CHARACTERISING EPIGENETIC ALTERATIONS FOLLOWING COCAINE CONSUMPTION

BY

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DECLARATION

I, **Duyilemi Chris Ajonijebu (215077684)**, hereby declare that the work described in this thesis entitled:

"Characterising epigenetic alterations following cocaine consumption"

is the result of my own investigations and has not been submitted in part or in full to any other university or tertiary institution for the purpose of obtaining a degree. Information taken from the work of others were duly acknowledged. This research project was carried out under the supervision of Professor WMU Daniels and Professor MV Mabandla.

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Date: 24/01/2018

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LIST OF ABBREVIATIONS

AGS3	Activator of G-protein signalling 3
ALDH	Aldehyde dehydrogenase
AMPA	Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMY	Amygdala
ANT	Alternate nose-poke tasks
AREC	Animal Research Ethics Committee
BDNF	Brain derived neurotrophic factor
cAMP	3', 5' – cyclic adenosine monophosphate
CBP	CREB-binding protein
Cdk5	Cyclin dependent kinase 5
c-Fos	Cellular oncogene Fos
CocE	Cocaine esterase
CpG	5' – Cytocine – phosphate – Guanine – 3'
CPP	Conditioned place preference
CRE	cAMP response element
CREB	cAMP response element binding protein
CREM	cAMP response element modulator
Cutl2	Cut-like 2
Cyp4f13	Cytochrome P450, family 4, subfamily f, polypeptide 13
DA	Dopamine

DARRP-32	Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa
DAT	Dopamine transporter
Dgcr2	DiGeorge syndrome critical region gene 2
DNMT	DNA methyltransferase
DSM-5	Diagnostic and Statistical Manual of Mental Disorders 5
Efemp1	epidermal growth factor–containing fibulin-like extracellular matrix protein 1
Elavl2	Embryonic lethal, abnormal vision, Drosophila – like RNA binding protein 2
ERK	Signal regulated kinase
FE	Offspring from females exposed to drug
F_0	Drug exposed male mice
GABAergic	gamma-aminobutyric acid-ergic
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GWA	Genome wide assays
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
Hist1h3d	Histone cluster 1 H3 family member d
HPC	Hippocampus
H3K9me2	Anti-Histone H3 (di methyl K9) antibody
IC	IntelliCage
Igf1	Insulin-like growth factor 1

Igf2r	Insulin Like Growth Factor 2 Receptor
i.p	Intraperitoneally
LCT	Lick contact time
ME	Offspring from males exposed to drug
MeCP2	Methyl-CpG-binding protein-2
MEF2	Myocyte enhancing factor 2
MFE	Offspring from drug-exposed parent mice
M_0	Drug exposed female mice
NaBut	Sodium Butyrate
NAc	Nucleus accumbens
NAC	N-acetylcysteine
NEP	Offspring from non-drug exposed parent mice
Nlgn3	Neuroligin–3
NMDA	N-methyl-D-aspartate
NMR	Nuclear Magnetic Resonance
NOR	Novel object recognition
NP	Nose-poke
OFT	Open field test
OPC	Operant conditioning chambers
PCR	Polymerase chain reaction
PFC	Prefrontal cortex

PKA	Protein Kinase A
PKC	Protein Kinase C
PP-1	Protein phosphatase – 1
qPCR	quantitative PCR
RFID	Radio frequency identification
SAHA	Suberoylanilide hydroxamic acid
Sim1	Single-minded homolog 1
siRNA	Small interfering ribonucleic acid
Smarca2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 2
SORT	spontaneous object recognition task
Sox 7	SRY-box-containing gene 7
Sox21	SRY-box-containing gene 21
Sp1	Specificity Protein 1
SWI/SNF	SWItch/Sucrose Non-Fermentable
TSA	Trichostatin A
VTA	Ventral tegmental area
X(c) system	Cystine-glutamate antiporter system

THESIS OUTLINE

The principal findings of this PhD research study have been compiled into an article format and presented as a thesis by manuscript.

Chapter One Provides background information with a brief review of selected topics relevant to the study. Study aims and objectives, hypotheses and potential benefits of this research are also highlighted.

Chapter Two Forms part of the literature review and describes "Epigenetics" as a link between drug abuse/addiction and social environment. This review has been published in Cellular and Molecular Life Sciences, an ISI accredited journal.

Chapter Three Reports on cocaine-induced alterations in DNA methylation within target gene promoters in the brains of male mice socially engaged in the conditioned place preference apparatus. This forms study 1 of the PhD experimental research and is currently under review by the International Journal of Developmental Neuroscience.

Chapter Four Forms part of study 2 that characterised drug-taking behaviours in home–caged female mice given prolonged access to multi-rewards (drugs *vs* water), while it featured comparative consumption preferences between rewards and executive learned nose-poke tasks.

Chapter Five Titled "Differential epigenetic changes in the hippocampus and prefrontal cortex of female mice that had free access to cocaine" also forms part of study 2 and has been published in Metabolic Brain Disease.

Chapter Six Describes the inheritance of cocaine-induced epigenetic changes by non-drug exposed offspring mice and examined the impact of postnatal fostering on both neurobiological and behavioural alterations including locomotion and memory performance. This chapter constitutes study 3 which has been submitted for peer review to Behavioural Brain Research.

Chapter Seven Provides general conclusions and social implications of the research findings. It also highlights major limitations and make recommendations for future studies.

ABSTRACT

Complementary data from clinical and animal research have converged on the hypothesis that persistent use of psychostimulant drugs such as cocaine may not only involve pathological alterations in neural processes that subserve reward–related learning, but also complex interactions between genes and the environment through epigenetic modifications. *Fosb* and *Crem* (cAMP response element modulator) are among the central trans-factors suspected to mediate these long–term neurobiological changes due to their potential roles in drug reward. However, the critical question that concerns inheritance of epigenetic marks associated with parental cocaine experience in social settings, offspring vulnerability and modifying maternal–foetal environment by fostering, remains poorly understood. The present study therefore aimed to investigate possible associations between cocaine–induced behavioural changes in social contexts and DNA methylation patterns of inducible transcription factors in the prefrontal cortex (PFC) and hippocampus (HPC) of the exposed parent mice. We further examined whether the induced epigenetic changes were inheritable and then determined the impact of early postnatal (PN) fostering on associated neurobiological changes in the offspring of drug-exposed parents. In doing so, we were able to investigate the interaction between epigenetic and environmental factors in relation to drug consumption.

Behavioural response of C57BL/6 mice to cocaine treatments were examined using conditioned place preference (CPP) and IntelliCage (IC) phenotyping techniques. In the CPP experiment, male mice received 6 cocaine injections (10mg/kg, *i.p.*) on alternate days followed by 6 days of extinction learning. A subthreshold dose of cocaine (5mg/kg, *i.p*) was later injected to reinstate CPP behaviour. In the IC, female mice were group–housed and initially had free access to drugs (300mg/L cocaine and 12% v/v ethanol in their drinking bottles) and water for 30 days to investigate consumption preference. Subsequently, withdrawal effect and alternate nose-poke learning tasks (ANT) were examined in the following 28 days with concurrent access to cocaine and water. In both experiments, locomotor activity and novelty exploration/recognition memory of mice were examined post cocaine treatments. DNA methylation status of *Crem* and *Fosb* gene promoters, within the HPC and PFC, were also assessed using quantitative real–time polymerase chain reaction. Thereafter, cocaine exposed or unexposed male and female mice were matched for mating to produce offspring with lineal phenotypes. At birth, some of the offspring were cross-fostered to further examine the impact of PN fostering on drug-induced epigenetic changes. Locomotion, memory competence and DNA methylation status were also evaluated in the offspring similar to the parent mice.

In male mice, cocaine treatment resulted in significant changes in CPP during conditioning. After extinction learning, the subthreshold dose of cocaine did not reinstate the conditioned behaviour. The

treatment increased locomotor activity in the open field but decreased novelty exploration in the object recognition task. These changes were characterized by significant hypomethylation in *Crem* and *Fosb* gene promoters only in the PFC. During the first 30 days of free access to cocaine, ethanol or water in the IC, the female mice spent significantly more time licking and consuming cocaine than ethanol, whereas consumption of either drugs was significantly less than water. Overall, the mice exhibited motivational deficits as manifested by their inability to learn the ANT. Our data also showed that prolonged access to cocaine in the IC decreased locomotor activity while recognition memory remained intact in the cocaine–experienced mice compared to their controls. These changes were accompanied by hypomethylation or hypermethylation in the promoters of *Fosb* and *Crem* genes in the PFC and HPC of the cocaine–experienced mice, respectively. In the offspring, memory performance and locomotor activity were not affected by parental cocaine exposure, except that recognition memory was impaired by early PN fostering in offspring lineally inclined to either paternal and/or combined parental cocaine experience. *Crem* was hypomethylated only in the PFC of offspring of cocaine–exposed parent mice, while fostering the offspring reversed the expression. Significant change in *Fosb* methylation was only observed in the HPC of fostered offspring.

Together, these findings suggest differential responses of the substrate brain regions to the converging environmental stimuli and dynamic regulation of induced neurobiological changes via DNA methylation. Overall, the data also provide some evidence that cocaine–induced epigenetic marks can be inherited by the non-drug exposed offspring while early PN fostering may enhance molecular switching that may render the individual vulnerable to drug consumption.

Key words: Cocaine; social environment; IntelliCage, conditioned place preference; *Crem*; *Fosb*; DNA methylation; hippocampus; prefrontal cortex; epigenetic inheritance; postnatal fostering.

CHAPTER ONE

LITERATURE REVIEW

1.1 Background of the Study

Drug abuse continues to be a serious psychiatric and social problem worldwide. It is a known fact that approximately 250 million people in the world are problem drug users [1]. Besides alcohol, cannabis and heroin, cocaine is one of the most widely used substances in South Africa [2, 3]. Since independence from the white minority rule ("Apartheid") in 1994, there has been a continuous increase in demand and use of cocaine and other illicit drugs in some South African subpopulations thus making the country one of the transhipment routes for drug trafficking in Africa [2, 4]. Compounding this global challenge is the osmotic relationship with crime which further breeds the consequences of security breach and governmental discord. The scourge of this pandemic and debilitating disorder ravages local communities and strips them of their health, social and economic values, making it difficult to escape the vicious cycle of drug abuse-criminal activity-drug abuse. Currently, there is no effective treatment to relieve this global burden and most valiant attempts to understand the neurobiological sequelae that underpin transition to substance dependence have been limited by inadequate experimental protocols and lack of appropriate animal models that validate the precise condition in humans.

Laboratories that use animal models to study drug abuse have mainly utilised rodents or primates within an isolated context. For instance, in place preference behaviour, individual rodents are placed within a two-compartment chamber and the preference for one compartment is assessed prior to and after drug administration [5, 6]. Similarly, in experiments using primates, an individual animal is placed within a self-administration apparatus where cue-matched lever pressing results in the administration of the drug of interest [7]. In both these widely-used protocols animals are studied in isolation, unlike the actual behaviour in humans where drug consumption is often occurring within a social environment. To improve on the existing methodologies and address the current need, modern investigators have developed efficient and standardized behavioural testing paradigms used to evaluate behavioural phenotyping and other executive brain functions in rodents using the automated IntelliCage (IC) system [8-10]. The IC system is also known as the "social home cage" apparatus, which allows mice to be housed and reared in social groups over extended periods of time while spontaneous behaviours are automatically monitored and analysed [11, 12]. Empirically, it is still a great challenge to validate in animal models the dimensionality of drug taking in free–living environments and the psychometric analysis of substance use to dependence in a manner that mirrors dependence criteria and ethologic conditions in humans. To this end, we used a free choice-drinking paradigm to characterize drug intake behaviour in IC–group housed

C57BL/6 mice. The paradigm initially incorporated a multiple–solution (polydrug *vs.* water) preference approach that measured drug consumption and comparative preference between drugs (cocaine *vs.* ethanol) and water concurrently presented to the animals. Another rationale for this approach was to also shed light on the "gateway drug hypothesis" in the case of sequential drug consumption (from ethanol to cocaine or cocaine to ethanol). Subsequently, persistence in cocaine seeking was demonstrated using scheduled alternate nose-poke learning tasks imposed before and after a brief period of drug withdrawal (*see Figure 2 in chapter 4 for detailed description of the protocol*). This in-depth phenotyping allowed us to identify natural "high" and "low" drug consumers as well facilitated the critical evaluation of sophisticated goal directed behaviours.

Conventionally, long-term exposure to addictive drugs or psychostimulants such as cocaine may induce impaired-control and vulnerable prone phenotypes characterized by hyperactive dopaminergic system, long-lasting loss of synaptic plasticity in reward brain areas and a dysfunctional prefrontal cortex [13]. These neuroadaptive changes are further modified by various environmental factors such as epigenetic changes and social influences [14]. Recent investigations have shifted focus to how these factors combined with drug stimuli converge on the genome to alter specific gene programs. This understanding is fundamental to drug pathogenesis, the molecular basis of progression from drug use to dependence and vulnerable phenotypes.

Alterations in gene and protein expression e.g Δ*FosB, CREB* (cAMP response element binding protein) and *CREM* (cAMP response element modulator) have been suggested to be fundamental to the development of drug abuse and vulnerability [15, 16]. As shown in Figure 1, changes in these gene-specific programs, within the rewarding circuitry i.e projections from ventral tegmental area (VTA) to nucleus accumbens (NAc), as well as the prefrontal cortex (PFC) and hippocampus (HPC) which mediates executive functions and context-specific memories of drug reward related events, are regulated in part by transcriptional alterations in downstream signalling pathways as well via epigenetic mechanisms such as DNA methylation or histone acetylation [17, 18].

It is also evident that certain epigenetic marks can be inherited which affect developmental and cellular features over generations. A study by Arai et al. (2009) showed that enhanced memory performance and synaptic plasticity that resulted from exposure to an enriched environment was not only transmitted from mother to the offspring but also persisted till adulthood [19]. Relatively, maternal cocaine exposure and early life events such as maternal separation and/or postnatal fostering, have been shown to also promote these long-term neurobiological changes [20-22]. Epigenetic mechanisms underlying the observed effects have been suggested to involve persistent chemical modifications such as

DNA methylation [17]. However, there have been several controversies in the literature regarding the germ line through which drug–induced heritable traits are transmitted across generations which requires further investigation. Likewise, the causal relationship between genetic predisposition to parental epigenetic imprints associated with cocaine experience and the exact impact of early postnatal fostering on the heritable traits, are yet to be established.

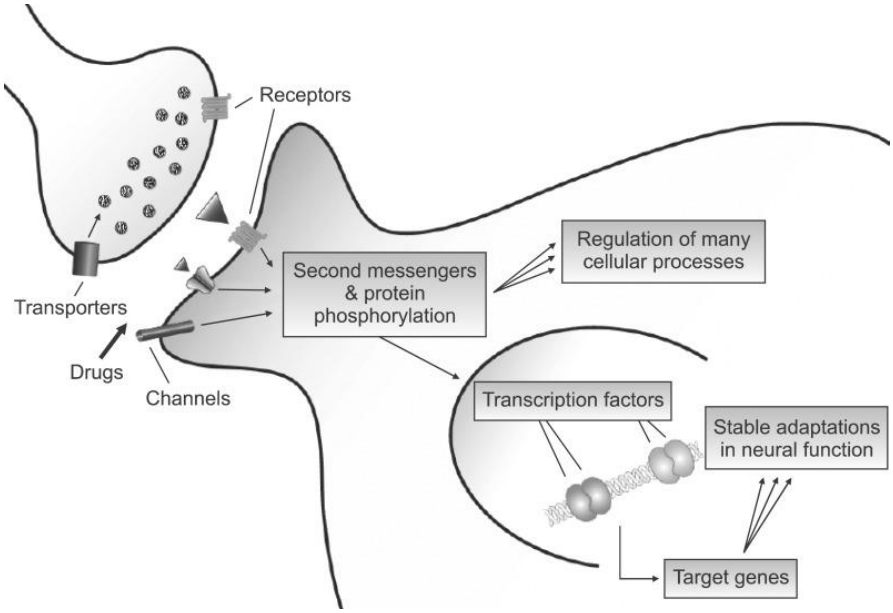

Figure 1: Regulation of downstream trans-factors and intracellular signalling pathways associated with long-term effects of drugs of abuse. Source: Nestler EJ (2012) [18].

1.2 **Cocaine**

Cocaine, also known as benzoylmethylecgonine or coke, is one of the most widely abused drugs in the world [23]. Its use is most prevalent in North America, Europe and South America [23]. According to South African Community Epidemiological Network on Drug Abuse (SACENDU), cocaine is also one of the primary substance of abuse in the country with approximately 9.6% of admission cases reported in 2007 [24]. Treatment indicators further point to significant increase in cocaine related admissions in South Africa with potential implications for public health unless appropriate surveillance systems for cocaine abuse control are installed [2].

Cocaine is a tropane alkaloid derived from *Erythroxylum coca Lam* leaves [25] and often used as recreational drug due to its euphoric and stimulant effects [26]. It is mostly consumed as hydrochloride salt (“crystalline powder”), which can be snorted intranasally, injected intravenously or ingested orally. Cocaine hydrochloride salt can also be converted to an alkaloid form (“crack cocaine”) by adding sodium

bicarbonate. This base form of cocaine is usually smoked by users and characteristically makes a cracking noise when heated [27]. Structurally, cocaine contains hydrophylic methyl and lipophylic benzoyl ester moieties which promote its rapid crossing of the blood brain barrier as well as the nasal mucosa membranes. Depending on the form, amount taken, route of exposure and concomitant use with other drugs, cocaine evokes varying physiological responses. In general, its rapid onset of action begins at 3 to 5 seconds and may last up to 60 to 90 minutes after which it is rapidly metabolized to breakdown products, such as benzoylecgonine and ecgonine methyl ester, by plasma or liver cholinesterases [27, 28].

Like other addictive drugs, cocaine targets the mesocorticolimbic dopamine (DA) system, which originates in the VTA and projects mainly to the NAc and PFC, affecting both glutamatergic and GABAergic synaptic transmission in all three brain areas resulting in synaptic plasticity [29]. These changes may persist long after the drug has been removed from the brain and consequently affect structural organization of the neural circuits [30]. In most cases, these early changes are not sufficient to engender drug dependence, but with repeated exposure, their effects accumulate resulting in addictive behaviour. Understanding these plastic changes has been the goal of many drug abuse related studies as it is thought to underlie drug craving and relapse [29].

Cocaine acts via competitive inhibition of DA re-uptake from the synapse by blocking DA transporter (DAT) action, thus leading to increased concentration and intensity of action of DA at postsynaptic receptors [31] (Figure 2). Just like DAT, transporters for other neurotransmitters (e.g serotonin and norepinephrine) can also be inhibited by cocaine, but its pharmacological action on DA system is considered most important [32].

Cocaine is a potent vasoconstrictor and also produces local anaesthetic and sympathomimetic effects [28]. It causes vasoconstriction either by stimulating α-adrenergic receptors in the smooth muscles of the arterioles or through simultaneous increase or decrease in blood concentrations of endothelin-1 and nitric oxide, respectively [33]. A number of pathologies and toxicities caused by chronic cocaine use are associated with its sympathomimetic potential [27]. Overall, cocaine produces euphoria, increased energy and self-confidence at lower doses. Delirium, agitations, depression, sexual problems and severe organ dysfunctions are generally associated with its prolonged use. Studies have also reported the incidence of congenital cardiovascular and brain malformations in offspring born to mothers with a history of cocaine abuse [34].

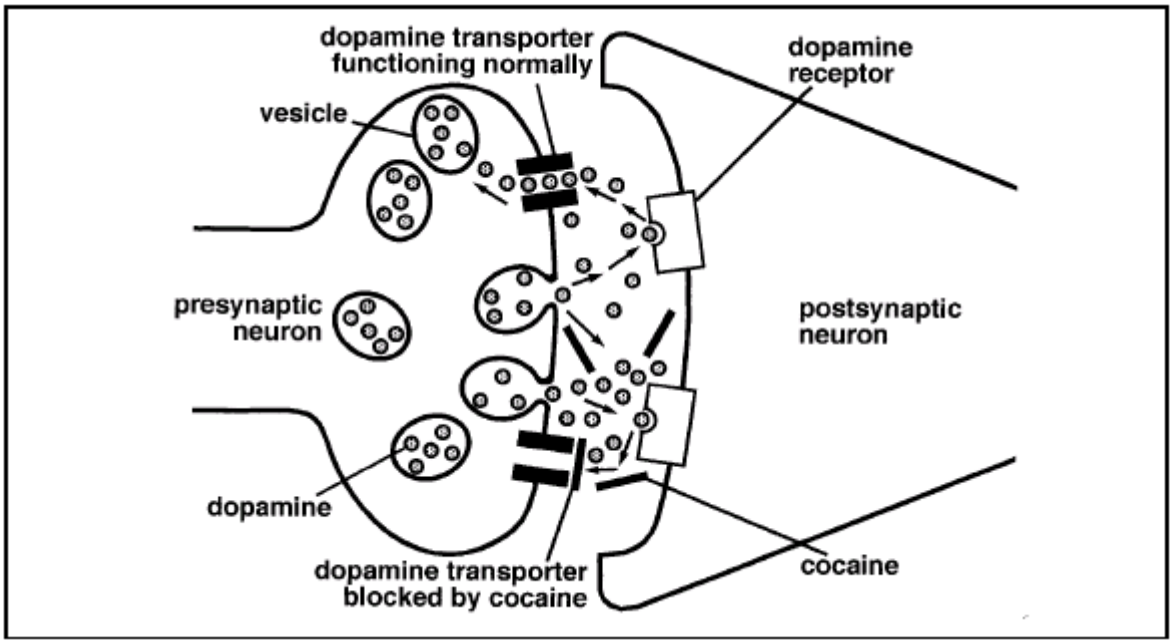

Figure 2: Schematic showing how cocaine blocks the action of dopamine transporter (DAT) re-uptake system at the synapse, leading to heightened dopaminergic neurotransmission in neurons. (Source: *https://science.education.nih.gov/supplements/webversions/BrainAddiction/guide/lesson3-1.html*).

1.3 **Synergistic interactions between cocaine and alcohol**

The majority of cocaine users frequently consume ethanol as well [35, 36]. However, it has been established that concurrent use of these drugs produces a subjective 'high' mostly attributed to the effects of cocaethylene, an active cocaine metabolite formed by hepatic carboxylesterases [26], with consequent neurotoxic and/or neuropsychiatric complications which include cerebral hypo-perfusion, maladaptive behaviour, cognitive impairment and also dopamine dysregulation mainly in the mesocorticolimbic system of the brain [26, 37-40]. Attempts to understand the cross reactivity between cocaine and ethanol effects have yielded comparable reports. For instance, cocaine rewarding effects were potently enhanced by alcohol in rodents and invertebrate animal models tested in the conditioned place preference (CPP) [41]. On the reverse, another study found that cocaine influenced alcohol seeking behaviours and relapse drinking in rats [42]. It has been shown that the reinforcing properties of these psychoactive substances converge within the posterior VTA where synergistic effects are mediated in part by activation of local serotonin receptors [43]. However, continuous use of these addictive drugs or psychostimulants either alone or combined may cause pathological forms of neuroplasticity and contribute to the emergence of aberrant behaviours that involve several distinct forms of learning in both humans and animals [44]. Conditioned responses as previously described by O'Brien et al. (1992) further support the relationship

between the associative learning and drug relapse, which further depends on psychologically and neurobiologically dissociable phenomena such as reinforcement, approach and motivation [45, 46].

1.4 Impact of cocaine on neurocognitive functions

It is a common belief that acute or prolonged exposure to substance abuse causes cognitive impairments [47-49]. A recent study that evaluated neuropsychological functioning in cocaine users suggest that cocaine use is associated with pronounced neuropsychological alterations characterized by general impairments of sustained attention, memory performance and executive functions [50]. In contrast, previous findings also suggest that memory functioning is intact in patients who abuse cocaine. With these controversies, it is therefore unclear the exact impact of cocaine on executive and cognitive functions.

1.5 Dopamine signalling and gene transcription

Complementary data from animal and post-mortem studies have shown that cellular and molecular responses to addictive drugs clearly depend on multiple factors determined by amount and extent of drug exposure, individual vulnerability and the substrate brain regions where observations are made [13, 51]. In general, molecular response to drug stimuli begins with dopamine binding with D1 receptors at the postsynaptic cleft leading to increased production of intracellular cAMP with consequent activation of protein kinase A (PKA) that in turn phosphorylates *CREB* (*pCREB*) in the nucleus. Ca^{2+}-calmodulin kinase II/IV pathway is an alternative pathway for *CREB* signaling. This pathway is activated by ligand binding to AMPA and NMDA receptors [52, 53]. Subsequently, *pCREB* facilitates transcription of downstream genes e.g *BDNF* which, in addition to *Fos* proteins, mediate drug-induced behaviours (Figure 3).

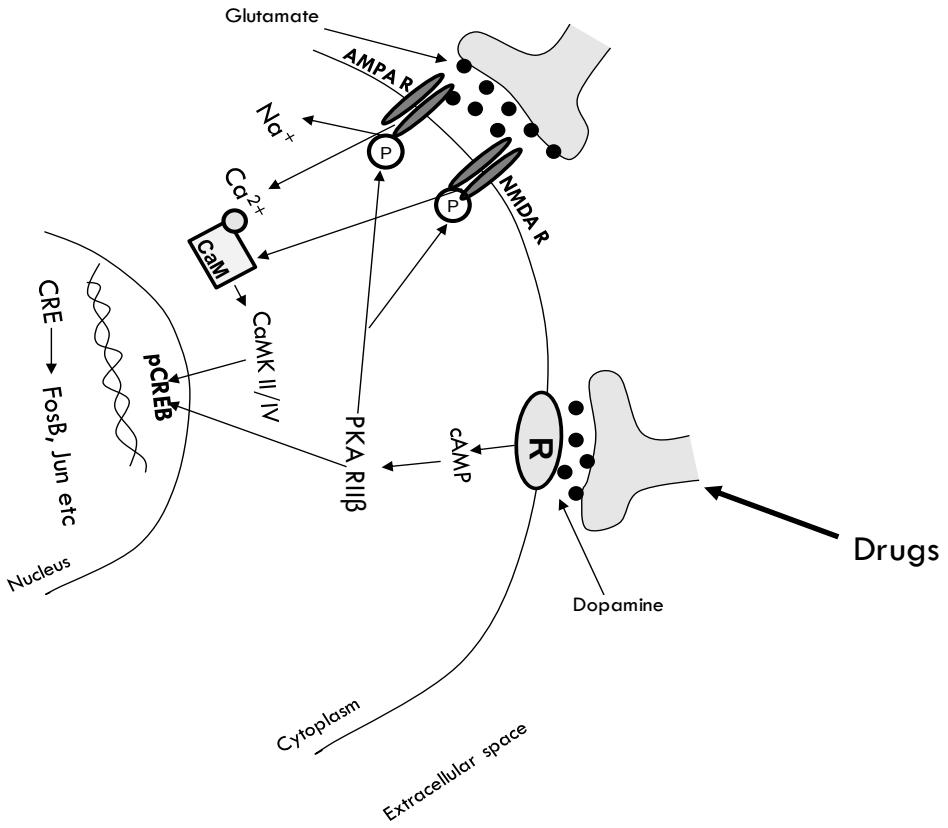

Figure 3: Schematic illustration showing dopamine signalling and transcription factors.

1.6 **Regulation of gene expression by transcription factors**

The classic mechanism for regulating gene expression is through the actions of transcription factors [54]. *Fos* family protein (e.g *FosB* and its splice variant Δ*FosB*) and cAMP response element binding protein (*CREB*) are among the prominent transcription factors identified to play key roles in drug abuse [18]. Δ*FosB* is the pace-setting chemical that begins to accumulate after acute or chronic exposure to cocaine. Together with other *Fos* family members (*c-fos* and *FosB*), they act as master control proteins that regulate expression of certain genes [55]. This is believed to constitute an important molecular "switch" in transiting from recreational use or abuse to full dependence [32]. Research evidence has shown that repeated exposure to cocaine results in a continuous build-up of these control proteins (especially Δ*FosB*) leading to increased sensitivity and enhanced behavioural response to cocaine rewarding effects (Figure 4). These changes are mediated by increased expression of AMPA receptor subunit GluR2 [56] and decreased expression of dynorphin which exerts its suppressive effect on dopaminergic signalling by activating *k* opioid receptors in the VTA [57].

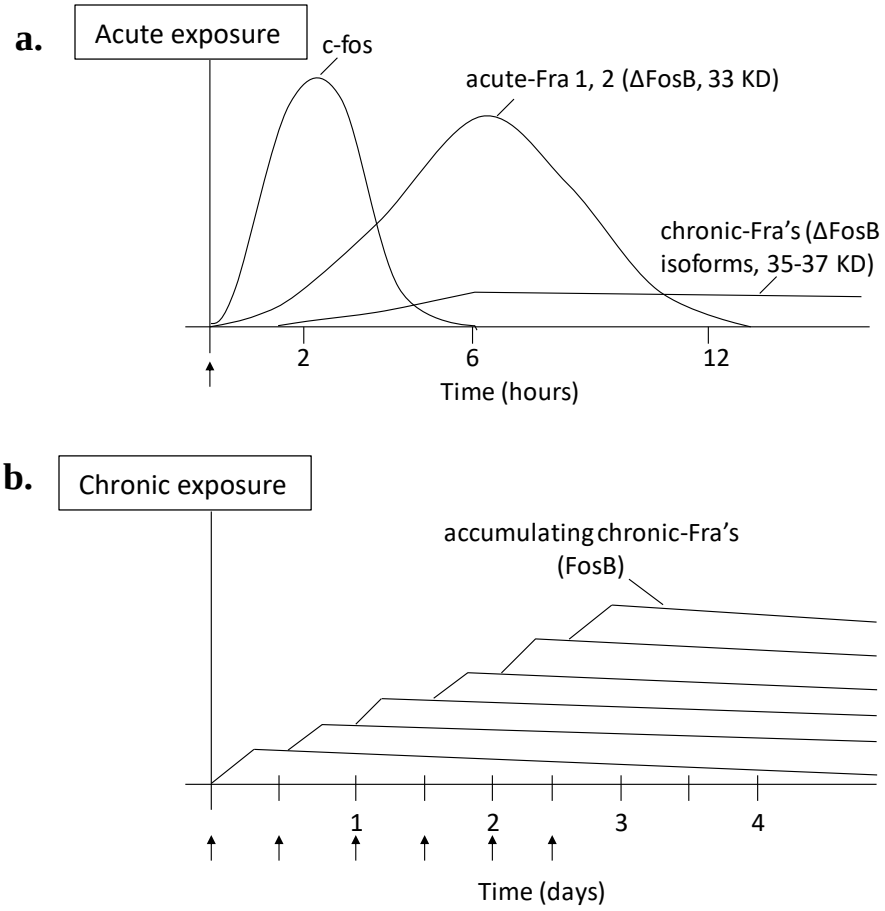

Figure 4: Graphs depicting Expression waves of *Fos* family proteins in the reward brain, NAc (a) after initial high dose of drug, expression levels of *c-Fos* followed by other genes within the *Fos* family sharply increase within the first few hours and persists for relatively short period before returning to baseline levels after drug withdrawal (b) repeated exposure to drugs caused progressive increase in Δ*FosB* expression which may persist for about 6 to 8 weeks. (Adapted from Nestler E.J, 2004) [58].

CREB is another upstream transcription factor critically implicated in neural plasticity, memory formation and drug related events. Dramatic alterations in *CREB* levels have been observed in specific brain areas such as NAc and VTA which are associated with drug reward. In contrast to Δ*FosB*, experimental induction of *Creb* overexpression in the NAc of mice resulted in decreased rewarding effects of cocaine as assessed in the CPP [59, 60]. Also, chronic administration of either drugs activated *Creb* within dopaminergic and non-dopaminergic neurons of the VTA, an effect which either potentiated or attenuated the rewarding responses to drugs of abuse depending on the substrate region of the VTA affected [18]. It has also been established that drug stimuli initiate intracellular signalling events that activate *CREB* binding to DNA sequence, cAMP response element (CRE), in the target gene promoters where it modulates transcription of certain downstream genes such as *c-fos* and brain derived

neurotrophic factor (*BDNF*) [61]. In addition, studies have shown that cocaine-induced expression of *Fos* proteins and *Creb* enhanced *Bdnf* activity in the NAc and PFC which in turn increased the number of dendritic branches and spines in these brain regions [61]. *CREB* is structurally and functionally similar to *CREM* and *ATF-1* (activating transcription factor-1), collectively referred to as members of *CREB* family. Structurally, they contain two domains, a C-terminal and Leucine Zipper that mediate DNA binding and dimerization [62]. Amongst alternatively spliced transcripts of *CREM* (repressor α and β isoforms), the powerful activator *CREMτ* has been reported to bear close structural and functional similarities with *CREB* and both factors mediate transcriptional activation of intracellular machinery [63]. Cascade events initiated by *CREB* or *CREM* activation are usually preceded by PKA phosphorylation at serine residue 133 or 117, respectively [64]. For many years, expression of *CREM* isoforms was thought to be restricted to neuroendocrine nuclei but it has now been found to be expressed ubiquitously throughout the brain [65]. *CREB* deletion studies have reported simultaneous upregulation of *CREM* in the brain and found to confound the predictable effects of diminished *CREB* on behaviour, learning and memory as well as gene transcription [66, 67]. Moreover, a previous study revealed that neurological impairments and progressive atrophies observed in the dorsolateral striatum, hippocampal neurons and in some regions of thalamus, amygdala and PFC, caused by postnatal central loss of *CREB* and *CREM*, were prevented by a single copy of *Crem* [68]. These findings support that *CREM* does not only encode the potential to effectively compensate for *CREB* deficiencies but also complement its functionality. *CREM*-null mice are also known to exhibit hyperlocomotion and altered emotional states [69]. Despite versatility and widespread effects of *CREM*, its role in drug addiction or drug abuse related disorders remains poorly understood. Although, a more recent research study highlighted the relevance of *CREM* in mediating neuroplasticity and impulsivity related to substance abuse and further proposed that targeting this neuromodulator protein and its regulated networks may be of therapeutic potential [15].

1.7 Impact of early postnatal fostering on drug–induced behavioural phenotypes and epigenetic profiles

It is well documented that prenatal/gestational exposure to stress or potential toxicants such as cocaine may cause behavioural modifications in dams which in turn interrupt maternal–offspring interactions and subsequently influence offspring neurobehavioral development [21] including cognition [20]. These changes in maternal behaviour may result from altered activities in the endocrine system [70] and neural circuits which involve media preoptic area, VTA and substantial nigra [71, 72]. However, fostering is a common practice used to dissociate the relative influences of genetic factors from impact of postnatal alterations in maternal and/or fetal environment on various neurobiological phenotypes [73], as previously demonstrated in rodents [74, 75] and primates [76]. Frankling and colleagues (2010) have

shown that maternal separation altered DNA methylation profiles in specific gene promoters in germlines and brains of separated male mice [77]. Most studies that considered the exact effects of early postnatal fostering on maternal/offspring interactions in rodents have mostly focused on stress/anxiety, aggression and social related factors [73, 78], whereas data on drug induced epigenetic profiles and impact on the vulnerability of the offspring are scarce.

1.8 **Hypotheses**

Four hypotheses were tested in this study. At first, we predicted that ethanol concurrently presented with cocaine in the IC would act as a "gateway" drug and greatly influence cocaine consumption to a point that engender dependence. Secondly, we thought that the background social interaction episodes associated with both IC and CPP paradigms would intercept both behavioural and epigenetic response of mice to cocaine's rewarding effects; the extent of interception was not envisaged, but we thought that molecular changes in the IC–treated animals would greatly differ due to extended voluntary drinking access to cocaine as opposed to experimenter–forced injections in the CPP. Thirdly, we also hypothesized that direct exposure to drugs would not only cause alterations in gene functions and marked changes in DNA methylation patterns of the cAMP-neuromodulator and control protein, *Crem* and *Fosb*, respectively, within the neurocognition-regulated brain structures (PFC and HPC) of the parent mice, but could also be passed down the germ lines to be present in the same brain regions of their non-drug exposed offspring. We further speculated that inheritance of the parental epigenetic imprints is more likely to be paternal given the dominant role male chromosomes play at fertilization, while behavioural phenotypes may be attributed to maternal experience during dam–to–pub interaction. Lastly, overall impact of early postnatal fostering was also envisaged, particularly in altering gene–specific programs and/or epigenetic profiles as well as inducible phenotypes through modifications in the environment.

1.9 **Overall aim of the study**

Generally, this study aimed at investigating associations of cocaine–induced behavioural changes in social context with DNA methylation patterns of inducible transcription factors in neurocognitive–dependent brain regions of drug-exposed mice, with a quest to understand whether the induced epigenetic changes are inheritable and further established the impact of early postnatal fostering on the neurobiological sequalae and associated functional components.

1.10 **Study objectives**

Specific objectives formulated for the studies (Chapters three, four, five and six) included in this thesis are:

1- to establish a mouse model of drug abuse within a social "home–cage" environment in a manner that mirrors the ethologic conditions in humans, as opposed to experimenter–forced injections in the CPP.
2- to characterize drug taking behaviours in a free–living environment and determine the sequential "gateway" effect of ethanol on cocaine consumption or vice-versa in the IC system.
3- to identify and compare epigenetic changes (alterations in DNA methylation) within the gene promoters of *Crem* and *Fosb* in the PFC and HPC of male and female mice that were either treated or had free access to cocaine. These brain areas were chosen as they are important mediators of executive brain functions, conditioned learning and context-specific memories associated with rewarding effects of drugs.
4- to examine whether these epigenetic changes are inheritable, therefore present in non-drug exposed offspring and establish whether the inheritance is paternal or maternal.
5- to examine the modulatory impact of early postnatal fostering on locomotion/exploratory behaviour, cognitive and neurobiological changes.

1.11 **Study design**

To achieve the specific aims highlighted above, study plans / treatment protocols were developed and enclosed as schematic illustrations in chapters three (Figure 1), four (Figure 2) and six (Figure 1) of this thesis.

1.12 **Potential benefits of this research**

Despite intensive research and significant advances, drug abuse remains a substantial public health problem and constitutes an increasing emotional and economic burden to local societies. South Africa is not an exemption. One of the reasons for this pandemic is that the underlying pathophysiological mechanism of this disorder is not fully understood. For example, the inheritability of drug–induced phenotypes, its impact on the vulnerability of the offspring, or the roles of trans-factors involved, remains unclear. Our study addressed some of these uncertainties. To date, there is no effective treatment for drug–related disorders. Also, our study is among the first to showcase the positive impact of social interaction in warding off the addictive potential of cocaine. Hence, critical assessment of social status in drug users may improve therapeutic outcomes possibly by breaking the link between substance use and transition to dependence. Investigating novel drugs for this disorder should consequently be high on the priority list of any laboratory in this field. As obtained in our study, *CREM* appears to encode some therapeutic potential based on its active molecular response to drug induced gene transcription that paralleled behavioural control. Unravelling the pharmacological importance of this trans-factor may set a

new direction in the search for improved drug abuse therapy. Also, we speculate that children of foster mothers may be at risk of increased vulnerability to drug effects due to perturbations in maternal–fetal interaction and/or environment, as substantiated by neurobiological changes caused by early PN fostering in our study.

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CHAPTER TWO

The current chapter forms part of my literature review and has been published in Cellular and Molecular Life Sciences. The article is titled: **"Epigenetics: a link between addiction and social environment"**. All figures with legends as well as tables with footnotes have been included for easy reading and understanding.

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Abstract

The detrimental effects of drug abuse are apparently not limited to individuals but may also impact the vulnerability of their progenies to develop addictive behaviours. Epigenetic signatures, early life experience and environmental factors converge to influence gene expression patterns in addiction phenotypes and consequently may serve as mediators of behavioural trait transmission between generations. The majority of studies investigating the role of epigenetics in addiction do not consider the influence of social interactions. This shortcoming in current experimental approaches necessitates developing social models that reflect the addictive behaviour in a free-living social environment. Furthermore, this review also reports on the advancement of interventions for drug addiction and takes into account the emerging roles of histone deacetylase (HDAC) inhibitors in the etiology of drug addiction and that HDAC may be a potential therapeutic target at nucleosomal level to improve treatment outcomes.

Keywords: Cocaine and alcohol abuse; DNA methylation; Chromatin remodeling; Epigenomic programming and inheritance; Environmental stimuli; Social stress.

Introduction

Drug addiction is a chronic relapsing disorder where the individual is unable to control his or her drug-seeking and drug-taking behaviours despite severe adverse consequences [1]. Clinically, drug addiction presents with behavioural, cognitive and physiological symptoms that reflect the involvement of complex control systems of the brain [2]. Due to the increase in risky behaviours, this disorder is often associated with trauma and death [3]. Epidemiological surveys have shown that not only is addiction highly prevalent on its own, but that the disorder also frequently occurs as a comorbidity with other mental disorders [4]. The hundreds of research projects conducted all over the world underline the importance of studying addictive behaviour. In fact, many of the current trends of addiction research now have a well-defined focus that attempts to understand the neural mechanisms that underpin the conventional change from recreational drug use to a chronic addicted state with persistent addictive behaviours even after long abstinence and relapse propensity in drug addicts [5]. Many studies on chromatin remodelling and transcriptional regulation have shown that exposure to drugs of abuse induces changes in the expression of specific genes such as brain-derived neurotrophic factor (*BDNF*), activator of G-protein signaling 3 (*AGS3*) and transcription factors (e.g Δ*FosB*) in key reward areas of the brain such as the ventral tegmental area (VTA), nucleus accumbens (NAc) and prefrontal cortex (PFC) [6-10]. Despite the substantial progress that has been made, our understanding of the molecular underpinnings of drug addiction remains incomplete. For instance, the impact of epigenetic alterations in combination with social environmental factors within the context of addictive behaviour appears underexplored. The purpose of this article is therefore to provide an overview of the current status of research in the field of drugs of abuse with specific reference to these factors, with a focus on how they might play a role in the etiology of drug abuse and its inheritance. We also report on current pharmacotherapies and highlight possible novel treatments that could be considered for drug-related disorders (Table 1).

Table 1 Overview of studies on the epigenetic regulation by drugs of abuse

Drug of Abuse	*Brain region*	*Epigenetic Mechanism*	*Residues*	*Implication*	*References*
Ethanol	AMY	Methylation	H3K27 / H3K4	Susceptibility to alcohol dependence	[118]
	AMY	Acetylation	H3K9	Susceptibility to alcohol dependence	[118]
	VTA	Acetylation	H3K9	Ethanol withdrawal	[119]
	NAc	Acetylation	H4K12	Persistence of ethanol-related behaviours	[120]
Cocaine	NAc	Acetylation	Gen H3	Cocaine addiction-related behaviours	[105, 121, 122]
	VTA	Acetylation	Gen H3	Motivation for drug reinforcement	[123]
	NAc	Acetylation	H3K14 / H4K12	Cocaine addiction-related behaviours	[105, 124]
	NAc	Methylation	H3K9	Cocaine addiction-related behaviours	[75, 105]
	NAc	Acetylation	Gen H4	Motivation for drug reinforcement	[74, 125]

Abbreviations: AMY (Amygdala); VTA (Ventral tegmental area); NAc (Nucleus accumbens); DG/ Hipp (Dentate gyrus/Hippocampus).

Epigenetic mechanisms

Both genes and the environment are important determinants of developmental processes. Subtle differences in the interaction between genes and the environment may be responsible for altering developmental trajectories that confer vulnerability or resilience to mental conditions such as addiction. At the molecular level, epigenetics provides invaluable insight into the interaction between an individual's genome and the environment [11]. Epigenetics, also known as chromatin remodeling, is defined as heritable chemical modifiations to DNA capable of influencing transcriptional activity independent of DNA coding sequence [12]. Principally, epigenetic mechanisms include DNA methylation and various chemical modifications of the histone proteins (H2A, H2B, H3 and H4). In general, histones have a core (C-terminal) where other signalling proteins assemble and a tail (N-terminal) which is mainly made up of two amino acids, lysine and arginine (see Fig. 1a). Most complex and coordinated series of epigenetic modifications such as acetylation, methylation, phosphorylation, ubiquitylation and ADP ribosylation occur at the N-terminal tail of histones [13-15]. It is worth noting that recent findings have revealed additional mechanisms involving RNA interference and prion proteins which also contribute to epigenetic regulation [16]. In general, *DNA methylation* and *histone acetylation* remain the most

recognized, widely studied and established epigenetic modifications [17]. These two mechanisms work in tandem to cause chromatin remodeling and thus regulate gene expression.

DNA methylation

DNA methylation is generally linked to gene silencing due to its repressive effect on gene transcription. It either disrupts association of DNA binding factors with their target sequence or recruits transcriptional co-repressors by binding to methyl-CpG-binding protein-2 (*MeCP2*) [18] to induce an inactivated, condensed (silenced) chromatin state. Studies have shown that MeCP2 mediates behavioural responses to addictive properties of alcohol and cocaine mainly by altering *BDNF* expression in specific regions of the brain [19-21]. DNA methylation modification occurs only within specific gene promoters to produce stable epigenetic changes as opposed to histone tail modifications which are readily reversible [22, 23]. DNA methyltransferases (*Dnmt*) catalyze and maintain the sequence of gene expression events induced by DNA methylation. *Dnmt1*, *Dnmt3a* and *Dnmt3b* are highly expressed in postmitotic nerve cells [24]. Altered expression of these *Dnmt* have been implicated in drug dependence and other related psychiatric disorders. For instance, *Dnmt1* is highly expressed in GABAergic neurons whose dysfunction characterizes development of schizophrenia [25, 26]. Both *Dnmt3a and Dnmt3b,* are capable of inducing methylation on naked DNA [27]. Expression of *Dnmt3a* in the NAc was increased by chronic social defeat stress and cocaine infusion suggesting its importance in regulating emotional behaviour and cocaine addiction [17, 28]. In response to cocaine treatment, it appears that *Dnmt3a* expression is biphasically regulated. Evidence from quantitative PCR analysis of NAc tissue from mice acutely and chronically pretreated with cocaine suggests that early withdrawal (4h after injection of cocaine) upregulated *Dnmt3a* expression, whereas after 24h it was downregulated [28]. Mice lacking both *Dnmt3a* and *3b* in mature forebrain neurons displayed impaired long-term plasticity and performed poorly in learning and memory tasks which was attributed in part to dysregulated gene expression. Other studies have shown that murine *Dnmt3b* knockouts exhibited neural tube defects that led to early lethality [29].

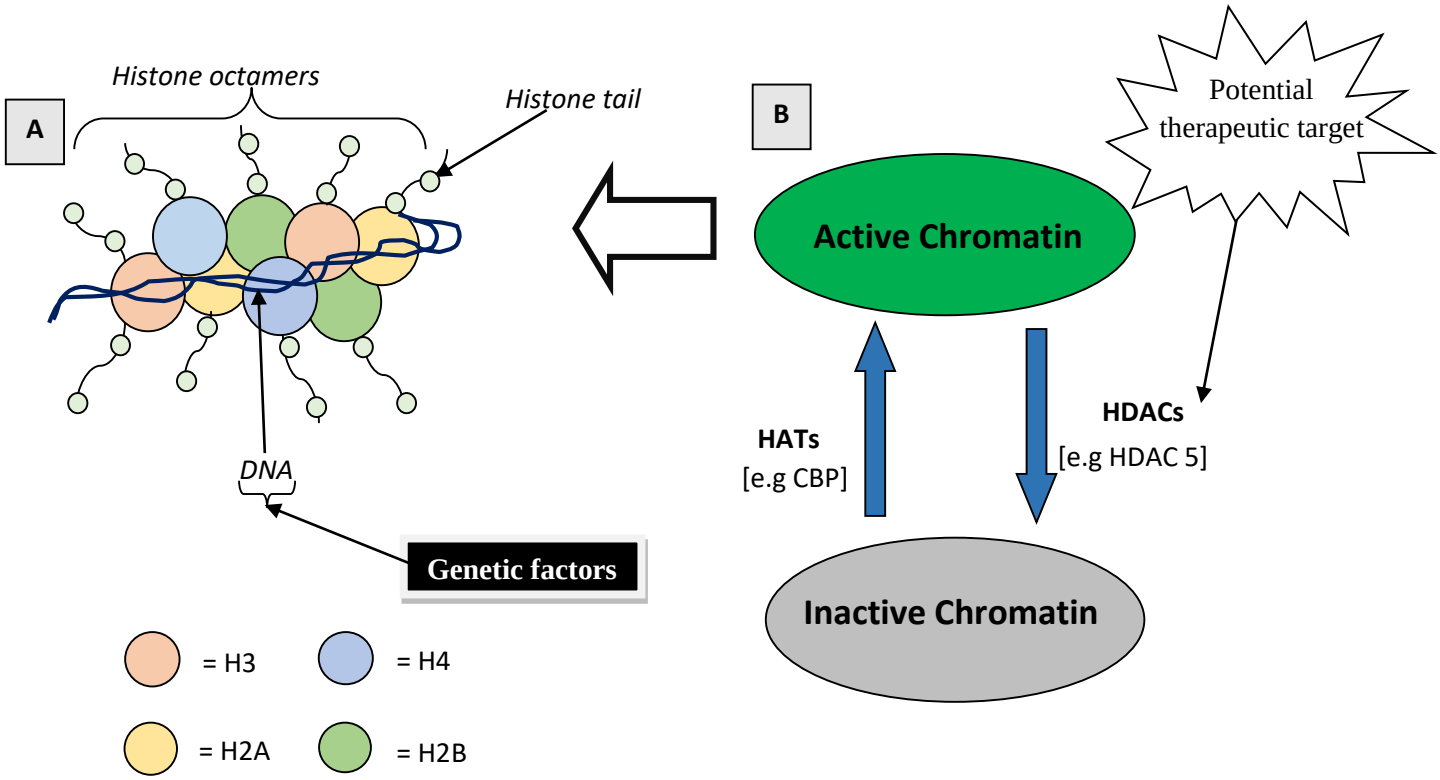

Fig. 1: Schematic view of post-translational modifications of histones. **a** In eukaryotic cells, the DNA (consists of 147 base pairs) wraps around histone octamers (two copies each of H2A, H2B, H3 and H4) to form the nucleosome which is the functional unit of the chromatin. Projecting from the core of the histone octamers is the amino acid (-N-) terminal tails on which significant transcriptional modification occurs. **b** Transcriptional active or inactive states of the chromatin are balanced by the opposing actions of HATs and HDACs, respectively. Enhanced action of HATs promotes histone acetylation which allows assessibility of the DNA to the transcription factors by relaxing the chromatin resulting in an enhanced gene activity. Conversely, increased action of HDACs represses transcription (gene silencing) by deacetylating the histones. HDAC5 is one the II HDACs that shuttles between the nucleus and the cytoplasm and actively mediates gene silencing mechanism by binding hormone co-repressors. Consequently, nuclear export of HDAC5 which results in histone hyperacetylation as well as increased mRNA expression of its target genes (e.g substance P and neurokinin 1) have been critically implicated in sensitized behavioural responses to addictive drugs [57]. Also, toxicants such as drugs or endocrine disruptors can induce epimutations of the histone octamers [73] resulting in chromosomal abnormalities that are fundamental to addiction phenotypes and disease.

Histone acetylation

Histone acetylation is considered a good marker of actively transcribed genes [30]. Most genomic studies involving histone acetylation have focussed on the amino acid (-N-) terminal lysine residues in histones H3 and H4 [15]. Following acetylation at lysine residues, the electrostatic interaction between the DNA and histone proteins reduces, resulting in a relaxed chromatin state that allows transcriptional regulators to access the DNA [31]. It therefore implies that increased or low levels of histone acetylation within specific promoter regions correlates with enhanced or repressed gene activity, respectively. These dynamic processes are actively controlled by two key enzymes histone acetyltransferases (HATs) and histone deacetylases (HDACs) (see Fig. 1b). The roles that HDACs and their inhibitors play in drug addiction are described below.

Social environmental factors that increase susceptibility to drug addiction

Human and animal studies suggest that there may be a direct relationship between environmental stress prior to drug exposure and the development of addiction-like behaviours. A meta-analysis showed that a degraded home environment significantly increases the risk for drug-related disorders to develop [31], while an enriched environment, such as positive family relationships, involvement and attachment, appear to discourage drug use and prevent drug addiction [32]. The relationship between an adverse environment and drug dependence is underpinned by the interaction of multiple endocrine, paracrine and intracellular systems [33], since these systems have been shown to be sensitive to social experience. It is therefore not surprising that drug addiction is highly prevalent in vulnerable populations undergoing social stress [34]. Many factors confound the study of drug addiction-related social stress and physiology in human populations, including long life spans, inaccuracy of self-reporting, ethical considerations, and high levels of genetic variation [35]. Animal models circumvent many of these challenges and provide more tractable systems to study the interplay of social factors and drug addiction. For example exposure to stress *in utero* has been shown to modify the behavioural reactivity of rats to drugs with addictive potential [36]. Similarly, an increase in drug seeking has been observed during adulthood in offspring of maternally stressed dams. Experiments utilising isolation stress and social deprivation yielded comparable results [37]. Poor maternal behaviour towards the pups and/or maternal separation during the early postnatal period increased ethanol consumption in adult rats [38]. Maternal licking and grooming correlated negatively with vulnerability to cocaine and ethanol use in rats [39]. Repeated social defeat during adolescence increased cocaine self-administration and cocaine-induced conditioned place preference (CPP) in rodents [40-43]. Furthermore, these stress events were suggested to share common epigenetic

process with drugs of abuse to influence addiction-like behaviours. Indeed, it has been demonstrated that both early life stress and methamphetamine alter the expression of the epigenetic regulator methyl CpG binding protein 2 (MeCP2) in the nucleus accumbens to influence the motivational effects of methamphetamine and natural reinforcement [44-46]. These basic studies offer valuable insights into the effect of adverse environmental conditions on addictive behaviour. Similar studies have demonstrated the involvement of epigenetic changes in these effects [43, 47] such as the epigenetic mechanisms responsible for dysregulation of the hippocampal glucocorticoid receptors [48] and upregulation of histone acetylation by social defeat [43]. Epigenetic alterations induced by early life experiences have been shown to accumulate over time and have consequently been considered serious risk factors for mental disease development [49, 50]. This line of work on epigenetic regulation related to environmental stimuli has raised many questions, including and among others, (i) how the pharmacological effects of drugs with addictive potential may vary depending on the associated environmental conditions to which subjects may be exposed, or (ii) to what extent social stress influences drug consumption, or (iii) which genes are altered and in what way, by various environmental circumstances.

Mild stress and environmental enrichment have also been shown to protect and sometimes reverse addictive phenotypes. The chronic mild stress of neonatal handling prevented reinstatement of morphine CPP in adulthood [51] and environmental enrichment blocked reinstatement of ethanol-induced CPP [52]. It also reduced cocaine seeking and reinstatement induced by cues and stress [53, 54]. Similar to environmental enrichment, overexpression of ΔFosB decreased cocaine self-administration, enhanced extinction of cocaine seeking, and decreased cocaine-induced reinstatement of intravenous cocaine self-administration [55].

Interestingly there is evidence that suggests that neurons do not only respond to various environmental signals via dynamic changes in epigenetic modifications [31], but also vary in sensitivity to drugs of abuse subject to altered states in the environment (see Figure 2) [32]. So, while there is no doubt of an interaction between the environment and addictive behaviour, the underlying mechanisms that facilitate this interaction continue to be poorly understood.

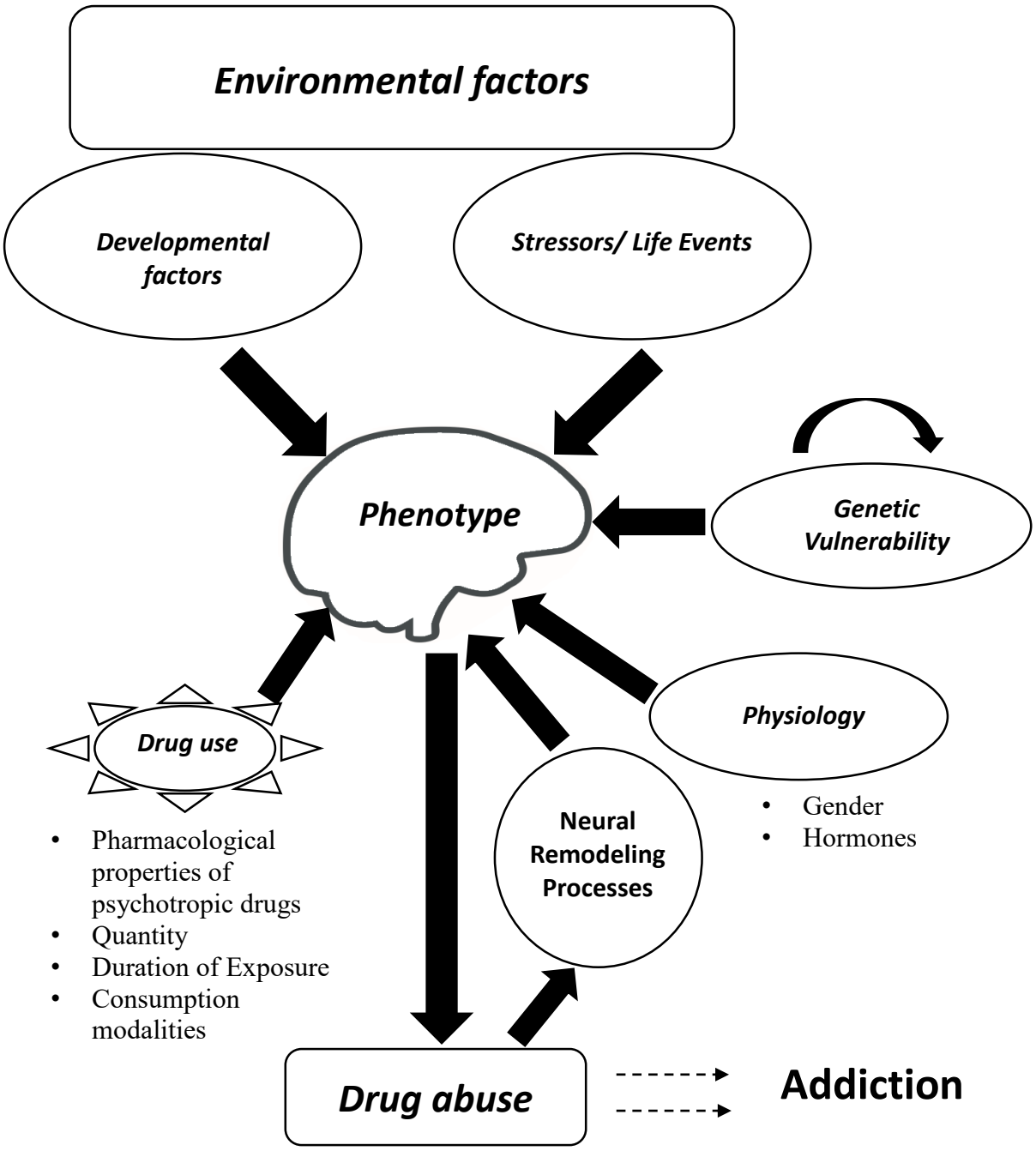

Fig. 2: A model of possible factors that influence drug intake. Altered states in the environment influenced by various factors like physical contact, gender, family history, social or early life experience all converge to impact on the individual's sensitivity and vulnerability to addictive compounds. In contrast, escalated or chronic drug intake especially at high doses possibly induces changes that accumulate over time to promote further drug use or addictive behaviours and sometimes may be passed down the germline to the next generation. Invariably, behavioural (drug intake) and psychological (vulnerability) balance largely depends on the level of exposure to drugs and associated factorial states within the environment.

Inheritance of epigenetic imprints and trans-generational effects

The vulnerability of progeny to drug-induced maladaptive behaviours or neural plasticity are jointly influenced by both genetic and non-genetic factors. Many authors share the opinion that drugs of abuse are likely to induce epigenetic changes in parent sex cells (ova and sperm) which are passed down to future generations [18] and thereby predispose the offspring to subsequent drug effects and/or addiction. The fact that some epigenetic imprints, particularly maternal DNA methylation, can escape the epigenomic reprogramming that occurs during gametogenesis and fertilization [56] provides a mechanism that may enable the transgenerational transfer of parental traits. Some epigenetic imprints may therefore accumulate over a lifetime and be conserved between generations. This implies that the inheritance of acquired traits resulting from environmental exposure that alters a phenotype in one generation can be transmitted epigenetically to unexposed offspring [57]. This notion has profound implications for understanding how diseases may be prevalent in families as well as how the genome functions as an etiological factor in hereditary diseases.

The impact of social environment on the epigenome and its transgenerational transfer

Drug intake related to social context involves long-lasting epigenetic underpinnings that may be transmissible from one generation to another. The genes affected by social factors, are mostly related to regulatory networks that control the hypothalamic-pituitary-gonadal axis and a variety of social behaviours [58]. Paternal transmission of epigenetic variation may manifest only in later life, when the social environment changes [59]. In male mice, chronic social stress (achieved through instability of social hierarchy) experienced during adolescence through to adulthood induced social deficits and increased anxiety-like behaviours in up to two succeeding generations [60].

These observations in animal models find their counterpart in human studies. Recently, it was shown that differences in socio-economic status early in life are imprinted on the epigenome and maintained into adulthood. Several hundred promoters showed different levels of DNA methylation in blood profiles of adults who experienced social adversity early in life when compared to those that did not [61]. Similarly, global DNA hypomethylation was observed in blood samples of socio-economically deprived subjects [62]. These clinical observations highlighted the ‘epigenome’ as an interface between the social environment and the genome. However, genome-wide assays (GWA) of epigenetic changes in different regions and cell types of the brain are necessary to fully understand how specific epigenetic modifications may both influence and be caused by social behaviour. These previous studies indicate that the social component may have tremendous impact on the physiological and behavioural responses of an individual to environmental factors.

The impact of substances of abuse on the genome and its transgenerational transfer

Adding to the transgenerational effects of social conditions, chronic exposure to several drugs of abuse, including alcohol and cocaine, have been reported to induce epigenetic changes which points to a dysregulation of gene expression in both the brain and periphery [5, 57].

Alcohol has been shown to interfere with the epigenetic regulation of gene expression. A recent transcriptome study, comparing human post-mortem brain samples of alcoholics and age-matched controls, showed profound epigenetic effects of alcohol abuse. A notable difference between the two groups was that endogenous retroviral sequences that maintain DNA methylation throughout gametogenesis and fertilization, which are normally silenced by DNA methylation, were less methylated, coinciding with dramatically increased transcription of their host genes [63]. *In utero* studies in mice have shown that exposure to alcohol induces teratogenic effects related to epigenetic changes in the foetus. In female mice, free access to 10% (v/v) ethanol for four days per week for ten weeks affected the adult offspring phenotype by altering the epigenotype of the early embryo. These alterations in the epigenome were associated with postnatal growth restriction and craniofacial dysmorphology reminiscent of foetal alcohol syndrome [64]. Alcohol exposure *in utero* also reduced DNA methylation at the differentially methylated domain of the paternally imprinted growth-related gene *H19* in the sperm of exposed mice (F1). Most notably, a similar decrease at the same CpG sites was observed in the brains of the offspring (F2) [65]. These epigenetic changes caused by alcohol exposure resulted in a variety of developmental disturbances ranging from reduced litter size and birth weight to behavioural alterations such as lower fearfulness and higher aggression. These findings from animal studies demonstrating how parental alcohol consumption may induce epigenetic aberrations that negatively affect the normal structure and function of their offspring were corroborated by clinical observations. For example, moderate and heavy drinkers show subtle reductions in DNA methylation at the *H19* imprinted gene in their sperm compared to non-drinkers [66], and alcohol-induced parental epigenome changes such as these have been suggested to have detrimental effects on the cognitive performance of their children [67].

Changes in histone acetylation in the PFC of ethanol-exposed adolescent rats are associated with ethanol-induced place conditioning [68]. Intermittent alcohol exposure upregulated HAT activity in adolescent rat PFC and increased histone acetylation and dimethylation in the promoter region of *cFos*, *Cdk5* and *FosB* [69]. Alcohol exposure during adulthood has been shown to be associated with downregulation of genes implicated in neural plasticity such as cut-like 2 (*cutl2*), insulin-like growth factor 1 (*Igf1*), epidermal growth factor–containing fibulin-like extracellular matrix protein 1 (*Efemp1*), SRY-box-containing gene 7 (*Sox 7*) and many others, as well up-regulation of *SWI/SNF* [*an ATP dependent chromatin remodelling complex that mobilizes histone octamers*] related, matrix associated, actin dependent regulator of

chromatin, subfamily a, member 2 (*Smarca2*), the cytosolic enzyme DiGeorge syndrome critical region gene 2 (*Dgcr2*) and *Pard6a* which are implicated in the migration of immature granule neurons and neuroblast cell polarity [57]. In addition, cocaine and alcohol exposure have been associated with significant decreases in the mRNA levels of enzymes responsible for DNA methylation in the testes and sperm of adult male rodents, presenting a high risk induction factor for heritable epigenetic changes [70, 71]. Altered methylation related to alcohol was associated with dysregulation of genes known to play a role in metabolism such as (*Cyp4f13*) and decreased methylation of genes associated with development (*Nlgn3*, *Elavl2*, *Sox21* and *Sim1*), imprinting (*Igf2r*) and chromatin (*Hist1h3d*) which contribute to abnormal foetal development [70].

Cocaine has a profound effect on chromatin remodelling in brain areas such as the NAc and PFC – brain regions key in processing reward and implicated in addiction [72]. Chronic cocaine treatment and self-administration induced epigenetic dysregulation of expression of several genes associated with neural plasticity such as the immediate-early gene *c-Fos*, *BDNF*, cyclin-dependent kinase 5 (*cdk5*) and myocyte enhancing factor 2 (*MEF2*) [5, 73]. Chronic cocaine-induced hyperlocomotor activity and expression of CPP in rats was associated with a decrease in gene expression in the NAc caused by an increase in DNA methylation and a decrease in global levels of histone H3 acetylation [74]. Moreover, using conditional mutagenesis and viral mediated gene transfer, Maze et al. [27] showed that histone methyltransferase *G9a* downregulation increases dendritic spine plasticity of NAc neurons and enhances rewarding responses to cocaine by decreasing repressive *H3K9me2* at specific target genes thus increasing the expression of those genes [75]. Vassoler et al. [28], using histone H3 acetylation in *BDNF* promoters as an epigenetic marker, showed that voluntary paternal ingestion of cocaine resulted in epigenetic reprogramming of the germline that changed medial PFC gene expression to such an extent that the male offspring became resistant to cocaine reinforcement [76]. This observation suggests that epigenetic re-programming of the genome in offspring of addicted parents may serve as a protective mechanism in rendering the next generation less susceptible to future addiction vulnerability. However, administration of cocaine during gestation has been shown to alter global DNA methylation in several promoter regions for genes implicated in crucial cellular functions [77]. For instance, maternal cocaine consumption during gestation caused increased CpG methylation at two *SP1* binding sites in the promoter region of protein kinase C (*PKC*) that precipitated down-regulation of *PKC* expression in the heart of adult offspring. This change in *PKC* expression rendered these animals more sensitive to ischemia and reperfusion injury [78]. The genomic effects of parental drug intake and how these effects impact on the well-being of the offspring therefore remains controversial.

Animal models of drug addiction

Animal models of drug addiction have enabled the implementation of protocols that are used to characterise addictive behaviour, as well as facilitating the study of trans-generational effects over short periods. Over time these models have undergone a number of refinements that allowed a deeper understanding of the circuitry involved in drug craving, relapse and loss of control in several behavioural paradigms, in particular, tests of behavioural sensitization, CPP and drug self-administration. Despite the advances made, these classical paradigms do not allow a continuous assessment of addictive behaviour, nor do they consider the social contexts of addicts in the neuropathology of addiction [79]. It has been repeatedly shown that social factors that trigger craving and drug-seeking behaviour in animals and humans induce lasting behavioural and neurogenic changes. Moreover, recent behavioural sensitization studies of housing conditions demonstrated that social isolation alters the neuronal functioning of the dopaminergic and serotonergic systems, evoking changes in sympathetic neurotransmission [80-82]. Such environmentally induced changes may potentiate the post-sensitization conditioned locomotor response to cocaine that is said to be mediated by alterations in dopamine D2 receptor density and can be further modified by ethanol treatment. Furthermore, animals subjected to overcrowded conditions appear to consume more ethanol than isolated animals that in turn drink more than animals housed under standard conditions (4 animals per cage). In line with these studies, McCormick et al. [37] have shown that daily social isolation for one hour followed by pair housing with an unfamiliar partner induced anxiety and endocrine changes [83] that was associated with suppressed hippocampal cell proliferation and impaired adult object recognition in a spatial memory test [84]. One neural mechanism that may underlie the role of social context in increased risk for drug abuse is the imbalance between mineralocorticoid and glucocorticoid receptor levels in the limbic system and in the hypothalamic-pituitary-gonadal axis [57, 85-87]. Thus, development of laboratory animal models for drug addiction should allocate more consideration to the social component in order to generate data that ethically and practically represent a valid construct of the human condition and closely resemble the componential behaviours of an addicted subject in a free-living state.

Pharmacological treatment of addictive disorders

Current medications and novel therapeutic approaches

Attempts to manage drug addiction have included behavioural and psychosocial interventions [88, 89] as well as various medications [90-93] (See Table 2 for tested medications for cocaine and alcohol addiction). Biologics such as monoclonal antibodies, vaccines and engineered enzymes are currently being proposed as alternatives to addiction pharmacotherapy [94-96]. For example, cocaine esterases (CocE-L169K/G173Q or CocE-T172R/G173Q) have been shown to robustly antagonize cocaine rewarding effects in rats [97-99]. Most biologics prevent central rewarding effects of drugs but fail to address the question of drug craving and relapse [100]. This may be attributed to their inability to rectify impaired components of the addiction neurocircuitry and/or neuroplasticity. A more recent therapeutic approach employed the use of small interfering RNA (siRNA) coupled with gold nanorods to silence DARRP-32 (dopamine and cyclic-AMP regulated protein phosphatase inhibitor) gene, in dopaminergic cells [101], generally known as one of the key regulators of histone phosphorylation. Studies have demonstrated that genetic disruption of DARPP-32 has dramatic effects on behavioural responses to cocaine [15], whereas inhibition breaks the addiction cycle possibly by down-regulating extracellular signal-regulated kinase (ERK) and protein phosphatase-1 (PP-1) - factors that play key roles in the addiction signalling pathway [101]. siRNA-complexes may therefore serve as pharmaceutical vehicles that could enable effective delivery of specific compounds, such as biologics or approved drugs, directly into brain sites relevant to addictive behaviour.

Table 2 Summary of pharmacotherapeutics for drug addiction

Drugs of Abuse	*Medications*	*Class*	*Action*	*Consequences/ Clinical implications*	*FDA Approved? (+/-)*	*Indications/ Remarks*	*References*
Alcohol	Naltrexone	Opioid receptor antagonist	Blocks *mu*-opioid receptors	Not effective in all patients	+	Less effective against stress-induced relapse	[126-130]
	Disulfiram	ALDH inhibitor	Inhibits ALDH &prevents acetaldehyde metabolism	Low compliance due to aversive reactions & other side effects	+	Effective against alcohol and cocaine dependence	[100, 127, 129-132]
	Acamprosate	NMDA glutamate receptor antagonist	Normalizes hyperglutamatergic states	Adverse reactions e.g diarrhea, suicidal ideation	+	Reduces withdrawal symptoms & prevents relapse	[127, 130, 131, 133, 134]
	Topiramate, Ondansetron & Baclofen,				-	Have promising therapeutic potential	[130, 133, 135, 136]
Cocaine	NAC	Thiol antioxidant	Regulates X(c) system activity & GSH biosynthesis	Reduces respiratory burst & may cause liver damage	-	Low bio-distribution	[137-139]
	Modafinil	GABA/glutaminergic agent	Upregulates brain glutamate system	Causes euphoria	-	Approved for other indications	[127]
	Topiramate	Antiepileptic drug	Mainly antagonizes AMPA receptors & kainate glutamate receptors	Causes anorexia, paresthesia, reversible cognitive impairment & taste aversion	-	Approved for other indications	[127, 135, 136]
	Baclofen, Propranolol, α-adrenergic agonists & Rimonabant				-	Have promising therapeutic potential	[127, 140-142]

Drug addiction, also called substance use disorder, is generally considered a brain disease. It is measured on a continuum scale that ranges from mild to severe. According to *SAMHSA*, diagnosis using *DSM-5* criteria is based on evidence of social and control impairments, risky use and pharmacological implications [143]. However, treatment approaches should involve biological, behavioural and social context components [144] to produce effective results. Abbreviations: *X(c)* system, Cystine-glutamate antiporter system; *NAC*, N-acetylcysteine; *GSH*, Glutathione; *DA*, Dopamine; *ALDH*, Aldehyde dehydrogenase; *AMPA*, Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; *NMDA*, N-methyl-D-aspartate; *FDA*, Food and drug administration; +/-, Yes/No; *DSM-5*, Diagnostic and Statistical Manual of Mental Disorders 5; *SAMHSA*, Substance Abuse and Mental Health Services Administration.

HDAC Inhibitors (HDACi): A Target Therapeutic For Drug Addiction

Recent advances in the field of chromatin remodelling and epigenetic regulation improved our understanding of how genes interact and are regulated by the environment. Although pathological alterations in the brain transcriptome that underlie psychiatric and neurodegenerative disorders are incompletely understood, GWA studies have established genetic associations between specific genes or chromosomal regions with various brain diseases which have in common the hallmark of cognitive impairment [102]. Genetic-based studies have suggested critical roles for the epigenetic modifiers – HATs and HDACs in maintaining brain homeostasis in various disease conditions. Targeting histone acetylation may provide benefit for the treatment of a broad range of human diseases such as depression, schizophrenia, anxiety disorders and drug addiction [103]. Of the various HATs, CREB-binding protein (CBP) has been implicated in drug addiction but the evidence is contradictory [104]. CBP deletion in the NAc attenuated cocaine sensitivity and CPP [105] whereas striatal deletion increased sensitivity to cocaine and amphetamine [106]. In contrast, evidence to support a role for HDACS in drug addiction is convincing. Previous studies have shown that HDAC activity was increased in the PFC and NAc of rodents following cocaine-self administration. Now, much attention is given to HDACs, especially HDAC5 due to its antidepressant activity [103, 107] and unique response to chronic cocaine administration which critically implicates its involvement in behavioural transitions from drug experimentation to compulsive drug use [15]. Moreover, HDAC5 has been identified to centrally integrate chromatin changes and gene alterations induced by drugs and stress stimuli [57]. Also, the regulation of saliency circuit, which mediates behavioural responses to various environmental stimuli, critically implicates HDAC5. For example, increased response to rewarding effects of chronic cocaine exposure as well as chronic neuropathic pain and social defeat stress were observed in HDAC5 knocked out mice [5, 57, 108]. Although the effects of HDAC5 on cocaine rewarding effects still remains unclear, but it has previously been shown that its overexpression blunts cocaine induced place conditioning and locomotor activating effects [57]. However, the behavioural effects of HDAC5 on cocaine induced reward and locomotion requires further investigation since it is uncertain whether its action is due to interaction with HDAC3 on the same catalytic deacetylase domain and blocking this site prevents its inhibitory action on cocaine reward [109].

Currently, there is considerable and growing interest in the use of HDAC inhibitors to activate the expression of mRNAs that are downregulated in various neurological disorders and psychiatric conditions [31] which include drug addiction. HDAC inhibitors have been considered for many years as potent anticancer agents [110, 111]. HDAC inhibitors competitively inhibit HDACs from deacetylating lysines on the histone tails resulting in hyperacetylated and transcriptionally active chromatin states – giving rise to increased gene expression in the cell. Trichostatin A (TSA), valproic acid (VPA), sodium butyrate (NaBut) and suberoylanilide hydroxamic acid (SAHA) are among pharmaceutical compounds known to have HDAC inhibitor activity [112]. The usefulness of these

compounds is exemplified by administration of SAHA restoring memory function in mice lacking appropriate CREB-binding protein (CBP) activity [102]. Similarly, it has also been reported that the HDAC inhibitor TSA improved long-term memory and synaptic plasticity in a mouse model of *Rubinstein-Taybi Syndrome* that is characterized by mental retardation due to mutations of CBP and p300 [113]. Another HDAC inhibitor NaBut has been used to strengthen memory associated with learning events [114]. Malvaez et al. (2010) has demonstrated that transgenic mice treated with NaBut extinguished cocaine-induced CPP more quickly and in a more persistent manner than their vehicle-treated controls [115]. An exciting application of these findings that incorporate pharmacologic enhancement of extinction learning by modulating memory components of substance use disorders via HDAC inhibitor is of potential therapeutic interest. Besides, it was previously shown that HDAC inhibitors reversed long term chromatin changes and persisted behavioural alterations at adulthood in maternally stressed rats [12]. This action coupled with other evidences of its neuroprotective and neuroregenerative properties in animal models [102] further support the speculations that HDAC inhibitors might be useful in the treatment of neuropsychiatric diseases.

Some controversial reports about the therapeutic action of HDAC inhibitors have also been recorded in the literature. It is therefore important to stress that HDAC inhibitors may sometimes exhibit opposing actions on drug seeking behaviours subject to the manner in which they are administered. For instance, HDAC inhibitors increased drug intake in animals trained to self-administer cocaine but reduced intake when given before drug acquisition [116, 117]. The bioavailability and half-life of HDAC inhibitors may also account for some of their disparate actions *in vivo* [110]. Hence, relatively high or low concentrations may be required for desired action when HDAC inhibitors with short (e.g valproic acid) or longer plasma half-lives (e.g SAHA and TSA) are used.

Valproic acid which mimics the action of the HDAC inhibitor, TSA, has been used for the treatment of schizophrenia and bipolar disorder in humans for decades and other HDAC inhibitors are currently in different phases of human clinical trials for CNS disorders [103].

Moreover, it is beyond any doubt that altered histone acetylation is one of the main contributors to transition to an addicted state. However, research in the field of drug addiction needs to focus more on specific HDAC inhibitors that target extinction memory of drugs as this may later translate to effective medications for preventing drug relapse.

Conclusion/Future directions

It has been shown that the majority of drug–induced behavioural alterations result not only from genetic but also epigenetic interactions giving rise to a breaking point situation that is beyond the individual's adjustment capacity to curtail further use [5]. Some authors have suggested that these changed behaviours are inheritable by succeeding generations through mechanisms that also implicate epigenetic modifications. Other proposed strategies include epigenetic mechanisms which may be at

play in the germline and subsequently interfere with normal embryonic-epigenomic programming. In the addicted parents, this suggests an intriguing and potentially alarming possibility that exposure to drugs of abuse may produce transmissible epigenetic changes that result in profound alterations to the physiology and behaviour of the offspring, raising the interesting question as to whether non-exposed children of addicts are "programmed" to become addicts themselves. The few studies that have looked beyond the first generation suggest that many phenotypes persist. Regardless of the number of future generations, the impact of drug use on the first generation offspring alone is sufficient to justify further research defining the extent of epigenetic heritability of phenotypes associated with parental drug abuse and the specific mechanisms underlying these effects. However, preventing, curtailing or even rolling back the scourge of cocaine or alcohol-induced maladaptive disorders still remains a notable challenge today. It is therefore imperative that the search for better treatment outcomes continues. We therefore propose that combining psychosocial intervention with gene therapy involving pharmacological manipulations of HDACs, especially HDAC5, may further enhance current therapies and perhaps result in a more successful management of drug addiction.

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Conflict of Interest

All authors have no conflict of interest to disclose.

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CHAPTER THREE

The study described in this chapter has been submitted for publication in the International Journal of Developmental Neuroscience. The article is titled: **"DNA hypomethylation of the promoter regions of *Crem, Fosb* and *Bdnf* in the prefrontal cortex of male mice treated with cocaine"**.

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Abstract

Little is known about the role of *Crem* (cAMP response element modulator), *Fosb*, and *Bdnf* (brain derived neurotrophic factor) in DNA methylation regulated–gene functions in the hippocampus (HPC) and prefrontal cortex (PFC) of mice exposed to cocaine in an interacting social state. Using a social interaction mouse model, the present study investigated cocaine-induced alterations in the promoter regions of *Crem*, *Fosb* and *Bdnf* in the HPC and PFC of mice. C57BL/6 were treated with cocaine (10mg/kg; i.p.) on 6 occasions, 1 injection every alternate day. This was followed by extinction learning for 6 days after which the animals later received cocaine at a lower dose of 5mg/kg, i.p. Locomotor activity, mood states and novelty exploration were assessed 24 hours after this last cocaine injection using the open field test (OFT) and novel object recognition (NOR) test. 48 hours after the behavioral tests, the PFC and HPC were dissected for subsequent determination of DNA methylation status in the promoter regions of *Crem*, *Fosb* and *Bdnf* genes. During the conditioning phase, administration of cocaine resulted in a significant change in place preference that was not observed after the period of extinction learning. Treating the mice with a priming dose of cocaine did not reinstate place preference behavior. Mice exposed to cocaine displayed significantly increased locomotor activity in the OFT, but decreased novelty exploration in the NOR test. These behavioral changes were associated with significant reduction in DNA methylation of *Crem*, *Fosb* and *Bdnf* promoters in the PFC, while a significant decrease in methylation was only observed in the *Bdnf* promoter region in the HPC. Together, these data suggested that cocaine induced elements of drug seeking behavior in socially housed mice, but at a lower dose, was unable to reinstate similar behavior. These behavioral changes were accompanied by alterations in the transcription rate of proteins that are implicated in cognition and plasticity. Furthermore, our data suggest that the PFC may be one of the brain areas that show early responses to cocaine.

Key words: Cocaine; social interaction; DNA methylation; hippocampus; prefrontal cortex.

1. Introduction

Cocaine use, repeatedly paired with environmental cues, with or without cage mates, has profound implications on conditioned emotional responses and memory consolidation in mice (Tzeng et al., 2016). Even after long periods of abstinence, the associated drug cues are usually strong enough to induce feelings of craving and drug-seeking (Paolone et al., 2009). However, extinction of drug associated cues is believed to prevent relapse apparently by reducing the motivational properties of drugs (Torregrossa et al., 2010).

The impact of social interaction has been a major thrust in drug abuse research. In a recent pre-clinical study, it was established that the presence of three cage mates decreased conditioned place preference (CPP) magnitudes induced by either moderate (10mg/kg) or high (20mg/kg) doses of cocaine (Tzeng et al., 2016). Similarly, amphetamine conditioning failed to induce CPP in pair-bonded male *Prairie vole* (a socially monogamous rodent) (Liu et al., 2011). These demonstrations support a previous highlight that social interaction, if offered in a context, may potentially decrease the incentive salience of drug-associated contextual stimuli (Fritz et al., 2011b). Furthermore, a study that examined the effects of mutual exclusive stimuli (social interaction *vs* cocaine injection) on CPP also reported that both stimuli yielded comparable reward strength (Fritz et al., 2011a). These studies therefore suggest that the environment in which drugs are consumed (e.g social *vs* isolated contexts) may have a significant effect on the extent to which a drug will ultimately impact the neurobiology and behavior of the consumer.

One of the mechanisms by which drugs mediate their effects is by modifying the expression of certain genes. DNA methylation represents one of the key post-transcriptional mechanisms through which expression levels of genes can be modulated. Within regulatory and/or promoter regions, DNA methylation interferes with the binding of transcription factors to their target sequences (Comb and Goodman, 1990) or initiates recruitment of co-repressors to cause chromatin repression. The result of these biochemical processes may ultimately lead to either the promotion of gene transcription or the silencing of genes (Novikova et al., 2008, Renthal and Nestler, 2009, Massart et al., 2015, Ajonijebu et al., 2017). The *Fos* family of transcription factors e.g *cFos*, Δ*Fosb* and *Fosb* have been implicated as master control proteins that moderate drug-induced changes in gene expression (Kumar et al., 2005, Larson et al., 2010). Accumulation of these immediate early gene products in brain areas related to reward such as nucleus accumbens (NAc), ventral tegmental area (VTA) and the striatum, have been observed following repeated cocaine use (Kelz et al., 1999, Perrotti et al., 2005, Lhuillier et al., 2007). Cocaine-induced elevated levels of Δ*Fosb* in the brain have also been shown to enhance *Bdnf* activity which in turn increases the number of dendritic branches and spines present on neurons in NAc and cortical areas of the brain. Interestingly, acute withdrawal from repeated cocaine exposure were also found to elevate this neurotrophin in the HPC, amygdala and other mesolimbic areas

(McGinty et al., 2010). The neuroplastic changes attributed to *Bdnf* upregulation have subsequently been proposed to be one of the major drivers of long-term potentiation, one of the fundamental neurobiological processes that underlies the development and persistence of addictive behavior (Boulle et al., 2012). However a study that utilized rat models of cocaine self-administration reported increased *Bdnf* expression in the PFC which was associated with compensatory neuroadaptations that reduced the reinforcing effect of the psychostimulant (Sadri-Vakili et al., 2010). The exact role of molecular factors such as *Fosb* and *Bdnf* therefore remains unresolved, justifying further investigation into their involvement in drug-induced effects.

Upstream of the *Fos* family of proteins, another group of signaling proteins that has widely been implicated as part of the intracellular machinery that drives addictive behavior, involves *Creb* (cAMP response element binding protein). Binding of agonists to dopamine D1 receptors leads to increases in intracellular levels of cAMP and the subsequent phosphorylation of *Creb* via the action of protein kinase A. Dimers of phosphorylated *Creb* then bind to the promoter regions of various genes (e.g. *c-fos* and *Bdnf*) to facilitate their transcription (Ortega-Martínez, 2015). Other pathways besides dopamine D1 receptor activation, can also trigger *Creb* signaling. These include the calcium/calmodulin/CaMkinase IV pathway activated by ligand binding to AMPA and NMDA receptors, as well as the p38/ERK pathway triggered by growth factor and cytokine binding to their respective tyrosine kinase receptors (Della Fazia et al., 1997, Servillo et al., 1997).

Crem (cAMP response element modulator) is another b-ZIP (leucine zipper) transcription factor that is related to *Creb*. *Crem* can act either as a repressor (*Crem* isoforms α, β and γ) or as an activator (*Crem* isoform τ) of cAMP-induced transcription depending on the particular isoform that is present within a specific cell type (Masquilier and Sassone-Corsi, 1992, Laoide et al., 1993, Mellström et al., 1993). Previous studies have shown that *Crem* play significant roles in gene expression (Storvik et al., 2000) and mediates neuroplasticity and impulsivity linked to addiction vulnerability in humans (Miller et al., 2017). In turn, transcription of the *Crem* gene is subject to epigenetic regulation that includes both histone acetylation as well as the methylation of specific promoter regions (Rauen et al., 2013).

The majority of studies that explored cocaine-induced epigenetic changes have focused mainly on histone modifications, and to a lesser extent DNA methylation, in the promoter region of the *Bdnf gene* in brain areas such as the NAc, VTA and striatum. So far little attention has been given to other candidate factors such as *Fosb* and *Crem* that have also been identified as central players in modulating gene expression programs associated with drug-induced behavioral and neuroplastic changes. To our knowledge, data indicating how these central factors interact and mediate cocaine-related effects is limited in the current literature. Therefore, the broad goal of the present study was to investigate the association of cocaine-induced behavioral changes with the methylation status of the promoter regions of certain genes that have previously been implicated in drug-mediated effects. Our

aim was therefore to delineate the role of the *Crem-Fos-Bdnf* signaling pathway in the PFC and HPC of socially engaged mice that have been treated with cocaine.

2. Materials and Methods

2.1 Animals and husbandry

Sixteen C57BL/6 male mice (9 – 10 weeks old) from the Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal, were used for this study. All animals were housed in plastic cages within the test room and allowed to acclimatize prior the start of the experiment. Standard laboratory conditions of 23±2 ˚C room temperature, 50-60% humidity and 12h light/dark cycle with lights on at 06h00 were maintained throughout the study period. The animals had unrestricted access to food and water. The Animal Ethics Subcommittee of the University of KwaZulu-Natal approved all procedures, and these were performed according to the guidelines of National Institutes of Health for the Care and Use of laboratory animals.

2.2 Drugs/Treatment

Cocaine powder – salt form (provided by the Forensic Science Laboratory of the South African Police Services) was dissolved in sterile 0.9% saline and injected intraperitoneally (i.p.) in volumes ≤ 1ml/kg of body weight. After acclimatization, the mice were randomly divided into two groups of eight (n=8) and were administered either 0.9% saline or cocaine (10mg/kg). Our choice for using the moderate dose of 10mg/kg was influenced by a previous report indicating that this dose has the potential to establish reliable place preference within a CPP paradigm (Tzeng et al., 2016). One of the unique features of our experimental design was that unlike many other similar studies, the mice during all phases of the experiment, i.e. the conditioning session, extinction and reinstatement, were never isolated but rather were exposed to cocaine within a group context. For example, there were 4 animals in the less preferred compartment during the conditioning phase i.e. two that received cocaine and two that were injected with saline. This design created a social context within which drug-associated conditioning could occur.

2.3 Apparatus

We assessed the conditioned appetitive response of mice to cocaine (Botreau et al., 2006) through place preference screening (Subiah et al., 2012, Tzeng et al., 2016) in a locally made conditioned place preference (CPP) box. The CPP box had dimensions of 45 x 45 x 45 cm and was divided by a sliding partitioned door into two compartments of equal sizes. Each side compartment had distinctive visual and tactile cues. One compartment had a white checked pattern on black surface walls and a grid floor whereas the surface of the walls of the other compartment was black and it had a smooth

floor. The compartments were cleaned with 70% ethanol after each test and then allowed to dry completely before the next animal was introduced. The essence of this exercise was to prevent interference of any confounding olfactory stimuli that may bias the animals to a specific compartment.

2.4 Experimental protocol

2.4.1 Baseline preference/Cocaine Conditioning

The experimental design and timeline of the various procedures are outlined in Figure 1. Unconditioned (Pre-test) place preference of each mouse was determined prior to cocaine conditioning. During this phase, the connecting partitioned door between the two compartments was opened allowing the animal free access to the entire apparatus for 15 minutes. The time spent in each compartment was recorded manually using a stop watch. Cocaine conditioning sessions were conducted from day 1 to 11 and throughout this period the animals were confined to the less preferred compartment. On day 1, 3, 5, 7, 9 and 11, animals in the treatment group received i.p. cocaine injections (10mg/kg/conditioning session) and those in the control group received 0.9% normal saline. The mice were placed in their less-preferred compartments for 20 minutes. In addition, we also administered 0.9% normal saline to all groups on day 2, 4, 6, 8 and 10. On these days the mice were placed in their preferred compartments also for 20 minutes. We adopted this strategy to ensure that the animals associated the less-preferred compartment with the drug and not the consequences of receiving an injection (Subiah et al., 2012). Twenty hours after the last cocaine injection (day 12), we removed the sliding door and measured CPP by allowing each mouse to once again freely explore both compartments for 15 minutes. The time spent in each compartment of the box was again recorded. CPP was considered when the time spent in the drug-paired compartment was greater than the time spent in the saline-paired compartment (Test) (Botreau et al., 2006, Yu et al., 2011a).

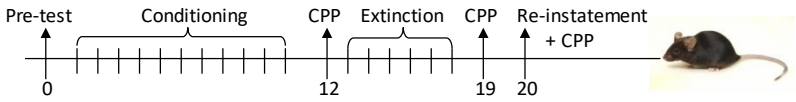

Figure 1: Showing experimental protocol and timeline of activities of mice tested for cocaine-induced conditioned place preference (CPP).

2.4.2 Extinction training

Extinction training was carried out on day 13 to 18. On day 13, 14 and 15, each animal was alternately placed in either compartment without any injection. This exercise was repeated on day 16 to 18, with all the animals receiving a saline injection before being placed in a particular compartment. The importance of restrained saline injection during the initial 3 days of extinction procedure was to maintain procedures that were related to injectioning the animals with the only

difference being that no drug was administered during this time period. After the washout period, we again assessed the place preference of each mouse on day 19 (Extinction) using the same procedure as that used during the Pre-test and Test sessions.

2.4.3 Reinstatement

48 hours (day 20) after the last extinction trial, a priming injection of 5mg/kg cocaine was given to the mice and immediately tested for re-establishment of CPP (Reinstatement). The testing schedule again consisted of free access to the entire apparatus with the partitioned door opened. The time spent in each compartment was recorded over a total period of 15 minutes.

2.4.4 Measurement of compartment changes

The total number of entries into an opposite compartment of the CPP apparatus was measured as an indirect, crude indicator of locomotor activity and drug-compartment association. We hypothesized that untreated animals would explore the CPP apparatus much more than drug-treated animals, as the latter was expected to spend more time in the drug-associated compartment and be less interested in exploring the entire apparatus.

2.5 Locomotor activity/anxiety related behaviors

Horizontal locomotor activity and anxiety related behaviors of mice in response to the effects of cocaine or saline injections were assessed using the open field test (OFT) 24 hours after the priming injection. The open field apparatus made of Plexiglas has 25cm (width) x 25cm (length) dimensional square floor surrounded by 20cm walls. Each mouse was placed at the center arena and allowed to explore for 5 minutes. Measurements included total number of squares crossed, central square visits, time spent in the central square, rearing (vertical exploration) and time spent grooming (facial, body and pubic cleaning). The apparatus was cleaned with 70% ethanol after each test and allowed to dry before placement of the next mouse. The tests were recorded using a high resolution digital video camera for subsequent scoring.

2.6 Novel object recognition (NOR) test

24 hours after OFT, novelty exploration of individual mice was assessed using NOR test which has previously been used to study learning and memory in rodents (Bevins and Besheer, 2006, Blaser and Heyser, 2015). The test was conducted in a transparent rectangular cage with dimensions 16cm wide x 24cm long x 12cm high. After habituation, mice were placed in the cage with two identical objects for 5 minutes. 24 hours later the animals were returned to the test arena to explore the objects, however this time one of the familiar objects (T_f) was replaced with a different or novel (T_n) object. Time spent

exploring both T_n and T_f objects were recorded for 5 minutes. Exploring included touching, sniffing or directing the nose less than 1cm away from the objects, while sitting on the objects was ignored.

2.7 DNA Methylation and real-time PCR analysis

DNA was isolated from mouse PFC and hippocampal tissues using ZR Genomic DNA MiniPrep kit (Inqaba Biotec, South Africa). The nuclear extract was treated with bisulphite and processed for CT conversion using the EZ DNA methylation kit (Zymo Research, USA) according to the manufacturer's guidelines. To determine DNA methylation status of *Crem*, *Fosb* and *Bdnf* genes, we used quantitative real-time PCR. PCR amplification was performed using the LightCycler system 2.0 (Roche Diagnostics Ltd, South Africa) with a total reaction mixture of 20μL (5μL bisulphite treated DNA template, 10μL SYBR Green Master mix, 2μL gene-specific 15μM primer pair stock and 1μL H_20). Primers previously used to detect specific promoter regions of the target genes or control locus of the reference gene were used to amplify the methylated DNA samples (Table 1) with standard PCR conditions (95^{o}C for 10 min; 95^{o}C for 15s, 60^{o}C for 30s and 72^{o}C for 30s for 45 cycles). The PCR assay plate included methylated DNA tissue samples, water blanks (as negative controls) and seven-fold serial dilutions of input methylated DNA control samples (as standard controls). All reactions were performed in duplicate while threshold cycle (Cq) values were chosen within a linear range of 1. To calculate differences in methylation between samples, the results were normalized to the housekeeping gene *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase) using comparative -ΔΔCq raised to a power of two as previously described (Schmittgen and Livak, 2008).

Table 1: Specific Primers Used for DNA Methylation Assay

Target gene	**Primer Sequence**
Crem	CAGAGGAAGAAGGGACACCA (Forward)
	TTGTATTGCCCCGTGCTAGT (Reverse)
Fosb	TATAGAAGCGCTGTGAATGGA (Forward)
	GACCATCTCCGAAATCCTACA (Reverse)
Bdnf	TAACGGCGGCAGACAAAAAGACT (Forward)
	GTGTCTATCCTTATGAATCGCCAGCCAA (Reverse)
Gapdh	GCCAAAAGGGTCATCATCTCCGC (Forward)
	GGATGACCTGCCCACAGCCTTG (Reverse)

2.8 Statistical analysis

Preconditioning/conditioning (CPP tests) and object exploration (NOR test) data were analyzed using Two-Way ANOVA, while analysis of compartmental changes was done with Two-Way repeated measures ANOVA, both followed by Bonferroni's post *hoc-test*. Other behavioral data and differences in qPCR elicited DNA methylation patterns were analyzed using Student's *t-test*. All statistical analyses were performed with GraphPad Prism 5 and results are expressed as group mean ± standard error of the mean (SEM). $p<0.05$ was considered statistically significant.

3. Results

3.1 Cocaine acquired in an interactive social state changed the place preference in CPP test but had no effect on drug reinstatement.

Two-way repeated measures analysis of variance (RM ANOVA) showed significant effects with respect to treatment ($F_{1,14}$ = 33.52, $p<0.0001$), CPP–conditioning ($F_{1,14}$ = 2.72, $p<0.0031$) and treatment *vs* CPP-test interaction ($F_{1,14}$ =16.61, $p=0.0011$) (Figure 2a). Bonferroni post *hoc-test* showed that cocaine-treated mice displayed a significant change in compartment preference during the CPP test by spending significantly more time in the initially less preferred compartment (Figure 2a, $p<0.001$). Saline-treated animals did not show this change in compartment preference (Figure 2a). After extinction training, comparable CPP was observed in both groups of animals (Figure 2b). Animals previously treated with cocaine that received a prime injection of 5mg/kg cocaine after the extinction period spent more time in the drug-paired compartment compared to the saline-treated controls, but this increase in time spent was not significant (Figure 2c, Student's *t-test*, $P=0.2612$).

While cocaine-treated animals displayed a greater number of compartment changes than saline-treated controls, this difference was not found to be significant as two-way RM ANOVA showed that there was only significant main effect of compartmental-exploration, while interactive effect of treatment and compartmental-exploration was absent (Figure 2d, treatment effect $F_{1,42}$ = 2.953, $p=0.1078$, compartmental-exploration effect $F_{3,42}$ = 4.300, $p=0.0098$, interaction $F_{3,42}$ = 0.6582, $p=0.5824$).

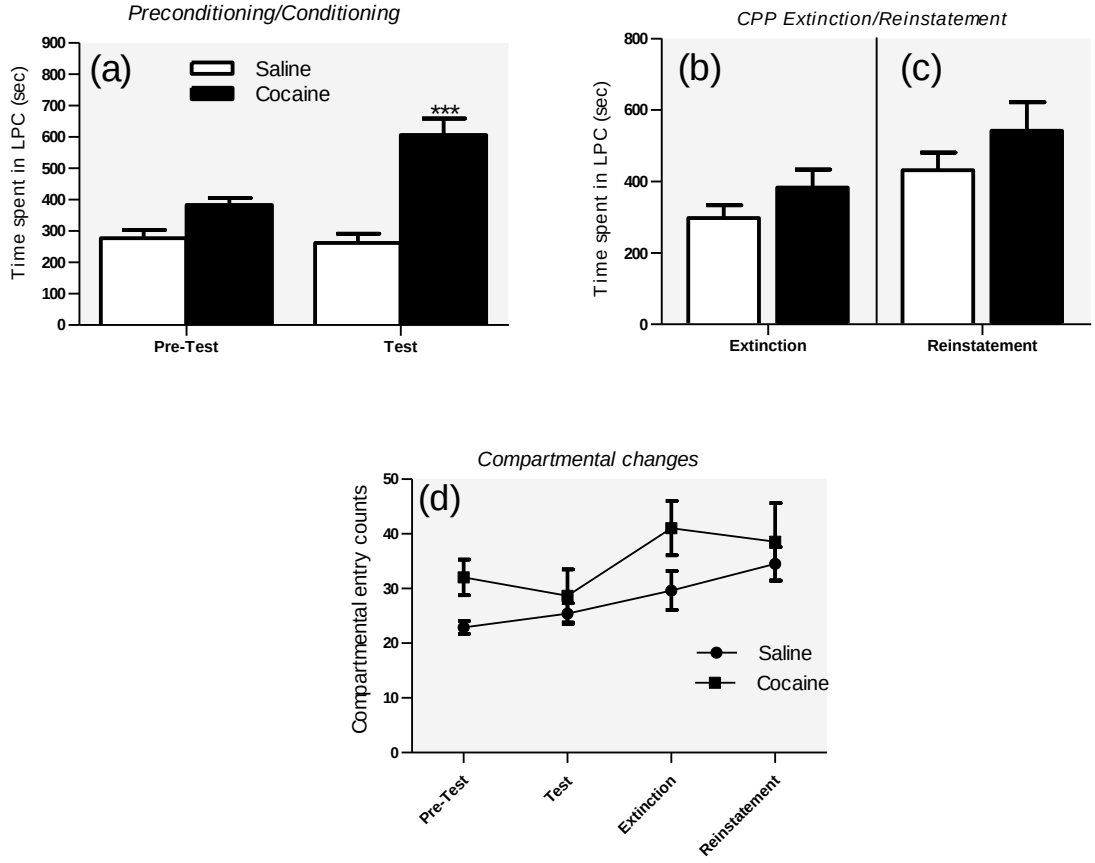

Figure 2: CPP score showing time spent in the less preferred compartment (LPC) before and after cocaine treatment (a) pre-test and test showing preference for cocaine-paired compartment following cocaine conditioning in the initial LPC (b) CPP after extinction training (c) reinstatement of cocaine-CPP by prime injection (d) total exploration of the CPP box compartments. Two-Way ANOVA, Two-Way RM ANOVA followed by Bonferroni posttest, Student's *t-test*, **p<0.001, ***p<0.0001, compared with saline, n=8. Error bars indicate SEM.

3.2 Cocaine increased locomotor activity, decreased novelty exploration, but did not alter anxiety in socially engaged mice.

In the present study, we assessed spontaneous locomotor activity and anxiety related behavior of mice in the open field arena 24h after a subthreshold dose of 5mg/kg cocaine was injected to re-establish cocaine-CPP. Our data showed that the frequency and total number of peripheral and central squares crossed was significantly higher in cocaine-CPP than the saline treated mice (Figure 3a, Student's *t-test*, p=0.0092). As shown in Table 2, the majority of parameters used to characterize anxiety related behavior of mice in the field arena were unaltered after cocaine-CPP, except a significant decrease in time spent grooming compared to saline ($p<0.05$).

We used NOR test to examine object exploratory behavior of the animals by allowing them to discriminate between familiar and novel objects in the test arena. Two-way ANOVA demonstrated significant main effects of treatments ($F_{1,28}$ =12.45, p=0.0015) on object exploration ($F_{1,28}$ = 28.46,

p<0.0001). Interaction between these variables (treatments x object exploration) was also statistically significant ($F_{1,28}$ = 6.663, p=0.0154) (Figure 3b). Post *hoc-test* analyses showed that the saline-treated animals spent significantly more time exploring the novel than the familiar objects (p<0.001), while novel object exploration by the cocaine-treated mice was significantly reduced (p>0.01) (Figure 3b).

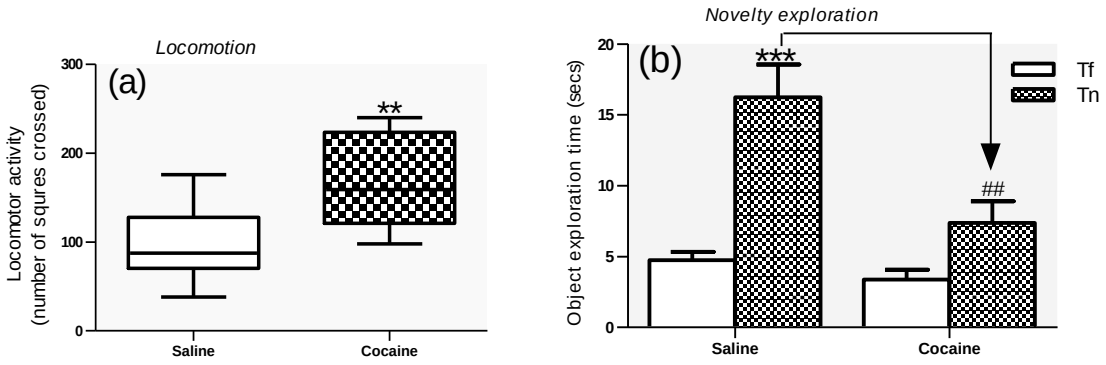

Figure 3: Effects of cocaine treatment on locomotor behavior of mice in the OF and object exploration assessed using the NOR test (a) treatment enhanced locomotor activity by increasing the number of squares crossed in the OF (b) shows differences in time spent by mice discriminating novel from familiar objects. Student's *t-test*; Two-Way ANOVA followed by Bonferroni posttest, **p<0.01 compared to saline, ***p<0.001 compared between objects; ##p<0.01 compared to saline, n=8. Error bars indicate SEM.

Table 2: Observable behaviors of socially engaged C57BL/6 mice in the open field in response to cocaine challenge.

Behavioral parameter	OF data	
	Saline	Cocaine
Locomotion (squares crossed)	97.38±15.14	170.00±18.71**
Central square visit	8.50±1.96	15.38±2.82
Central square visit time (seconds)	13.13±2.08	16.38±2.75
Vertical exploration (seconds)	20.38±3.40	26.75±3.55
Grooming (seconds)	34.88±5.91	20.13±3.40*

Data represents mean±SEM. Student's *t-test*, *p<0.05, ***p<0.0001 compared to saline, n=8.

3.3 Cocaine treatment resulted in DNA hypomethylation in the promoter region of all three genes in the prefrontal cortex but only Bdnf in the hippocampus.

The data generated from qPCR demonstrated that repeated cocaine treatment resulted in significant DNA hypomethylation in the promoter regions of *Crem*, *Fosb* and *Bdnf* genes in the PFC (Figure 4a,

4b, 4c, $p<0.05$, $p<0.001$), while in the HPC a significant decrease in DNA methylation was only seen in the *Bdnf* promoter region (Figure 4c, $p<0.001$).

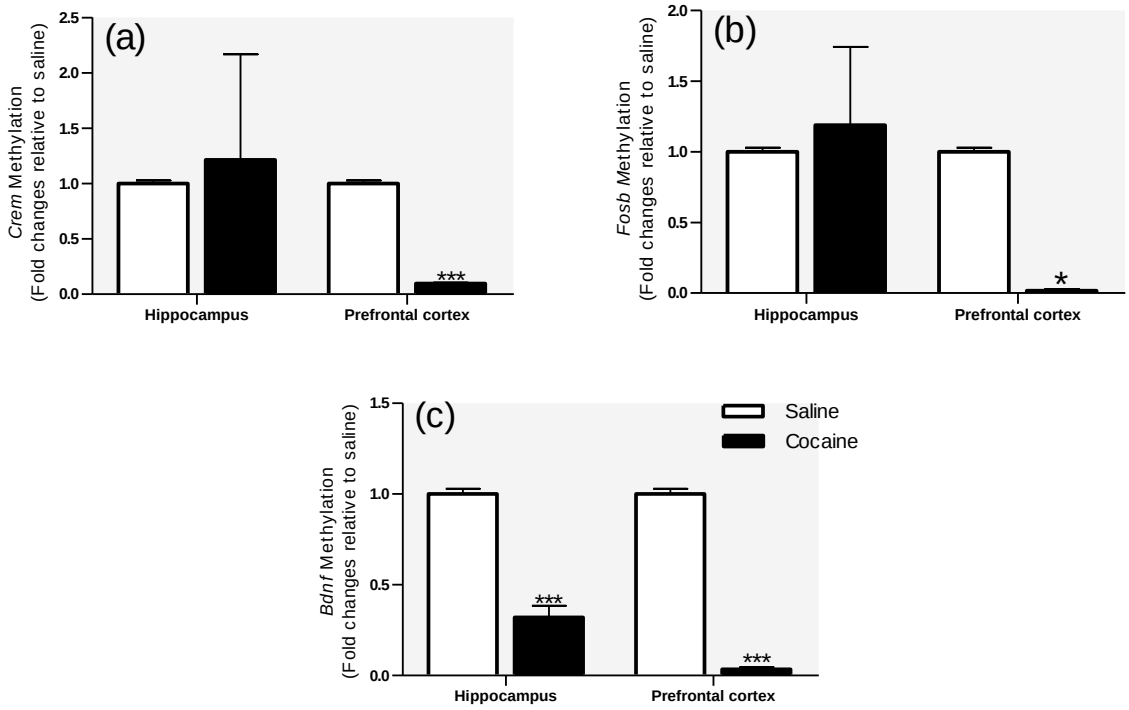

Figure 4: DNA methylation patterns in the HPC and PFC of mice after repeated cocaine injections. (a-b) show that the treatment caused DNA hypomethylation in *Crem* and *Fosb* promoter genes only in the PFC, while methylation status of these neurotrophic factors was unaltered in the HPC (c) *Bdnf* was hypomethylated in both regions of the brain. Student's *t-test*, *p<0.05, ***p<0.0001, compared to saline, n=4. Error bars indicate SEM.

4. Discussion

In this study, cocaine-induced CPP was performed within a social context, based on the premise that positive social interaction has the potential to shift preference of dependent individuals away from drug-related activities, and that cocaine and social stimuli have equal reward strength (Fritz et al., 2011a). The fact that both these factors activate similar reward-related brain regions in rodents, provided additional justification for this approach (El Rawas et al., 2012, Kummer et al., 2014). Determining whether cocaine acquired in an interactive social environment would affect place preference in CPP test and drug reinstatement after extinction or washout period therefore appeared to be a natural progression away from the usual investigation of a change in place preference in isolated mice. We also investigated alterations in methylation status within the promoter regions of *Crem, Fosb* and *Bdnf* in the PFC and HPC in order to see whether the gene-specific transcriptional changes were related to the observed behaviors. The principal findings of this study were that (i) reliable cocaine-induced CPP was acquired after repeated injections within a social setting, (ii) drug-seeking behavior was reduced after extinction learning, (iii) mice that received a priming injection of cocaine tended to spend more time in the drug-associated compartment but this was not significant, and (iv)

the promoter regions of *Crem, Fosb* and *Bdnf* were all hypomethylated in the PFC, whereas only the *Bdnf* promoter region was hypomethylated in the HPC.

The experimental paradigm of cocaine-CPP in a social environment using C57BL/6 mice has previously been used to understand the neurobiological mechanisms underlying drug abuse within a communal context (Kummer et al., 2014). In our study, mice were conditioned in the presence of other animals and displayed normal acquisition of cocaine-CPP following cocaine injections. This place preference (arguably drug seeking behavior) was not observed after extinction learning, neither following a subsequent priming injection of cocaine. These observations were comparable to findings in a previous study that examined the influence of different social experiences on acquisition, extinction and reinstatement of cocaine-induced CPP. The authors demonstrated that reinstatement of cocaine-CPP after extinction was profoundly affected by different housing conditions such as crowding or co-habiting with a female, while CPP acquisition remains unaffected (Ribeiro Do Couto et al., 2009). A similar study by Fritz et al (2011) also reported that 15-minutes of dyadic social interaction in same sex rats reversed cocaine-induced place preference and further prevented re-acquisition of cocaine-CPP (Fritz et al., 2011b). Our data is therefore in line with these observations showing that cue-induced learned behavior related to cocaine CPP-acquisition is possible within a social state while background social experience may diminish the predictive cocaine reward or reacquisition. A proposed mechanism by which the social context could prevent reinstatement may include the blocking of associative learning following cocaine injections and possible augmentation of consolidated extinction learning. To confirm this proposition, we assessed novelty exploration as a measure of memory competence after cocaine priming injection. We found that cocaine treatment decreased novelty exploration without preference for the familiar stimulus 24h after the initial exposure trial. These findings suggest that combination of extinction learning and social experience may be considered as a promising therapeutic approach for breaking the link between cocaine acquisition and drug seeking behavior.

DNA methylation is an essential regulator of drug-induced neural plasticity, including learning and memory (Yu et al., 2011b), and other experience-dependent behavioral changes (Day et al., 2013). We subsequently investigated gene-specific transcriptional changes that may characterize PFC and hippocampal activity in cocaine-treated mice. We found that hypomethylation at *Bdnf* promoter was evident in the HPC of cocaine-experienced mice without significant changes in the promoters of *Crem* and *Fosb*, while all three promoter regions were significantly hypomethylated in the PFC. This finding implied increased transcription of *Crem, Fosb* and *Bdnf* genes within the PFC in comparison to the HPC following cocaine exposure and suggested that the PFC may have been more responsive to establish neuroplastic changes than the HPC. The apparent enhanced transcription of *Crem, Fosb* and *Bdnf* may also reflect a tendency to favor cortical activation and enhanced behavioral control in the mice as manifested by lack of CPP response to priming injection after

extinction learning. Furthermore, the increased gene regulation and strong cortical activation may further be attributed to indirect effects of background social influence during conditioning and extinction periods. Consistent with this reasoning is a previous report that the insular cortex is activated post drug conditioned learning, while transiting from drug conditioning to non-drug stimuli such as social interaction, is preferentially mediated by activation of prelimbic cortex–NAc core projections (El Rawas et al., 2012).

Han and colleagues (2010) previously injected 5-aza-2-deoxycytidine (5-aza), an inhibitor of DNA methyltransferases (DNMTs), into the prelimbic cortex and CA1 region of the HPC during cocaine acquisition and CPP expression to demonstrate the relevance of DNA methylation in drug-induced learning and memory. They found that 5-aza in the HPC restrained acquisition but did not affect expression of cocaine-CPP, whereas in the prelimbic cortex the inhibitor drug blocked CPP expression but failed to alter acquisition (Han et al., 2010). The authors concluded that DNA methylation in the HPC and the prelimbic cortex is necessary for cocaine-induced memory acquisition or memory retrieval, respectively (Han et al., 2010). To correlate these results with our findings, it appears that epigenetic regulation of genes that encodes molecular memory of cocaine acquisition to extinction involves transcriptional activation of *Bdnf* in the HPC and integration in the PFC for maintenance of long-term memory of the predominant experience, that is background social interaction, during the extinction phase. This argument is supported by a previous report showing an association between the extinction of conditioned fear and a significant increase in histone H4 acetylation around *Bdnf* P4 gene promoter and increases in *Bdnf* exon I and IV mRNA expression in the PFC (Bredy et al., 2007). In addition, dose-response experiments have also indicated that a single or repeated low dose of cocaine (5mg/kg) is sufficient to upregulate *Bdnf* mRNA levels selectively in the PFC two hours after the last injection and may persists for about 72 hours (Fumagalli et al., 2007). Also, *Bdnf* responsiveness was attributed to initial *Creb* phosphorylation induced by cocaine. However, unexplainable is insignificant *Crem* activity in the HPC following cocaine exposure because its dynamic regulation of *Creb* functions and downstream *Creb*-target gene *Bdnf* have also been established (Gundersen, 2010). Specifically, multiple studies have shown that deletion or decreased expression of *Creb* in the HPC upregulates *Crem* activity while viral overexpression of activator isoform of *Crem*, *Cremτ*, increased hippocampal neurogenesis and enhanced antidepressant treatment (Gundersen et al., 2013, Briand et al., 2015). Since many of these studies that investigated *Crem* activity in the HPC were not specific on its epigenetic regulation and our study being among the first to consider this, further studies are required to delineate epigenetic regulation of *Crem* activity in the HPC in response to cocaine. Moreover, real time PCR and western blotting analyses revealed that *Crem* mRNA expression and protein levels were decreased in testicular tissues of rats that were chronically administered cocaine for 90 days (Li et al., 2003). Similarly, heroin sensitive rats were found to exhibit decreased *CREM* expression in their NAc core (Miller et al., 2017). In contrast, our

data demonstrated *Crem* hypomethylation in the PFC post cocaine experience providing some evidence for its transcriptional activation in this region of the brain relative to HPC and its possible epigenetic regulation within the cortical region. *Crem* induction in the PFC may further suggest its involvement in synaptic plasticity and neurotransmission as previously posited (Miller et al., 2017) and as further supported by simultaneous activation of *Fosb* and downstream *Bdnf*. Due to complexity of *Crem* isoforms for which its activator or repressor activities are not fully understood (Laoide et al., 1993), it will be important that future studies access the impact of each isoform of *Crem* on cocaine reward.

Among other few studies that explored specific dynamic changes of DNA methylation at *Fosb* promoter region associated with cocaine-induced neuroadaptations is that of Anier et al. (Anier et al., 2010). These authors demonstrated that animals treated repeatedly with cocaine showed an upregulation of *Fosb* in the NAc due to hypomethylation and decreased binding of MeCP2 at *Fosb* promoter site. Similar studies focusing on chromatin-epigenetic alterations, reported increased striatal *Fosb* levels in response to cocaine administration (Kumar et al., 2005). Consistent with these findings we also found that repeated cocaine injections were associated with DNA hypomethylation at *Fosb* promoter but only in the PFC, and in addition to hypomethylation of *Bdnf* and *Crem* suggesting that epigenetic changes following cocaine exposure are more evident in this brain region. Decreased global DNA hypomethylation in the PFC following chronic cocaine treatments (Tian et al., 2012) supports the view of heightened signaling activity within the PFC following cocaine in comparison to other brain structures. This generalized activation within the prefrontal cortical network may also account for the significant changes in locomotor activity of cocaine-treated mice in the open field. However, it is unclear why other behavioral parameters, except grooming, that characterize mood states of the mice were unaltered by the treatment. The lack of additional behavioral effects may be attributed to the indirect effects of the background mouse interaction as supported by previous reports that factors such as cognitive control, choice making, reward related behaviors and behavioral inhibition are all associated with social play interaction which involve cortico-striatal projections from PFC to the striatum (Bell et al., 2009, Van Kerkhof et al., 2013).

In conclusion, our study demonstrated that cue-induced learning associated with cocaine experience can be achieved in social states, whereas re-establishing the experience after extinction learning may be interrupted possibly due to blunting of drug related memories. The increase in transcriptional activation of *Bdnf* in both brain regions, suggested an importance of this growth factor in the encoding of the predominant memory, i.e background social interaction, during cocaine-CPP. The extensive hypomethylation of *Crem*, *Fosb* and *Bdnf* in the PFC suggested that cocaine either has a profound effect on this brain region or that the PFC may be more responsive to cocaine treatment in comparison to other brain structures. A major limitation of this study is that the effects of cocaine-induced CPP and associated DNA methylation changes were evaluated only in mice that were

subjected to enriched social contexts. However, further studies are required to compare the impact of social enrichment versus social isolation.

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Disclosure

The authors have no competing financial or other interests to disclose.

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CHAPTER FOUR

This chapter forms part of my PhD research work and will be submitted for publication in Behavioural Neuroscience. The article is titled: **"Long-term study of drug intake behaviour in home-caged mice using a multiple-choice drug preference approach".**

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Abstract

Few studies have characterized drug-taking behaviour in a multi-choice social paradigm. However, comparative learning and behavioural response associated with prolonged access to polydrug vs. water in free living conditions have not yet been established. Here, we first investigated voluntary drinking behaviour and choice preference in young adult C57BL/6 female mice (2 times n=16) that were concurrently exposed to water and drugs (300mg/L cocaine vs. 12% v/v ethanol) in the automated IntelliCage (IC) system for 30 days. Subsequently, persistent cocaine–seeking behaviours were assessed through spontaneous learning of alternate nose-poke tasks (ANT) imposed for 15 days before and 9 days (preceded by 4 days of uninterrupted cocaine vs. water access) after a drug withdrawal period of 7 days. In all task sessions, motivation for drug (visits with or without nose-pokes, NP) and fluid consumption (lick contact time or total licks from bottles) were automatically recorded and analysed. During this period, body weight changes of both drug exposed (M_0) and control (a separate group of eight mice exposed to the IC apparatus without access to drugs) mice were recorded weekly over 56 days, and thereafter, anxiety-related behaviours were assessed in the open field. We show that cocaine was visited and licked more often than ethanol during the initial 30 days access period. Ethanol licking was high only when cocaine licking was low, which suggests an interchanged pattern of drug taking. Overall, the animals preferred to visit water providing corners than drug containing ones during all test sessions. Visiting proportion in all levels of ANT were insignificant, while progressive improvement in nose-poking action (from NP1 to NP5) was observed only during the 9-day ANT post drug withdrawal. Also, prolonged access to the drugs had no significant impact on body weight due to proportionate weight gain in both groups of mice. Following drug experience, exploratory behaviours of the M_0 mice were comparable to that of their controls. Taken together, our data confirm cocaine preference over ethanol when simultaneously presented and further established that continuous water intake occurs probably to maintain hydration status in the animals. These results reflect episodic drug use in humans and highlights the complexity of studying drug-taking behaviour within a social environment.

Key words: cocaine, ethanol, alcohol, social home-cage, drug addiction, voluntary choice drinking, consumption preference, behavioral phenotype.

Introduction

Substance use disorder is a complex condition that is characterized mainly by loss of control of drug intake and a strong prevalence of drug seeking behavior during abstinence. A variety of animal models have been used to unravel the neurobiological mechanisms underpinning these behaviors. However these models have been critically challenged based on the inadequacies of experimental protocols and the resultant shortfalls in its validation of the condition in humans (Piazza & Deroche-Gamonet, 2013). For instance research approaches in experimental psychology and analysis of behavior to modern investigations have mostly modeled the non-pathological recreational use of drugs (Goldberg, Woods, & Schuster, 1969; Weeks, 1962) that basically highlights the reinforcing power of substances and its sustained escalated intake (Koob & Kreek, 2007; Koob et al., 2004). This focus however only partially accounts for the pathological state of addiction. Piazza and Deroche-Gamonet (2013) further stressed the relevance of models that take into consideration the "behavioral crystallization" which is not only caused by long term exposure to drugs but also characterized by alterations in synaptic plasticity in brain reward areas making drugs pathologically craved after abstinence. The intrinsic neuroplasticity induced by drugs of abuse usually results from abnormalities in neural signaling within the mesocorticolimbic system and the hypothalamic-pituitary-adrenal axis (HPA) (Kauer, 2004; Kauer & Malenka, 2007; Koob & Le Moal, 2005; Van den Oever, Spijker, Smit, & De Vries, 2010), whose dysregulation are central to the ensuing maladaptive behaviors. Koob and Kreek (2007) reported that cocaine infusion induced activation of the HPA axis which became evident during acute drug withdrawal and then suggested that this drug-induced response may depend on feed-forward activation of the mesolimbic dopaminergic system (Koob & Kreek, 2007).

It is well known that most of the biological systems that mediate drug dependence are also sensitive to social and/or environmental stress (Ajonijebu, Abboussi, Russell, Mabandla, & Daniels, 2017; Koob & Le Moal, 2005). For instance, increased drug seeking and taking have been observed in primates and rodent models of isolation stress and those that were socially deprived (Haney, Maccari, Le Moal, Simon, & Piazza, 1995; Sinha, 2001). These environmental conditions generally activate the release of corticotropin releasing factor (CRF), mainly from extended amygdala during protracted abstinence or acute drug withdrawal states, that subsequently triggers the brain stress systems (Koob & Kreek, 2007).

Although a great deal of evidence still implicates hyperactive dopaminergic system in the rewarding or psychomotor effects of cocaine and ethanol (Bahi, 2015; Bello et al., 2011; Kaun, Azanchi, Maung, Hirsh, & Heberlein, 2011; Ramchandani et al., 2011), the question of cross-behavioral sensitization (Itzhak & Martin, 1999) ensuing from concurrent exposure to these compounds remains a long-standing debate. Previous studies have mostly hypothesized that enhanced psychological and euphorigenic effects of combined cocaine-ethanol abuse may promote ingestion of larger amounts of

individual substances and are associated with increased risk of cocaethylene (ethyl ester of benzoylecgonine mostly detected in urine of patients that co-abuse ethanol and cocaine) – induced toxicity (McCance-Katz, Kosten, & Jatlow, 1998; McCance-Katz et al., 1993). Another study that measured plasma concentrations of ethanol and cocaine in human cohorts reported that ethanol administration significantly increased concentration of cocaine in plasma, whereas cocaine administration had no effect on ethanol concentration (Perez-Reyes & Jeffcoat, 1992). Conversely, a postmortem brain study later established a reciprocal relationship between ethanol and cocaine effects in influencing dorsal raphe functions (Little et al., 1998). Despite these reliable evidences, the impact of alcohol serving as a gateway drug in the foray of sensitizing cocaine consumption and vice-versa has not been investigated in animal model. Also, the understanding of rodent's behavioral response to water and polydrug (cocaine and ethanol) concurrently presented in the social home-cage setting in a manner that represents substance abuse in humans, is still underexplored. Since a high percentage of individuals concurrently abuse cocaine and ethanol (Pereira, Andrade, & Valentão, 2015; Tallarida et al., 2014), it is pertinent that animal models are designed to evaluate the interacting factors that converge to influence the drug-seeking effects of these compounds, as this would contribute meaningfully to our understanding of substance use in vulnerable populations.

One of the initial constraints in developing an effective model in this regard is the general belief that cocaine would be hydrolysed within the gastrointestinal environment when consumed orally, as opposed to orally administered alcohol (Stromberg, Mackler, Volpicelli, O'Brien, & Dewey, 2001). However, it has been established that oral cocaine is more potent than those consumed intravenously (Ma, Falk, & Lau, 1999) and produces comparable bioavailability and a greater subjective "high" compared to intranasal cocaine (Van Dyke, Jatlow, Ungerer, Barash, & Byck, 1978). On the other hand, most traditional behavioural testing have been considered labour intensive and difficult to interpret (Vannoni et al., 2014). Besides, many of these protocols are designed for individual testing of rodents usually in a non-free-living state. A few years back, comprehensive behavioral testing within a social environment began employing the use of automated tools such as the IC system. This computerized system has been used by many researchers to study spontaneous learning tasks, behavioral characterization and phenotyping of genetically modified mouse lines, as well as assessment of executive brain functions of mice (Endo et al., 2011; Smutek et al., 2014; Vannoni et al., 2014).

The aim of the present study was to evaluate sequential drug consumption and associated goal-directed learned behaviours in mice subjected to an enriched social context. To achieve this, we used alcohol-preferring phenotype of transgenic mice in a group-housing setting to model transition from recreational drug use to escalated intake. We then assessed executive neurocognitive functions related to drug-induced maladaptive behaviors by measuring learning performances against imposed

resistance tasks in the operant chambers of the IC and further examined exploratory behavior of the animals in the longitudinal open field (OF) arena. Our model of a free choice paradigm utilizing the IC was developed in a manner that allowed extended access to both cocaine and ethanol, measured comparative consumption preferences between drugs and water, and modeled neurobiological challenges that are similar to the etiologies and symptoms of drug abuse in humans.

Materials and Methods

Animals and facilities

Forty young adult C57BL/6 female mice (9 – 10 weeks old) were used in this experiment. They were obtained from the breeding colony of the Biomedical Resource Unit (BRU) at the University of KwaZulu-Natal and kept in the animal holding facilities throughout the experiment. The mice were divided into two groups, drug exposed M_0 mice (2 times n=16) and baseline control mice (n=8). Both groups of animals were housed in transparent plastic rodent cages (8 mice per cage) within the test room for approximately one month before the testing began. They were maintained under standard laboratory conditions of 23±2 ˚C room temperature, 50±5% humidity, a 12h light/dark cycle (lights on at 0600 h) and had access to food and water *ad-libitum*. All experiments were conducted humanely in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and all protocols were approved (*Ethics clearance number is AREC/071/015*) by the Animal Ethics Subcommittee of the University of KwaZulu-Natal, South Africa.

The IntelliCage (IC) system

In this study, we used the IC (TSE Systems GmbH, Bad Homburg, Germany; http://www.tse-systems.com/products/behavior/intellicage/index.htm) for behavioural testing and monitoring of the mice. Female mice were used because a previous study has shown that IC is not appropriate for males due to interference with social hierarchy resulting from group housing (Gapp et al., 2014). IC is an automated apparatus used for behavioural characterization of transponder tagged mice (Endo et al., 2011). The apparatus, designed to fit into a large standard plastic home cage (20.5cm high, 58 x 40cm on top and 55 x 37.5cm base) (Krackow et al., 2010), consists of *food delivery space* on top, four *triangular operant conditioning chambers (OPC)* (15cm x 15cm x 21cm), four red plastic *shelters* that coalesce to a mini house at the center, a *controller* which provides an interface for connecting the cage to a central computer (PC) that has the operating application software, tubular *radio frequency identification (RFID) reader* and *other monitoring sensors* (Endo et al., 2011; Wolfer, Voikar, Vannoni, Colacicco, & Lipp, 2012). Leading to the OPC is a plastic tube which is moderately wide (30mm) to easily accommodate mice between 20-50g. Each operant conditioning chamber contains

two drinking bottles (*bottle licks are monitored by lickometers*) that are accessed via two round openings (13mm in diameter) guarded by motorized doors. The IC application on the PC has three components – designer, controller and analyser, which allow protocol design, run and data analysis, respectively. (*Refer to (Vannoni et al., 2014) for further details about the IC system*) (*Fig. 1 and 2*).

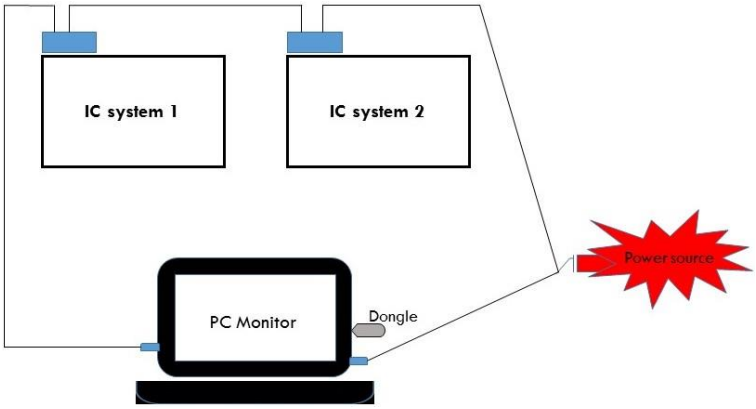


Figure 1: IC system connection with the computer interface. Programmed behavioral tasks in the IC systems were directed through the controller software installed on the computer. The IC system was connected using AC connecting cable and the hardware controller was linked to the computer port using serial converter. A dongle was required for effective running of the computer applications.

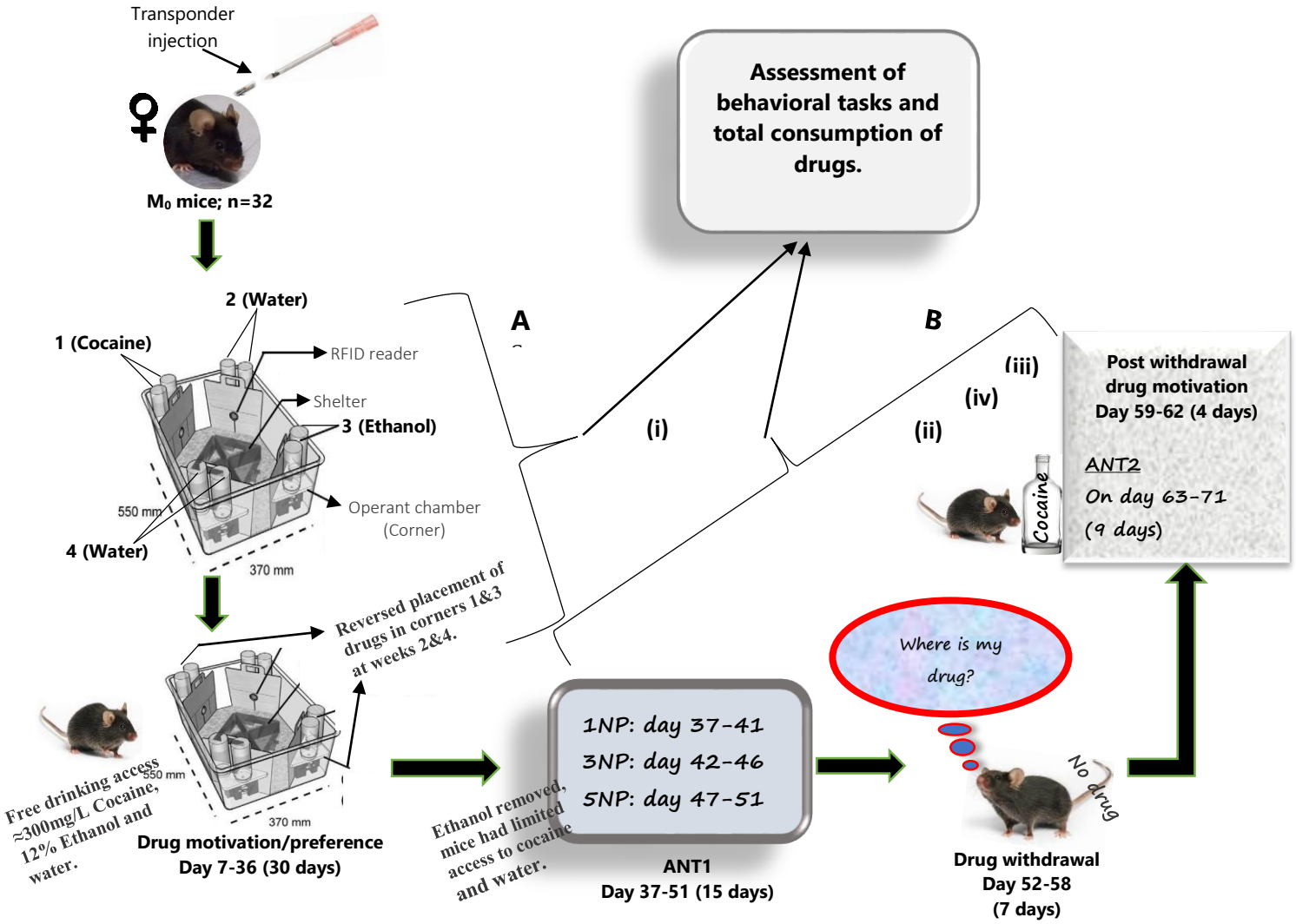


Figure 2: Overview of experimental procedures in the IC. After RFID tagging and habituation, the protocol was divided into two phases: (A) Initial 30-day period of motivation for rewards (water, cocaine and ethanol) and preference. Besides, various components of the automated IC system, also known as the home cage (Endo et al., 2011; Krackow et al., 2010), were shown and these include plastic shelters, operant chambers, drinking bottles and different monitoring sensors, such as RFID readers, light sensors and lickometers which allow effective tracking of changes in circadian rhythm and behavioural activities of group-housed transponder tagged mice in the home cage**.** (B) Cocaine-seeking and resisted drug-taking were demonstrated in four steps: (i) initial resistance learning tasks (NP1, NP3 and NP5) which examined persistence of M_0 mice for drug seeking/taking (ii) withdrawal of cocaine from the IC for 7 days (iii) post drug withdrawal motivation for 4 days (iv) post drug withdrawal ANT. Overall, the parameters recorded included corner visits with or without NP and lick contact time at bottles and total licks which were analysed and used for assessment of behavioural tasks given.

Drugs and Chemicals

Cocaine powder – salt form [*Issue Ref Number 26/57/4 (19/15)*] was obtained from the Forensic Science Laboratory [*Lab number 282409/15*] of the South African Police Service (SAPS) with permit [*POS 389/2015/2016*] issued by the Department of Health, Pretoria, South Africa. Both cocaine and ethanol (BDH, England) were dissolved in distilled water and presented to mice in drinking bottles as a free choice access as described below.

Experimental protocol

RFID Transponder Implantation

Before IC testing began, RFID transponders (*Planet ID GmbH, Essen, Germany*) with unique identification codes were injected subcutaneously into the posterior portion of the neck of all mice. This procedure was performed under light anaesthesia with halothane. The injected site was sealed with a small drop of tissue glue to prevent the transponder from slipping out. The animals were allowed to recover for at least 3 days before IC monitoring commenced. RFID tagging is essential for individual mice to be monitored by the automated apparatus during the test period.

Acclimatization

After RFID tagging recovery period, mice were placed in the IC for continuous monitoring. Initially the animals were allowed to freely explore and familiarize themselves with the IC environment for six days without restriction to food and water-containing bottles in all corners of the IC system. After this, the behavioural tasks for M_0 mice were divided into two phases.

Phase 1: Motivation/Preference for drugs

After the acclimatization period, water-containing bottles in two opposite corners of the IC housing the M_0 animals were replaced with ethanol (12% v/v) and cocaine (300 mg/L), respectively. These concentrations were used based on reports from Sershen et al. (1994) (Sershen, Hashim, & Lajtha, 1994), where a decreased consumption rate was observed when low doses <200mg/L or high doses >400mg/L of cocaine were used. Also, our choice of ethanol dose was due to previous studies that demonstrated pharmacologically significant ethanol (12% v/v) drinking in C57BL/6 mice with limited access (Le, Ko, Chow, & Quan, 1994; Rhodes, Best, Belknap, Finn, & Crabbe, 2005). Other authors have also suggested ethanol concentration in the range of 8 – 12% as a suitable standard for rodent's consumption (Sanchis-Segura & Spanagel, 2006).

In the first week of preference testing, corners 1 and 3 were assigned to hold bottles containing cocaine or ethanol solutions, respectively. We reversed drug placement every week to prevent

independent place preferences. On the other hand, both corners 2 and 4 were permanently assigned to hold bottles containing water throughout the experiment. All motorized doors to the operant chambers that give access to the nipples of the drinking bottles were kept opened continuously so that mice could enter any preferred corner and have unlimited drinking access time. The purpose of this test was to ascertain whether the strain of mice used in this study display a choice preference for either of the two drug solutions. Another advantage this protocol has over previous attempts that utilized a drug free-choice paradigm, is that among drug options we allowed access to water in order to maintain water balance in the rodents and to prevent drug-induced dehydration which could result from escalated drug consumption. This phase lasted for 30 days.

Phase 2: Drug-seeking and alternate drug-taking tasks

Alternate drug-taking tasks allowed strict evaluation of persistent behaviour directed towards drug taking even when access is resisted, restricted or denied. In this phase, we used the same mice that were previously exposed to cocaine and ethanol. Here, we imposed three levels of alternate nose-poke (NP) tasks, NP1 (1 nose-poke), NP3 (3 nose-pokes) and NP5 (5 nose-pokes) on the mice. Each task lasted for only five days. In each task, motorized doors were programmed to stay closed in all corners. The mice were expected to learn to differentiate cocaine (in corners 1 and 3) from water (in corners 2 and 4), make correct NP at the doors to access cocaine bottles. Once the correct NP was made, the door would open and stayed active for just 5 five seconds for the mouse to get rewarded (reward = opportunity to drink cocaine solution) after which the corner becomes inactive for the opposite door to become active. The implication of this strategy was that each mouse had to shuttle between two opposite cocaine corners within the active periods to get successive rewards. The alternating corner assignment was specifically controlled for each animal in the cage using the automated software. After the initial alternate nose-poke tasks (ANT1) were completed, cocaine solutions were removed from the IC for 7 days – a period which the mice only had free access to water and food. Following interruption of drug access, we again re-introduced cocaine for thirteen days. During the first 4 days, the IC system was programmed to allow unrestricted access to water and cocaine in their respective corners while the lick behaviour of the animals was measured. For the remaining nine days, persistent directed behaviours towards cocaine were again tested using the alternate nose-poke tasks (ANT2), NP1, NP3 and NP5 imposed (3 days/task) as previously described. The rationale for this alternate drug-taking approach stems from drug seeking behaviour observed in addicts where the drive to obtain the drug often requires overcoming severe obstacles and/or development of sophisticated goal-directed behaviours.

Scoring IC system behavioural tasks

Data indicating motivation (visits with or without NP) and actual consumption of drugs (lick contact time and total licks from bottles) in all task sessions were recorded and later extracted from the archives for subsequent analysis using FlowR software (XBehavior GmbH, Bänk, Switzerland). Overall spontaneous learning of scheduled ANT was also recorded and presented as proportion of correct visits to cocaine corners (shown as whisker plots and smooth curves with confidence interval).

Measurement of body weight

We recorded body weight changes in mice weekly over fifty-six days of behavioural testing in the IC using an electronic scale (Sartorius, Zeiss West Germany Pty. Ltd).

Post IC behavioural test battery: The open field test (OFT)

OFT was used to assess the anxiety-like behaviour of both M_0 and baseline control mice as previously described (Zhang et al., 2008), such that the exploratory activity was performed in a black plexiglass chamber (50 x 50 x 25cm) which has its floor divided into 16 identical squares, illuminated with bright lamp suspended 2m above the field arena. Each mouse was placed at the centre of the chamber and allowed to explore for 5 minutes. Mouse behaviour was recorded by a video camera placed above the structure for subsequent analyses. The parameters assessed included central square (CSq) visits, total time spent visiting the CSq, rears (the total time the animal stood on its rear paws or lean against the walls) and grooming behaviour.

Statistical analysis

Behavioural variables or measured parameters obtained from all testing sessions were extracted from the archives, averaged and analysed using FlowR software. Drug(s) versus water lick behavior were plotted as time-bin graphs while sequential learning of drug-taking tasks was presented as whisker plots or smooth curves. Data set for body weight changes and mood behavior in the OF were analyzed with GraphPad prism 5 software using repeated measures (RM) ANOVA or Student's two-sample *t-test*, respectively. The data were presented as mean ± SEM. $p<0.05$ was considered as criterion for statistical significance.

Results

Body weight changes of M_0 and control mice.

Repeated measures (RM) One-Way ANOVA revealed that the time of exposure to drugs presented significant main effects ($F_{(8,120)}$=70.32, $p<0.0001$) on body weight of the animals (Figure. 3). In general, we observed an increase in body weight of both groups of animals, with no significant differences ($p>0.05$) between them, throughout the testing period. This observation of weight gain in our model opposed the findings from many studies which vastly reported that chronic cocaine and/or ethanol consumption negatively affect body weights in animals (Bozarth & Wise, 1985; Larsen, Vrang, Petersen, & Kristensen, 2000; Tran, Cronise, Marino, Jenkins, & Kelly, 2000).

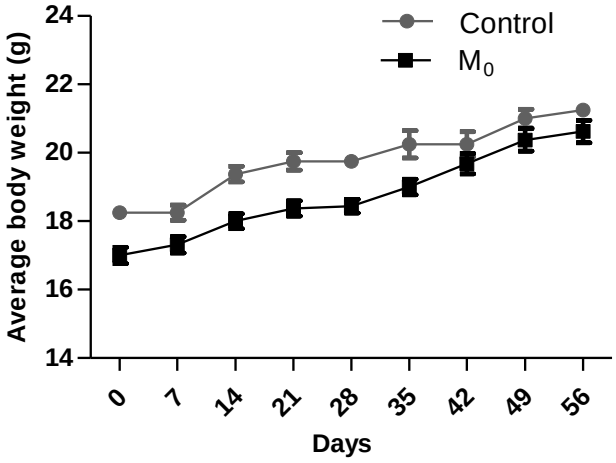


Figure 3: Body weight profile of group-housed mice recorded over fifty-six days access to the IC. The data shows that both the baseline control and M_0 mice gained weight throughout the testing period. Data shown represents Mean±SEM; control, ball shape; M_0, square shape. RM ANOVA.

Phase 1: Behavioural indices of drug motivation and consumption preferences

As shown in Figure 4, our data demonstrated that the M_0 mice licked more often from water (in corners 2 and 4) than either of the drug bottles (in corners 1 and 3) during the 30-day period of unlimited drinking access. This suggested that a greater amount of water was generally consumed by the animals relative to drugs throughout the access period, except on days 22 to 28 when cocaine and water licks were comparably similar. Between drugs, lick patterns were also significant as cocaine solution was moderately licked compared to few licks at the ethanol bottles. We recorded 1147.19 licks at cocaine bottles on day 1 which later averaged to 631 licks over day 6 to 7 of drug exposure compared to insignificant licks at ethanol bottles during this time. Cocaine licks then sharply dropped on day 8 to almost the same levels as ethanol, moderately increasing from day 9 to 13 and then

decreasing again on day 14 to levels comparable with ethanol. On days 18 to 20, ethanol licks also moderately increased and then returned to about initial levels on day 23. As ethanol licks decreased, cocaine licks markedly increased (day 23 – 27) to about the starting lick-level on day 1. Surprisingly, as licks in cocaine bottles dropped on day 28, ethanol licks sharply increased to about the same cocaine lick-level in the first week of exposure (Figure 4). See table 1 for overall descriptive statistics of visiting frequencies, pokes at specific corners and bottles as well as averaged lick contact time during the first 30 days of unrestricted access to polydrugs and water.

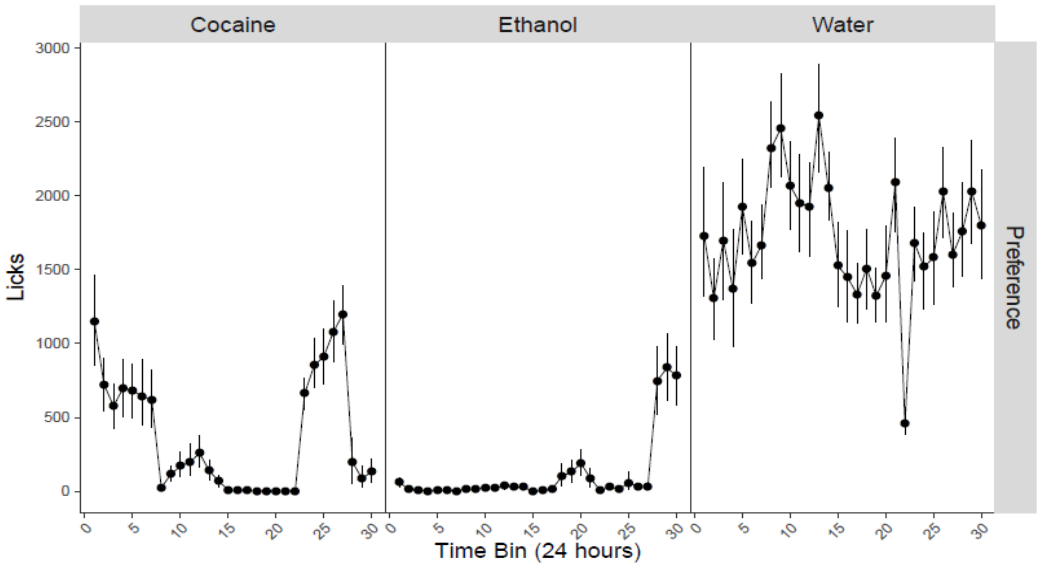
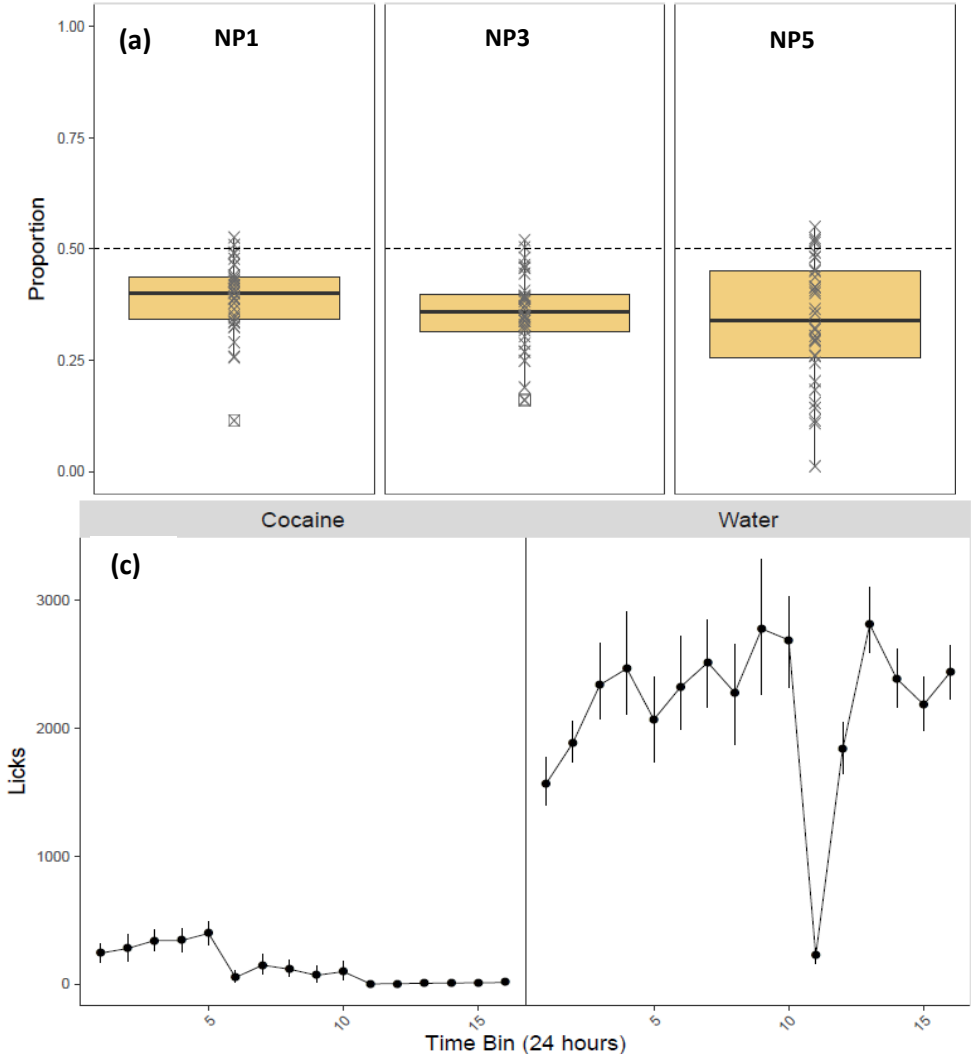


Figure 4: Behavioural characterization of M_0 mice during 30 days of voluntary choice drinking access to cocaine, ethanol and water in the IC. The 24h time bin graph demonstrates that the mice drank more from water than cocaine bottles and much less from ethanol. All data were extracted and analysed using FlowR software. Further analysis, RM ANOVA followed by Tukey Multiple Comparison test. n=32 (cocaine, ethanol and water).

Phase 2: Learning outcomes of the scheduled alternate nose-poke tasks

Figures 5a and 5b show that the animals exhibited a relative avoidance of cocaine for 15 days ANT1 that followed the initial drug motivation period. This is because most of the animals failed to attain a random expected 50% criterion for correctly visiting cocaine corners as demonstrated by an insignificant visit proportion in all three levels of NP tasks. Lick behaviour data further showed that the M_0 mice consumed relatively lower amounts of cocaine compared to water during this period as indicated on the 24h time bin graph in Figure 5c.



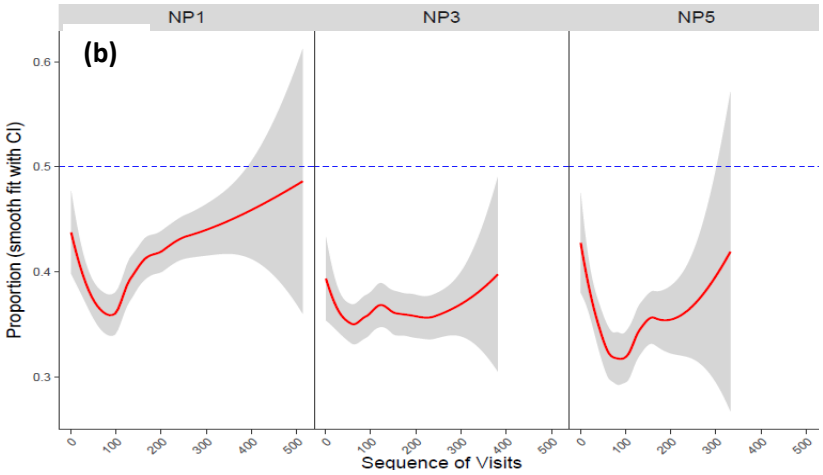


Figure 5: Proportion of correct visits to cocaine corners and lick patterns at bottles during 15 days of initial ANT1 (a-b) box plot graphs and confidence interval (CI) smooth fit curves showing visiting proportion at correct corners relative to the scheduled learning tasks (c) 24h time bin of M_0 mice lick-behaviour indicating higher licks at water bottles than cocaine during the learning task period. All data were extracted and analysed using FlowR software; n=32.

Subsequent to ANT1 and 7 days of drug withdrawal, 4 days of uninterrupted cocaine access was introduced and our findings showed that the mice progressively increased their consumption of cocaine daily throughout the access period, whereas water consumption initially increased in the first three days after which it slightly decreased but not at comparable levels with cocaine (Figure 6). Overall, our data demonstrated that M_0 mice still drank more from water than cocaine bottles in this period (Figure 6).

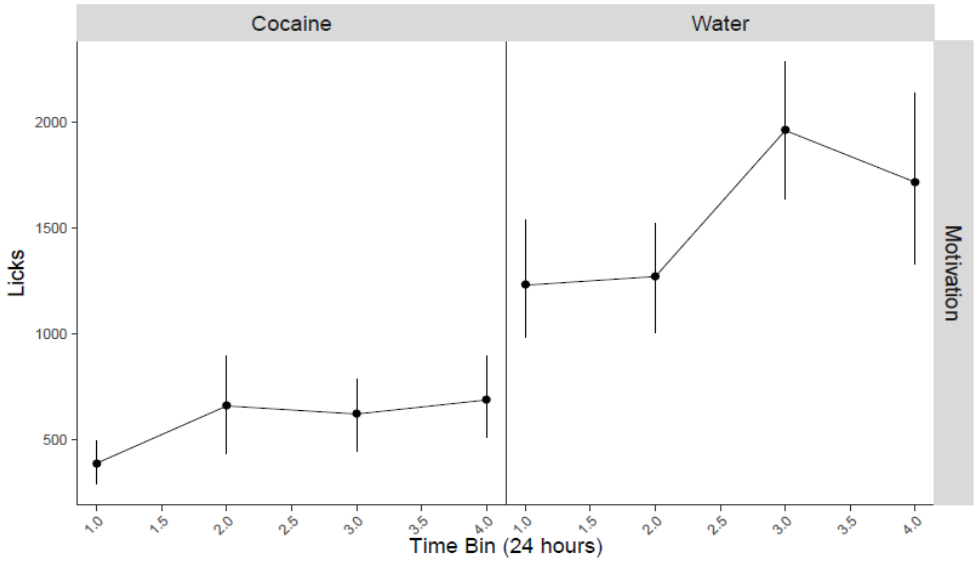
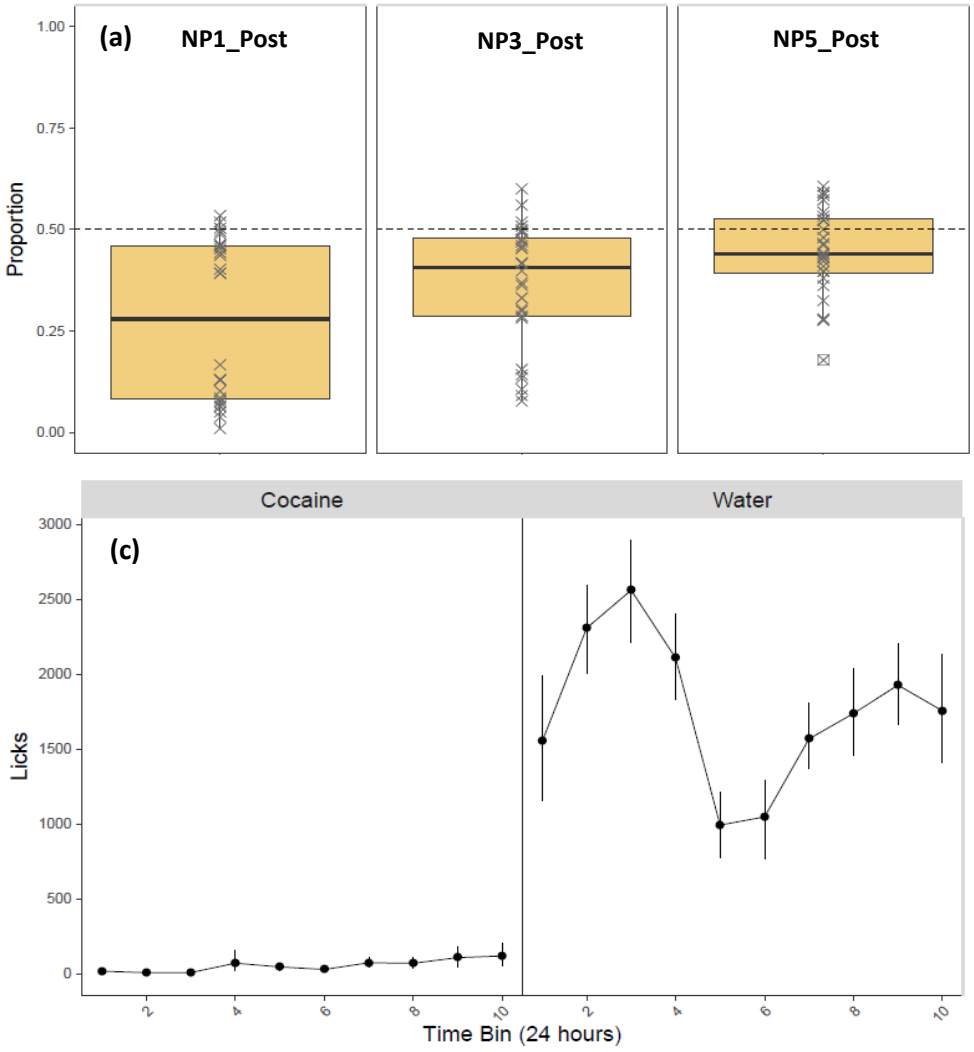


Figure 6: Behavioural characterization of M_0 mice during 4 days post withdrawal motivation period. The time bin graphs indicate that the amount of water drunk by the animals was greater than cocaine throughout the brief period of reintroducing the drug. All data were extracted and analysed using FlowR software. Further analysis, Student's *t-test.* n=32 (cocaine *vs* water).

As shown in Figures 7a and 7b, the set criterion for learning across task sessions was also not met in the second ANT that lasted 9 days. Although, there was progressive improvement in the nose-poking action (from NP1 to NP5) but the average proportion of correct visits to cocaine corners was still considered insignificant due to the expected 50% chance of visiting either drug or non-drug corners. During this period, the animals also drank less from cocaine corners compared to water (Figure 6) which indicated a decreased success rate in acquiring the drug. Statistics of other relevant behavioural parameters, i.e visits, pokes and lick contact time at corners, as measured during phase 2 are also shown in Table 1.



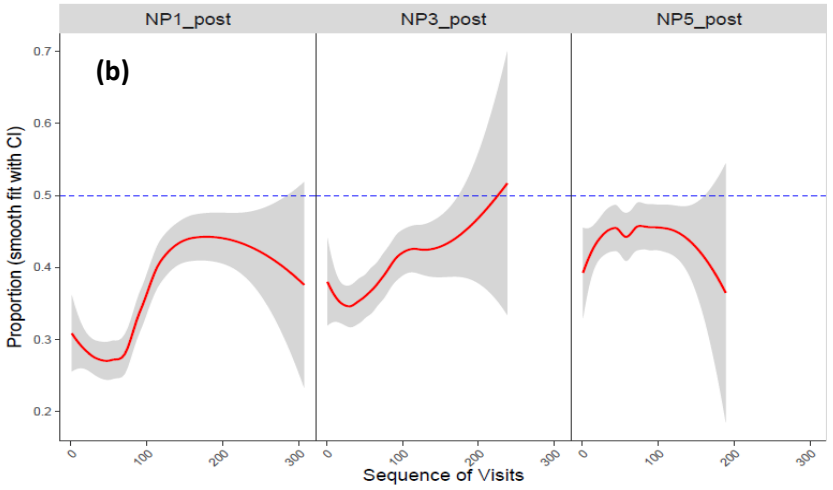


Figure 7: Proportion of correct visits to cocaine corners and lick patterns at bottles for 9 days of ANT2 (a-b) box plot graphs and confidence interval (CI) smooth fit curves showing visiting proportion at correct corners relative to the scheduled learning tasks (c) 24h time bin graphs indicating that the M_0 mice still drank more from water bottles than cocaine during the second learning task schedule. All data were extracted and analysed using FlowR software; n=28.

Table 1: Descriptive statistics of IC measured parameters (averaged across days). LCT= lick contact time; ANT= alternate NP task; x/n=total observations/days

		Mean	SEM	Percentile				
				10	25	50	75	90
Phase 1	Visits to cocaine corners (%)	28.44	5.336	7.907	18.54	32.94	36.09	38.32
	Visits to ethanol corners (%)	14.15	4.964	4.444	6.398	8.398	24.77	32.06
	Visits to water in corner 2 (%)	33.97	4.044	18.60	25.80	38.78	39.73	40.25
	Visits to water in corner 4 (%)	23.44	4.209	17.50	17.97	20.33	30.47	40.10
	Pokes in cocaine corners (%)	29.18	5.353	11.08	16.98	35.37	38.28	39.29
	Pokes in ethanol corners (%)	17.04	4.974	5.494	7.298	12.95	28.83	30.15
	Pokes in water corner 2 (%)	32.95	3.074	21.19	26.95	35.68	37.59	37.74
	Pokes in water corner 4 (%)	20.83	3.044	16.00	16.45	17.47	26.88	32.45
	Cocaine licks (%)	16.06	7.677	0.1238	2.582	6.211	34.47	38.27
	Ethanol licks (%)	7.364	5.227	0.6488	0.7463	1.188	17.07	27.85
	Water licks in corner 2 (%)	52.83	9.001	20.27	33.30	63.84	66.86	68.02
	Water licks in corner 4 (%)	23.75	9.167	3.000	11.89	24.58	35.18	40.62
	LCT at cocaine bottles (x/30, sec)	14.75	1.163	3.572	9.578	13.61	20.08	30.32
	LCT at ethanol bottles (x/30, sec)	2.584	0.4125	0.08427	0.3543	2.266	4.957	7.343
	LCT at water bottles (x/30, sec)	23.35	0.9955	13.39	17.84	24.19	27.18	32.57
	Total cocaine licks (x/30)	373.60	72.48	0.0625	8.391	183.60	686.20	1199.00
	Total ethanol licks (x/30)	111.10	42.63	0.3750	7.328	25.91	66.97	835.30
	Total water licks (x/30)	1722.00	74.27	459.40	1495.00	1687.00	2025.00	2540.00
Phase 2	Total cocaine licks in ANT1 (x/15)	140.40	36.56	1.500	8.813	96.81	277.80	396.10
	Total water licks in ANT1 (x/15)	2155.00	165.00	222.30	1885.00	2323.00	2512.00	2809.00
	LCT at cocaine bottles (x/4, sec)	10.25	1.393	1.041	5.299	8.433	13.19	33.34
	LCT at water bottles (x/4, sec)	20.62	2.129	4.757	12.23	17.20	26.60	54.29
	Total cocaine licks during motivation (x/4)	587.50	68.17	387.10	445.20	638.50	678.80	685.80
	Total water licks during motivation (x/4)	1543.00	177.3	1229.00	1239.00	1492.00	1899.00	1960.00
	Total cocaine licks in ANT2 (x/9)	46.98	11.66	6.571	12.00	45.00	71.36	107.10
	Total water licks in ANT2 (x/9)	1755.00	177.50	992.20	1299.00	1737.00	2207.00	2560.00

Exploratory behaviour was maintained after drug experience in the IC

Evaluation of exploratory behaviour of mice in the OF arena indicated that the number of central square entries (Figure 8a), the total time spent in the center (Figure 8b) and grooming behaviour (Figure 8d) did not differ significantly when drug-exposed animals were compared to baseline control mice (p>0.05). However, M_0 mice engaged in higher rearing behaviour indicated by increased vertical exploration compared to control animals (Student's *t-test*, p<0.05) (Figure 8c).

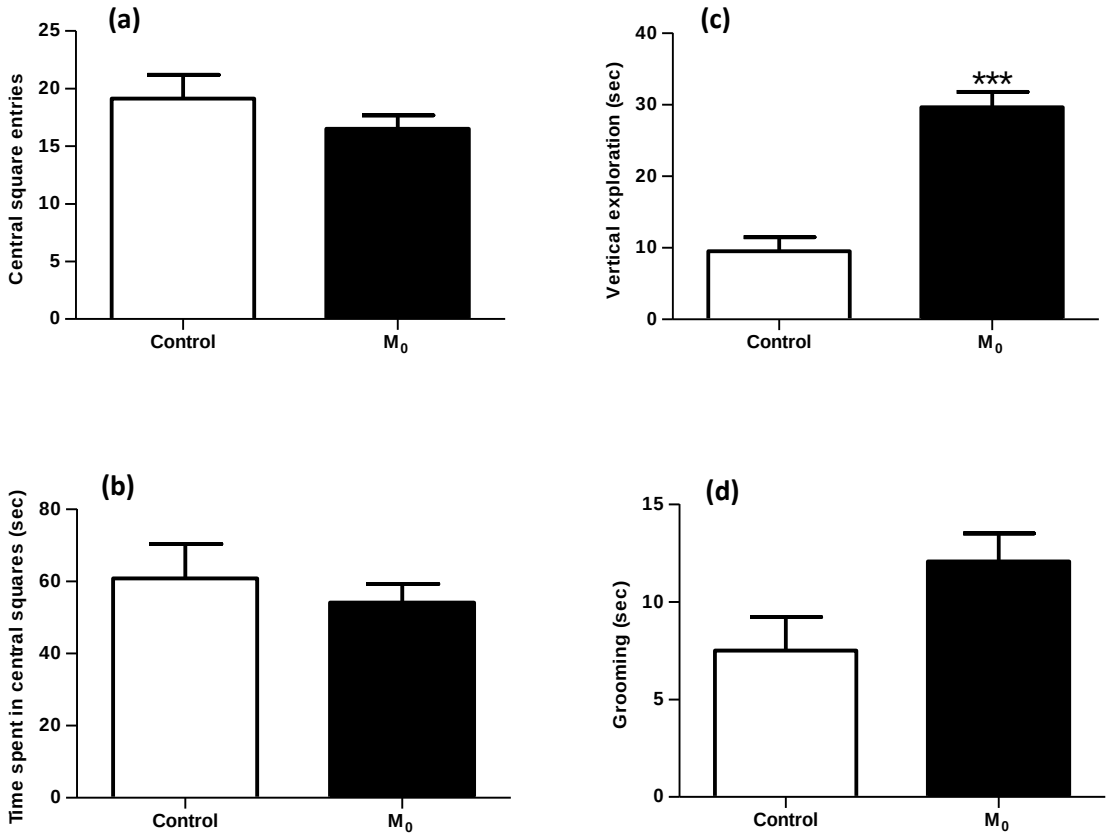


Figure 8: Open field activity of M_0 mice indicated unaltered anxiety-like behaviours due to insignificant changes in (a-b) number and duration of central square entries and (d) grooming behaviour. However, the mice somewhat displayed increases in exploration characterized by (c) significant vertical exploration or time spent rearing. Data shown represents Mean±SEM; n=8 (BS control), n=14 (M_0); ***p<0.001. (Student's *t-test*).

Discussion and Conclusion

In this study, we used home-cage free choice drinking paradigm that employed a multiple-solution preference approach which allowed group-housed mice to exhibit free choice drinking from multiple water bottles versus bottles containing ethanol or cocaine solutions. Our data showed greater drinking and exploratory activities in water-containing corners than either drug during the first 30 days of preference testing. The lick preference attributed to water was not surprising as most human drug addicts are known to also consume water while they are abusing drugs. Unexplained, however, is that water and drug solutions were generally licked less on day 22 of the preference testing.

Between the two drugs, preference data indicated that cocaine was visited and licked more often than ethanol. However, increased activity and consumption of ethanol observed just after switching corners for drugs in the second week (day 9 – 13) may be attributed to a lick preference for cocaine previously tied to that corner. Previous studies have reported ethanol escalation in rats following every-other-day (EOD) or intermittent access (Melendez, 2011; Rosenwasser, Fixaris, Crabbe, Brooks, & Ascheid, 2013). Another demonstration with C57BL/6 (B6) mice revealed that uninterrupted access to ethanol, water and food blocked EOD-elicited ethanol escalation (Melendez, 2011). These reports parallel our findings as mice given voluntary and unrestricted drinking access to drugs and water demonstrated relatively low ethanol consumption but with gradual increase over time. Similarly, water-penalized ethanol intake was previously proposed in a study by Pildaín et al (2013). These authors presented varying concentrations of ethanol with accompanying water in drinking bottles and later measured alcohol intake by ethanol preference. They showed that overall ethanol intake decreased with increasing alcohol concentrations (Pildaín, Vengeliene, & Matthäus, 2013). In this study, the overall decrement in voluntary ethanol consumption may further be attributed to a similar effect of using a moderately high concentration of ethanol (12% v/v). It can therefore be expected that continuous free choice access to ethanol rarely cause voluntary ethanol consumption at levels sufficient to induce intoxication or to engender dependence (Crabbe, Harkness, Spence, Huang, & Metten, 2012; Rosenwasser et al., 2013), except when sweetened solutions are added (Ristuccia & Spear, 2008; Sanchis-Segura & Spanagel, 2006). Resting on this knowledge, we chose an ethanol–preferring C57BL/6 mouse phenotype (Rosenwasser et al., 2013; Szumlinski, Ary, Lominac, Klugmann, & Kippin, 2008) for our study to evaluate the hypothesis suggesting that ethanol use serves as a "gateway" drug for cocaine (Levine et al., 2011).

Due to substantial low preference for ethanol and moderate consumption of cocaine, our findings do not unequivocally support the "gateway" effect of ethanol despite preference for cocaine. Besides, it appears that cocaine consumption versus ethanol intake observed throughout the initial preference phase occurs in an alternating periodic manner, that is when the mice licked more from cocaine bottles they also did not lick or licked less from the ethanol bottles and vice-versa. These dynamic

intake behaviour is typical of binge drinkers in social settings. These findings are also in agreement with a previous study which demonstrated that unlimited access to cocaine hydrochloride induced a pattern of episodic drug intake characterized by periods of excessive cocaine self-administration alternating with brief periods of abstinence with a gradual increase in daily drug intake over the first two weeks of testing (Bozarth & Wise, 1985). It has also been established in literature that rat models of drug-self administration remarkably maintained consistent levels of cocaine intake when limited access was given, whereas they exhibited variable patterns of intake behaviours that were subject to change over time (Roberts, Brebner, Vincler, & Lynch, 2002). The authors further alluded that motivation for drugs can decrease under certain conditions of extended access thus constituting another reason for the alternating pattern of drug taking behaviour in our mice.

However, it was unexpected that the mice did not peak on cocaine intake to a full state of dependence within the thirty days of free choice access based on previous demonstrations that reported that combined intake of cocaine and ethanol reduced retreat behaviour in rats given the opportunity to self-administer intravenous cocaine in a runaway test (Knackstedt & Ettenberg, 2005). It has also been reported that the euphoria experienced by cocaine users is further enhanced by concurrent ingestion of ethanol (McCance-Katz et al., 1998; McCance-Katz et al., 1993). Similarly, we expected that the reward stemming from both drugs would strengthen approach behaviour and increase cocaine intake relative to water. Moreover, research evidence have also indicated progressive increases in reward threshold of rats differentially exposed to cocaine self-administration on long term access compared to those on short term access (Ahmed, Kenny, Koob, & Markou, 2002). Exquisitely, this dramatic effect may contribute to the insufficient intake of cocaine observed in our model (Koob, 2017). It has been established that social and environmental factors are also important determinants of drug consumption in rodents. For instance, a recent study demonstrated that isolated mice had greater preference for ethanol than those housed in groups or in the IC (Holgate, Garcia, Chatterjee, & Bartlett, 2017). This supports the impact of converging social behaviour and/or group interaction/housing enrichment on the animal's choice thus constituting another possible reason for low consumption of drugs in the mice used for this study. Furthermore, the enrichment factors (social and environment) and water may also serve as alternative rewards which tend to shift the animal's attention away from drugs as they are found to be more rewarding than the pharmacological effects of either drugs. For example, studies have shown that cocaine addicted rats will forgo cocaine if offered alternative rewards like sugars, saccharin (Zernig, Kummer, & Prast, 2013) and snuffing or snuggling cage mates (Pickard, Ahmed, & Foddy, 2015).

We further seek to determine whether prior drug sensitization and 7 days of drug withdrawal will provoke drug seeking and intake behaviours in mice that had previously experienced oral cocaine as previously posited (Ahmed & Koob, 1998; Ahmed & Cador, 2006). Surprisingly, we found that

cocaine and water consumption during the brief period of 4-day uninterrupted access was much comparable to that during the initial 30 days, thus suggesting that previous drug exposure and/or withdrawal experience had no impact on intake behaviour of the IC group-housed mouse. This confirms that the animals only maintained their drinking status but not to escalated levels, as we also expect that there would have been a frenzy in the intake behaviours if they attained cocaine dependence.

Failure of mice learning and persistence in acquiring cocaine during alternate NP tasks resulted in decreased success learning rates. This decrease meant an insufficient consumption of cocaine and further reinforced our reasoning of the negative impact of possible confounding consequences of alternative rewards, low impulsivity or reduced ability to reach threshold for cocaine reward. In order to rule out learning disability in our mice during performance of progressive ANT schedules, we subjected the same animals to memory test using novel object recognition task. We found that memory performance was intact in the animals (data not shown) which further confirms that the amount of drugs consumed over 58 days of oral voluntary access in the home-cage appears insufficient to cause severe neurobiological alterations and/or cognitive dysfunctions as expected. However, this did not mean that the mice had not exhibited drug-seeking behaviours, (as evidenced for example by increased shuttling visits between drug corners), rather they only failed to transit to a full addicted state.

Accumulating evidence from clinical and preclinical studies have shown that females are more susceptible to the reinforcing effects of psychostimulants mainly due to ovarian hormone influences (Lynch, Roth, & Carroll, 2002; Roth, Cosgrove, & Carroll, 2004). Although we did not consider gender-based effects in the present study, but since the mice used were females evaluated for a long time we suppose that many of the observed changes in drug seeking and consumption, especially during the transition phase, may partly be influenced by the hormonal waves. Moreover, animal studies have further shown that females regularly self-administer addictive drugs at lower doses than males but rapidly escalate to addiction (Becker & Hu, 2008). Therefore, non-compulsive drug consumption after prolonged exposure was unexpected in our model, which suggests that further studies may be required to untangle the interlink between ovarian hormones, environmental and neurobiological factors that mediate transition from steady-state maintenance of drug use to dysregulated-escalated states which contribute to drug abuse progression and vulnerability.

It is long established that co-morbidity exists between substance use and anxiety-related disorders with consequent modulations in neural functions geared towards attention and enhanced consumption of natural rewards for survival (Russo & Nestler, 2013). In addition, animal studies that utilized depression models of C57BL/6J mice have reported that social isolation or chronic social defeat stress causes grooming deficits and increased anhedonia or anxiety-like symptoms (Krishnan et al., 2007;

Warren et al., 2013). In this study, longitudinal exploratory activities measured in the OF revealed increased rearing whereas other exploratory-related parameters remained unaltered. At least, we expected reciprocity in grooming and rearing behaviours to help our judgement on the exploratory states of the animals. Due to the nature of our result, we imply that exploratory behaviours were maintained even after cocaine experience while increased rearing may reflect some of the anxiolytic-like behaviours that accompanied previous social interaction episodes and not necessarily result from drug effects due to the observed decrease in anhedonia manifested by increased intake of food and water.

In conclusion, this is the first study to report on the behaviour of mice housed under social conditions in an IC with voluntary choice consumption of cocaine, ethanol and water. The data showed that the social components of the IC system and water presented as an alternative reward may possibly conflict with the animals' choice and preference for drugs. Between drugs, cocaine consumption was higher except in the last two days of preference testing. Overall, there was an alternating pattern of intake behaviour in the mice. Our data also showed that both baseline control and drug exposed mice gained body weight within the 58-day bracket of drug exposure. Despite no evidence of memory loss, drug-exposed mice still failed to learn the ANT in order to gain access to cocaine suggesting that the animals did not develop strong drive to seek the drug. The clinch on sustained exploratory behaviour in the OF following drug experience and the paradox of anhedonia-like symptoms further suggest converging psychological impact of social interaction. In contrast to expectation, the mice did not show any signs of addictive behaviour. Since the fundamental difference between our study and that of others revolve around the nature of our animal model (free choice of drug consumption in a social environment), further studies are required to tease out the impact of the environment on the development of addictive behaviour and compare changes with animal models of social isolation.

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Author contributions

Originally, this project is part of an ongoing PhD research by D.C, who carried out the experimental work and wrote the manuscript. Project planning and development was by W.M.U, D.C and O. S. analysed the IntelliCage data and produced the graphs, while S., H.P, M.V and W.M.U assisted with data interpretation and manuscript editing. W.M.U and M.V supervised the research. The results were discussed, and comments carefully challenged by all authors.

Conflict of interest

All authors declare that they have no competing financial or any other conflict of interests to disclose.

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CHAPTER FIVE

This chapter forms part of my PhD research work and has been published in Metabolic Brain Disease. The article is titled: **"Differential epigenetic changes in the hippocampus and prefrontal cortex of female mice that had free access to cocaine".** All figures with legends have been included for easy reading and understanding.

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Abstract

Alterations in gene expression within the neural networks of prefrontal cortex (PFC) and hippocampus (HPC) are known to contribute to behavioural phenotypes associated with drug intake. However, the functional consequences of regulated expression patterns of *Fosb* and *Crem* (cAMP response element modulator) in both brain regions in response to volitional intake of cocaine in social environment is yet to be explored. Here, we first exposed young adult mice to cocaine (300mg/L) and water concurrently for 30 days in the IntelliCage to investigate consumption preference, and subsequently for 28 days during which persistent motivated drug seeking behaviours were examined. Thereafter, locomotor activity and memory performance of the mice were assessed. DNA methylation status in the promoters of *Fosb* and *Crem* genes were also evaluated. We show that mice that had extended access to cocaine exhibited motivational deficit and demonstrated decreased locomotor activity and intact recognition memory. These changes were accompanied by hypomethylation or hypermethylation in the promoters of *Fosb* and *Crem* genes in the PFC and HPC of the cocaine-experienced mice, respectively. Together, these findings correlate the molecular changes to behavioural effects of the treatment and further suggest a possible activation of prefrontal cortical networks by social interaction episodes in the IntelliCage which possibly enhanced behavioural control that dampens mice sensitivity to cocaine rewards. Furthermore, our data delineate the molecular response of *Crem* and *Fosb* to oral cocaine in group-housed mice and demonstrates differential regulation of activities within the substrate brain regions studied.

Keywords: Cocaine; IntelliCage; Social environment; DNA methylation; *Fosb*; *Crem*.

Introduction

Neuroadaptive changes and alterations in gene expression within brain structures of the mesocorticolimbic circuit, especially the PFC (that mediates executive functions) and the HPC (that is involved in context-specific memories associated with reinstatement of drug seeking), represent attractive candidates for studying the molecular underpinnings of drug abuse. Interestingly many molecular changes may not necessarily be directly related to the neurochemical effects of drugs, but may stem from other factors that may also influence behavioural phenotypes and/or physiological responses to drug intake (Parkitna and Engblom 2012). For instance some authors have attributed phenomena such as decreased sensitivity to drugs to alterations in the environment rather than as part of the neurobiological sequelae of chronic drug intake, suggesting that context of drug consumption may itself have profound biological and behavioural consequences (Piazza and Le Moal 1998, Morgan et al. 2002).

Drugs of abuse commonly lead to heightened dopamine neurotransmission. Binding of dopamine to postsynaptic D1 receptors results in an increase in intracellular cAMP levels, that in turn lead to the activation of protein kinase A (Self 2004). Protein kinase A (PKA RIIβ) phosphorylates glutamatergic receptors (NMDA and AMPA) causing an influx of calcium into the cell. The increased calcium binds to calmodulin that subsequently leads to the activation of the enzyme CaMKinase II/IV, that in turn phosphorylates *Creb*. Phosphorylated *Creb*, as a transcription factor, promotes the transcription of many genes, including the immediate early gene, *Fos* (Levine et al. 2005, Kida and Serita 2014). *Creb* and *Fos* are therefore key factors known to play significant roles in regulating the expression of genes related to drug reward and vulnerability (McClung and Nestler 2003, Miller et al. 2017). However experiments involving the use of viral vectors or bi-transgenic mice have shown that either acute or chronic exposure to cocaine increased *Creb* activity in the nucleus accumbens and dorsal striatum, and this observation was associated with a decrease in the rewarding effect of cocaine (Carlezon et al. 1998, Robison and Nestler 2011). In contrast, Larson et al. (2011) reported increased cocaine reinforcement in self-administering Sprague-Dawley rats following *Creb* overexpression in the nucleus accumbens shell. These reports clearly indicate that the exact nature of the role of *Creb* in cocaine-mediated effects still appears to be uncertain.

Crem is a related family member that is similar to *Creb* due to its specific binding to cAMP-responsive element (CRE) sequences and may either act as an inducible cAMP repressor (Robison and Nestler 2011) or activator (Sassone-Corsi 1995). Phosphorylation of multiple serine residues of *Crem* has been shown to elicit its activator functions by enhancing DNA binding (De Groot et al. 1993). In contrast, several *Crem* transcripts were reportedly increased following hippocampal *Creb* deletion in mice (Hummler et al. 1994). Besides, *Crem* is also considered a nuclear effector where

signalling pathways may converge and/or cross-talk (Masquilier and Sassone-Corsi 1992, De Groot et al. 1993).

A recent study has implicated a role for *Crem* in drug-induced behavioural effects. Miller et al (2017) demonstrated that impulsive rats that were neurochemically and behaviourally sensitive to heroin exhibited decreased striatal *Crem*, but when *Crem* was virally expressed in this brain region, heroin-associated impulsivity was decreased (Miller et al. 2017). This finding suggested a possible modulatory role for *Crem* in the precipitation of heroin-induced behavioural effects.

Despite the extensive literature on the function of *Fos* and *Creb* in mediating behavioural effects and physiological responses to drugs, the role of *Fosb* and *Crem* in relation to cocaine consumption within a social environment has not yet been studied. Here, we investigated the DNA methylation status of the promoter regions of *Fosb* and *Crem* genes in the PFC and HPC of mice that were group housed and had free access to cocaine to simulate voluntary drug consumption (as opposed to experimenter-forced drug intake) within a social context. We also assessed the animals' recognition memory and locomotor activity as indicators of overall functioning of the PFC and HPC.

Methods

Animals and treatment protocol

All experiments were performed in accordance with the National Institute of Health guidelines on the ethical use of animals, using approved protocols by the Animals Ethics Subcommittee of the University of KwaZulu-Natal (UKZN) [*Ethical clearance, AREC/071/015*]. A total of forty C57BL/6 female mice (9 – 10 weeks old) were used for this study. They were obtained from the Biomedical Resource Unit (UKZN, Durban) and maintained in temperature (23±2 ˚C) and humidity (50±5%) controlled rooms with 12h light – dark cycle (light from 0600 to 1800 h). Of the total sample size (n=40), thirty-two mice, (2 times n=16) were subsequently housed in an IntelliCage apparatus (NewBehavior, TSE Systems, Germany). A major advantage of this system is that animals can be studied in a "social home cage" environment (Rudenko et al. 2009), and there was no need for any handling by the experimenter except for normal housekeeping of the apparatus. The animals were kept in the Intellicage for 71 days. During this period, the animals were initially allowed to familiarize themselves with the IntelliCage environment for 6 days after which they had free access to drug (300 mg/L cocaine solution) and water concurrently for the next 30 days. This was followed by a 15-day interval during which animals had to execute tasks (which consisted of increasing number of nose-pokes: NP1, NP3 and NP5) to gain access to the drug solution. Subsequent to this phase, a 7-day period of drug withdrawal followed. Cocaine was then re-introduced to the animals for the next 4 days

after which they were subjected to second period of task-mediated access to drugs (9 days). The tasks and withdrawal period were used to stimulate and/or reveal persistent, motivated drug-seeking behaviors. Cocaine consumption of the animals were subsequently assessed under two sets of circumstances, i.e. (1) after having prolonged access to the drug (first 30 days), and (2) after a period of drug withdrawal followed by a brief period of drug access.

The 32 animals that were housed in the Intellicage were implanted with transponders to facilitate the automated continuous recording of the behavior of each individual mouse. A separate group of eight mice (n=8) were only exposed to the apparatus without access to drugs and thus served as controls.

Drugs and Chemicals

Cocaine powder (salt form) was generously provided by South African Police Service (SAPS). Cocaine purity was confirmed by nuclear magnetic resonance (NMR) at the Catalysis and Peptide Unit, Discipline of Pharmaceutical Sciences, University of KwaZulu-Natal. The NMR spectra (Fig. 1a, b) were recorded on a Bruker Avance 400Mhz instrument in deuterated methanol as the solvent. The proton and carbon spectra confirmed the molecular formula and splitting patterns characteristic of the molecular structure of cocaine in accordance with the literature (Allen et al. 1981). Cocaine was dissolved in distilled water and presented to mice in drinking bottles.

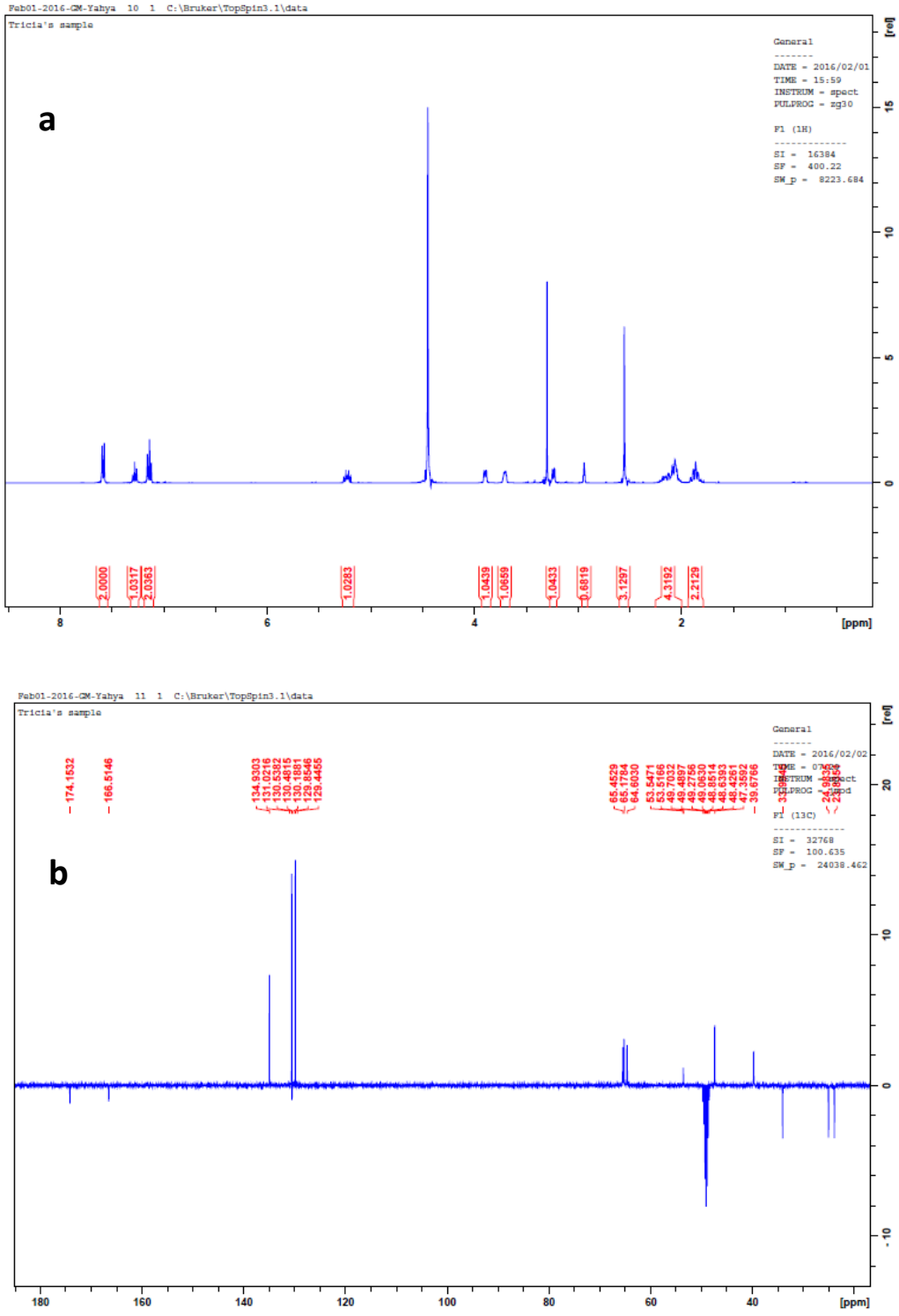

Fig. 1: NMR spectra showing the purity analysis of cocaine powder which indicates (a) the proton signals and (b) the carbon signals compared to standard chemical structure. It was recorded on Bruker Avance 400 Mhz instrument using deuterated methanol as the solvent. All signals were accounted for, hence need no further purification.

Assessment of cocaine consumption

Cocaine and water consumption were monitored everyday throughout the test period. To determine the amount of drug ingested by the mice, lick data were extracted from the archives and analysed using FlowR software (XBehavior GmbH, Bänk, Switzerland). In each archive, lick contact times at each bottle were summed for each subject and divided by the duration of the session to give lick contact times per day (in seconds). The mean bottle lick contact time was then taken as a measure of drug seeking behaviour. The average consumption of drug solutions was calculated by multiplying the bottle licks with number of available bottles. Drug consumption was calculated at two intervals, i.e. after the first 30 days of continuous drug access, and then again after 4 days drug access that was preceded by a drug withdrawal phase.

Behavioural tests

After drug exposure in the IntelliCage, overall locomotor abilities of the animals were assessed in the open field (OF), while memory competence was tested using the novel object recognition (NOR) test.

OF Test

In this test, locomotor activity was assessed in a black plexiglass chamber (50 x 50 x 25cm) which has its floor divided into 16 identical squares, illuminated with a bright lamp suspended 2m above the field arena. Each mouse was placed at the centre of the chamber and allowed to explore for 5 minutes. Mouse behaviour was recorded by a video camera that was positioned above the OF apparatus for scoring at a later stage. Total number of peripheral and central squares crossed was used as a measure of locomotor activity.

NOR test

Recognition memory performance of individual mice was assessed using the NOR task. This testing paradigm enables an animal to discriminate between novel and familiar objects, a process that is dependent on prefrontal cortex (Ennaceur et al. 1997) and hippocampal functioning (Broadbent et al. 2010). The task procedure which consists of familiarization and retention/test sessions, was conducted over two days. During the familiarization phase, each mouse was placed at the center of a transparent rectangular cage (16cm x 24cm x 12cm) that had objects A and B. The animals were allowed to explore the objects for 5 minutes after which they were returned to their home cage. 24 hours later (test phase), object B was replaced with object C (the novel object), whereas object A (familiar object) was retained. Mice returned to the test arena to perform the recognition task. The amount of

time spent exploring the novel (T_n) and familiar (T_f) objects were recorded in 5 minutes. We constantly changed beddings and thoroughly cleaned objects with 70% ethanol in-between trials to eliminate precipitates of olfactory stimuli. Object exploration was scored when the animal made a directional approach towards the object such as touching, sniffing or directing its nose <1cm away from the objects, while climbing or sitting on the objects was ignored.

Tissue isolation, DNA extraction and Bisulfite conversion

Mice were decapitated using a guillotine. Brains were removed from the skull and the PFC and HPC tissues were dissected on an ice-cold surface, immediately frozen in liquid nitrogen and stored at -80°C until DNA extracts were prepared. We isolated DNA from the mouse brain tissues using ZR Genomic DNA™-Tissue MiniPrep kit, D3050 (Inqaba Biotec, South Africa). The purity and yield of the extracted DNA was determined by spectrophotometry using Nanodrop 1000 (Thermo Fisher Scientific Inc., USA). DNA extracts were later processed for bisulfite conversion using the EZ DNA methylation™ kit, D5002 (Zymo Research, USA) according to the manufacturer's protocols. After denaturing the input DNA, 100μL of light-sensitive CT conversion reagent was added. The resultant mixtures were incubated twice; first at 50^{o}C for 16 hours in the dark and later on ice (4^{o}C) for 10 minutes. After incubation, we transferred the samples to zymo-spin IC columns containing 400μL M-binding buffer and subsequently mixed. After centrifugation at 12000 rpm, the samples were washed with 100μL M-wash and desulphonated with 200μL M-desulphonation buffer after which they were incubated at room temperature for 20 minutes. After two more wash steps, the converted DNA was eluted with 10μL of M-elution buffer added to the column placed on a micro-centrifuge tube.

Evaluation of DNA Methylation status by real-time quantitative polymerase chain reaction (qPCR).

DNA methylation status of *Fosb* and *Crem* genes were determined by qPCR. PCR amplification was performed on a LightCycler 2.0 (Roche Diagnostics (Pty) Ltd, South Africa) using 20μL total reaction volume which consisted of 5μL DNA template (bisulfite modified), 10μL SYBR Green I (Roche Diagnostics), 1μL DNA water and 2μL each of forward and reverse primers at 15μM concentration. Methylated DNA samples were amplified using the following set of primers:

Fosb: TATAGAAGCGCTGTGAATGGA (forward),
GACCATCTCCGAAATCCTACA (reverse);
Crem: CAGAGGAAGAAGGGACACCA (forward),

TTGTATTGCCCCGTGCTAGT (reverse);

Gapdh: GCCAAAAGGGTCATCATCTCCGC (forward),

GGATGACCTGCCCACAGCCTTG (reverse).

PCR cycling conditions were as follows: initial 10 minutes at 95ºC, followed by 45 cycles of 95ºC for 15s, 60ºC for 30s and 72ºC for 30s. The samples were placed in a 96-well PCR plate in which each sample was assayed in duplicate and each run included water blanks (as negative controls). For each target gene, serial dilutions (seven-fold) of methylated DNA control samples were prepared and used to construct linear standard curves. Data were analysed by comparing C(q) values of the treated samples to control and both normalized to *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase) using the $2^{-\Delta\Delta Cq}$ comparative method (Schmittgen and Livak 2008). A value of 1 was assigned to the control (Anier et al. 2010) and fold changes were calculated.

Data analysis

GraphPad prism 5 software was used for the statistical analysis. All datasets were subjected to the Shapiro-Wilk normality test to determine whether data displayed a Gaussian distribution or not. Lick contact and drug consumption data, Real-time qPCR data and all behavioral experiments were analyzed using Student's *t test*, except analysis of the novel object recognition data that was done using Two-Way ANOVA. All data are reported as mean±SEM, and criterion for significance was set at $p<0.05$.

Results

Cocaine acquisition and total consumption before and after drug withdrawal

During the first 30 days when the mice had free access to the drinking bottles containing either cocaine or water, the animals spent significantly less time licking (Fig. 2a) and consuming (Fig. 2b) cocaine than water ($p<0.0001$). However, similar pattern of cocaine intake was observed when drug consumption was assessed after the second brief period of drug access (Fig. 3). Cocaine licks and consumption levels were comparable to that during the first 30 days, and remained significantly less than water intake (Fig. 3a, b*;* Student's *t-test, p*<0.0001).

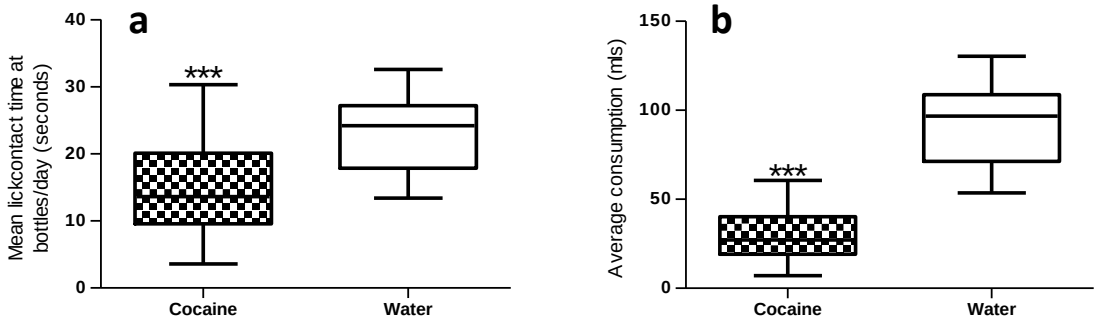

Fig. 2: Analysis of lick behavior of drug exposed (M_0) mice and compound consumption in the IntelliCage system during the first 30 days of exposure (a) test shows that subjects spent less time drinking from cocaine bottles compared to water (b) indicates that the total amount of cocaine consumed were less than water. Data shown represents Mean±SEM; n=32. ***p<0.0001 compared to water; Two-tailed unpaired Student's *t-test*.

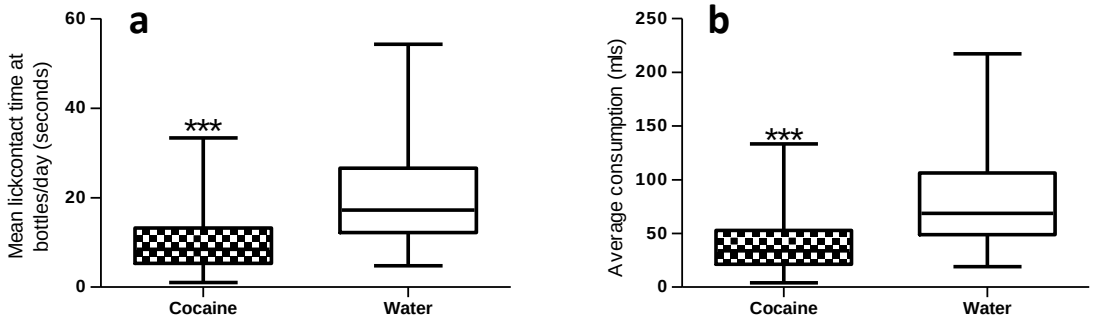

Fig. 3: Bottle lick behaviour of drug exposed (M_0) mice and cocaine *vs.* water consumption in the IntelliCage system during 4 days of cocaine reintroduction after a brief drug withdrawal period (a) demonstrates that lick contact time at cocaine bottles was decreased compared to water and (b) indicates that the total amount of cocaine consumed was significantly less than water. Data shown represents Mean±SEM; n=32. ***p<0.0001 compared to water; Two-tailed unpaired Student's *t-test*.

Figure 4a, b indicate the learning outcomes of ANP tasks imposed on the M_0 mice for 15 days before drug withdrawal and for 9 days after reintroducing cocaine post-drug withdrawal. The advantage of the ANP design was to see how well the mice learn to alternate visiting cocaine corners in order to maximize cocaine intake. The learning graphs show that most of the animals repeatedly made wrong visits to cocaine corners especially at NP1 and NP3 prior and after drug withdrawal periods. However, some of the animals still visited correctly by crossing the upper red line but made fewer visits especially during NP5 of both test periods. Even those with probable sequential learning ability later fell back within few days of the test (Fig. 4a, b). In overall, the graphs show that none of the animals learned the ANP tasks given due to failure to correctly execute the activity.

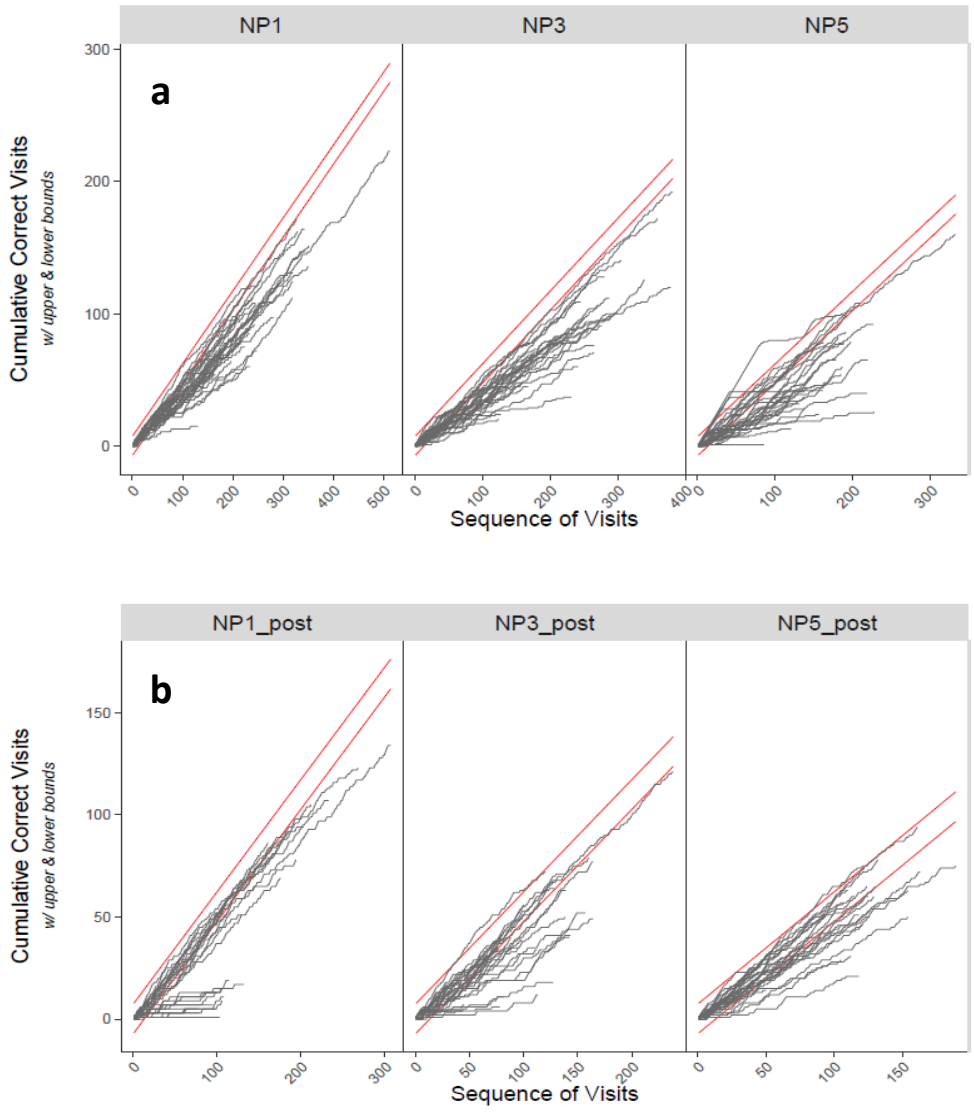

Fig. 4: Sequential probability ratio learning plot indicating cumulative correct visits to cocaine corners during (a) 15 days of alternate nosepoke (ANP) learning tasks imposed after initial motivation period (b) 9 days of ANP learning tasks given after post withdrawal cocaine reintroduction. (If the grey lines cross the lower or upper red lines, it indicates that learning is significant at 50% or 60%, respectively; criterion=0.6, expectation=0.5). All data were extracted and analysed using FlowR software; n=32, 15 days ANP; n=28, 9 days ANP.

Cocaine consumption is associated with DNA hypomethylation in the prefrontal cortex and DNA hypermethylation in the hippocampus.

After acquisition and/or exposure to cocaine in the IntelliCage apparatus, we assessed DNA methylation changes in the promoter regions of *Fosb* and *Crem* genes in the brain. Using bisulfite modification and qPCR analysis, our data demonstrated that mice that consumed cocaine had significant decreases in methylation at *Fosb* ($p<0.05$) and *Crem* ($p<0.0001$) gene promoters in their

PFC compared to controls (p<0.001; Fig. 5a, b). Consequently, the altered patterns of gene expression indicate transcriptional activation of associated genes in animals that had access to cocaine.

Examination of DNA methylation status at *Fosb* and *Crem* promoter regions in the HPC showed that DNA methylation was increased at both *Fosb* and *Crem* promoters in the HPC of mice that consumed cocaine when compared to controls (p<0.05; Fig. 5c, d).

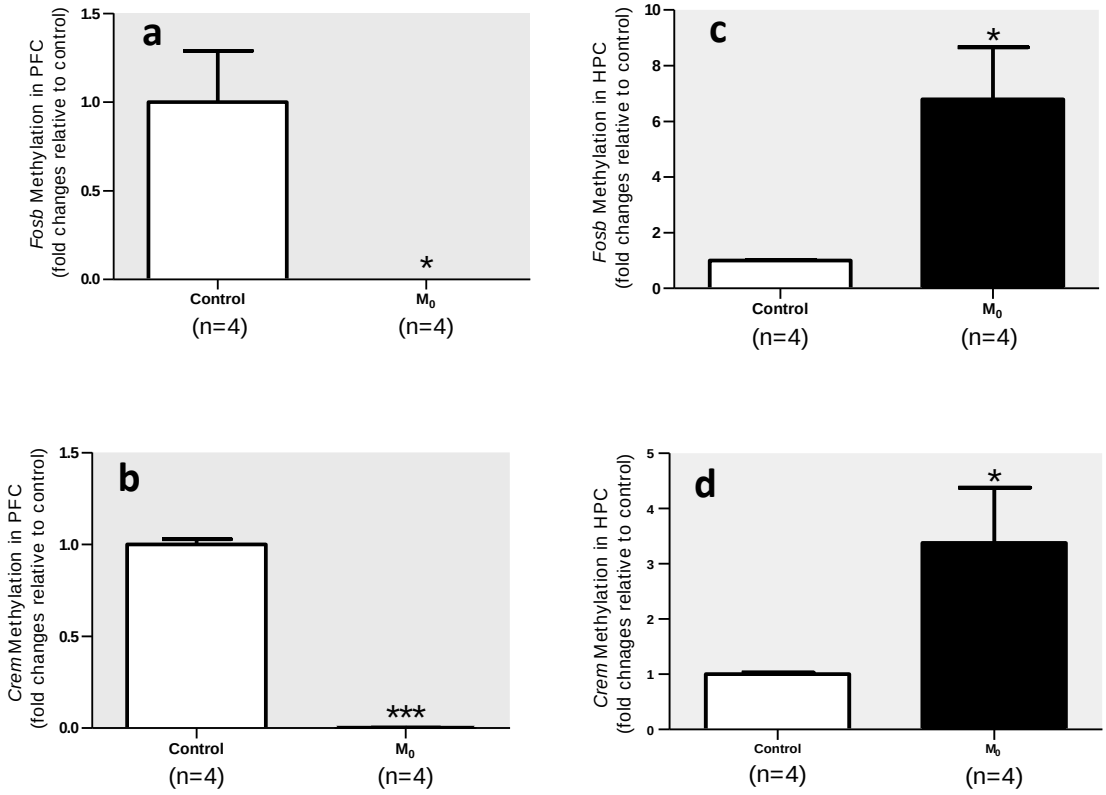

Fig. 5: Regulated gene expression changes after prolonged exposure to cocaine in the IntelliCage and altered patterns of DNA methylation at *Fosb* and *Crem* promoter regions in the brain (a-b) cocaine treatment induced DNA hypomethylation in the promoters of *Fosb* and *Crem* in the PFC of M_0 mice compared to their controls (c-d) In the HPC, treatment was associated with significant hypermethylation at both promoters indicating transcriptional silencing of *Fosb* and *Crem* genes. Data shown represents Mean±SEM; *p<0.05, ***p<0.0001. Two-tailed unpaired Student's t-test.

Object recognition memory was intact while locomotor activity was decreased in mice that consumed cocaine.

We conducted the NOR test in order to evaluate the memory functioning of all the mice. A Two-Way ANOVA indicated significant main effects of the treatments ($F_{(1,40)}$=4.307, p=0.04) and total exploration time ($F_{(1,40)}$=20.54, p<0.0001). Drug exposure x exploratory time accounts for approximately 0.99% of the variance, hence indicated no significant interaction ($F_{(1,40)}$=0.6433,

p=0.4272) between factors. Both groups of mice (cocaine exposed and controls) spent significantly more time exploring the novel object (Tn) compared to the familiar object (Tf) ($p<0.001$; Fig. 6a).

The locomotor activity of all mice was assessed in the open field arena. The total number of central and peripheral squares crossed was used as a measure of locomotion (Abboussi et al. 2016). The data showed that the total squares crossed by the mice that had access to cocaine was significantly decreased compared to controls ($p<0.05$; Fig. 6b).

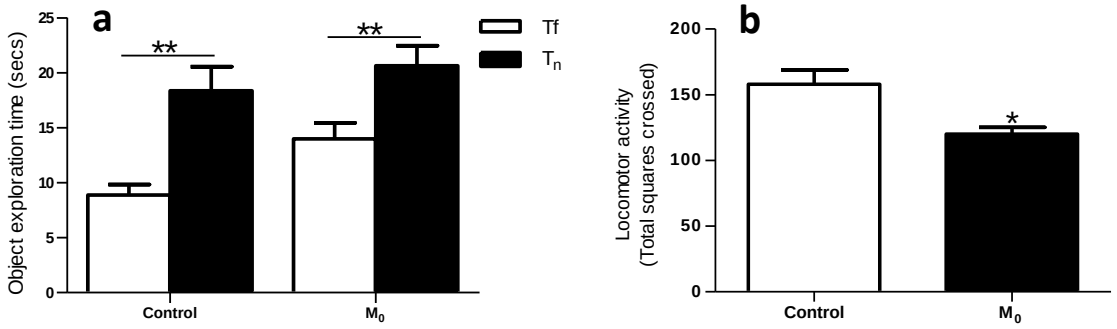

Fig. 6: Object recognition memory and locomotor response following prolonged exposure to cocaine (a) time spent exploring familiar (Tf) and novel (Tn) objects in the novel apparatus (b) locomotor activity of M_0 mice tested after cocaine-experience in the IntelliCage. Data shown represents Mean±SEM**p<0.01, *p<0.05, Two-Way ANOVA or Two-tailed unpaired Student's *t-test*.

Discussion

In the first part of this study, we demonstrated preference consumption of compounds in the social home-cage and examined motivation for cocaine rewards by scheduled learning tasks. We show that M_0 mice licked and drank more water than cocaine in all test sessions (Figs. 2 and 3). This may be expected as most human drug addicts are also known to consume water while on drugs. Consistent with previous reports from animal research, it has been suggested that rats presented with alcohol or cocaine among other drugs of abuse and water, on choice trials drank more water than drugs with consequent behavioural and characteristic patterns of drinking responses without evidence of dependence (Stolerman et al. 1971). It is therefore possible that higher preference for water in this study may suggest an increasing cellular demand for maintaining fluid balance which may further account for the lower consumption of cocaine. Holgate and colleagues (2017) recently examined the effect of social and environmental enrichment on ethanol and sucrose consumption in C57BL/6 mice. These authors concluded that enrichment in the IntelliCage may have the potential to decrease ethanol preference while favouring sucrose consumption (Holgate et al. 2017). This suggested that the decreased drug consumption observed in our mice may similarly result from the impact of the enriched environment offered by the IntelliCage system such that the consumption of a natural reward (water) is preferred above the pharmacological effects of cocaine when concurrently presented. We

further showed that the cocaine-experienced mice performed poorly in all tasks given as they exhibited increased activity at cocaine corners to get at sufficient drug but failed because they did not follow the design alternation pattern. Similarly, a previous study that employed place learning paradigm in the IntelliCage reported that C57BL/6J mice initially failed to learn to discriminate rewarded from non-rewarded corners until negative air-puff punishment was applied. The authors further showed that young mice later learned the task significantly better than the middle-aged mice (Mechan et al. 2009). In our study, time-constraint reinforcement was applied in drug corners during the nose-poke learning tasks. Only correct visits and nose-pokes were rewarded (access to cocaine) by active opening of the motorized doors which lasted 5 seconds. Therefore, some possible reasons for the observed learning deficit in our model could be related to age factor and that the negative reinforcement used may also be considered not strong enough, as opposed to the conventional air-puff, to influence motivational learning in the animals. Converging theories of reward dependent learning have previously suggested that learning is also dependent on how predictable a reward is relative to associated cues (Schultz et al. 1997). Learning deficits in this study may further be attributed to inaccurate nose-poke sequence and time prediction in the animals.

Altered gene expression in specific reward areas of the brain are thought to provide great insight into the neurobiology of drug abuse (Renthal and Nestler 2008). After behavioural phenotyping, we then set out to identify changes in the DNA methylation status of the promoter regions of *Fosb/Crem* in the PFC and HPC. We found that *Fosb* and *Crem* were hypomethylated in the PFC and hypermethylated in the HPC. Electrical stimulation studies have revealed induction of *Fosb* mRNA in the HPC in response to signalling agents in the brain (Nakabeppu and Nathans 1991**).** Anier and co-workers reported DNA hypomethylation at *Fosb* promoter in the NAc following acute and repeated cocaine administration and associated these changes with transcriptional induction of *Fosb* in this brain region (Anier et al. 2010). In our study cocaine–experienced mice had increases in methylation at *Fosb* gene promoters in the HPC suggesting transcriptional silencing of the gene in this region. It may therefore be possible that reduced *Fosb* expression may reflect decreased sensitization towards the reinforcing or motivational properties of cocaine when concurrently presented with water in the IntelliCage system. Moreover, transcriptional induction of *Fosb* in the PFC of cocaine exposed mice may on the other hand provide evidence for enhanced behavioural control that explains desensitization towards cocaine.

Our findings also showed that mice that consumed cocaine in the IntelliCage displayed decreased locomotor activity. While unexpected, this results was nevertheless in line with a previous study by Hiroi et al (Hiroi et al. 1997) who showed that *Fosb* mutant mice lacking 35-to-37 KDa *Fos* proteins in their striatum had exaggerated locomotor activation in response to initial cocaine exposures compared to their wild type littermates. The data therefore points to some role of *Fosb* as one of the

key regulators of gene expression changes recruited in the functional activation of neural networks that mediate locomotor response to long-term cocaine effects. Further support stems from another study reporting inhibition of sensitization of post-amphetamine-stereotypy locomotion in prefrontal cortical lesioned rats (Wolf et al. 1995), while a more recent study demonstrated the expression of *Crem* in the core of NAc, a brain region that may be involved in the regulation of impulsive actions related to reward behaviour (Miller et al. 2017). It is therefore possible that decreased locomotor activity following cocaine exposure, as observed in our study, might also be associated with *Crem* perturbations in both brain areas studied. In addition, the impact of social interaction episodes previously experienced by the animals may further account for their post-treatment diminished locomotor activity.

Like *Creb*, *Crem* is also known to play significant physiological roles in memory and long-term potentiation (Silva et al. 1998, De Cesare et al. 1999). There is compelling evidence that DNA methylation regulates gene transcription necessary for memory function (Mikaelsson and Miller 2011). Our investigation indicated that cocaine-induced *Crem* hypermethylation in the HPC of mice, suggesting transcriptional silencing of memory-linked genes regulated by *Crem* in the HPC. This further provided evidence for a lack of drug-induced neural plasticity despite prolonged exposure to cocaine in the IntelliCage. This is in line with a previous finding that established that long-term potentiation is unaltered in *Crem* mutants (Maldonado et al. 1999). However, the observed molecular changes seem to correlate with the overall performance of the animals in the NOR tasks which demonstrated that cocaine consumption did not alter recognition memory but rather promoted novelty-seeking behaviour. Furthermore, in a study that used conditioned taste aversion as learning and memory paradigm, *Fosb* and *Crem* were among the proteins whose increased expression were detected in several brain regions after lithium chloride administration (Lamprecht and Dudai 1995). In our study, it appears that there was a form of memory shift from HPC to PFC in the cocaine-experienced mice as evidenced by transcriptional activation of *Crem* and *Fosb* within the prefrontal cortical network. This is in agreement with the concept of cortical integration previously posited (Mikaelsson and Miller 2011) and further corroborated the argument that HPC has a time-related role in memory consolidation. Hence long term memories require activities of neocortical regions which may include the prefrontal cortex (Squire et al. 2001).

It is widely reported that persistently enhanced sensitization and behavioural response are associated with most drugs of abuse including cocaine (Henry and White 1995). Since the mice used for this study had extended voluntary oral access to cocaine, we expected that the rewarding effects of cocaine would evoke robust euphoria and precipitate addictive behaviour as previously reported (Horger et al. 1990, Shippenberg and Heidbreder 1995). On the contrary, our observations proved otherwise as the mice housed in the IntelliCage only consumed cocaine at modest levels despite

having free access for extended periods. We think that this low consumption rate may be due to the impact of social interaction amongst cage mates in the group housing system of the IntelliCage apparatus. In a study that examined how environmental conditions and social factors impact on cocaine reward in early adolescence, the investigators showed that cocaine treatment established reliable conditioned place preference (CPP) in socially isolated rats housed alone whereas the presence of other cage mates with toys abolished CPP. They further added that enrichment plus group housing increased dopamine transporter protein levels in the NAc (Zakharova et al. 2009). Also, Fritz and co-authors (Fritz et al. 2011) previously investigated the effect of social interaction offered as an alternative stimulus to cocaine conditioning in Sprague-Dawley rats, and found that CPP induced by intraperitoneal cocaine (15mg/kg) injection and molecular induction of Zif268 in the NAc shell, VTA, central and basolateral amygdala, were completely reversed by the social interaction episodes (Fritz et al. 2011). Since our previous observations about cocaine reward in the IntelliCage were comparable to the findings of others (Zakharova et al. 2009, Fritz et al. 2011), it is evident that C57BL/6 mice used in our study only experienced cocaine in social settings but were not addicted to it. On the other hand, insignificant cocaine-seeking behaviour in the animals can further be attributed to changes in gonadal hormones in the female mice used for this study as they were evaluated over long periods. A study by Russo et al (2003) demonstrated that cocaine-induced CPP was decreased in ovariectomized rats pre-treated with progesterone whereas when combined with estrogen, the magnitude of CPP was enhanced (Russo et al. 2003). The reciprocity or opposing effects of these ovarian hormones on cocaine consumption has also been demonstrated in self-administration experiments (Larson et al. 2007). It is therefore logical to reason that the physiological response of mice to cocaine in this model is partly dependent on phasic changes in their ovarian cycles. Although it is unclear whether there is a direct relationship between the hormonal changes and social interaction experience, we speculate that the lack of addictive behaviours and low impulsivity in our mice likely result from the impact of these factors.

We postulate that the experiences of drug intake and that of the social environment seems to consolidate into new memories in the PFC that regulate cocaine reward to social engagement in the animals. A recent study that employed pharmacological inactivation techniques, highlighted the importance of functional integrity of PFC and striatal circuits for expression of social play behaviour, a form of social interaction in animals (Van Kerkhof et al. 2013). Many factors such as cognitive control, choice making, reward related behaviours and behavioural inhibition were all associated with social play interaction which involved cortico-striatal projections from PFC to the striatum (Bell et al. 2009, Van Kerkhof et al. 2013). Van Kerkhof et al. (van Kerkhof et al. 2014) further established that the activation of the cortico-striatal pathway after social play behaviour correlated with *c-fos* positive cell density in the prefrontal regions. Resting on these knowledge, transcriptional activation of *Crem*

and *Fosb* in the PFC of cocaine–experienced mice further support the involvement of both transcription factors in the inhibition of drug related behaviours by activating social networks in the PFC which dampens the sensitivity of the animals to cocaine rewards and thus enhanced behavioural control. This further accounts for insignificant motivational deficits observed in our mice.

Taken together, this study established the significant role of DNA methylation in identifying epigenetic changes that are associated with prolonged intake of cocaine in the IntelliCage. Our findings show that *Fosb* and *Crem* transcription factors function in similar directions in both HPC and PFC when mediating molecular responses to cocaine-rewarding effects intercepted by social behaviours, whereas these specific brain regions exhibited differential responses to the converging environmental stimuli (drug and social interaction) resulting in a memory shift that favours behavioural control, further manifested by decreased locomotor response in the open field.

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Disclosure

All authors declare that they have no competing financial or any other conflict of interests to disclose.

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CHAPTER SIX

This chapter forms part of my PhD research work and has been formatted as a short communication submitted for publication in Behavioural Brain Research. The short communication article is titled: **"Early postnatal fostering altered memory performance and DNA methylation in the prefrontal cortex of offspring mice with lineal phenotypes related to parental cocaine exposure".**

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Abstract

Here, we explored the hypothesis that parental cocaine exposure could alter epigenetic machinery in the unexposed offspring while early postnatal (PN) fostering may further modify the accompanied neurochemical and functional components. Cocaine exposed or unexposed C57BL/6 female mice were matched with their male counterpart for mating to produce variant pups with lineal phenotypes. The pups were initially examined for locomotor activity and memory performance and subsequently for changes in DNA methylation in promoter regions of cAMP response element modulator (*Crem*) and *Fosb* genes in the prefrontal cortex (PFC) and hippocampus (HPC) at 48 days postnatum. The impact of PN fostering on these parameters was also investigated to evaluate the influence of the environment on epigenetic expression. We found that (1) early PN fostering impaired recognition memory in pups lineally inclined to paternal and/or parental cocaine experience; (2) neither parental cocaine exposure nor fostering affected locomotor behaviour in the pups; (3) the offspring of cocaine–exposed parent mice showed decreased methylation of *Crem* in their PFC, while fostering the offspring produced an opposite effect on methylation in this substrate brain region without any significant change recorded in the HPC, (4) fostering also decreased *Fosb* methylation in the HPC of pups linked to progenitor's cocaine exposure without a significant change in the PFC. Our data provide some evidence that cocaine may cause epigenetic changes that can be inherited by the offspring, that mediation by *Crem* signalling in the PFC may be beneficial, while early PN fostering may further engineer molecular switching that may predispose the individual to future risky behaviours.

Keywords: Cocaine; DNA methylation; *Crem*; *Fosb*; Epigenetic inheritance; Fostering

Communication

Despite evidence for epigenetic inheritance of gene profiles, the exact molecular and circuit-linked regulatory mechanisms that underlie behavioural responses to drug-taking in socially engaged parents, the transfer of drug-induced epigenetic marks to succeeding generations and how offspring of non – addicted drug taking parents are programmed to exhibit some of these transferred traits, are not fully understood. This knowledge is crucial for understanding the molecular basis of drug abuse and the accompanied heritable changes.

DNA methylation represents an important regulatory mechanism that induces long term stable changes in gene expression patterns mainly in the reward pathways during repeated exposure to drugs of abuse or psychostimulants such as cocaine. It has been postulated that maternal DNA methylation may possibly mediate inheritance of parental phenotypes due to escape of epigenomic reprograming at gametogenesis [1]. For example, maternal exposure to high doses of cocaine in the second and third trimester of gestation caused profound alterations in global DNA methylation in neonatal and prepubertal offspring mice with evidence of structural and functional modifications in the postnatal brain [2]. Similarly, there is also growing evidence that supports a link between future expression of paternal epigenetic signatures and social changes in the environment [3]. Fostering and/or variation in maternal care represent some of the factors that have profound capacity to modify the juvenile environment and thereby may induce developmental plasticity that further renders the offspring vulnerable or susceptible to drug acquired traits. Persistence of these early life experiences are also associated with DNA methylation with consequent effects on gene expression and behaviour [4].

Our previous studies have demonstrated that repeated cocaine administration was associated with characteristic patterns of methylation in the promoters of *Crem* and *Fosb* genes within the PFC and HPC of C57BL/6 mice. We also found that the transcriptional changes within the PFC are consistent in both free-living (in the IntelliCage) and conditioned learned paradigms tested. Additional to other established roles of *Crem* and *Fosb* in drug reward and vulnerability [5-7] is that they also mediate gamete functions [8] and nurturing effects [9] in mice, respectively. These data led us to investigate whether (1) parental cocaine exposure could affect DNA methylation in specific promoters of *Crem* and *Fosb* genes in the PFC and HPC of non-drug exposed offspring mice; we chose these substrate brain regions because they have been critically implicated in cocaine abuse [10-12] and previously investigated in the progenitors used for this study; (2) whether the expected epigenetic changes are maternally or paternally inherited; (3) examined whether the transmitted epigenetic changes can be altered by early PN fostering and (4) whether these changes paralleled with memory performance and locomotor behaviour of the unexposed mice.

This study began with twelve young adult mice (C57BL/6, *Biomedical Resource Unit, UKZN, Durban*) reared in group – housed settings. Of this sample size, four dams were exposed to cocaine (300mg/L) in free living conditions in the IntelliCage (IC) (TSE Systems GmbH, Bad Homburg, Germany; http://www.tse-systems.com/products/behavior/intellicage/index.htm) for 58 days, while two male mice received intraperitoneal injections of cocaine (10mg/kg) in the conditioned place preference (CPP) apparatus for 6 alternate days. The remaining mice received only water in drinking bottles or 0.9% saline injections like their counterparts and served as controls (See Figure 1a). It is worth noting that social hierarchy might interfere with cocaine consumption in male mice when grouped together, hence the reason for choosing an alternative method of cocaine exposure. To obtain pups that were studied for genetic predisposition to either paternal and/or maternal drug-induced neurological marks, we paired cocaine – exposed and control dams with their male counterparts for one sexual cycle that lasted four days. The mating protocol was as follows: control female mice and a male mouse paired together – all non-drug exposed parent mice [NEP group], females exposed to drug paired with control male [FE group], male exposed to drug paired with control females [ME group], drug exposed male and females paired together [MFE group]. In all mating groups, two nulligravid dams were paired with one male in the same cage (See Figure 1b). The pregnant dams were separated and housed in separate cages until delivery. The male mice were never in contact again with both the pregnant dams and their pups after birth. Within 3 – 4 days of birth, pups from one of the treated dams in FE and MFE groups were exchanged for pups from inception-matched untreated dams in NEP and ME groups, respectively. Whereas, other primiparous dams retained their pups and reared them until weaning at postnatal day 21. The weaned pups were reared in groups of 4 – 5 animals (same sex) per cage until young adulthood. Both non-fostered and fostered pups were used for subsequent behavioral testing and molecular experiments. All animal procedures were approved by the University of KwaZulu-Natal's Animal Ethics Subcommittee [*Licence, AREC/071/015*].

At postnatal day (PND) 44 – 47, memory functioning and locomotor activity of the offspring mice of both genders were assessed using novel object recognition (NOR) and open field (OF) tests, respectively. NOR test is based on the premise that novelty elicits approach behavior in rodents [13]. We used a previously described method [14] which included two sessions (familiarization and test) conducted over two days. In our protocol, two identical objects (A and B) were first presented to the animals to explore in 5 minutes. 24h later, object B was replaced with object C (novel) and the time spent exploring the novel object was measured. Index of recognition memory as a measure of time taken to discriminate the novel object in the 5–minute test session, was calculated using this mathematical expression: $[(T_n / T_n + T_f) \times 100]$[15]. For OFT, we used a locally made black plexiglass (50 x 50 x 25cm) apparatus. The floor was divided into sixteen equal sized-squares. Locomotor

activity was assessed by recording the total number of squares crossed using a video camera affixed to a wooden holder positioned just above the apparatus.

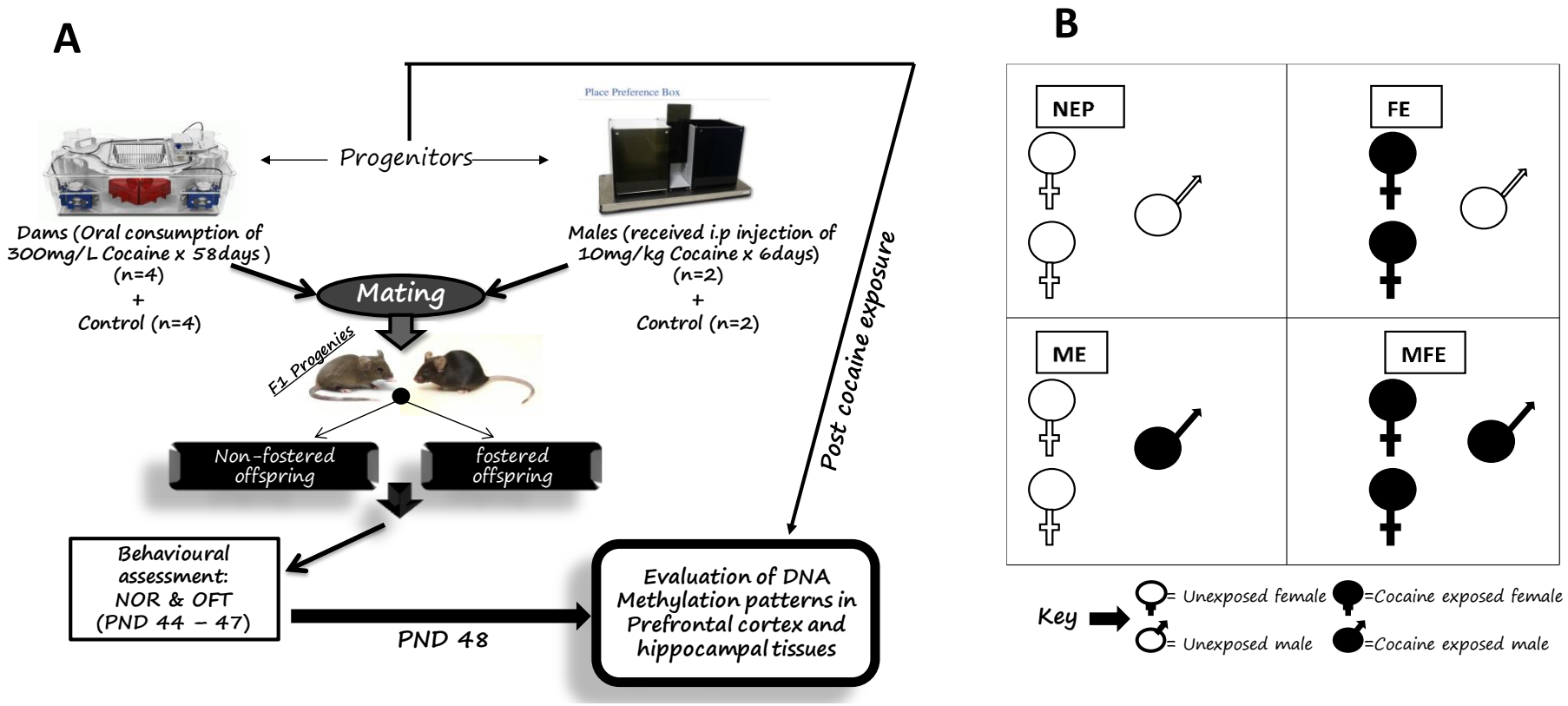

Figure 1: Schematics summarizing parental drug history, mating protocols, and overall behavioural and molecular assay techniques used in this study. (A) Four dams (female progenitors) that experienced 58 days of choice access to cocaine (300mg/L) solutions via drinking bottles in the IntelliCage apparatus, two male progenitors that had previously received cocaine (10mg/kg, i.p) injection for 6 days and their controls (dams, n=4 and males, n=2) were paired for mating to produce the F1 generation offspring mice (progenies). Subsequently, DNA methylation in the promoters of *Crem* and *Fosb* was assessed in the PFC and HPC of the progenies. (B) Indicate patterns of parental pairing for mating resulting in various groups of offspring used in this study. Shaded and unshaded sex symbols indicate drug exposed and non-drug exposed progenitor mice, respectively.

Exploration of familiar objects in all fostered and non-fostered ME and FE adolescent mice of cocaine exposed progenitors were insignificant and comparable to controls (NEP). In contrast, familiar object exploration was significantly decreased in both ME and FE compared to the MFE subgroup which spent more time exploring the objects than others (Figure 2a). Also, all the tested groups preferentially explored the novel objects but not to significant levels, except MFE and fostered FE subgroups that demonstrated either a slight decrease or matched status in their novelty exploration (Figure 2b) relative to time spent with the familiar objects. As shown in Figure 2c, memory index of object discrimination or novelty preference further confirmed that memory functioning was normal in non-fostered ME and FE, and fostered FE pups genetically predisposed to paternal and/or maternal drug experience. Moreover, recognition memory was slightly decreased in fostered ME offspring but greatly impaired in fostered MFE offspring (two–way ANOVA; group $F_{(3,38)}=7.637$, $p=0.0004$; condition x group interaction $F_{(3,38)}=3.501$, $p=0.0245$). In the OF, we found that the frequency and total number of lines crossed were similar across all groups which indicate that neither parental cocaine exposure nor fostering after birth affected locomotor behaviour of the offspring mice (Figure 2d, $F_{(3,38)}=0.04799$, $p=0.9859$).

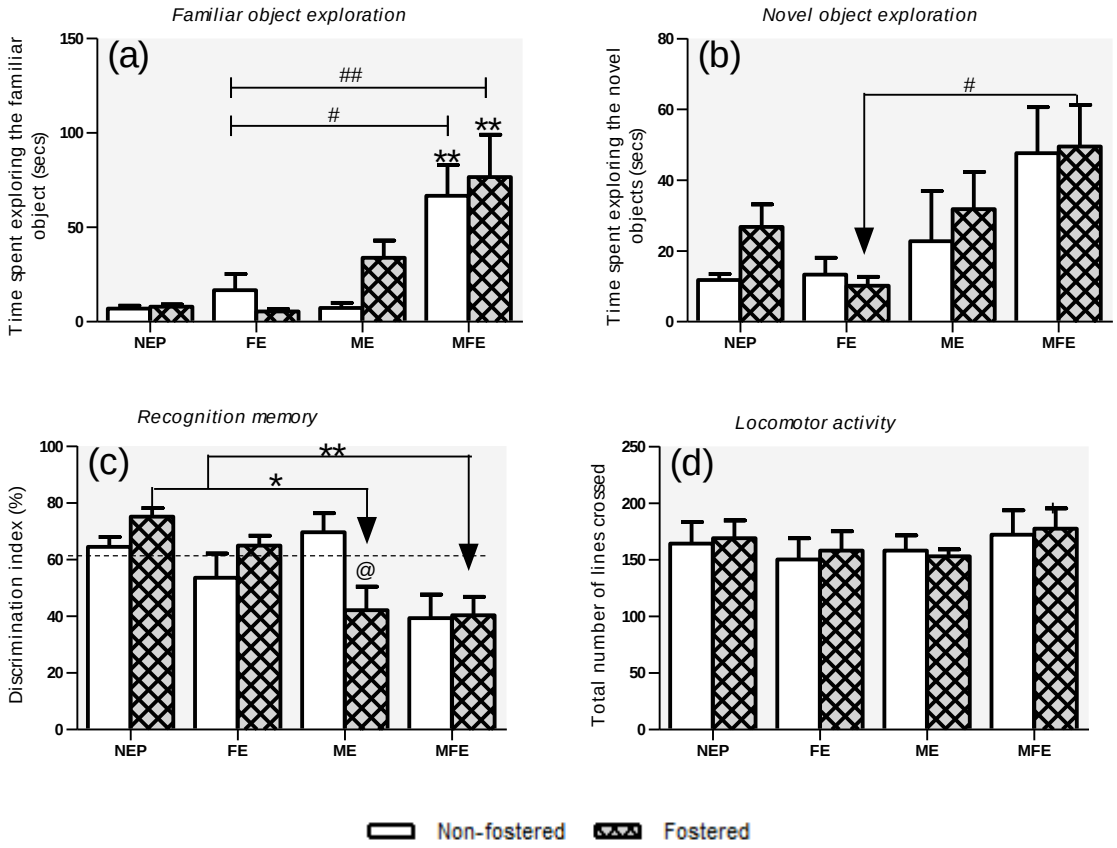

Figure 2: Impact of parental cocaine exposure and fostering on memory functioning and locomotor activity of non-drug exposed F1 generation offspring mice. (a-b) Indicate the average time spent exploring either familiar or novel objects in the exploratory arena. (c) Showing the % discrimination between novel and familiar objects, an index of memory performance. Overall, both non-fostered and fostered offspring demonstrated intact recognition memory although with slight concerns for fostered ME and MFE offspring that attained <50% memory score, thus indicating significant decreases in their memory competence compared to control. (d) Indicates no change in locomotor activity of all groups of mice tested in the OF. Two-Way ANOVA, Bonferroni's comparison posttest. *p<0.05, **p<0.001 (subgroups *vs.* control); $^{\#}p$<0.05, $^{\#\#}p$<0.001 (comparison within subgroups); $^{@}p$<0.05 (non-fostered *vs.* fostered). For non-fostered groups, n=6 (NEP, FE, MFE) and n=4 (ME). For fostered groups, n=6 (NEP, FE, ME, MFE). Error bars indicate SEM.

At PND 48, the offspring were sacrificed and their PFC and hippocampal tissues were removed on cold ice followed by DNA extraction (ZR Genomic DNA^{TM}-Tissue MiniPrep, Inqaba biotec) and bisulfite conversion (EZ DNA methylation kit, Zymo Research), performed according to the manufacturer's instructions. Methylated DNA was then subjected to quantitative real-time polymerase chain reaction (qPCR) on LightCycler 2.0 (Roche) using the following primers: *Fosb*: TATAGAAGCGCTGTGAATGGA (forward), GACCATCTCCGAAATCCTACA (reverse), *Crem:* CAGAGGAAGAAGGGACACCA (forward), TTGTATTGCCCCGTGCTAGT (reverse) and *Gapdh*: GCCAAAAGGGTCATCATCTCCGC (forward), GGATGACCTGCCCACAGCCTTG (reverse).

The PCR reaction mixture consisted of 5μL DNA template, 10μL SYBR Green I (Roche Diagnostics GmbH), 2μL each of 15μM primer pair stock and 1μL DNA free water. Seven-fold serial dilutions of methylated DNA control samples for each target gene was used to construct standard curves from which PCR efficiency was extrapolated. Cq values were chosen within linear range while differences in methylation between samples were determined using comparative Cq method as previously described [7, 16]**.**

We found that DNA methylation in *Crem* promoter genes within the HPC of all non-fostered and fostered offspring were not significantly different from their controls (NEP) (Figure 3a; $p>0.05$). Although, there was evidence of marked increase or decrease in methylation in the brains of non-fostered ME and MFE mice, respectively, while changes in fostered ME and MFE tended towards hypomethylation but also not to significant levels. (Figure 3a; $p>0.05$). qPCR assessment further showed that hippocampal methylation changes in *Fosb* promoter genes were similar to *Crem* profiles except that interaction between conditions (non-fostering *vs* fostering) and groups were statistically significant (Figure 3c; $F_{(3,35)}=3.144$, $p=0.0437$). Likewise, *Fosb* was hypomethylated in the HPC of fostered MFE subgroup compared to NEP and in ME pups compared to their non-fostered counterparts (Figure 3c; $p<0.01$). In the PFC, *Crem* methylation was significantly decreased in all non-fostered pups compared to NEP (Figure 3b; $p=0.001$). These changes were reversed by fostering effect in the FE and ME subgroups as indicated by significant hypermethylation in the corresponding fostered groups compared to their non-fostered counterparts (Figure 3b; Two-Way ANOVA, condition x group interaction $F_{(3,35)}=4.996$, $p=0.0055$). DNA methylation patterns in *Fosb* promoter genes in the PFC of both non-fostered and fostered pups were also comparable to *Crem* alterations except that the observed transcriptional changes did not differ statistically from their controls (Figure 3d; $p>0.05$).

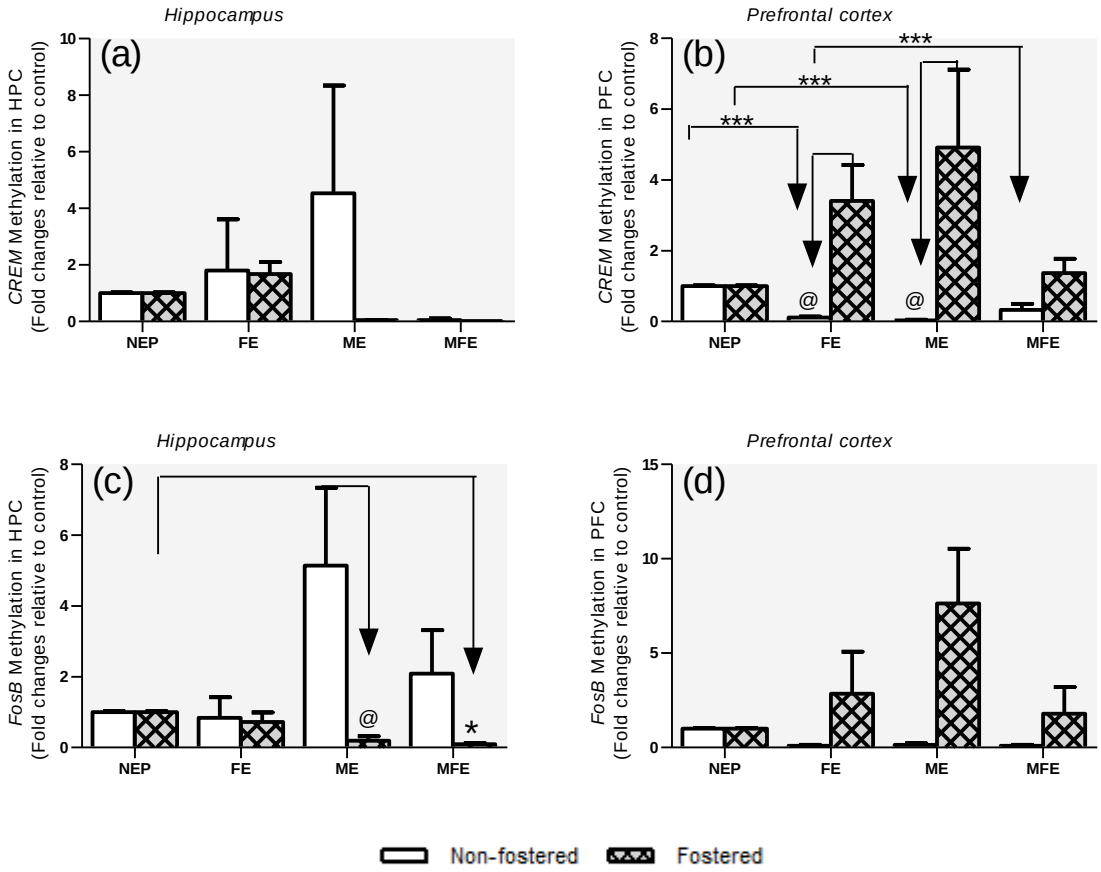

Figure 3: DNA methylation at *Crem* and *Fosb* promoters in the PFC and hippocampal brain regions of P48 offspring from non-drug exposed or cocaine – experienced sires/dams and their fostered counterparts. (a) No evidence of significant alterations in methylation status of *Crem* in the HPC of both non-fostered and fostered offspring. (b) All non-fostered offspring with parental history of cocaine use exhibited significant decreases in methylation at *Crem* promoter gene in their prefrontal cortices compared to control (NEP) while changes were unaffected by fostering. (c) Methylation status at *Fosb* promoters in the HPC of all non-fostered subgroup offspring were similar to controls except ME that tended towards hypermethylation when compared to its fostered counterpart. Also, *Fosb* was hypomethylated in the HPC of MFE fostered subgroup compared to control. (d) Unaltered DNA methylation patterns were found at *Fosb* promoters in the PFC of all pups except for significant methylation differences between non-fostered and fostered ME subgroup offspring. Two-Way ANOVA, Bonferroni's comparison posttest. *p<0.05, ***p<0.0001 (subgroups *vs.* control), $^{@}p$<0.01 (non-fostered, n=4 *vs.* fostered, n=6). Error bars indicate SEM.

We have previously demonstrated that cocaine acquired in social settings by the progenitor mice used in this study was associated with hypomethylation or transcriptional activation of *Crem* and *Fosb* in the PFC, and with *Fosb* transcriptionally downregulated in the HPC of dams. The pronounced cortical activation was linked to enhanced behavioural control evidenced by motivational and/or reinstatement deficits (data not shown) previously observed in the dams and male mice, respectively. In this study, we used DNA methylation to investigate inheritance of the parental epigenetic marks in non-drug

exposed offspring and found that similar methylation patterns associated with *Crem* promoters in the PFC of cocaine – experienced progenitor mice were replicated in the same brain region of all groups of non-fostered pups. Similar to this study, Vassoler et al [17] recently delineated heritable phenotypes in rats that self-administered cocaine and reported that male offspring, not females, were resistant to cocaine reinforcement which parallels activity of *Bdnf* mRNA and proteins levels in the medial prefrontal cortex (mPFC). The authors further alluded these heritable changes to paternal cocaine experience and increased association of histone H3 and *Bdnf* promoters in the sperm [17]. At first, the current study established inheritance of cocaine – induced phenotypes in the mouse PFC and implicates *Crem* in the regulation of these phenotypic changes which possibly result from epigenetic reprogramming in the germ lines. Since our data further indicated no change in *Crem* activation within the experimental groups, it therefore means that the suggested transmission may have occurred without preference attributed to any of the germ lines. Assuming these changes were peculiar to any of the subset groups, for instance if FE dams display significant hypomethylation compared to other test groups, we would have argued that the observed gene expression changes were solely influenced by maternal cocaine exposure either by genetic means or via direct interaction of those pups with their biological mothers. Consistent with a previous report that the putative binding sites of *Crem*, among other transcription factors that operate in testicular function, were strongly enriched by promoter regions hypomethylated in sperm cells [18], if subset ME was also directly implicated in our findings, sole inheritance of paternal single-copy genes would have been suspected. Since transcriptional induction of *Crem* in the PFC occurs in all groups, the data demonstrates possible epigenetic transfer of encoding factors through equal contributions from both germ lines. Analysis of DNA methylation have shown that alleles inherited maternally or paternally were treated differently and that there is lack of evidence for DNA methylation in the blastocyst [19]. Conversely, other studies that considered methylation profiling, have demonstrated epigenetic inheritance of sperm DNA methylomes in humans [18] and zebra fish [20], except that most oocyte methylomes gradually faded off through cell division stages and progressively reprogrammed to match changes in the sperm [20]. A major caveat in our research is that the discrepancies concerning preferential link between parental methylation patterns and inheritance was not assessed directly by examining gene expression changes in both maternal and paternal gametes.

Secondly, we propose that the molecular changes associated with *Crem* signalling in the PFC of non-fostered pups may also be beneficial, similar to their progenitors, in decreasing their vulnerability to future drug abuse. This argument can be supported by recent findings by Miller et al. (2017) where decreased NAc *Crem* expression levels was associated with increased neurochemical and behavioural sensitivity to heroin whereas an opposite response was observed when *Crem* levels was virally increased in the same brain region [5].

Fostering and/or maternal stress have been identified as one of the early life stressors that toggles psychological balance of individuals to factorial states in the environment and largely determines vulnerability to future drug abuse [21, 22]. In the present study, we also examined the impact of early PN fostering on the transmitted epigenetic marks and found that the decreased methylation associated with *Crem* promoter genes in the PFC of non-fostered pups appears reversed by early fostering, an effect observed in FE and ME pups. Since alterations induced by fostering are not directly linked to activated gene expression changes in the non-fostered subgroups, the exact relationship between the conditions remains unclear. Hence, further studies are required to unravel these uncertainties. Nevertheless, we propose that alterations in maternal environment due to fostering may provoke molecular sensitization in the non-exposed offspring which has accumulative potential to increase future risk of psychopathy. Previously it has been shown that mice lacking immediate early gene *Fosb* in the preoptic area of the hypothalamus exhibited nurturing deficit. Hypomethylation in *Fosb* promoters in the HPC of MFE and ME fostered pups, showed that the regulation mediated by DNA-methylation of nurturing and/or maternal care response to early life stress induced by fostering, is not only region specific but also sensitive to genetic predisposition. Moreover, lack of *Crem*–mediated gene expression changes in the HPC and unaltered recognition memory in the non-fostered pups, further support the notion of poor consolidated cocaine memory or plastic changes owing to reduced activation in the reward system. Weaver (2009) previously reported that the early nurturing environment persistently influenced developmental programming of inter-individual differences in metabolic and endocrine functions that contribute to emotional and cognitive performance throughout life [23]. Our findings showed that fostered pups, with either paternal or a double history of parental cocaine exposure, exhibited memory decline, suggesting the negative impact of the nurturing medium on the cognitive ability of fostered mice. Previous studies have also implicated enhanced *Fos* expression especially in the NAc and *Crem* promoter variant in the striatum, as positive markers of locomotor sensitization or hyperactivity [5, 24, 25]. Here, we demonstrated that locomotor activities of both non-fostered and fostered pups were not different from their controls, a finding that paralleled *Fosb* and *Crem*–mediated molecular changes in the PFC and HPC. These observations may also be attributed to tapered behavioural sensitization in the progenitors.

Taken together, our data from DNA methylation evaluations showed that some epigenetic marks associated with cocaine reward were passed down to the next generation. These dynamic changes, mediated by significant alterations in the epigenetic machinery domicile in the PFC, were likely reversed by early PN fostering reflecting the interplay between epigenetic modifications and the environment. Furthermore, associations between the epigenetic molecular events, locomotor behaviour and cognitive performance of the unexposed offspring were also established.

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Disclosure

The authors declare no conflict of interest.

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CHAPTER SEVEN

SYNTHESIS AND CONCLUSION

One of the major objectives of our study was to gain more insight into the contribution of the epigenome to the pathogenesis and persistence of substance abuse and its inheritance. We particularly focused on the epigenome as the interface where drugs and factors in the environment interact to influence gene expression to eventually determine drug-related phenotypes. In this regard the present study was limited to DNA methylation as the key mechanism responsible for epigenetic changes and for the transgenerational transfer of epigenetic imprints.

A review of the literature identified some shortcomings in the conventional experimental approaches adopted to study drug-induced effects in animal models of addictive behaviour. Most notably was the use of rodents and primates in isolation, rather than in a social context. This necessitated the present study to develop social models that mirror the aetiologies and substance use behaviours in humans better. To build on previous knowledge, the current study subsequently employed two distinct mouse models to characterize experimenter–forced injections in place preference conditioning (CPP) as well as voluntary drug consumption in a free–living environment (IC system), both within a social context. In doing so we developed a multiple–choice preference approach to study the behavioural repertoire of transponder tagged female mice (dams) in the automated IC system for 58 days, initially to investigate the preferred consumption between polydrugs (cocaine *vs.* ethanol) and water and subsequently to examine persistence in cocaine seeking and correct sequential learning of the scheduled ANT measured before and after drug withdrawal period. Thereafter, the role of DNA methylation as an epigenetic modifier of the underpinned molecular responses delineated by flux in *Crem* and *Fosb* signalling in the PFC and HPC brain regions were investigated and correlated to behavioural effects. Our findings showed that:

1- consumption of water was preferred above the pharmacological effects of either drug solutions when concurrently presented, whereas cocaine was moderately licked more often than ethanol during the initial period of voluntary drinking access.
2- overall drug intake in the animals reflected episodic drug use.
3- drug experienced dams could not learn the ANT given, despite intact recognition memory.
4- 58 days of prolonged drug access with concurrent water option in the IC led to decreased locomotor activity but anxiety-like behaviours were unaltered in these mice.
5- the decreased response to drug reward in this model was associated with transcriptional activation (hypomethylation) or repression (hypermethylation) of both *Crem* and *Fosb* promoters in the PFC and HPC, respectively.

Simultaneous to behavioural characterization of dams in the IC, we utilized a CPP–social paradigm to examine cocaine–induced behavioural changes in male mice. Our aim here was to investigate the association between cocaine-induced behavioral changes and the methylation status of the promoter regions of *Crem, Fosb* and *Bdnf* that have previously been implicated in drug-mediated effects. We further established patterns of transcriptional signaling of these trans-factors in the PFC and HPC of socially engaged mice that have been treated with cocaine. We also examined locomotor activity, anxiety-like behaviour and memory performance of the animals. We found that:

1- behaviourally, repeated cocaine injections acquired in an interactive social state produced reliable place preference after conditioning but had no effect on drug reinstatement.
2- the experience was also associated with hyperlocomotion, decreased memory performance and unaltered anxiety-like behaviours.
3- neurochemically, these changes were characterized by decreased DNA methylation at *Bdnf* promoters in both PFC and HPC, while *Fosb* and *Crem* promoters were hypomethylated only in the PFC without significant changes in the HPC.

Accumulating evidence have indicated varying responses to drugs of abuse based on sex differences. For instance, studies have shown that females rats are more sensitive to behavioural effects of cocaine than males [1]. The varied responses are mainly due to influences by gonadal hormones [2]. In the present study, the data obtained from both male and female mice showed that these animals also responded differently in some respects to the effects of cocaine. The observed differences may be due to hormonal changes in the female mice that were exposed to drugs over extended periods. However, the overall behavioural and subjective responses to cocaine appear not to be related to gender factors only but may also be influenced by the different routes of drug administration and duration of exposure.

We further explored the hypothesis that parental cocaine exposure could alter these epigenetic sequelae in the unexposed offspring and sought to ascertain whether the epigenetic inheritance is paternal or maternal. Gross impact of early PN fostering on locomotor activity, memory performance and DNA methylation changes in the offspring were also investigated. We reported these main findings:

1- drug induced hypomethylation found in *Crem* promoter genes in PFC of the parent mice were similarly expressed in the same brain region of the unexposed offspring mice without a significant change in the HPC.
2- early PN fostering caused an opposite effect on methylation in the same promoter and brain region.

3- inheritance of the epigenetic marks was likely attributed to contributions from both germ lines.
4- recognition memory was impaired by early PN fostering only in the offspring linked to either paternal (ME) or combined parental (MFE) cocaine experience.
5- neither parental cocaine exposure nor PN fostering affected locomotion in the offspring.

Overall, these findings were interpreted knowing that both dams and male mice studied did not attain full dependence on cocaine. This lack of drug dependence behaviour was ascribed, at least partially, to the impact of social interaction as planned in our experimental design. The accompanied epigenetic changes however indicated a dynamic interplay between the genome in drug-related brain regions and converging environmental stimuli. The strong effects in the PFC compared to the HPC led us to speculate that cortical structures may initially be more responsive to cocaine and therefore social factors that intercept these responses may indeed prevent or delay the development of addictive behaviour.

To the best of our knowledge, this is the first study to produce some evidence that cocaine–induced epigenetic marks can be inherited and that early PN fostering may provoke molecular sensitization towards future risky behaviors.

In conclusion, the social implications of these investigations are (1) occasional and/or frequent drug use in an interacting social environment may not engender dependence, irrespective of oral or intravenous administration mode. This may account for one of the reasons why only approximately 15% of drug users progress from recreational use to substance related disorder [3, 4]; (2) this type of drug experience may also be associated with less pronounced behavioural phenotypes, but it may cause epigenetic changes in the reward brain areas that can be transmitted across generations; (3) alterations in maternal environment due to early PN fostering can engineer molecular switching that may further predispose the non-drug exposed offspring to future risk of drug use.

One major limitation in this research is that we did not examine epigenetic profiles in maternal and paternal gametes which may further help clarify the discrepancies concerning preferential link between parental methylation patterns and inheritance of drug–induced epigenetic marks. We recommend that future studies should identify epigenetic changes in the parent mice gametes, induced under the same experimental settings and conditions, and match these to transmitted traits down the germ lines. Coherent with our findings is a recent study that suggests the therapeutic potential of *Crem* in mediating impulsivity relevant to substance abuse vulnerability [5]. Hence, further studies are required to fully establish the role of *Crem* in cocaine abuse vulnerability. We also acknowledge that the present research studies did not directly compare the effects of cocaine observed in conditions of

social enrichment with social isolation, however future investigations utilizing similar paradigms are required to untangle the relationship.

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APPENDIX I

ZYMO RESEARCH
The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

ZR Genomic DNA™-Tissue MiniPrep

Catalog Nos. **D3050 & D3051**

Highlights

- For high quality DNA purification from solid tissues (e.g., tailsnips, earpunches, adipose tissue, etc.), whole blood, plasma, serum, buffy coat, lymphocytes, cultured cells, buccal cells, FFPE tissues, semen, hair, and other biological sources.
- Combines Proteinase K digestion with innovative *Fast*-Spin column purification technology.
- Isolated DNA is ideal for PCR, endonuclease digestion, Southern blotting, bisulfite conversion/methylation detection, sequencing, genotyping, etc.

Contents

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Satisfaction of all Zymo Research products is guaranteed. If you are not satisfied with this product please call 1-888-882-9682.

Product Contents

ZR Genomic DNA™-Tissue MiniPrep (Kit Size)	D3050 (50 Preps.)	D3051 (200 Preps.)	Storage Temperature
Proteinase K & Storage Buffer*	2 x 5 mg	2 x 20 mg	-20°C(after mixing)
2X Digestion Buffer**	5 ml	20 ml	Room Temp.
Genomic Lysis Buffer***	50 ml	2 x 100 ml	Room Temp.
DNA Pre-Wash Buffer**	15 ml	50 ml	Room Temp.
g-DNA Wash Buffer	50 ml	100 ml	Room Temp.
DNA Elution Buffer	10 ml	50 ml	Room Temp.
Zymo-Spin™ IIC Columns	50 columns	200 columns	Room Temp.
Collection Tubes	100 tubes	400 tubes	Room Temp.
Instruction Manual	1	1	-

Note - Integrity of kit components is guaranteed for up to one year from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide maximal performance and reliability.

* The Proteinase K is stable as shipped. Add 260 µl (1,040 µl for D3051) **Proteinase K Storage Buffer** to each **Proteinase K** tube prior to use. The final concentration of **Proteinase K** after the addition of **Proteinase K Storage Buffer** is ~20 mg/ml.

** The **2X Digestion Buffer** and **DNA Pre-Wash Buffer** may have formed a precipitate. If this is the case, incubate at 37°C to solubilize.

*** *Recommended*: Add beta-mercaptoethanol to 0.5%(v/v) i.e., 250 µl per 50 ml or 500 µl per 100 ml.

Specifications

- **Sample Sources** – Solid tissues (e.g., tailsnips, earpunches, adipose tissue, etc.), whole blood, plasma, serum, buffy coat, lymphocytes, cultured cells, buccal cells, FFPE tissues, semen, hair, and other biological sources are effectively processed using this kit.
- **DNA Purity** – High quality DNA for PCR, endonuclease digestion, Southern blotting, bisulfite conversion/methylation detection, sequencing, genotyping, etc., is eluted with **DNA Elution Buffer** or water. ($A_{260}/A_{280} \geq 1.8$)
- **DNA Size** – Capable of recovering genomic and mitochondrial DNA sized fragments from 100 bp to ≥40 kb. If present, parasitic, microbial, and viral DNA will also be recovered. Typical fragment sizes range from 25 kb-35 kb.
- **DNA Yield** – The DNA binding capacity of the column is 25 µg. Typically, mammalian tissues yield: 1-3 µg DNA per mg skeletal, heart, and brain tissues and 3-5 µg DNA per mg liver, kidney and lung tissues. Human whole blood will yield 3-7 µg DNA per 100 µl blood sampled. DNA is eluted into ≥30 µl **DNA Elution Buffer** or water.
- **Product Detergent Tolerance** – ≤5% Triton X-100, ≤5% Tween-20, ≤5% Sarkosyl, ≤0.1% SDS.
- **Equipment** – Water bath or heat block (55°C), microcentrifuge, and vortex.

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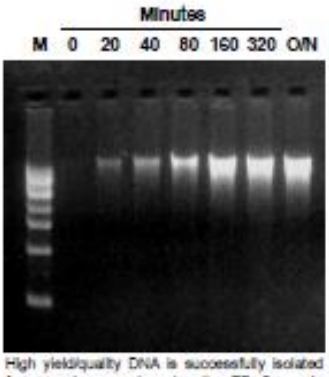
Product Description

The ZR Genomic DNA™-Tissue MiniPrep is a simple procedure for the rapid isolation of total DNA (e.g., genomic, mitochondrial, parasitic, microbial, viral) from a variety of solid tissues. This product has been optimized for maximal recovery of ultra-pure DNA without RNA contamination and is also compatible with buffy coat, bone marrow, cells from culture, whole blood (fresh or stored), serum, plasma, and many biological liquid samples. For processing, simply digest the sample with the supplied **Proteinase K** then add the **Genomic Lysis Buffer**, vortex, and transfer the mixture to the supplied **Zymo-Spin™ Column**. PCR inhibitors are effectively removed during the purification process and purified DNA is suitable for downstream applications including: PCR, Southern blotting, DNA sequencing, endonuclease digestion, bisulfite conversion/methylation analysis, etc.



High yield/quality DNA is successfully isolated from porcine muscle using the **ZR Genomic DNA™-Tissue MiniPrep**. Equivalent amounts (25 mg) of muscle tissue were processed using the **ZR Genomic DNA™-Tissue MiniPrep** after incubation with Proteinase K at 55°C for the indicated times (in minutes) or overnight (O/N). Equal volumes of eluted DNA were then analyzed in a 0.8% (w/v) TAE/agarose/ethidium bromide gel. The size marker "M" is a 1 kb ladder (Zymo Research).

The ZR-96 Genomic DNA™-Tissue MiniPrep (D3055, D3056, D3057) provides high-throughput (i.e., 96-well plate) processing of solid tissue samples.

Zymo Research offers the following for rapid, precise DNA methylation detection...

1.) **EZ DNA Methylation™ Kit** (D5001, D5002, D5003)

2.) **EZ DNA Methylation-Gold™ Kit** (D5005, D5006, D5007, D5008)

3.) **EZ DNA Methylation-Direct™ Kit** (D5020, D5021, D5022, D5023)

General Considerations When Purifying Genomic DNA

Please visit: www.zymoresearch.com for a comprehensive list of genomic DNA purification products.

Zymo Research offers a range of genomic DNA isolation kits that are suitable for extracting high molecular weight DNA from a wide variety of sample types. Kits are tailor-made for specific applications and feature chemical, Proteinase K, and/or mechanical lysis technologies depending on the starting material (see table below).

DNA Extraction Method	Applications
Chemical	*Soft* tissue samples from humans, mice, etc., including: whole blood, plasma, serum, cells, buffy coat, buccal cells, biological liquids, crude homogenates, etc.
Proteinase K & Chemical	*Solid* tissue samples from humans, mice, etc., including: tailsnips, earpunches, hair*, feathers*, and FFPE* samples, as well as all of the above.
Mechanical Homogenization & Chemical	*Tough* tissues and organisms including: insects, arthropods, fungi, gram (+/-) bacteria, and microorganisms in soil, sludge, feces, or water, as well as most of the above.

The **ZR Genomic DNA™-Tissue MiniPrep** includes Proteinase K digestion and chemical lysis for the rapid, efficient purification of DNA (up to 25 µg/prep.) from soft and solid tissues, cells, and a range of biological liquids (see table below for sample types and protocol recommendations).

Recommended Protocol	Sample Types
Solid Tissue	Solid tissue samples from humans, mice, etc., including: tailsnips, earpunches, hair*, feathers*, and FFPE* samples. **(pg. 4)**
Whole Blood, Serum, and Plasma	Whole blood, plasma, and serum. **(pg. 5)**
Cell Monolayer	Monolayer cells (≤5 x10^6) from culture. **(pgs. 5-6)**
Biological Liquids and Cell Suspensions	Biological liquids including: semen, CSF, buffy coat, body fluids. Cell suspensions containing less than 5x10^6 cells (e.g., buffy coat, suspension cultured cells, etc.) **(pg. 7)**

* With protocol modification. See Alternative Protocols **(pg. 8.)**

Starting Material: The quality of the sampled material will affect both the yield and quality of the purified DNA. Freshly sampled tissues and cells yield the highest quantity/ quality DNA. If sampling from "stored" sources and/or if samples have been subject to repeated freeze/thawing, yields may decrease and the purified DNA may be degraded (e.g., FFPE).

The **ZR Soil Microbe DNA MiniPrep™** (D6001), **ZR Fecal DNA MiniPrep™** (D6010), and **ZR Plant/Seed DNA MiniPrep™** (D6020) can be used for the purification of inhibitor-free DNA from soil, feces, and plants, respectively.

Removal of PCR Inhibitors: The **ZR Genomic DNA™-Tissue MiniPrep** has been designed for the efficient removal of PCR inhibitors during DNA purification from the samples listed in the tables above. However, some environmental samples including soil, plants, and manure (feces) will require alternative technologies (see sidebar) for the effective removal of polyphenolic PCR inhibitors.

Storage of Purified DNA: The eluted DNA can be used immediately for molecular-based applications or stored ≤-20°C.

Reagent Preparation

- ✓ Add 260 µl (1,040 µl for D3051) **Proteinase K Storage Buffer** to each **Proteinase K** tube prior to use. The final concentration of **Proteinase K** after the addition of **Proteinase K Storage Buffer** is ~20 mg/ml.
- ✓ *Recommended*: Add beta-mercaptoethanol (user supplied) to the **Genomic Lysis Buffer** to a final dilution of 0.5%(v/v) i.e., 250 µl per 50 ml *or* 500 µl per 100 ml.

Protocols

Solid Tissue **– Including: tailsnips, earpunches, biopsies, etc.**

The following is for the purification of DNA from up to 25 mg fresh or frozen tissue. Typical yields are: 1-3 µg DNA per mg skeletal, heart, and brain tissues and 3-5 µg DNA per mg liver, kidney, and lung tissues. For hair, feathers and FFPE tissues follow **Alternative Protocols I** and **II** on page 8, respectively.

1. To a tissue sample (≤ 25 mg) in a microcentrifuge tube add a solution of...

H_2O	95 µl
2X Digestion Buffer	95 µl
Proteinase K	10 µl

2. Mix and then incubate the tube at 55°C for 1-3 hours.

   Note: If required (e.g., FFPE samples), digesting samples overnight at 55°C with Proteinase K is possible without affecting the integrity of the DNA.

3. Add 700 µl **Genomic Lysis Buffer** to the tube and mix thoroughly by vortexing. Centrifuge at 10,000 *x g* for one minute to remove insoluble debris.
4. Transfer the supernatant to a **Zymo-Spin™ IIC Column** in a **Collection Tube**. Centrifuge at 10,000 *x g* for one minute.
5. Add 200 µl of **DNA Pre-Wash Buffer** to the spin column in a new **Collection Tube**. Centrifuge at 10,000 *x g* for one minute.
6. Add 400 µl of **g-DNA Wash Buffer** to the spin column. Centrifuge at 10,000 *x g* for one minute.
7. Transfer the spin column to a clean microcentrifuge tube. Add ≈50 µl **DNA Elution Buffer** or water (e.g., add 200 µl if sampling 25 mg tissue) to the spin column. Incubate 2-5 minutes at room temperature, then centrifuge at top speed for 30 seconds to elute the DNA. The eluted DNA can be used immediately for molecular based applications or stored ≤-20°C for future use.

For **Technical Assistance**, please contact 1-888-882-9682 or E-mail tech@zymoresearch.com.

Incubate 12-16 hours for formalin-fixed deparaffinized samples.

The column capacity is ~1 ml.

Elution of DNA from the column is dependent on pH and temperature. If water is used, ensure the pH is >6.0. Also, the total yield may be improved by eluting the DNA with Elution Buffer or water pre-equilibrated to 60-70°C or by performing and pooling sequential elutions.

Human whole blood should yield between 3-7 µg DNA per 100 µl.

Whole Blood, Serum and Plasma

The following is for the purification of DNA from up to 100 µl whole blood, serum or plasma (the volumes can be adjusted depending on your requirements). Fresh, frozen, or preserved blood (in EDTA, citrate, or heparin) can be used.

1. Adjust total volume of sample (blood, serum, or plasma) to 100 µl with water in a microcentrifuge tube and then add the following...

2X Digestion Buffer	95 µl
Proteinase K	5 µl

   Example: Add 40 µl H_2O to 60 µl blood, serum, or plasma prior to adding the 2X Digestion Buffer and Proteinase K.

2. Mix and then incubate the tube at 55°C for 20 minutes.

3. Add 700 µl **Genomic Lysis Buffer** to the tube and mix thoroughly by vortexing.

The column capacity is ~1 ml.

4. Transfer the mixture to a **Zymo-Spin™ IIC Column** in a **Collection Tube**. Centrifuge at 10,000 *x g* for one minute.

5. Add 200 µl of **DNA Pre-Wash Buffer** to the spin column in a new **Collection Tube**. Centrifuge at 10,000 *x g* for one minute.

6. Add 400 µl of **g-DNA Wash Buffer** to the spin column. Centrifuge at 10,000 *x g* for one minute.

Elution of DNA from the column is dependent on pH and temperature. If water is used, ensure the pH is >6.0. Also, the total yield may be improved by eluting the DNA with Elution Buffer or water pre-equilibrated to 60-70°C or by performing and pooling sequential elutions.

7. Transfer the spin column to a clean microcentrifuge tube. Add ≥50 µl **DNA Elution Buffer** or water (e.g., add 100 µl if sampling 100 µl blood, serum, or plasma) to the spin column. Incubate 2-5 minutes at room temperature, then centrifuge at top speed for 30 seconds to elute the DNA. The eluted DNA can be used immediately for molecular based applications or stored ≤-20°C for future use.

Cell Monolayer

Generally, no more than $5x10^6$ cells should be sampled, for larger samples will exceed the binding capacity of the spin column.

The following procedure is designed for up to $5x10^6$ monolayer cells. Although cell types and culture conditions may vary, the protocol will work with high-density growth cells (e.g., HeLa cells) as well as with low-density growth cells (e.g., neuronal cells).

1. Trypsinize or scrape adherent cells from a culture flask or plate. Centrifuge the suspension at approximately 500 x *g* for 5 minutes. Remove the supernatant and resuspend the cell pellet in 1 ml PBS (Phosphate Buffered Saline) and then transfer suspension to a microcentrifuge tube. Centrifuge the suspension at approximately 500 x *g* for 5 minutes. Remove the supernatant and resuspend the pellet in a solution of...

H_2O	95 µl
2X Digestion Buffer	95 µl
Proteinase K	5 µl

(continued on next page)

2. Incubate the tube at 55°C for 20 minutes.
3. Add 700 µl **Genomic Lysis Buffer** to the tube and mix thoroughly by vortexing. Centrifuge at 10,000 *x g* for one minute to remove insoluble debris.
4. Transfer the supernatant to a **Zymo-Spin™ IIC Column** in a **Collection Tube**. Centrifuge at 10,000 *x g* for one minute.
5. Add 200 µl of **DNA Pre-Wash Buffer** to the spin column in a new **Collection Tube**. Centrifuge at 10,000 *x g* for one minute.
6. Add 400 µl of **g-DNA Wash Buffer** to the spin column. Centrifuge at 10,000 *x g* for one minute.
7. Transfer the spin column to a clean microcentrifuge tube. Add ≥50 µl **DNA Elution Buffer** or water (e.g., add 200 µl if sampling $5x10^6$ cells) to the spin column. Incubate 2-5 minutes at room temperature, then centrifuge at top speed for 30 seconds to elute the DNA. The eluted DNA can be used immediately for molecular based applications or stored ≤-20°C for future use.

The column capacity is ~1 ml.

Elution of DNA from the column is dependent on pH and temperature. If water is used, ensure the pH is >8.0. Also, the total yield may be improved by eluting the DNA with Elution Buffer or water pre-equilibrated to 60-70°C or by performing and pooling sequential elutions.

Guidelines for Monolayer Cell DNA Isolation: Cell numbers (growth densities) can vary between different cell types. Table 1 (below) provides an approximation of the cell numbers that can be recovered from different culture containers for "high-density" growth cells like CV1 and HeLa cells.

Table 1: Culture Plate/Flask Growth Area (cm^2) and Cell Number

Culture Container	Well /Flask Surface Area	Cell Number
96-well plate	0.32-0.6 cm^2	$4-5x10^4$
24-well plate	2 cm^2	$1-3x10^5$
12-well plate	4 cm^2	$4-5x10^5$
6-well plate	9.5 cm^2	$0.5-1x10^6$
T25 Culture Flask	25 cm^2	$2-3x10^6$
T75 Culture Flask	75 cm^2	$0.6-1x10^7$
T175 Culture Flask	175 cm^2	$2-3x10^7$

Biological Liquids and Cell Suspensions

Cells should be processed directly from biological fluids or from suspension in PBS, TE, or compatible buffers.

The following protocol is designed for up to 100 µl of biological liquid sample including semen, CSF, buffy coat, body fluids, and cell suspensions containing less than $5x10^6$ cells.

1. Adjust total volume of liquid sample to 100 µl with water in a microcentrifuge tube and then add the following...

2X Digestion Buffer	95 µl
Proteinase K	5 µl

2. Mix and then incubate the tube at 55°C for 20 minutes.
3. Add 700 µl **Genomic Lysis Buffer** to the tube and mix thoroughly by vortexing.

The column capacity is ~1 ml.

4. Transfer the mixture to a **Zymo-Spin™ IIC Column** in a **Collection Tube**. Centrifuge at 10,000 *x g* for one minute.
5. Add 200 µl of **DNA Pre-Wash Buffer** to the spin column in a <u>new</u> **Collection Tube**. Centrifuge at 10,000 *x g* for one minute.
6. Add 400 µl of **g-DNA Wash Buffer** to the spin column. Centrifuge at 10,000 *x g* for one minute.

Elution of DNA from the column is dependent on pH and temperature. If water is used, ensure the pH is >6.0. Also, the total yield may be improved by eluting the DNA with Elution Buffer or water pre-equilibrated to 60-70°C or by performing and pooling sequential elutions.

7. Transfer the spin column to a clean microcentrifuge tube. Add ≥50 µl **DNA Elution Buffer** or water (e.g., add 200 µl if sampling liquids containing $5x10^6$ cells) to the spin column. Incubate 2-5 minutes at room temperature, then centrifuge at top speed for 30 seconds to elute the DNA. The eluted DNA can be used immediately for molecular based applications or stored ≤-20°C for future use.

Alternative Protocols:

I.) For Samples Collected onto Storage Papers/Cards: Rapid purification of inhibitor-free, PCR-quality DNA from blood, saliva, and cells collected onto Guthrie, FTA®, and other storage papers/cards.

1. Add card samples (punches) to a **ZR BashingBead™ Lysis Tube (2.0 mm)**[1]. Add 400 µl **Lysis Solution**[2] to the tube.
2. Secure lysis tube in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed.

   Note: Required processing time will vary depending on the device and application and therefore should be evaluated on a case by case basis.

   For example, processing times may be as little as 3 minutes when using high-speed cell disrupters (e.g., the portable TerraLyzer™Sample Processor, FastPrep® -24, or similar) or as long as 20 minutes when using lower speeds (e.g., Disruptor Genie™, or standard benchtop vortexes). See manufacturer's literature for operating information.
3. Centrifuge the ZR BashingBead™ Lysis Tube (2.0 mm) for one minute.
4. To the lysate in the ZR BashingBead™ Lysis Tube (2.0 mm), add:

Proteinase K	10 µl
2X Digestion Buffer	390 µl
5. Mix and then incubate the tube at 55°C for 20 minutes.
6. Centrifuge the ZR BashingBead™ Lysis Tube (2.0 mm) for one minute. Transfer 400 µl supernatant to a tube that can hold up to 2 ml.
7. Add 1,200 µl **Genomic Lysis Buffer** to the tube and mix thoroughly.
8. Transfer 800 µl of the supernatant to a Zymo-Spin IIC Column in a Collection Tube. Centrifuge at 10,000 x *g* for one minute. Discard the flow through.
9. Repeat Step 8 and discard the Collection Tube.
10. Add 200 µl of **DNA Pre-Wash Buffer** to the spin column in a new Collection Tube. Centrifuge at 10,000 x *g* for one minute.
11. Add 500 µl of **g-DNA Wash Buffer** to the spin column and centrifuge at 10,000 x *g* for one minute.
12. Transfer the spin column to a clean microcentrifuge tube. Add ≈50 µl **DNA Elution Buffer** or water (e.g., add 200 µl if sampling $5x10^6$ cells) to the spin column. Incubate 2-5 minutes at room temperature, then centrifuge at top speed for 30 seconds to elute the DNA. The eluted DNA can be used immediately for molecular based applications or stored ≤-20°C for future use.

[1]ZR BashingBead Lysis Tube (2.0 mm) [Catalog No. S6003-50]

[2]Lysis Solution (Catalog No. S6001-3-40)

Alternative Protocols (continued):

II.) **For Hair, Feathers, or Related Samples:** Freshly prepared DTT (dithiolthreitol) (not provided) needs to be added to Step 1 of the Solid Tissue Protocol (page 3) as follows...

H_2O	90 µl
2x Digestion Buffer	90 µl
DTT (1 M)	10 µl
Proteinase K	10 µl

Then follow with the rest of the procedure as indicated.

III.) **For FFPE Samples:** Tissues need to be deparaffinized prior to Step 1 of the Solid Tissue Protocol (page 4) by...

i. Removing (trimming) as much paraffin from the sample(s) as possible.
ii. Transfer samples to 1.5 ml microcentrifuge tubes. Add 750 µl xylene (not provided) to the samples.
iii. Vortex and incubate samples at room temperature for 1 hour with gentle rocking.
iv. Centrifuge for 1 minute at 10,000 *x g* and remove the xylene from the sample. Repeat steps 2-4.
v. Wash two times with 1 ml EtOH (100%) for 5 minutes with gentle rocking.
vi. Wash two times with 1 ml EtOH (95%) for 5 minutes with gentle rocking.
vii. Wash two times with 1 ml EtOH (75%) for 5 minutes with gentle rocking.
viii. Wash once with 1 ml ddH_2O for 5 minutes with gentle rocking. Remove as much water from the sample as possible
ix. Use sample or store at -80°C.

Note: For steps v-viii, add the wash, vortex briefly, and incubate for 5 minutes with gentle rocking. Remove wash from the sample after centrifugation at 10,000 *x g* for 1 minute.

Troubleshooting:

1. *DNA degradation:* Check for DNase contamination. All reagents and components supplied with the **ZR Genomic DNA™-Tissue MiniPrep** are DNase-free. However, DNase contamination can result during the processing of some samples. Check pipets, pipet tips, microcentrifuge tubes, etc., and exercise the appropriate precautions during the DNA purification procedure. Make sure Proteinase K digestions are performed at 55°C as indicated.

2. *DNA is not performing well in subsequent experiments:* Ensure the correct volume of **Genomic Lysis Buffer** has been added to the sample. Also, make sure all centrifugation steps are completed for the indicated times and speeds (rcfs). Failure to do so may result in incomplete washing, which may cause salts to be eluted with the DNA affecting quantitation and subsequent experiments including enzymatic processes like PCR.

3. *RNA contamination:* The buffers and spin columns provided in this kit are designed to efficiently remove RNA during the DNA purification procedure. However, additional RNA removal (e.g., digestion with RNase A) may be necessary for subsequent applications sensitive to trace amounts of RNA.

Ordering Information

Product Description	Catalog No.	Kit Size
ZR Genomic DNA™-Tissue MicroPrep	D3040 D3041	50 preps. 200 preps.
ZR Genomic DNA™-Tissue MiniPrep	D3050 D3051	50 preps. 200 preps.
ZR-96 Genomic DNA™-Tissue MiniPrep	D3055 D3056 D3057	2x96 preps. 4x96 preps. 10x96 preps.

For Individual Sale	Catalog No.	Amount
Proteinase K & Storage Buffer	D3001-2-5 D3001-2-20	5 mg set 20 mg set
2X Digestion Buffer	D3050-1-5 D3050-1-20	5 ml 20 ml
Genomic Lysis Buffer	D3004-1-50 D3004-1-100	50 ml 100 ml
DNA Pre-Wash Buffer	D3004-5-15 D3004-5-30 D3004-5-50	15 ml 30 ml 50 ml
g-DNA Wash Buffer	D3004-2-50 D3004-2-100	50 ml 100 ml
DNA Elution Buffer	D3004-4-4 D3004-4-10 D3004-4-50	4 ml 10 ml 50 ml
Zymo-Spin™ IIC Columns	C1011-50 C1011-250	50 columns 250 columns
Collection Tubes	C1001-50 C1001-500 C1001-1000	50 tubes 500 tubes 1,000 tubes

THE Epigenetics COMPANY™

Popular Products From Zymo Research

Product	Description	Kit Size (Preps)	Catalog No. (Format)
	Fragment DNA Purification		
DNA Clean & Concentrator™-5	Clean and concentrate up to 5µg DNA into ≥6 µl elution volume in as little as 2 minutes with no wash residue carryover.	50 200 50 200	D4003 (uncapped) D4004 (uncapped) D4013 (capped) D4014 (capped)
DNA Clean & Concentrator™-25	Clean & concentrate 25 µg of DNA into ≥25 µl elution volume in as little as 2 minutes with no wash residue carryover.	50 200 50 200	D4005 (uncapped) D4006 (uncapped) D4033 (capped) D4034 (capped)
ZR-96 DNA Clean & Concentrator™-5	Quick (15 minute), high-output recovery of up to 5 µg pure DNA into 10-15 µl minimum elution volume allows for highly concentrated DNA.	2x96 4x96	D4023 D4024
Genomic DNA Clean & Concentrator™	Quick (5 minute) clean-up of up to 10 µg high molecular weight DNA (≤200 kb) from any enzymatic reaction or impure preparation without precipitations.	25 100	D4010 (capped) D4011 (capped)
Zymoclean™ Gel DNA Recovery Kit	Purify DNA from high and low-melting agarose gels in minutes	50 200 50 200	D4001 (uncapped) D4002 (uncapped) D4007 (capped) D4008 (capped)
ZR-96 Zymoclean™ Gel DNA Recovery Kit	High-throughput DNA purification from high and low-melting agarose gels.	2x96 4x96	D4021 D4022
Zymoclean™ Large Fragment DNA Recovery Kit	Purify high molecular weight DNA (≤200 kb) from high and low-melting agarose gels in minutes	25 100	D4045 (capped) D4046 (capped)
OneStep™ PCR Inhibitor Removal Kit	Fast, one step procedure for removal of PCR inhibitors such as polyphenolics, humic/fulvic acids, melanin, etc. for successful PCR and other downstream applications.	50 2x96	D6030 D6035
	Plasmid DNA Purification		
Zyppy™ Plasmid Miniprep Kit	Pellet-Free™ plasmid DNA purification in less than 10 minutes. Recover up to 25 µg DNA in as low as 30 µl.	50 100 400	D4036 D4019 D4020
Zyppy™-96 Plasmid Miniprep	The fastest and simplest high-throughput method for plasmid purification.	2x96 4x96 8x96	D4041 D4042 D4043
Zyppy™ Plasmid Midiprep Kit	Pellet-Free™ plasmid DNA purification in 15 minutes in a 150 µl minimum elution volume).	25 50	D4025 D4026
ZR Plasmid MiniPrep™ Classic	Plasmid DNA purification in minutes: (alkaline lysis/spin column format for low 30 µl elution volume).	50 100 400	D4036 D4019 D4020
	Genomic DNA Purification		
Quick-gDNA™ MiniPrep	Easy purification of genomic DNA from whole blood, plasma, serum, body fluids, buffy coat, lymphocytes, tissue, swabs or cultured cells in as little as 15 minutes without the use of Proteinase K or organic denaturants.	50 200 50 200	D3006 (uncapped) D3007 (uncapped) D3024 (capped) D3025 (capped)
ZR-96 Quick-gDNA™	Simple, high throughput (96-well) purification of DNA from whole blood, plasma, serum, body fluids, buffy coat, lymphocytes, tissue, swabs, or cultured cells in about 30 minutes.	2x96 4x96 10x96	D3010 D3011 D3012
ZR-Genomic DNA™-Tissue MiniPrep	For high quality DNA purification from solid tissues (e.g., tail snips, ear punches, adipose tissue, etc.), body fluids, cultured cells, buccal cells, FFPE tissues, hair, and other biological sources using Proteinase K and Fast.	50 200	D3050 D3051
Environmental DNA Purification Kits	Unique BashingBead™ technology allows isolation of DNA from samples refractory to conventional lysis procedures including tough-to-lyse tissues, soil samples, feces, plants, seeds, insects, bacteria, yeast, filamentous fungi, unicellular and filamentous algae, and protozoa		Visit website for a comprehensive list

Please visit our website to see our complete line-up of products.

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APPENDIX II

ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

EZ DNA Methylation™ Kit

Catalog Nos. **D5001 & D5002**

Highlights

- Streamlined, proven procedure for bisulfite conversion of DNA.
- Desulphonation and recovery of bisulfite-treated DNA with a spin column.
- Recovered DNA is ideal for downstream analyses including PCR, endonuclease digestion, sequencing, microarrays, etc.

Contents

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Product Contents:

EZ DNA Methylation™ Kit	D5001 50 rxns.	D5002 200 rxns.	Storage Temperature
CT Conversion Reagent*	5 tubes	20 tubes	Room Temp.
M-Dilution Buffer	1.3 ml	5.2 ml	Room Temp.
M-Binding Buffer	20 ml	80 ml	Room Temp.
M-Wash Buffer**	6 ml	24 ml	Room Temp.
M-Desulphonation Buffer	10 ml	40 ml	Room Temp.
M-Elution Buffer	1 ml	4 ml	Room Temp.
Zymo-Spin™ IC Columns	50 columns	200 columns	Room Temp.
Collection Tubes	50 tubes	200 tubes	Room Temp.
Instruction Manual	1	1	–

Note - Integrity of kit components is guaranteed for one year from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide maximal performance and reliability.

* 750 µl water and 210 µl **M-Dilution Buffer** are added per tube of **CT Conversion Reagent** and mixed prior to use.

** Add 24 ml of 100% ethanol to the 6 ml **M-Wash Buffer** concentrate (D5001) or 96 ml of 100% ethanol to the 24 ml **M-Wash Buffer** concentrate (D5002) before use.

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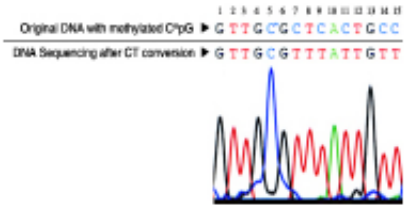
Introduction to DNA Methylation:

DNA methylation is a naturally occurring event in both prokaryotic and eukaryotic organisms. In prokaryotes DNA methylation provides a way to protect host DNA from digestion by restriction endonucleases that are designed to eliminate foreign DNA, and in higher eukaryotes DNA methylation functions in the regulation/control of gene expression (1). It has been demonstrated that aberrant DNA methylation is a widespread phenomenon in cancer and may be among the earliest changes to occur during oncogenesis (2). DNA methylation has also been shown to play a central role in gene imprinting, embryonic development, X-chromosome gene silencing, and cell cycle regulation. In many plants and animals, DNA methylation consists of the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring via a methyltransferase enzyme (3). The majority of DNA methylation in mammals occurs in 5'-CpG-3' dinucleotides, but other methylation patterns do exist. In fact, about 80 percent of all 5'-CpG-3' dinucleotides in mammalian genomes are found to be methylated, whereas the majority of the twenty percent that remain unmethylated are within promoters or in the first exons of genes.

The ability to detect and quantify DNA methylation efficiently and accurately has become essential for the study of cancer, gene expression, genetic diseases, as well as many other important aspects of biology. To date, a number of methods have been developed to detect/quantify DNA methylation including: high-performance capillary electrophoresis (4) and methylation-sensitive arbitrarily primed PCR (5). However, the most common technique used today remains the bisulfite conversion method (6). This technique involves treating methylated DNA with bisulfite, which converts unmethylated cytosines into uracil. Methylated cytosines remain unchanged during the treatment. Once converted, the methylation profile of the DNA can be determined by PCR amplification followed by DNA sequencing (see below).



DNA sequencing results following bisulfite treatment. DNA with methylated C*pG at nucleotide position #5 was processed using the **EZ DNA Methylation™ Kit**. The recovered DNA was amplified by PCR and then sequenced directly. The methylated cytosine at position #5 remained intact while the unmethylated cytosines at positions #7, 9, 11, 14 and 15 were completely converted into uracil following bisulfite treatment and detected as thymine following PCR.

References:

1. Costello JF, Plass CJ. Med. Genet. 2001; 38(5): 285-303.

2. Stirzaker C. Cancer Res. 1997; 57(11): 2229-2237.

3. Adams RL. Bioessays. 1995; 17(2): 139-145.

4. Fraga MF, *et al.* Electrophoresis. 2000; 21(14): 2990-2994.

5. Gonzalgo ML. Cancer Res. 1997; 57(4): 594-599.

6. Frommer M. Proc. Natl. Acad. Sci. USA. 1992; 89(5): 1827-1831.

Note: 96-Well spin-plate formats are available for processing larger numbers of samples. Also, MagPrep kits are available (p. 8) for adaptation to liquid handling robots (e.g., Tecan – Freedom EVO®) and automated sample prep.

Selected EZ DNA Methylation™ Kit Citations:

1. Ehrich M, *et al.* Nuc. Acids Res. 2007; 35 (5): e29

2. Kaneda M, *et al.* Nature. 2004; 429: 900-903

3. Zhang F, *et al.* Proc. Natl. Acad. Sci. USA. 2007; 104 (11): 4395-4400.

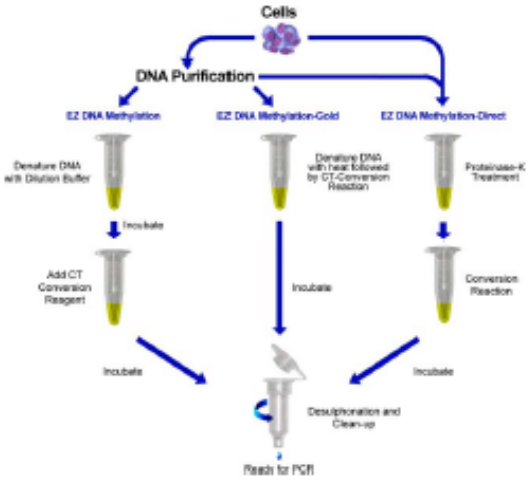
4. Oda M, *et al.* Genes & Dev. 2006; 20: 3382-3394.

5. England RPM, *et al.* Nature Meth. 2005; 2: 1-2.

Product Description:

The **EZ DNA Methylation™ Kit** features a simplified procedure that streamlines bisulfite conversion of DNA. The kit is based on the three-step reaction that takes place between cytosine and sodium bisulfite where cytosine is converted into uracil. The product's innovative in-column desulphonation technology eliminates otherwise cumbersome precipitations. The kit is designed to reduce template degradation, minimize DNA loss during treatment and clean-up, while ensuring complete conversion of the DNA. Purified, converted DNA is ideal for PCR amplification for downstream analyses including endonuclease digestion, sequencing, microarrays, etc.

An outline comparing the **EZ DNA Methylation™ Kit** procedure to Zymo Research's other methylation kits is shown below.



Outline of the EZ DNA Methylation™, EZ DNA Methylation-Gold™ and EZ DNA Methylation-Direct™ Kit procedures.

Specifications:

- **DNA Input**: Samples containing 500 pg - 2 µg of DNA. For optimal results, the amount of input DNA should be from 200 to 500 ng.
- **Conversion Efficiency**: > 99% of non-methylated C residues are converted to U; > 99% protection of methylated cytosines.
- **DNA Recovery**: > 80%

Reagent Preparation:

- **Preparation of CT Conversion Reagent**

  The **CT Conversion Reagent** supplied within this kit is a solid mixture and must be prepared prior to first use. Prepare as follows:

  1. Add 750 µl water and 210 µl of **M-Dilution Buffer** to a tube of **CT Conversion Reagent**.
  2. Mix at room temperature with frequent vortexing or shaking for 10 minutes.

  Note: It is normal to see trace amounts of undissolved reagent in the **CT Conversion Reagent**. Each tube of **CT Conversion Reagent** is designed for 10 separate DNA treatments.

  Storage: The **CT Conversion Reagent** is light sensitive, so minimize its exposure to light. For best results, the **CT Conversion Reagent** should be used immediately following preparation. If not used immediately, the **CT Conversion Reagent** solution can be stored overnight at room temperature, one week at 4°C, or up to one month at -20°C. Stored **CT Conversion Reagent** solution must be warmed to 37°C, then vortexed prior to use.

- **Preparation of M-Wash Buffer**

  Add 24 ml of 100% ethanol to the 6 ml **M-Wash Buffer** concentrate (D5001) or 96 ml of 100% ethanol to the 24 ml **M-Wash Buffer** concentrate (D5002) before use.

Protocol:

1. Add 5 µl of **M-Dilution Buffer** to the DNA sample and adjust the total volume to 50 µl with water. Mix the sample by flicking or pipetting up and down.
   Example: For 14 µl of a DNA sample add 5 µl M-Dilution Buffer and 31 µl water.
2. Incubate the sample at 37°C for 15 minutes.
3. After the above incubation, add 100 µl of the prepared **CT Conversion Reagent** to each sample and mix.
4. Incubate the sample in the dark at 50°C for 12-16 hours.
   Please see Appendix (page 6) for alternative incubation conditions (e.g., when using the Illumina Infinium® Methylation Assay)
5. Incubate the sample at 0-4°C (e.g., on ice) for 10 minutes. Samples may be kept at 4°C for up to 20 hours.
6. Add 400 µl of **M-Binding Buffer** to a **Zymo-Spin™ IC Column** and place the column into a provided **Collection Tube**.
7. Load the sample (from Step 5) into the **Zymo-Spin™ IC Column** containing the **M-Binding Buffer**. Close the cap and mix by inverting the column several times.
8. Centrifuge at full speed (≥10,000 x *g*) for 30 seconds. Discard the flow-through.
9. Add 100 µl of **M-Wash Buffer** to the column. Centrifuge at full speed for 30 seconds.
10. Add 200 µl of **M-Desulphonation Buffer** to the column and let stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at full speed for 30 seconds.
11. Add 200 µl of **M-Wash Buffer** to the column. Centrifuge at full speed for 30 seconds. Add another 200 µl of **M-Wash Buffer** and centrifuge for an additional 30 seconds.
12. Place the column into a 1.5 ml microcentrifuge tube. Add 10 µl of **M-Elution Buffer** directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA.

    The DNA is ready for immediate analysis or can be stored at or below -20°C for later use. For long term storage, store at or below -70°C. We recommend using 1-4 µl of eluted DNA for each PCR, however, up to 10 µl can be used if necessary. The elution volume can be > 10 µl depending on the requirements of your experiments, but small elution volumes will yield more concentrated DNA.

The **CT Conversion reagent** is light sensitive, so try to minimize the reaction's exposure to light whenever possible.

The capacity of the collection tube with the column inserted is 800 µl. Empty the collection tube whenever necessary to prevent contamination of the column contents by the flow-through.

Alternatively, water or TE (pH ≥ 8.0) can be used for elution if required for your experiments.

Appendix: Bisulfite Conversion and PCR Optimization

1. **Incomplete C to T Conversion.**

   A. Increase temperature in Step 2 of the **Protocol** to 42°C and extend the incubation time to 30 minutes. If the problem persists, use modified conversion conditions (see B, below).

   B. In Step 1 of the **Protocol**, add 7.5 µl **M-Dilution Buffer** instead of 5 µl (the total volume should remain 50 µl). If this change is made, the preparation of the **CT-Conversion Reagent** must also be modified by reducing the volume of **M-Dilution Buffer** from 210 µl to 185 µl. In Step 3 of the **Protocol**, add 97.5 µl prepared **CT-Conversion Reagent** per reaction instead of 100 µl.

2. **Bisulfite Conversion of Double Stranded DNA Templates.** The following illustrates what occurs to a DNA template during bisulfite conversion.

   Template: A: 5′-GACGTTCCAGGTCCAGCAGTGCGCT-3′
   B: 3′-CTGGCAAGGTCCAGGTCGTCACGCGA-5′

   Bisulfite Converted: A: 5′-GATCGTTTTAGGTTTAGTAGTGCGTT-3′
   B: 3′-TTGGCAAGGTTTAGGTTGTTATGCGA-5′

   Note: Methylated "C" is underlined in the examples.

   Note: Following bisulfite conversion, the strands are no longer complementary.

3. **PCR Primer Design.** Generally, primers 26 to 32 bases are required for amplification of bisulfite converted DNA. In general, all Cs should be treated as Ts for primer design purposes, unless they are in a CpG context. See example below.

   Bisulfite Converted: A: 5′-GATCGTTTTAGGTTTAGTAGTGCGTT-3′
   Primers: Reverse: 3′-ATCATCACRCAA-5′ R= G/A
   Forward: 5′-GATTGTTTTAGGT-3′ Y= C/T

   Zymo Research provides primer design assistance with its Bisulfite Primer Seeker Program, available at: www.zymoresearch.com/tools/bisulfite-primer-seeker

   Note: Only one strand (A) is amplified by a given primer set. Only the reverse primer binds to the converted DNA, the forward primer will bind the strand generated by the reverse primer.

   If the primer contains CpG dinucleotides with uncertain methylation status, then mixed bases with C and T (or G and A) can be used. Usually, there should be no more than one mixed position per primer and it should be located toward the 5′ end of the primer. It is not recommended to have mixed bases located at the 3′ end of the primer.

4. **Amount of DNA Required for Bisulfite Conversion.** The minimal amount of human or mouse genomic DNA required for bisulfite treatment and subsequent PCR amplification is 100 pg. The optimal amount of DNA per bisulfite treatment is 200 to 500 ng. Although, up to 2 µg of DNA can be processed, it should be noted that high input levels of DNA may result in incomplete bisulfite conversion for some GC-rich regions.

5. **PCR Conditions.** Usually, 35 to 40 cycles are required for successful PCR amplification of bisulfite converted DNA. Optimal amplicon size should be between 150-300 bp; however larger amplicons (up to 1 kb) can be generated by optimizing the PCR conditions. Annealing temperatures between 55-60°C typically work well.

   As most non-methylated cytosine residues are converted into uracil, the bisulfite-treated DNA usually is AT-rich and has low GC composition. Non-specific PCR amplification is relatively common with bisulfite treated DNA due to its AT-rich nature. PCR using "hot start" polymerases is strongly recommended for the amplification of bisulfite-treated DNA.

   Zymo Taq™ is a "hot start" DNA polymerase specifically designed for the amplification of bisulfite treated DNA. (see page 9 for details)

6. **Alternative Incubation Conditions When Using the Illumina Infinium® Methylation Assay.** For Steps 4 & 5 of the protocol, incubate the sample(s) in a thermocycler at...
   (95°C for 30 sec., 50°C for 60 min.) x 16 cycles, then "hold" at 4°C

Frequently Asked Questions:

Q: Should the Input DNA be dissolved in TE, water, or some other buffer prior to its conversion?

A: Water, TE or modified TE buffers can be used to dissolve the DNA and do not interfere with the conversion process.

Q: Which *Taq* polymerase(s) do you recommend for PCR amplification of converted DNA?

A: We recommend a "hot start" DNA polymerase (e.g., ZymoTaq™, page 9).

Q: Why are there two different catalog numbers for the EZ-96 DNA Methylation™ Kit?

A: The two different catalog numbers are used to differentiate between the binding plates that are included in the kit. Deep and shallow-well binding plates are available to accommodate most rotors and microplate carriers. Below is a comparison of the two binding plates.

Binding Plate	Silicon-A™ Plate	Zymo-Spin™ I-96 Plate
Style	Shallow-Well	Deep-Well
Height of Binding Plate	19 mm (0.75 inches)	35 mm (1.38 inches)
Binding Plate/Collection Plate Assembly	43 mm (1.69 inches)	60 mm (2.36 inches)
Binding Cap./Minimum Elution Volume	5 µg/30 µl	5 µg/15 µl
Catalog Numbers	D6003	D6004

Ordering Information:

Product Description	Catalog No.	Kit Size
EZ DNA Methylation™ Kit	D5001 D5002	50 rxns. 200 rxns.
EZ-96 DNA Methylation™ Kit (Shallow-Well)	D5003	2 x 96 rxns.
EZ-96 DNA Methylation™ Kit (Deep-Well)	D5004	2 x 96 rxns.
EZ-96 DNA Methylation™ MagPrep*	D5040 D5041	4 x 96 rxns. 8 x 96 rxns.

For Individual Sale	Catalog No.	Amount(s)
CT Conversion Reagent	D5001-1 D5003-1	1 tube 1 bottle
M-Dilution Buffer	D5001-2 D5002-2	1.3 ml 5.2 ml
M-Binding Buffer	D5005-3 D5006-3 D5040-3	30 ml 125 ml 250 ml
M-Wash Buffer	D5001-4 D5002-4 D5007-4 D5040-4	6 ml 24 ml 38 ml 72 ml
M-Desulphonation Buffer	D5001-5 D5002-5 D5040-5	10 ml 40 ml 80 ml
M-Elution Buffer	D5001-6 D5002-6 D5007-6 D5041-6	1 ml 4 ml 8 ml 40 ml
Zymo-Spin™ IC Columns (capped)	C1004-50 C1004-250	50 columns 250 columns
Collection Tubes	C1001-50 C1001-500 C1001-1000	50 tubes 500 tubes 1,000 tubes
MagBinding Beads	D4100-2-6 D4100-2-8 D4100-2-12 D4100-2-18 D4100-2-24	6ml 8 ml 12 ml 18 ml 24 ml
Zymo-Spin™ I-96 Binding Plates	C2004	2 plates
Silicon-A™ Binding Plates	C2001	2 plates
Conversion Plates w/ Pierceable Cover Film	C2005	2 plates/films
Collection Plates	C2002	2 plates
Elution Plates	C2003	2 plates

* MagPrep kits are adaptable to liquid handling robots (e.g., Tecan – Freedom EVO®) making them ideal for automated sample prep.

ZYMO RESEARCH CORP.
Phone: (949) 679-1190 • Toll Free: (888) 882-9682 • Fax: (949) 266-9452 • info@zymoresearch.com • www.zymoresearch.com

THE Epigenetics COMPANY™

Epigenetics Products From Zymo Research

Product	Description	Kit Size	Cat No. (Format)
	Bisulfite Kits for DNA Methylation Detection		
EZ DNA Methylation™ Kit	For the conversion of unmethylated cytosines in DNA to uracil via the chemical-denaturation of DNA and a specially designed CT Conversion Reagent. *Fast-Spin* technology ensures ultra-pure, converted DNA for subsequent DNA methylation analysis. Magnetic bead format for adaptation to automated liquid handling platforms.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns. 4x96 Rxns. 8x96 Rxns.	**D5001** (spin column) **D5002** (spin column) **D5003** (shallow-well plate) **D5004** (deep-well plate) **D5040** (magnetic bead) **D5041** (magnetic bead)
EZ DNA Methylation-Gold™ Kit	For the fast (3 hr.) conversion of unmethylated cytosines in DNA to uracil via heat/chemical-denaturation of DNA and a specially designed CT Conversion Reagent. *Fast-Spin* technology ensures ultra-pure, converted DNA for subsequent DNA methylation analysis. Magnetic bead format for adaptation to automated liquid handling platforms.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns. 4x96 Rxns. 8x96 Rxns.	**D5005** (spin column) **D5006** (spin column) **D5007** (shallow-well plate) **D5008** (deep-well plate) **D5042** (magnetic bead) **D5043** (magnetic bead)
EZ DNA Methylation-Direct™ Kit	Features simple and reliable DNA bisulfite conversion directly from blood, tissue (FFPE/LCM), and cells without the prerequisite for DNA purification in as little as 4-8 hrs. The increased sensitivity of this kit makes it possible to amplify bisulfite converted DNA from as few as 10 cells or 50 pg DNA. Magnetic bead format for adaptation to automated liquid handling platforms.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns. 4x96 Rxns. 8x96 Rxns.	**D5020** (spin column) **D5021** (spin column) **D5022** (shallow-well plate) **D5023** (deep-well plate) **D5044** (magnetic bead) **D5045** (magnetic bead)
EZ DNA Methylation-Lightning™ Kit	Complete bisulfite conversion in about an hour using a unique liquid format conversion reagent that requires no preperation. *Fast-Spin* technology ensures ultra-pure, converted DNA for subsequent DNA methylation analysis. Magnetic bead format for adaptation to automated liquid handling platforms.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns. 4x96 Rxns. 8x96 Rxns.	**D5030** (spin column) **D5031** (spin column) **D5032** (shallow-well plate) **D5033** (deep-well plate) **D5046** (magnetic bead) **D5047** (magnetic bead)
EZ DNA Methylation-Startup™ Kit	Designed for the first time user requiring a consolidated product to perform DNA methylation analysis. Includes technologies for sample processing, bisulfite treatment of DNA, and PCR amplification of "converted" DNA for methylation analysis.	1 Kit	**D5024**
	Methylated DNA Standards		
Universal Methylated Human DNA Standard	Human (male) genomic DNA having all CpG sites methylated. To be used for the evaluation of bisulfite-mediated conversion of DNA. Supplied with a control primer set.	1 set	**D5011**
Universal Methylated Mouse DNA Standard	Mouse (male) DNA having all CpG sites methylated. To be used for the evaluation of bisulfite-mediated conversion of DNA. Supplied with a control primer set.	1 set	**D5012**
	Other...		
ChIP DNA Clean & Concentrator™	Clean and concentrate DNA from any reaction or "crude" preperation in 2 min. A 6 µl minimum elution volume allows for highly concentrated DNA. Designed for samples containing up to 5 µg of DNA.	50 Preps. 50 Preps.	**D5201** (uncapped column) **D5205** (capped column)
Genomic DNA Clean & Concentrator™	Genomic DNA clean-up in minutes. Unique spin column technology for recovery of ultra-pure large-sized DNA (100 bp to ≥200 kb) DNA from any impure preperation (e.g., Proteinase K digestion).	25 Preps. 100 Preps.	**D4010** **D4011**
ZymoTaq™ DNA Polymerase	ZymoTaq™ "hot start" DNA Polymerase is specifically designed for the amplification of "difficult" DNA templates including: bisulfite-treated DNA for methylation detection. The product generates specific amplicons with little or no by-product formation. Available either as a single buffer premix or as a polymerase system with components provided seperately.	50 Rxns. 200 Rxns. 50 Rxns. 200 Rxns.	**E2001** (system) **E2002** (system) **E2003** (premix) **E2004** (premix)
Methylated-DNA IP Kit	IP with a highly specific anti-5-methylcytosine monoclonal antibody. Designed for the enrichment of 5-methylcytosine-containing DNA from any pool of fragmented genomic DNA for use in genome-wide methylation analysis.	10 Rxns.	**D5101**
	Services		
Available for **DNA Methylation** and **Hydroxymethylation** at http://www.zymoresearch.com/services or inquire at services@zymoresearch.com. *...powered by the latest Next-Gen Sequencing technologies!*			

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APPENDIX III

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

25 August 2015

Mr Duyilemi Chris Ajonijebu (215077684)
Department of Human Physiology
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Mr Ajonijebu,

Protocol reference number: AREC/071/015D
Project title: Establishing and characterising an animal model of addiction within a social context

Full Approval – Research Application

With regards to your revised application received on 13 July 2015. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

CONDITION:

1. **Animals can be housed in the intellicage only during the test period, otherwise they have to be housed in the usual rodent cages as specified by BRU.**
2. **Pain, discomforts and distress to animals must be monitored at least every 8 hours during the entire experimental period.**
3. **All sophisticated surgical and non-surgical procedures must be done by expert veterinary persons at BRU or under their direct supervision.**

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 25 August 2016.

I take this opportunity of wishing you everything of the best with your study.

Yours faithful[redacted]

.....

Dr Shahidul Islam
Chair: Animal Research Ethics Committee

/ms

Cc Superivsor: Professor W Daniels
Cc Registrar: Mr Baatile Poo
Cc NSPCA: Ms Lebo Sentle
Cc BRU – Dr Sanil Singh

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

APPENDIX IV

GW 12/22

DEPARTMENT OF HEALTH
Private Bag X828
PRETORIA, 0001
Republic of South Africa

UMNYANGO WEZEMPILO
LEFAPHA LA MAPHELO

health

Department:
Health
REPUBLIC OF SOUTH AFRICA

APPLICATION TO ACQUIRE ANY SCHEDULE DRUGS IN TERMS OF SECTION 22A(9)(a)(i) OF THE MEDICINES AND RELATED SUBSTANCES ACT, 1965 (ACT 101 OF 1965)

plication to acquire, possess or use any specified Schedule 5, Schedule 6 or Schedule 7 substances for educational, analytical or research rposes in terms of section 22A(9)(a)(i) of the Medicines and Related Substances Act, 1965 (Act 101 of 1965)

N • TO:
The Registrar of Medicines
Department of Health
Private Bag X828
Pretoria 0001

Particulars of applicant:

Surname DANIELS

Full name WILLIAM MARK URON

Identity number 610502 5079 089

Permanent residential address UNIT 20, GLEN ELGIN ESTATE, 5 CAMP ROAD, GILLITTS, DURBAN, SOUTH AFRICA Tel. No: 083 320 7020

Occupation PROFESSOR

Status PERMANENT EMPLOYER

Name and address of employer UNIVERSITY OF KWAZULU-NATAL, UNIVERSITY DRIVE, WESTVILLE, DURBAN

Tel no.

Qualifications:

Qualifications	Dates	Major subjects	Institutions	Dissertations / Theses
PhD	1993	CHEMICAL PATHOLOGY	UNIV OF STELLENBOSCH	CENTRAL NEUROTRANSMITTER REGULATION OF HPA AXIS ACTIVITY

rticulars of publications and research projects already completed by you. (Use a separate sheet if necessary and attach it to this form.)

Particulars of the research project: The impact of street drugs on the genome of the individual

Aim ① Validate animal model of addiction
② Identify epigenetic changes in certain brain areas following drug administration.
③ Investigating the heritability of above epigenetic changes.

Proposed procedure
Rats are housed in Intellicages where they will have free access to either drinking water or drug solution. Animal behaviour will be studied for 90 days.

Adres waar die navorsing onderneem sal word • Address at which research will be undertaken Discipline of Human Physiology, Westville campus, Univ. of KwaZulu-Natal

Geraamde duur van projek • Estimated duration of project 3 years

Naam Name	Rang Status	Instansie Institution
WILLIAM DANIELS	PROF	UKZN

Wie anders sal u met hierdie projek bystaan? (Behulpsaam wees) • Who else will assist you in this project?

Naam Name	Rang Status	Instansie Institution
OUAFID ABBOUSSI	POST DOCTORAL FELLOW	UKZN
CHRIS AJONIJOBU	DOCTORAL STUDENT	UKZN

Besonderhede van afhanklikheidsvormende middels • Particulars of dependence-producing drugs:

Tipe Type	Totale hoeveelheid wat in voorraad gehou sal word per jaar Total quantity to be kept in stock per annum	Voorsieningsbron Source of supply
Cocaine powder	50 grams	SAPS / SIGMA

aar en hoe beoog u om die betrokke middels veilig te bewaar? • Where and how do you intend to store the drugs concerned safely?
The drug will be stored in a locked cabinet in the office of the project leader. A register will be kept to record utilization of drug.

Algemeen • General

(a) Indien u voorheen 'n soortgelyke aansoek aan die Departement voorgelê het, meld wanneer (asook die verwysingsnommer indien bekend.) • If you have previously submitted a similar application to the Department, state when (also give the Department's reference number if known):
No

(b) Het u aansoek gedoen vir 'n bydrae by die Suid-Afrikaanse Mediese Navorsingsraad of is u van voorneme om dit te doen? • Have you applied for a grant from the South African Medical Research Council or do you intend to apply?
We have applied for a grant from the National Research Foundation

Indien u aansoek gedoen het, wat was die resultaat? • If you have applied, what was the result?
Result pending

VERKLARING • STATEMENT

• I WILLIAM DANIELS

rklaar hiermee dat die inligitng wat hier verstrek is, waar en juis is en indien hierdie aansoek suksesvol is, onderneem ek - • hereby declare that e information furnished herein is true and correct and if this application is successful, I undertake -

(a) om die afhanklikheidsvormende middels waarvoor aansoek gedoen is, op 'n plek en wyse te bewaar sodat geen ongemagtigde persoon toegang daartoe sal verkry nie. • to store the dependence-producing drugs applied for in such a place and manner that no unauthorised person shall have access thereto;

(b) om die betrokke middels slegs te gebruik vir die doel soos gespesifiseer in paragraaf 2 • to use the drugs concerned only for the purpose specified in paragraph 2; en • and

(c) om na voltooing van die navorsingsprojek met enige oorblywende voorraad van die middel te handel soos deur die Departement gespesifiseer word • on completion of the research project, to deal with any surplus quantity of the drug in the manner to be specified by the authority.

eteken te Westville hierdie 2nd dag van October 20 15
gned this day of

[redacted]
Handtekening • Signature

etuies • Witness
DR HLENGANE [redacted]

res • Address FO 411 WESTVILLE CAMPUS, UKZN, UNIVERSITY ROAD, DURBAN, 4001

[redacted]

MR. JACOBUS HENDRICKS
50 Annerley Rd. 17 Walnut Grove, Pinetown, 3610

APPENDIX V

health
Department:
Health
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH
Private Bag X828
PRETORIA, 0001
Republic of South Africa

UMNYANGO WEZEMPILO
LEFAPHA LA MAPHELO

PERMIT IN TERMS OF SECTION 22A(9)(a)(i) OF THE MEDICINES AND RELATED SUBSTANCES ACT, 1965 TO ACQUIRE, POSSESS AND USE SCHEDULE 6 AND 7 SUBSTANCES FOR THE PURPOSE OF EDUCATION.

Date of Issue: 11 November 2015	Expiry Date: 10 November 2016	Permit No: POS 389/2015/2016

Authority is hereby granted in terms of Section 22A (9)(a)(i) of the above-mentioned Act to **Prof W M U Daniels** of **University of Kwazulu-Natal, Durban** to acquire, possess and use, subject to the conditions stated, the under-mentioned Schedule 6 and Schedule 7 substances in respect of which the quoted quantity should not be exceeded during the period **11 November 2015** to **11 November 2016.**

Name of Scheduled Substance(s)	Schedule	Total quantity of substance(s) and/or preparation(s) allocated per calendar year
Cocaine	Schedule-6	50 g [ten grams]

Total Items: 1

The acquisition, possess and use the relevant substances are subject to the following conditions:

1. The substances shall be used for **training of Sniffer dogs** only.
2. The control over the substances shall be the responsibility of:

   Full Name & Surname: **Prof W M U Daniels**

   ID Number: **610502 5079 089**
3. Complete details of the substances acquired and used shall be recorded in registers designed specifically for this purpose in accordance with the provisions of the relevant regulations to the Medicines and Related Substance Act, 1965.
4. Orders for the substances shall be signed for by:

   Full Name & Surname: **Prof W M U Daniels**

   ID Number: **610502 5079 089**
5. When the substances are acquired, the name and address of the supplier, the date supplied, the quantity supplied and the number of the relevant invoice shall be recorded **on this permit**.
6. The register referred to in paragraph 3, as well as copies of orders and invoices pertaining to the supply of the substances, shall be available at the offices of the **University of Kwazulu-Natal, Durban** for a period of at least three years and shall be subject to inspection by Inspectors appointed in terms of the Medicines and Related Substances Act, 1965.
7. This permit expires on **10 November 2016** and shall on expiry be returned to the Department of Health for cancellation and shall be accompanied by a statement reflecting the quantity of substances on stock at expiry.

[redacted]

DEPARTMENT OF HEALTH

DATE: 11/11/2015

APPENDIX VI

health
Department:
Health
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH
Private Bag X828
PRETORIA, 0001
Republic of South Africa

UMNYANGO WEZEMPILO
LEFAPHA LA MAPHELO

PERMIT IN TERMS OF SECTION 22A(9)(a)(i) OF THE MEDICINES AND RELATED SUBSTANCES ACT, 1965 TO ACQUIRE, POSSESS AND USE SCHEDULE 6 AND 7 SUBSTANCES FOR THE PURPOSE OF EDUCATION.

Date of Issue: 28 March 2017	**Expiry Date: 27 March 2018**	**Permit No: POS 173/2017/2018**

Authority is hereby granted in terms of Section 22A (9)(a)(i) of the above-mentioned Act to **Prof W M U Daniels** of **University of Kwazulu-Natal, Durban** to acquire, possess and use, subject to the conditions stated, the under-mentioned Schedule 6 and Schedule 7 substances in respect of which the quoted quantity should not be exceeded during the period **28 March 2017** to **27 March 2018.**

Name of Scheduled Substance(s)	Schedule	Total quantity of substance(s) and/or preparation(s) allocated per calendar year
Cocaine	Schedule-6	50 g [fifty grams]

Total Items: 1

The acquisition, possess and use the relevant substances are subject to the following conditions:

1. The substances shall be used for **research purposes** only.
2. The control over the substances shall be the responsibility of:

   Full Name & Surname: **Prof W M U Daniels**

   ID Number: **610502 5079 089**
3. Complete details of the substances acquired and used shall be recorded in registers designed specifically for this purpose in accordance with the provisions of the relevant regulations to the Medicines and Related Substance Act, 1965.
4. Orders for the substances shall be signed for by:

   Full Name & Surname: **Prof W M U Daniels**

   ID Number: **610502 5079 089**
5. When the substances are acquired, the name and address of the supplier, the date supplied, the quantity supplied and the number of the relevant invoice shall be recorded **on this permit**.
6. The register referred to in paragraph 3, as well as copies of orders and invoices pertaining to the supply of the substances, shall be available at the offices of the **University of Kwazulu-Natal, Durban** for a period of at least three years and shall be subject to inspection by Inspectors appointed in terms of the Medicines and Related Substances Act, 1965.
7. This permit expires on **27 March 2018** and shall on expiry be returned to the Department of Health for cancellation and shall be accompanied by a statement reflecting the quantity of substances on stock at expiry.

[redacted]

DIRECTOR-GENERAL:
DEPARTMENT OF HEALTH

DATE: 28/03/2017

[redacted]

APPENDIX VII

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

School of Laboratory Medicine and Medical Sciences
College of Health Sciences
Nelson R Mandela School of Medicine
719 Umbilo Road, Congella, 4013, Durban

Private Bag x7, Congella, 4013, Durban, South Africa
Tel no (27)31 260 4603/8116/7132

3 September 2015

South African Police Services (SAPS)
KwaZulu-Natal,
Durban,
South Africa.

Dear Sir/Madam,

RE: REQUEST FOR COCAINE POWDER FOR USE IN A PHD STUDY AT THE UNIVERSITY OF KWAZULU-NATAL

The School of Laboratory Medicine and Medical Sciences within the College of Health Sciences requires the assistance from the SAPS with regards to sourcing cocaine for a PhD study. The study focuses on the neurological and trans-generational effects of cocaine addiction known to contribute substantially to global public health problems. We therefore engage this study which attempts to address some uncertainties with respect to the transfer of addictive traits from parents to offspring, its impact on the vulnerability of the offspring and search for improved addiction therapy.

We have an obligation to provide research tools and materials for our research students. As a means to risc to our immediate need, we hereby wish to kindly request that SAPS provides us with **50g** of highly concentrated **Cocaine powder** with a certificate of analysis if possible, as the purity of the compound will determine the amount to use in the experimental study.

The 50g of cocaine will be used at a concentration of 300 mg/litre of water to treat 64 mice in a free-living environment for 13 weeks to model drug addiction. The Cocaine compound

will be safely stored in a locked cabinet throughout the study period and the remainder will be returned back to your service station at the completion of the study. We will also keep a log book showing the accurate consumption of the drug during the experiment which will be presented to the SAPS upon completion of the study.

Assistance in this regard will be highly appreciated as our findings will add to a better understanding of the aetiology of drug addiction pathology and will shed more light on possible new therapeutic targets. The outcomes of the study will also be published and will be available to SAPS on request.

I hope this request will receive a favourable response.

Find attached a copy of this study ethics clearance approval certificate for your perusal.

Thanking you in advance.

Yours Faithfully,

[redacted]

Prof Willie M.U. Daniels
Dean and Head: School of Laboratory Medicine and Medical Sciences
danielsw@ukzn.ac.za

APPENDIX VIII

FORENSIC SCIENCE LABORATORY FORMS MANUAL CHEMISTRY SECTION			
ISSUING OF DRUG SUBSTANCES			
Revision no: 00	Effective Date: June 2015	Ref: DRG0055F_1	Page 1 of 2

Verw/Ref	**Provincial ISSUE Ref No 26/57/4 (19/15)**
Navrae/Enq	Prof W M U Daniels
Telno	031 2604603/8116/7132
Faks/Fax	

THE OFFICE OF THE CHIEF
FORENSIC SCIENCE LABORATORY
PRIVATE BAG X 620
PRETORIA
0001

Date : 2015-12-07

TO: THE HEAD FORENSIC SCIENCE LABORATORY
270 Pretoria Road
Pretoria - Silverton
0182

ISSUING OF DRUG SUBSTANCES : HEAD of FSL ISSUE Reference no: 26/57/4 (19/15) Lab no: 282409 /15

Application to issue the following:

- 1 x 20.00 g of powder and solid substance containing Cocaine and originated from lab no. 170038/07.

to University of KZN.

COMMENT/PURPOSE: Research.

[redacted]
Provincial CHEMISTRY : COMMANDER :

COMMENT _____

Recommended / ~~NOT Recommended~~

[redacted]
BRIGADIER.....BRIG
0444109-5
J. ALLEN

COMMENT

.....

.....

Approved / ~~NOT Approved~~

[redacted]

Verw/Ref	**Provincial ISSUE Ref No.26/57/4 (19/15)**
Navrae/Enq	Capt Smith
Telno	031 319 2270
Faks/Fax	0313192265

THE OFFICE OF THE CHIEF
FORENSIC SCIENCE LABORATORY
PRIVATE BAG X 620
PRETORIA
0001

Date:2015-12-07

ISSUE NO: 26/57/4 (12/15)

1. Issuing of scheduled substances by Forensic Science Laboratory
2. Substances to be issued according to Prov. Issue Ref no **26/57/4 (19/15)** and Lab no. **282409/15.**

2.1 Sealed in FSL exhibit bag no PA5001962727

- 1 x 20.00 g of powder and solid substance containing Cocaine and originated from lab no. 170038/07.

3. Issued by: Lt M P Modise 7160398-1

2016 -01- 15 .....(Date)

09 - 30 .....(Time)

[redacted] .....(Signature)

3.1 The following documents were verified and copies attached:-

3.1.1 Original authorisation (DPP/Medicine Control Council/Permits) ❑

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4. **All the substances received must be handed back and a copy of the receipt form must be forwarded (faxed/ e-mailed) to the issuer.**

5. Received by:- William Daniels .....(Name)

.....(Rank)

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APPENDIX IX

CONGRESS CONTRIBUTIONS

Abstract

UNIVERSITY OF KWAZULU-NATAL
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EARLY POSTNATAL FOSTERING ALTERED MEMORY PERFORMANCE AND DNA METHYLATION IN THE PREFRONTAL CORTEX OF OFFSPRING MICE WITH LINEAL PHENOTYPES RELATED TO PARENTAL COCAINE EXPOSURE

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Here, we explored the hypotheses that parental cocaine exposure could alter epigenetic machinery in the unexposed offspring while early postnatal (PN) fostering may further modify the accompanied neurochemical and functional components. Cocaine exposed or unexposed C57BL/6 female mice were matched with their male counterpart for mating to produce variant pubs with lineal phenotypes. The pubs were initially examined for locomotor activity and memory performance and subsequently for changes in DNA methylation in promoter regions of cAMP response element modulator (CREM) and FosB genes in the prefrontal cortex (PFC) and hippocampus (HPC) at 48 days postnatum. The impact of PN fostering on these parameters were also investigated to evaluate the influence of the environment on epigenetic expression. We found that (1) early PN fostering impaired recognition memory in pubs lineally inclined to paternal and/or parental cocaine experience; (2) neither parental cocaine exposure nor fostering affected locomotor behaviour in the pubs; (3) the offspring of cocaine-exposed parent mice showed decreased methylation of CREM in their PFC, while fostering the offspring produced opposite effect on methylation in this substrate brain region without any significant change recorded in the HPC (4) fostering also decreased FosB methylation in the HPC of pubs linked to progenitor's cocaine exposure without significant change in the PFC. Our data provide some evidence that cocaine may cause epigenetic changes that can be inherited by the offspring, that mediation by CREM signalling in the PFC may be beneficial, while early PN fostering may further engineer molecular switch for future risky behaviours.

APPENDIX X

PUBLISHED ARTICLES

Article (a)

Epigenetics: a link between addiction and social environment

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Cellular and Molecular Life Sciences

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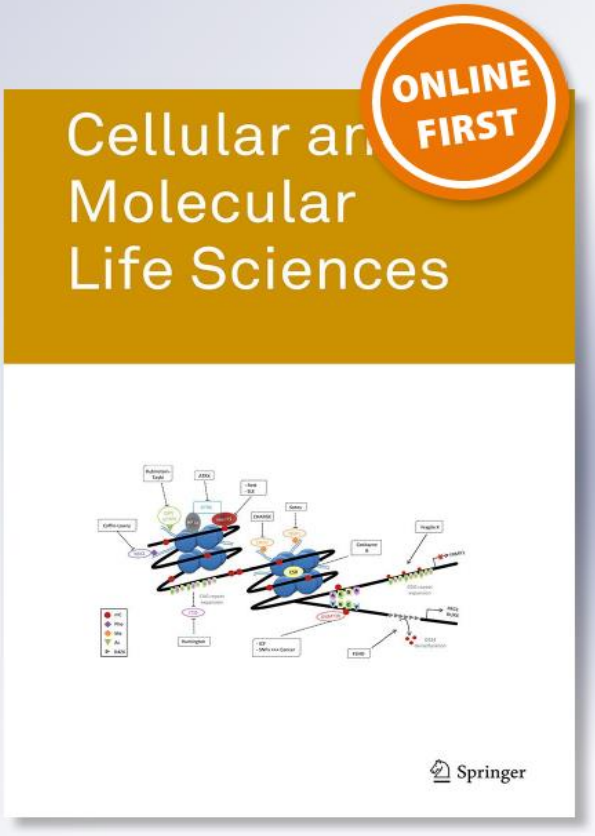

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Cellular and Molecular Life Sciences

REVIEW

Epigenetics: a link between addiction and social environment

Duyilemi C. Ajonijebu[1] · Oualid Abboussi[1] · Vivienne A. Russell[1] · Musa V. Mabandla[1] · William M. U. Daniels[1,2]

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Abstract The detrimental effects of drug abuse are apparently not limited to individuals but may also impact the vulnerability of their progenies to develop addictive behaviours. Epigenetic signatures, early life experience and environmental factors, converge to influence gene expression patterns in addiction phenotypes and consequently may serve as mediators of behavioural trait transmission between generations. The majority of studies investigating the role of epigenetics in addiction do not consider the influence of social interactions. This shortcoming in current experimental approaches necessitates developing social models that reflect the addictive behaviour in a free-living social environment. Furthermore, this review also reports on the advancement of interventions for drug addiction and takes into account the emerging roles of histone deacetylase (HDAC) inhibitors in the etiology of drug addiction and that HDAC may be a potential therapeutic target at nucleosomal level to improve treatment outcomes.

Keywords Cocaine and alcohol abuse · DNA methylation · Chromatin remodeling · Epigenomic programming and inheritance · Environmental stimuli · Social stress

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Introduction

Drug addiction is a chronic relapsing disorder, where the individual is unable to control his or her drug-seeking and drug-taking behaviours despite severe adverse consequences [1]. Clinically, drug addiction presents with behavioural, cognitive, and physiological symptoms that reflect the involvement of complex control systems of the brain [2]. Due to the increase in risky behaviours, this disorder is often associated with trauma and death [3]. Epidemiological surveys have shown that not only is addiction highly prevalent on its own, but that the disorder also frequently occurs as a comorbidity with other mental disorders [4]. The hundreds of research projects conducted all over the world underline the importance of studying addictive behaviour. In fact, many of the current trends of addiction research now have a well-defined focus that attempts to understand the neural mechanisms that underpin the conventional change from recreational drug use to a chronic addicted state with persistent addictive behaviours even after long abstinence and relapse propensity in drug addicts [5]. Many studies on chromatin remodeling and transcriptional regulation have shown that exposure to drugs of abuse induces changes in the expression of specific genes, such as brain-derived neurotrophic factor (*BDNF*), activator of G-protein signaling 3 (*AGS3*), and transcription factors (e.g., Δ*FosB*) in key reward areas of the brain, such as the ventral tegmental area (VTA), nucleus accumbens (NAc), and prefrontal cortex (PFC) [6–10]. Despite the substantial progress that has been made, our understanding of the molecular underpinnings of drug addiction remains incomplete. For instance, the impact of epigenetic alterations in combination with social environmental factors within the context of addictive behaviour appears underexplored. The

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purpose of this article is, therefore, to provide an overview of the current status of research in the field of drugs of abuse with specific reference to these factors, with a focus on how they might play a role in the etiology of drug abuse and its inheritance. We also report on current pharmacotherapies and highlight possible novel treatments that could be considered for drug-related disorders (Table 1).

Epigenetic mechanisms

Both genes and the environment are important determinants of developmental processes. Subtle differences in the interaction between genes and the environment may be responsible for altering developmental trajectories that confer vulnerability or resilience to mental conditions, such as addiction. At the molecular level, epigenetics provides invaluable insight into the interaction between an individual's genome and the environment [11]. Epigenetics, also known as chromatin remodeling, is defined as heritable chemical modifications to DNA capable of influencing transcriptional activity independent of DNA-coding sequence [12]. Principally, epigenetic mechanisms include DNA methylation and various chemical modifications of the histone proteins (H2A, H2B, H3, and H4) (see Fig. 1a). Most complex and coordinated series of epigenetic modifications, such as acetylation, methylation, phosphorylation, ubiquitylation, and ADP ribosylation, occur at the N-terminal tail of histones [13–15]. It is worth noting that recent findings have revealed additional mechanisms involving RNA interference and prion proteins which also contribute to epigenetic regulation [16]. In general, *DNA methylation* and *histone acetylation* remain the most recognized, widely studied, and established epigenetic modifications [17]. These two mechanisms work in tandem to cause chromatin remodeling and thus regulate gene expression.

DNA methylation

DNA methylation is generally linked to gene silencing due to its repressive effect on gene transcription. It either disrupts association of DNA binding factors with their target sequence or recruits transcriptional co-repressors by binding to methyl-CpG-binding protein-2 (*MeCP2*) [18] to induce an inactivated, condensed (silenced) chromatin state. Studies have shown that MeCP2 mediates behavioural responses to addictive properties of alcohol and cocaine mainly by altering *BDNF* expression in specific regions of the brain [19–21]. DNA methylation modification occurs only within specific gene promoters to produce stable epigenetic changes as opposed to histone tail modifications which are readily reversible [22, 23]. DNA methyltransferases (*Dnmt*) catalyze and maintain the sequence of gene expression events induced by DNA methylation. *Dnmt1*, *Dnmt3a*, and *Dnmt3b* are highly expressed in postmitotic nerve cells [24]. Altered expression of these *Dnmt* has been implicated in drug dependence and other related psychiatric disorders. For instance, *Dnmt1* is highly expressed in GABAergic neurons whose dysfunction characterizes development of schizophrenia [25, 26]. Both *Dnmt3a and Dnmt3b* are capable of inducing methylation on naked DNA [27]. Expression of *Dnmt3a* in the NAc was increased by chronic social defeat stress and cocaine infusion suggesting its importance in regulating emotional behaviour and cocaine addiction [17, 28]. In response to cocaine treatment, it appears that *Dnmt3a* expression is biphasically regulated. Evidence from quantitative PCR analysis of NAc tissue from mice acutely and chronically pretreated with cocaine suggested that early withdrawal (4 h after injection of cocaine) upregulated *Dnmt3a* expression, whereas after 24 h, it was downregulated [28]. Mice lacking both *Dnmt3a* and *3b* in mature forebrain neurons

Table 1 Overview of studies on the epigenetic regulation by drugs of abuse

Drug of abuse	Brain region	Epigenetic mechanism	Residues	Implication	References
Ethanol	AMY	Methylation	H3K27/H3K4	Susceptibility to alcohol dependence	[119]
	AMY	Acetylation	H3K9	Susceptibility to alcohol dependence	[119]
	VTA	Acetylation	H3K9	Ethanol withdrawal	[120]
	NAc	Acetylation	H4K12	Persistence of ethanol-related behaviours	[121]
Cocaine	NAc	Acetylation	Gen H3	Cocaine addiction-related behaviours	[105, 122, 123]
	VTA	Acetylation	Gen H3	Motivation for drug reinforcement	[124]
	NAc	Acetylation	H3K14/H4K12	Cocaine addiction-related behaviours	[105, 125]
	NAc	Methylation	H3K9	Cocaine addiction-related behaviours	[75, 105]
	NAc	Acetylation	Gen H4	Motivation for drug reinforcement	[74, 122]

AMY amygdala, *VTA* ventral tegmental area, *NAc* nucleus accumbens, *DG/Hipp* dentate gyrus/Hippocampus

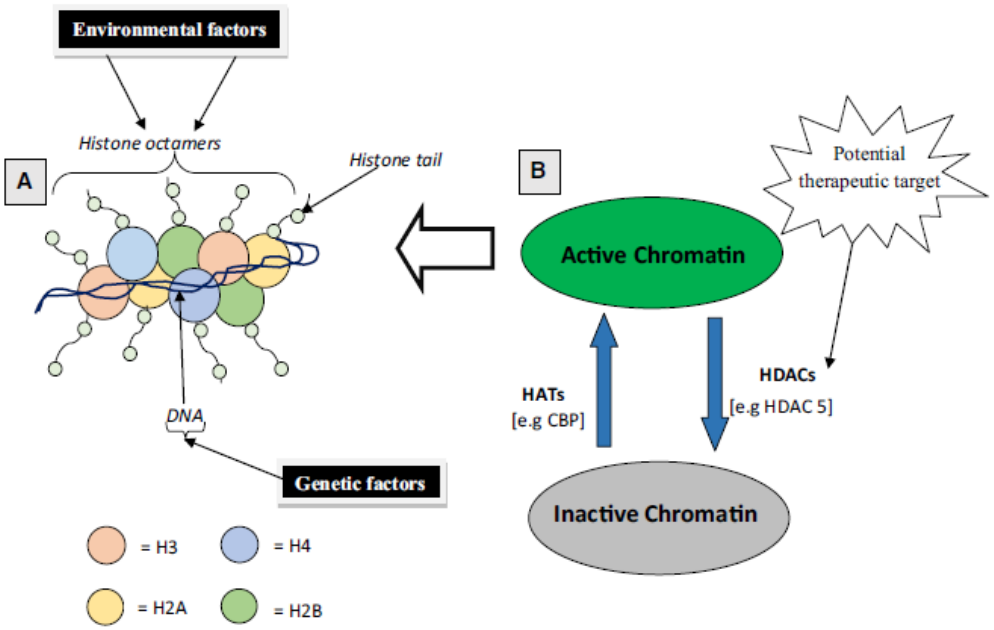

Fig. 1 Schematic view of post-translational modifications of histones. **a** In eukaryotic cells, the DNA (consists of 147 base pairs) wraps around histone octamers (two copies each of H2A, H2B, H3, and H4) to form the nucleosome which is the functional unit of the chromatin. Projecting from the core of the histone octamers is the amino acid (-N-) terminal tails on which significant transcriptional modification occurs. **b** Transcriptional active or inactive states of the chromatin are balanced by the opposing actions of HATs and HDACs, respectively. Enhanced action of HATs promotes histone acetylation which allows assessibility of the DNA to the transcription factors by relaxing the chromatin resulting in an enhanced gene activity. Conversely, increased action of HDACs represses transcription (gene silencing) by deacetylating the histones. HDAC5 is one the II HDACs that shuttles between the nucleus and the cytoplasm and actively mediates gene silencing mechanism by binding hormone co-repressors. Consequently, nuclear export of HDAC5 which results in histone hyperacetylation as well as increased mRNA expression of its target genes (e.g., substance P and neurokinin 1) have been critically implicated in sensitized behavioural responses to addictive drugs [57]. In addition, toxicants such as drugs or endocrine disruptors can induce epimutations of the histone octamers [73] resulting in chromosomal abnormalities that are fundamental to addiction phenotypes and disease

displayed impaired long-term plasticity and performed poorly in learning and memory tasks which were attributed in part to dysregulated gene expression. Other studies have shown that murine *Dnmt3b* knockouts exhibited neural tube defects that led to early lethality [29].

Histone acetylation

Histone acetylation is considered a good marker of actively transcribed genes [30]. Most genomic studies involving histone acetylation have focussed on the amino acid (-N-) terminal lysine residues in histones H3 and H4 [15]. Increased or low levels of histone acetylation within specific promoter regions correlates with enhanced or repressed gene activity, respectively [31]. These dynamic processes are actively controlled by two key enzymes histone acetyltransferases (HATs) and histone deacetylases (HDACs) (see Fig. 1b). The roles that these enzymes play in drug addiction are described below.

Social environmental factors that increase susceptibility to drug addiction

Human and animal studies suggest that there may be a direct relationship between environmental stress prior to drug exposure and the development of addiction-like behaviours. A meta-analysis showed that a degraded home environment significantly increases the risk for drug-related disorders to develop [31], while an enriched environment, such as positive family relationships, involvement, and attachment, appear to discourage drug use and prevent drug addiction [32]. The relationship between an adverse environment and drug dependence is underpinned by the interaction of multiple endocrine, paracrine, and intracellular systems [33], since these systems have been shown to be sensitive to social experience. It is, therefore, not surprising that drug addiction is highly prevalent in vulnerable populations undergoing social stress [34]. Many factors confound the study of drug addiction-related social stress

and physiology in human populations, including long life spans, inaccuracy of self-reporting, ethical considerations, and high levels of genetic variation [35]. Animal models circumvent many of these challenges, and provide more tractable systems to study the interplay of social factors and drug addiction. For example, exposure to stress *in utero* has been shown to modify the behavioural reactivity of rats to drugs with addictive potential [36]. Similarly, an increase in drug seeking has been observed during adulthood in offspring of maternally stressed dams. Experiments utilising isolation stress and social deprivation yielded comparable results [37]. Poor maternal behaviour towards the pups and/or maternal separation during the early postnatal period increased ethanol consumption in adult rats [38]. Maternal licking and grooming correlated negatively with vulnerability to cocaine and ethanol use in rats [39]. Repeated social defeat during adolescence increased cocaine self-administration and cocaine-induced conditioned place preference (CPP) in rodents [40–43]. Furthermore, these stress events were suggested to share common epigenetic process with drugs of abuse to influence addiction-like behaviours. Indeed, it has been demonstrated that both early life stress and methamphetamine alter the expression of the epigenetic regulator methyl CpG binding protein 2 (MeCP2) in the nucleus accumbens to influence the motivational effects of methamphetamine and natural reinforcement [44–46]. These basic studies offer valuable insights into the effect of adverse environmental conditions on addictive behaviour. Similar studies have demonstrated the involvement of epigenetic changes in these effects [43, 47], such as the epigenetic mechanisms responsible for dysregulation of the hippocampal glucocorticoid receptors [48] and upregulation of histone acetylation by social defeat [43]. Epigenetic alterations induced by early life experiences have been shown to accumulate over time and have consequently been considered serious risk factors for mental disease development [49, 50]. This line of work on epigenetic regulation related to environmental stimuli has raised many questions, including and among others, (1) how the pharmacological effects of drugs with addictive potential may vary depending on the associated environmental conditions to which subjects may be exposed, or (2) to what extent social stress influences drug consumption, or (3) which genes are altered and in what way, by various environmental circumstances.

Mild stress and environmental enrichment have also been shown to protect and sometimes reverse addictive phenotypes. The chronic mild stress of neonatal handling prevented reinstatement of morphine CPP in adulthood [51] and environmental enrichment blocked reinstatement of ethanol-induced CPP [52]. It also reduced cocaine seeking and reinstatement induced by cues and stress [53, 54]. Similar to environmental enrichment, overexpression of ΔFosB decreased cocaine self-administration, enhanced extinction of cocaine seeking, and decreased cocaine-induced reinstatement of intravenous cocaine self-administration [55].

Interestingly, there is evidence that suggests that neurons do not only respond to various environmental signals via dynamic changes in epigenetic modifications [31], but also vary in sensitivity to drugs of abuse subject to altered states in the environment (see Fig. 2) [32]. Therefore, while there is no doubt of an interaction between the environment and addictive behaviour, the underlying mechanisms that facilitate this interaction continue to be poorly understood.

Inheritance of epigenetic imprints and trans-generational effects

The vulnerability of progeny to drug-induced maladaptive behaviours or neural plasticity is jointly influenced by both genetic and non-genetic factors. Many authors share the opinion that drugs of abuse are likely to induce epigenetic changes in parent sex cells (ova and sperm) which are passed down to future generations [18] and thereby predispose the offspring to subsequent drug effects and/or addiction. The fact that some epigenetic imprints, particularly maternal DNA methylation, can escape the epigenomic reprogramming that occurs during gametogenesis and fertilization [56] provides a mechanism that may enable the transgenerational transfer of parental traits. Some epigenetic imprints may, therefore, accumulate over a lifetime and be conserved between generations. This implies that the inheritance of acquired traits resulting from environmental exposure that alters a phenotype in one generation can be transmitted epigenetically to unexposed offspring [57]. This notion has profound implications for understanding how diseases may be prevalent in families as well as how the genome functions as an etiological factor in hereditary diseases.

The impact of social environment on the epigenome and its transgenerational transfer

Drug intake related to social context involves long-lasting epigenetic underpinnings that may be transmissible from one generation to another. The genes affected by social factors are mostly related to regulatory networks that control the hypothalamic-pituitary-gonadal axis and a variety of social behaviours [58]. Paternal transmission of epigenetic variation may manifest only in later life when the social environment changes [59]. In male mice, chronic social stress (achieved through instability of social hierarchy) experienced during adolescence through to adulthood induced social deficits and increased anxiety-like behaviours in up to two succeeding generations [60].

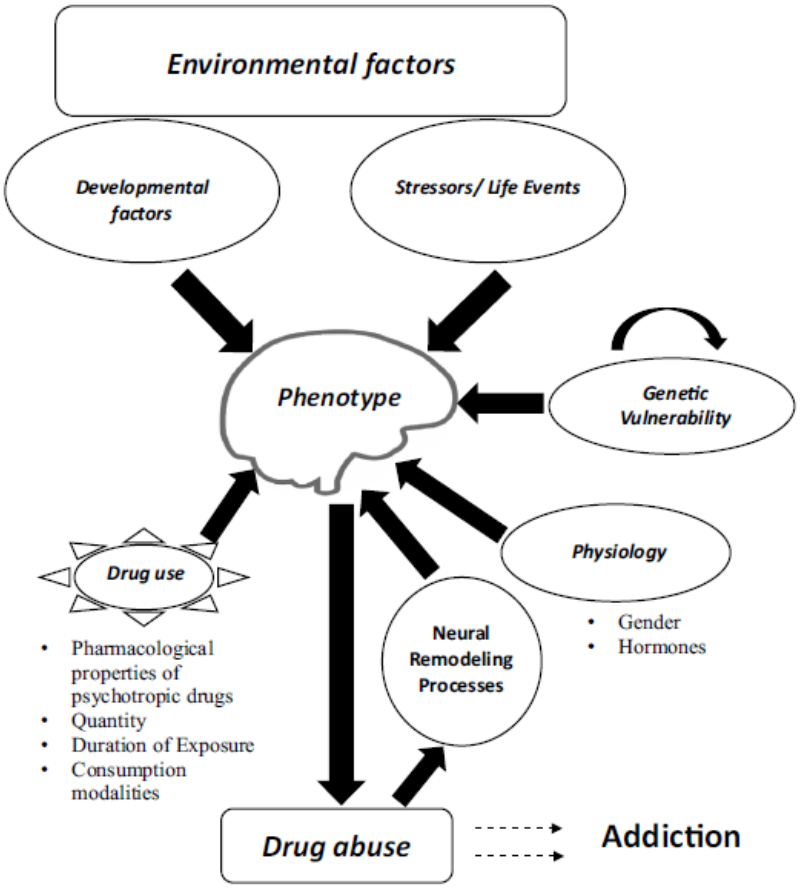

Fig. 2 Model of possible factors that influence drug intake. Altered states in the environment influenced by various factors, such as physical contact, gender, family history, social or early life experience all converge to impact on the individual's sensitivity and vulnerability to addictive compounds. In contrast, escalated or chronic drug intake especially at high doses possibly induces changes that accumulate over time to promote further drug use or addictive behaviours and sometimes may be passed down the germline to the next generation. Invariably, behavioural (drug intake) and psychological (vulnerability) balance largely depends on the level of exposure to drugs and associated factorial states within the environment

These observations in animal models find their counterpart in human studies. Recently, it was shown that differences in socio-economic status early in life are imprinted on the epigenome and maintained into adulthood. Several hundred promoters showed different levels of DNA methylation in blood profiles of adults who experienced social adversity early in life when compared to those that did not [61]. Similarly, global DNA hypomethylation was observed in blood samples of socio-economically deprived subjects [62]. These clinical observations highlighted the 'epigenome' as an interface between the social environment and the genome. However, genome-wide assays (GWA) of epigenetic changes in different regions and cell types of the brain are necessary to fully understand how specific epigenetic modifications may both influence and be caused by social behaviour. These previous studies indicate that the social component may have tremendous impact on the physiological and behavioural responses of an individual to environmental factors.

The impact of substances of abuse on the genome and its transgenerational transfer

Adding to the transgenerational effects of social conditions, chronic exposure to several drugs of abuse, including alcohol and cocaine, has been reported to induce epigenetic changes which points to a dysregulation of gene expression in both the brain and periphery [5, 57].

Alcohol has been shown to interfere with the epigenetic regulation of gene expression. A recent transcriptome study, comparing human post-mortem brain samples of alcoholics and age-matched controls, showed profound epigenetic effects of alcohol abuse. A notable difference between the two groups was that endogenous retroviral sequences that maintain DNA methylation throughout gametogenesis and fertilization, which are normally silenced by DNA methylation, were less methylated, coinciding with dramatically increased transcription of their host genes [63]. *In utero* studies in mice have shown that exposure to alcohol induces teratogenic effects related

to epigenetic changes in the foetus. In female mice, free access to 10% (v/v) ethanol for 4 days per week for 10 weeks affected the adult offspring phenotype by altering the epigenotype of the early embryo. These alterations in the epigenome were associated with postnatal growth restriction and craniofacial dysmorphology reminiscent of foetal alcohol syndrome [64]. Alcohol exposure *in utero* also reduced DNA methylation at the differentially methylated domain of the paternally imprinted growth-related gene *H19* in the sperm of exposed mice (F1). Most notably, a similar decrease at the same CpG sites was observed in the brains of the offspring (F2) [65]. These epigenetic changes caused by alcohol exposure resulted in a variety of developmental disturbances ranging from reduced litter size and birth weight to behavioural alterations, such as lower fearfulness and higher aggression. These findings from animal studies demonstrating how parental alcohol consumption may induce epigenetic aberrations that negatively affect the normal structure and function of their offspring were corroborated by clinical observations. For example, moderate and heavy drinkers show subtle reductions in DNA methylation at the *H19* imprinted gene in their sperm compared to non-drinkers [66], and alcohol-induced parental epigenome changes such as these have been suggested to have detrimental effects on the cognitive performance of their children [67].

Changes in histone acetylation in the PFC of ethanol-exposed adolescent rats are associated with ethanol-induced place conditioning [68]. Intermittent alcohol exposure upregulated HAT activity in adolescent rat PFC and increased histone acetylation and dimethylation in the promoter region of *cFos, Cdk5*, and *FosB* [69]. Alcohol exposure during adulthood has been shown to be associated with downregulation of genes implicated in neural plasticity, such as cut-like 2 (*cutl2*), insulin-like growth factor 1 (*Igf1*), epidermal growth factor–containing fibulin-like extracellular matrix protein 1 (*Efemp1*), SRY-box-containing gene 7 (*Sox 7*) and many others, as well upregulation of *SWI/SNF* (an ATP dependent chromatin remodeling complex that mobilizes histone octamers) related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2 (*Smarca2*), the cytosolic enzyme DiGeorge syndrome critical region gene 2 (*Dgcr2*), and *Pard6a* which are implicated in the migration of immature granule neurons and neuroblast cell polarity [57]. In addition, cocaine and alcohol exposure have been associated with significant decreases in the mRNA levels of enzymes responsible for DNA methylation in the testes and sperm of adult male rodents, presenting a high risk induction factor for heritable epigenetic changes [70, 71]. Altered methylation related to alcohol was associated with dysregulation of genes known to play a role in metabolism, such as (*Cyp4f13*) and decreased methylation of genes associated with development (*Nlgn3, Elavl2, Sox21*, and *Sim1*), imprinting (*Igf2r*), and chromatin (*Hist1h3d*) which contribute to abnormal foetal development [70].

Cocaine has a profound effect on chromatin remodeling in brain areas, such as the NAc and PFC—brain regions key in processing reward and implicated in addiction [72]. Chronic cocaine treatment and self-administration induced epigenetic dysregulation of expression of several genes associated with neural plasticity, such as the immediate–early gene *cFos, BDNF*, cyclin-dependent kinase 5 (*cdk5*), and myocyte enhancing factor 2 (*MEF2*) [5, 73]. Chronic cocaine-induced hyperlocomotor activity and expression of CPP in rats was associated with a decrease in gene expression in the NAc caused by an increase in DNA methylation and a decrease in global levels of histone H3 acetylation [74]. Moreover, using conditional mutagenesis and viral mediated gene transfer, Maze et al. [27] showed that histone methyltransferase *G9a* downregulation increases dendritic spine plasticity of NAc neurons and enhances rewarding responses to cocaine by decreasing repressive *H3K9me2* at specific target genes thus increasing the expression of those genes [75]. Vassoler et al. [28], using histone H3 acetylation in *BDNF* promoters as an epigenetic marker, showed that voluntary paternal ingestion of cocaine resulted in epigenetic reprogramming of the germline that changed medial PFC gene expression to such an extent that the male offspring became resistant to cocaine reinforcement [76]. This observation suggests that epigenetic reprogramming of the genome in offspring of addicted parents may serve as a protective mechanism in rendering the next generation less susceptible to future addiction susceptibility. However, administration of cocaine during gestation has been shown to alter global DNA methylation in several promoter regions for genes implicated in crucial cellular functions [77]. For instance, maternal cocaine consumption during gestation caused increased CpG methylation at two *SP1* binding sites in the promoter region of protein kinase C (*PKC*) that precipitated downregulation of *PKC* expression in the heart of adult offspring. This change in *PKC* expression rendered these animals more sensitive to ischemia and reperfusion injury [78]. The genomic effects of parental drug intake and how these effects impact on the well-being of the offspring, therefore, remain controversial.

Animal models of drug addiction

Animal models of drug addiction have enabled the implementation of protocols that are used to characterise addictive behaviour, as well as facilitating the study of transgenerational effects over short periods. Over time, these models have undergone a number of refinements that allowed a deeper understanding of the circuitry involved in

drug craving, relapse and loss of control in several behavioural paradigms, in particular, tests of behavioural sensitization, CPP, and drug self-administration. Despite the advances made, these classical paradigms do not allow a continuous assessment of addictive behaviour, nor do they consider the social contexts of addicts in the neuropathology of addiction [79]. It has been repeatedly shown that social factors that trigger craving and drug-seeking behaviour in animals and humans induce lasting behavioural and neurogenic changes. Moreover, recent behavioural sensitization studies of housing conditions demonstrated that social isolation alters the neuronal functioning of the dopaminergic and serotonergic systems, evoking changes in sympathetic neurotransmission [80–82]. Such environmentally induced changes may potentiate the post-sensitization conditioned locomotor response to cocaine that is said to be mediated by alterations in dopamine D2 receptor density, and can be further modified by ethanol treatment. Furthermore, animals subjected to overcrowded conditions appear to consume more ethanol than isolated animals that in turn drink more than animals housed under the standard conditions (four animals per cage). In line with these studies, McCormick et al. [37] have shown that daily social isolation for 1 h followed by pair housing with an unfamiliar partner induced anxiety and endocrine changes [83] that was associated with suppressed hippocampal cell proliferation and impaired adult object recognition in a spatial memory test [84]. One neural mechanism that may underlie the role of social context in increased risk for drug abuse is the imbalance between mineralocorticoid and glucocorticoid receptor levels in the limbic system and in the hypothalamic-pituitary-gonadal axis [57, 85–87]. Thus, development of laboratory animal models for drug addiction should allocate more consideration to the social component in order to generate data that ethically and practically represent a valid construct of the human condition and closely resemble the componential behaviours of an addicted subject in a free-living state.

Pharmacological treatment of addictive disorders

Current medications and novel therapeutic approaches

Attempts to manage drug addiction have included behavioural and psychosocial interventions [88, 89] as well as various medications [90–93] (see Table 2 for tested medications for cocaine and alcohol addiction). Biologics, such as monoclonal antibodies, vaccines, and engineered enzymes, are currently being proposed as alternatives to addiction pharmacotherapy [94–96]. For example, cocaine esterases (CocE-L169K/G173Q or CocE-T172R/G173Q) have been shown to robustly antagonize cocaine rewarding effects in rats [97–99]. Most biologics prevent central rewarding effects of drugs but fail to address the question of drug craving and relapse [100]. This may be attributed to their inability to rectify impaired components of the addiction neurocircuitry and/or neuroplasticity. A more recent therapeutic approach employed the use of small interfering RNA (siRNA) coupled with gold nanorods to silence DARRP-32 (dopamine and cyclic-AMP regulated protein phosphatase inhibitor) gene, in dopaminergic cells [101], generally known as one of the key regulators of histone phosporylation. Studies have demonstrated that genetic disruption of DARPP-32 has dramatic effects on behavioural responses to cocaine [15], whereas inhibition breaks the addiction cycle possibly by down-regulating extracellular signal-regulated kinase (ERK) and protein phosphatase-1 (PP-1)—factors that play key roles in the addiction signaling pathway [101]. siRNA complexes may, therefore, serve as pharmaceutical vehicles that could enable effective delivery of specific compounds, such as biologics or approved drugs, directly into brain sites relevant to addictive behaviour.

HDAC inhibitors (HDACi): a target therapeutic for drug addiction

Recent advances in the field of chromatin remodeling and epigenetic regulation improved our understanding of how genes interact and are regulated by the environment. Although pathological alterations in the brain transcriptome that underlie psychiatric and neurodegenerative disorders are incompletely understood, GWA studies have established genetic associations between specific genes or chromosomal regions with various brain diseases which have in common the hallmark of cognitive impairment [102]. Genetic-based studies have suggested critical roles for the epigenetic modifiers—HATs and HDACs in maintaining brain homeostatsis in various disease conditions. Targeting histone acetylation may provide benefit for the treatment of a broad range of human diseases, such as depression, schizophrenia, anxiety disorders, and drug addiction [103]. Of the various HATs, CREB-binding protein (CBP) has been implicated in drug addiction, but the evidence is contradictory [104]. CBP deletion in the NAc attenuated cocaine sensitivity and CPP [105], whereas striatal deletion increased sensitivity to cocaine and amphetamine [106]. In contrast, evidence to support a role for HDACS in drug addiction is convincing. Previous studies have shown that HDAC activity was increased in the PFC and NAc of rodents following cocaine self-administration. Now, much attention is given to HDACs, especially HDAC5 due to its antidepressant activity [103, 107] and unique response to chronic cocaine administration which critically implicates its involvement in behavioural

Table 2 Summary of pharmacotherapeutics for drug addiction

Drugs of abuse	Medications	Class	Action	Consequences/clinical implications	FDA approved? (±)	Indications/remarks	References
Alcohol	Naltrexone	Opioid receptor antagonist	Blocks *mu*-opioid receptors	Not effective in all patients	+	Less effective against stress-induced relapse	[126–130]
	Disulfiram	ALDH inhibitor	Inhibits ALDH and prevents acetaldehyde metabolism	Low compliance due to aversive reactions and other side effects	+	Effective against alcohol and cocaine dependence	[100, 127, 129–132]
	Acamprosate	NMDA glutamate receptor antagonist	Normalizes hyperglutamatergic states	Adverse reactions e.g., diarrhea, suicidal ideation	+	Reduces withdrawal symptoms and prevents relapse	[127, 130, 131, 133, 134]
	Topiramate, Ondansetron and Baclofen				–	Have promising therapeutic potential	[130, 133, 135, 136]
Cocaine	NAC	Thiol antioxidant	Regulates X(c) system activity and GSH biosynthesis	Reduces respiratory burst and may cause liver damage	–	Low bio-distribution	[137–139]
	Modafinil	GABA/glutaminergic agent	Upregulates brain glutamate system	Causes euphoria	–	Approved for other indications	[127]
	Topiramate	Antiepileptic drug	Mainly antagonizes AMPA receptors and kainate glutamate receptors	Causes anorexia, paresthesia, reversible cognitive impairment and taste aversion	–	Approved for other indications	[127, 135, 136]
	Baclofen, Propranolol, α-adrenergic agonists and Rimonabant				–	Have promising therapeutic potential	[127, 140–142]

Drug addiction, also called substance use disorder, is generally considered a brain disease. It is measured on a continuum scale that ranges from mild to severe. According to SAMHSA, diagnosis using DSM-5 criteria is based on evidence of social and control impairments, risky use, and pharmacological implications [143]. However, treatment approaches should involve biological, behavioural, and social context components [144] to produce effective results

X(c) system cystine–glutamate antiporter system, *NAC* N-acetylcysteine, *GSH* glutathione, *DA* dopamine, *ALDH* aldehyde dehydrogenase, *AMPA* alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, *NMDA* N-methyl-D-aspartate, *FDA* food and drug administration, ± yes/no, *DSM-5* diagnostic and statistical manual of mental disorders 5, *SAMHSA* substance abuse and mental health services administration

transitions from drug experimentation to compulsive drug use [15]. Moreover, HDAC5 has been identified to centrally integrate chromatin changes and gene alterations induced by drugs and stress stimuli [57]. In addition, the regulation of saliency circuit, which mediates behavioural responses to various environmental stimuli, critically implicates HDAC5. For example, increased response to rewarding effects of chronic cocaine exposure as well as chronic neuropathic pain and social defeat stress were observed in HDAC5 knockout mice [5, 57, 108]. Although the effects of HDAC5 on cocaine rewarding effects still remains unclear, it has previously been shown that its overexpresion blunts cocaine-induced place conditioning and locomotor-activating effects [57]. However, the behavioural effects of HDAC5 on cocaine-induced reward and locomotion requires further investigation, since it is uncertain whether its action is due to interaction with HDAC3 on the same catalytic deacetylase domain and blocking this site prevents its inhibitory action on cocaine reward [109].

Currently, there is considerable and growing interest in the use of HDAC inhibitors to activate the expression of mRNAs that are downregulated in various neurological disorders and psychiatric conditions [31] which include drug addiction [110]. HDAC inhibitors have been considered for many years as potent anticancer agents [111, 112]. HDAC inhibitors competitively inhibit HDACs from deacetylating lysines on the histone tails resulting in hyperacetylated and transcriptionally active chromatin states—giving rise to increased gene expression in the cell. Trichostatin A (TSA), valproic acid (VPA), sodium butyrate (NaBut), and suberoylanilide hydroxamic acid (SAHA) are among pharmaceutical compounds known to have HDAC inhibitor activity [113]. The usefulness of these compounds is exemplified by administration of SAHA restoring memory function in mice lacking appropriate CBP activity [102]. Similarly, it has also been reported that the HDAC inhibitor TSA improved long-term memory and synaptic plasticity in a mouse model of *Rubinstein-Taybi Syndrome* that is characterized by mental retardation due to mutations of CBP and p300 [114]. Another HDAC inhibitor NaBut has been used to strengthen memory associated with learning events [115]. Malvaez et al. have demonstrated that transgenic mice treated with NaBut extingished cocaine-induced CPP more quickly and in a more persistent manner than their vehicle-treated controls [116]. An exciting application of these findings that incorporate pharmacologic enhancement of extinction learning by modulating memory components of substance use disorders via HDAC inhibitor is of potential therapeutic interest. Besides, it was previously shown that HDAC inhibitors reversed long-term chromatin changes and persistent behavioural alterations at adulthood in maternally stressed rats [12]. This action coupled with other evidence of its neuroprotective and neuroregenerative properties in animal models [102] further support the speculations that HDAC inhibitors might be useful in the treatment of neuropsychiatric diseases.

Some controversial reports about the therapeutic action of HDAC inhibitors have also been recorded in the literature. It is, therefore, important to stress that HDAC inhibitors may sometimes exhibit opposing actions on drug-seeking behaviours subject to the manner in which they are administered. For instance, HDAC inhibitors increased drug intake in animals trained to self-administer cocaine but reduced intake when given before drug acquisition [117, 118]. The bioavailability and half-life of HDAC inhibitors may also account for some of their disparate actions *in vivo* [111]. Hence, relatively high or low concentrations may be required for desired action when HDAC inhibitors with short (e.g., valproic acid) or longer plasma half-lives (e.g., SAHA and TSA) are used.

Valproic acid which mimics the action of the HDAC inhibitor, TSA, has been used for the treatment of schizophrenia and bipolar disorder in humans for decades and other HDAC inhibitors are currently in different phases of human clinical trials for CNS disorders [103].

Moreover, it is beyond any doubt that altered histone acetylation is one of the main contributors to transition to an addicted state. However, research in the field of drug addiction needs to focus more on specific HDAC inhibitors that target extinction memory of drugs as this may later translate to effective medications for preventing drug relapse.

Conclusion/future directions

It has been shown that the majority of drug–induced behavioural alterations result not only from genetic but also epigenetic interactions giving rise to a breaking point situation that is beyond the individual's adjustment capacity to curtail further use [5]. Some authors have suggested that these changed behaviours are inheritable by succeeding generations through mechanisms that also implicate epigenetic modifications. Other proposed strategies include epigenetic mechanisms which may be at play in the germline and subsequently interfere with normal embryonic–epigenomic programming. In the addicted parents, this suggests an intriguing and potentially alarming possibility that exposure to drugs of abuse may produce transmissible epigenetic changes that result in profound alterations to the physiology and behaviour of the offspring, raising the interesting question as to whether non-exposed children of addicts are "programmed" to become addicts themselves. The few studies that have looked beyond the first generation suggest that many phenotypes persist. Regardless of the number of future generations, the impact of drug use

on the first generation offspring alone is sufficient to justify further research defining the extent of epigenetic heritability of phenotypes associated with parental drug abuse and the specific mechanisms underlying these effects. However, preventing, curtailing, or even rolling back the scourge of cocaine or alcohol-induced maladaptive disorders still remains a notable challenge today. It is, therefore, imperative that the search for better treatment outcomes continues. We, therefore, propose that combining psychosocial intervention with gene therapy involving pharmacological manipulations of HDACs, especially HDAC5, may further enhance current therapies and perhaps result in a more successful management of drug addiction.

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Compliance with ethical standards

Conflict of interest All authors have no conflict of interest to disclose.

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Article (b)

Differential epigenetic changes in the hippocampus and prefrontal cortex of female mice that had free access to cocaine

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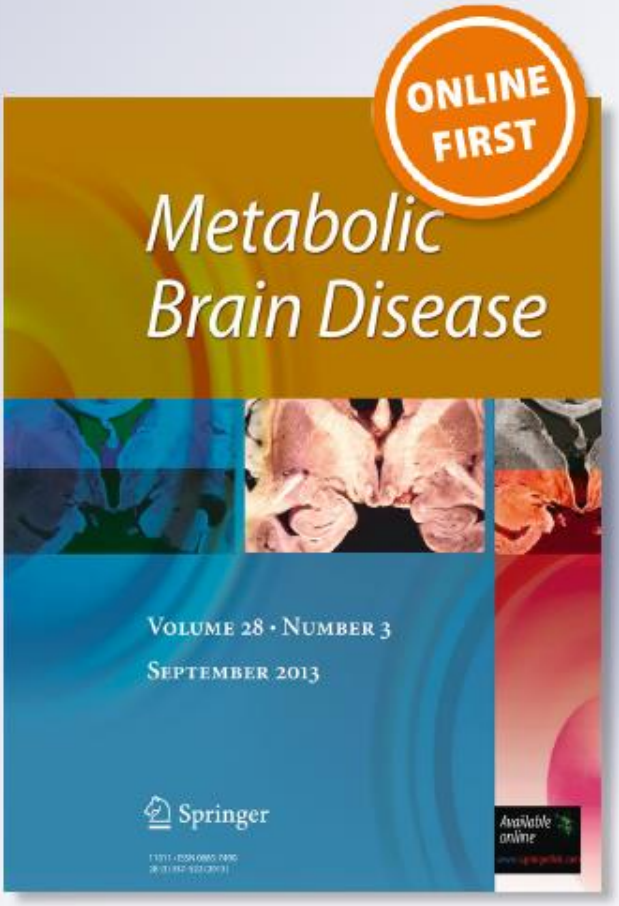

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ORIGINAL ARTICLE

Differential epigenetic changes in the hippocampus and prefrontal cortex of female mice that had free access to cocaine

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Abstract Alterations in gene expression within the neural networks of prefrontal cortex (PFC) and hippocampus (HPC) are known to contribute to behavioural phenotypes associated with drug intake. However, the functional consequences of regulated expression patterns of *Fosb* and *Crem* (cAMP response element modulator) in both brain regions in response to volitional intake of cocaine in social environment is yet to be explored. Here, we first exposed young adult mice to cocaine (300 mg/L) and water concurrently for 30 days in the IntelliCage to investigate consumption preference, and subsequently for 28 days during which persistent motivated drug seeking behaviours were examined. Thereafter, locomotor activity and memory performance of the mice were assessed. DNA methylation status in the promoters of *Fosb* and *Crem* genes were also evaluated. We show that mice that had extended access to cocaine exhibited motivational deficit and demonstrated decreased locomotor activity and intact recognition memory. These changes were accompanied by hypomethylation or hypermethylation in the promoters of *Fosb* and *Crem* genes in the PFC and HPC of the cocaine-experienced mice, respectively. Together, these findings correlate the molecular changes to behavioural effects of the treatment and further suggests a possible activation of prefrontal cortical networks by social interaction episodes in the IntelliCage which possibly enhanced behavioural control that dampens mice sensitivity to cocaine rewards. Furthermore, our data delineate the molecular response of *Crem* and *Fosb* to oral cocaine in group-housed mice and demonstrates differential regulation of activities within the substrate brain regions studied.

Keywords Cocaine · IntelliCage · Social environment · DNA methylation · *Fosb* · *Crem*

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Introduction

Neuroadaptive changes and alterations in gene expression within brain structures of the mesocorticolimbic circuit, especially the PFC (that mediates executive functions) and the HPC (that is involved in context-specific memories associated with reinstatement of drug seeking), represent attractive candidates for studying the molecular underpinnings of drug abuse. Interestingly many molecular changes may not necessarily be directly related to the neurochemical effects of drugs, but may stem from other factors that may also influence behavioural phenotypes and/or physiological responses to drug intake (Parkitna and Engblom 2012). For instance some authors have attributed phenomena such as decreased sensitivity to drugs to alterations in the environment rather than as part of the neurobiological sequelae of chronic drug intake, suggesting that context of drug consumption may itself have profound biological and behavioural consequences (Piazza and Le Moal 1998; Morgan et al. 2002).

Drugs of abuse commonly lead to heightened dopamine neurotransmission. Binding of dopamine to postsynaptic D1 receptors result in an increase in intracellular cAMP levels, that in turn leads to the activation of protein kinase A (Self 2004). Protein kinase A (PKA RIIβ) phosphorylates

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glutamatergic receptors (NMDA and AMPA) causing an influx of calcium into the cell. The increased calcium binds to calmodulin that subsequently leads to the activation of the enzyme CaMKinase II/IV, that in turn phosphorylates *Creb*. Phosphorylated *Creb*, as a transcription factor, promotes the transcription of many genes, including the immediate early gene, *Fos* (Levine et al. 2005; Kida and Serita 2014). *Creb* and *Fos* are therefore key factors known to play significant roles in regulating the expression of genes related to drug reward and vulnerability (McClung and Nestler 2003; Miller et al. 2017). However experiments involving the use of viral vectors or bi-transgenic mice have shown that either acute or chronic exposure to cocaine increased *Creb* activity in the nucleus accumbens and dorsal striatum, and this observation was associated with a decrease in the rewarding effect of cocaine (Carlezon et al. 1998; Robison and Nestler 2011). In contrast, Larson et al. (Larson et al. 2011) reported increased cocaine reinforcement in self-administering Sprague-Dawley rats following *Creb* overexpression in the nucleus accumbens shell. These reports clearly indicate that the exact nature of the role of *Creb* in cocaine-mediated effects still appears to be uncertain.

Crem is a related family member that is similar to *Creb* due to its specific binding to cAMP-responsive element (CRE) sequences and may either act as an inducible cAMP repressor (Robison and Nestler 2011) or activator (Sassone-Corsi 1995). Phosphorylation of multiple serine residues of *Crem* has been shown to elicit its activator functions by enhancing DNA binding (De Groot et al. 1993). In contrast, several *Crem* transcripts were reportedly increased following hippocampal *Creb* deletion in mice (Hummler et al. 1994). Besides, *Crem* is also considered a nuclear effector where signalling pathways may converge and/or cross-talk (Masquilier and Sassone-Corsi 1992; De Groot et al. 1993).

A recent study has implicated a role for *Crem* in drug-induced behavioural effects. Miller et al. (2017) demonstrated that impulsive rats that were neurochemically and behaviourally sensitive to heroin exhibited decreased striatal *Crem*, but when *Crem* was virally expressed in this brain region, heroin-associated impulsivity was decreased (Miller et al. 2017). This finding suggested a possible modulatory role for *Crem* in the precipitation of heroin-induced behavioural effects.

Despite the extensive literature on the function of *Fos* and *Creb* in mediating behavioural effects and physiological responses to drugs, the role of *Fosb* and *Crem* in relation to cocaine consumption within a social environment has not yet been studied. Here, we investigated the DNA methylation status of the promoter regions of *Fosb* and *Crem* genes in the PFC and HPC of mice that were group housed and had free access to cocaine to simulate voluntary drug consumption (as opposed to experimenter-forced drug intake) within a social context. We also assessed the animals' recognition memory and locomotor activity as indicators of overall functioning of the PFC and HPC.

Methods

Animals and treatment protocol

All experiments were performed in accordance with the National Institute of Health guidelines on the ethical use of animals, using approved protocols by the Animals Ethics Subcommittee of the University of KwaZulu-Natal (UKZN) [*Ethical clearance, AREC/071/015*]. A total of forty C57BL/6 female mice (9–10 weeks old) were used for this study. They were obtained from the Biomedical Resource Unit (UKZN, Durban) and maintained in temperature (23 ± 2 °C) and humidity (50 ± 5%) controlled rooms with 12 h light – dark cycle (light from 0600 to 1800 h). Of the total sample size ($n = 40$), thirty-two mice, (2 times $n = 16$) were subsequently housed in an IntelliCage apparatus (NewBehavior, TSE Systems, Germany). A major advantage of this system is that animals can be studied in a "social home cage" environment (Rudenko et al. 2009), and there was no need for any handling by the experimenter except for normal housekeeping of the apparatus. The animals were kept in the Intellicage for 71 days. During this period, the animals were initially allowed to familiarize themselves with the IntelliCage environment for 6 days after which they had free access to drug (300 mg/L cocaine solution) and water concurrently for the next 30 days. This was followed by a 15-day interval during which animals had to execute tasks (which consisted of increasing number of nose-pokes: NP1, NP3 and NP5) to gain access to the drug solution. Subsequent to this phase, a 7-day period of drug withdrawal followed. Cocaine was then re-introduced to the animals for the next 4 days after which they were subjected to second period of task-mediated access to drugs (9 days). The tasks and withdrawal period were used to stimulate and/or reveal persistent, motivated drug-seeking behaviors. Cocaine consumption of the animals were subsequently assessed under two sets of circumstances, i.e. (1) after having prolonged access to the drug (first 30 days), and (2) after a period of drug withdrawal followed by a brief period of drug access.

The 32 animals that housed in the Intellicage, were implanted with transponders to facilitate the automated continuous recording of the behavior of each individual mouse. A separate group of eight mice ($n = 8$) was only exposed to the apparatus without access to drugs and thus served as controls.

Drugs and chemicals

Cocaine powder was generously provided by South African Police Service (SAPS). Cocaine purity was confirmed by nuclear magnetic resonance (NMR) at the Catalysis and

Peptide Unit, Discipline of Pharmaceutical Sciences, University of KwaZulu-Natal. The NMR spectra (Fig. 1a, b) were recorded on a Bruker Avance 400Mhz instrument in deuterated methanol as the solvent. The proton and carbon spectra confirmed the molecular formula and splitting patterns characteristic of the molecular structure of cocaine in accordance with the literature (Allen et al. 1981). Cocaine was dissolved in distilled water and presented to mice in drinking bottles.

Assessment of cocaine consumption

Cocaine and water consumption were monitored everyday throughout the test period. To determine the amount of drug ingested by the mice, lick data were extracted from the archives and analysed using FlowR software (XBehavior GmbH, Bänk, Switzerland). In each archive, lick contact times at each bottle were summed for each subject and divided by the duration of the session to give lick contact times per day

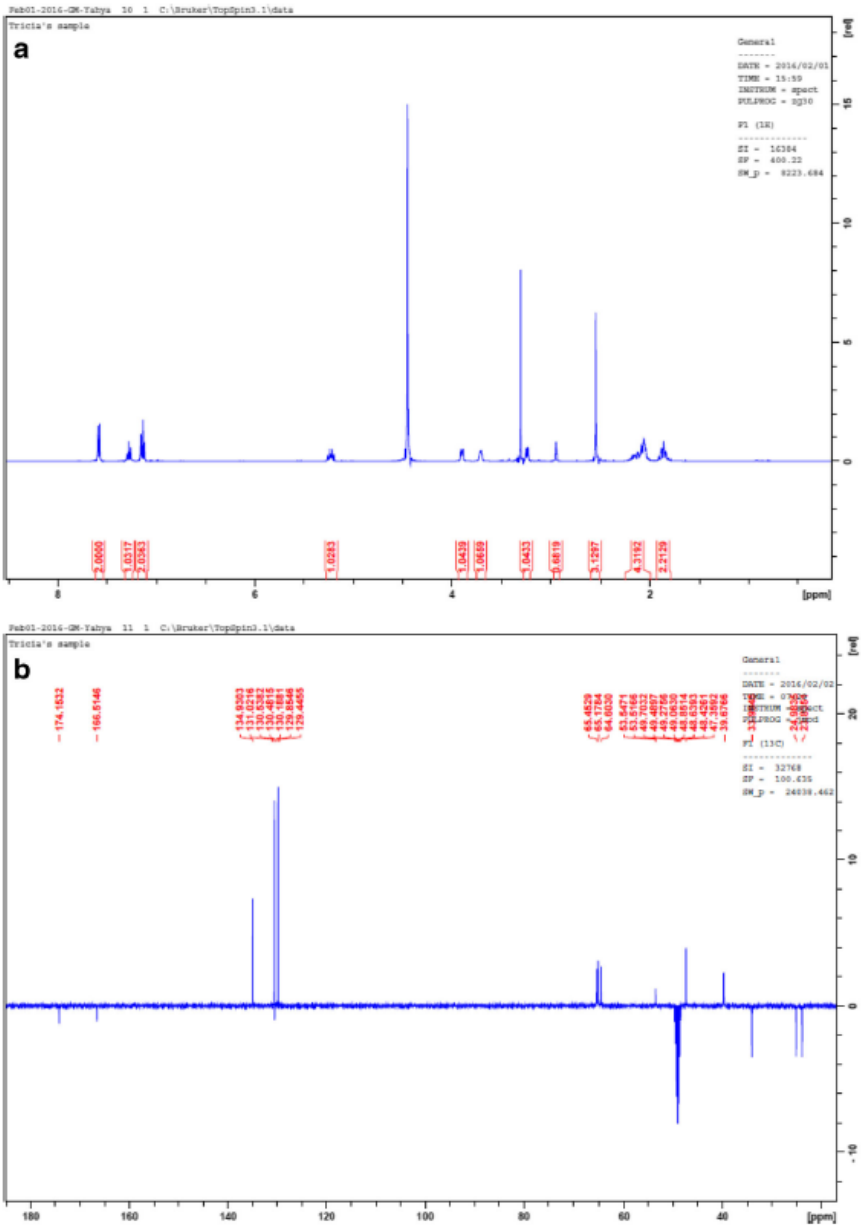

Fig. 1 NMR spectra showing the purity analysis of cocaine powder which indicates (**a**) the proton signals and (**b**) the carbon signals compared to standard chemical structure. It was recorded on Bruker Avance 400 Mhz instrument using deuterated methanol as the solvent. All signals were accounted for, hence need no further purification

(in seconds). The mean bottle lick contact time was then taken as a measure of drug seeking behaviour. The average consumption of drug solutions was calculated by multiplying the bottle licks with number of available bottles. Drug consumption was calculated at two intervals, i.e. after the first 30 days of continuous drug access, and then again after 4 days drug access that was preceded by a drug withdrawal phase.

Behavioural tests

After drug exposure in the IntelliCage, overall locomotor abilities of the animals were assessed in the open field (OF), while memory competence was tested using the novel object recognition (NOR) test.

OF test

In this test, locomotor activity was assessed in a black plexiglass chamber (50 × 50 × 25 cm) which has its floor divided into 16 identical squares, illuminated with a bright lamp suspended 2 m above the field arena. Each mouse was placed at the centre of the chamber and allowed to explore for 5 min. Mouse behaviour was recorded by a video camera that was positioned above the OF apparatus for scoring at a later stage. Total number of peripheral and central squares crossed was used as a measure of locomotor activity.

NOR test

Recognition memory performance of individual mice was assessed using the NOR task. This testing paradigm enables an animal to discriminate between novel and familiar objects (Abboussi et al. 2016), a process that is dependent on prefrontal cortex (Ennaceur et al. 1997) and hippocampal functioning (Broadbent et al. 2010). The task procedure which consists of familiarization and retention/test sessions, was conducted over two days. During the familiarization phase, each mouse was placed at the center of a transparent rectangular cage (16 cm × 24 cm × 12 cm) that had objects A and B. The animals were allowed to explore the objects for 5 min after which they were returned to their home cage. 24 h later (test phase), object B was replaced with object C (the novel object), whereas object A (familiar object) was retained. Mice returned to the test arena to perform the recognition task. The amount of time spent exploring the novel (T_n) and familiar (T_f) objects were recorded in 5 min. We constantly changed beddings and thoroughly cleaned objects with 70% ethanol in-between trials to eliminate precipitates of olfactory stimuli. Object exploration was scored when the animal made a directional approach towards the object such as touching, sniffing or directing its nose <1 cm away from the objects, while climbing or sitting on the objects was ignored.

Tissue isolation, DNA extraction and bisulfite conversion

Mice were decapitated using a guillotine. Brains were removed from the skull and the PFC and HPC tissues were dissected on an ice-cold surface, immediately frozen in liquid nitrogen and stored at −80 °C until DNA extracts were prepared. We isolated DNA from the mouse brain tissues using ZR Genomic DNA™-Tissue MiniPrep kit, D3050 (Inqaba Biotec, South Africa). The purity and yield of the extracted DNA was determined by spectrophotometry using Nanodrop 1000 (Thermo Fisher Scientific Inc., USA). DNA extracts were later processed for bisulfite conversion using the EZ DNA methylation™ kit, D5002 (Zymo Research, USA) according to the manufacturer's protocols. After denaturing the input DNA, 100μL of light-sensitive CT conversion reagent was added. The resultant mixtures were incubated twice; first at 50 °C for 16 h in the dark and later on ice (4 °C) for 10 min. After incubation, we transferred the samples to zymo-spin IC columns containing 400μL M-binding buffer and subsequently mixed. After centrifugation at 12000 rpm, the samples were washed with 100μL M-wash and desulphonated with 200μL M-desulphonation buffer after which they were incubated at room temperature for 20 min. After two more wash steps, the converted DNA was eluted with 10μL of M-elution buffer added to the column placed on a micro-centrifuge tube.

Evaluation of DNA methylation status by real-time quantitative polymerase chain reaction (qPCR)

DNA methylation status of *Fosb* and *Crem* genes were determined by qPCR. PCR amplification was performed on a LightCycler 2.0 (Roche Diagnostics (Pty) Ltd., South Africa) using 20μL total reaction volume which consisted of 5μL DNA template (bisulfite modified), 10μL SYBR Green I (Roche Diagnostics), 1μL DNA water and 2μL each of forward and reverse primers at 15μM concentration. Methylated DNA samples were amplified using the following set of primers:

Fosb: TATAGAAGCGCTGTGAATGGA (forward),
GACCATCTCCGAAATCCTACA (reverse);
Crem: CAGAGGAAGAAGGGACACCA (forward),
TTGTATTGCCCCGTGCTAGT (reverse);
Gapdh: GCCAAAAGGGTCATCATCTCCGC (forward),
GGATGACCTGCCCACAGCCTTG (reverse).

PCR cycling conditions were as follows: initial 10 min at 95 °C, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30s and 72 °C for 30s. The samples were placed in a 96-well PCR plate in which each sample was assayed in duplicate and each run included water blanks (as negative controls). For each target gene, serial dilutions (seven-fold) of methylated DNA control samples were prepared and used to construct linear standard curves. Data were analysed by comparing C(q)

values of the treated samples to control and both normalized to *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase) using the $2^{-\Delta\Delta Cq}$ comparative method (Schmittgen and Livak 2008). A value of 1 was assigned to the control (Anier et al. 2010) and fold changes were calculated.

Data analysis

GraphPad prism 5 software was used for the statistical analysis. All datasets were subjected to the Shapiro-Wilk normality test to determine whether data displayed a Gaussian distribution or not. Lick contact and drug consumption data, Real-time qPCR data and all behavioral experiments were analyzed using Student's *t test*, except analysis of the novel object recognition data that was done using Two-Way ANOVA. All data are reported as mean ± SEM, and criterion for significance was set at $p < 0.05$.

Results

Cocaine acquisition and total consumption before and after drug withdrawal

During the first 30 days when the mice had free access to the drinking bottles containing either cocaine or water, the animals spent significantly less time licking (Fig. 2a) and consuming (Fig. 2b) cocaine than water ($p < 0.0001$). However, similar pattern of cocaine intake was observed when drug consumption was assessed after the second brief period of drug access (Fig. 3). Cocaine licks and consumption levels were comparable to that during the first 30 days, and remained significantly less than water intake (Fig. 3a, b; Student's *t-test*, $p < 0.0001$).

Figure 4a, b indicate the learning outcomes of ANP tasks imposed on the M_0 mice for 15 days before drug withdrawal and for 9 days after reintroducing cocaine post-drug withdrawal. The advantage of the ANP design was to see how well the mice learn to alternate visiting cocaine corners in order to maximize cocaine intake. The learning graphs show that most of the animals repeatedly made wrong visits to cocaine corners especially at NP1 and NP3 prior and after drug withdrawal periods. However, some of the animals still visited correctly by crossing the upper red line but made fewer visits especially during NP5 of both test periods. Even those with probable sequential learning ability later fell back within few days of the test (Fig. 4a, b). In overall, the graphs show that none of the animals learned the ANP tasks given due to failure to correctly execute the activity.

Cocaine consumption is associated with DNA hypomethylation in the prefrontal cortex and DNA hypermethylation in the hippocampus

After acquisition and/or exposure to cocaine in the IntelliCage apparatus, we assessed DNA methylation changes in the promoter regions of *Fosb* and *Crem* genes in the brain. Using bisulfite modification and qPCR analysis, our data demonstrated that mice that consumed cocaine had significant decreases in methylation at *Fosb* ($p < 0.05$) and *Crem* ($p < 0.0001$) gene promoters in their PFC compared to controls ($p < 0.001$; Fig. 5a, b). Consequently, the altered patterns of gene expression indicate transcriptional activation of associated genes in animals that had access to cocaine.

Examination of DNA methylation status at *Fosb* and *Crem* promoter regions in the HPC showed that DNA methylation was increased at both *Fosb* and *Crem* promoters in the HPC of mice that consumed cocaine when compared to controls ($p < 0.05$; Fig. 5c, d).

Object recognition memory was intact while locomotor activity was decreased in mice that consumed cocaine

We conducted the NOR test in order to evaluate the cognitive functioning of all the mice. A Two-Way ANOVA indicated significant main effects of the treatments ($F_{(1,40)} = 4.307$, $p = 0.04$) and total exploration time ($F_{(1,40)} = 20.54$, $p < 0.0001$). Drug

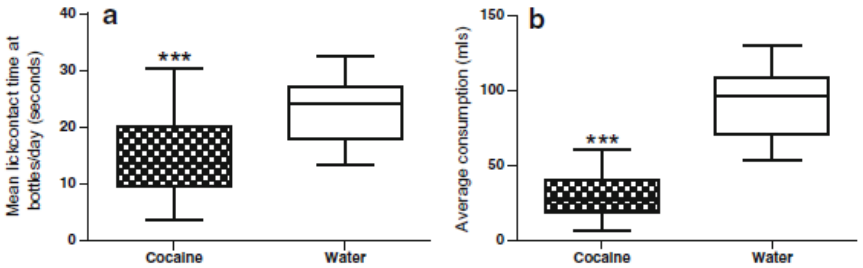

Fig. 2 Analysis of lick behavior of M_0 mice and compound consumption in the IntelliCage system during the first 30 days of exposure (**a**) test shows that subjects spent less time drinking from cocaine bottles compared to water (**b**) indicates that the total amount of cocaine consumed were less than water. Data shown represents Mean ± SEM; $n = 32$. ***$p < 0.0001$ compared to water; Two-tailed unpaired Student's *t-test*

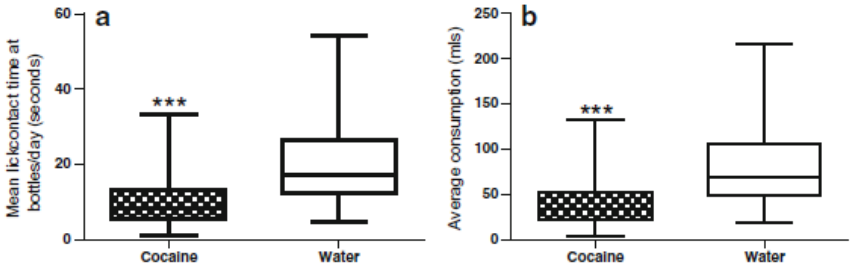

Fig. 3 Bottle lick behaviour of M_0 mice and cocaine vs water consumption in the IntelliCage system during 4 days of cocaine reintroduction after a brief drug withdrawal period (a) demonstrates that lick contact time at cocaine bottles was decreased compared to water and (b) indicates that the total amount of cocaine consumed was significantly less than water. Data shown represents Mean ± SEM; n = 32. ***$p < 0.0001$ compared to water; Two-tailed unpaired Student's *t-test*

exposure x exploratory time accounts for approximately 0.99% of the variance, hence indicated no significant interaction ($F_{(1,40)} = 0.6433$, $p = 0.4272$) between factors. Both groups of mice (cocaine exposed and controls) spent significantly more time exploring the novel object (Tn) compared to the familiar object (Tf) ($p < 0.001$; Fig. 6a).

The locomotor activity of all mice was assessed in the open field arena. The total number of central and peripheral squares

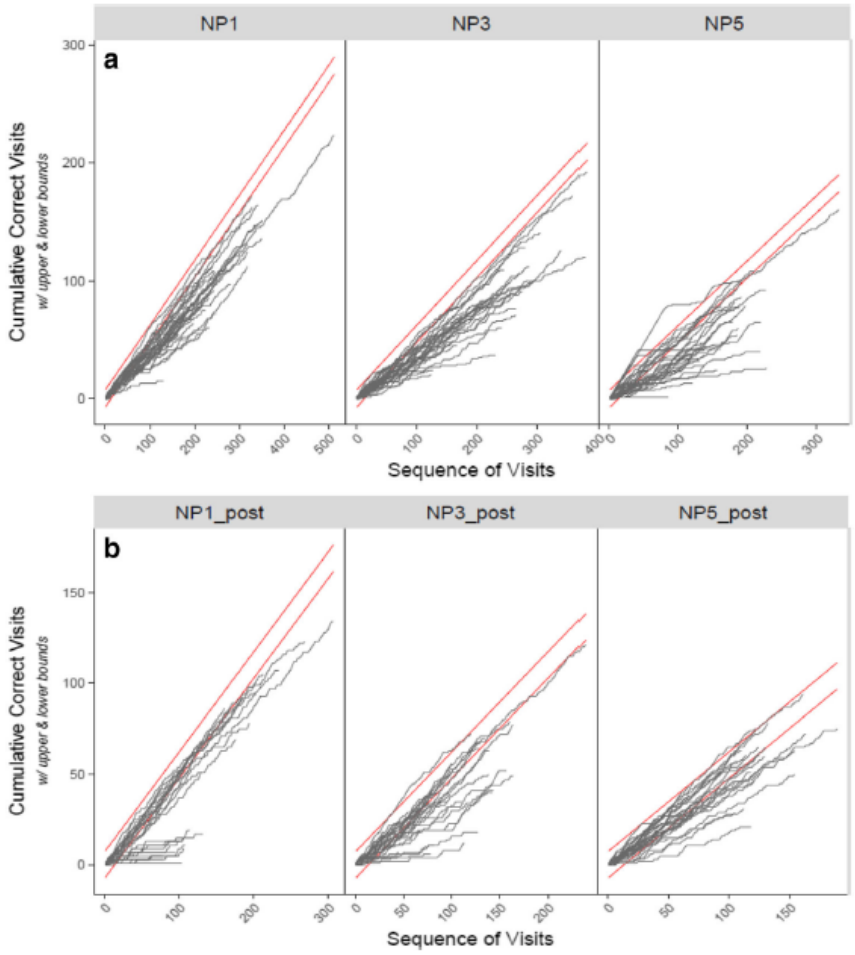

Fig. 4 Sequential probability ratio learning plot indicating cumulative correct visits to cocaine corners during (a) 15 days of alternate nosepoke (ANP) learning tasks imposed after initial motivation period (b) 9 days of ANP learning tasks given after post withdrawal cocaine reintroduction. (If the grey lines cross the lower or upper red lines, it indicates that learning is significant at 50% or 60%, respectively; criterion = 0.6, expectation = 0.5). All data were extracted and analysed using FlowR software; n = 32, 15 days ANP; $n = 28$, 9 days ANP

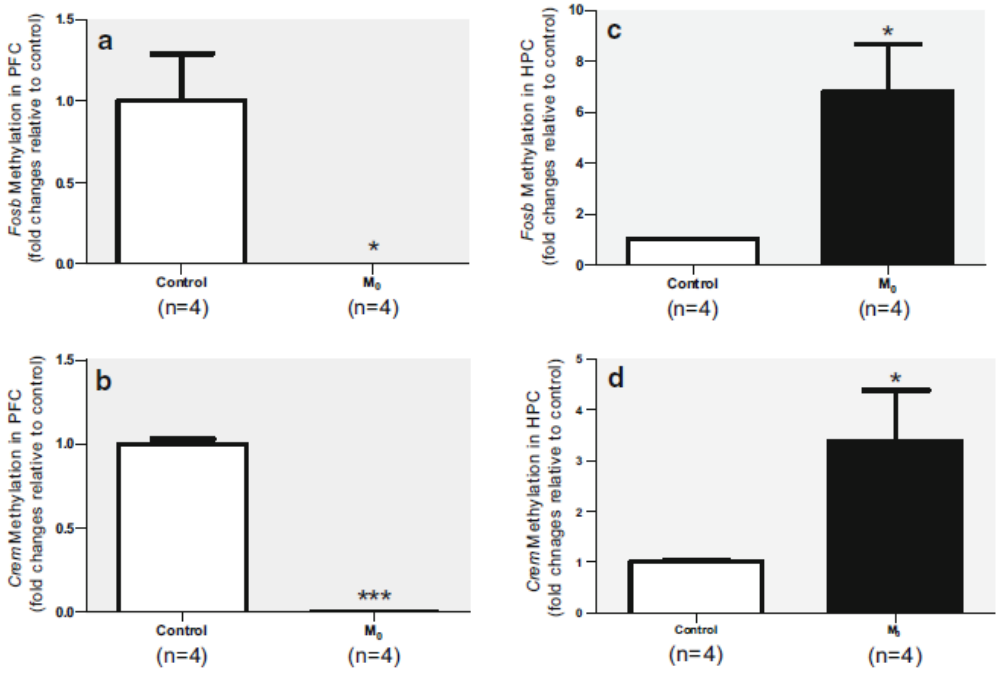

Fig. 5 Regulated gene expression changes after prolonged exposure to cocaine in the IntelliCage and altered patterns of DNA methylation at *Fosb* and *Crem* promoter regions in the brain (**a-b**) cocaine treatment induced DNA hypomethylation in the promoters of *Fosb* and *Crem* in the PFC of M_0 mice compared to their controls (**c-d**) In the HPC, treatment was associated with significant hypermethylation at both promoters indicating transcriptional silencing of *Fosb* and *Crem* genes. Data shown represents Mean ± SEM; $*p < 0.05$, $***p < 0.0001$. Two-tailed unpaired Student's t-test

crossed was used as a measure of locomotion (Abboussi et al. 2016). The data showed that the total squares crossed by the mice that had access to cocaine was significantly decreased compared to controls ($p < 0.05$; Fig. 6b).

Discussion

In the first part of this study, we demonstrated preference consumption of compounds in the social home-cage and examined motivation for cocaine rewards by scheduled learning tasks. We show that M_0 mice licked and drank more water than cocaine in all test sessions (Figs. 2 and 3). This may be expected as most human drug addicts are also known to consume water while on drugs. Consistent with previous reports from animal research, it has been suggested that rats presented with alcohol or cocaine among other drugs of abuse and water, on choice trials drank more water than drugs with consequent behavioural and characteristic patterns of drinking responses without evidence of dependence (Stolerman et al. 1971). It is

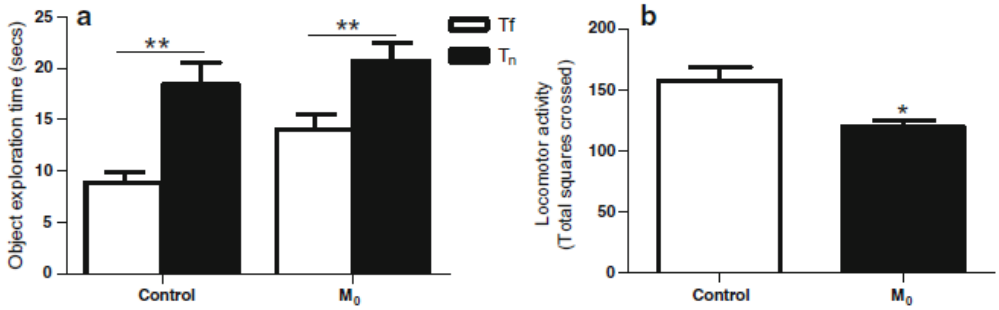

Fig. 6 Object recognition memory and locomotor response following prolonged exposure to cocaine (**a**) time spent exploring familiar (Tf) and novel (Tn) objects in the novel apparatus (**b**) locomotor activity of M_0 mice tested after cocaine-experience in the IntelliCage. Data shown represents Mean ± SEM$**p < 0.01$, $*p < 0.05$, Two-Way ANOVA or Two-tailed unpaired Student's *t-test*

therefore possible that higher preference for water in this study may suggest an increasing cellular demand for maintaining fluid balance which may further account for the lower consumption of cocaine. Holgate and colleagues (2017) recently examined the effect of social and environmental enrichment on ethanol and sucrose consumption in C57BL/6 mice. These authors concluded that enrichment in the IntelliCage may have the potential to decrease ethanol preference while favouring sucrose consumption (Holgate et al. 2017). This suggested that the decreased drug consumption observed in our mice may similarly result from the impact of the enriched environment offered by the IntelliCage system such that the consumption of a natural reward (water) is preferred above the pharmacological effects of cocaine when concurrently presented. We further showed that the cocaine-experienced mice performed poorly in all tasks given as they exhibited increased activity at cocaine corners to get at sufficient drug but failed because they did not follow the design alternation pattern. Similarly, a previous study that employed place learning paradigm in the IntelliCage reported that C57BL/6 J mice initially failed to learn to discriminate rewarded from non-rewarded corners until negative air-puff punishment was applied. The authors further showed that young mice later learned the task significantly better than the middle-aged mice (Mechan et al. 2009). In our study, time-constraint reinforcement was applied in drug corners during the nose-poke learning tasks. Only correct visits and nose-pokes were rewarded (access to cocaine) by active opening of the motorized doors which lasted 5 s. Therefore, some possible reasons for the observed learning deficit in our model could be related to age factor and that the negative reinforcement used may also be considered not strong enough, as opposed to the conventional air-puff, to influence motivational learning in the animals. Converging theories of reward dependent learning have previously suggested that learning is also dependent on how predictable a reward is relative to associated cues (Schultz et al. 1997). Learning deficits in this study may further be attributed to inaccurate nose-poke sequence and time prediction in the animals.

Altered gene expression in specific reward areas of the brain are thought to provide great insight into the neurobiology of drug abuse (Renthal and Nestler 2008). After behavioural phenotyping, we then set out to identify changes in the DNA methylation status of the promoter regions of *Fosb*/*Crem* in the PFC and HPC. We found that *Fosb* and *Crem* were hypomethylated in the PFC and hypermethylated in the HPC. Electrical stimulation studies have revealed induction of *Fosb* mRNA in the HPC in response to signalling agents in the brain (Nakabeppu and Nathans 1991). Anier and co-workers reported DNA hypomethylation at *Fosb* promoter in the NAc following acute and repeated cocaine administration and associated these changes with transcriptional induction of *Fosb* in this brain region (Anier et al. 2010). In our study cocaine–experienced mice had increases in methylation at *Fosb* gene promoters in the HPC suggesting transcriptional silencing of the gene in this region. It may therefore be possible that reduced *Fosb* expression may reflect decreased sensitization towards the reinforcing or motivational properties of cocaine when concurrently presented with water in the IntelliCage system. Moreover, transcriptional induction of *Fosb* in the PFC of cocaine exposed mice may on the other hand provide evidence for enhanced behavioural control that explains desensitization towards cocaine.

Our findings also showed that mice that consumed cocaine in the IntelliCage displayed decreased locomotor activity. While unexpected, this results was nevertheless in line with a previous study by Hiroi et al. (Hiroi et al. 1997) who showed that *Fosb* mutant mice lacking 35-to-37 KDa *Fos* proteins in their striatum had exaggerated locomotor activation in response to initial cocaine exposures compared to their wild type littermates. The data therefore points to some role of *Fosb* as one of the key regulators of gene expression changes recruited in the functional activation of neural networks that mediate locomotor response to long-term cocaine effects. Further support stems from another study reporting inhibition of sensitization of post-amphetamine-stereotypy locomotion in prefrontal cortical lesioned rats (Wolf et al. 1995), while a more recent study demonstrated the expression of *Crem* in the core of NAc, a brain region that may be involved in the regulation of impulsive actions related to reward behaviour (Miller et al. 2017). It is therefore possible that decreased locomotor activity following cocaine exposure, as observed in our study, might also be associated with *Crem* perturbations in both brain areas studied. In addition, the impact of social interaction episodes previously experienced by the animals may further account for their post-treatment diminished locomotor activity.

Like *Creb*, *Crem* is also known to play significant physiological roles in memory and long-term potentiation (Silva et al. 1998; De Cesare et al. 1999). There is compelling evidence that DNA methylation regulate gene transcription necessary for memory function (Mikaelsson and Miller 2011). Our investigation indicated that cocaine-induced *Crem* hypermethylation in the HPC of mice, suggesting transcriptional silencing of memory-linked genes regulated by *Crem* in the HPC. This further provided evidence for a lack of drug-induced neural plasticity despite prolonged exposure to cocaine in the IntelliCage. This is in line with a previous finding that established that long-term potentiation is unaltered in *Crem* mutants (Maldonado et al. 1999). However, the observed molecular changes seem to correlate with the overall performance of the animals in the NOR tasks which demonstrated that cocaine consumption did not alter recognition memory but rather promoted novelty-seeking behaviour. Furthermore, in a study that used conditioned taste aversion as learning and memory paradigm, *Fosb* and *Crem* were among the proteins whose increased expression were detected

in several brain regions after lithium chloride administration (Lamprecht and Dudai 1995). In our study, it appears that there was a form of memory shift from HPC to PFC in the cocaine-experienced mice as evidenced by transcriptional activation of *Crem* and *Fosb* within the prefrontal cortical network. This is in agreement with the concept of cortical integration previously posited (Mikaelsson and Miller 2011) and further corroborate the argument that HPC has a time-related role in memory consolidation, hence long term memories require activities of neocortical regions which may include the prefrontal cortex (Squire et al. 2001).

It is widely reported that persistently enhanced sensitization and behavioural response are associated with most drugs of abuse including cocaine (Henry and White 1995). Since the mice used for this study had extended voluntary oral access to cocaine, we expected that the rewarding effects of cocaine would evoke robust euphoria and precipitate addictive behaviour as previously reported (Horger et al. 1990; Shippenberg and Heidbreder 1995). On the contrary, our observations proved otherwise as the mice housed in the IntelliCage only consumed cocaine at modest levels despite having free access for extended periods. We think that this low consumption rate may be due to the impact of social interaction amongst cage mates in the group housing system of the IntelliCage apparatus. In a study that examined how environmental conditions and social factors impact on cocaine reward in early adolescence, the investigators showed that cocaine treatment established reliable conditioned place preference (CPP) in socially isolated rats housed alone whereas the presence of other cage mates with toys abolished CPP. They further added that enrichment plus group housing increased dopamine transporter protein levels in the NAc (Zakharova et al. 2009). Also, Fritz and co-authors (Fritz et al. 2011) previously investigated the effect of social interaction offered as an alternative stimulus to cocaine conditioning in Sprague-Dawley rats, and found that CPP induced by intraperitoneal cocaine (15 mg/kg) injection and molecular induction of Zif268 in the NAc shell, VTA, central and basolateral amygdala, were completely reversed by the social interaction episodes (Fritz et al. 2011). Since our previous observations about cocaine reward in the IntelliCage were comparable to the findings of others (Zakharova et al. 2009; Fritz et al. 2011), it is evident that C57BL/6 mice used in our study only experienced cocaine in social settings but were not addicted to it.

We postulate that the experiences of drug intake and that of the social environment seems to consolidate into new memories in the PFC that regulate cocaine reward to social engagement in the animals. A recent study that employed pharmacological inactivation techniques, highlighted the importance of functional integrity of PFC and striatal circuits for expression of social play behaviour, a form of social interaction in animals (Van Kerkhof et al. 2013). Many factors such as cognitive control, choice making, reward related behaviours and behavioural inhibition were all associated with social play interaction which involved cortico-striatal projections from PFC to the striatum (Bell et al. 2009; Van Kerkhof et al. 2013). Van Kerkhof et al. (van Kerkhof et al. 2014) further established that the activation of the cortico-striatal pathway after social play behaviour correlated with *c-fos* positive cell density in the prefrontal regions. Resting on these knowledge, transcriptional activation of *Crem* and *Fosb* in the PFC of cocaine–experienced mice further support the involvement of both transcription factors in the inhibition of drug related behaviours by activating social networks in the PFC which dampens the sensitivity of the animals to cocaine rewards and thus enhanced behavioural control. This further accounts for insignificant motivational deficits observed in our mice.

Taken together, this study established the significant role of DNA methylation in identifying epigenetic changes that are associated with prolonged intake of cocaine in the IntelliCage. Our findings show that *Fosb* and *Crem* transcription factors function in similar directions in both HPC and PFC when mediating molecular responses to cocaine-rewarding effects intercepted by social behaviours, whereas these specific brain regions exhibited differential responses to the converging environmental stimuli (drug and social interaction) resulting in a memory shift that favours behavioural control, further manifested by decreased locomotor response in the open field.

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Compliance with ethical standards

Disclosure All authors declare that they have no competing financial or any other conflict of interests to disclose.

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APPENDIX XI

PRESENTATIONS AT LOCAL AND NATIONAL MEETINGS

1. **Ajonijebu D.C**., Abboussi O., Mabandla M.V., Daniels W.M.U. *Early postnatal fostering altered memory performance and DNA methylation in the prefrontal cortex of offspring mice with lineal phenotypes related to parental cocaine exposure*. College of Health Sciences Annual Research Symposium, K-Rith, Nelson Mandela Medical Campus, University of KwaZulu-Natal, South Africa. October 2017.
2. Daniels W.M.U., **Ajonijebu D.C**. *Epigenetic mechanisms in drug addiction*. 45th conference of the Physiological Society of Southern Africa (PSSA), University of Pretoria, South Africa. August 2017.
3. **Ajonijebu D.C**., Abboussi O., Mabandla M.V., Daniels W.M.U. *Characterizing drug intake in socially engaged mice* (Postal Session). Southern Africa Neuroscience Symposium (SANS), University of Cape Town, South Africa. December 2016.
4. **Ajonijebu D.C**., Abboussi O., Mabandla M.V., Daniels W.M.U. *Characterizing drug intake in socially engaged mice*. College of Health Sciences Annual Research Symposium, K-Rith, Nelson Mandela Medical Campus, University of KwaZulu-Natal, South Africa. September 2016.
5. **Ajonijebu D.C**., Abboussi O., Mabandla M.V., Daniels W.M.U. *Interrupting alcohol and drug consumption – good or bad news*? School of Laboratory Medicine and Medical Science (SLMMS) Research Day, GSB, Westville Campus, University of KwaZulu-Natal, South Africa. August 2016.
6. **Ajonijebu D.C**., Abboussi O., Mabandla M.V., Daniels W.M.U. *Characterizing drug intake in group-housed mice*. 2nd African College of Neuropsychopharmacology (AfCNP) Congress, Stellenbosch, Western Cape, South Africa. July 2016.
7. **Ajonijebu D.C**., Abboussi O., Mabandla M.V., Daniels W.M.U. *Establishing and characterising an animal model of addiction within a social context* (Postal Session). 3rd Africa and Middle East Congress on Addiction (AMECA) conference, Gaborone, Botswana. October 2015.