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CHANGES IN THE LEVELS OF DNA METHYLTRANSFERASES AND DEMETHYLASES IN THE NUCLEUS ACCUMBENS IN COCAINE-INDUCED BEHAVIOURAL SENSITISATION IN RATS WITH DIFFERENT EXPLORATORY ACTIVITY

Research Paper

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Running head: Exploratory activity, cocaine, and DNA methylation

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Changes in the levels of DNA methyltransferases and demethylases in the nucleus accumbens in cocaine-induced behavioural sensitisation in rats with different exploratory activity

Abstract

Epigenetic modifications, such as DNA methylation, are involved in the development of cocaine-induced behavioural sensitisation. This study investigated the development and expression of cocaine-induced behavioural sensitisation in rats with high and low exploratory activity (HE and LE, respectively), and the drug-induced changes in the mRNA levels of *Dnmt* and *Tet* family genes in the nucleus accumbens. Only LE-animals had increased locomotor activity over continuous test sessions in response to repeated cocaine (12 mg/kg) administration and they developed a more pronounced behavioural sensitisation. Repeated cocaine treatment resulted in upregulated mRNA of *Dnmt3b* in HE-, but not in LE-rats, and upregulated mRNA levels of *Tet3* in both. Thus, our results suggest that LE- and HE-rats differ in the development and expression of cocaine-induced behavioural sensitisation. The aim of future research is to further examine, whether the observed changes in *Dnmt* and *Tet* levels could explain the behavioural differences between HE- and LE-animals in response to cocaine.

Keywords: DNA methyltransferase, DNA demethylase, cocaine, behavioural sensitisation, nucleus accumbens, exploratory activity

Muutused DNA metüültransferaaside ja demetülaaside tasemetes naalduvas tuumas kokaiini sensitisatsioonil erineva uudistamisaktiivsusega rottidel

Kokkuvõte

Epigeneetilised mehhanismid, nagu DNA metüleerimine, mängivad rolli kokaiinist tingitud käitumusliku sensitisatsiooni tekkimisel. Käesolevas töös uuriti kokaiinist tingitud käitumusliku sensitisatsiooni tekkimist ja avaldumist palju- ja väheuudistavatel rottidel (vastavalt HE ja LE) ning kaasuvaid muutusi *Dnmt*-de ja *Tet*-de mRNA tasemetes naalduvas tuumas. Ainult LE-loomadel tõusis lokomotoorne aktiivsus katsepäevade jooksul vastusena korduvale kokaiini manustamisele (12 mg/kg) ja neil arenes tugevam käitumuslik sensitisatsioon. Korduv kokaiini manustamine suurendas *Dnmt3b* ekspressiooni HE- aga mitte LE-rottidel ning *Tet3* ekspressiooni mõlemas uudistamisgrupis. Seega viitavad tulemused, et kokaiini sensitisatsiooni tekkimine ja avaldumine on erineva uudistamisaktiivsusega loomadel erinev. Edasiste uuringute eesmärk on teha kindlaks, kas leitud muutused *Dnmt* ja *Tet* tasemetes võiksid selgitada LE- ja HE-loomade käitumuslikke erinevusi vastusena kokaiinile.

Märksõnad: DNA metüültransferaas, DNA demetülaas, kokaiin, käitumuslik sensitisatsioon, naalduv tuum, uudistamisaktiivsus

1. Introduction

1.1. Exploratory behaviour

Individual differences in behavioural traits are considered to be related to differences in susceptibility to neurochemical and psychopharmacological manipulations. Exploratory behaviour, an activity that permits the detection of novel environmental stimuli and unfamiliar parts of the environment, is critical for survival as it promotes dispersion and improves the chances of finding life necessities such as food and shelter (Crusio & van Abeelen, 1986; Crusio, 2001). An animal's behaviour in a novel environment is simultaneously influenced by its natural curiosity or motivation to explore the potentially dangerous environment and by fear of the unknown which motivates the animal to stay within the secure and familiar surroundings or simply freeze on spot if the environment is alien in total (Harro, 1993). Extreme expressions of these traits – low motivation and high anxiety – are considered to be the core features of depression.

The exploration box test, developed for simultaneous detection of the various effects that psychoactive substances can elicit on exploratory behaviour (Harro, Oreland, Vasar, & Bradwejn, 1995; Otter et al., 1997), enables to separate the rats into two groups according to their novelty-related behaviour: high motivation to explore/low neophobia (high exploratory, HE) and low motivation to explore/high neophobia (low exploratory, LE). It has previously been shown that these differences in spontaneous exploratory activity are stable and persist with repeated testing, and can also predict behaviour in several other behavioural experiments (Mällo et al., 2007). For example, when compared to HE-rats, LE-rats are less active and more anxious in the elevated plus-maze test, display passive coping mechanisms in the forced swimming test, and acquire a more persistent association between neutral and stressful stimuli in the fear conditioning test (Mällo et al., 2007). However, many of such differences disappear upon repeated handling, while the defining phenotype is extremely resistant (Matrov, Vonk, Herm, Rinken, & Harro, 2011, and unpublished findings). It has also been shown that HE- and LE-rats do not differ in their social anxiety as they show similar behaviour in active social interaction tests and they do not differ in activity in a novel home-cage like environment (Mällo et al., 2007).

Various experiments using a number of different behavioural paradigms have shown that individual differences in responding to novel stimuli are associated with several neurochemical features. With regard to dopamine (DA), using an inescapable new environment paradigm it has been shown that there are higher basal and evoked DA release in the nucleus accumbens (NAc) in those animals with high locomotor response to novelty (high responders) compared to those with low response (low responders) (Hooks, Colvin, Juncos, & Justice, 1992). However, using the exploration box test, research has shown that HE-animals have higher basal and stimulated

extracellular DA levels in the striatum but not in NAc (Alttoa, Seeman, Kõiv, Eller, & Harro, 2009; Mällo et al., 2007), and that there is a higher proportion of DA-D_2 receptors in the functional high-affinity state in HE-animals compared to LE-animals (Alttoa et al., 2009). In addition, research has shown differences in other neurotransmitter systems between high/low responders and LE/HE-animals. These include differences in serotonin (5-HT) (e.g. Mällo et al., 2008; Thiel, Müller, Huston, & Schwarting, 1999) and noradrenaline systems (e.g. Alttoa et al., 2005; Rosario & Abercrombie, 1999).

Furthermore, in LE- and HE-animals, previous research has shown differences in the expression of genes related to neurotransmitter systems that are predicted to be biologically relevant to modulating affective symptomatology, especially in depression and anxiety (Alttoa, Kõiv, Hinsley, Brass, & Harro, 2010). For example, there is downregulation of three genes involved in 5-HT neurotransmission in LE-rats including the expression of *Tph2* (tryptophan hydroxylase 2 which has a fundamental role in brain 5-HT synthesis (Zhang, Beaulieu, Sotnikova, Gainetdinov, & Caron, 2004)) and *Htrc2c* (5-HT_{2C} receptor) in the raphe, and *Htr1a* (5-HT_{1A} receptor) in the hippocampus, posing an increased risk for mood disorders (Alttoa et al., 2010). In addition, several GABA and glutamate system related genes are also differentially expressed in LE- and HE-animals.

1.2. Effects of psychostimulants

Dopaminergic neurotransmission, especially DA release in the NAc, increases in response to naturally existing rewards and novel stimuli (Heffner, Hartman, & Seiden, 1980; Pfaus et al., 1990; Rebec, Grabner, Johnson, Pierce, & Bardo, 1996; Young, Joseph, & Gray, 1992), and plays an important role in the mechanisms of addictive drugs (Di Chiara & Imperato, 1988). Psychostimulants such as amphetamine and cocaine activate the core of the brain reward circuitry, the mesolimbocortical DA system. For example, cocaine achieves its main primary psychological effect by inhibiting DA transporters, causing a build-up of DA in the synaptic cleft, especially producing the feelings of pleasure and satisfaction through its effect on NAc (Nestler, 2001; 2005). Addictive drugs, although differing in their primary/acute actions in the brain, converge in producing some common actions, particularly the activation of the mesolimbic DA system. This activation involves increased firing of DA neurons in the ventral tegmental area (VTA) and a subsequent increase of DA released into the NAc and other limbic forebrain areas such as the prefrontal cortex (PFC) (Nestler, 2001). It is proposed that addictive behaviour is largely caused by progressive and long-lasting adaptations in the nervous system that take place after repeated drug use and that these changes are evident in molecular, cellular, and behavioural levels by the phenomenon of sensitisation (Robinson & Berridge, 1993).

On acute administration, psychostimulants stimulate locomotor activity, and chronic administration of psychostimulants induces psychomotor or behavioural sensitisation, characterised by an enhanced locomotor response to subsequent drug exposure (Anier, Malinovskaja, Aonurm-Helm, Zharkovsky, & Kalda, 2010; Pierce & Kalivas, 1997; Segal & Mandell, 1974). In rodents, psychostimulant-induced locomotor sensitisation can be a good model for studying addictive behaviours associated with excessive drug craving and relapse (Robinson & Berridge, 1993).

1.3. Psychostimulants and novelty related behaviour

The sensitivity to psychostimulants and the vulnerability to drug abuse are also influenced by the individual's reactions to new environments (Hooks, Jones, Smith, Neill, & Justice, 1991; Piazza, Deminiére, Le Moal, & Simon, 1989). Rodents classified as high responders compared to low responders to novelty are shown to be more sensitive to the stimulating and rewarding effects of psychostimulants, but these results seem to be highly dependent on the behavioural selection tests used, the doses of the drug, and methods used to evaluate the effects of the drug (Hooks et al., 1992; Klebaur & Bardo, 1999; Klebaur, Bevins, Segar, & Bardo, 2001; Piazza et al., 1989). For example, it has been shown using an inescapable novel environment that high responders acquire amphetamine self-administration more readily (Piazza et al., 1989) and develop a stronger behavioural sensitisation to repeated amphetamine administration (Hooks et al., 1992) when compared to low responders. In contrast to the latter, an experiment using the exploration box test showed, that only LE-rats develop behavioural sensitisation after repeated low dose amphetamine administration and a behavioural desensitisation was observed in the drug-treated HE-animals (Alttoa, Eller, Herm, Rinken, & Harro, 2007). The same study showed that the proportional increase in exploratory activity following an acute low-dose administration of amphetamine is similar in HE- and LE-rats, and the activity levels of HE-rats remain significantly higher when compared to LE-animals, but the differences in behavioural activity in drug-treated and control LE-rats disappeared with repeated testing. Also, in a study using a free-choice novelty test, high seeking activity predicted greater amphetamine-conditioned place preference (CPP), but not changes in locomotor activity after either a single or repeated administration of amphetamine (Klebaur & Bardo, 1999).

1.4. Epigenetics and epigenetic mechanisms

It has been shown that behavioural sensitisation can persist for months (Henry & White, 1995; Robinson & Berridge, 1993), indicating short- and long-term changes in gene expression following repeated drug administration (Nestler & Aghajanian, 1997). Epigenetic changes may be a key molecular mechanism of lasting changes in brain plasticity related to addiction (Anier et

al., 2010; Maze et al., 2010; Robison & Nestler, 2011; Tian et al., 2012) and recent research suggest that epigenetic mechanisms are involved in the development of behavioural sensitisation following repeated psychostimulant administration (e.g. Anier et al., 2010). As epigenetic processes can change dynamically in response to external factors, this provides a mechanism how environment and drugs can mediate long-term changes in gene expression and therefore also the phenotype (Wong, Mill, & Fernandes, 2011).

Epigenetics can be defined as long-lived and reversible modifications to nucleotides or chromosomes that do not change the sequence of DNA, but can alter gene expression and therefore the phenotype (Lasalle, Powell, & Yasui, 2013). Epigenetic mechanisms are important for normal development and differentiation of the cells, and allow the long-term regulation of gene function through non-mutagenic mechanisms (Henikoff & Matzke, 1997). Epigenetic mechanisms organize the genome in a manner that allows regulated gene expression in the appropriate cell type in response to appropriate cellular stimuli (LaPlant & Nestler, 2011).

The chromatin (the complex of DNA, histones and non-histone proteins in the cell nucleus) structure is highly condensed, meaning that control over gene expression occurs in part by permitting the transcriptional factors to access DNA (Renthal & Nestler, 2008). The structure of chromatin and therefore also access to DNA is regulated by posttranslational modifications of histones and DNA (Kouzarides, 2007).

1.4.1 Histone modifications

The main location for posttranslational covalent modifications (acetylation, methylation, phosphorylation, ubiquitination, sumoylation) are specific amino acid residues in amino-terminal tails of histones: modifications that weaken or disrupt the contact between histones and DNA (e.g. acetylation) and modifications that strengthen the histone-DNA bonds (e.g. methylation) correlate with transcriptionally active sites and repressed sites respectively (Strahl & Allis, 2000). It is also hypothesized that the combination of different modifications summate to influence gene expression (Kouzarides, 2007; Renthal & Nestler, 2008; Strahl & Allis, 2000).

1.4.2 DNA methylation

The methylation of cytosine (C), the addition of a methyl group to position 5 of the C pyrimidine ring, is one of the most stable epigenetic modification that regulates the transcriptional plasticity in mammalian genomes (Wong et al., 2011). This occurs primarily when C is next to guanine (G) in the DNA sequence (C-G dinucleotides, CpG) (Wong et al., 2011). The CpG-s are not evenly distributed in the genome, but are rather clustered in so-called CpG islands, which are over-represented in the promoter regulatory regions of many genes (Strahl & Allis, 2000). Methylation at these sites displaces the binding of transcription factors by attracting co-repressor

complexes that instigate chromatin compaction and gene silencing (Robison & Nestler, 2011; Wong et al., 2011).

1.4.2.1 DNA methyltransferases

The methylation of C is catalysed by a group of DNA methyltransferases (DNMTs) which mediate the transfer of a methyl group from S-adenosylmethionine (SAM) to C (Goll & Bestor, 2005). The product S-adenosylhomocysteine acts as a DNMT inhibitor (Chiang, 1998; Detich, Hamm, Just, Knox, & Szyf, 2003). The two main groups of DNMTs are DNMT1 and DNMT3 families. DNMT1 is found to be important for maintaining DNA methylation patterns in proliferating cells as it has higher affinity to hemimethylated DNA: it copies DNA methylation patterns from the old/matrix strand to newly synthesized DNA strand and therefore maintains the methylation pattern during cell replication, but it is also involved in *de novo* methylation (establishing of new DNA methylation patterns) (Goll & Bestor, 2005; Probst, Dunleavy, & Almouzni, 2009). The enzymes in the DNMT3 family, DNMT3a and DNMT3b, are essential in *de novo* methylation (Li, 2002). All three DNMTs are essential in the development of an embryo and the expression of *Dnmt*-s is much reduced by the time cells reach terminal differentiation, but postmitotic mammalian brain cells still express substantial levels of *Dnmt*-s (Moore, Le, & Fan, 2012).

1.4.3 DNA demethylation

Although information has accumulated on DNA methylation, DNA demethylation remains largely unsolved area of research (Kapoor, Agius, & Zhu, 2005; Wu et al., 2010). This process can either be passive or active, or a combination of both. Passive DNA demethylation involves dilution of 5-methylcytosine (5mC) through progressive cell division by inhibition or lack of maintenance of DNMTs, active DNA demethylation requires specific enzymes (Moore et al 2013, Ooi & Bestor 2008).

1.4.3.1 DNA demethylases

It has been demonstrated that ten-eleven translocation (TET) family enzymes TET1, TET2 and TET3 are able to catalyse the addition of hydroxyl group onto the methyl group of 5mC. Therefore they catalyse oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) and further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (He et al., 2011; Ito et al., 2010, 2011; Kriaucionis & Heitz, 2009). Thymine DNA glycosylase has been shown to excise 5fC and 5caC and therefore creates abasic sites which can be repaired by nitrogenous base excision repair mechanism to restore the unmodified cytosine residue (He et al., 2011; Shen et al., 2013). Recent research also suggests that 5hmC is not only a DNA demethylation intermediate, but that it could

also function as a stable epigenetic mark within gene promoter regions, gene bodies and transcription factor binding sites, therefore influencing gene expression (Hahn et al., 2013; Kaas et al., 2013; Mellén, Ayata, Dewell, Kriaucionis, & Heintz, 2012; Szulwach et al., 2011).

1.5 DNA methylation and psychostimulant addiction

Accumulating research has shown that in addition to posttranslational histone modifications, psychostimulant-induced changes in gene expression are also regulated by changes in DNA methylation in brain cells. It has been shown that acute cocaine exposure increases the expression of *Dnmt3a* and *Dnmt3b* genes in NAc (Anier et al., 2010). The same study demonstrated that acute and chronic cocaine exposure induces hypomethylation and decreased binding of MeCP2 (methyl CpG binding protein 2) at the transcription factor *fosB* promoter, which is associated with transcriptional upregulation of *fosB* in the NAc (Anier et al., 2010). However, in cocaine CPP procedure, the global levels of DNA methylation and also the expression of *Dnmt3b* are decreased in the PFC (Tian et al., 2012). This study also showed that repeated administration of a methyl group donor, methionine, inhibits the establishment of cocaine CPP and reverses the downregulation of *Dnmt3b* in PFC during cocaine CPP, and could also attenuate the rewarding effects of cocaine.

The changes in DNA methylation also seem to be lasting, as *Dnmt3a* expression is shown to be increased during protracted periods of drug abstinence in cocaine-treated animals (LaPlant et al., 2010). In addition, decreased DNMT3a function enhances behavioural responses to cocaine, implicating that decreases in DNA methylation promote increased gene transcription in response to repeated cocaine administration and contribute to drug-induced behavioural plasticity (LaPlant et al., 2010).

Furthermore, pharmacological inhibition of DNMTs decreases cocaine-induced increases in DNA methylation and attenuates drug-induced decreases in gene expression in the NAc (Carouge, Host, Aunis, Zwiller, & Anglard, 2010). For example, intracerebroventricular administration of zebularine, an inhibitor of DNMT, decreases cocaine-induced DNA hypermethylation at the *PP1c* (protein phosphatase 1 catalytic subunit) promoter, resulting in attenuated *PP1c* downregulation in the NAc, and zebularine and cocaine co-treatment delays the development of cocaine-induced behavioural sensitisation (Anier et al., 2010).

Moreover, a study investigating the role of exogenous SAM treatment demonstrated that SAM pre-treatment modifies the development and expression of cocaine-induced locomotor sensitisation, as SAM pre-treatment does not affect acute cocaine-induced locomotor response, but potentiates the development and expression of cocaine-induced locomotor sensitisation in mice (Anier, Zharkovsky, & Kalda, 2013). The authors also demonstrated altered cocaine-elicited

gene expression following SAM pre-treatment, and that cocaine-treatment increases and SAM pre-treatment decreases the levels of *Dnmt3a* and *Dnmt3b* mRNA levels in the NAc. This study supports the findings that reduced methyltransferase activity in the NAc positively regulates cocaine-induced locomotor sensitisation (LaPlant et al., 2010). In general, altered DNA methylation in the NAc may play a critical role in the development and expression of cocaine-induced behavioural sensitisation (Anier et al., 2013).

In addition, the withdrawal periods are associated with enhancement of alterations of DNA methylation in the NAc (Massart et al., 2015). The authors demonstrated that administration of a DNMT inhibitor abolishes cue-induced cocaine seeking and that administration of SAM has the opposite effect. This suggests that changes in DNA methylation in the NAc seem to be an important mechanism also in other addiction related behaviours such as cue-induced cocaine craving.

With regard to active DNA demethylation in response to psychostimulants, the research is limited. Recently it has been shown that *Tet1* is regulated by cocaine and it is involved in cocaine CPP (Feng et al., 2015). This study showed that repeated cocaine injections result in decreased *Tet1* mRNA and protein levels in the NAc, but the levels of *Tet2* and *Tet3* remain unchanged. Repeated cocaine administration increases the enrichment of 5hmC in a large subset of genes involved in drug addiction, which positively correlates with increased expression of these genes and also with their alternative splicing in response to cocaine. In addition, cocaine addicted humans have also reduced *Tet1* mRNA levels (Feng et al., 2015). The same study demonstrated that *Tet1* knockdown and overexpression in the NAc respectively facilitates and weakens the acquisition of CCP. These results indicate that TET1 is involved in negative regulation of cocaine reward. However, TET2 and TET3 are more highly expressed in the NAc, which could also potentially compensate for this observed reduction in TET1 levels (Alaghband, Bredy, & Wood, 2016).

In conclusion, acute and repeated administration of cocaine as well as drug abstinence induce changes in epigenetic mechanisms in the NAc which in turn influence the development and persistence of psychostimulant-induced addiction. Therefore, understanding the epigenetic modifications is important to understand the neurobiology of a subject's sensitivity to psychostimulants.

1.6 Aim of the study

The aim of the current research is to investigate whether LE- and HE-animals differ in the development and expression of cocaine-induced sensitisation, whether acute and repeated cocaine treatment changes expression of *Dnmt* and *Tet* family genes in the NAc, and whether these

changes differ between LE- and HE-animals. As no previous research has investigated epigenetic differences between LE- and HE-animals, three hypotheses could be proposed based on previous literature.

Hypothesis 1: the locomotor activity of HE- and LE-rats upon drug exposure depends on the habituation levels.

Hypothesis 2: acute and repeated cocaine exposure increases mRNA levels of *Dnmt3a* and *Dnmt3b* genes in NAc.

Hypothesis 3: repeated cocaine exposure decreases *Tet1* mRNA levels in the NAc.

The author wrote the review of the literature and methodological part, performed all experimental work (behavioural experiments, tissue analysis), statistical analysis, and interpreted the results based on the research questions and hypothesis.

2. Materials and methods

2.1. Animals

Male Wistar rats (N = 48, weighing 322 – 480 g at the beginning of the experiments; Wistar Hannover GALAS™ strain from Taconic) were housed two to five per standard transparent polypropylene cage and maintained in temperature- and humidity-controlled rooms with 12 hours light-dark cycle (lights on from 7:00 – 19:00). Animals had free access to tap water and food pellets. The experiments were conducted in three sets, with 16 animals per set. The animals were allowed to acclimate to laboratory conditions and were handled at least four days before the behavioural experiments. All experiments were carried out between 13:00 and 19:00. Animals were submitted to behavioural experiments after 2 months of age. All experiments were performed in accordance with the EU guidelines (directive 86/609/EEC) on the ethical use of animals using the experimental protocol approved by the Ministry of Rural Affairs (licence # 99).

2.2 General procedures

Rats were observed in the exploration box and tested for their spontaneous exploratory activity. They were divided into low exploratory (LE) and high exploratory (HE) activity groups on the basis of the median value of the sum of exploratory activity during the second exposure to the exploratory box (Figure 1).

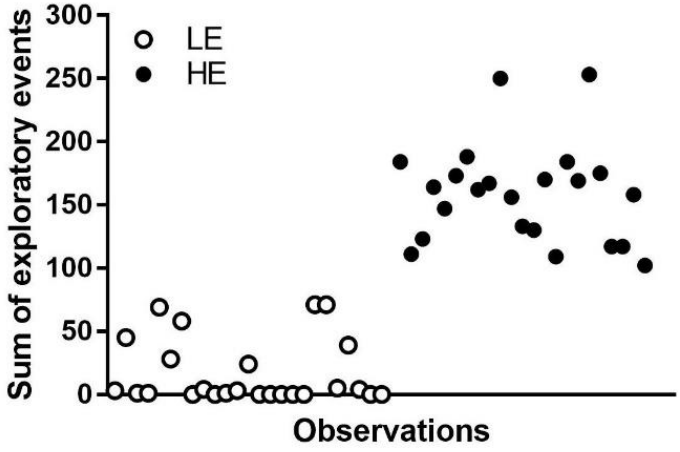

Figure 1. *Scattergram of individual exploratory activity levels during the second exposure to the exploration box. The animals were designated as HE or LE based on the median split of the sum of exploratory events.*

The two groups were randomly assigned to the following treatment groups, therefore forming a 2 × 3 between-subjects experimental design:

1) Sal – rats were treated for 7 days and on the challenge day (two weeks after the first 7 days of drug administration) with sterile saline (0.9% sodium chloride) 0.05 ml per 10 g body weight intraperitoneally (i.p.);
2) acute cocaine AC – rats were treated for 7 days with saline and on the challenge day with cocaine (7 mg/kg, i.p.);
3) repeated cocaine RC – rats were treated for 7 days with cocaine (12 mg/kg, i.p.) and on the challenge day received the challenge dosage (7 mg/kg, i.p.).

Exact group sizes are reported in Table 1.

Table 1. *Experimental group sizes by exploratory activity and treatment group*

	Exploratory activity	
Treatment	**Low-exploratory**	**High-exploratory**
Saline	*n* = 8	*n* = 7
Acute cocaine	*n* = 8	*n* = 7
Repeated cocaine	*n* = 9	*n* = 9
Total (*N* = 48)	*n* = 25	*n* = 23

During the induction period of cocaine-induced behavioural sensitisation, on the first, fourth and seventh day, horizontal locomotor activity was recorded for 60 min right after the drug injection. On the second, third, fifth, and sixth day, rats were injected with the drugs and placed in the test cages for 60 min without locomotor recording. On the challenge day, fourteen days after the last injection (after the induction period), the animals were injected with the drugs and their horizontal locomotor activity was recorded for 60 min. After 24 hours the animals were

anaesthetised with carbon dioxide (CO_2) and decapitated. The brains were quickly dissected on ice. Thereafter the analysis of the tissues and statistical analysis were carried out.

2.3 Behavioural experiments

Exploration box test

The exploration box test was conducted as described previously (Mällo et al., 2007). The exploration box was made of metal and consisted of an open area 0.5 m × 1 m (height of side walls 40 cm) with a small compartment 20 cm × 20 cm × 20 cm attached to one of the shorter sides of the open area. The open area was divided into eight squares of equal size. In the open area, four objects, three unfamiliar and one familiar (a glass jar, a cardboard box, a wooden handle and a food pellet) were situated in certain places (which remained the same throughout the experiment). The small compartment, which had its floor covered with wood shavings, was directly linked to the open area through an opening (size 20 cm × 20 cm). The apparatus was cleaned with dampened laboratory tissue after each animal. The exploration test was initiated by placing a rat into the small compartment, which was then covered with a lid. The following measures were taken by an observer: (a) latency of entering the open area with all four paws on it; (b) entries into the open area; (c) line crossings, (d) rearings; (e) exploration of the three unfamiliar objects in the open area; (f) the time spent exploring the open area. To provide an index of exploration considering both the elements of inquisitive and inspective exploration, the scores of line crossing, rearing and object investigation were summed for each animal. A single test session lasted 15 min and experiments were carried out under dim light conditions (3–7 lx in the open area).

Drug and locomotor activity

Cocaine hydrochloride was dissolved in sterile saline and administered intraperitoneally immediately prior to locomotor activity training. Horizontal locomotor activity was monitored in 16 separate PhenoTyper 4500 cages (Noldus Information Technology, The Netherlands), with floor area of 45 cm × 45 cm and transparent wall height of 55 cm, in a separate room, uniformly illuminated with dim lightning. Horizontal distance travelled was recorded with the assistance of the PhenoTyper top unit that contains an infrared sensitive camera with three arrays of infrared LED lights.

2.4 Tissue isolation

Dissection of the tissues (nucleus accumbens, prefrontal cortex, hippocampus, habenula, cerebellum, raphe, locus coeruleus) was performed by two experienced researchers with the rat

brain atlas of Paxinos and Watson as a guide. The tissues were immediately frozen in liquid nitrogen and stored at -80°C until RNA extracts were prepared.

2.5 Measuring mRNA levels by real-time PCR

RNA extraction from tissues

Total RNA was extracted from the NAc tissues using RNeasy Mini Kit (QIAGEN, Hilden, Germany) and according to the provided protocol. Extracted RNA was stored at -80°C. RNA quantity and quality were assessed using the NanoDrop-1000 spectrophotometer directly before the cDNA synthesis.

cDNA synthesis

Oligo-dT first-strand cDNA was synthesized from 200 ng of total RNA using the First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) and respective protocol provided by the manufacturer.

Real-time PCR

Quantitative real-time PCR (qPCR) was performed using QuantStudio 12K Flex Real-Time PCR System equipped with QuantStudio 12K Flex Software (Thermo Scientific, Waltham, MA, USA). The primers (*Dnmt1, Dnmt3a, Dnmt3b, Tet1, Tet2, Tet3, Gapdh*) were designed using Primer3 with BLAST sequence verification (Table 2). Primers were synthesised by TAG Copenhagen AS (Copenhagen, Denmark), and each primer was optimised prior to use to ensure specificity of the PCR product.

Table 2. *qPCR primer sequences for gene expression analysis*

Target gene	Forward primer (5′–3′)	Reverse primer (3′–5′)
Dnmt1	AACGGAACACTCTCTCTCACTCA	TCACTGTCCGACTTGCTCCTC
Dnmt3a	CAGCGTCACACAGAAGCATATCC	GGTCCTCACTTTGCTGAACTTGG
Dnmt3b	GAATTTGAGCAGCCCAGGTTG	TGAAGAAGAGCCTTCCTGTGCC
Tet1	TGTCACCTGTTGCATGGATT	TTGGATCTTGGCTTTCATCC
Tet2	GAGGAGCAGAAGGAAGCAAG	CACCGTAGCAGAACAGGAAC
Tet3	CAGGGACCAAGCAACAGAAC	AGGGTGTGAGAGGAAAGAGG
Gapdh	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

PCR amplification was performed in a total reaction volume of 10 µl in three parallels. The PCR reaction mixture consisted of 1 µl first-strand cDNA diluted template, 5 µl 2 × Master SYBR Green qPCR Master Mix (Applied Biosystems Inc, USA), 3 µl H_20, and 1 µl gene-specific PCR primers (final concentration 1 µM). The PCR amplification was performed as follows:

denaturation step at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min, repeated for 40 cycles. SYBR Green fluorescence was measured after each extension step and amplification specificity was confirmed by melting curve analyses and gel electrophoresis of the PCR products. Results were normalized to *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase) using the comparative C_T ($2^{-\Delta\Delta C_T}$) method (Schmittgen & Livak, 2008).

2.6 Statistical analysis

All behavioural data were analysed using the IBM SPSS Statistics 20 software. Three- or two-way mixed analysis of variance (ANOVA) was performed where appropriate, followed by Bonferroni post-hoc test for pairwise comparisons. Gene expression data were analysed using GraphPad Prism software for each gene separately. Two-way ANOVA was performed, followed by Bonferroni post-test. Basal mRNA level differences were calculated using t-tests. All data were expressed as mean ± SEM, and significance was set at $p < 0.05$.

Most of the assumptions for ANOVA were met but in a few cells of the design the assumptions of normality, absence of outliers, homogeneity of variances, and sphericity were violated. Removing the outliers and transforming the dependent variable ruled out these violations. As there are no nonparametric alternatives to three-way ANOVA, all statistical analysis were performed on the original data as well as on the data without outliers and using the transformed dependent variable. As no remarkable differences were observed, the results of the statistical analysis performed on original data are reported in this work in terms of the informativity of the results. In addition, three- and two-way mixed ANOVAs are considered "robust" to violations of normality and heterogeneity of variance (Laerd Statistics, 2015).

3. Results

3.1 Individual differences in exploratory activity in the exploration box

After LE- and HE-animals were divided into the three treatment groups, an analysis was performed to ensure that there were no differences in the sum of the exploratory events between the treatment groups for both exploratory activity groups. The two-way interaction of exploratory activity and treatment groups was not statistically significant ($F(2, 42) = 0.873$, $p = 0.425$, partial $\eta 2 = 0.04$). There was only a significant main effect of exploratory activity group ($F(1, 41) = 207.5$, $p < 0.001$, partial $\eta 2 = 0.832$) as in all the treatment groups the sum of the exploratory events was significantly higher in HE-rats compared with LE-rats (Figure 2; $p < 0.001$). In both LE- and HE-animals, there were no statistically significant differences in the sum of the exploratory events between the treatment groups ($p > 0.05$).

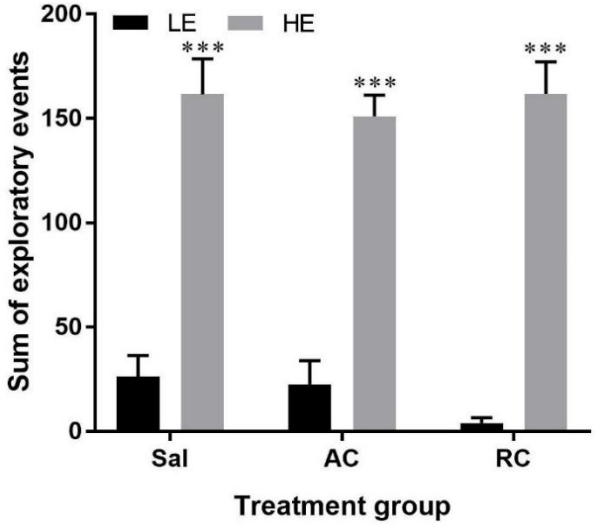

Figure 2. *Exploratory behaviour in the exploration box according to exploratory activity and treatment group. Two-way ANOVA, followed by Bonferroni post-test. Error bars indicate SEM. *** p < 0.001 HE vs LE in the same treatment group.*

3.2 Individual differences in locomotor activity during the induction period of cocaine-induced behavioural sensitisation

Here, the effect of repeated cocaine administration for seven days (induction period of cocaine-induced behavioural sensitisation) on adult rats with different exploratory activity was evaluated. In this analysis, the data of Sal and AC groups were combined in the exploratory activity groups as both Sal and AC groups were administred saline for the first seven days of the cocaine sensitisation experiment. The three-way interaction between exploratory activity, treatment and time was statistically significant ($F(2, 88) = 3.33$, $p = 0.04$, partial $\eta 2 = 0.70$). The simple two-way interaction of exploratory activity group and treatment group was not statistically significant on any of the days ($p = 0.267$, $p = 0.352$, $p = 0.286$ for the first, fourth, and seventh day respectively). However, there were statistically significant two-way interactions of time and exploratory group ($F(2, 88) = 3.76$, $p = 0.027$, partial $\eta 2 = 0.079$), and also time and treatment ($F(2, 88) = 18.6$, $p < 0.001$, partial $\eta 2 = 0.297$). There was a significant simple main effect of treatment on the fourth and seventh day ($F(1, 44) = 43.0$, $p < 0.001$, partial $\eta 2 = 0.494$ and $F(1, 44) = 46.5$, $p < 0.001$, partial $\eta 2 = 0.514$ respectively) when the locomotor activity was significantly higher in the RC groups compared to the Sal groups ($p < 0.001$). At any time point there was no statistically significant simple main effect of the exploratory activity group ($p = 0.270$, $p = 0.366$, $p = 0.170$ for the first, fourth and seventh day respectively).

3.2.1. The effects of treatment and time on locomotor activity in the exploratory groups

In LE-animals, there was a statistically significant interaction of time and treatment ($F(2, 46) = 18.8$, $p < 0.001$, partial $\eta 2 = 0.450$), but this was not observed in HE-animals, ($F(2, 42) = 3.20$, $p = 0.051$, partial $\eta 2 = 0.132$).

In both LE- and HE-animals, there was a statistically significant difference in locomotor activity between treatment groups on the fourth ($F(1, 23) = 25.1$, $p < 0.001$, partial $\eta 2 = 0.522$ in LE- and $F(1, 21) = 18.4$, $p < 0.001$, partial $\eta 2 = 0.467$ in HE-animals) and seventh day ($F(1, 23) = 24.4$, $p < 0.001$, partial $\eta 2 = 0.515$ in LE- and $F(1, 21) = 24.4$, $p < 0.001$, partial $\eta 2 = 0.537$ in HE-animals) of the cocaine sensitisation experiment as the locomotor activity was significantly higher in the RC groups compared to the Sal groups on the fourth and seventh day of the cocaine administration (Figure 3; $p < 0.001$), indicating that repeated cocaine administration increased the locomotor activity.

In LE-animals there was a significant effect of time in both RC and Sal groups ($F(2, 16) = 8.12$, $p = 0.004$, partial $\eta 2 = 0.504$ and $F(2, 30) = 9.16$, $p = 0.001$, partial $\eta 2 = 0.379$ respectively): in the LE × RC group the locomotor activity was significantly higher on the fourth and seventh day compared to the first day (Figure 3; $p = 0.014$ and $p = 0.027$ respectively), indicating the development of a behavioural sensitisation, and in the LE × Sal group, the locomotor activity was significantly lower on the fourth and seventh day compared to the first day (Figure 3; $p = 0.021$ and $p = 0.008$ respectively), referring to a behavioural habituation. In HE-animals there was a significant effect of time only in the Sal group ($F(2, 26) = 9.29$, $p = 0.001$, partial $\eta 2 = 0.417$), but not in the RC group ($F(2, 16) = 1.02$, $p = 0.383$, partial $\eta 2 = 0.113$): in the HE × Sal group, the locomotor activity was significantly lower on the seventh day compared to the first day (Figure 3; $p = 0.004$).

3.2.2 The effects of exploratory group and time on locomotor activity in the treatment groups

When analysing the results of the RC groups only, there was no statistical interaction between time and exploratory group ($F(2, 32) = 2.24$, $p = 0.123$, partial $\eta 2 = 0.123$) and the locomotor activity did not differ significantly between LE- and HE-rats in repeated cocaine group on any of the days during the cocaine sensitisation experiment (Figure 3; $p = 0.348$, $p = 0.463$, $p = 0.318$ for the first, fourth, and seventh day respectively). The analysis of only the Sal groups showed that there was no statistical interaction between time and exploratory group ($F(2, 56) = 0.353$, $p = 0.704$, partial $\eta 2 = 0.012$), and the locomotor activity did not differ significantly between LE and HE control animals on any of the days during the cocaine sensitisation experiment (Figure 3; $p = 0.348$, $p = 0.463$, $p = 0.318$ for the first, fourth, and seventh day respectively).

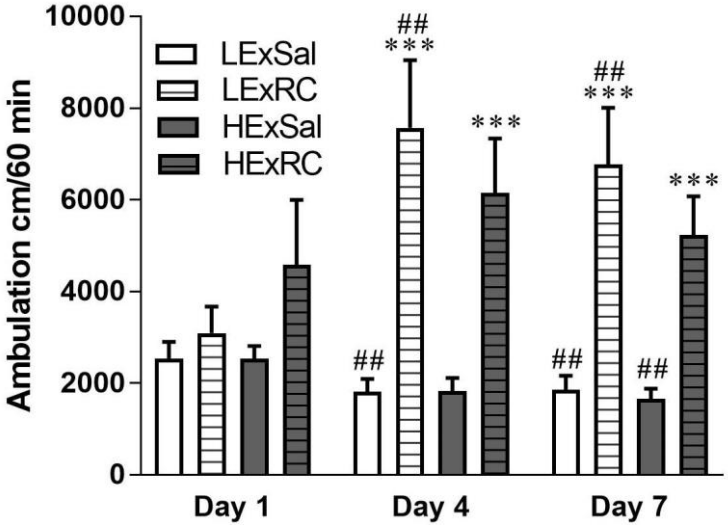

Figure 3. *Differences in locomotor activity during the induction period of cocaine-induced behavioural sensitisation experiment. Data of Sal and AC groups were combined in the exploratory activity groups as both were administred saline. Three-way mixed ANOVA, followed by Bonferroni post-test. Error bars indicate SEM. *** $p < 0.001$ RC vs Sal group in the same exploratory activity group on the same experimental day, ## $p < 0.05$ with the same exploratory and treatment group on the first experimental day.*

3.3. The effects of exploratory activity and treatment on the challenge day

In this analysis, the Sal and AC groups were kept as separate groups because of the difference in the received drugs on the challenge day. The interaction effect between exploratory activity and treatment group on locomotor activity was not statistically significant ($F(2, 42) = 0.536$, $p = 0.589$, partial $\eta^2 = 0.025$). The main effect of treatment group was statistically significant (Figure 4; $F(2, 42) = 13.5$, $p < 0.001$, partial $\eta^2 = 0.391$). The analysis of the main effect for exploratory group showed no statistically significant effects ($F(1, 42) = 0.371$, $p = 0.545$, partial $\eta^2 = 0.009$).

In both HE- and LE-animals, there was a significant effect of treatment group on the locomotor activity ($F(2, 20) = 4.39$, $p = 0.026$, partial $\eta^2 = 0.305$ and $F(2, 22) = 9.59$, $p = 0.001$ partial $\eta^2 = 0.466$ in HE- and LE-rats respectively). The locomotor activity in the HE × RC group was significantly higher than in the HE × Sal group ($p = 0.026$), but there were no statistically significant differences between other treatment groups in HE-animals. The locomotor activity in the LE × RC group was significantly higher compared to the LE × AC and LE × Sal groups ($p = 0.033$ and $p = 0.001$ respectively), but there was no significant difference between the LE × AC and LE × Sal groups ($p = 0.463$).

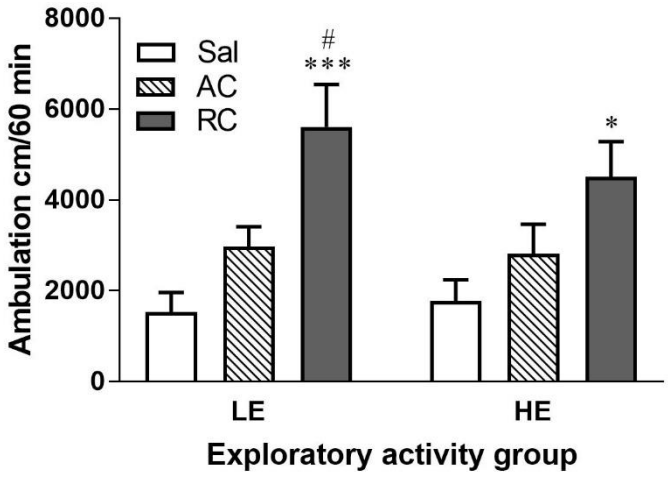

Figure 4. *The locomotor activity on the challenge day. Two-way ANOVA, followed by Bonferroni post-test. Error bars indicate SEM. * p = 0.026 RC vs Sal in the same exploratory activity group, *** p = 0.001 RC vs Sal in the same exploratory activity group, # p = 0.033 RC vs AC in the same exploratory activity group.*

3.4. The effects of exploratory activity, treatment group, and time on locomotor activity between the first and challenge day

In this analysis, the data of the Sal and AC groups were kept separate in order to compare the locomotor activity on the first day to the challenge day. This was necessary because AC groups were administered cocaine only on the challenge day. In general, a three-way interaction between time, exploratory activity and treatment was not statistically significant ($F(2, 42) = 2.24$, $p = 0.119$, partial $\eta^2 = 0.096$). In LE-rats, there was a statistically significant two-way interaction of time and treatment ($F(2, 22) = 13.9$, $p < 0.001$, partial $\eta^2 = 0.558$). In the LE × AC group, the effect of time was not statistically significant, but the locomotor activity in the LE × RC group was statistically higher on the challenge day than on the first day (Figure 5; $p = 0.003$) indicating the expression of a behavioural sensitisation. In the LE × Sal group, the locomotor activity was statistically lower on the challenge day compared with the first day (Figure 5; $p = 0.037$). In HE-rats, there was not a statistically significant two-way interaction of time and treatment ($F(2, 20) = 0.461$, $p = 0.637$, partial $\eta^2 = 0.044$). The locomotor activity was not statistically higher on the challenge day when compared to the first day in the HE × AC and HE × RC groups, but in the HE × Sal groups, the locomotor activity was statistically lower on the challenge day compared to the first day (Figure 5; $p = 0.031$).

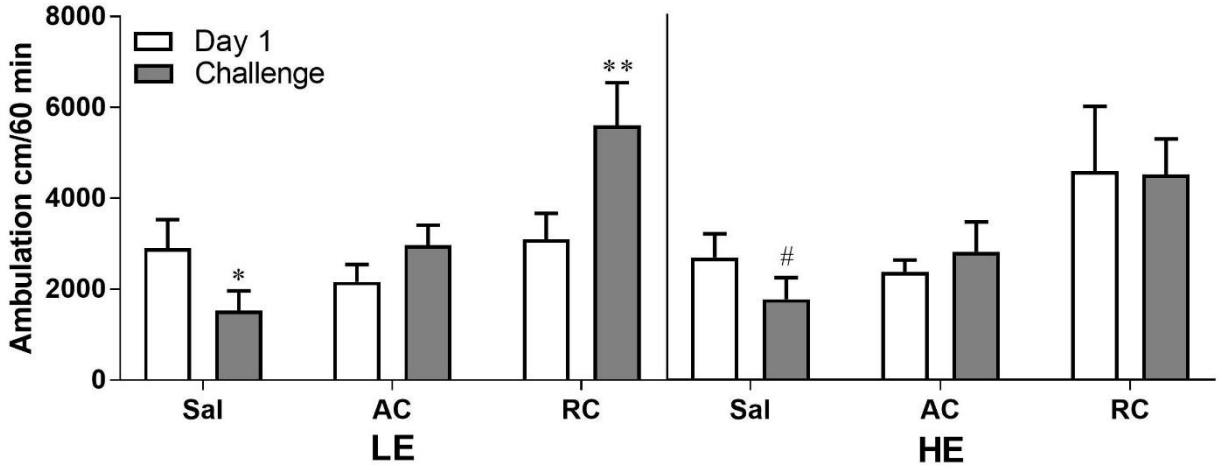

Figure 5. *The locomotor activity on the first and challenge days. Three-way mixed ANOVA, followed by Bonferroni post-test. Error bars indicate SEM. * p = 0.037 LE × Sal first vs challenge day in the same exploratory activity, ** p = 0.003 LE × RC first vs challenge day in the same exploratory activity group, # p = 0.031 HE × Sal first vs challenge day in the same exploratory activity group.*

3.5. Changes in the mRNA levels of investigated methylation and demethylation related genes

We investigated whether *Dnmt* and *Tet* mRNA levels are altered by acute and repeated cocaine treatment in the NAc, and whether these changes differ between LE- and HE-rats. mRNA levels of *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Tet1*, *Tet2*, and *Tet3* were assayed at 24 hours after administration of challenge dose of cocaine (7 mg/kg) to previously saline- or cocaine-treated (12 mg/kg) animals. The preliminary results of our study showed no differences on basal mRNA levels of any of the genes between LE- and HE-animals (p = 0.367, p = 0.482, p = 0.792, p = 0.177, p = 0.180, p = 403 for *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Tet1*, *Tet2*, and *Tet3* respectively). Therefore, the mRNA levels of both control groups (Sal groups) were equalised with 1 for data analysis.

3.5.1. Changes in mRNA levels of *Dnmt* family genes

We observed no changes in the mRNA levels of *Dnmt1* and *Dnmt3a* in response to acute or repeated cocaine administration (Figure 6A-B). However, changes in the levels of *Dnmt3b* mRNA were observed (Figure 6C). Namely, there was a significant two-way interaction of exploratory activity and treatment (F(2, 28) = 4.38, p = 0.0221, 15.94% of the total variance) and there was a significant main effect of treatment group (F(2, 28) = 7.38, p = 0.0027, 26.84% of the total variance). The treatment effect was only observed in HE-animals as the *Dnmt3b* mRNA levels were increased in the RC group compared with the Sal and AC groups (p = 0.0003 and p = 0.0038 respectively). In turn, the mRNA levels of *Dnmt3b* were increased in the HE × RC group compared to the LE × RC group (p = 0.0016). No changes in the mRNA levels of *Dnmt3b* were observed for LE-animals. A single administration of 7 mg/kg of cocaine did not significantly alter

the mRNA levels of *Dnmt* genes in comparison with the saline-receiving animals. Moreover, there were no differences regarding mRNA levels of *Dnmt* genes between the LE × AC and HE × AC groups ($p = 0.505$, $p = 0.668$, $p > 0.999$, for *Dnmt1*, *Dnmt3a*, and *Dnmt3b* respectively).

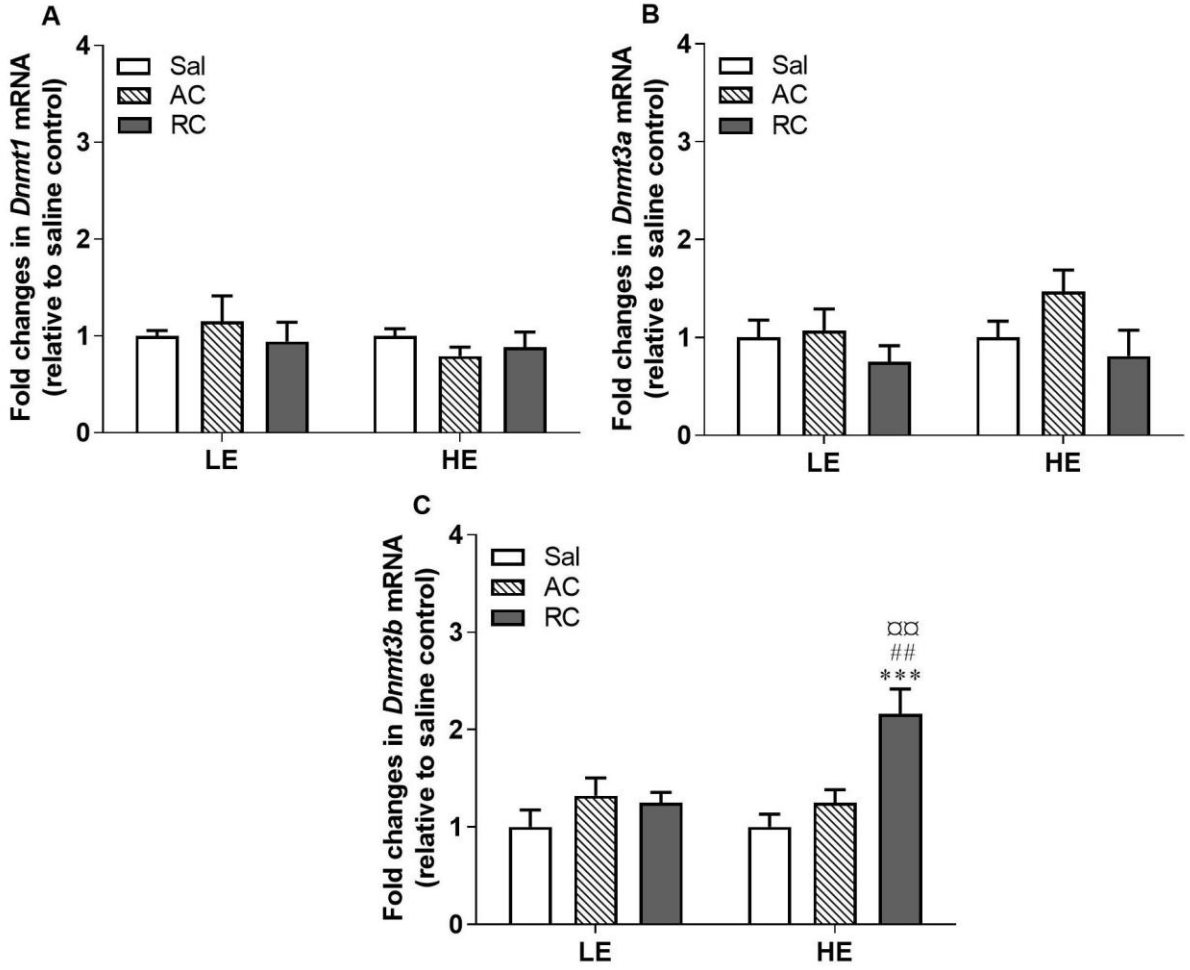

Figure 6. *mRNA levels of Dnmt1 (A), Dnmt3a (B), and Dnmt3b (C) in the NAc at 24 h after last treatment. Two-way ANOVA, followed by Bonferroni post-test. Error bars indicate SEM. *** p = 0.003 RC vs Sal in the same exploratory activity group, ## p = 0.0038 RC vs AC in the same exploratory activity group, ¤¤ p = 0.0016 HE × RC vs LE × RC group.*

3.5.2. Changes in mRNA levels of *Tet* family genes

No changes were observed in the mRNA levels of *Tet1* and *Tet2* in response to acute or repeated cocaine (Figure 7A-B). With regard to *Tet3* mRNA levels (Figure 7C), the two-way interaction of exploratory activity and treatment was not statistically significant, but there was a significant main effect of treatment ($F(2, 28) = 25.1$, $p < 0.0001$, 60.51% of the total variance). In both LE- and HE-rats, the *Tet3* mRNA levels were significantly increased in response to repeated cocaine in comparison with the saline group ($p = 0.0019$ and $p < 0.0001$ respectively). Although no statistical differences were observed between the LE × RC and HE × RC groups, this effect was more pronounced in HE-animals. In HE-animals, mRNA levels of *Tet3* were also elevated in the RC group compared with AC group ($p < 0.0001$); similar tendency was also observed in LE-animals, but this increase was not statistically significant ($p = 0.0521$). A single administration of

cocaine did not significantly alter the mRNA levels of any of the *Tet* genes when compared to the saline group, and no differences were observed between the LE × AC and HE × AC groups at any mRNA level ($p = 0.0580$, $p = 0.993$, $p = 0.702$ *Tet1*, *Tet2*, and *Tet3* respectively).

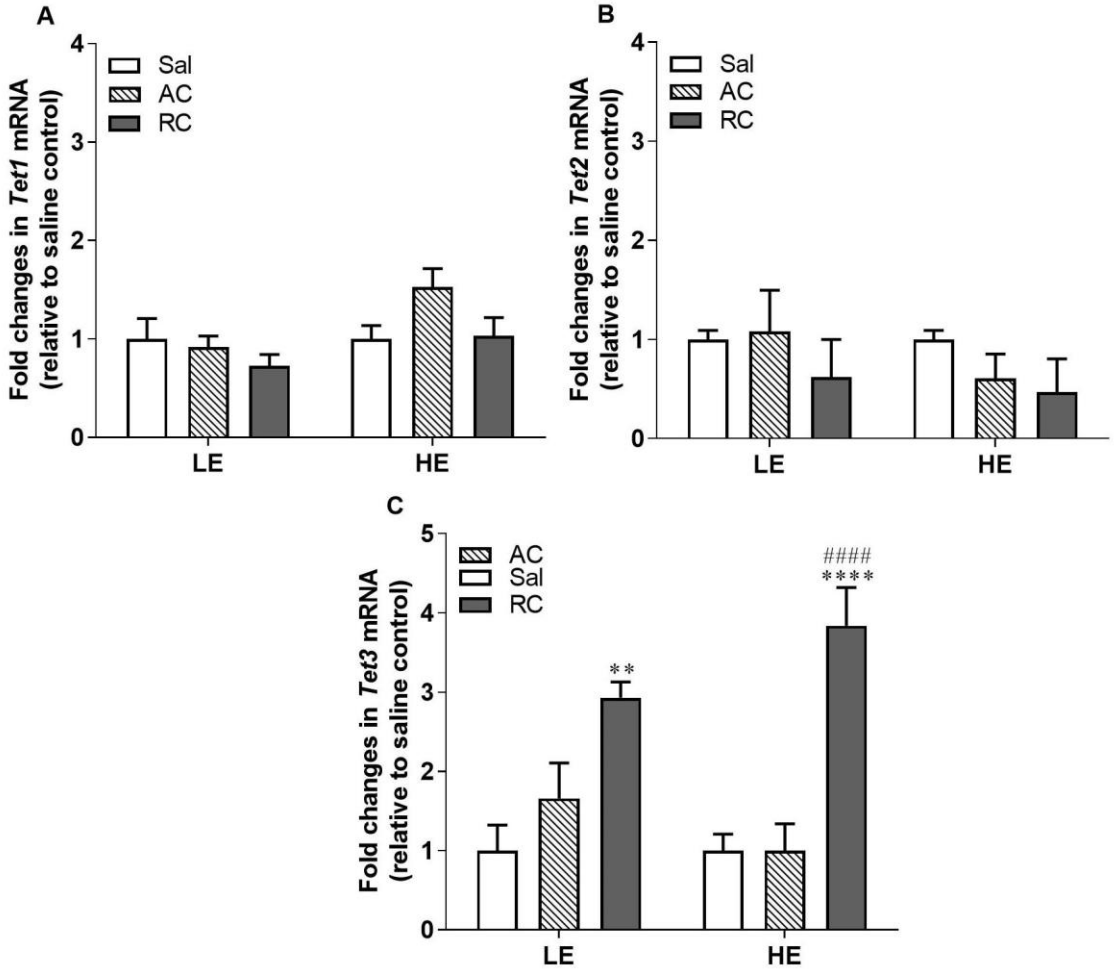

Figure 7. *mRNA levels of Tet1 (A), Tet2 (B), and Tet3 (C) in the NAc at 24 h after last treatment. Two-way ANOVA, followed by Bonferroni post-test. Error bars indicate SEM. ** p = 0.0019 RC vs Sal in the same exploratory activity group, **** p < 0.0001 RC vs Sal in the same exploratory activity group, #### p < 0.0001 RC vs AC in the same exploratory activity group.*

4. Discussion

In recent years, several studies have paid attention to the role of DNA methylation in psychostimulant addiction. The aim of this study was to further investigate how individual differences in exploratory activity are reflected firstly on the behavioural level in response to acute and repeated cocaine administration, and secondly, how the drug-induced changes on the molecular level depend on these individual traits. We evaluated the effect of acute and repeated cocaine exposure on DNA modifying enzymes (DNA methyltransferases and demethylases) in the nucleus accumbens, a brain structure that plays significant role in drug addiction, and compared the effects between low- (LE-) and high-exploratory (HE-) animals.

This study produced four interesting while preliminary findings. First, we found that LE-animals develop a more pronounced behavioural sensitisation in response to repeated cocaine exposure. Second, we observed no differences in the basal mRNA levels of DNA methyltransferases and demethylases between the two exploratory groups. Third, it was observed that repeated cocaine treatment resulted in upregulated mRNA of *Dnmt3b* in HE-animals, but not in LE-rats. Fourth, it was found that both LE- and HE-animals had elevated *Tet3* mRNA levels in response to repeated cocaine exposure. These observations suggest that DNA methylation and demethylation play a role in the development and expression of cocaine-induced behavioural sensitisation. In addition, the differences observed on the molecular level could shed light on the reasons behind the behavioural differences between HE- and LE-animals in response to cocaine, but this remains to be investigated further.

In the last few decades, several studies have investigated neurochemical differences in sensitivity to psychostimulants in rats preselected for their responsiveness in novel environments (e.g. Alttoa et al., 2005, 2007; Cain et al., 2005; Hooks et al., 1992, 1991; Piazza et al., 1989; Thiel et al., 1999). However, it is difficult to compare all previous results with the current research due to the different experimental paradigms that have been used for behavioural categorization.

The results of the present study showed that during the induction period of cocaine-induced behavioural sensitisation, repeated cocaine exposure significantly increased locomotor activity in both LE- and HE-rats in comparison with the control animals, indicating the development of a behavioural sensitisation. Namely, a single dose of 12 mg/kg on the first day of cocaine administration did not significantly increase the locomotor activity when compared to animals receiving saline solution. On the fourth and seventh administration, the locomotor activity was significantly increased in comparison with the saline-receiving animals in both LE- and HE-animals. Our results do not exactly correspond to some previous findings. Alttoa et al (2007) showed that differences in exploratory activity between HE- and LE-animals were still present during the 5-day amphetamine injection experiment, but we observed an opposite trend: on the fourth and seventh day of cocaine administration, LE-animals had a higher, although not statistically significant, locomotor activity in response to repeated cocaine exposure than HE-animals. However, one-to-one comparisons are complicated because we used cocaine instead of amphetamine and they used exploration box to evaluate changes in activity levels in response to the drug while we simply measured locomotor activity.

Interestingly, although LE- and HE-animals did not significantly differ in their locomotor activity in response to repeated cocaine exposure on any of the days, the development and expression of behavioural sensitisation was more evident in LE-rats. Only in LE-animals the

locomotor activity was significantly increased upon repeated cocaine administration on the course of the experiment when compared to the first cocaine exposure, and their locomotor activity was also higher than that of cocaine-receiving HE-animals. In addition, even on the administration of the challenge dose (7 mg/kg), only the LE-animals in the repeated cocaine group showed elevated locomotor activity when compared to the effects of the first administration of a much larger dose (12 mg/kg) on the first day of the cocaine sensitisation experiment, which further shows that LE-animals developed a more pronounced behavioural sensitisation.

This confirms the results of previous research (e.g. Alttoa et al., 2007; Piazza et al., 1989), but the comparisons are complicated. Piazza and colleagues showed that only low responders became sensitised to repeated psychostimulant exposure, but they used a circular inescapable corridor to classify animals as high or low responders to novelty and measured locomotor activity in the same environment, while we used the exploration box to classify animals as high- or low-exploratory and used a simple locomotor activity measurement method. Our findings also confirm the results of a more recent work from our research group (Alttoa et al., 2007) as they observed expression of behavioural sensitisation only in LE-rats. However, our results do not confirm the findings from some other previous studies, which have demonstrated that animals classified as high responders to novelty based on their locomotor activity in a simple Plexiglas cage are more active following cocaine administration and only high responders develop behavioural sensitisation (Hooks et al., 1991). These comparisons further confirm that locomotor activity in an inescapable environment reflects different aspects of novelty-related behaviour than exploratory behaviour measured by the emergence paradigm like the exploration box (Alttoa et al., 2007).

We observed that already a single dose of 12 mg/kg cocaine has a higher effect on the locomotor activity of HE-animals compared to LE-rats. Although this difference was not statistically significant, it could explain why we did not observe increase in locomotor activity over time in the HE-animals upon repeated cocaine exposure. Piazza and colleagues (1989) also showed that high responders respond faster to first amphetamine injection, but it is questionable, whether these results are comparable. Moreover, Alttoa et al. (2007) observed that first injection of amphetamine increased the exploratory activity of HE-animals more than of LE-animals. In addition, Anier et al. (2014) showed that maternal separation increased exploratory activity in the exploration box and these animals also had a higher locomotor response to acute cocaine treatment. Although they made use of the maternal separation paradigm, it still acts as evidence that animals with higher exploratory activity also have higher locomotor response to acute cocaine exposure.

Moreover, the variation in locomotor activity in the HE × RC group was much larger than in the LE × RC group. This could also explain why no significant increases in locomotor activity upon repeated cocaine administration were observed in HE-rats. It is possible that HE-animals developed behavioural sensitisation at a faster rate compared to LE-animals, which could not be detected with the current experimental design, and elevated locomotor activity was later replaced with stereotypies. Indeed, it is well known that repeated cocaine administration may result in repetitive sequences of movements (e.g. Johanson & Fischman, 1989; Post & Rose, 1976; Schlussman, Ho, Zhou, & Curtis, 1998). In some animals, stereotypical behavioural patterns were observed in the current research as well.

In response to the challenge dose of cocaine on the challenge day, the locomotor activity in the repeated cocaine groups was higher in both LE- and HE-animals in comparison with the control group. Therefore it is still possible to assume that both HE- and LE-animals developed a behavioural sensitisation. However, as on the challenge day, LE × RC animals had elevated activity levels in comparison with the LE × AC group as well, it is further indicated that LE-animals developed a more noticeable behavioural sensitisation. Therefore we can conclude that LE- and HE-animals differ in the way behavioural sensitisation is manifested.

The effects of the acute administration of a smaller dose of cocaine on the challenge day on locomotion were equivalent in LE- and HE-animals. This result does not confirm previous findings of Alttoa et al. (2005, 2007) who found that the differences in the activity levels between LE- and HE-rats were still present after amphetamine treatment and that HE-rats remain significantly more active after acute administration of amphetamine compared to LE-animals. However, it is difficult to compare these results with the current research as Alttoa et al. (2005, 2007) used the exploration box to measure activity, but we performed a simple locomotor activity measurement in this study. This further shows that exploratory behaviour differs from general motor activity and drugs of abuse can have differential effects of these behaviours.

It is important to distinguish between the acute doses of cocaine on the first day of the experiment, when the animals in the RC groups received 12 mg/kg of cocaine, and on the challenge day, when AC groups were administered 7 mg/kg cocaine. On the first day we observed a quantitative difference, although not statistically significant, in locomotor activity in response to first administration of cocaine between LE- and HE-animals, but no differences upon acute cocaine exposure were observed on the challenge day. This might be the case, because on one hand, the administered acute doses were different, but on the other hand, it is important to acknowledge the differences in habituation levels.

Even though we observed no drug effect in HE-animals on the first day of the experiment, an increasing trend in locomotor activity in response to cocaine exposure was still evident which was not observed in LE-rats. The animals were allowed to get used to the PhenoTypers and injections for four days before the start of the experiment, which is not enough to ensure a full habituation. Therefore, this indicates that in a somewhat novel environment, LE- and HE-animals differ in their locomotor response upon cocaine administration. On the challenge day however, the animals were much more habituated with the laboratory environment and getting injections than on the first day, and their reaction to acute cocaine exposure was equivalent. We observed no differences in locomotor activity between LE and HE control animals, and both LE- and HE-rats receiving saline became even more habituated to the environment as they were less active already on the fourth and the seventh day as well as on the challenge day compared to the first day of locomotor activity recording. This data confirmed what has been observed previously, that LE- and HE-rats do not differ in their activity in a familiar environment (Mällo et al., 2007, and unpublished results).

Our results therefore confirm the first hypothesis, as we showed that although LE- and HE-animals do not differ in locomotor activity in a familiar environment in the absence of a psychostimulant, the locomotor activity of LE- and HE-rats upon acute cocaine exposure depends on the habituation levels. Namely, LE- and HE- animals do not differ in their activity upon cocaine exposure in a familiar environment, but have different, although not statistically significant, activity levels in a more novel environment in response to cocaine. The present findings significantly extend previous knowledge as never before has the locomotor activity of the LE- and HE-rats been compared by using automatic activity recording, and for such long periods of time.

The interpretation of the preliminary qPCR data of the mRNA levels of DNA methyltransferase and demethylase genes is complicated. In this study we observed no baseline differences in mRNA levels of *Dnmt* and *Tet* genes between LE- and HE-rats.

With regard to DNA methyltransferases, it is interesting that we only observed changes in the expression levels of *Dnmt3b* as it is poorly expressed by the majority of differentiated tissues as well as in the brain (Moore et al., 2012). Previous research suggests that *Dnmt3b* is more essential during early development and the main *de novo* methyltransferase in adult mammalian cells is *Dnmt3a*, which is required for normal cellular development (Moore et al., 2012).

Our results partly contradicted previous findings and our second hypothesis about the elevated levels of *Dnmt3a* and *Dnmt3b* mRNA following acute and repeated cocaine exposure was only partly confirmed. Namely, in this study we showed alterations only in the mRNA levels of *Dnmt3b* gene in response to cocaine, whereas Anier et al. (2010) showed elevated mRNA levels

of *Dnmt3a* gene as well. Moreover, they showed upregulation of both *Dnmt3a* and *Dnmt3b* in response to acute cocaine treatment and that this effect was diminished after repeated cocaine administration, while we observed increased mRNA levels of *Dnmt3b* gene only after repeated cocaine exposure, and this effect was only evident for high exploratory animals. In agreement with previous findings by Anier et al. (2010), we also observed no alterations in the mRNA levels of *Dnmt1* gene. However, it may be difficult to compare these results with the current data because their methods did not include the 2-week abstinence and injection of a challenge dose of cocaine. Moreover, a mice behavioural sensitisation model was used by Anier and colleagues, whereas we used rats. This is also a reason, why we cannot make one-to-one comparisons.

With regard to *Dnmt3a*, one study found upregulation at 4 hours and downregulation at 24 hours following both acute and chronic administration of cocaine, and downregulation also at 24 hours following cocaine self-administration for 13 days (LaPlant et al., 2010). In our study, in both LE- and HE-animals the mRNA levels of *Dnmt3a* were very slightly reduced at 24 hours after last cocaine treatment following repeated cocaine exposure, but this decrease was not statistically significant. In contrast with the aforementioned research, we observed an upward trend in the mRNA levels of *Dnmt3a* following acute cocaine exposure in HE-animals only, but this effect was not statistically significant. In addition, they observed no change from control values at 48 hours after last chronic injection of cocaine, which is comparable with our results at 24 hours.

LaPlant et al. (2010) also found no significant alterations in the mRNA levels of *Dnmt1* following repeated cocaine exposure – a result which was also confirmed in this study, but they did observe reduced expression of *Dnmt1* at 24 hours in response to acute cocaine treatment, which was not found in our experiment. It is also interesting to note that they found that *Dnmt1* mRNA levels 24 hours after cocaine self-administration were significantly reduced. Moreover, we only found alterations in the *Dnmt3b* expression levels, while LaPlant et al. (2010) observed no differences in *Dnmt3b* mRNA levels in any conditions. However, it is difficult to compare our results with theirs as we only measured the mRNA levels at 24 hours after last injection of cocaine and could not therefore investigate the dynamic changes in time. In addition, the LaPlant et al. (2010) used a larger dose of cocaine in their research (20 mg/kg) and investigated the mRNA levels only after administering drugs for seven days or made use of the self-administration paradigm.

With regard to active DNA demethylation, only one study has investigated the role of *Tet1* family enzymes in cocaine addiction. Feng et al. (2015) found *Tet1* to be downregulated in response to repeated cocaine exposure. They did not observe any changes in *Tet2* or *Tet3* mRNA

levels. Our results do not confirm these findings and therefore our third hypothesis, that repeated cocaine administration decreases *Tet1* mRNA levels, was not confirmed. We only found significant alterations regarding *Tet3*. In our experiment, *Tet3* was upregulated following repeated cocaine administration compared with saline control in both LE- and HE-rats. Although we observed no significant differences between LE- and HE-animals, this effect was more apparent in HE-animals. In RC × HE animals, a significant difference in *Tet3* mRNA levels was also observed in comparison with acute administration of cocaine and a similar non-significant trend was apparent also in LE-rats. We found no significant alterations in response to acute cocaine administration, although for LE-animals, mRNA levels of *Tet3* were also slightly increased. With regard to *Tet1* mRNA levels, we observed a slight downward trend in response to acute and chronic cocaine exposure, but this was only seen in LE-animals and was not statistically significant. However, as the experimental procedure and drug dose used in the study conducted by Feng and colleagues were different compared to these used in this research, the comparisons of the results are complicated.

In conclusion, our study further confirms what has been found previously that animals with differential exploratory activity differ in their behavioural responses to acute and repeated psychostimulant exposure, and that DNA methylation and demethylation are involved in the development of psychostimulant-induced sensitisation. Therefore it is important to understand the epigenetic modifications behind drug addiction. As this study yielded only preliminary findings regarding the molecular level, the research questions remain to be further investigated in order to draw more profound conclusions on whether the observed changes on the molecular level could explain the observed behavioural differences between LE- and HE-animals in response to cocaine. In addition, so far we have only measured the alterations in mRNA levels which are extremely difficult to implicate by themselves. We have already started new experiment series to increase the sizes of experimental groups and molecular analyses also need to be repeated. Moreover, questions regarding enzyme activity and the exact loci where DNA methylation and demethylation occurs, remain to be answered in future molecular analyses.

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