation and clinical responses to decitabine have not been

clearly established. Several factors contribute to this discrepancy, such as elimination of demethylated clones, clonal replacement by resistant cells and others. Azacytidine was first approved for the treatment of myelodysplastic syndromes in 2004 by the US Food and Drug Administration (Raynal and Issa, 2016).

AZN have poor chemical stability in solution and are sensitive to enzymatic degradation, thus zebularine and a dinucleotide, guadecitabine, were synthesized for improved aqueous stability. Zebularine has a weak DNMT inhibition activity, but due to toxicity in primates the clinical trials were blocked. Guadecitabine (SGI-110) is composed of decitabine and guanosine linked with a phosphodiester bond that has shown improved DNA demethylation activity and a prolonged half-life as compared to decitabine (Raynal and Issa, 2016). SGI-110 is currently under evaluation in clinical trials across multiple cancer types including advanced solid tumors (www.clinicaltrials.gov).

Non-nucleosidic inhibitors were developed to overcome the toxicity and non-specificity of nucleosidic compounds, such as RG108 which directly blocks the active site of DNMT1. RG108 has a significantly longer half-life in aqueous solution compared to 5-azacitidine (~20 days versus 17 hours). Moreover, RG108 does not cause covalent DNMT trapping in human cell lines which is associated with lower levels of toxicity in comparison with AZN (Brueckner et al., 2005). RG108 is highly lipophilic, but safe for *in vivo* use, therefore it is currently assessed at the preclinical stage (Schneeberger et al., 2016).

9. Early life stress triggers predisposition and vulnerability to drug addiction

9.1. Stress and cocaine addiction

Several preclinical and clinical studies have shown that exposure to early life stress is a major risk factor for the development of various adult psychopathologies including drug dependence (Enoch, 2011). Early life stress causes chronic activation of the hypothalamus-pituitary-adrenal (HPA) axis, morphological adaptations and transcriptional changes in the DA reward circuitry that are all associated with the development of drug abuse. Furthermore, many studies show a correlation between child's neurodevelopmental disturbances and maternal stress and anxiety during pregnancy (Glover, 1997; Koss and Gunnar, 2018). Some pre- and early postnatal studies in humans have pointed to differential DNA methylation in multiple cortisol regulatory genes (Koss and Gunnar, 2018).

Central control of glucocorticoid release is regulated by a population of neurosecretory neurons in the hypothalamic paraventricular nucleus (PVN). Stress triggers the release of two neuropeptides from these neurons, corticotrophin-releasing hormone (CRH) and arginine vasopressin. The subsequent increases in adrenocorticotropin (ACTH) from the anterior pituitary stimulate

the adrenal cortex synthesis and release of cortisol in humans, and corticosteroids in rodents, that can directly send negative feedback to the PVN. During prolonged or intense stressors the input to the PVN can override the negative feedback and continue corticosteroid secretion. In the brain, glucocorticoids exert their action through mineralocorticoid (MR) and glucocorticoid receptors (GR). MR has high and GR has low affinity to glucocorticoids, thus GR bind at the peak of the daily rhythm and as well as glucocorticoid levels rise during an acute stressor. Acute HPA axis responses are necessary on functioning, however, under chronic stress, a dysregulation of the axis occurs that leads to elevated plasma corticosterone levels (Herman and Cullinan, 1997). Chronic stress can cause neuronal exitotoxicity leading to reduction of dendritic morphology in the hippocampus that modulates the stress response (Magariños et al., 1996). Glucocorticoids upon binding to GR form homo- or heterodimers and bind glucocorticoid response elements (GRE) in target genes to regulate their transcription (Oakley and Cidlowski, 2013). Negative GREs have been described, where GR after binding recruits corepressors and GR can also influence target gene transcription indirectly by interfering with upstream signaling pathways of other transcription factors (Oakley and Cidlowski, 2013).

Postnatal environment influences HPA axis activity. The postnatal period in rodents coincides with the stress hypo-responsive period during the first 2 weeks of life, while the baseline plasma glucocorticoid levels are lower than normal (Levine, 1994). Nevertheless, one of the most potent stressors for pups is the separation from the dam. Maternal separation (MS) is an animal model of chronic stress, where rat pups are separated from the mothers and littermates for long periods (3 hours or more) per day for several subsequent days from PND3 to PND20. MS induces molecular and functional changes in GABA receptors and CRH, and in repeated prolonged MS (360 min), alterations in glutamate receptors (Miczek et al., 2008). MS leads to an increased HPA activity by increased plasma ACTH and corticosterone (CORT) secretion and a decrease of growth hormone, whereby they lose weight (Kuhn et al., 1990). MS rats also display decreased hippocampal GR binding and increased hypothalamic CRH mRNA in adulthood (Plotsky and Meaney, 1993; Vázquez et al., 1996). Brief handling of pups (10–15 min per day) decreases HPA responses to stressors encountered in adulthood that may be related to the increased expression of GR in the hippocampus and prefrontal cortex (Meaney et al., 1996).

Exposure to stress at a young age in humans is associated with an increased risk to drug abuse. Depression has been associated with the dysregulation of the HPA axis (Bruijnzeel et al., 2004; Rao et al., 1999). Epidemological studies show high prevalence in both depression and SUD and also indicate a high comorbidity between these diseases (Bruijnzeel et al., 2004; Kessler et al., 1994). In cocaine-dependent individuals, higher lifetime stress was positively correlated with addiction severity (Mahoney et al., 2013). These studies implicate a role of HPA axis dysregulation on drug abuse behavior, although the causal relationship remains unclear. Experimental evidence support the idea that stress reactivity is altered in drug abusers, although CRF antagonists have not

been found effective in the treatment of drug addiction (Fosnocht and Briand, 2016). Thus, stress in early life can affect vulnerability to drug addictive behavior in later life.

Many studies have described the potential role of GR in initiating cocaine-induced neuroplasticity. The putative neural circuit for stress-induced cocaine use includes of dopaminergic projections to the PFC from the VTA (Mantsch et al., 2014). Many other stress- and drug-cue related inputs converge on the VTA as a key integration site. The glutamate projections from PFC to the NAc core that form part of the mesocortical pathway have been implicated for stress-induced reinstatement (McFarland et al., 2004). However, other clinical and preclinical reports show that stressors themselves do not directly trigger cocaine-seeking behavior, but rather function as increasing reactivity to drug related cues (Mantsch et al., 2014).

GR is expressed in many neurons that comprise the stress-induced cocaine seeking circuit, including the stress responsive neurons in the PFC (Ostrander et al., 2003) and GR regulation of stress-responsive processes has been demonstrated in these brain regions. In the PFC, chronic stress induces reorganization in the dendritic morphology (Cook and Wellman, 2004) and blocking GR locally in the PFC reduces stress-induced dopaminergic signaling efflux (Butts et al., 2011). The glutamatergic neurotransmission from the PFC to the NAc has also been shown to be regulated by GR (Campioni et al., 2009). The role of GR in establishing and reinforcing effects of cocaine has also been examined on the behavioral level. Whole brain GR knock-out reduced behavioral sensitization and a GR antagonist weakens the motivation to self-administer cocaine (Deroche-Gamonet et al., 2003). Selective deletion of GR in dopaminergic neurons in the NAc decreases cocaine-induced behavioral sensitization (Barik et al., 2010), while overexpression of GR in the PFC increases behavioral sensitization (Wei et al., 2004). The GR transgenic mice do not have altered activity or reactivity to acute cocaine, but the increased susceptibility to sensitization of GR over-expressing mice implies changes in hyperresponsivity to strong emotional stimuli. Therefore, GRs are well positioned as potential mediators of glucocorticoid-mediated neuroplasticity in addiction, although the mechanisms are yet unclear.

The medial PFC may play different roles in the regulation of the HPA axis. PFC consists of a dorsal part including the anterior cingulate cortex and a ventral part including prelimbic and infralimbic cortex. Both parts are implicated in executive functioning and enable flexibility in affective processing (Bush et al., 2000). In rat, the prelimbic subdivision of PFC is involved in reward and stress response inhibition through projections that include NAc and basolateral amygdala, whereas the infralimbic PFC influences visceral/autonomic activity and is implicated in stress excitation (Herman et al., 2005; Vertes, 2004). The development of HPA responses to stress is altered by adverse events in early life. MS triggers a decrease in dendritic length, spine density (Monroy et al., 2010) and impairs long-term potentiation processes in the PFC (Baudin et al., 2012; Chocyk et al., 2013). The development of HPA responses during stress is affected also through alterations in gene transcription, including the GR. GR

functions to repress stress-induced responses, however it's activation in the forebrain can also lead to delayed inhibition of HPA activity through negative feedback inhibition (Kloet et al., 2005). The network properties are altered in a way that experiences are remembered and stress responsiveness is preserved. GR knockdown in the prelimbic PFC increases basal morning corticosterone release suggesting its involvement in the basal tone of the HPA axis under chronic stress (McKlveen et al., 2013). Conversely, overexpression of GR in the forebrain leads to a emotionally labile phenotype (Kloet et al., 2005). GR in the infralimbic PFC may be involved in the sensitization to chronic stress that indicates to a functional diversity of GR within the PFC (McKlveen et al., 2013).

9.2. Epigenetic mechanisms in stress

Studies on the mechanisms that shape stress reactivity and regulation have also pointed to DNA methylation. Translation studies in humans suggest an effect of parental care on the epigenetic regulation of hippocampal GR expression, where greater methylation of the exon 1_7 *GR* promoter was detected in the offspring of the low-licking/grooming mothers (McGowan et al., 2009; Weaver et al., 2004). Differences in the methylation status of GR developed between PND1 and PND6. Therefore, early environmental events may decrease hippocampal GR expression and increase HPA activity that has been associated with higher risk to psychopathology.

However, mixed results have been found in other studies depending on the adverse experience and age of offspring. Reduced methylation of *GR* promoter was associated with childhood maltreatment and substance-use disorders in adults (Tyrka et al., 2016). Differential DNA methylation of HPA-related genes, such as *CRH*, *GR* and *FKBP5* (FKBP prolyl isomerase 5), have been associated in a prenatal study with chronic and war-related stressors (Kertes et al., 2016). These epigenetic mechanisms may continue operating the stress regulation also postnatally.

MS seems to induce changes in the overall status of DNA methylation in the genome. Our previous studies have shown that MS increased the expression of *Dnmt1, Dnmt3a, Dnmt3b* that persisted into adulthood (Anier et al., 2014) and the augmented expression levels of *Dnmt* resulted in alterations in the DNA methylation both at a global and local levels on neuronal plasticity-related genes (Anier et al., 2014). However, global changes in DNA methylation may remain unaffected. MS does not alter global methylation in the adult PFC (Anier et al., 2014; McCoy et al., 2016). Early life stress affects local alterations also in GABA and glutamate signaling possibly accounting for the cognitive phenotype after stress. For example, the hippocampal and PFC expression of some glutamate receptor subunits decrease after MS indicating that early life stress reprograms inhibitory and excitatory signaling (Burns et al., 2018). BDNF (brain-derived neurotrophic factor) plays an important role in neurodevelopment, neurogenesis and synaptic connectivity throughout life and its signaling has been associated

with early life stress, although some epigenetic changes in BDNF are transcriptionally silent (Burns et al., 2018).

Local MS-induced alterations in DNA methylation have also been shown in the HPA axis-associated genes, including GR in adult mice (Kember et al., 2012), although the role of GR remains unclear as other studies show no alteration in the methylation of GR promoter (Daniels et al., 2009). The increased expression of a key regulator in the HPA axis, CRF, along with decreased DNA methylation at the *Crf* promoter in the PVN of mice in the coincides with social stress behavior and both effects are reversed by chronic antidepressant treatment (Elliott et al., 2010), thus antidepressant treatment can change DNA methylation levels and this affects both transcriptional and behavioral outcomes. Moreover, knockdown of *Crf* decreased stress-induced social avoidance, therefore taken together, direct epigenetic regulation of *Crf* could be a mechanism that regulates long-term behavioral response to stress (Elliott et al., 2010).

Epigenetic modifications occurring in early life may be reversible with epigenetic modulators. Trichostatin A, a HDAC (histone deacetylase) inhibitor, reversed the hypermethylated status of *GR* exon 1_7 promoter in adult offspring of low licking/grooming mothers and re-established HPA responsiveness to stress (Cervoni and Szyf, 2001; Weaver et al., 2004). Conversely, methionine injections to the offspring of high licking/grooming mothers resulted in increased methylation of *GR* and higher HPA responsiveness to stress (Weaver et al., 2005). However, in humans, the *GR* 1_F promoter hypermethylation in infants of depressed mothers was not reversed by antidepressant treatment (Bagot et al., 2014).

Interestingly, it was recently proposed that patterns of neuronal activity by early life experience restricts the amount of DNMT3A-mediated non-CpG methylation on gene bodies (Stroud et al., 2017). DNMT3A expression trajectories may differ in the brains of primates and rodents, therefore DNMT3A-dependent epigenetic gene expression may still have different strategies in rodents and humans, especially in postnatal brain development (Cardoso-Moreira et al., 2019; Pruunsild and Bading, 2019). In rodents, *Dnmt3a* expression levels increase transiently after birth, whereas in the human brain, its expression is retained in high levels in early embryonic stage and decrease to low levels postnatally (Cardoso-Moreira et al., 2019). Whether the DNMT3A2 isoform is activity-regulated in humans is yet unknown.

AIMS OF THE STUDY

The general aim of the dissertation is to assess the involvement of DNA methylation in cocaine-induced changes of neuroplasticity in the predisposition, expression and abstinence of cocaine abuse. The specific aims of the thesis are as follows:

1. In the predisposition phase, to study the molecular mechanism of GR-mediated genomic regulation of DNA methyltransferases on a maternal separation model of early life stress.

2. In the expression and abstinence phase, to describe the effect of acute and chronic cocaine on DNA methylation enzymes in an addiction-specific (NAc) and non-specific regions (cerebellum, peripheral blood cells).

3. In the expression and abstinence phase, to characterize the role of DNMT3A in cocaine-induced behavioral sensitization.

MATERIALS AND METHODS

1. Animals

Wistar rats were obtained from Harlan Laboratories (Netherlands) and adult male C57BL/6NTac mice were purchased from Taconic Biosciences (Denmark). Animals were housed in standard polypropylene cages under 12-hour light-dark cycle with lights on at 7:00 a.m. Animals were allowed free access to food and water except during experimental procedures. All behavioral experiments were performed during the light phase in an isolated room.

2. Drug treatments

Cocaine hydrochloride (8 mg/kg or 15 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) or procaine hydrochloride (15 mg/kg or 45 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) was injected to mice intraperitoneally (i.p.). Compounds were diluted in 0.9% NaCl (B. Braun Melsungen AG, Germany). Saline group animals received an equal volume of 0.9% NaCl i.p. RG108 (0.6 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9% NaCl containing 10% dimethyl sulphoxide (DMSO, Sigma-Aldrich, St. Louis, USA).

Corticosterone (1 mM, Sigma-Aldrich, St. Louis, USA) and mifepristone (0.4 mM, Sigma-Aldrich, St. Louis, USA) stock solutions were dissolved in DMSO and subsequently diluted in Neurobasal-A (Invitrogen, Carlsbad, USA). On 5th day *in vitro* (DIV5) corticosterone 1, 5, 10 and 50 μM (final concentration in the medium) alone or in combination (50 μM corticosterone) with mifepristone 20 μM (final concentrations in the medium) was added to primary cells for 3 hours. Cells were washed with an ice-cold phosphate buffered saline (PBS) and immediately lysed for RNA isolation.

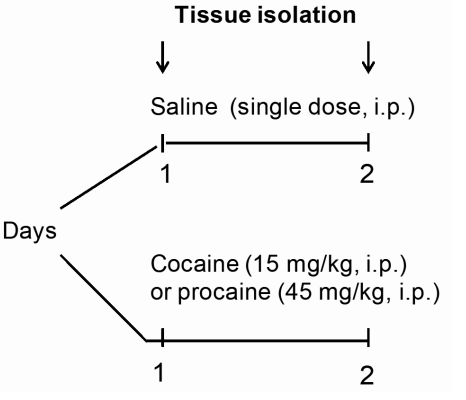

Figure 1. Experimental timeline. Adult mice were injected (i.p.) a single dose of cocaine (15 mg/kg) or procaine (45 mg/kg). Control mice received saline injections. Tissues were isolated 0.5 h and 24 h after injections.

3. Locomotor activity and tissue isolation

Horizontal locomotor activity was detected with a PhenoTyper (Noldus Information Technology, Netherlands) during 60 min after the i.p. injections on the 1st, 7th, 28th and 35th day. Each animal was tracked individually.

The NAc, cerebellum (Cer) and peripheral blood cells (PBC) were isolated at 0.5 h and 24 h after acute cocaine injection and 0.5 h and 24 h after procaine treatment (Figure 1, experimental design), and in repeated cocaine experiments, tissues were isolated 2 h or 24 h after cocaine challenge dose (Figure 2, experimental design). Trunk blood samples were collected in EDTA-containing tubes. Serial 1-mm thick sections were prepared using a brain matrix and NAc was dissected with a round-shape puncher (inner diameter 1.5 mm) on chilled microscope slides. Tissues were frozen immediately in liquid nitrogen and stored at –80 °C.

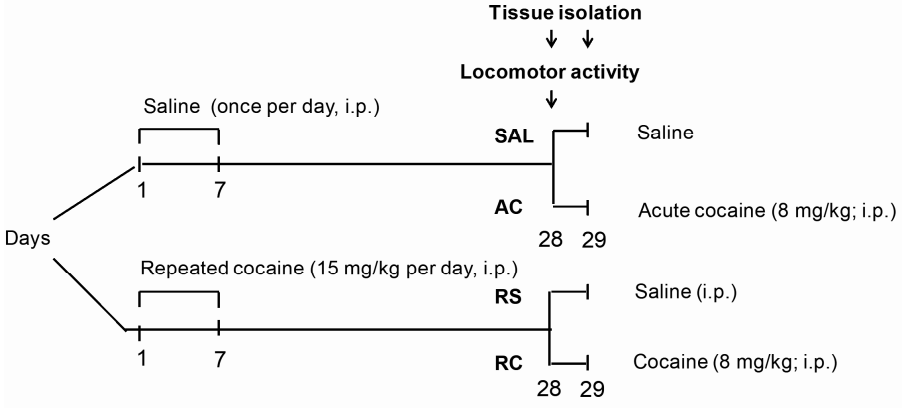

Figure 2. Experimental timeline. Adult mice were injected (i.p.) cocaine (15 mg/kg per day) or saline for 7 consecutive days. On day 28, animals received cocaine (8 mg/kg) or saline injections. Tissues were isolated 0.5 h and 24 h after injections. Locomotor activity was measured for 60 min after last injection.

4. Induction and expression of behavioral sensitization

Induction of behavioral sensitization. Animals were randomly assigned to following groups: (1) saline (SS), mice received saline for 7 days; (2) acute cocaine (SC), mice received saline for 6 days and cocaine (15 mg/kg) on the 7th day; (3) repeated cocaine (CC), mice received cocaine (15 mg/kg) for 7 days.

Expression of behavioral sensitization. Animals were assigned to one of the following groups: (1) saline + saline (SS), mice received saline for 7 days and were treated with saline on the 28th (experimental design 0) or the 35th (experimental design I) day; (2) saline + cocaine (SC), mice received saline for 7 days and were treated with a challenge dose of cocaine (8 mg/kg) on the 28th day; (3) cocaine + saline (CS), mice received cocaine (15 mg/kg) for 7 days and saline on the 28th day; (4) cocaine + cocaine (CC), mice received

cocaine (15 mg/kg) for 7 days and a challenge dose of cocaine (8 mg/kg) on the 28th day.

5. Maternal separation

Maternal separation was performed as described previously (Anier et al., 2014). Briefly, male pups were randomly assigned to three experimental groups: animal facility reared (AFR), handled (MS15) and maternally-separated (MS180) group. MS15 pups were handled and individually separated for 15 min and then returned to the home cage. MS180 pups were individually placed into new cages for 180 min per day from PND2 to PND14 and then returned to the home cage. The bedding (aspen chips) and nesting material (aspen wool) was changed on PND10 in all cages. The procedure for MS15 and MS180 groups began at 10:00 a.m. and the dam and pup behavior was oberved in their home cage for 10 min. AFR is considered as the control group and the comparison of MS15 and MS180 groups shows the effect brought about by maternal separation. The researchers were not blinded to the experimental group during the experiments, decapitation and data analysis. Decaptitation was performed in a separate room in a randomized manner on PND15. Trunk blood samples were collected in EDTA-containing tubes between 10:00 and 11:00 a.m. Medial PFC, including the prelimbic region, was dissected by using a brain matrix according to rat brain atlas landmarks (Paxinos and Watson, 1986), frozen in liquid nitrogen and stored at –80 °C.

6. Rat primary neuronal culture

Rat primary cultures were prepared from male and female newborn (1st postnatal day) Wistar rats or from embryonic day 21 Sprague Dawley rats as described previously (Jaako et al., 2016; Koppel et al., 2015). Briefly, cortices were dissected in ice-cold Krebs-Ringer solution (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.4 mM K_2HPO_2, 15 mM glucose, 20 mM HEPES, pH 7.4) containing 0.3% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, USA). Tissue was trypsinized in 0.8% trypsin (Sigma-Aldrich, St. Louis, USA) for 10 min at 37 °C and triturated in 0.008% DNase (Sigma-Aldrich, St. Louis, USA) solution containing 0.05% soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, USA). Cells were resuspended in Basal Medium Eagle with Earle's salts (Sigma-Aldrich, St. Louis, USA) containing 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, USA), 25 mM KCl, 2 mM GlutaMAX^{TM}-I (Invitrogen, Carlsbad, USA), 100 µg/ml gentamicin (KRKA, Novo Mesto, Slovenia) and plated onto poly-L-lysine-coated (Sigma-Aldrich, St. Louis, USA) 35-mm dishes (Nunc, Thermo Fisher Scientific, MA, USA). The medium was changed to Neurobasal-A supplemented with B-27 (both from Invitrogen, Carlsbad, USA), 2 mM GlutaMAX^{TM}-I, and 100 µg/ml gentamicin three hours later and cultured at 37 °C in a humidified 5% CO2 incubator.

7. Adeno-associated virus (AAV) vector generation and transduction *in vitro*

The shRNAs were designed to target all known mouse and rat *Dnmt3a* transcripts equivalent to the target sequence of Silencer Select siRNA s65079 (Thermo Scientific, Waltham, MA, USA). A scrambled shRNA sequence was used for specificity control that corresponded to Silencer Select negative control no. 2 siRNA (Thermo Scientific, Waltham, MA, USA). Oligonucleotides containing 21 base sense and antisense sequences linked with a hairpin loop (5′-TTCAAGAGA-3′) followed by a poly (dT) termination signal and complementary reverse oligonucleotides were purchased from Microsynth AG (Switzerland) and are listed in Table 1.

Table 1. List of *Dnmt3a*-specific shRNA oligonucleotides sequences.

Oligonucleotide name	**Sequence (5' –> 3')**
Dnmt3a shRNA sense	GATCCCCCGAGGACATTTGTATCTCATTCAAGAGA TGAGATACAAATGTCCTCGTTTTTGGAAA
Dnmt3a shRNA antisense	GGGGCTCCTGTAAACATAGAGTAAGTTCTCTACTC TATGTTTACAGGAGCAAAAACCTTTTCGA
Scrambled shRNA sense	GATCCCCACTACCGTTGTTATAGGTGTTCAAGAGA CACCTATAACAACGGTAGTTTTTTGGAAA
Scrambled antisense	AGCTTTTCCAAAAAACTACCGTTGTTATAGGTGTCT CTTGAACACCTATAACAACGGTAGTGGG

The corresponding complementary oligonucleotides were annealed and the double-stranded DNA was ligated via BamHI and HindIII into an AAV expression vector (kind gift from H. Bading) under the U6 promoter. The AAV vector also contains mCherry under the Camk2a promoter. The shRNA encoding AAV vectors, serotype 1, were purchased from the University of Pennsylvania Vector Core (Philadelphia, PA, USA).

On DIV2, cultured neurons were transduced with 7×10^{12} genome copies/mL, simultaneously with the replacement of one half of the medium. RNA and protein isolation was performed on DIV8.

8. Intra-NAc AAV injections

Mice were anesthetized with a mix of ketamine and xylazine. 3.5×10^{10} genome copies of AAV-Dnmt3a-shRNA (AAV-Dnmt3a) or AAV-Scrambled-shRNA (AAV-Scr) per hemisphere were injected bilaterally into mouse NAc (–1.3 mm anteroposterior, ±1.0 mm lateral, and –4.5 mm dorsoventral relative to bregma) using a Hamilton syringe (10 µl) connected to a motorized unit. Mice were

killed by decapitation 24 hours (experimental design I) and 2 hours (experimental design II) after cocaine challenge dose. Brains were cut on a brain matrix after the verification of injection coordinates and NAc was dissected using a round-shape puncher (inner diameter 1.5 mm). Tissues were frozen immediately in liquid nitrogen and stored at –80 °C. Mice with incorrect injection sites were excluded from further analysis.

9. Immunohistochemistry

For TUJ1 (tubulin beta 3 class III, TUBB3) immunohistochemistry, free-floating coronal sections were incubated for 24 h at 4 °C with rabbit anti-TUJ1 monoclonal antibody (1:500, Covance, MRB-435P, USA) that was followed by incubation with rabbit secondary antibody conjugated with Alexa-488 (1:1000, Thermo Fisher Scientific, USA) as described previously (Jaako et al., 2011). Nuclei were stained with 300 nM DAPI solution (4′,6-Diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich, USA) in PBS. Representative images were captured with a confocal microscope (LSM 510 Duo, Zeiss, Germany) using a 63× oil objective and processed using Zeiss LSM Image Browser and Adobe Photoshop.

10. RNA isolation and quantitative PCR

Total RNA was extracted from primary neuronal cells, rat PFC, mouse NAc and cerebellum using RNeasy Mini Kit (QIAGEN, Hilden, Germany) and mouse trunk blood using QIAamp RNA Blood Mini kit (Qiagen, Hilden, Germany). RNA was treated with DNase I (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from 750 ng rat RNA and 200 ng mouse RNA using RevertAid First Strand cDNA Synthesis Kit with oligo$(dT)_{18}$ primers (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Quantitative PCR (qPCR) was performed using an ABI PRISM 7000 Sequence Detection System equipped with ABI Prism 7000 SDS Software (Applied Biosystems Inc, USA). Agarose gel electrophoresis and sequencing was used to confirm the specificity of PCR products. Results were calculted using the ΔΔCT method (Schmittgen and Livak, 2008) and were normalized to *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase). Rat primers for *Dnmt1, Dnmt3a, Dnmt3b, Gapdh* were designed to amplify all known transcripts of a given gene (Microsynth AG, Balgach, Switzerland). The primers used for amplifying specific rat *Dnmt3a* transcripts as well as primers for total mRNA are listed in Table 2 (Microsynth AG, Balgach, Switzerland). Mouse primers for *Dnmt1, Dnmt3a, Dnmt3b, Gapdh, Esr1, Grin1, Ppp1cc* and *Gal* were designed to target all known transcripts, were synthesized by TAG Copenhagen AS (Copenhagen, Denmark) are listed in Table 2.

Table 2. List of rat and mouse RT-qPCR primer sequences.

Organism	Gene name	Sequence (5' –>3')
Rat	*Dnmt1*	Forward: AACGGAACACTCTCTCTCACTCA Reverse: TCACTGTCCGACTTGCTCCTC
Rat	*Dnmt3a*	Forward: CAGCGTCACACAGAAGCATATCC Reverse: GGTCCTCACTTTGCTGAACTTGG
Rat	*Dnmt3b*	Forward: GAATTTGAGCAGCCCAGGTTG Reverse: TGAAGAAGAGCCTTCCTGTGCC
Rat	*Gapdh*	Forward: TGCACCACCAACTGCTTAGC Reverse: GGCATGGACTGTGGTCATGAG
Rat	*Dnmt3a1*	Forward: CATCACTTTCCGAGGGCTTGA Reverse: ATCGTGGTCTTTGTGAGCAA
Rat	*Dnmt3a2*	Forward: GGCTGCACCTGGCCTTAT Reverse: GCTGCCTTGGTAGCATTCTTG
Mouse	*Dnmt1*	Forward: CCCATGCATAGGTTCACTTCCTTC Reverse: TGGCTTCGTCGTAACTCTCTACCT
Mouse	*Dnmt3a*	Forward: GCCGAATTGTGTCTTGGTGGATGACA Reverse: CCTGGTGGAATGCACTGCAGAAGGA
Mouse	*Dnmt3b*	Forward: TTCAGTGACCAGTCCTCAGACACGAA Reverse: TCAGAAGGCTGGAGACCTCCCTCTT
Mouse	*Gapdh*	Forward: GTCATATTTCTCGTGGTTCACACC Reverse: CTGAGTATGTCGTGGAGTCTACTGG
Mouse	*Esr1*	Forward: GAAGGCCGAAATGAAATGGGTG Reverse: GGTCAGCTGTCAAGGACAAG
Mouse	*Grin1*	Forward: ACTCCCAACGACCACTTCAC Reverse: GTAGACGCGCATCATCTCAA
Mouse	*Ppp1cc*	Forward: CACGAGACCTGTCACACCAC Reverse: CAGTAATCAAATGGAGGAGGGC
Mouse	*Gal*	Forward: GTGCAGTAAGCGACCATCCA, Reverse: TCAGTGCAGGATCAAGGCTC

11. Chromatin immunoprecipitation assay

PFC ChIP was performed as described previously (Pruunsild et al., 2011). Primary neuron ChIP experiments were performed from newborn Wistar rats using 5×10^6 cells per sample and according to ChIP kit instructions (ab500, Abcam, Cambridge, UK) with minor modifications. Primary neurons were

treated with CORT for 1 h, washed with 1× PBS, harvested by scraping and then cross-linked with 1% formaldehyde for 10 min at room temperature and the reaction was quenched by adding 0.12 M glycine. After obtaining a nuclear lysate (supplemented with protease inhibitors cOmplete Protease Inhibitor Cocktail from Roche, Basel, Switzerland), chromatin was then sonicated (Diagenode Bioruptor) with the following settings to obtain DNA fragments of an average length of 500 bp: high (H) power output, 30 sec ON/30 sec OFF pulses, total sonication time 8 min, 4 °C water bath with no floating ice. The cell debris was pelleted by centrifugation (17900 g for 15 min at 4 °C). For ChIP, the supernatant was diluted with 1x ChIP buffer and samples were rotated with 3 µg GR antibody (ab3579, Abcam, Cambridge, UK) or no antibody overnight at 4 °C. On the following day, 50 µl of 50% protein A Sepharose CL-4B (GE Healthcare, Little Chalfont, UK) slurry was added to each sample and the mix was rotated for 1 h at 4 °C. After washes, the immune complexes were eluted from Sepharose three times using 50 µl elution buffer (1% SDS, 100 mM $NaHCO_3$); eluates from the same samples were combined. Cross-linking was reversed by incubating eluates with 250 mM NaCl at 65 °C overnight. DNA was purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Immunoprecipitated target genomic regions were quantified by qPCR using LightCycler 480 SYBR Green I Master qPCR mix (Roche, Basel, Switzerland) and the following primers (synthesized by Microsynth AG, Balgach, Switzerland): *Mb*: forward (5'-TAGTGTGCATCCAGCAGAGG-3'), reverse (5'-ACACTGTGGCCTTTTTGTCC-3'); *Per1*: forward (5'-GGGTTGGGGGAG GCGCCAA-3'), reverse (5'-GGCGGCCAGCGCACTAGG-3'); *Dnmt3a* intron 3: forward (5'-TAAGCCAGAGCTTTCAGGGC-3'), reverse (5'-CGGTACAAA TGCAGGCTGTC-3'); *Dnmt3a* intron 5: forward (5'-AGAGGATAGACACTGG GCCG-3'), reverse (5'-AGCTGAAGCTGGAAGAAGGC-3'); *Dnmt3a* promoter: forward (5'-GACACGCTACACCTTCGAGT-3'), reverse (5'-CCCAGTC TCACCAACACCTC-3'); *Dnmt3b* promoter: forward (5'-AAAGCAGGAAGC AGTACGGG-3'), reverse (5'-CACACCGCATCAGCTTTTCG-3') on a LightCycler 480 II Real Time PCR System (Roche, Basel, Switzerland). ChIP was performed over 4 independent experiments for primary neurons and 5–7 animals per group were used for PFC ChIP. Data were presented as the percentage of starting material (% of input). Results were log-transformed for statistical analysis and back-transformed for graphical representation as a box blot. Whiskers represent max and min data points. MatInspector 8.4.1 (Cartharius et al., 2005) was used for promoter and intragenic analysis.

12. Western immunoblotting

For total protein isolation, prefrontal cortices were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 1 mM PMSF, protease inhibitor cocktail (Roche, Switzerland)] and 0.1% SDS was added subsequently for

complete lysis. Primary neurons were lysed in ice-cold RIPA buffer (including 0.1% SDS). Lysates were sonicated briefly and cell debris was removed by centrifugation (16 000 g, 15 min, 4 °C). Total protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). 30 µg of total protein in 1 × Laemmli sample buffer was separated in 7.5% SDS–polyacrylamide gel and transferred to PVDF membrane (pore size 0.45 µm, Merck Millipore, Darmstadt, Germany). The membranes were blocked with 3% bovine serum albumin (BSA) in TBS containing 0.1% Tween-20 (TBST) for 1 h at room temperature and incubated with primary antibodies, anti-DNMT3A (1:500, ab2850, Abcam, Cambridge, UK), anti-GAPDH (1:2500, MAB374, Merck Millipore, Darmstadt, Germany), diluted with 5% BSA in TBST. The following secondary antibodies were used, Stabilized Peroxidase Conjugated Goat anti-rabbit and anti-mouse (1:5000, both from Thermo Scientific, Waltham, MA, USA), diluted with 2% skim milk in TBST. Chemo luminescence signal was detected using SuperSignal West Femto Chemi-luminescent Substrate (Thermo Scientific, Waltham, MA, USA) and ImageQuant LAS 4000 bioimager (GE Healthcare, Finland). ImageQuantTL software (GE Healthcare, Finland) was used to quantify signals from non-saturated exposures. DNMT3A levels were normalized to GAPDH and expressed as mean optical density (OD) ratio relative to control group ± SEM. Immunoblotting of PFC was performed in duplicates per animal and using 7 animals per group.

13. DNMT activity measurements

Nuclear proteins were isolated from mouse NAc, Cer and PBCs and rat PFC using Nuclear Extraction Kit (Abcam, Cambridge, UK). EpiQuik DNMT activity assay kit (Epigentek Group, Brooklyn, NY, USA) was used to detect DNMT activity. Each experiment was performed in duplicate with 5 µg of nuclear protein extracts for each assay. DNMT activity levels were measured at 450 nm using a Tecan Sunrise plate reader (Tecan Group Ltd., Switzerland) and expressed as OD/h/mg according to the formula provided in manufacturers' protocol.

14. Plasma corticosterone assay

Rat blood samples were centrifuged at 4000 × g for 5 min for plasma extraction and stored at −80 °C. Plasma corticosterone concentrations were measured using corticosterone ELISA kit (Abcam, Cambridge, UK) according to the manufacturers' instructions. OD was measured at 450 nm using Tecan Sunrise microplate reader (Tecan Group Ltd., Switzerland). Sensitivity for the corticosterone assay was 0.3 ng/ml, and all samples were analyzed in duplicate in a single assay; the intra-assay coefficient of variation was 6.2%. Total corticosterone concentrations are expressed as nanogram per ml plasma (ng/ml).

15. Global 5-mC quantification

Genomic DNA was isolated from the NAc using a QIAamp DNA Micro kit (Qiagen, Hilden, Germany) and DNA was digested into individual nucleosides using DNA Degradase Plus (Zymo Research, Irvine, CA, USA) according to manufacturer's protocols. 10 µl of the DNA was used for liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) to measure 5-mC and 5-hmC levels.

Chemicals. Methanol was purchased from J. T. Baker (Deventer, Netherlands) and ammonia was obtained from Riedel-de-Haën (Seelze, Germany). 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). DNA standards containing cytosine, 5-mC or 5-hmC were from Zymo Research (Irvine, CA, USA), and cytosine, 5-mC standards were from Sigma-Aldich (St. Louis, MO, USA).

LC-ESI-MS/MS assay. Chromatographic separation of the analytes was performed using an Agilent Series 1100 LC-MSD Trap XCT (Agilent Technologies, Santa-Clara, CA, USA) equipped with a binary pump, a degasser, an auto-sampler and a column thermostat. Validation and quantification of cytosine, standard solutions of cytosine, 5-mC in water and digested cytosine, 5-mC and 5-hmC standards were performed. Standards were diluted in water, DNA hydrolysis standards and digested samples (10 µL) were injected onto a reverse phase liquid chromatography column (Waters XBridge C18 column (150 mm × 3 mm, 3.5 µm) equipped with a Waters Guard Cartridge (20 mm × 4.6 mm) (Waters, Milford, MA, USA) and were eluted at a flow rate of 0.3 mL/min. ESI-MS detection was performed in positive ion detection mode. Analytes were eluted using a two component eluent with 5 mM HFIP buffer at pH 9 (adjusted using ammonia) and methanol with the following gradient program: eluation started at 4% methanol, followed by increase to 12% methanol in 5 minutes and further increase to 100% methanol within the next 5 minutes. Methanol content was maintained at 100% for 3 minutes and was then reduced to 4% in the course of 2 minutes. For column equilibration, a 5-minute post-run was used. The column temperature was 30 °C. MS detection of the analytes was performed in the multiple reaction monitoring (MRM) mode using previously optimized conditions (5 mC m/z 242→126, 5-hmC m/z 258→142 and deoxycytidine (Cyt) m/z 228→112). Quantifications of 5-mC and 5-hmC were performed as previously described (Kaas et al., 2013).

16. 5-mC quantification at *Esr1* and *Grin1*

Genomic DNA (gDNA) from mouse NAc and PBC was isolated using DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany) according to manufacturer's protocol. For PBC, the DNA of 2 mice was pooled and three pools were used for the assay. For the NAc, no pools were used; thus, each array corresponded to 1 single mouse. In total, 6 mice were used per group for the NAc. The 5-hmC

and 5-mC levels at *Esr1* were detected using the EpiMark 5-hmC and 5-mC Analysis Kit (New England Biolabs, MA, US). Briefly, 2.3 mg of NAc and 7 mg of blood gDNA were treated with T4 Phage b-glucosyltransferase according to the manufacturer's instructions. Glucosylated gDNA was digested using 100 U of MspI, 50 U of HpaII or no enzyme (negative control for digestion) at 37 °C for 16 h. Digestion was terminated using Proteinase K at 40 C for 30 min and the reaction was inactivated at 95 C for 10 min. The qPCR primers for specific 5-mC detection are the following: *Esr1*: forward, (5'-GAAGCTAGCG GTTCCTTGGG-3'), reverse, (5'-TGCGATTAGAGAAGGCGCAG-3'), *Grin1:* forward, (5'-CCGCTC AACCTCTCAGATCC-3'), reverse, (5'- CTCATCAG TGTCCTTGGCCG-3'). Quantification of the methylation status of the inner C at the specific CCGG site was calculated by subtraction of the Ct values according to the manufacturer's instructions.

17. Statistical analysis

The sample size was predetermined during preliminary and previously published experiments (Anier et al., 2014, 2013) and statistical significance levels were set to $p < 0.05$. Definition for unit of analysis (n) for all experiments is provided in the results and in the figure legends. Data points outside of 99% confidence intervals were considered outliers and excluded from further data analysis unless stated otherwise at figure legend. Exact numbers for all experiments are provided in the results and in the figure legends. For statistical analysis, all data were log-transformed to obtain normal distribution of the data. RT-qPCR, Western blot, ELISA, DNMT activity and global 5-mC data were analyzed using Student's t-test (in the case with data in two groups) or one-way ANOVA, followed by Bonferroni post-hoc test. For graphical representation data were back-transformed into linear scale and presented as mean ± SEM. Student's t-tests followed by False Discovery Rate correction (Q was set to 5%) was performed on *in vitro* and *in vivo* ChIP-RT-qPCR data and for graphical representation data were back-transformed into linear scale and presented as a box plot, whiskers showing max to min data points. Locomotor activity data were analyzed using one-way or two-way ANOVA, followed by Bonferrori post-hoc test. Only hypotheses specified *a priori* were tested for statistical significance. GraphPad Prism 7 (GraphPad, San Diego, CA, USA) was used for statistical analysis.

18. Ethics

All experiments were performed in accordance with EU guidelines (Directive 2010/63/EU) and with experimental protocols approved by the Animal Welfare Committee of the Ministry of Rural Affairs, Republic of Estonia.

RESULTS

1. Glucocorticoid receptor stimulation resulting from early life stress affects the expression of DNMT in rat prefrontal cortex (Paper I)

1.1. Maternal separation increases corticosterone levels at PND15

Stress is a well known risk factor for drug abuse disorder and PFC is one of the principal targets of glucocorticoids (McEwen, 2007). GR regulation is involved in stress-induced dopaminergic signaling in the PFC and in the glutamatergic neurotransmission from the PFC to the NAc (Butts et al., 2011; Campioni et al., 2009), but the GR-dependant mechanism remains unclear. We have previously shown an early life stress-induced increase in *Dnmt1, Dnmt3a* and *Dnmt3b* expression in the NAc (Anier et al., 2014) and thus we hypothesized that GR as a transcription factor mediates the stress response by genomic regulation of *Dnmt*.

We used the rat MS model of early life stress to study the role of GR stimulation on *Dnmt* expression (Fig. 3A). We assessed the plasma CORT levels at PND15 that indicates the development of HPA axis hyperactivity in response to stress. Increase in CORT was evident in the handling control group (MS15) and in the MS group separated from the dam and littermates for 180 min per day 24 hours after the last separation event (Fig. 3B, $F_{(2, 15)} = 322.2$, ****$p < 0.0001$, AFR vs. MS15; ****$p < 0.0001$, AFR vs. MS180). CORT levels were markedly increased in the MS180 pups in comparison with MS15 group indicating higher HPA axis activity after MS (Fig. 3B, ####$p < 0.0001$, MS15 vs. MS180).

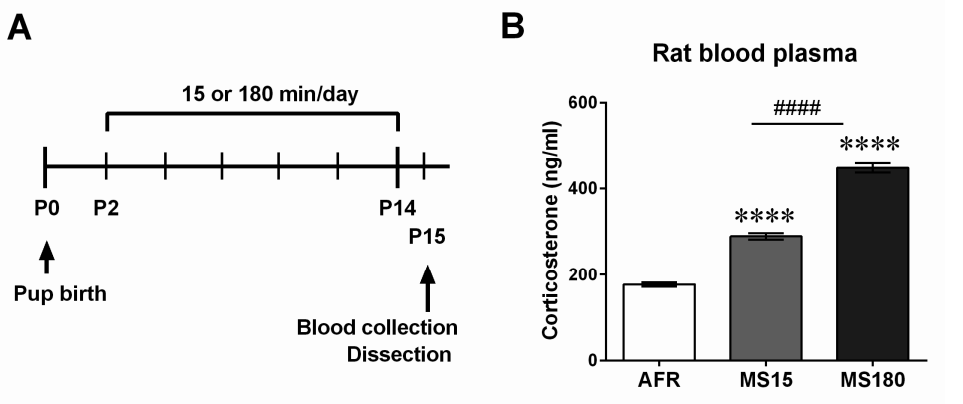

Figure 3. Maternal separation increases plasma corticosterone levels. (A) Experimental timeline. Male pups were separated to individual cages on PND2-14 for 15 min or 180 min/daily. (B) Plasma corticosterone was increased 24 h after last separation event both in MS15 and MS180 pups (Bonferroni post-hoc test after one-way ANOVA, ****$p < 0.0001$, in comparison with AFR, ####$p < 0.0001$, MS15 vs MS180); error bars indicate SEM; n = 6.

1.2. Maternal separation is associated with increased *Dnmt1, Dnmt3a, Dnmt3b* expression and DNMT activity

We studied *Dnmt* expression after MS in the PFC of male rats at PND15. *Dnmt3a, Dnmt3b* mRNA was increased in the PFC of both MS15 and MS180 groups, while *Dnmt1* mRNA increased in MS180 animals (Fig. 4A–C; Dnmt1, $F_{(2,\ 16)} = 66.19$, ****p < 0.0001, AFR vs. MS180; Dnmt3a, $F_{(2,\ 16)} = 90.00$, ****p < 0.0001, AFR vs. MS180; Dnmt3b, $F_{(2,\ 16)} = 121.5$, **p < 0.01, AFR vs. MS15, ****p < 0.0001, AFR vs. MS180). There was a notable increase in the expression of *Dnmt1, Dnmt3a, Dnmt3b* in the PFC after MS (Fig. 4A–C; MS15 vs. MS180, Dnmt1, ####p < 0.0001; Dnmt3a, ####p < 0.0001, Dnmt3b, ####p < 0.0001).

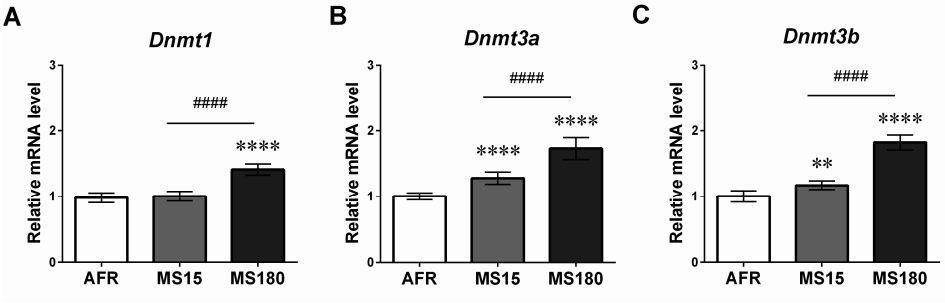

Figure 4. Maternal separation increases total mRNA of (A) *Dnmt1,* (B) *Dnmt3a,* (C) *Dnmt3b* in the PFC. Bonferroni post-hoc test after one-way ANOVA, **p < 0.01, ****p < 0.0001, in comparison with AFR, ####p < 0.0001, MS15 vs MS180); error bars indicate SEM; n = 6 – 7.

To date, rat *Dnmt1* and *Dnmt3b* have been described with a single transcript each. Two transcripts are known of rat *Dnmt3a* gene (Fig. 5A). *Dnmt3a* transcripts were measured in another cohort of MS littermates that had an increase in total mRNA (Fig. 5B, $F_{(2,\ 13)} = 11.72$, **p = 0.002, AFR vs. MS15, **p = 0.0073, AFR vs. MS180). *Dnmt3a1* mRNA was induced in the handling (MS15) group (Fig. 5C, $F_{(2,\ 12)} = 4.022$, *p = 0.0457, AFR vs. MS15). Interestingly, transcript 2 was greatly increased in MS180, but not in MS15 rats and also augmented by MS stress alone (Fig. 5D, $F_{(2,\ 12)} = 5.745$, *p = 0.0329, AFR vs. MS180, #p = 0.043, MS15 vs. MS180). *Dnmt3a* has a considerable contribution to DNA methylation in postmitotic cells, as the expression of *Dnmt1* and *Dnmt3b* is greatly decreased in postnatal neural cells (Yue et al., 2014). At the protein level, DNMT3A1 expression was increased in the MS180 group (Fig. 5E–F, $F_{(2,\ 18)} = 5.798$, p = 0.0095, AFR vs. MS180).

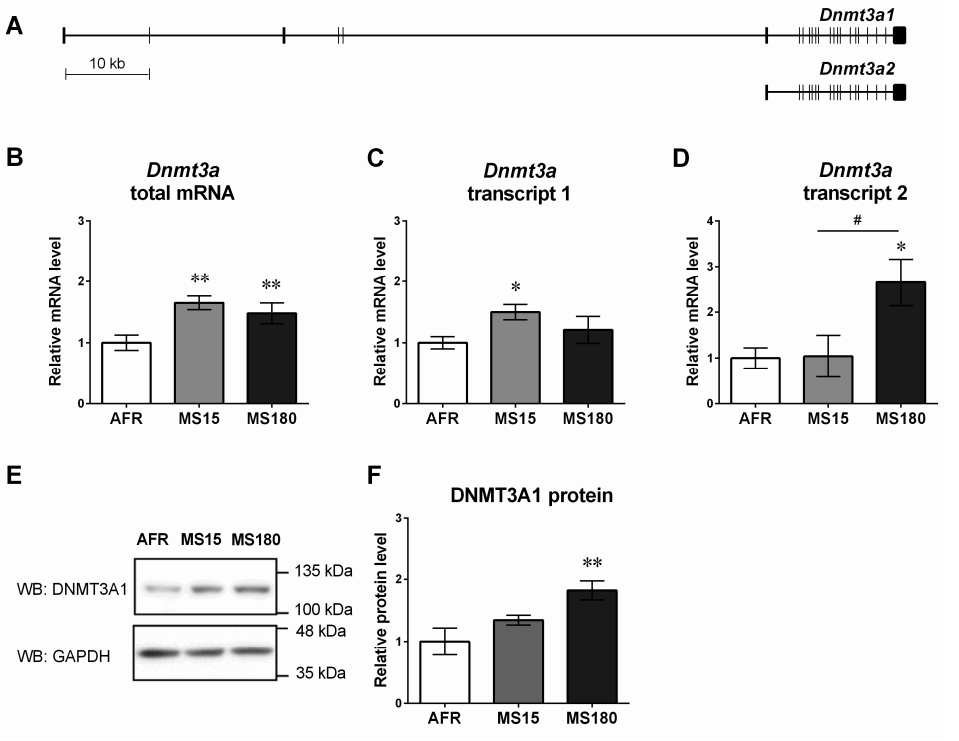

Figure 5. *Dnmt3a* expression changes after MS procedure. (A) Genomic structure (chr6:28235695-28,340,577) of *Dnmt3a* with introns drawn in scale displaying the two encoded transcripts. Total mRNA of (B) *Dnmt3a*, (C) *Dnmt3a* transcript 1 and (D) *Dnmt3a* transcript 2 after MS for 15 min or 180 min. (E – F) Representative Western blot bands of DNMT3A1 expression and quantitative data analysis normalized to GAPDH. Results are presented as mean OD ratio relative to control group. (B–D) n = 5–7; (F) n = 9;

Next, we detected DNMT activity using a DNMT enzyme activity assay that showed an increase in the PFC of MS180 rats that was induced by MS (Fig. 6, $F_{(2, 18)} = 10.90$, ***p = 0.0006, AFR vs. MS180, #p = 0.0358, MS15 vs. MS180). Taken together, our data shows that maternal separation induces *Dnmt3a* expression and DNMT activity in rat PFC at PND15.

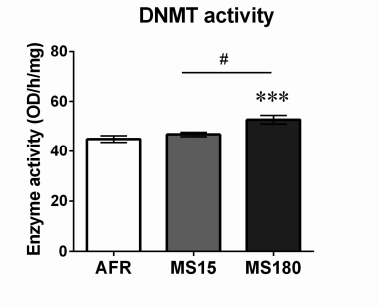

Figure 6. DNMT activity increases after MS, presented as OD/h/mg of protein. Bonferroni post-hoc test after one-way ANOVA, *p < 0.05, **p < 0.01, ****p < 0.0001, in comparison with AFR, #p < 0.05, MS15 vs MS180); error bars indicate SEM; n = 7.

1.3. *In silico* identified GR-binding elements in *Dnmt3a* and *Dnmt3b* are biologically relevant

To determine the biologically relevant GR binding sites of *Dnmt1, Dnmt3a, Dnmt3b*, we studied the effects of GR stimulation in primary cortical neurons. Our preliminary observations in primary neurons indicated that stress-induced increase in *Dnmt1, Dnmt3a, Dnmt3b* mRNA may be mediated by GR. To determine an optimal CORT concentration for studying *Dnmt* expression in primary cells, we treated cortical neurons with increasing concentrations of CORT (1, 5, 10, 50 μM). *Dnmt1, Dnmt3a, Dnmt3b* expression were all upregulated with 50 μM CORT (Fig. 7A–C, Dnmt1, *p = 0.0156; Dnmt3a, §p < 0.0001; Dnmt3b, §p < 0.0001, Vehicle vs. CORT). As 1 μM CORT treatment only had an effect on *Dnmt3b* mRNA and no changes were seen with 5 μM CORT (Fig. 7C; Dnmt3b, *p = 0.0218, Vehicle vs. 1 μM CORT), 50 μM CORT was chosen for molecular studies that indicated GR-mediated regulation for CORT-induced expression of *Dnmt1, Dnmt3a* and *Dnmt3b* (Fig. 7D–F, Dnmt1, $F_{(2, 3)}$ = 55.29, ##p = 0.0043; Dnmt3a, $F_{(2, 3)}$ = 31.44, #p = 0.0137; Dnmt3b, $F_{(2, 3)}$ =12.02, #p = 0.0479, CORT vs. CORT + Mif).

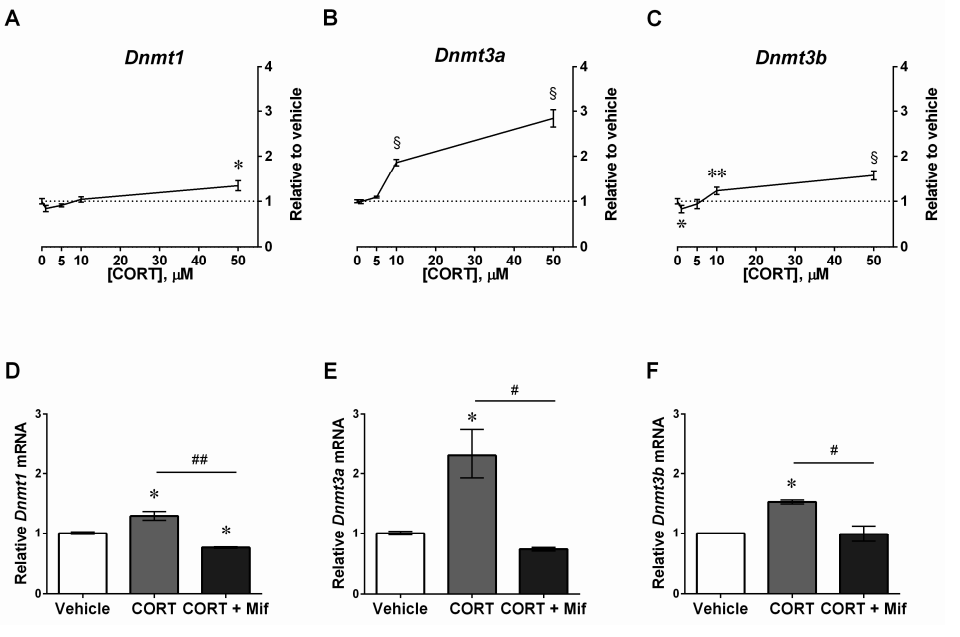

Figure 7. GR mediates CORT-induced increase in *Dnmt1, Dnmt3a, Dnmt3b* expression. CORT was added in increasing concentrations to primary cortical neurons and 50 μM CORT upregulated *Dnmt1, Dnmt3a* and *Dnmt3b* mRNA (A–C). GR antagonist mifepristone abolished the CORT-induced increase in *Dnmt1* (D), *Dnmt3a* (E), *Dnmt3b* (F) expression. Bonferroni post-hoc test after one-way ANOVA, *p < 0.05, §p < 0.05, in comparison with vehicle, #p < 0.05, in comparison with CORT; error bars indicate SEM; (A–C) n = 4, (D–F) n = 2.

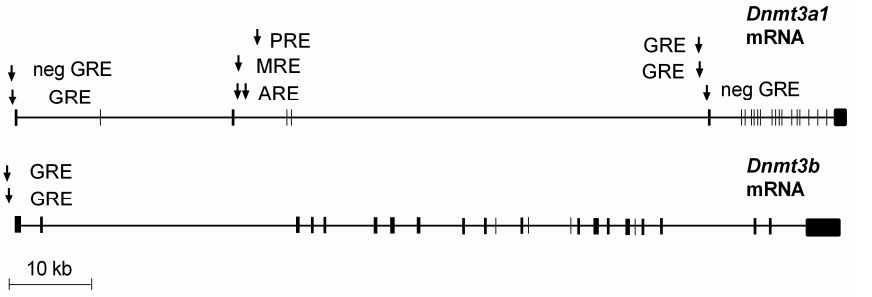

Figure 8. Genomic structure of rat *Dnmt3a* and *Dnmt3b* (chr3:149,131,541–149,170,061) with introns drawn in scale showing the in silico found locations of glucocorticoid (GRE), progesterone (PRE), mineralocorticoid (MRE) and androgen response elements (ARE).

Based on transcription factor binding site analysis, four loci were selected for study that included putative genomic GR-binding regions: *Dnmt3a* and *Dnmt3b* promoters, *Dnmt3a* intron 3 and intron 5 (Fig. 8A). GR immunoprecipitated from all selected regions except *Dnmt3a* intron 5 in comparison with the negative control region (Fig. 9A, **p = 0.004, Dnmt3b promoter Vehicle vs. Negative control Vehicle; **p = 0.007, Dnmt3b promoter 1 h CORT vs. Negative control 1 h CORT, ***p = 0.0002; Dnmt3a promoter Vehicle vs. Negative control Vehicle; ***p = 0.0005, Dnmt3a promoter 1 h CORT vs. Negative control 1 h CORT; **p = 0.005, Dnmt3a intron 3 Vehicle vs. Negative control Vehicle; *p = 0.06, Dnmt3a intron 3 1 h CORT vs. Negative control 1 h CORT). GR binding remained unaltered in all selected loci after CORT treatment (in comparison with vehicle). These results confirm the biological relevance of the in silico predicted GR binding sites at *Dnmt3a* and *Dnmt3b*.

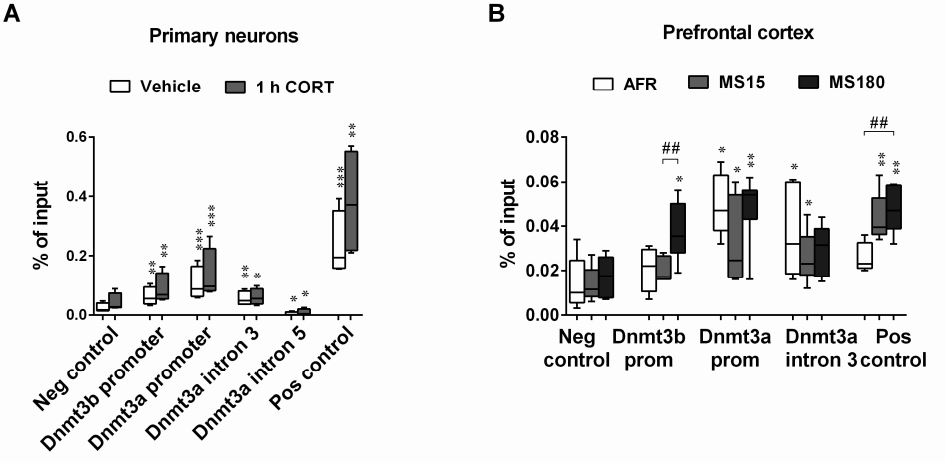

Figure 9. GR binds to *Dnmt3a* and *Dnmt3b* promoters. ChIP-qPCR analysis of immunoprecipitated DNA with GR antibodies of (A) primary cortical neurons and (B) PFC. Data are presented as percent of total DNA. *Per1* promoter was used as positive and *Mb* intron as a negative control region. False discovery rate correction after Student's t-tests, *p < 0.05, **p < 0.01, ****p < 0.0001, in comparison with respective group's binding to Neg control; ##p < 0.01, in comparison with AFR or MS15 group; error bars indicate SEM; (A) n = 4; (B) n = 5–7.

1.4. Glucocorticoid receptor binding increases at *Dnmt3b* in the PFC after maternal separation

We assessed GR recruitment at *Dnmt3a* and *Dnmt3b* in the PFC after maternal separation. Interestingly, GR binding was detected by chromatin immunoprecipitation at the *Dnmt3b* promoter in the PFC of MS180 rats, but not in the control groups (Fig. 9B, *p = 0.018, Dnmt3b promoter MS180 vs. Negative control MS180). GR binding at the *Dnmt3b* promoter increased after maternal separation (##p = 0.0096, MS15 vs. MS180). We also measured GR binding at the *Dnmt3a* promoter as well as *Dnmt3a* intron 3 independently of the presence of stress (Dnmt3a promoter, *p = 0.016, Dnmt3a promoter AFR vs. Negative control AFR; *p = 0.024, Dnmt3a promoter MS15 vs. Negative control MS15; **p = 0.007, Dnmt3a promoter MS180 vs. Negative control MS180; Dnmt3a intron 3, *p = 0.047, Dnmt3a intron 3 AFR vs. Negative control AFR; *p = 0.036, Dnmt3a intron 3 MS15 vs. Negative control MS15). In addition, we detected no change in GR mRNA after maternal separation (Fig. 10, $F_{(2, 12)} = 0.4318$), therefore the increased binding of GR at *Dnmt3b* promoter was not due to transcriptional activation of GR. Our results show that GR can directly regulate *Dnmt3a* and *Dnmt3b* expression in the PFC and suggests that the increased binding of GR at *Dnmt3b* promoter after MS leads to its transcriptional activation.

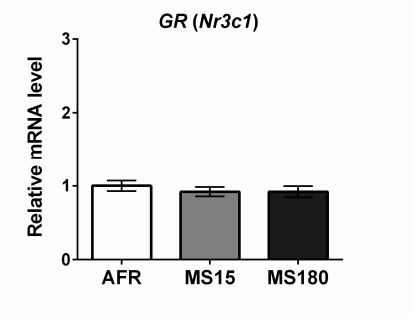

Figure 10. GR mRNA does not change after MS. Bonferroni post-hoc test after one-way ANOVA; error bars indicate SEM; n = 7.

2. Cocaine-induced epigenetic DNA modification in mouse addiction-specific and non-specific tissues (Paper II)

2.1. The effect of acute drug treatments on *Dnmt* expression and enzyme activity in the brain and peripheral blood cells (PBC)

The alterations in *Dnmt1* and *Dnmt3a* expression in the NAc after cocaine injection has been described previously on the behavioral sensitization and self-administration models (Anier et al., 2010; LaPlant et al., 2010). Firstly, we studied whether cocaine-induced changes in the expression of epigenetic

modifiers in the NAc can be detected in peripheral blood. Secondly, since cocaine as well as procaine inhibit voltage-gated sodium channels, we hypothesized that cocaine may affect DNA methylation enzymes by blocking voltage-sensitive sodium channels in the NAc and in PBC. The effects of cocaine and procaine treatments were assessed in adult mice.

During the first half an hour of a single cocaine dose treatment *Dnmt1, Dnmt3a* expression decreased in the NAc (Fig. 11A, Dnmt1, $F_{(3,20)} = 4.438$, $^{*}p = 0.0151$; Dnmt3a, $F_{(3,20)} = 6.244$, $^{**}p = 0.0036$) and interestingly, the decrease is also reflected in peripheral blood cells (Fig. 11B, Dnmt1, $F_{(3,20)} = 3.792$, $^{*}p = 0.0265$; Dnmt3a, $F_{(3,20)} = 3.999$, $^{*}p = 0.0221$). No changes were seen in a reference brain region outside of the mesolimbic dopaminergic system, cerebellum (Cer) that is not directly related to cocaine-induced behavioral sensitization (Fig. 11C). However, 24 hours after the single cocaine injection, *Dnmt1*, *Dnmt3a* expression increased in the NAc (Fig. 11A, Dnmt1, $t_{(12)} = 5.893$, $^{***}p < 0.0001$; Dnmt3a, $t_{(12)} = 6.988$, $^{***}p < 0.0001$) and an upregulation in *Dnmt3a* mRNA was also detected in the PBC (Fig. 11B, $t_{(12)} = 2.374$, $^{*}p = 0.0351$).

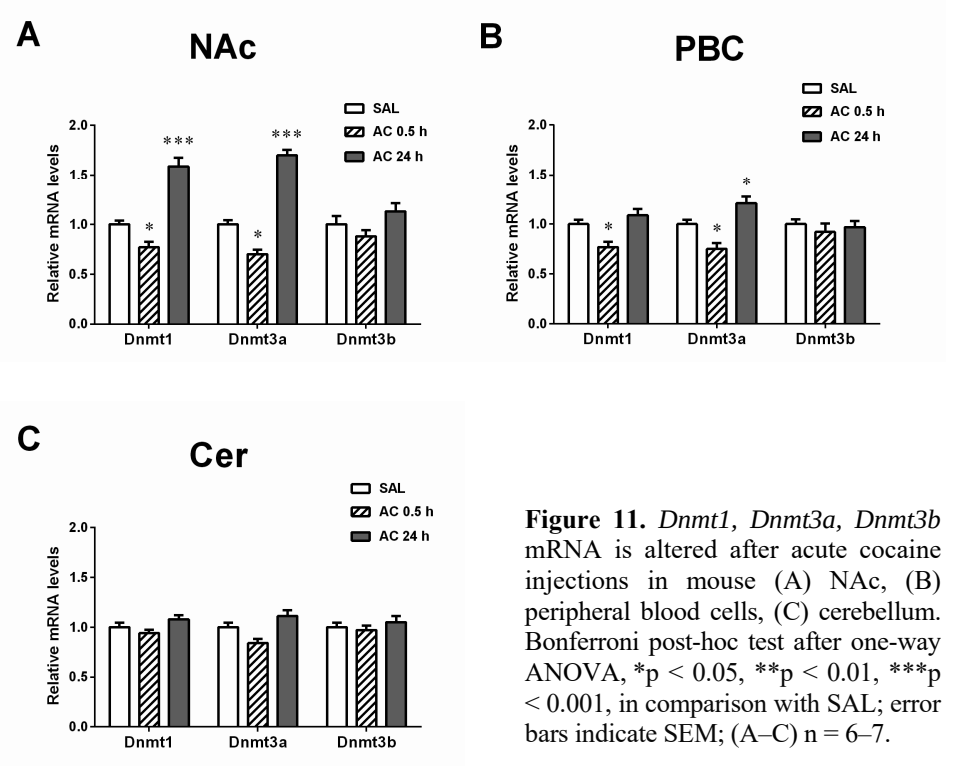

Figure 11. *Dnmt1, Dnmt3a, Dnmt3b* mRNA is altered after acute cocaine injections in mouse (A) NAc, (B) peripheral blood cells, (C) cerebellum. Bonferroni post-hoc test after one-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, in comparison with SAL; error bars indicate SEM; (A–C) n = 6–7.

Moreover, DNMT activity levels increased in the NAc and PBC (Fig. 12A–B, NAc, $t_{(10)} = 4.347$, **p = 0.0015; PBC, $t_{(10)} = 2.494$, *p = 0.0317) and positive correlation was found between the DNMT activity increase in the NAc and PBC (Fig. 12C, r = 0.632, *p = 0.0274). In the NAc, *Dnmt1, Dnmt3a* had a bi-phasical change in expression and remained increased 24 h after acute cocaine injection that is reflected also in PBC. The elevated DNMT enzyme activity in the NAc after acute cocaine correlates with its increase in PBC.

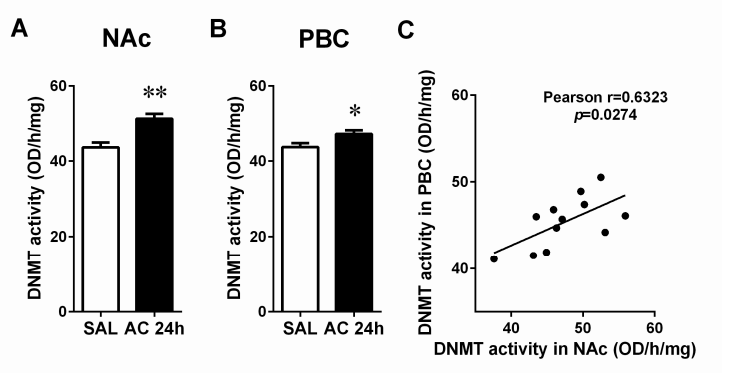

Figure 12. DNMT activity increases 24 h after acute cocaine injections both in the (A) NAc and in (B) PBC. (C) Pearson's correlation of DNMT activity levels in the NAc and in PBC. Student's t-test, *p < 0.05, **p < 0.01, in comparison with SAL; error bars indicate SEM; (A–C) n = 7.

Cocaine may affect DNA methyltransferases by inhibiting voltage-gated sodium channels, thus we used another ester-type local anesthetic to study the observed effects. A single dose of procaine (45 mg/kg) affected *Dnmt3a* expression in an analogous, biphasical manner in the PBC, but increasing *Dnmt3a* mRNA 0.5 h (Fig. 13A, $t_{12} = 2.910$, *p = 0.0131) and decreasing it 24 h after injection (Fig. 13B, $t_{12} = 2.791$, *p = 0.0163). Procaine treatment did not change Dnmt expression in the NAc (Fig. 13B). These results indicate that a single dose of procaine affects the dynamics of *Dnmt3a* expression in similar biphasic manner as cocaine, but in an opposite direction.

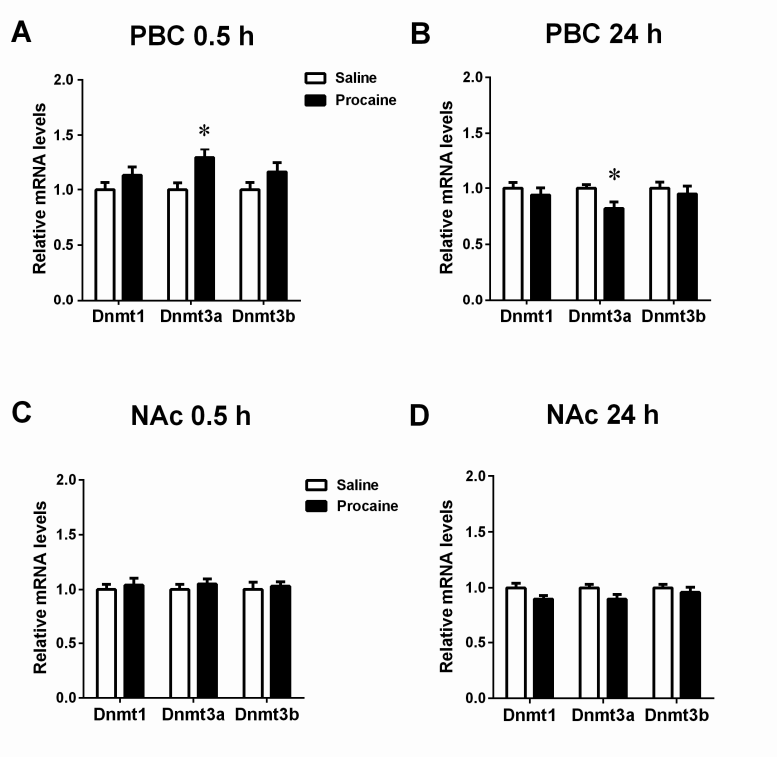

Figure 13. Acute procaine administration alters *Dnmt3a* mRNA in mouse PBC (A) 0.5 h and (B) 24 h after i.p. injections. Procaine administration did not change *Dnmt* expression in the NAc (C) 0.5 h and (D) 24 h later. Student's t-test, *p < 0.05, in comparison with SAL; error bars indicate SEM; (A–D) n = 6–7.

2.2. Repeated cocaine administration increases locomotor activity in mice

Our next aim was to study the effect of withdrawal from repeated cocaine on DNA methyltransferases in the brain and PBC. An increase in *Dnmt3a* mRNA in the NAc after withdrawal from chronic cocaine has been previously shown on the behavioral sensitization and self-administration models (LaPlant et al., 2010). We hypothesized that the acute cocaine-induced changes in *Dnmt* expression and activity observed both in the NAc and PBC persist after chronic cocaine in the behavioral sensitization model.

Cocaine treatment for 7 consecutive days (AC) increased locomotor activity in mice indicating the development of behavioral sensitization (Fig. 14A, $F_{(1,37)} = 87.19$, $p < 0.0001$; time $F_{(1,37)} = 8.36$, $p = 0.0064$; interaction $F_{(1,37)} = 16.46$, $p = 0.0002$; ***$p < 0.001$ cocaine 1st vs. 7th day). Mice that received a challenge dose of cocaine (RC) after three weeks of withdrawal from cocaine displayed an enhanced locomotion response implying behavioral sensitization (Fig. 14B, $F_{(3,41)} = 27.61$, $p < 0.0001$; ***$p < 0.001$ SAL vs. RC).

The increased locomotor activity of RC mice in comparison with AC mice indicates the persistence of behavioral sensitization (#p < 0.001 AC vs. RC). A group of mice previously receiving repeated cocaine treatment and injected with saline (RS) three weeks later showed increased locomotor activity (*p < 0.05 SAL vs. RS) that implicates the presence of psychomotor response and the extent of placebo with saline challenge dose.

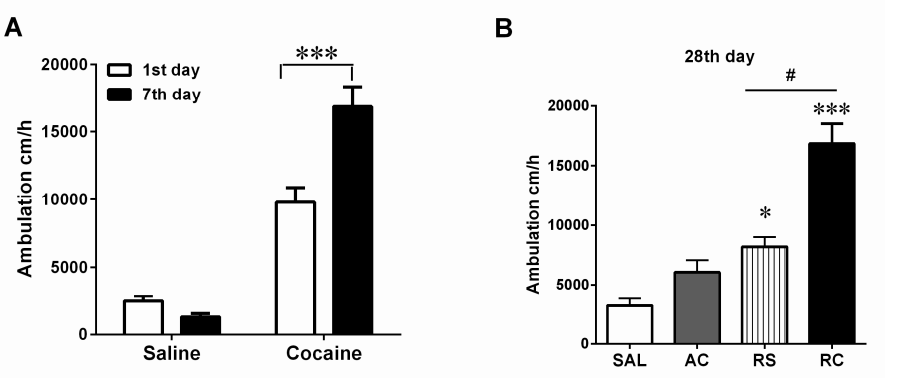

Figure 14. Behavioral sensitization develops and persists in mice with chronic cocaine administration. Repeated cocaine injections increase locomotion response on (A) day 7 in comparison with day 1. Bonferroni post hoc test after two-way ANOVA, ***p < 0.001 cocaine group 1st vs 7th day, n = 8–16. A challenge dose of cocaine after withdrawal (RC) enhances locomotor activity (B) on day 28 in comparison with acute cocaine administration (AC). Bonferroni post hoc test after one-way ANOVA, *p < 0.05, ***p < 0.001 in comparison with SAL; #p < 0.001, AC vs RC and RS vs RC; error bars indicate SEM, n = 8–12.

2.3. The effect of repeated cocaine treatment and withdrawal on *Dnmt* expression in the brain and PBC

We measured *Dnmt* expression levels 24 h after the repeated cocaine injections in order to see the long-term effect after cocaine has been metabolized (half life about 1 h). We describe an increased expression of *Dnmt1, Dnmt3a, Dnmt3b* in the NAc after cocaine withdrawal that persists up to 24 h (Fig. 15, main effect of group: Dnmt1, $F_{(3, 43)} = 35.94$, $p < 0.0001$; Dnmt3a, $F_{(3,43)} = 61.44$, $p < 0.0001$; Dnmt3b, $F_{(3,43)} = 13.27$, $p < 0.0001$; ***p < 0.001, **p < 0.01, *p < 0.05 compared with SAL; ##p < 0.001, #p < 0.05 AC vs. RC and RS vs. RC).

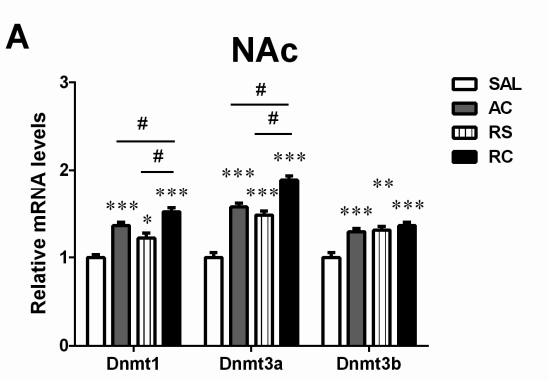

Figure 15. Withdrawal after repeated cocaine and a challenge dose increases *Dnmt* mRNA in the NAc 24 h after last dose. Bonferroni post hoc test after one-way ANOVA, *p < 0.05, **p > 0.01, ***p < 0.001 in comparison with SAL; #p < 0.001, AC vs RC and RS vs RC; error bars indicate SEM, n = 6–14.

In PBC, *Dnmt1, Dnmt3a* and *Dnmt3b* mRNA were also increased 24 h after the cocaine challenge (Fig. 16A, main effect of group: Dnmt1, $F(3,39) = 8.187$, $p = 0.0002$; Dnmt3a, $F(3,39) = 11.25$, $p < 0.0001$; ***p < 0.001, **p < 0.01, *p < 0.05 compared with SAL; #p < 0.05 RS vs. RC). The long-term increase in *Dnmt1* and *Dnmt3a* expression in PBC seems to be independent of withdrawal and sensitization as observed in the NAc.

Interestingly, *Dnmt1, Dnmt3a* and *Dnmt3b* expression was also increased in the Cer at 24 h (Fig. 16B, Dnmt1, $F_{(3,37)} = 11.17$, $p < 0.0001$; Dnmt3a, $F_{(3,37)} = 12.13$, $p < 0.0001$; Dnmt3b, $F_{(3,20)} = 8.572$, $p = 0.0002$; ***p < 0.001, **p < 0.01 compared with SAL). *Dnmt1* and *Dnmt3a* mRNA were upregulated independently of withdrawal and sensitization as in NAc, but the increase in *Dnmt3b* expression may be associated with withdrawal in the Cer.

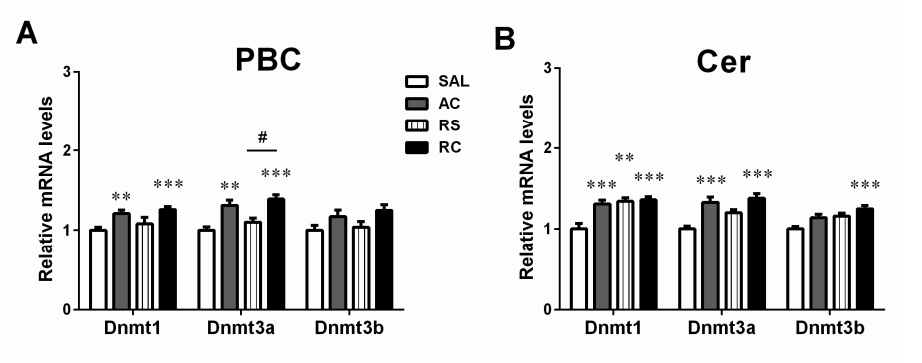

Figure 16. *Dnmt1, Dnmt3a* and *Dnmt3b* mRNA increases in (A) PBC and (B) Cer 24 h after a 3-week withdrawal period and a cocaine challenge. Bonferroni post hoc test after one-way ANOVA, **p > 0.01, ***p < 0.001 in comparison with SAL; #p < 0.001, RS vs RC; error bars indicate SEM, n = 6–14.

2.4. Repeated cocaine treatment and withdrawal affects DNMT enzyme activity in NAc and PBC

As we found cocaine-induced changes in *Dnmt* expression, we asked whether repeated cocaine treatment also affects the enzyme activitiy of DNMT. DNMT activity was increased in RC group in the NAc 24 h after the cocaine challenge dose (Fig. 17A, main effect of group: $F_{(3,23)} = 19.43$, $p < 0.0001$; ***p < 0.001 compared with SAL; #p < 0.001 AC vs. RC and RS vs. RC). We found a notable increase in DNMT activity levels in the PBC of AC and RC mice, while the augmented activity level was higher in the NAc than in PBC (Fig. 17B, main effect of group: $F_{(3,20)} = 6.741$, $p = 0.0025$; **p < 0.01, *p < 0.05 compared with SAL; #p < 0.05 RS vs. RC).

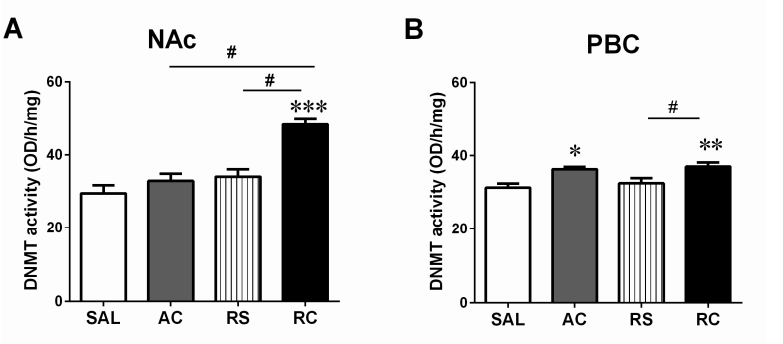

Figure 17. Cocaine challenge enhances DNMT activity 24 h later in the (A) NAc and in (B) PBC. Bonferroni post hoc test after one-way ANOVA, *p > 0.05, **p > 0.01, ***p < 0.001 in comparison with SAL; #p < 0.001, AC vs RC and RS vs RC; error bars indicate SEM, n = 7.

Our data showed a strong positive correlation between *Dnmt3a* mRNA and DNMT activity in the NAc (Fig. 18A, r = 0.7758, ***p < 0.0001). *Dnmt1* mRNA increase contributes partially (49% in the NAc and 47% in PBC) to augmented DNMT activity in the NAc and PBC (Fig 18B–C, NAc, r = 0.6993, ***p < 0.0001; PBC, r = 0.6883, ***p = 0.0002).

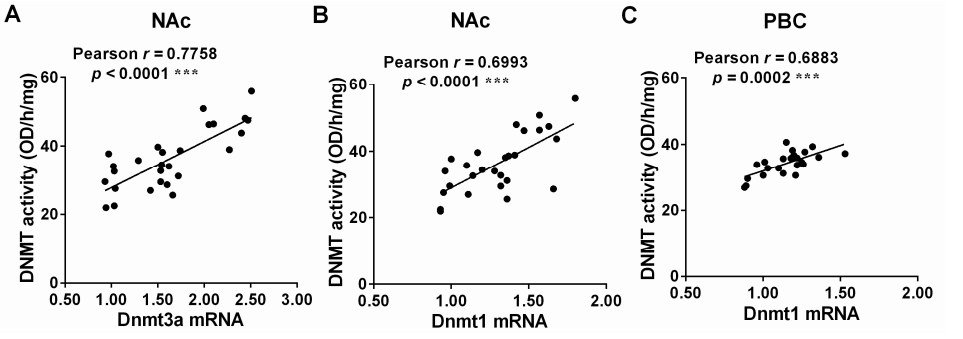

Figure 18. Correlations between (A) *Dnmt3a* mRNA and DNMT activity in the NAc and (B–C) *Dnmt1* mRNA and DNMT activity in (B) NAc and (C) PBC were analyzed 24 h after cocaine challenge.

2.5. Cocaine withdrawal affects global levels of 5-mC

Since cocaine withdrawal affected DNMT activity in the NAc, we quantified the global levels of 5-mC after cocaine withdrawal. Interestingly, we observed an increase in both 5-mC level after cocaine withdrawal and a challenge dose (Fig. 19, 5-mC, main effect of group: $F_{(2, 12)} = 9.329$, $p = 0.0036$; *$p < 0.05$ compared with SAL; #$p < 0.01$ AC vs. RC). These results show that withdrawal from repeated cocaine induces global alterations in DNA methylation in the NAc.

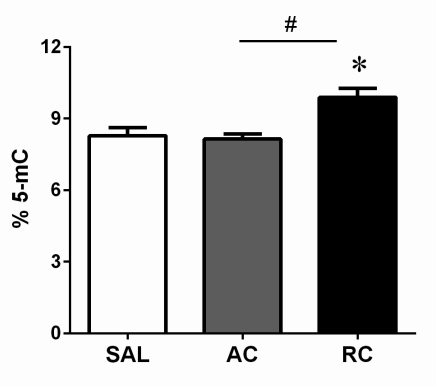

Figure 19. Global levels of 5-methylcytosine (5-mC) in the NAc were increased with withdrawal after repeated cocaine and a challenge dose (RC). Bonferroni post hoc test after one-way ANOVA, *$p > 0.05$, in comparison with SAL; #$p < 0.001$, AC vs RC; error bars indicate SEM, n = 5.

2.6. The regulation of the expression of cocaine-induced genes after cocaine withdrawal

We studied the DNA methylation status and the expression of several genes previously associated with repeated cocaine, namely estrogen receptor-1 (*Esr1*) and glutamate receptor subunit zeta-1 (*Grin1*) (Massart et al., 2015). The expression of *Esr1* decreased and *Grin1* mRNA increased in AC and RC mice in the NAc (Fig. 20A–B, Esr1, main effect of group: $F_{(3,19)} = 5.193$, $p = 0.0087$; *$p < 0.05$ compared with SAL; Grin1, main effect of group: $F_{(3,19)} = 7.385$, $p = 0.0018$, **$p < 0.01$, compared with SAL). Our results show that the expression levels of *Esr1*, *Gal* and *Grin1* were also markedly affected in the PBC after cocaine withdrawal (Fig. 20C–D, Esr1, main effect of group: $F_{(3, 19)} = 4.446$, $p = 0.0158$; *$p < 0.05$ compared with SAL; Grin1, main effect of group: $F_{(3, 19)} = 3.575$, $p = 0.0333$, *$p < 0.05$, compared with SAL).

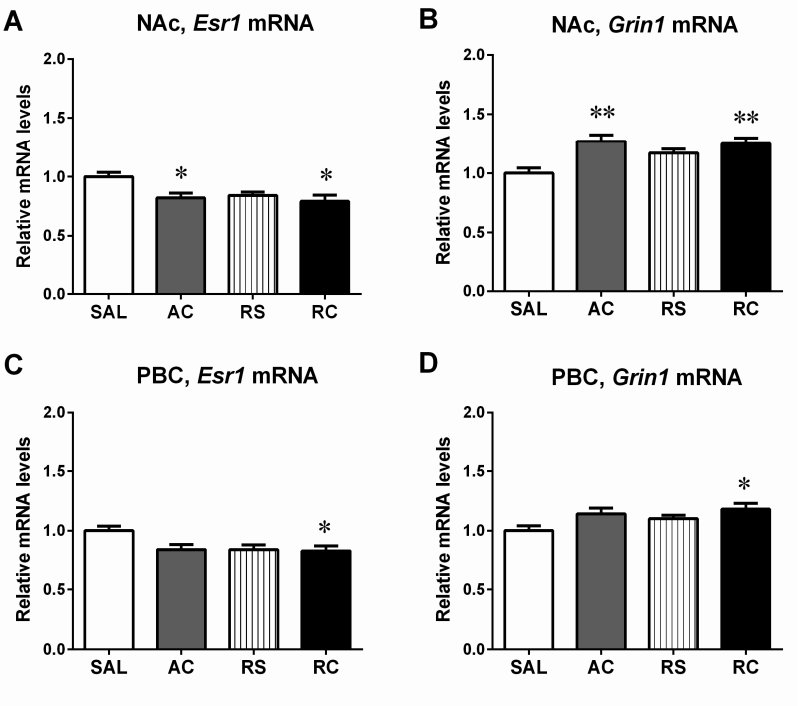

Figure 20. Cocaine challenge after prolonged withdrawal decreased *Esr1* mRNA in the (A) NAc and (C) PBC and increased *Grin1* mRNA in the (B) NAc and (D) PBC. Bonferroni post hoc test after one-way ANOVA, *p > 0.05, **p > 0.01, in comparison with SAL; error bars indicate SEM, n = 5–7.

We performed a site specific detection of the methylation of a GC dinucleotide in *Esr1* promoter. We used a glycosylation-mediated restriction enzyme approach and quantified the result with qPCR. DNA methylation analysis in the NAc revealed that withdrawal from repeated cocaine increases site-specific methylation in *Esr1* promoter (Fig. 21A, main effect of group: $F_{(3, 11)} = 68.31$, $p < 0.0001$; ***p < 0.001 compared with SAL), a decrease in 5-mC was detected in the AC mice. No changes were seen in the promoter methylation in PBC (Fig. 21B). Thus, withdrawal from repeated cocaine is associated with an increase in a specific GC dinucleotide methylation in the *Esr1* promoter in the NAc and a decrease in its expression that is also reflected in PBC.

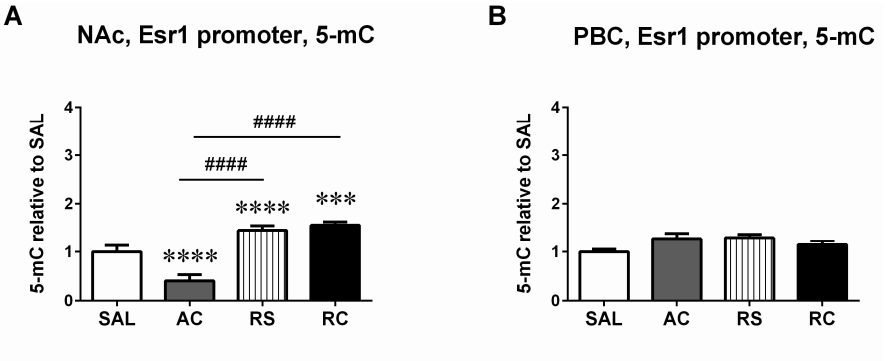

Figure 21. Specific CG dinucleotide methylation in the *Esr1* promoter increased in the (A) NAc, but not in (B) PBC with withdrawal from repeated cocaine. Bonferroni post hoc test after one-way ANOVA, ***p > 0.001, in comparison with SAL; ####p > 0.001, AC vs RS and AC vs RC; error bars indicate SEM, n = 3–6.

We also confirm an increase in the methylation in a 3' untranslated region (UTR) of *Grin1* in the NAc after repeated cocaine injections that indicates a positive regulation between the site-specific DNA methylation and *Grin1* expression (Fig. 22A, ****p < 0.0001 compared with SAL). In the PBC, we detected a decrease in methylation at the 3' UTR of *Grin1* that indicates a negative regulation between DNA methylation and *Grin1* expression in a tissue outside of the mesolimbic dopaminergic system (Fig. 22B, *p < 0.05, **p < 0.01, ****p < 0.0001 compared with SAL). Therefore, withdrawal from repeated cocaine is associated with an increase in the GC dinucleotides in the gene body and an increase in *Grin1* expression in the NAc, but the methylation pattern differs in the PBC.

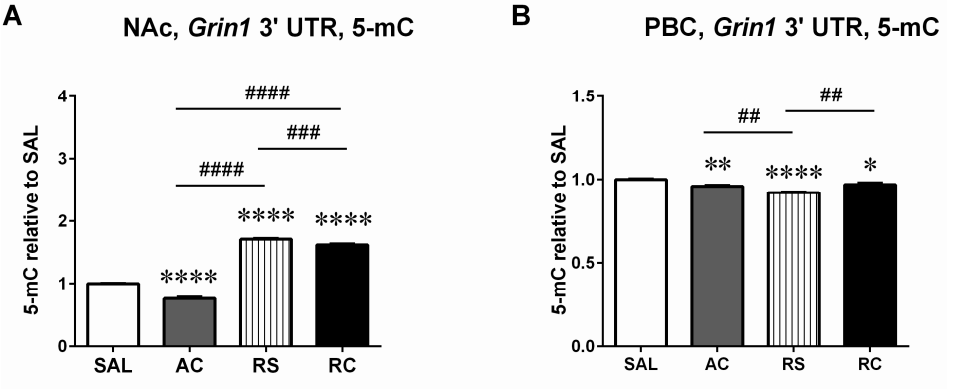

Figure 22. Methylation in *Grin1* gene body is altered in the (A) NAc and (B) PBC after cocaine administration. Bonferroni post hoc test after one-way ANOVA, ***p > 0.001, ****p < 0.0001, in comparison with SAL; ##p < 0.01, ###p < 0.001, ####p < 0.0001, AC vs RS, AC vs RC, RS vs RC, error bars indicate SEM, n = 3–6.

3. The role of DNA methyltransferase activity in cocaine treatment and withdrawal in the NAc of mice (Paper III)

3.1. AAV-mediated *Dnmt3a* silencing is selective

Previous studies suggest that DNMT inhibition affects cocaine seeking behavior, also in the abstinence phase of cocaine abuse (LaPlant et al., 2010; Massart et al., 2015). Thus, we hypothesized that DNMT activity has a crucial role in the cocaine-induced sensitization during withdrawal. Among the DNMT enzymes, *Dnmt3a* has an important role in the neuroadaptations during cocaine treatment (Anier et al., 2010; LaPlant et al., 2010). Thus, we also studied the role of DNMT3A in cocaine-induced sensitization for which we designed a shRNA to silence *Dnmt3a* mRNA in the mouse NAc.

AAV encoded shRNA had a 100% similarity between the selected target sequence of *Dnmt3a* shRNA in mouse and rat to silence *Dnmt3a*. The specificity of the AAV-Dnmt3a-specific shRNA (AAV-Dnmt3a) was verified in rat primary neuronal culture, where *Dnmt3a* mRNA and protein levels were reduced 90% in comparison with a scrambled shRNA (AAV-scrambled) and *Dnmt3b, Dnmt1* expression was not affected (Figure 23A–B, $F_{(1,14)} = 225.8$, **p = 0.0025; $t_{(2)} = 16.29$, **p = 0.003).

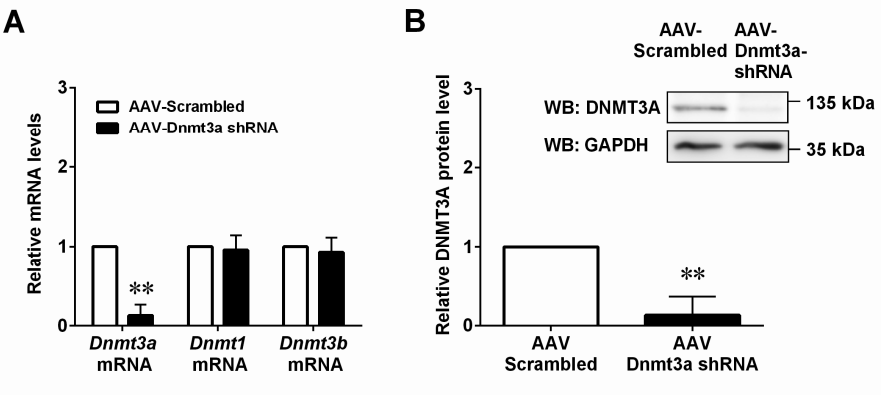

Figure 23. Specific silencing of *Dnmt3a* on (A) mRNA and (B) protein level with AAV-*Dnmt3a*-shRNA in primary neuronal culture. (A) Dunnett's post-hoc test after one-way ANOVA, **p < 0.01, in comparison with AAV-scrambled; (B) Student's t-test, **p < 0.01, in comparison with AAV-scrambled; error bars indicate SEM; (A–B) n = 3.

In the NAc of adult mice not sensitized to cocaine, bilateral injections of AAV-Dnmt3a for 4 weeks decreased *Dnmt3a* expression, however, there was an increase in *Dnmt3b* mRNA (Figure 24A, Dnmt3a, $t_{(12)} = 4.498$, ***p = 0.0007; Dnmt3b, $t_{(12)} = 3.280$, **p = 0.0066). *Dnmt1* expression did not change in the AAV-Dnmt3a group. AAV-Dnmt3a-mediated silencing in the NAc of saline-injected mice also reduced the enzyme activity of DNMT (Figure 24B, $t_{(10)} = 8.961$, ***p < 0.0001).

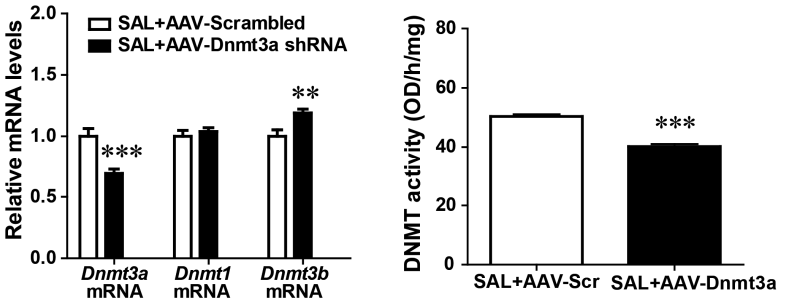

Figure 24. *Dnmt3a* silencing in the NAc of cocaine naïve mice decreases (A) *Dnmt3a* mRNA, increases *Dnmt3b* mRNA and reduces (B) DNMT activity. Student's t-test, $^{**}p < 0.01$, $^{***}p < 0.001$, in comparison with SAL+AAV-scrambled; error bars indicate SEM; (A–B) n = 6–8.

3.2. AAV-shRNA-mediated silencing decreases *Dnmt3a* expression in the NAc at the initiation phase of cocaine-induced behavioral sensitization

We first studied the role of *Dnmt3a* in the initiation phase of cocaine-induced behavioral sensitization in the NAc. The establishment of behavioral sensitization was verified on mice receiving cocaine intraperitoneally for 7 consecutive days that increased locomotor activity in comparison with the 1st day of treatment (Fig. 25, treatment $F_{(1,58)} = 308.75$, $p < 0.0001$; time $F_{(1,58)} = 16.33$, $p = 0.0002$; interaction $F_{(1,58)} = 34.21$, $p < 0.0001$; $^{***}p < 0.001$ cocaine 1st vs. 7th day).

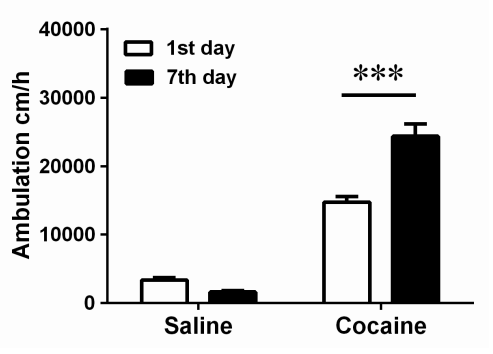

Figure 25. Repeated cocaine administration for 7 consecutive days induced behavioral sensitization in mice. Bonferroni post hoc test after two-way ANOVA, $^{***}p < 0.001$ cocaine 1st vs. 7th day; error bars indicate SEM; n = 15–16.

A four-week period was included into the study design to silence *Dnmt3a* mRNA with AAV-Dnmt3a before repeated cocaine treatments (Fig. 26A). AAV-Dnmt3a decreased *Dnmt3a* expression in the saline-treated group (Fig. 26B, $\#p < 0.001$, AAV-scrambled vs. AAV-Dnmt3a shRNA) that was further decreased 24 h after acute and repeated cocaine treatment ($^{**}p < 0.01$, compared with SAL group of AAV-scrambled injections). However, silencing

Dnmt3a before the initiation of behavioral sensitization also resulted in an induction of *Dnmt1* expression after repeated cocaine treatments (Fig. 26C, *p < 0.05, ***p < 0.001 compared with SAL group of AAV-scrambled injections). *Dnmt3b* mRNA increased further after acute and repeated cocaine treatment in AAV-Dnmt3a mice (Fig. 26D, **p < 0.01, ***p < 0.001 compared with SAL group of AAV-scrambled injections).

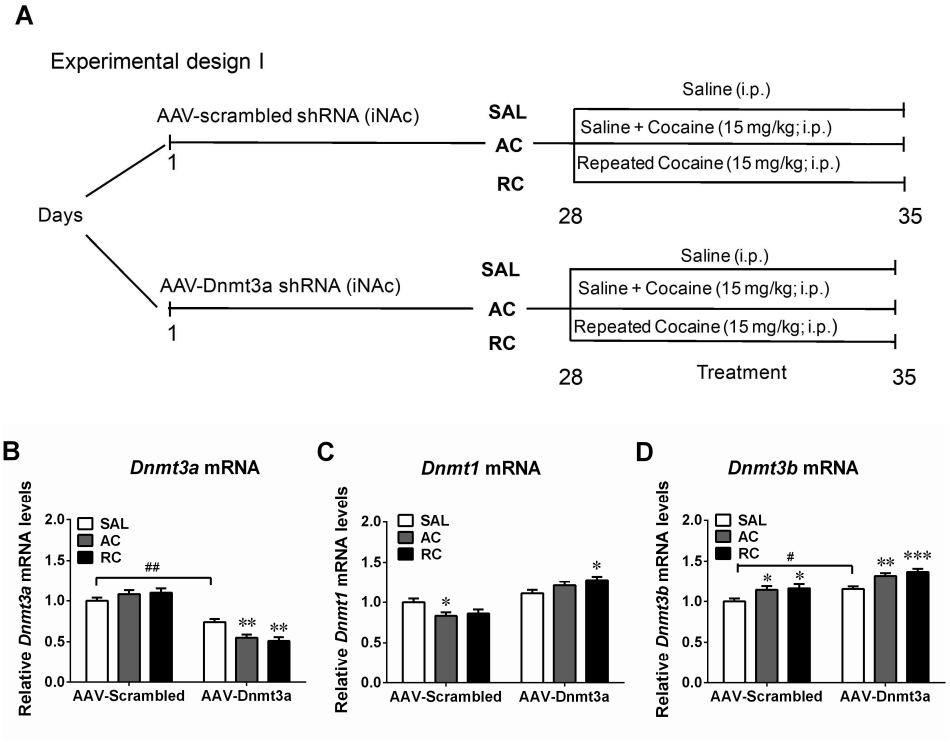

Figure 26. Bilateral iNAc injection of AAV-Dnmt3a shRNA alters *Dnmt* mRNA after chronic cocaine administration. (A) Experimental design of *Dnmt3a* silencing in the NAc for 4 weeks before repeated cocaine administration for 7 days. (B) *Dnmt3a* expression reduced and (C) *Dnmt1,* (D) *Dnmt3b* mRNA increased in the NAc after mice received repeated cocaine injections. Bonferroni post hoc test after two-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001 in comparison with SAL; #p < 0.01, ##p < 0.001, AAV-Scrambled-SAL vs AAV-Dnmt3a-SAL, error bars indicate SEM; n = 7–8.

3.3. Dnmt3a silencing in the NAc increases the behavioral sensitization and decreases DNMT activity at the initiation phase

Mice receiving repeated cocaine i.p. displayed increased locomotor activity after bilateral iNAc injections of AAV-Dnmt3a in comparison with AAV-scrambled mice (Fig. 27A, treatment $F_{(2,36)} = 9.90$, $p = 0.0040$; genotype $F_{(1,36)} = 6.48$, $p = 0.0154$; interaction $F_{(2,36)} = 1.67$, $p = 0.2025$; #p < 0.05

AAV-Scrambled RC vs. AAV-Dnmt3a RC). Bilateral iNAc injections of AAV-Dnmt3a reduced DNMT enzyme activity further with acute and repeated cocaine treatments (Fig. 27B, #p < 0.001, AAV-scrambled vs. AAV-Dnmt3a). We correlated the decreased DNMT activity and increased locomotion of AAV-injected mice that were administered cocaine for 7 days. Pearson's correlation analysis showed a negative correlation between the DNMT activity and suggesting that DNMT activity, including DNMT3A, contributes to the development of behavioral sensitization (Fig. 27C, r = –0.06569, *p = 0.0107).

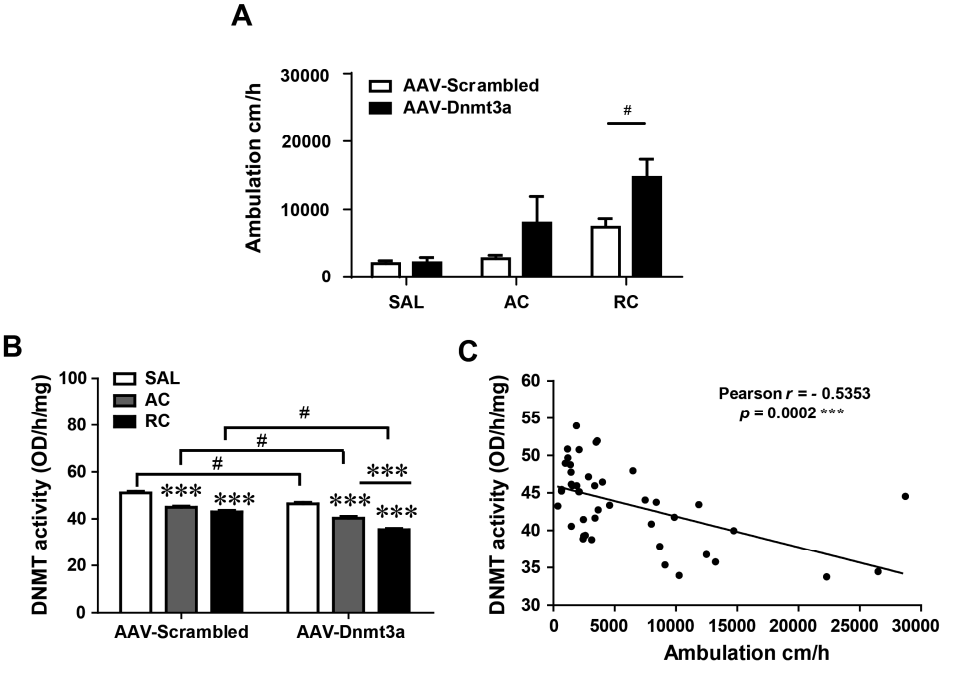

Figure 27. *Dnmt3a* inhibition in the NAc increased (A) locomotor activity in the initiation phase of sensitization and decreased (B) DNMT activity levels 24 h after cocaine administration. (C) Correlation between DNMT activity and locomotion of RC group injected with AAV-Scrambled or AAV-Dnmt3a. (A) Bonferroni post hoc test after two-way ANOVA, #p < 0.05, AAV-Scrambled-SAL vs AAV-Dnmt3a-SAL. (B) Bonferroni post hoc test after two-way ANOVA, ***p < 0.001 in comparison with SAL; #p < 0.001, AAV-Scrambled-SAL vs AAV-Dnmt3a-SAL, error bars indicate SEM; (A–B) n = 7–8, (C) n = 14.

3.4. Withdrawal from repeated cocaine alters DNMT expression and enzyme activity

To study the effect of withdrawal from repeated cocaine on *Dnmt* mRNA and activity in the expression phase of behavioral sensitization, mice were withdrawn from repeated cocaine administration for three weeks and injected with a

challenge dose of cocaine to manifest sensitization. A three-week abstinence period from chronic cocaine treatment induced locomotor activity (Fig. 28, main effect of group: $F_{(3,27)} = 46.14$, $p < 0.0001$; ***$p < 0.001$ SAL vs. RC) and behavioral sensitization persisted after withdrawal and a challenge dose of cocaine in comparison with the AC and RS groups (#$p < 0.001$ AC vs. RC, RS vs. RC).

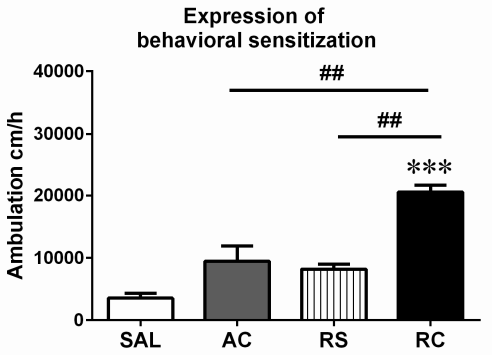

Figure 28. A challenge dose of cocaine increases locomotor activity after withdrawal from repeated cocaine for 7 days. Bonferroni post hoc test after one-way ANOVA, ***$p < 0.001$ in comparison with SAL; ##$p < 0.001$, AC vs RC and RS vs RC, error bars indicate SEM; n = 7.

We found *Dnmt1, Dnmt3a, Dnmt3b* mRNA upregulated after withdrawal from repeated cocaine (Fig. 29A, main effect of group: Dnmt3a, $F_{(3,27)} = 6.755$, $p = 0.0015$; Dnmt1, $F_{(3,27)} = 4.581$, $p = 0.0102$; Dnmt3b, $F_{(3,27)} = 3.717$, $p = 0.0233$, **$p < 0.01$, *$p < 0.05$ compared with SAL). The expression of *Dnmt3a* and *Dnmt3b* increased also in the RS mice, but not *Dnmt1* mRNA that indicates their participation in cocaine associated cues during withdrawal. Cocaine also increased DNMT activity in all treatment groups (Fig. 29B, ***$p < 0.001$, compared with SAL) and a further induction in DNMT activity was detected with a cocaine challenge after repeated treatment (#$p < 0.05$ AC vs. RC; ##$p < 0.01$ RS vs. RC).

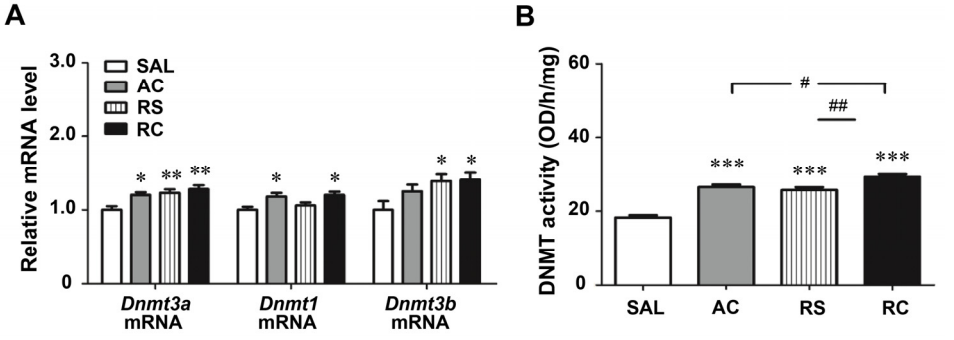

Figure 29. A challenge dose of cocaine increases (A) *Dnmt1, Dnmt3a, Dnmt3b* mRNA and (B) DNMT activity after withdrawal from repeated cocaine for 7 days. Bonferroni post hoc test after one-way ANOVA, *$p < 0.05$, **$p < 0.01$ in comparison with SAL; #$p < 0.05$, AC vs RC, ##$p < 0.01$, RS vs RC, error bars indicate SEM; n = 7 – 8.

3.5. Effect of *Dnmt3a* silencing on the expression of cocaine-induced behavioral sensitization

Our experimental design included 7 days of repeated cocaine treatment followed by iNAc AAV-Dnmt3a or AAV-scrambled injections and saline or cocaine challenge on the 35th day (Figure 30A). AAV infection was verified in the ventral striatum by mCherry protein expression encoded by the viral vector (Fig. 30B–C). TUJ1 (tubulin beta 3 class III, TUBB3) and mCherry double positive cells confirmed the neuronal transduction by AAV-Dnmt3a injected mice (Fig. 30D).

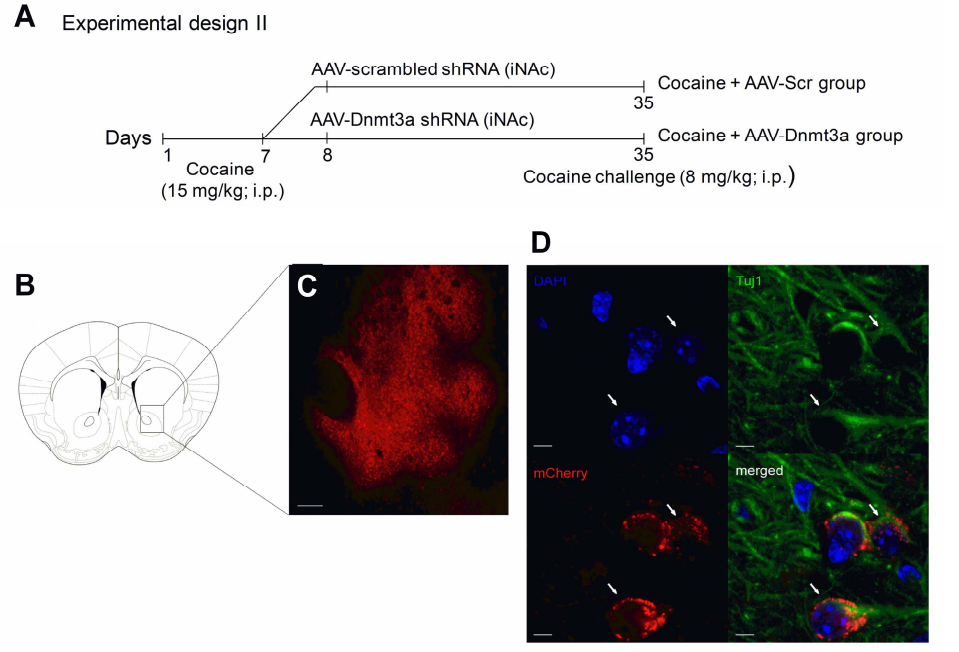

Figure 30. Anatomical and cellular verification of AAV-Dnmt3a-shRNA-mCherry infection in mouse NAc. (A) Experimental design II of repeated cocaine treatments, followed by *Dnmt3a* silencing in the NAc and a 4-week withdrawal before a challenge dose of cocaine administration. The (B–C) anatomical location and (D) infection of TUJ1+ (green) cells in the NAc by AAV-Dnmt3a-shRNA-mCherry (red) was verified immunohistochemically. DAPI (blue) labels nuclei. (B) Cartoon adapted from atlas of Mouse Brain in Stereotaxic Coordinates (Franklin and Paxinos, 2001), figure 21, 1.18 mm Bregma. Scale bar: (C) 100 µm, (D) 5 µm.

iNAc injections of AAV-Dnmt3a decreased *Dnmt3a* mRNA and increased *Dnmt3b* mRNA after withdrawal from repeated cocaine, while *Dnmt1* expression remained unaltered 2 h after the challenge dose (Figure 31A, Dnmt3a, $t_{(26)} = 5.328$, ***$p < 0.0001$; Dnmt3b, $t_{(26)} = 2.484$, *$p = 0.0197$ compared with the cocaine + AAV-Scrambled group). AAV-Dnmt3a did not alter the loco-

motor activity on the 35th day of behavioral sensitization (Fig. 31B, treatment $F_{(1,65)} = 0.21$, $p = 0.6464$; time $F_{(2,65)} = 7.24$, $p = 0.0015$; interaction $F_{(2,65)} = 0.36$, $p = 0.6986$). Finally, we detected no decrease in DNMT activity in the AAV-Dnmt3a mice in comparison with AAV-scrambled mice 2 h after cocaine challenge (Figure 31C, $t_{(19)} = 1.129$, $p = 0.2730$). These results suggest that the selective silencing of *Dnmt3a* in the NAc does not alter DNMT activity and behavioral sensitization in its expression phase.

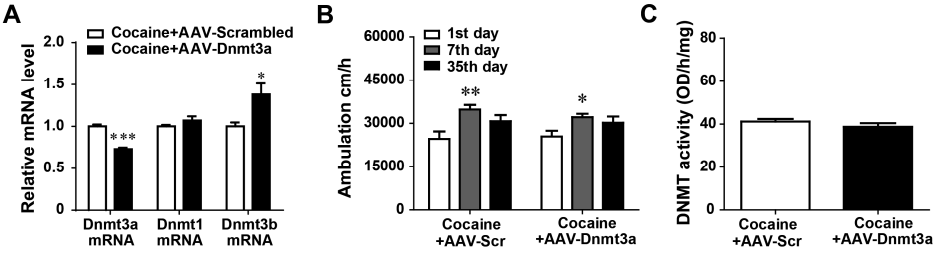

Figure 31. *Dnmt3a* inhibition in the NAc alters (A) *Dnmt3a, Dnmt3b* mRNA in the NAc, but does not change (B) locomotor activity or (C) DNMT activity in the NAc after the challenge dose of cocaine. (A) Student's t-test, *p < 0.05, ***p < 0.001 in comparison with cocaine+AAV-scrambled, n = 13–15; (B) Bonferroni post hoc test after two-way ANOVA, *p < 0.05, **p < 0.01 in comparison with SAL, n = 13–15; (C) Student's t-test, n = 10–11, error bars indicate SEM.

DISCUSSION

1. Impact of DNA methylation on GR signaling in early life stress

Despite increasing evidence of stress-induced changes in DNA methylation, the role of DNMT in stress response remains poorly understood. GR signaling is one possible pathway by which stress may modulate DNA methylation and further downstream events on specific genes. The epigenetic impact of early life stress has been studied in many signaling pathways, including HPA axis, BDNF, GABA, glutamate, estrogen, oxytocin signaling (Jawahar et al., 2015). HPA axis pathway has largely remained in the focus due to its effect on physiological and behavioral response to stressful events.

Individuals with a history of childhood abuse who committed suicide had altered DNA methylation within the promoters 1B, 1C, and 1F of GR compared with nonabused suicides and healthy controls (Labonte et al., 2012; McGowan et al., 2009). These changes resulted in lower expression of the respective transcripts of GR that may affect the GRE-regulated gene expression e.g. *Dnmt3b*. Similar findings in the alterations of GR have been reported in association of different features of child abuse in individuals with depressive disorders and these changes seem to be specific to early life adversity (Bagot et al., 2014). GR promoter methylation status seems to be specific to the paradigm as no alterations were found with the maternal separation model at PND21 (Daniels et al., 2009). GR mRNA remained unchanged in our study and has close-to-adult expression level (Kalinyak et al., 1989). We found that genomic signaling of GR increases *Dnmt3b* expression after maternal separation in the PFC. DNMT3A and DNMT3B cooperate to methylate the genome in embryos. However, DNMT3B also has non-redundant roles in embryonic development and DNMT3A can deposit non-CpG methylation in neurons after birth showing unique roles for either enzymes during development (Greenberg and Bourc'his, 2019). Therefore, we speculate that *Dnmt3b* induction after early life stress to alter the methylation pattern of specific genes may be different from the target genes of *Dnmt3a*.

The outcome of methylation patterns after early adverse events may have several layers of regulation. The study from Labonte and colleagues also showed a complex pattern of methylation across other CpG sites in the *GR* gene of abused suicide completers that did not result in transcriptional alterations in comparison with controls (Labonte et al., 2012). Differential methylation of 1B, 1C, and 1F was detected in non-abused suicides that did not result in a change of *GR* expression (Labonte et al., 2012; McGowan et al., 2009). The relevance of these transcriptionally silent epigenetic modifications remains unknown, but it has been speculated to mediate an increase in speed of regulatory responses to activity or environmental cues (Burns et al., 2018). A term "epigenetic priming" is referred to as permanent changes in neuronal chromatin structure that govern

silent dysregulation of transcription that remain silent in NAc until triggered by re-exposure to the drug or to drug cues (Mews et al., 2018). Epigenetic priming or desensitization affects genes that regulate AMPA or NMDA receptor levels among other synaptic and neural-signaling proteins to induce plasticity in the synapse and the neural circuit (Mews et al., 2018). A set of genes were distinguished that are up- or downregulated by cocaine re-exposure and not cocaine cue alone after abstinence from repeated cocaine and the authors hypothesized that these genes are primed or desensitized during withdrawal and activated or repressed by drug re-exposure (Mews et al., 2018).

Dnmt3b is linked to *de novo* methylation; its role has been less studied than *Dnmt3a. Dnmt3b* expression is induced in the intestinal epithelium to functionally compensate *Dnmt1*-deficient tissue (Elliott et al., 2016). The expression of *Dnmt3b* in the nervous system decreases dramatically after birth to very low mRNA levels (Yue et al., 2014). We speculate that the induction of *Dnmt3b* after MS may be a stress-related neuroadaptation to maintain or alter the methylation patterns in specific genes (Paper I). However, further studies could elucidate the interaction partners of GR that induce *Dnmt3b* and *Dnmt3a* expression after early life stress. Given the complexity of PFC, *Dnmt3a* and *Dnmt3b* may be differentially regulated in this region. Knockdown of *Dnmt3a* in the PFC displayed an anxiogenic phenotype, while the overexpression of *Dnmt3a* induced an anxiolytic effect (Elliott et al., 2016). Therefore, the authors suggested that *Dnmt3a*-mediated methylation patterns in the PFC may mediate chronic stress-induced anxiety behavior (Elliott et al., 2016).

Mifepristone abolishes the CORT-induced increase in *Dnmt* expression indicating GR involvement in the transcriptional regulation of *Dnmt1, Dnmt3a* and *Dnmt3b* after CORT exposure (Paper I). A supersaturating dose of CORT was used to study the mechanism in primary neurons. Astrocytes are more sensitive to GR stimulation than neurons (Slezak et al., 2013); the lack of glial cells in our primary cultures may have reduced the CORT-induced effect on *Dnmt3a* and *Dnmt3b* in comparison with the upregulation seen in the PFC (Paper I). GR binds to the GRE-containing regions in *Dnmt3a* in the PFC in all rearing conditions (Paper I). These results suggest that genomic signaling of GR regulates *Dnmt* expression. GR signaling causes increased chromatin accessibility in many genomic sites that enables other steroid receptors such as estrogen receptor to bind at the same site (Wiley et al., 2016). Although, we did not study ESR1 binding levels, maternal behavior is known to affect gene-specific gene methylation, including *Esr1* in female rodents (Champagne et al., 2006), thus the causal relationship between *Dnmt3a* induction and *Esr1* regulation in male rats after early life stress remains to be clarified. Alternatively, since GR immunoprecipitation levels remained unaltered in MS180 animals, other transcription factor response elements may have greater influence on *Dnmt3a* mRNA increase after maternal separation. We speculate that genomic signaling of GR may also be involved in the transcriptional regulation of *Dnmt3a* and *Dnmt3b* in the NAc after maternal separation (Anier et al., 2014).

Early life stress-associated changes in epigenetic modifiers in the brain may be region and age specific. *Dnmt1, Dnmt3a* and *Dnmt3b* increases in other brain regions after maternal separation (Anier et al., 2014; Boku et al., 2015; Park et al., 2018). *Dnmt3a* expression is increased by MS (Anier et al., 2014) and by chronic social stress (LaPlant et al., 2010) in the NAc, an key region in reward-associated behavior. *Dnmt1* expression, that is representative of maintenance methylation, is affected after MS in the hippocampal tissue (Park et al., 2018), as well as in neural precursor cells (Boku et al., 2015). In the hippocampus, MS prevents the neural progenitors to differentiate into neurons that was reversed by a DNMT inhibitor (Boku et al., 2015). In the PFC at PND15, maternal separation increased *Dnmt1, Dnmt3a, Dnmt3b* expression (Paper I). Maltreatment affected DNA methylation in the loci of *Dnmt1, Dnmt3a, Mecp2* and *Gadd45b* (a protein required for activity-induced DNA demethylation) in an age-specific manner. Namely, alterations in the expression of these genes did not occur in the second week after birth, but were present in the adult PFC (Blaze and Roth, 2013). Changes in the enzyme activity and global methylation levels in the PFC are also age-dependent. MS does not alter global methylation (Anier et al., 2014; McCoy et al., 2016) nor DNMT activity (Ignácio et al., 2017) in the adult PFC, but DNMT activity was increased at PND15 in the PFC (Paper I).

The activity of the HPA axis is also regulated intracellularly by *Fkbp5*. FKBP5 is a co-chaperone of the GR complex that decreases GR binding affinity to glucocorticoids. *Fkbp5* is affected by the DNA methylation-demethylation machinery in HPA dysregulation. Childhood stress-related demethylation of a GRE in intron 7 of *FKBP5* in the context of GR stimulation results in increased expression of *FKBP5* (Klengel et al., 2013). The authors speculated that lower expression of *FKBP5* leads to reduced negative feedback and hyperactivity of the HPA axis. This particular epigenetic modification only emerges in early life, suggesting that the functioning of certain GREs is restricted to this period in life. The two GREs in rat *Dnmt3b* promoter suggest responsiveness to GR stimulation in early life, although their functionality in adulthood is unclear (Paper I). It would be important to ascertain in future studies which GREs or their modifications in *Dnmt* genes are restricted to early life.

The activity-regulated *Dnmt3a2* transcript has been previously linked with neuronal activity (Oliveira et al., 2012) and the regulation of drug cue memory (Cannella et al., 2018). Cannella *et al* showed that silencing of *Dnmt3a2* in the NAc shell reduced the cue-induced cocaine seeking, thus *Dnmt3a2* promotes cocaine seeking. Dnmt3a2 also affected immediate early genes that participate in memory consolidation. The authors suggest that reduced levels of *Dnmt3a2* mRNA allow for a less permissive genomic state to strengthen drug-associated memories. Maternal separation can influence activity-regulated transcriptional activation and we showed that *Dnmt3a2* transcript is affected by maternal separation in the PFC (Paper I). Future studies can elucidate the role of *Dnmt3a2* transcript in stress-induced adaptations on memory function. Interestingly, it was recently proposed that activity-regulated gene expression in early life can

be mediated in a Dnmt3a-dependent manner and the phenomenon is long-lasting (Stroud et al., 2017). Decreased DNMT3A binding along with lower levels of non-CG methylation in the immediate early genes can be detected 8 weeks after the early activity-dependent experience. However, the causality of non-CpG methylation and gene expression was not assessed in this study, thus the impact on the neuronal function by experience-dependent changes on non-CpG methylation remains to be clarified.

It is clear that early life stress-induced changes in DNA methylation do not occur only in a subset of candidate genes e.g. *GR*. Genome-wide studies in animals mapping alterations in DNA methylation after stress are beginning to emerge. Most recent genome-wide studies show that early life stress impacted methylation patterns in species and sex dependent manner (Burns et al., 2018). McCoy and colleagues found global hypermethylation in the hippocampus of male rats exposed to maternal separation and clusters of hypomethylation at specific genomic loci (McCoy et al., 2016). The biological pathways with altered DNA methylation included cell proliferation, insulin signaling, axonal guidance, tyrosine kinase signaling, synaptogenesis and synaptic transmission. However, the resilient phenotype they observed in MS rats with reduced levels of anxiety- and depressive-like behavior is contrary to what is normally observed in MS exposed rodents. Thus, caution should be taken in interpreting the consequences of specific epigenomic alterations within different genomic backgrounds, a stress reactive rat strain in this case. Interestingly, recent evidence has been provided of differential epigenetic programming of individuals with history of child abuse in neuronal cells and oligodendrocytes (Burns et al., 2018). The functional impact of differentially methylated loci is yet unclear due to variety of methods used and multiple individual factors, such as cell type, genetic background, species, sex and stress-resilient phenotype that interact with early life stress. Therefore, candidate gene approaches still are a crucial supportive component in the assessment of epigenetic contribution to early life stress.

2. The cross-tissue alterations in cocaine-induced DNA methylation modifiers and the role of DNMT3A in repeated cocaine administration and withdrawal

2.1. The behavioral response to cocaine action

The studies with pharmacological manipulations on DNA methylation have shown that the interaction between DNA methylation and the behavioral response to cocaine action is complex and involve region and abstinence specificity, the latter will be discussed in the following chapter. Methyl supplementation, such as methionine and SAM provide the methyl group substrates to DNMT enzymes that could increase DNA methylation (Zeisel, 2009). In the following

studies, behavior was assessed up to 24 h after last cocaine administration. Systemic administration of methyl supplementation increased sensitization to cocaine (Anier et al., 2013). Conversely, intracerebroventricular injections of zebularine, a DNMT inhibitor, delayed cocaine-induced behavioral sensitization (Anier et al., 2010). However, in the NAc, local *Dnmt3a* overexpression decreases cocaine seeking behavior and conversely, DNMT inhibition increases cocaine seeking and behavioral sensitization (LaPlant et al., 2010). Therefore, the decrease or increase in behavioral response to cocaine depends on the region where DNA methylation is altered. Pharmacological manipulations of DNA methylation in the VTA have not been studied in cocaine action, but circuit-specific (NAc vs. VTA) epigenetic alterations seem to differ between a natural reward and cocaine reward in the NAc (Day et al., 2013; Massart et al., 2015). The work from Wright and colleagues showed no effect of methyl supplementation on the acute locomotor response to cocaine, although *Dnmt3a* and *Dnmt3b* mRNA was specifically increased in the NAc, which suggests that the role of DNA methylation in the NAc becomes more important as cocaine dependence develops (Wright et al., 2015).

Dnmt3a is considered to have a prominent role in the NAc during cocaine abuse. LaPlant and colleagues demonstrated a negative regulation of *Dnmt3a* in cocaine seeking behavior as described above (LaPlant et al., 2010). In addition, the authors used Cre-mediated knockdown of *Dnmt3a* in the NAc that also increased cocaine seeking behavior, which is in line with the results obtained with a DNMT inhibitor, while *Dnmt1* knockdown had no effect on cocaine seeking behavior (LaPlant et al., 2010). Silencing *Dnmt3a* in the NAc enhanced the behavioral sensitization in the induction phase and also showed a negative correlation between *Dnmt3a* expression and reward behavior (Paper III). Our results with the behavioral sensitization model add evidence to the hypothesis that increased DNA methylation impairs rewarding signaling in the NAc at the binge/intoxication stage, although, as seen in the aforementioned study, this mechanism may have the side-effect of increasing depressive-like symptoms.

Dnmt1 and *Dnmt3a* mRNA were upregulated both in AC and RC groups in the NAc (Paper II and III), thus their role is not necessarily associated with withdrawal and sensitization. *Dnmt3a* could play a role in behavioral sensitization from the acquisition phase and upon silencing *Dnmt3a* expression before repeated cocaine injections, sensitization increased as discussed above (Paper III). We speculate that *Dnmt3b* may have a unique role in the withdrawal phase, since acute cocaine does not affect its expression (Paper II and III). *Dnmt3b* was found downregulated in the hippocampus of rats withdrawn from cocaine self-administration on Illumina microarray platform (García-Fuster et al., 2011).

2.2. Gene specific alterations in DNA methylation during withdrawal from repeated cocaine

The abovementioned studies (Anier et al., 2010; LaPlant et al., 2010) are limited on two aspects. Firstly, a withdrawal period was not included in the design of these studies to investigate the effect of abstinence on DNA methylation. Secondly, none of these studies used a genome-wide analysis of methylation. Global levels of 5-mC and the methylation status and expression of some genes were assessed. These two limitations were addressed in the study by Massart and colleagues (Massart et al., 2015). The "incubation of craving" method in the self-administration model was used with treatments of RG108 or SAM to measure the genome-wide DNA methylation and the gene expression in the NAc after a short (1 day) or long (30 days) period of withdrawal. Their analysis of functionally related pathways with differential promoter methylation during long withdrawal period highlighted the following pathways: cancer, morphology of cells and ephrin receptor signaling, neurotrophin signaling, GR signaling, synaptic long-term depression. The members involved in these pathways (e.g. *GR*) have been associated previously with motivation to self-administer cocaine (Deroche-Gamonet et al., 2003). Different directions of the methylation status over time were also examined by Massart and colleagues (Massart et al., 2015). Some methylation marks remained stable (e.g. *Creb1*) over prolonged withdrawal. Other DNA methylation alterations were enhanced during withdrawal, becoming more hypermethylated or hypomethylated while abstinence progressed (e.g. *Dnmt1, Dnmt3a, Dnmt3b, GR, Grin1*). Our study on *Grin1* 3' UTR methylation mark indicated an increase in hypermethylation after a long period of withdrawal (unpublished results, Fig. 20B, AC vs RC), although the methylation site may be species specific for mouse as no alterations downstream of the transcription start site were seen in the rat model (Massart et al., 2015). Interestingly, cue-induced cocaine seeking generally reversed alterations in methylation triggered by abstinence (Massart et al., 2015), some examples of such genes are involved in glutamate signaling (*Gad1, Grm3*). We suggest that *Grin1* may also be involved in cue-induced processes after a withdrawal period in mice.

Massart et al reasoned that RG108 blocks the "incubated" or heightened cocaine craving by demethylating target genes. *Esr1* was hypothesized to be one of critical genes that is affected by DNA methylation changes and could alter the incubation of craving. In fact, RG108 injections to the NAc resulted in increased *Esr1* expression and decreased methylation at its promoter after 30 day withdrawal from repeated cocaine in comparison with the opposite effects of SAM injections (Massart et al., 2015). A previous study shows that *Esr1* knock-out mice display enhanced cocaine sensitization and a trend toward increased cocaine seeking in the place preference paradigm (Lasek et al., 2011). In addition, estrogen contributes to cocaine-induced behaviors both in females and males (Lasek et al., 2011). Thus, conversely, an ESR1 agonist caused a dramatic decrease in cue-induced cocaine seeking behavior after a withdrawal period (Massart et al., 2015). The data presented in our publications confirm the

methylation and expression changes of *Esr1* induced by withdrawal from repeated cocaine and a challenge dose in comparison with a saline group (Paper II). Interestingly, the alteration in methylation and hydroxymethylation at the *Esr1* promoter occurs in the cue-induced RS group that did not receive cocaine without changes in expression (Paper II). Transcriptionally silent DNA modifications in promoters have been described also in humans in a stress-related context that will be further discussed below (Labonte et al., 2012).

2.3. Cocaine-induced changes in DNA methylation across tissues

The relevance of DNA methylation changes in peripheral tissues for neuropsychiatric diseases has recently gained much attention, because DNA methylation patterns are cell-type specific. However, an increasing number of studies show a differential methylation that correlates across brain and peripheral tissues within subjects (Lax and Szyf, 2018). In the field of addiction, the human studies performed so far have been on alcohol abusers (Lax and Szyf, 2018). In cocaine abuse, our study (Paper II) presents evidence on the dynamic alterations in *Dnmt* genes across three tissues in mice. Acute cocaine treatment decreased *Dnmt1, Dnmt3a* mRNA levels in the NAc and PBC, whereas 24 h later, *Dnmt3a* mRNA and DNMT enzyme activity levels were upregulated in both tissues (Paper II). Dopamine transporter (DAT) is expressed in various peripheral immune cells and cocaine blocks the DAT-dependent uptake of dopamine in lymphocytes, suggesting that the DAT present in lymphocytes functions similarly as the DAT expressed in the CNS (Mackie et al., 2018). Cocaine as well as procaine has a local anesthetic effect. Functional voltage-gated sodium channels are expressed in various immune cells and erythrocytes (Black and Waxman, 2013). Acute procaine injections resulted in an opposite effect on *Dnmt3a* expression in PBC (Paper II). The contrasting outcomes of cocaine and procaine on *Dnmt3a* mRNA may be caused by different pharmacokinetic properties; procaine (half-life 7.7 min in human plasma) has a shorter duration of action in comparison with cocaine (half life 1 h), suggesting that voltage-gated sodium channels may be involved in the effect of cocaine on *Dnmt* expression in PBC.

On gene specific level, the decrease in the expression of *Esr1* correlated across mouse NAc and PBC after an abstinence period and a cocaine challenge (Paper II). Furthermore, hydroxymethylation levels were induced at the *Esr1* promoter that correlated across tissues in mice exposed to cocaine associated cues (RS, Paper II). Cross-tissue studies on a large cohort of alcohol abusers have found few differentially methylated genes that have been replicated (Lax and Szyf, 2018). However, there is mainly a need for human studies on drug abusers that assess the treatment outcome of DNA methylation inhibitors and the effect on drug relapse. Additionally, it may be beneficial to screen for differentially methylated loci as biomarkers in recreational drug users that would aid in identifying the individuals at high risk for developing drug abuse disorder.

In our study we chose Cer as a reference tissue to study epigenetic changes in the mesolimbic dopaminergic pathway (NAc). However, withdrawal from repeated cocaine also increased in *Dnmt1, Dnmt3a, Dnmt3b* mRNA in the Cer (Paper II). Recently it was shown that different subsets of granule cells respond to reward delivery, anticipation and omission (Wagner et al., 2017). Thus, the question arises of how is reward related information contributing to the cerebellar function. Classical models postulate that granule cells are activated with sensorimotor signals. The fact that granule cells are able to integrate sensorimotor information as well as reward-omission and reward-anticipation signals indicates cerebellar involvement in cognitive processing. Although the input pathways that activate these populations of neurons in the reward context are currently unknown, we speculate that DNA methylation may participate in the cocaine-induced changes in the Cer. We noted that *Dntm3b* expression may be associated with withdrawal in the Cer (Paper II), thus *Dnmt3b* would be an interesting candidate to study in the withdrawal phase from repeated cocaine in the Cer.

DNA methylation is a source of new type of biomarkers. Levels of DNA methylation at specific CpG locations and inter-individual variation in DNA methylation are known to vary across tissues, especially due to the cellular heterogeneity (Bakulski et al., 2016). Furthermore, epigenetic modifications vary also between different cell types within the same tissue (Mo et al., 2015b). However, the expression of *Dnmt* as well as their enzyme activity was more pronounced in NAc than in other tissues outside the mesolimbic dopaminergic system (Paper II). We also noted an increase in DNMT activity in the NAc of RC mice in comparison with AC group 24 hours after cocaine challenge, suggesting an association with withdrawal (Paper II, Fig. 7; Paper III, Fig. 5). Although the cell type specific alterations in DNA methylation in the Cer and in PBC needs further study, DNMT activity seems a promising candidate to identify cocaine exposure across tissues after the drug has been metabolized. The disease-related changes in other epigenetic protein levels (e.g. histone deacetylases), as well as enzyme activity are currently under study in neurodegenerative disorders (Sartor, 2019).

SUMMARY AND CONCLUSIONS

The biochemical evidence presented in this dissertation shows that a stress-related neuroadaptation occurs during maternal separation inducing DNA methylation in the PFC. Our data suggests that genomic signaling of GR contributes to the regulation of DNA methylation-mediated neural (mal)adaptations during early life stress. DNA methylation-induced maladaptations (may sensitize the stress responsivity that) can underlie the predisposition to cocaine abuse.

In the expression and abstinence of cocaine abuse, chronic cocaine increased DNMT activity in the NAc. *Dnmt1* and *Dnmt3a* contribute to repeated cocaine-induced cellular adaptations across tissues. *Dnmt3a* silencing increased behavioral sensitization in the induction phase, indicating that DNMT3A-mediated methylation inhibits reward behavior from the acquisition of behavioral sensitization.

The results suggest the following conclusions:

1. Our study showed that MS leads to the stimulation of the glucocorticoid receptor genomic pathway and thus could be involved in the regulation of DNA methylation in the brain. MS increased *Dnmt1, Dnmt3a, Dnmt3b* expression and DNMT activity suggesting that early life stress can affect DNA methylation in the PFC and the increased DNMT activity in the PFC may be age-specific. Results with GR-antagonist on primary neurons and increased GR binding at *Dnmt3b* promoter suggest that *Dnmt3b* is regulated by genomic signaling of GR after early life stress, although the specific regulatory elements remain to be determined. Genomic signaling of GR also may regulate *Dnmt3a* in the PFC and affect the activity-regulated transcriptional activation of *Dnmt3a*. The results of this study suggest that early life stress-induced changes in DNMT enzyme activity may lead to persistent changes in gene expression that predisposes to cocaine abuse.

2. Acute cocaine increases *Dnmt1* and *Dnmt3a* expression across tissues that are specific for psychostimulant abuse and non-specific tissues. The effect of acute cocaine and acute procaine on *Dnmt3a* expression suggests that *Dnmt3a* regulation involves voltage-gated sodium channels in PBC. Repeated cocaine administration increased DNMT activity in behavioral sensitization expression phase and in abstinence, therefore DNMT activity is involved in both the expression phase and abstinence. Both acute and chronic cocaine induce DNMT enzyme activity in NAc and PBC that support the idea of a biomarker that correlates across tissues. Acute and chronic cocaine have opposing effects on the methylation of both *Esr1* and *Grin1* in the NAc. Our study showed that some cocaine-induced changes on *Dnmt3a, Dnmt3b, Dnmt1* in the NAc occur also in leucocytes.

3. Our study suggests a role for *Dnmt3a* in the acquisition phase of cocaine-induced sensitization. Silencing of *Dnmt3a* in the NAc reduced DNMT enzyme activity and repeated cocaine administration increased behavioral sensitization in comparison with control group mice, therefore *Dnmt3a* has an important role in the acquisition phase of behavioral sensitization. However, *Dnmt3a* silencing in NAc did not affect behavioral sensitization in the abstinence phase. *Dnmt3b* induction during *Dnmt3a* silencing indicated that *Dnmt3b* may compensate the reduced *Dnmt3a* expression.

REFERENCES

Administration, S.A. and M.H.S., 2016. Substance Use Disorders. Substance Abuse and Mental Health Services Administration (US).

Albertson, D.N., Schmidt, C.J., Kapatos, G., Bannon, M.J., 2006a. Distinctive Profiles of Gene Expression in the Human Nucleus Accumbens Associated with Cocaine and Heroin Abuse. Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol. 31, 2304–2312. https://doi.org/10.1038/sj.npp.1301089

Albertson, D.N., Schmidt, C.J., Kapatos, G., Bannon, M.J., 2006b. Distinctive Profiles of Gene Expression in the Human Nucleus Accumbens Associated with Cocaine and Heroin Abuse. Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol. 31, 2304–2312. https://doi.org/10.1038/sj.npp.1301089

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. https://doi.org/10.1038/npp.2010.128

Anier, K., Malinovskaja, K., Pruus, K., Aonurm-Helm, A., Zharkovsky, A., Kalda, A., 2014. Maternal separation is associated with DNA methylation and behavioural changes in adult rats. Eur. Neuropsychopharmacol. 24, 459–468. https://doi.org/10.1016/j.euroneuro.2013.07.012

Anier, K., Zharkovsky, A., Kalda, A., 2013. S-adenosylmethionine modifies cocaine-induced DNA methylation and increases locomotor sensitization in mice. Int. J. Neuropsychopharmacol. 16, 2053–2066. https://doi.org/10.1017/S1461145713000394

Bagot, R.C., Labonté, B., Peña, C.J., Nestler, E.J., 2014. Epigenetic signaling in psychiatric disorders: stress and depression. Dialogues Clin. Neurosci. 16, 281–295.

Bakulski, K.M., Halladay, A., Hu, V.W., Mill, J., Fallin, M.D., 2016. Epigenetic Research in Neuropsychiatric Disorders: the "Tissue Issue." Curr. Behav. Neurosci. Rep. 3, 264–274. https://doi.org/10.1007/s40473-016-0083-4

Barau, J., Teissandier, A., Zamudio, N., Roy, S., Nalesso, V., Hérault, Y., Guillou, F., Bourc'his, D., 2016. The DNA methyltransferase DNMT3C protects male germ cells from transposon activity. Science 354, 909–912. https://doi.org/10.1126/science.aah5143

Barik, J., Parnaudeau, S., Saint Amaux, A.L., Guiard, B.P., Golib Dzib, J.F., Bocquet, O., Bailly, A., Benecke, A., Tronche, F., 2010. Glucocorticoid Receptors in Dopaminoceptive Neurons, Key for Cocaine, Are Dispensable for Molecular and Behavioral Morphine Responses. Biol. Psychiatry, Mechanisms of Cocaine Addiction 68, 231–239. https://doi.org/10.1016/j.biopsych.2010.03.037

Barrot, M., Olivier, J.D.A., Perrotti, L.I., DiLeone, R.J., Berton, O., Eisch, A.J., Impey, S., Storm, D.R., Neve, R.L., Yin, J.C., Zachariou, V., Nestler, E.J., 2002. CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. Proc. Natl. Acad. Sci. 99, 11435–11440. https://doi.org/10.1073/pnas.172091899

Baudin, A., Blot, K., Verney, C., Estevez, L., Santamaria, J., Gressens, P., Giros, B., Otani, S., Daugé, V., Naudon, L., 2012. Maternal deprivation induces deficits in temporal memory and cognitive flexibility and exaggerates synaptic plasticity in the rat medial prefrontal cortex. Neurobiol. Learn. Mem. 98, 207–214. https://doi.org/10.1016/j.nlm.2012.08.004

Bayraktar, G., Kreutz, M.R., 2018. Neuronal DNA Methyltransferases: Epigenetic Mediators between Synaptic Activity and Gene Expression? The Neuroscientist 24, 171–185. https://doi.org/10.1177/1073858417707457

Belin-Rauscent, A., Fouyssac, M., Bonci, A., Belin, D., 2016. How Preclinical Models Evolved to Resemble the Diagnostic Criteria of Drug Addiction. Biol. Psychiatry, Animal Models of Psychiatric Disease 79, 39–46. https://doi.org/10.1016/j.biopsych.2015.01.004

Berlin, G.S., Hollander, E., 2014. Compulsivity, impulsivity, and the DSM-5 process. CNS Spectr. 19, 62–68. https://doi.org/10.1017/S1092852913000722

Berlucchi, G., Buchtel, H.A., 2009. Neuronal plasticity: historical roots and evolution of meaning. Exp. Brain Res. 192, 307–319. https://doi.org/10.1007/s00221-008-1611-6

Berridge, K.C., 2007. The debate over dopamine's role in reward: the case for incentive salience. Psychopharmacology (Berl.) 191, 391–431. https://doi.org/10.1007/s00213-006-0578-x

Bird, A., 2002. DNA methylation patterns and epigenetic memory. Genes Dev. 16, 6–21. https://doi.org/10.1101/gad.947102

Black, J.A., Waxman, S.G., 2013. Noncanonical Roles of Voltage-Gated Sodium Channels. Neuron 80, 280–291. https://doi.org/10.1016/j.neuron.2013.09.012

Blaze, J., Roth, T.L., 2013. Exposure to caregiver maltreatment alters expression levels of epigenetic regulators in the medial prefrontal cortex. Int. J. Dev. Neurosci. 31, 804–810. https://doi.org/10.1016/j.ijdevneu.2013.10.001

Boku, S., Toda, H., Nakagawa, S., Kato, A., Inoue, T., Koyama, T., Hiroi, N., Kusumi, I., 2015. Neonatal Maternal Separation Alters the Capacity of Adult Neural Precursor Cells to Differentiate into Neurons Via Methylation of Retinoic Acid Receptor Gene Promoter. Biol. Psychiatry, Early Life Stress, Epigenetics, and Resilence 77, 335–344. https://doi.org/10.1016/j.biopsych.2014.07.008

Bostick, M., Kim, J.K., Estève, P.-O., Clark, A., Pradhan, S., Jacobsen, S.E., 2007. UHRF1 Plays a Role in Maintaining DNA Methylation in Mammalian Cells. Science 317, 1760–1764. https://doi.org/10.1126/science.1147939

Bourc'his, D., Bestor, T.H., 2004. Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. Nature 431, 96–99. https://doi.org/10.1038/nature02886

Brueckner, B., Boy, R.G., Siedlecki, P., Musch, T., Kliem, H.C., Zielenkiewicz, P., Suhai, S., Wiessler, M., Lyko, F., 2005. Epigenetic Reactivation of Tumor Suppressor Genes by a Novel Small-Molecule Inhibitor of Human DNA Methyltransferases. Cancer Res. 65, 6305–6311. https://doi.org/10.1158/0008-5472.CAN-04-2957

Bruijnzeel, A.W., Repetto, M., Gold, M.S., 2004. Neurobiological mechanisms in addictive and psychiatric disorders. Psychiatr. Clin. North Am., Addictive Disorders 27, 661–674. https://doi.org/10.1016/j.psc.2004.06.005

Bühler, K.-M., Giné, E., Echeverry-Alzate, V., Calleja-Conde, J., de Fonseca, F.R., López-Moreno, J.A., 2015. Common single nucleotide variants underlying drug addiction: more than a decade of research. Addict. Biol. 20, 845–871. https://doi.org/10.1111/adb.12204

Burns, S.B., Szyszkowicz, J.K., Luheshi, G.N., Lutz, P.-E., Turecki, G., 2018. Plasticity of the epigenome during early-life stress. Semin. Cell Dev. Biol., Arc/ARg3.1 77, 115–132. https://doi.org/10.1016/j.semcdb.2017.09.033

Bush, G., Luu, P., Posner, M.I., 2000. Cognitive and emotional influences in anterior cingulate cortex. Trends Cogn. Sci. 4, 215–222. https://doi.org/10.1016/S1364-6613(00)01483-2

Butts, K.A., Weinberg, J., Young, A.H., Phillips, A.G., 2011. Glucocorticoid receptors in the prefrontal cortex regulate stress-evoked dopamine efflux and aspects of executive function. Proc. Natl. Acad. Sci. U. S. A. 108, 18459–18464. https://doi.org/10.1073/pnas.1111746108

Caine, S.B., Negus, S.S., Mello, N.K., Patel, S., Bristow, L., Kulagowski, J., Vallone, D., Saiardi, A., Borrelli, E., 2002. Role of Dopamine D2-like Receptors in Cocaine Self-Administration: Studies with D2 Receptor Mutant Mice and Novel D2 Receptor Antagonists. J. Neurosci. 22, 2977–2988. https://doi.org/10.1523/JNEUROSCI.22-07-02977.2002

Caine, S.B., Thomsen, M., Gabriel, K.I., Berkowitz, J.S., Gold, L.H., Koob, G.F., Tonegawa, S., Zhang, J., Xu, M., 2007. Lack of Self-Administration of Cocaine in Dopamine D1 Receptor Knock-Out Mice. J. Neurosci. 27, 13140–13150. https://doi.org/10.1523/JNEUROSCI.2284-07.2007

Campioni, M.R., Xu, M., McGehee, D.S., 2009. Stress-Induced Changes in Nucleus Accumbens Glutamate Synaptic Plasticity. J. Neurophysiol. 101, 3192–3198. https://doi.org/10.1152/jn.91111.2008

Cannella, N., Oliveira, A.M.M., Hemstedt, T., Lissek, T., Buechler, E., Bading, H., Spanagel, R., 2018. Dnmt3a2 in the Nucleus Accumbens Shell Is Required for Reinstatement of Cocaine Seeking. J. Neurosci. 38, 7516–7528. https://doi.org/10.1523/JNEUROSCI.0600-18.2018

Cardoso-Moreira, M., Halbert, J., Valloton, D., Velten, B., Chen, C., Shao, Y., Liechti, A., Ascenção, K., Rummel, C., Ovchinnikova, S., Mazin, P.V., Xenarios, I., Harshman, K., Mort, M., Cooper, D.N., Sandi, C., Soares, M.J., Ferreira, P.G., Afonso, S., Carneiro, M., Turner, J.M.A., VandeBerg, J.L., Fallahshahroudi, A., Jensen, P., Behr, R., Lisgo, S., Lindsay, S., Khaitovich, P., Huber, W., Baker, J., Anders, S., Zhang, Y.E., Kaessmann, H., 2019. Gene expression across mammalian organ development. Nature 1. https://doi.org/10.1038/s41586-019-1338-5

Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M., Werner, T., 2005. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 21, 2933–2942. https://doi.org/10.1093/bioinformatics/bti473

Cervoni, N., Szyf, M., 2001. Demethylase Activity Is Directed by Histone Acetylation. J. Biol. Chem. 276, 40778–40787. https://doi.org/10.1074/jbc.M103921200

Champagne, F.A., Weaver, I.C.G., Diorio, J., Dymov, S., Szyf, M., Meaney, M.J., 2006. Maternal Care Associated with Methylation of the Estrogen Receptor-α1b Promoter and Estrogen Receptor-α Expression in the Medial Preoptic Area of Female Offspring. Endocrinology 147, 2909–2915. https://doi.org/10.1210/en.2005-1119

Chen, Chang, Q., Lin, Y., Meissner, A., West, A.E., Griffith, E.C., Jaenisch, R., Greenberg, M.E., 2003. Derepression of BDNF Transcription Involves Calcium-Dependent Phosphorylation of MeCP2. Science 302, 885–889. https://doi.org/10.1126/science.1086446

Chen, J.-F., Moratalla, R., Yu, L., Martín, A.B., Xu, K., Bastia, E., Hackett, E., Alberti, I., Schwarzschild, M.A., 2003. Inactivation of Adenosine A_{2A} Receptors Selectively Attenuates Amphetamine-Induced Behavioral Sensitization. Neuropsychopharmacology 28, 1086–1095. https://doi.org/10.1038/sj.npp.1300152

Chen, Z., Li, S., Subramaniam, S., Shyy, J.Y.-J., Chien, S., 2017a. Epigenetic Regulation: A New Frontier for Biomedical Engineers. Annu. Rev. Biomed. Eng. 19, 195–219. https://doi.org/10.1146/annurev-bioeng-071516-044720

Chen, Z., Li, S., Subramaniam, S., Shyy, J.Y.-J., Chien, S., 2017b. Epigenetic Regulation: A New Frontier for Biomedical Engineers. Annu. Rev. Biomed. Eng. 19, 195–219. https://doi.org/10.1146/annurev-bioeng-071516-044720

Chocyk, A., Bobula, B., Dudys, D., Przyborowska, A., Majcher-Maślanka, I., Hess, G., Wędzony, K., 2013. Early-life stress affects the structural and functional plasticity of the medial prefrontal cortex in adolescent rats. Eur. J. Neurosci. 38, 2089–2107. https://doi.org/10.1111/ejn.12208

Christoffel, D.J., Golden, S.A., Dumitriu, D., Robison, A.J., Janssen, W.G., Ahn, H.F., Krishnan, V., Reyes, C.M., Han, M.-H., Ables, J.L., Eisch, A.J., Dietz, D.M., Ferguson, D., Neve, R.L., Greengard, P., Kim, Y., Morrison, J.H., Russo, S.J., 2011. IκB Kinase Regulates Social Defeat Stress-Induced Synaptic and Behavioral Plasticity. J. Neurosci. 31, 314–321. https://doi.org/10.1523/JNEUROSCI.4763-10.2011

Colby, C.R., Whisler, K., Steffen, C., Nestler, E.J., Self, D.W., 2003. Striatal Cell Type-Specific Overexpression of ΔFosB Enhances Incentive for Cocaine. J. Neurosci. 23, 2488–2493. https://doi.org/10.1523/JNEUROSCI.23-06-02488.2003

Cook, S.C., Wellman, C.L., 2004. Chronic stress alters dendritic morphology in rat medial prefrontal cortex. J. Neurobiol. 60, 236–248. https://doi.org/10.1002/neu.20025

Covington, H.E., Maze, I., Sun, H., Bomze, H.M., DeMaio, K.D., Wu, E.Y., Dietz, D.M., Lobo, M.K., Ghose, S., Mouzon, E., Neve, R.L., Tamminga, C.A., Nestler, E.J., 2011. A Role for Repressive Histone Methylation in Cocaine-Induced Vulnerability to Stress. Neuron 71, 656–670. https://doi.org/10.1016/j.neuron.2011.06.007

Cremer, T., Cremer, M., Dietzel, S., Müller, S., Solovei, I., Fakan, S., 2006. Chromosome territories – a functional nuclear landscape. Curr. Opin. Cell Biol., Nucleus and gene expression 18, 307–316. https://doi.org/10.1016/j.ceb.2006.04.007

Crick, F., 1970. Central Dogma of Molecular Biology. Nature 227, 561–563. https://doi.org/10.1038/227561a0

Daniels, W.M.U., Fairbairn, L.R., van Tilburg, G., McEvoy, C.R.E., Zigmond, M.J., Russell, V.A., Stein, D.J., 2009. Maternal separation alters nerve growth factor and corticosterone levels but not the DNA methylation status of the exon 1(7) glucocorticoid receptor promoter region. Metab. Brain Dis. 24, 615–627. https://doi.org/10.1007/s11011-009-9163-4

Day, J.J., Childs, D., Guzman-Karlsson, M.C., Kibe, M., Moulden, J., Song, E., Tahir, A., Sweatt, J.D., 2013. DNA methylation regulates associative reward learning. Nat. Neurosci. 16, 1445–1452. https://doi.org/10.1038/nn.3504

Deroche-Gamonet, V., Sillaber, I., Aouizerate, B., Izawa, R., Jaber, M., Ghozland, S., Kellendonk, C., Moal, M.L., Spanagel, R., Schütz, G., Tronche, F., Piazza, P.V., 2003. The Glucocorticoid Receptor as a Potential Target to Reduce Cocaine Abuse. J. Neurosci. 23, 4785–4790. https://doi.org/10.1523/JNEUROSCI.23-11-04785.2003

Diesch, J., Zwick, A., Garz, A.-K., Palau, A., Buschbeck, M., Götze, K.S., 2016. A clinical-molecular update on azanucleoside-based therapy for the treatment of hematologic cancers. Clin. Epigenetics 8. https://doi.org/10.1186/s13148-016-0237-y

DiNieri, J.A., Nemeth, C., Parsegian, A., Carle, T., Gurevich, V.V., Gurevich, E., Neve, R.L., Nestler, E.J., Carlezon, W.A., 2009. Altered sensitivity to rewarding and aversive drugs in mice with inducible disruption of CREB function within the

nucleus accumbens. J. Neurosci. Off. J. Soc. Neurosci. 29, 1855–1859. https://doi.org/10.1523/JNEUROSCI.5104-08.2009
Donahoe, J., Palmer, D., Burgos, J., 1997. The Unit Of Selection: What Do Reinforcers Reinforce? J. Exp. Anal. Behav. 67, 259–273. https://doi.org/10.1901/jeab.1997.67-259
Dong, Y., Green, T., Saal, D., Marie, H., Neve, R., Nestler, E.J., Malenka, R.C., 2006. CREB modulates excitability of nucleus accumbens neurons. Nat. Neurosci. 9, 475–477. https://doi.org/10.1038/nn1661
Edwards, J.R., Yarychkivska, O., Boulard, M., Bestor, T.H., 2017. DNA methylation and DNA methyltransferases. Epigenetics Chromatin 10, 23. https://doi.org/10.1186/s13072-017-0130-8
Edwards, S., Koob, G.F., 2010. Neurobiology of dysregulated motivational systems in drug addiction. Future Neurol. 5, 393–401. https://doi.org/10.2217/fnl.10.14
Egervari, G., 2016. AMPA Receptor Plasticity in the Nucleus Accumbens Mediates Withdrawal-Related Negative-Affective States. J. Neurosci. 36, 10505–10507. https://doi.org/10.1523/JNEUROSCI.2329-16.2016
Elliott, E., Ezra-Nevo, G., Regev, L., Neufeld-Cohen, A., Chen, A., 2010. Resilience to social stress coincides with functional DNA methylation of the Crf gene in adult mice. Nat. Neurosci. 13, 1351–1353. https://doi.org/10.1038/nn.2642
Elliott, E., Manashirov, S., Zwang, R., Gil, S., Tsoory, M., Shemesh, Y., Chen, A., 2016. Dnmt3a in the Medial Prefrontal Cortex Regulates Anxiety-Like Behavior in Adult Mice. J. Neurosci. 36, 730–740. https://doi.org/10.1523/JNEUROSCI.0971-15.2016
Elliott, E.N., Sheaffer, K.L., Kaestner, K.H., 2016. The 'de novo' DNA methyltransferase Dnmt3b compensates the Dnmt1-deficient intestinal epithelium. eLife 5, e12975. https://doi.org/10.7554/eLife.12975
Enoch, M.-A., 2011. The role of early life stress as a predictor for alcohol and drug dependence. Psychopharmacology (Berl.) 214, 17–31. https://doi.org/10.1007/s00213-010-1916-6
Fan, G., Beard, C., Chen, R.Z., Csankovszki, G., Sun, Y., Siniaia, M., Biniszkiewicz, D., Bates, B., Lee, P.P., Kühn, R., Trumpp, A., Poon, C.-S., Wilson, C.B., Jaenisch, R., 2001. DNA Hypomethylation Perturbs the Function and Survival of CNS Neurons in Postnatal Animals. J. Neurosci. 21, 788–797. https://doi.org/10.1523/JNEUROSCI.21-03-00788.2001
Feng, J., Shao, N., Szulwach, K.E., Vialou, V., Huynh, J., Zhong, C., Le, T., Ferguson, D., Cahill, M.E., Li, Y., Koo, J.W., Ribeiro, E., Labonte, B., Laitman, B.M., Estey, D., Stockman, V., Kennedy, P., Couroussé, T., Mensah, I., Turecki, G., Faull, K.F., Ming, G., Song, H., Fan, G., Casaccia, P., Shen, L., Jin, P., Nestler, E.J., 2015. Role of Tet1 and 5-hydroxymethylcytosine in cocaine action. Nat. Neurosci. 18, 536–544. https://doi.org/10.1038/nn.3976
Feng, J., Zhou, Y., Campbell, S.L., Le, T., Li, E., Sweatt, J.D., Silva, A.J., Fan, G., 2010. Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. Nat. Neurosci. 13, 423–430. https://doi.org/10.1038/nn.2514
Fosnocht, A.Q., Briand, L.A., 2016. Substance use modulates stress reactivity: Behavioral and physiological outcomes. Physiol. Behav., The effects of somatic disease on the stress response 166, 32–42. https://doi.org/10.1016/j.physbeh.2016.02.024

Franklin, K., Paxinos, G., 2001. Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates, Compact – 5th Edition, 2nd Edition. ed. Academic Press, San Diego, CA, US.

Franklin, K.B.J., McCoy, S.N., 1979. Pimozide-induced extinction in rats: Stimulus control of responding rules out motor deficit. Pharmacol. Biochem. Behav. 11, 71–75. https://doi.org/10.1016/0091-3057(79)90299-5

Freeman, W.M., Nader, M.A., Nader, S.H., Robertson, D.J., Gioia, L., Mitchell, S.M., Daunais, J.B., Porrino, L.J., Friedman, D.P., Vrana, K.E., 2001. Chronic cocaine-mediated changes in non-human primate nucleus accumbens gene expression. J. Neurochem. 77, 542–549. https://doi.org/10.1046/j.1471-4159.2001.00252.x

Galupa, R., Heard, E., 2018. X-Chromosome Inactivation: A Crossroads Between Chromosome Architecture and Gene Regulation. Annu. Rev. Genet. 52, 535–566. https://doi.org/10.1146/annurev-genet-120116-024611

García-Fuster, M.J., Flagel, S.B., Mahmood, S.T., Mayo, L.M., Thompson, R.C., Watson, S.J., Akil, H., 2011. Decreased Proliferation of Adult Hippocampal Stem Cells During Cocaine Withdrawal: Possible Role of the Cell Fate Regulator FADD. Neuropsychopharmacology 36, 2303–2317. https://doi.org/10.1038/npp.2011.119

Glover, V., 1997. Maternal stress or anxiety in pregnancy and emotional development of the child. Br. J. Psychiatry 171, 105–106. https://doi.org/10.1192/bjp.171.2.105

Goldberg, A.D., Allis, C.D., Bernstein, E., 2007. Epigenetics: A Landscape Takes Shape. Cell 128, 635–638. https://doi.org/10.1016/j.cell.2007.02.006

Goll, M.G., Kirpekar, F., Maggert, K.A., Yoder, J.A., Hsieh, C.-L., Zhang, X., Golic, K.G., Jacobsen, S.E., Bestor, T.H., 2006. Methylation of tRNAAsp by the DNA Methyltransferase Homolog Dnmt2. Science 311, 395–398. https://doi.org/10.1126/science.1120976

Gontier, G., Iyer, M., Shea, J.M., Bieri, G., Wheatley, E.G., Ramalho-Santos, M., Villeda, S.A., 2018. Tet2 Rescues Age-Related Regenerative Decline and Enhances Cognitive Function in the Adult Mouse Brain. Cell Rep. 22, 1974–1981. https://doi.org/10.1016/j.celrep.2018.02.001

Greenberg, M.V.C., Bourc'his, D., 2019. The diverse roles of DNA methylation in mammalian development and disease. Nat. Rev. Mol. Cell Biol. 20, 590–607. https://doi.org/10.1038/s41580-019-0159-6

Grucza, R.A., Wang, J.C., Stitzel, J.A., Hinrichs, A.L., Saccone, S.F., Saccone, N.L., Bucholz, K.K., Cloninger, C.R., Neuman, R.J., Budde, J.P., Fox, L., Bertelsen, S., Kramer, J., Hesselbrock, V., Tischfield, J., Nurnberger, John.I., Almasy, L., Porjesz, B., Kuperman, S., Schuckit, M.A., Edenberg, H.J., Rice, J.P., Goate, A.M., Bierut, L.J., 2008. A Risk Allele for Nicotine Dependence in CHRNA5 Is a Protective Allele for Cocaine Dependence. Biol. Psychiatry 64, 922–929. https://doi.org/10.1016/j.biopsych.2008.04.018

Gu, T.-P., Guo, F., Yang, H., Wu, H.-P., Xu, G.-F., Liu, W., Xie, Z.-G., Shi, L., He, X., Jin, S., Iqbal, K., Shi, Y.G., Deng, Z., Szabó, P.E., Pfeifer, G.P., Li, J., Xu, G.-L., 2011. The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. Nature 477, 606–610. https://doi.org/10.1038/nature10443

Guo, Ma, D.K., Mo, H., Ball, M.P., Jang, M.-H., Bonaguidi, M.A., Balazer, J.A., Eaves, H.L., Xie, B., Ford, E., Zhang, K., Ming, G., Gao, Y., Song, H., 2011a. Neuronal activity modifies the DNA methylation landscape in the adult brain. Nat. Neurosci. 14, 1345–1351. https://doi.org/10.1038/nn.2900

Guo, Su, Y., Zhong, C., Ming, G., Song, H., 2011b. Hydroxylation of 5-Methylcytosine by TET1 Promotes Active DNA Demethylation in the Adult Brain. Cell 145, 423–434. https://doi.org/10.1016/j.cell.2011.03.022

Halder, R., Hennion, M., Vidal, R.O., Shomroni, O., Rahman, R.-U., Rajput, A., Centeno, T.P., Bebber, F. van, Capece, V., Vizcaino, J.C.G., Schuetz, A.-L., Burkhardt, S., Benito, E., Sala, M.N., Javan, S.B., Haass, C., Schmid, B., Fischer, A., Bonn, S., 2016. DNA methylation changes in plasticity genes accompany the formation and maintenance of memory. Nat. Neurosci. 19, 102–110. https://doi.org/10.1038/nn.4194

Han, J., Li, Y., Wang, D., Wei, C., Yang, X., Sui, N., 2010. Effect of 5-aza-2-deoxycytidine microinjecting into hippocampus and prelimbic cortex on acquisition and retrieval of cocaine-induced place preference in C57BL/6 mice. Eur. J. Pharmacol. 642, 93–98. https://doi.org/10.1016/j.ejphar.2010.05.050

Hasin, D.S., O'Brien, C.P., Auriacombe, M., Borges, G., Bucholz, K., Budney, A., Compton, W.M., Crowley, T., Ling, W., Petry, N.M., Schuckit, M., Grant, B.F., 2013. DSM-5 Criteria for Substance Use Disorders: Recommendations and Rationale. Am. J. Psychiatry 170, 834–851. https://doi.org/10.1176/appi.ajp.2013.12060782

Herman, J.P., Cullinan, W.E., 1997. Neurocircuitry of stress: central control of the hypothalamo–pituitary–adrenocortical axis. Trends Neurosci. 20, 78–84. https://doi.org/10.1016/S0166-2236(96)10069-2

Herman, J.P., Ostrander, M.M., Mueller, N.K., Figueiredo, H., 2005. Limbic system mechanisms of stress regulation: Hypothalamo-pituitary-adrenocortical axis. Prog. Neuropsychopharmacol. Biol. Psychiatry, Experimental Stress: from Basic to Clinical Aspects 29, 1201–1213. https://doi.org/10.1016/j.pnpbp.2005.08.006

Hervouet, E., Peixoto, P., Delage-Mourroux, R., Boyer-Guittaut, M., Cartron, P.-F., 2018. Specific or not specific recruitment of DNMTs for DNA methylation, an epigenetic dilemma. Clin. Epigenetics 10, 17. https://doi.org/10.1186/s13148-018-0450-y

Hutcheson, D.M., Everitt, B.J., Robbins, T.W., Dickinson, A., 2001. The role of withdrawal in heroin addiction: enhances reward or promotes avoidance? Nat. Neurosci. 4, 943. https://doi.org/10.1038/nn0901-943

Hyman, S.E., Malenka, R.C., Nestler, E.J., 2006. NEURAL MECHANISMS OF ADDICTION: The Role of Reward-Related Learning and Memory. Annu. Rev. Neurosci. 29, 565–598. https://doi.org/10.1146/annurev.neuro.29.051605.113009

Ignácio, Z.M., Réus, G.Z., Abelaira, H.M., Maciel, A.L., de Moura, A.B., Matos, D., Demo, J.P., da Silva, J.B.I., Gava, F.F., Valvassori, S.S., Carvalho, A.F., Quevedo, J., 2017. Quetiapine treatment reverses depressive-like behavior and reduces DNA methyltransferase activity induced by maternal deprivation. Behav. Brain Res. 320, 225–232. https://doi.org/10.1016/j.bbr.2016.11.044

Im, H.-I., Hollander, J.A., Bali, P., Kenny, P.J., 2010. MeCP2 controls BDNF expression and cocaine intake through homeostatic interactions with microRNA-212. Nat. Neurosci. 13, 1120–1127. https://doi.org/10.1038/nn.2615

Jaako, K., Aonurm-Helm, A., Kalda, A., Anier, K., Zharkovsky, T., Shastin, D., 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. Eur. J. Pharmacol. 666, 61–71. https://doi.org/10.1016/j.ejphar.2011.05.008

Jaako, K., Waniek, A., Parik, K., Klimaviciusa, L., Aonurm-Helm, A., Noortoots, A., Anier, K., Van Elzen, R., Gérard, M., Lambeir, A.-M., Roßner, S., Morawski, M.,

Zharkovsky, A., 2016. Prolyl endopeptidase is involved in the degradation of neural cell adhesion molecules *in vitro*. J. Cell Sci. 129, 3792–3802. https://doi.org/10.1242/jcs.181891

Jang, H.S., Shin, W.J., Lee, J.E., Do, J.T., 2017. CpG and Non-CpG Methylation in Epigenetic Gene Regulation and Brain Function. Genes 8. https://doi.org/10.3390/genes8060148

Jawahar, M.C., Murgatroyd, C., Harrison, E.L., Baune, B.T., 2015. Epigenetic alterations following early postnatal stress: a review on novel aetiological mechanisms of common psychiatric disorders. Clin. Epigenetics 7, 122. https://doi.org/10.1186/s13148-015-0156-3

Kaas, G.A., Zhong, C., Eason, D.E., Ross, D.L., Vachhani, R.V., Ming, G., King, J.R., Song, H., Sweatt, J.D., 2013. TET1 Controls CNS 5-Methylcytosine Hydroxylation, Active DNA Demethylation, Gene Transcription, and Memory Formation. Neuron 79, 1086–1093. https://doi.org/10.1016/j.neuron.2013.08.032

Kalda, A., Zharkovsky, A., 2015. Chapter Five – Epigenetic Mechanisms of Psychostimulant-Induced Addiction, in: Taba, P., Lees, A., Sikk, K. (Eds.), International Review of Neurobiology, The Neuropsychiatric Complications of Stimulant Abuse. Academic Press, pp. 85–105. https://doi.org/10.1016/bs.irn.2015.02.010

Kalinyak, J.E., Griffin, C.A., Hamilton, R.W., Bradshaw, J.G., Perlman, A.J., Hoffman, A.R., 1989. Developmental and hormonal regulation of glucocorticoid receptor messenger RNA in the rat. J. Clin. Invest. 84, 1843–1848. https://doi.org/10.1172/JCI114370

Kelz, M.B., Chen, J., Jr, W.A.C., Whisler, K., Gilden, L., Beckmann, A.M., Steffen, C., Zhang, Y.-J., Marotti, L., Self, D.W., Tkatch, T., Baranauskas, G., Surmeier, D.J., Neve, R.L., Duman, R.S., Picciotto, M.R., Nestler, E.J., 1999. Expression of the transcription factor ΔFosB in the brain controls sensitivity to cocaine. Nature 401, 272–276. https://doi.org/10.1038/45790

Kember, R.L., Dempster, E.L., Lee, T.H.A., Schalkwyk, L.C., Mill, J., Fernandes, C., 2012. Maternal separation is associated with strain-specific responses to stress and epigenetic alterations to Nr3c1, Avp, and Nr4a1 in mouse. Brain Behav. 2, 455–467. https://doi.org/10.1002/brb3.69

Kertes, D.A., Kamin, H.S., Hughes, D.A., Rodney, N.C., Bhatt, S., Mulligan, C.J., 2016. Prenatal Maternal Stress Predicts Methylation of Genes Regulating the Hypothalamic–Pituitary–Adrenocortical System in Mothers and Newborns in the Democratic Republic of Congo. Child Dev. 87, 61–72. https://doi.org/10.1111/cdev.12487

Kessler, R.C., McGonagle, K.A., Zhao, S., Nelson, C.B., Hughes, M., Eshleman, S., Wittchen, H.-U., Kendler, K.S., 1994. Lifetime and 12-Month Prevalence of DSM-III-R Psychiatric Disorders in the United States: Results From the National Comorbidity Survey. Arch. Gen. Psychiatry 51, 8–19. https://doi.org/10.1001/archpsyc.1994.03950010008002

Kim, G.-D., Ni, J., Kelesoglu, N., Roberts, R.J., Pradhan, S., 2002. Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. EMBO J. 21, 4183–4195. https://doi.org/10.1093/emboj/cdf401

Klengel, T., Mehta, D., Anacker, C., Rex-Haffner, M., Pruessner, J.C., Pariante, C.M., Pace, T.W.W., Mercer, K.B., Mayberg, H.S., Bradley, B., Nemeroff, C.B., Holsboer, F., Heim, C.M., Ressler, K.J., Rein, T., Binder, E.B., 2013. Allele-specific *FKBP5* DNA demethylation mediates gene–childhood trauma interactions. Nat. Neurosci. 16, 33–41. https://doi.org/10.1038/nn.3275

Kloet, E.R. de, Joëls, M., Holsboer, F., 2005. Stress and the brain: from adaptation to disease. Nat. Rev. Neurosci. 6, 463. https://doi.org/10.1038/nrn1683
Kõks, S., 2015. Chapter Six – Experimental Models on Effects of Psychostimulants, in: Taba, P., Lees, A., Sikk, K. (Eds.), International Review of Neurobiology, The Neuropsychiatric Complications of Stimulant Abuse. Academic Press, pp. 107–129. https://doi.org/10.1016/bs.irn.2015.03.002
Konorski, J., 1948. Conditioned reflexes and neuron organization, Conditioned reflexes and neuron organization. Cambridge University Press, New York, NY, US.
Koob, G.F., Moal, M.L., 2001. Drug Addiction, Dysregulation of Reward, and Allostasis. Neuropsychopharmacology 24, 97–129. https://doi.org/10.1016/S0893-133X(00)00195-0
Koob, G.F., Volkow, N.D., 2016. Neurobiology of addiction: a neurocircuitry analysis. Lancet Psychiatry 3, 760–773. https://doi.org/10.1016/S2215-0366(16)00104-8
Koob, G.F., Volkow, N.D., 2010. Neurocircuitry of Addiction. Neuropsychopharmacology 35, 217–238. https://doi.org/10.1038/npp.2009.110
Koppel, I., Tuvikene, J., Lekk, I., Timmusk, T., 2015. Efficient use of a translation start codon in BDNF exon I. J. Neurochem. 134, 1015–1025. https://doi.org/10.1111/jnc.13124
Koss, K.J., Gunnar, M.R., 2018. Annual Research Review: Early adversity, the hypothalamic–pituitary–adrenocortical axis, and child psychopathology. J. Child Psychol. Psychiatry 59, 327–346. https://doi.org/10.1111/jcpp.12784
Kriaucionis, S., Heintz, N., 2009. The Nuclear DNA Base 5-Hydroxymethylcytosine Is Present in Purkinje Neurons and the Brain. Science 324, 929–930. https://doi.org/10.1126/science.1169786
Kuhn, C.M., Pauk, J., Schanberg, S.M., 1990. Endocrine responses to mother-infant separation in developing rats. Dev. Psychobiol. 23, 395–410. https://doi.org/10.1002/dev.420230503
Labonte, B., Yerko, V., Gross, J., Mechawar, N., Meaney, M.J., Szyf, M., Turecki, G., 2012. Differential Glucocorticoid Receptor Exon 1B, 1C, and 1H Expression and Methylation in Suicide Completers with a History of Childhood Abuse. Biol. Psychiatry 72, 41–48. https://doi.org/10.1016/j.biopsych.2012.01.034
LaPlant, Q., Vialou, V., Covington, H.E., Dumitriu, D., Feng, J., Warren, B.L., Maze, I., Dietz, D.M., Watts, E.L., Iñiguez, S.D., Koo, J.W., Mouzon, E., Renthal, W., Hollis, F., Wang, H., Noonan, M.A., Ren, Y., Eisch, A.J., Bolaños, C.A., Kabbaj, M., Xiao, G., Neve, R.L., Hurd, Y.L., Oosting, R.S., Fan, G., Morrison, J.H., Nestler, E.J., 2010. Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens. Nat. Neurosci. 13, 1137–1143. https://doi.org/10.1038/nn.2619
Larson, E.B., Graham, D.L., Arzaga, R.R., Buzin, N., Webb, J., Green, T.A., Bass, C.E., Neve, R.L., Terwilliger, E.F., Nestler, E.J., Self, D.W., 2011. Overexpression of CREB in the Nucleus Accumbens Shell Increases Cocaine Reinforcement in Self-Administering Rats. J. Neurosci. 31, 16447–16457. https://doi.org/10.1523/JNEUROSCI.3070-11.2011
Lasek, A.W., Gesch, J., Giorgetti, F., Kharazia, V., Heberlein, U., 2011. Alk Is a Transcriptional Target of LMO4 and ERα That Promotes Cocaine Sensitization and Reward. J. Neurosci. 31, 14134–14141. https://doi.org/10.1523/JNEUROSCI.3415-11.2011
Lax, E., Szyf, M., 2018. The Role of DNA Methylation in Drug Addiction: Implications for Diagnostic and Therapeutics, in: Grayson, D.R. (Ed.), Progress in Molecular

Biology and Translational Science, Epigenetics and Psychiatric Disease. Academic Press, pp. 93–104. https://doi.org/10.1016/bs.pmbts.2018.01.003
Leith, N.J., Kuczenski, R., 1982. Two dissociable components of behavioral sensitization following repeated amphetamine administration. Psychopharmacology (Berl.) 76, 310–315.
Levine, S., 1994. The Ontogeny of the Hypothalamic-Pituitary-Adrenal Axis. The Influence of Maternal Factorsa. Ann. N. Y. Acad. Sci. 746, 275–288. https://doi.org/10.1111/j.1749-6632.1994.tb39245.x
Li, E., Bestor, T.H., Jaenisch, R., 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69, 915–926. https://doi.org/10.1016/0092-8674(92)90611-F
Lin, J.C., Jeong, S., Liang, G., Takai, D., Fatemi, M., Tsai, Y.C., Egger, G., Gal-Yam, E.N., Jones, P.A., 2007. Role of Nucleosomal Occupancy in the Epigenetic Silencing of the MLH1 CpG Island. Cancer Cell 12, 432–444. https://doi.org/10.1016/j.ccr.2007.10.014
Lister, R., Mukamel, E.A., Nery, J.R., Urich, M., Puddifoot, C.A., Johnson, N.D., Lucero, J., Huang, Y., Dwork, A.J., Schultz, M.D., Yu, M., Tonti-Filippini, J., Heyn, H., Hu, S., Wu, J.C., Rao, A., Esteller, M., He, C., Haghighi, F.G., Sejnowski, T.J., Behrens, M.M., Ecker, J.R., 2013. Global Epigenomic Reconfiguration During Mammalian Brain Development. Science 341, 1237905. https://doi.org/10.1126/science.1237905
Lubin, F.D., Roth, T.L., Sweatt, J.D., 2008. Epigenetic Regulation of bdnf Gene Transcription in the Consolidation of Fear Memory. J. Neurosci. 28, 10576–10586. https://doi.org/10.1523/JNEUROSCI.1786-08.2008
Lyko, F., 2018. The DNA methyltransferase family: a versatile toolkit for epigenetic regulation. Nat. Rev. Genet. 19, 81–92. https://doi.org/10.1038/nrg.2017.80
Ma, D.K., Jang, M.-H., Guo, J.U., Kitabatake, Y., Chang, M., Pow-anpongkul, N., Flavell, R.A., Lu, B., Ming, G., Song, H., 2009. Neuronal Activity–Induced Gadd45b Promotes Epigenetic DNA Demethylation and Adult Neurogenesis. Science 323, 1074–1077. https://doi.org/10.1126/science.1166859
Mackie, P., Lebowitz, J., Saadatpour, L., Nickoloff, E., Gaskill, P., Khoshbouei, H., 2018. The dopamine transporter: An unrecognized nexus for dysfunctional peripheral immunity and signaling in Parkinson's Disease. Brain. Behav. Immun. 70, 21–35. https://doi.org/10.1016/j.bbi.2018.03.020
Magariños, A.M., McEwen, B.S., Flügge, G., Fuchs, E., 1996. Chronic Psychosocial Stress Causes Apical Dendritic Atrophy of Hippocampal CA3 Pyramidal Neurons in Subordinate Tree Shrews. J. Neurosci. 16, 3534–3540. https://doi.org/10.1523/JNEUROSCI.16-10-03534.1996
Mahoney, J.J., Newton, T.F., Omar, Y., Ross, E.L., De La Garza, R., 2013. The relationship between lifetime stress and addiction severity in cocaine-dependent participants. Eur. Neuropsychopharmacol. 23, 351–357. https://doi.org/10.1016/j.euroneuro.2012.05.016
Maiti, A., Drohat, A.C., 2011. Thymine DNA Glycosylase Can Rapidly Excise 5-Formylcytosine and 5-Carboxylcytosine POTENTIAL IMPLICATIONS FOR ACTIVE DEMETHYLATION OF CpG SITES. J. Biol. Chem. 286, 35334–35338. https://doi.org/10.1074/jbc.C111.284620
Mantsch, J.R., Vranjkovic, O., Twining, R.C., Gasser, P.J., McReynolds, J.R., Blacktop, J.M., 2014. Neurobiological mechanisms that contribute to stress-related cocaine use. Neuropharmacology 76. https://doi.org/10.1016/j.neuropharm.2013.07.021

Martinowich, K., Hattori, D., Wu, H., Fouse, S., He, F., Hu, Y., Fan, G., Sun, Y.E., 2003. DNA Methylation-Related Chromatin Remodeling in Activity-Dependent Bdnf Gene Regulation. Science 302, 890–893. https://doi.org/10.1126/science.1090842

Massart, R., Barnea, R., Dikshtein, Y., Suderman, M., Meir, O., Hallett, M., Kennedy, P., Nestler, E.J., Szyf, M., Yadid, G., 2015. Role of DNA Methylation in the Nucleus Accumbens in Incubation of Cocaine Craving. J. Neurosci. 35, 8042–8058. https://doi.org/10.1523/JNEUROSCI.3053-14.2015

McCoy, C.R., Rana, S., Stringfellow, S.A., Day, J.J., Wyss, J.M., Clinton, S.M., Kerman, I.A., 2016. Neonatal maternal separation stress elicits lasting DNA methylation changes in the hippocampus of stress-reactive Wistar Kyoto rats. Eur. J. Neurosci. 44, 2829–2845. https://doi.org/10.1111/ejn.13404

McCreary, A.C., Müller, C.P., Filip, M., 2015. Chapter Four – Psychostimulants: Basic and Clinical Pharmacology, in: Taba, P., Lees, A., Sikk, K. (Eds.), International Review of Neurobiology, The Neuropsychiatric Complications of Stimulant Abuse. Academic Press, pp. 41–83. https://doi.org/10.1016/bs.irn.2015.02.008

McEwen, B.S., 2007. Physiology and Neurobiology of Stress and Adaptation: Central Role of the Brain. Physiol. Rev. 87, 873–904. https://doi.org/10.1152/physrev.00041.2006

McFarland, K., Davidge, S.B., Lapish, C.C., Kalivas, P.W., 2004. Limbic and Motor Circuitry Underlying Footshock-Induced Reinstatement of Cocaine-Seeking Behavior. J. Neurosci. 24, 1551–1560. https://doi.org/10.1523/JNEUROSCI.4177-03.2004

McFarland, K., Kalivas, P.W., 2001. The Circuitry Mediating Cocaine-Induced Reinstatement of Drug-Seeking Behavior. J. Neurosci. 21, 8655–8663. https://doi.org/10.1523/JNEUROSCI.21-21-08655.2001

McGowan, P.O., Sasaki, A., D'Alessio, A.C., Dymov, S., Labonté, B., Szyf, M., Turecki, G., Meaney, M.J., 2009. Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nat. Neurosci. 12, 342–348. https://doi.org/10.1038/nn.2270

McKlveen, J.M., Myers, B., Flak, J.N., Bundzikova, J., Solomon, M.B., Seroogy, K.B., Herman, J.P., 2013. Role of Prefrontal Cortex Glucocorticoid Receptors in Stress and Emotion. Biol. Psychiatry, Biomarkers of Posttraumatic Stress Disorder 74, 672–679. https://doi.org/10.1016/j.biopsych.2013.03.024

Meaney, M.J., Diorio, J., Francis, D., Widdowson, J., LaPlante, P., Caldji, C., Sharma, S., Seckl, J.R., Plotsky, P.M., 1996. Early Environmental Regulation of Forebrain Glucocorticoid Receptor Gene Expression: Implications for Adrenocortical Responses to Stress; pp. 49–60. Dev. Neurosci. 18, 49–60. https://doi.org/10.1159/000111395

Meehan, R.R., Lewis, J.D., McKay, S., Kleiner, E.L., Bird, A.P., 1989. Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. Cell 58, 499–507. https://doi.org/10.1016/0092-8674(89)90430-3

Meffert, M.K., Chang, J.M., Wiltgen, B.J., Fanselow, M.S., Baltimore, D., 2003. NF-κB functions in synaptic signaling and behavior. Nat. Neurosci. 6, 1072–1078. https://doi.org/10.1038/nn1110

Mews, P., Walker, D.M., Nestler, E.J., 2018. Epigenetic Priming in Drug Addiction. Cold Spring Harb. Symp. Quant. Biol. 83, 131–139. https://doi.org/10.1101/sqb.2018.83.037663

Miczek, K.A., Yap, J.J., Covington, H.E., 2008. Social stress, therapeutics and drug abuse: Preclinical models of escalated and depressed intake. Pharmacol. Ther. 120, 102–128. https://doi.org/10.1016/j.pharmthera.2008.07.006

Miller, C.A., Sweatt, J.D., 2007. Covalent Modification of DNA Regulates Memory Formation. Neuron 53, 857–869. https://doi.org/10.1016/j.neuron.2007.02.022

Mo, A., Mukamel, E.A., Davis, F.P., Luo, C., Henry, G.L., Picard, S., Urich, M.A., Nery, J.R., Sejnowski, T.J., Lister, R., Eddy, S.R., Ecker, J.R., Nathans, J., 2015a. Epigenomic Signatures of Neuronal Diversity in the Mammalian Brain. Neuron 86, 1369–1384. https://doi.org/10.1016/j.neuron.2015.05.018

Mo, A., Mukamel, E.A., Davis, F.P., Luo, C., Henry, G.L., Picard, S., Urich, M.A., Nery, J.R., Sejnowski, T.J., Lister, R., Eddy, S.R., Ecker, J.R., Nathans, J., 2015b. Epigenomic Signatures of Neuronal Diversity in the Mammalian Brain. Neuron 86, 1369–1384. https://doi.org/10.1016/j.neuron.2015.05.018

Moeller, F.G., Barratt, E.S., Dougherty, D.M., Schmitz, J.M., Swann, A.C., 2001. Psychiatric Aspects of Impulsivity. Am. J. Psychiatry 158, 1783–1793. https://doi.org/10.1176/appi.ajp.158.11.1783

Monroy, E., Hernández-Torres, E., Flores, G., 2010. Maternal separation disrupts dendritic morphology of neurons in prefrontal cortex, hippocampus, and nucleus accumbens in male rat offspring. J. Chem. Neuroanat. 40, 93–101. https://doi.org/10.1016/j.jchemneu.2010.05.005

Morris, M.J., Adachi, M., Na, E.S., Monteggia, L.M., 2014. Selective role for DNMT3a in learning and memory. Neurobiol. Learn. Mem., MCCS 2014 115, 30–37. https://doi.org/10.1016/j.nlm.2014.06.005

Morris, M.J., Na, E.S., Autry, A.E., Monteggia, L.M., 2016. Impact of DNMT1 and DNMT3a forebrain knockout on depressive- and anxiety like behavior in mice. Neurobiol. Learn. Mem., MCCS 2016 135, 139–145. https://doi.org/10.1016/j.nlm.2016.08.012

Münzel, M., Globisch, D., Brückl, T., Wagner, M., Welzmiller, V., Michalakis, S., Müller, M., Biel, M., Carell, T., 2010. Quantification of the Sixth DNA Base Hydroxymethylcytosine in the Brain. Angew. Chem. Int. Ed. 49, 5375–5377. https://doi.org/10.1002/anie.201002033

Nestler, E.J., 2016. Reflections on: "A general role for adaptations in G-Proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function." Brain Res., 50th Anniversary Issue 1645, 71–74. https://doi.org/10.1016/j.brainres.2015.12.039

Nestler, E.J., 2013. Cellular basis of memory for addiction. Dialogues Clin. Neurosci. 15, 431–443.

Nestler, E.J., 2012. Transcriptional Mechanisms of Drug Addiction. Clin Psychopharmacol Neurosci 10, 136–143. https://doi.org/10.9758/cpn.2012.10.3.136

Nestler, E.J., 2008. Transcriptional mechanisms of addiction: role of ΔFosB. Philos. Trans. R. Soc. B Biol. Sci. 363, 3245–3255. https://doi.org/10.1098/rstb.2008.0067

Nestler, E.J., Aghajanian, G.K., 1997. Molecular and Cellular Basis of Addiction. Science 278, 58–63. https://doi.org/10.1126/science.278.5335.58

Oakley, R.H., Cidlowski, J.A., 2013. The biology of the glucocorticoid receptor: New signaling mechanisms in health and disease. J. Allergy Clin. Immunol. 132, 1033–1044. https://doi.org/10.1016/j.jaci.2013.09.007

Okano, M., Bell, D.W., Haber, D.A., Li, E., 1999. DNA Methyltransferases Dnmt3a and Dnmt3b Are Essential for De Novo Methylation and Mammalian Development. Cell 99, 247–257. https://doi.org/10.1016/S0092-8674(00)81656-6

Okano, M., Xie, S., Li, E., 1998. Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. Nucleic Acids Res. 26, 2536–2540. https://doi.org/10.1093/nar/26.11.2536

Olds, J., Milner, P., 1954. Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. J. Comp. Physiol. Psychol. 47, 419–427.

Oliveira, A.M.M., 2016. DNA methylation: a permissive mark in memory formation and maintenance. Learn. Mem. 23, 587–593. https://doi.org/10.1101/lm.042739.116

Oliveira, A.M.M., Hemstedt, T.J., Bading, H., 2012. Rescue of aging-associated decline in Dnmt3a2 expression restores cognitive abilities. Nat. Neurosci. 15, 1111–1113. https://doi.org/10.1038/nn.3151

Ostrander, M.M., Richtand, N.M., Herman, J.P., 2003. Stress and amphetamine induce Fos expression in medial prefrontal cortex neurons containing glucocorticoid receptors. Brain Res. 990, 209–214. https://doi.org/10.1016/j.brainres.2003.07.001

Park, S.W., Seo, M.K., Lee, J.G., Hien, L.T., Kim, Y.H., 2018. Effects of maternal separation and antidepressant drug on epigenetic regulation of the brain-derived neurotrophic factor exon I promoter in the adult rat hippocampus. Psychiatry Clin. Neurosci. 72, 255–265. https://doi.org/10.1111/pcn.12609

Paulson, P.E., Camp, D.M., Robinson, T.E., 1991. Time course of transient behavioral depression and persistent behavioral sensitization in relation to regional brain monoamine concentrations during amphetamine withdrawal in rats. Psychopharmacology (Berl.) 103, 480–492.

Paxinos, G., Watson, C., 1986. The rat brain in stereotaxic coordinates (2nd ed). Trends Neurosci. 10, 439. https://doi.org/10.1016/0166-2236(87)90017-8

Peña, C.J., Neugut, Y.D., Champagne, F.A., 2013. Developmental Timing of the Effects of Maternal Care on Gene Expression and Epigenetic Regulation of Hormone Receptor Levels in Female Rats. Endocrinology 154, 4340–4351. https://doi.org/10.1210/en.2013-1595

Phillips, A.G., Fibiger, H.C., 1978. The role of dopamine in maintaining intracranial self-stimulation in the ventral tegmentum, nucleus accumbens, and medial prefrontal cortex. Can. J. Psychol. Can. Psychol. 32, 58–66. https://doi.org/10.1037/h0081676

Pierce, R.C., Fant, B., Swinford-Jackson, S.E., Heller, E.A., Berrettini, W.H., Wimmer, M.E., 2018. Environmental, genetic and epigenetic contributions to cocaine addiction. Neuropsychopharmacology 43, 1471–1480. https://doi.org/10.1038/s41386-018-0008-x

Pierce, R.C., Kalivas, P.W., 1997. A circuitry model of the expression of behavioral sensitization to amphetamine-like psychostimulants. Brain Res. Rev. 25, 192–216. https://doi.org/10.1016/S0165-0173(97)00021-0

Plotsky, P.M., Meaney, M.J., 1993. Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Mol. Brain Res. 18, 195–200. https://doi.org/10.1016/0169-328X(93)90189-V

Pruunsild, P., Bading, H., 2019. Shaping the human brain: evolutionary cis-regulatory plasticity drives changes in synaptic activity-controlled adaptive gene expression. Curr. Opin. Neurobiol. 59, 34–40. https://doi.org/10.1016/j.conb.2019.04.003

Pruunsild, P., Sepp, M., Orav, E., Koppel, I., Timmusk, T., 2011. Identification of cis-Elements and Transcription Factors Regulating Neuronal Activity-Dependent Transcription of Human BDNF Gene. J. Neurosci. 31, 3295–3308. https://doi.org/10.1523/JNEUROSCI.4540-10.2011

Qin, T., Castoro, R., Ahdab, S.E., Jelinek, J., Wang, X., Si, J., Shu, J., He, R., Zhang, N., Chung, W., Kantarjian, H.M., Issa, J.-P.J., 2011. Mechanisms of Resistance to Decitabine in the Myelodysplastic Syndrome. PLOS ONE 6, e23372. https://doi.org/10.1371/journal.pone.0023372

Rao, U., Ryan, N.D., Dahl, R.E., Birmaher, B., Rao, R., Williamson, D.E., Perel, J.M., 1999. Factors Associated With the Development of Substance Use Disorder in Depressed Adolescents. J. Am. Acad. Child Adolesc. Psychiatry 38, 1109–1117. https://doi.org/10.1097/00004583-199909000-00014

Raynal, N.J.-M., Issa, J.-P.J., 2016. Chapter 7 – DNA Methyltransferase Inhibitors, in: Egger, G., Arimondo, P. (Eds.), Drug Discovery in Cancer Epigenetics. Academic Press, Boston, pp. 169–190. https://doi.org/10.1016/B978-0-12-802208-5.00007-2

Ritz, M.C., Lamb, R.J., Goldberg, S.R., Kuhar, M.J., 1987. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science 237, 1219–1223. https://doi.org/10.1126/science.2820058

Robinson, T.E., Berridge, K.C., 1993. The neural basis of drug craving: An incentive-sensitization theory of addiction. Brain Res. Rev. 18, 247–291. https://doi.org/10.1016/0165-0173(93)90013-P

Robison, A.J., Vialou, V., Mazei-Robison, M., Feng, J., Kourrich, S., Collins, M., Wee, S., Koob, G., Turecki, G., Neve, R., Thomas, M., Nestler, E.J., 2013. Behavioral and Structural Responses to Chronic Cocaine Require a Feedforward Loop Involving ΔFosB and Calcium/Calmodulin-Dependent Protein Kinase II in the Nucleus Accumbens Shell. J. Neurosci. 33, 4295–4307. https://doi.org/10.1523/JNEUROSCI.5192-12.2013

Rudenko, A., Dawlaty, M.M., Seo, J., Cheng, A.W., Meng, J., Le, T., Faull, K.F., Jaenisch, R., Tsai, L.-H., 2013. Tet1 Is Critical for Neuronal Activity-Regulated Gene Expression and Memory Extinction. Neuron 79, 1109–1122. https://doi.org/10.1016/j.neuron.2013.08.003

Russo, S.J., Dietz, D.M., Dumitriu, D., Morrison, J.H., Malenka, R.C., Nestler, E.J., 2010. The addicted synapse: mechanisms of synaptic and structural plasticity in nucleus accumbens. Trends Neurosci. 33, 267–276. https://doi.org/10.1016/j.tins.2010.02.002

Russo, S.J., Wilkinson, M.B., Mazei-Robison, M.S., Dietz, D.M., Maze, I., Krishnan, V., Renthal, W., Graham, A., Birnbaum, S.G., Green, T.A., Robison, B., Lesselyong, A., Perrotti, L.I., Bolaños, C.A., Kumar, A., Clark, M.S., Neumaier, J.F., Neve, R.L., Bhakar, A.L., Barker, P.A., Nestler, E.J., 2009. Nuclear Factor κB Signaling Regulates Neuronal Morphology and Cocaine Reward. J. Neurosci. 29, 3529–3537. https://doi.org/10.1523/JNEUROSCI.6173-08.2009

Sanders, S.J., He, X., Willsey, A.J., Ercan-Sencicek, A.G., Samocha, K.E., Cicek, A.E., Murtha, M.T., Bal, V.H., Bishop, S.L., Dong, S., Goldberg, A.P., Jinlu, C., Keaney, J.F., Klei, L., Mandell, J.D., Moreno-De-Luca, D., Poultney, C.S., Robinson, E.B., Smith, L., Solli-Nowlan, T., Su, M.Y., Teran, N.A., Walker, M.F., Werling, D.M., Beaudet, A.L., Cantor, R.M., Fombonne, E., Geschwind, D.H., Grice, D.E., Lord, C., Lowe, J.K., Mane, S.M., Martin, D.M., Morrow, E.M., Talkowski, M.E., Sutcliffe, J.S., Walsh, C.A., Yu, T.W., Ledbetter, D.H., Martin, C.L., Cook, E.H., Buxbaum, J.D., Daly, M.J., Devlin, B., Roeder, K., State, M.W., 2015. Insights into Autism Spectrum Disorder Genomic Architecture and Biology from 71 Risk Loci. Neuron 87, 1215–1233. https://doi.org/10.1016/j.neuron.2015.09.016

Sartor, G.C., 2019. Epigenetic pharmacotherapy for substance use disorder. Biochem. Pharmacol. 168, 269–274. https://doi.org/10.1016/j.bcp.2019.07.012

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat. Protoc. 3, 1101–1108.
Schneeberger, Y., Stenzig, J., Hübner, F., Schaefer, A., Reichenspurner, H., Eschenhagen, T., 2016. Pharmacokinetics of the Experimental Non-Nucleosidic DNA Methyl Transferase Inhibitor N-Phthalyl-l-Tryptophan (RG 108) in Rats. Basic Clin. Pharmacol. Toxicol. 118, 327–332. https://doi.org/10.1111/bcpt.12514
Schneider, R., Grosschedl, R., 2007. Dynamics and interplay of nuclear architecture, genome organization, and gene expression. Genes Dev. 21, 3027–3043. https://doi.org/10.1101/gad.1604607
Schultz, W., 2002. Getting Formal with Dopamine and Reward. Neuron 36, 241–263. https://doi.org/10.1016/S0896-6273(02)00967-4
Self, D.W., Genova, L.M., Hope, B.T., Barnhart, W.J., Spencer, J.J., Nestler, E.J., 1998. Involvement of cAMP-Dependent Protein Kinase in the Nucleus Accumbens in Cocaine Self-Administration and Relapse of Cocaine-Seeking Behavior. J. Neurosci. 18, 1848–1859. https://doi.org/10.1523/JNEUROSCI.18-05-01848.1998
Sharif, J., Muto, M., Takebayashi, S., Suetake, I., Iwamatsu, A., Endo, T.A., Shinga, J., Mizutani-Koseki, Y., Toyoda, T., Okamura, K., Tajima, S., Mitsuya, K., Okano, M., Koseki, H., 2007. The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. Nature 450, 908–912. https://doi.org/10.1038/nature06397
Siegel, S., 1979. The role of conditioning in drug tolerance and addiction, in: Psychopathology in Animals: Research and Clinical Implications. Academic Press, San Diego, CA, US, pp. 143–168.
Slezak, M., Korostynski, M., Gieryk, A., Golda, S., Dzbek, J., Piechota, M., Wlazlo, E., Bilecki, W., Przewlocki, R., 2013. Astrocytes are a neural target of morphine action via glucocorticoid receptor-dependent signaling. Glia 61, 623–635. https://doi.org/10.1002/glia.22460
Steketee, J.D., Kalivas, P.W., 2011. Drug Wanting: Behavioral Sensitization and Relapse to Drug-Seeking Behavior. Pharmacol. Rev. 63, 348–365. https://doi.org/10.1124/pr.109.001933
Strecker, R.E., Roberts, D.C.S., Koob, G.F., 1982. Apomorphine-induced facilitation of intracranial self-stimulation following dopamine denervation of the nucleus accumbens. Pharmacol. Biochem. Behav. 17, 1015–1018. https://doi.org/10.1016/0091-3057(82)90487-7
Stroud, H., Su, S.C., Hrvatin, S., Greben, A.W., Renthal, W., Boxer, L.D., Nagy, M.A., Hochbaum, D.R., Kinde, B., Gabel, H.W., Greenberg, M.E., 2017. Early-Life Gene Expression in Neurons Modulates Lasting Epigenetic States. Cell 171, 1151-1164.e16. https://doi.org/10.1016/j.cell.2017.09.047
Symmank, J., Zimmer, G., 2017. Regulation of neuronal survival by DNA methyltransferases. Neural Regen. Res. 12, 1768–1775. https://doi.org/10.4103/1673-5374.219027
Terwilliger, R.Z., Beitner-Johnson, D., Sevarino, K.A., Crain, S.M., Nestler, E.J., 1991. A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. Brain Res. 548, 100–110. https://doi.org/10.1016/0006-8993(91)91111-D

Tyrka, A.R., Parade, S.H., Welch, E.S., Ridout, K.K., Price, L.H., Marsit, C., Philip, N.S., Carpenter, L.L., 2016. Methylation of the leukocyte glucocorticoid receptor gene promoter in adults: associations with early adversity and depressive, anxiety and substance-use disorders. Transl. Psychiatry 6, e848. https://doi.org/10.1038/tp.2016.112

Vázquez, D.M., Van Oers, H., Levine, S., Akil, H., 1996. Regulation of glucocorticoid and mineralocorticoid receptor mRNAs in the hippocampus of the maternally deprived infant rat. Brain Res. 731, 79–90. https://doi.org/10.1016/0006-8993(96)00465-9

Vertes, R.P., 2004. Differential projections of the infralimbic and prelimbic cortex in the rat. Synapse 51, 32–58. https://doi.org/10.1002/syn.10279

Volkow, N.D., Fowler, J.S., Wang, G.-J., 2003. The addicted human brain: insights from imaging studies. J. Clin. Invest. 111, 1444–1451. https://doi.org/10.1172/JCI200318533

Volkow, N.D., Wise, R.A., Baler, R., 2017. The dopamine motive system: implications for drug and food addiction. Nat. Rev. Neurosci. 18, 741–752. https://doi.org/10.1038/nrn.2017.130

Vorel, S.R., Ashby, C.R., Paul, M., Liu, X., Hayes, R., Hagan, J.J., Middlemiss, D.N., Stemp, G., Gardner, E.L., 2002. Dopamine D3 Receptor Antagonism Inhibits Cocaine-Seeking and Cocaine-Enhanced Brain Reward in Rats. J. Neurosci. 22, 9595–9603. https://doi.org/10.1523/JNEUROSCI.22-21-09595.2002

Wagner, M.J., Kim, T.H., Savall, J., Schnitzer, M.J., Luo, L., 2017. Cerebellar granule cells encode the expectation of reward. Nature 544, 96–100. https://doi.org/10.1038/nature21726

Walker, D.M., Nestler, E.J., 2018. Neuroepigenetics and addiction. Handb. Clin. Neurol. 148, 747–765. https://doi.org/10.1016/B978-0-444-64076-5.00048-X.

Weaver, I.C.G., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., Meaney, M.J., 2004. Epigenetic programming by maternal behavior. Nat. Neurosci. 7, 847. https://doi.org/10.1038/nn1276

Weaver, I.C.G., Champagne, F.A., Brown, S.E., Dymov, S., Sharma, S., Meaney, M.J., Szyf, M., 2005. Reversal of Maternal Programming of Stress Responses in Adult Offspring through Methyl Supplementation: Altering Epigenetic Marking Later in Life. J. Neurosci. 25, 11045–11054. https://doi.org/10.1523/JNEUROSCI.3652-05.2005

Weber, A.R., Krawczyk, C., Robertson, A.B., Kuśnierczyk, A., Vågbø, C.B., Schuermann, D., Klungland, A., Schär, P., 2016. Biochemical reconstitution of TET1–TDG–BER-dependent active DNA demethylation reveals a highly coordinated mechanism. Nat. Commun. 7, 10806. https://doi.org/10.1038/ncomms10806

Weeks, J.R., 1962. Experimental Morphine Addiction: Method for Automatic Intravenous Injections in Unrestrained Rats. Science 138, 143–144. https://doi.org/10.1126/science.138.3537.143

Wei, Q., Lu, X.-Y., Liu, L., Schafer, G., Shieh, K.-R., Burke, S., Robinson, T.E., Watson, S.J., Seasholtz, A.F., Akil, H., 2004. Glucocorticoid receptor overexpression in forebrain: A mouse model of increased emotional lability. Proc. Natl. Acad. Sci. 101, 11851–11856. https://doi.org/10.1073/pnas.0402208101

Wiley, J.W., Higgins, G.A., Athey, B.D., 2016. Stress and glucocorticoid receptor transcriptional programming in time and space: Implications for the brain–gut axis. Neurogastroenterol. Motil. 28, 12–25. https://doi.org/10.1111/nmo.12706

Wright, K.N., Hollis, F., Duclot, F., Dossat, A.M., Strong, C.E., Francis, T.C., Mercer, R., Feng, J., Dietz, D.M., Lobo, M.K., Nestler, E.J., Kabbaj, M., 2015. Methyl Supplementation Attenuates Cocaine-Seeking Behaviors and Cocaine-Induced c-Fos Activation in a DNA Methylation-Dependent Manner. J. Neurosci. 35, 8948–8958. https://doi.org/10.1523/JNEUROSCI.5227-14.2015

Wu, X., Zhang, Y., 2017. TET-mediated active DNA demethylation: mechanism, function and beyond. Nat. Rev. Genet. 18, 517–534. https://doi.org/10.1038/nrg.2017.33

Yap, E.-L., Greenberg, M.E., 2018. Activity-Regulated Transcription: Bridging the Gap between Neural Activity and Behavior. Neuron 100, 330–348. https://doi.org/10.1016/j.neuron.2018.10.013

Yue, F., Cheng, Y., Breschi, A., Vierstra, J., Wu, W., Ryba, T., Sandstrom, R., Ma, Z., Davis, C., Pope, B.D., Shen, Y., Pervouchine, D.D., Djebali, S., Thurman, B., Kaul, R., Rynes, E., Kirilusha, A., Marinov, G.K., Williams, B.A., Trout, D., Amrhein, H., Fisher-Aylor, K., Antoshechkin, I., DeSalvo, G., See, L.-H., Fastuca, M., Drenkow, J., Zaleski, C., Dobin, A., Prieto, P., Lagarde, J., Bussotti, G., Tanzer, A., Denas, O., Li, K., Bender, M.A., Zhang, M., Byron, R., Groudine, M.T., McCleary, D., Pham, L., Ye, Z., Kuan, S., Edsall, L., Wu, Y.-C., Rasmussen, M.D., Bansal, M.S., Keller, C.A., Morrissey, C.S., Mishra, T., Jain, D., Dogan, N., Harris, R.S., Cayting, P., Kawli, T., Boyle, A.P., Euskirchen, G., Kundaje, A., Lin, S., Lin, Y., Jansen, C., Malladi, V.S., Cline, M.S., Erickson, D.T., Kirkup, V.M., Learned, K., Sloan, C.A., Rosen-bloom, K.R., de Sousa, B.L., Beal, K., Pignatelli, M., Flicek, P., Lian, J., Kahveci, T., Lee, D., Kent, W.J., Santos, M.R., Herrero, J., Notredame, C., Johnson, A., Vong, S., Lee, K., Bates, D., Neri, F., Diegel, M., Canfield, T., Sabo, P.J., Wilken, M.S., Reh, T.A., Giste, E., Shafer, A., Kutyavin, T., Haugen, E., Dunn, D., Reynolds, A.P., Neph, S., Humbert, R., Hansen, R.S., De Bruijn, M., Selleri, L., Rudensky, A., Josefowicz, S., Samstein, R., Eichler, E.E., Orkin, S.H., Levasseur, D., Papayanno-poulou, T., Chang, K.-H., Skoultchi, A., Gosh, S., Disteche, C., Treuting, P., Wang, Y., Weiss, M.J., Blobel, G.A., Good, P.J., Lowdon, R.F., Adams, L.B., Zhou, X. Q., Pazin, M.J., Feingold, E.A., Wold, B., Taylor, J., Kellis, M., Mortazavi, A., Weissman, S.M., Stamatoyannopoulos, J., Snyder, M.P., Guigo, R., Gingeras, T.R., Gilbert, D.M., Hardison, R.C., Beer, M.A., Ren, B., 2014. A Comparative Encyclopedia of DNA Elements in the Mouse Genome. Nature 515, 355–364. https://doi.org/10.1038/nature13992

Zeisel, S.H., 2009. Epigenetic mechanisms for nutrition determinants of later health outcomes1234. Am. J. Clin. Nutr. 89, 1488S-1493S. https://doi.org/10.3945/ajcn.2009.27113B

Zhang, R.-R., Cui, Q.-Y., Murai, K., Lim, Y.C., Smith, Z.D., Jin, S., Ye, P., Rosa, L., Lee, Y.K., Wu, H.-P., Liu, W., Xu, Z.-M., Yang, L., Ding, Y.-Q., Tang, F., Meissner, A., Ding, C., Shi, Y., Xu, G.-L., 2013. Tet1 Regulates Adult Hippocampal Neurogenesis and Cognition. Cell Stem Cell 13, 237–245. https://doi.org/10.1016/j.stem.2013.05.006

Zhou, Z., Yuan, Q., Mash, D.C., Goldman, D., 2011. Substance-specific and shared transcription and epigenetic changes in the human hippocampus chronically exposed to cocaine and alcohol. Proc. Natl. Acad. Sci. 108, 6626–6631. https://doi.org/10.1073/pnas.1018514108

Zhu, H., Wang, G., Qian, J., 2016. Transcription factors as readers and effectors of DNA methylation. Nat. Rev. Genet. 17, 551–565. https://doi.org/10.1038/nrg.2016.83

SUMMARY IN ESTONIAN

DNA metülatsiooni roll kokaiini ravimsõltuvuse väljakujunemises, sõltuvuse avaldumises ja abstinentsis

Ravimsõltuvus on ajuhaigus, milles psühhoaktiivne aine kutsub esile füüsilisi, psüühilisi probleeme, sotsiaalse funktsiooni puudujääki või töövõimekuse halvenemist. Uuringud näitavad, et ligikaudu pooled psühhostimulaatorite (näiteks kokaiin, amfetamiin) regulaarsetest tarbijatest muutuvad ravimsõltuvateks. Inimestel on erinev riskitase psühhostimulaatoritest sõltuvuse tekkeks, nende riskide uurimine aitab paremini mõista ravimsõltuvuse mehhanisme. Hiljuti avaldatud teadustööd näitavad, et nii geneetilised tegurid kui ka keskkond (näiteks traumaatilised läbielamised varajases eas) mõjutavad indiviidi ravimsõltuvuse tekkeriski. Varasemad uuringud on näidanud, et psühhostimulandid põhjustavad muutusi geeniekspressioonis, mida osaliselt vahendavad epigeneetilised mehhanismid sh. DNA metülatsioon. Käesoleva doktoritöö eesmärk oli uurida, nii katseloomade käitumise kui ka molekulaarsel tasemel, kas kokaiini poolt põhjustatud DNA metülatsiooni muutused võivad olla ravimsõltuvuse indviduaalse tundlikkuse, sõltuvuse avaldumise ja abstinentsi üheks mehhanismiks. Samuti oli doktoritöö eesmärgiks hinnata, kas ravimsõltuvuse riskitegur stress mõjutab epigeneetilisi protsesse ajus.

DNA metülatsioon on epigeneetiline mehhanism rakus, mis vahendab geeniekspressiooni muutusi vastusena keskkonna- ja arengust tingitud mõjuritele. DNA metüleerimist viivad läbi ensüümid DNA metüültransferaasid (DNMT), mis on vajalikud imetajate arenguks. Mutatsioone DNMT3A geenis seostatakse Tatton-Brown-Rahman sündroomi ja autismiga. DNMT1 geeni mutatsioone on seostatud neuroloogiliste ning psühhiaatriliste häiretega sh tserebellaarataksia, sensorineuraalne kuulmislangus, narkolepsia, dementsus jt. *Dnmt3a*-puudulikkusega hiired, kellel see geen on väljalülitatud, surevad varajases eas peale sündi. Ka *Dnmt1*- ja *Dnmt3b*-puudulikkusega hiired ei ole elujõulised. Varasemad uuringud näitasid, et psühhostimulantide manustamisel algatatud DNA metüleerimise ja geeniekspressiooni muutused võivad luua olukorra, mis soodustavad nii ravimsõltuvuse teket kui ka püsimist. Farmakoloogiliste ja käitumiskatsete andmed viitavad, et *Dnmt3a* võib olla kõige olulisem DNA metüültransferaas psühhostimulantide sõltuvuses.

Dissertatsiooni I artikli tulemused näitasid, et maternaalne separatsioon (varajase eluea stressi mudel) põhjustab glükokortikoidi retseptori rakusisese (genoomse) signaalraja stimulatsiooni ja võib osaleda DNA metülatsiooni regulatsioonis ajus. Meie uuringud näitasid, et maternaalne separatsioon suurendab *Dnmt1, Dnmt3a, Dnmt3b* ekspressiooni ja DNMT ensümaatilist aktiivsust rottide prefrontaalses korteksis. Promootorala uuringud näitasid, et GR genoomne signaalirada reguleerib *Dnmt3b* geeniekspressiooni, ent konkreetsed regulatooralad on kindlaksmääramata. Meie katsed näitasid, et glükokortikoidi retseptori

genoomne signaalirada võib mõjutada ka *Dnmt3a* ekspressiooni ning neuraalsest aktiivsusest-sõltuvat *Dnmt3a* transkriptsiooni. Nende uuringute tulemustest võib järeldada, et varajase eluea stressist põhjustatud DNMTde ensümaatilise aktiivsuse muutused ajus võivad omakorda viia püsivate muutusteni geeniekspressioonis, mis võivad suurendada kokaiini tundlikkust.

Dissertatsiooni II ja III artiklis uurisime DNMTde rolli kokaiini korduval manustamisel kasutades hiirte käitumusliku sensitisatsiooni mudelit. Korduv kokaiini manustamine põhjustab katseloomadel võimendunud käitumisvastuse, mida nimetatakse käitumuslikuks sensitisatsiooniks. See käitumuslik fenomen modelleerib sõltuvuskäitumist ja kokaiini psühhootilisi komplikatsioone inimesel. Hiirtel eristatakse käitumusliku sensitisatsiooni kujunemisfaasi ning väljendumisfaasi. Käitumusliku sensitisatsiooniga on seostatud aju sarrustuspiirkonnad, näiteks naalduv tuum (*nucleus accumbens*) ja prefrontaalkorteks.

Meie uuringud näitasid, et korduva kokaiini manustamine hiirtele suurendab DNMTde ensümaatilist aktiivsust käitumusliku sensitisatsiooni väljendumisfaasis ja abstinentsi perioodil, seega on nii käitumusliku sensitisatsiooni väljendumisfaas kui ka abstinents seotud DNMT ensümaatilise aktiivsusega naalduvas tuumas. Meie katsete tulemused selgitasid, akuutne ja korduva kokaiini manustamine hiirtele põhjustas *Dnmt1* ning *Dnmt3a* geeniekspressiooni ning DNMT aktiivsuse suurenemise nii naalduvas tuumas, kui ka vererakkudes (täpsemalt leukotsüütides). Prokaiini (kokaiinisarnane aine) mõju vererakkudele viitab, et *Dnmt3a* ekspressiooni regulatsioon leukotsüütides võib olla seotud pingest-sõltuvate naatriumkanalitega. Nende uuringute tulemused näitasid, et osad kokaiinist tingitud DNMTde muutused aju naalduvas tuumas on sarnased muutustele katseloomade leukotsüütides.

Dissertatsiooni III artiklis vaigistasime hiire *Dnmt3a* geeni naalduvas tuumas, mille tulemusel vähenes DNMT ensümaatiline aktiivsus ja korduv kokaiini manustamine põhjustas käitumusliku sensitisatsiooni suurenemise võrreldes kontrollgrupi hiirtega, viidates *Dnmt3a* olulisele rollile käitumisliku sensitisatsiooni kujunemisfaasis. Samas ei mõjutanud *Dnmt3a* geeni vaigistamine naalduvas tuumas käitumusliku sensitisatsiooni abstinentsi perioodil. Meie tulemused viitasid, et *Dnmt3b* geeni ekspressiooni suurenemine naalduvas tuumas võis kompenseerida *Dnmt3a* vaigistamise efekti.

Kokkuvõttes annavad antud dissertatsiooni aluseks olevad artiklid meile uusi teadmisi ravimsõltuvuse molekulaarsete mehhanismide kohta ja näitavad uusi võimalusi ravimsõltuvuse ravi alasteks uuringuteks.

ACKNOWLEDGEMENTS

I have composed this dissertation with the aid of numerous great persons. First, I would like to begin by thanking my supervisor Anti Kalda. Over the years he has taught me to persevere with an unwavering optimism. He helped me to strive from details of molecular mechanisms to a clinical understanding of our work. I sincerely appreciate your confidence in granting me the possiblity to be part of your group often remotely. A giant thank you, Tõnis Timmusk as my co-supervisor. When I first walked into your lab, I had no idea it would take me to share your passion for life sciences. Tõnis and Mari Sepp gave me that early push as undergradute to continue in a field I felt most attracted to. For all this, plus your openness and willingness to guide in scientific and professional life's challenges, I am immensely grateful.

I would also like to thank my pre-examiners Urmas Arumäe and Kristina Mäemets-Allas. Their comments and questions have been helpful in preparation of this dissertation. My present and former colleagues in Tartu and Tallinn have made this path enganging, pleasant and endurable, Kaili, Kerly, Margret, Kerli, Aveli, Jane, Indrek (great lab recommendation, why not Tartu!), Kaja, Jürgen (technically, for all our thoughtprovoking discussions throughout the years), Laura, Angela, Mari and Jaan Palgi. Thank you Ulla and Epp for your excellent technical support. My heartfelt respect and gratitude goes to Aleksandr Žarkovski for the patience in mastering the teaching of Pharmacology and also for showing me the humanity of biomedical sciences. Külli Jaako and Malle Kuum, thank you for assisting with immunohistochemistry and confocal microscopy. Miriam Ann Hickey, I greatly appreciate your linguistic aid. Kai Kisand, thank you for your discussions and experimental guidance in working with donor blood.

I would especially like to thank my family and people most closest to me. You have been the wonderful source of affection and encouragement all these years. I feel incredibly blessed to have so many in my corner, thank you! Last but by far the least, I would like to thank Cristina, Teresa, Nati, Elisabeth, Silmara, Fabienne for all of your love and sacrifice. I will be never able to express my gratitude enough, but – aitäh, kiitos!

Finally, I sincerely appreciate the financial support from University of Tartu, the Doctoral School of Clinical Medicine, Nadia Walter Scholarship, Estonian Research Council and European Union through European Regional Development Fund that have funded both the research presented in this dissertation and several opportunities to present the work at conferences.

PUBLICATIONS

CURRICULUM VITAE

Name: Mari Urb
Date of Birth: 11.02.1988
Address: Haartmaninkatu 8, Helsinki, 00280, Finland
E-mail: mari.urb@helsinki.fi

Education

2014–present	Doctor of Philosophy, PhD, Neurosciences, University of Tartu
2011–2014	Master's degree, Gene technology, Tallinn University of Technology
2008–2011	Bachelor's degree, Gene technology, Tallinn University of Technology

Experience

Nov 2018–Present	Researcher/Lab manager, University of Helsinki, Finland
2018–2019	Researcher, Protobios LLC, Estonia
2014–2020	Researcher / PhD candidate, University of Tartu, Estonia
2014–2015	Researcher Tallinn University of Technology, Estonia
2012–2013	Intern, Cima Universidad de Navarra, Spain

Certifications

Certificate in Laboratory Animal Sciences	Estonian University of Life Sciences, University of Eastern Finland

Honors-awards

Nadia Walter Scholarship 2018	University of Tartu Foundation, Estonia
Best Research Article 2017 in Natural, Health and Exact Sciences category	Tallinn University of Technology, Estonia

Courses

8–12 May 2018	EMBO Workshop "Molecular, Neurobiology" Crete, Greece

Memberships and voluntary work

2016–2018	Tartu Hooldekodu OÜ, Estonia
2013–2018	Eakate Hooldekodu OÜ, Tallinn, Estonia

Member of European Society of Toxicology *In vitro*
Member of Estonian Society of Pharmacology

List of publications

Urb, M., Niinep, K., Matsalu, T., Kipper, K., Herodes, K., Zharkovsky, A., Timmusk, T., Anier, K., & Kalda, A. (2020). The role of DNA methyltransferase activity in cocaine treatment and withdrawal in the nucleus accumbens of mice. *Addiction Biology*, *25*(1), e12720. https://doi.org/10.1111/adb.12720

Urb, M., Anier, K., Matsalu, T., Aonurm-Helm, A., Tasa, G., Koppel, I., Zharkovsky, A., Timmusk, T., & Kalda, A. (2019). Glucocorticoid Receptor Stimulation Resulting from Early Life Stress Affects Expression of DNA Methyltransferases in Rat Prefrontal Cortex. *Journal of Molecular Neuroscience*, *68*(1), 99–110. https://doi.org/10.1007/s12031-019-01286-z

Anier, K., **Urb, M**., Kipper, K., Herodes, K., Timmusk, T., Zharkovsky, A., & Kalda, A. (2018). Cocaine-induced epigenetic DNA modification in mouse addiction-specific and non-specific tissues. *Neuropharmacology*, *139*, 13–25. https://doi.org/10.1016/j.neuropharm.2018.06.036

Sepp, M., Vihma, H., Nurm, K., **Urb, M**., Page, S. C., Roots, K., Hark, A., Maher, B. J., Pruunsild, P., & Timmusk, T. (2017). The Intellectual Disability and Schizophrenia Associated Transcription Factor TCF4 Is Regulated by Neuronal Activity and Protein Kinase A. *The Journal of Neuroscience*, *37*(43), 10516–10527. https://doi.org/10.1523/JNEUROSCI.1151-17.2017

Sepp, M., Kannike, K., Eesmaa, A., **Urb, M**., & Timmusk, T. (2011). Functional diversity of human basic helix-loop-helix transcription factor TCF4 isoforms generated by alternative 5' exon usage and splicing. *PloS One*, *6*(7), e22138. https://doi.org/10.1371/journal.pone.0022138

ELULOOKIRJELDUS

Nimi: Mari Urb
Sünniaeg: 11.02.1988
Aadress: Haartmaninkatu 8, Helsingi, 00280, Soome
E-mail: mari.urb@helsinki.fi

Haridus

2014–present	Filosoofiadoktor PhD, Neuroteadused, Tartu Ülikool
2011–2014	Magistrikraad, Geenitehnoloogia, Tallinna Tehnikaülikool
2008–2011	Bakalaureusekraad, Geenitehnoloogia, Tallinna Tehnikaülikool

Töökogemus

Nov 2018–Present	Teadur, University of Helsinki, Finland
2018–2019	Teadur, Protobios LLC, Estonia
2014–2020	Teadur / PhD kandidaat, University of Tartu, Estonia
2014–2015	Teadur, Tallinn University of Technology, Estonia
2012–2013	Praktikant, Cima Universidad de Navarra, Spain

Sertifikaadid

Certificate in Laboratory Animal Sciences	Eesti Maaülikool, University of Eastern Finland

Preemiad ja tunnustused

Nadia Walteri stipendium 2018	Tartu Ülikooli Sihtasutus
Parim teadusartikkel 2017 Loodus-, tervise ja täppisteaduste kategoorias	Tallinn Tehnikaülikool, Eesti

Kursused

8.–12. mai 2018	EMBO Workshop "Molecular, Neurobiology", Kreeka

Liikmelisus ja vabatahtlik töö

2016–2018	Tartu Hooldekodu OÜ, Estonia
2013–2018	Eakate Hooldekodu OÜ, Tallinn, Estonia

Euroopa *In vitro* Toksikoloogia Seltsi liige
Eesti Farmakoloogia Seltsi liige

Publikatsioonide loetelu

Urb, M., Niinep, K., Matsalu, T., Kipper, K., Herodes, K., Zharkovsky, A., Timmusk, T., Anier, K., & Kalda, A. (2020). The role of DNA methyltransferase activity in cocaine treatment and withdrawal in the nucleus accumbens of mice. *Addiction Biology*, *25*(1), e12720. https://doi.org/10.1111/adb.12720

Urb, M., Anier, K., Matsalu, T., Aonurm-Helm, A., Tasa, G., Koppel, I., Zharkovsky, A., Timmusk, T., & Kalda, A. (2019). Glucocorticoid Receptor Stimulation Resulting from Early Life Stress Affects Expression of DNA Methyltransferases in Rat Prefrontal Cortex. *Journal of Molecular Neuroscience*, *68*(1), 99–110. https://doi.org/10.1007/s12031-019-01286-z

Anier, K., **Urb, M**., Kipper, K., Herodes, K., Timmusk, T., Zharkovsky, A., & Kalda, A. (2018). Cocaine-induced epigenetic DNA modification in mouse addiction-specific and non-specific tissues. *Neuropharmacology*, *139*, 13–25. https://doi.org/10.1016/j.neuropharm.2018.06.036

Sepp, M., Vihma, H., Nurm, K., **Urb, M**., Page, S. C., Roots, K., Hark, A., Maher, B. J., Pruunsild, P., & Timmusk, T. (2017). The Intellectual Disability and Schizophrenia Associated Transcription Factor TCF4 Is Regulated by Neuronal Activity and Protein Kinase A. *The Journal of Neuroscience*, *37*(43), 10516–10527. https://doi.org/10.1523/JNEUROSCI.1151-17.2017

Sepp, M., Kannike, K., Eesmaa, A., **Urb, M**., & Timmusk, T. (2011). Functional diversity of human basic helix-loop-helix transcription factor TCF4 isoforms generated by alternative 5' exon usage and splicing. *PloS One*, *6*(7), e22138. https://doi.org/10.1371/journal.pone.0022138

DISSERTATIONES NEUROSCIENTIAE UNIVERSITATIS TARTUENSIS

1. **Sirli Raud.** Cholecystokinin$_2$ receptor deficient mice: changes in function of GABA-ergic system. Tartu, 2005.
2. **Kati Koido.** Single-nucleotide polymorphism profiling of 22 candidate genes in mood and anxiety disorders. Tartu, 2005.
3. **Dzhamilja Safiulina.** The studies of mitochondria in cultured cerebellar granule neurons: characterization of mitochondrial function, volume homeostasis and interaction with neurosteroids. Tartu, 2006.
4. **Tarmo Areda.** Behavioural and neurogenetic study of mechanisms related to cat odour induced anxiety in rodents. Tartu, 2006.
5. **Aleksei Nelovkov.** Behavioural and neurogenetic study of molecular mechanisms involved in regulation of exploratory behaviour in rodents. Tartu, 2006.
6. **Annika Vaarmann**. The studies on cystatin B deficient mice: neurochemical and behavioural alterations in animal model of progressive myoclonus epilepsy of Unverricht-Lundborg type. Tartu, 2007.
7. **Urho Abramov.** Sex and environmental factors determine the behavioural phenotype of mice lacking CCK_2 receptors: implications for the behavioural studies in transgenic lines. Tartu, 2008.
8. **Hendrik Luuk.** Distribution and behavioral effects of WFS1 protein in the central nervous system. Tartu, 2009.
9. **Anne Must.** Studies on molecular genetics of male completed suicide in Estonian population. Tartu, 2009.
10. **Kaido Kurrikoff**. Involvement of cholecystokinin in chronic pain mechanisms and endogenous antinociception. Tartu, 2009.
11. **Anu Aonurm-Helm.** Depression-like phenotype and altered intracellular signalling in neural cell adhesion molecule (NCAM)-deficient mice. Tartu, 2010.
12. **Silva Sütt.** Role of endocannabinoid system and *Wfs1* in regulation of emotional behaviour: behavioural, pharmacological and genetic studies. Tartu, 2010.
13. **Mari-Anne Philips.** Characterization of *Myg1* gene and protein: expression patterns, subcellular localization, gene deficient mouse and functional polymorphisms in human. Tartu, 2010.
14. **Ranno Rätsep.** Genetics of psoriasis and vitiligo, focus on IL10 family cytokines. Tartu, 2010.
15. **Kairit Joost.** Selective screening of metabolic diseases in Estonia: the application of new diagnostic methods. Tartu, 2012, 143 p.
16. **Monika Jürgenson.** A complex phenotype in mice with partial or complete deficiency of the NCAM protein. Tartu, 2012, 117 p.

17. **Ene Reimann.** Description of the cytokines and cutaneous neuroendocrine system in the development of vitiligo. Tartu, 2012, 117 p.
18. **Jürgen Innos.** Behavioural, pharmacological and neurochemical characterisation of limbic system-associated membrane protein (LSAMP) deficient mice. Tartu, 2013, 113 p.
19. **Kaili Anier.** The role of DNA methylation in the development of cocaine-induced behavioural sensitisation. Tartu, 2013, 147 p.
20. **Maarika Liik.** Cognitive functioning, perceived cognition, subjective complaints and symptoms of depression in patients with epilepsy: neuropsychological assessment and spet brain imaging study. Tartu, 2014, 124 p.
21. **Sten Ilmjärv.** Estimating differential expression from multiple indicators. Tartu, 2015, 125 p.
22. **Paula Reemann.** The effects of microenvironment on skin cells. Tartu, 2015, 146 p.
23. **Tanel Visnapuu.** Pharmacological and behavioral characterization of the monoaminergic and GABA-ergic systems of Wfs1-deficient mice. Tartu, 2015, 107 p.
24. **Indrek Heinla.** Behavioural and genetic comparison of B6 and 129Sv mouse lines focusing on the anxiety profile and the expression of *Lsamp* gene. Tartu, 2016, 115 p.
25. **Liina Haring.** Cognitive functioning after first psychotic episode. Tartu, 2017, 146 p.
26. **Triin Tekko.** Neurodevelopmental Approach in the Study of the Function of *Wfs1* and *Lsamp*, Potential Targets in the Regulation of Emotional Behaviour. Tartu, 2018, 194 p.
27. **Alina Altpere.** Targeting of mechanisms of elevated anxiety in female Wfs1-deficient mice. Tartu, 2018, 98 p.
28. **Maarja Toots.** Pharmacological challenge in rodent models of Wolfram syndrome with emphasis on diabetic phenotype. Tartu, 2018, 114 p.
29. **Katyayani Singh.** Neuropsychiatric endophenotypes – focusing on IgLON adhesion molecules in the mouse brain. Tartu, 2019, 148 p.
30. **Kattri-Liis Eskla.** Therapeutic strategies for ischemia reperfusion injury. Tartu, 2019, 138 p.
31. **Hardo Lilleväli**. Hyperphenylalaninaemias and neurophysiological disorders associated with the condition. Tartu, 2020, 134 p.
32. **Roman Balõtšev**. Interaction between the immune and metabolic systems in different stages of schizophrenia spectrum disorders. Tartu, 2020, 164 p.