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DNA methylation:

An epigenetic watermark of former cocaine self-administration

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Abstract

Converging lines of evidence suggest that cocaine commandeers traditional reward-related learning and memory pathways to instill pathologically persistent memories, which encode the association between stimuli in the drug use environment and the rewarding effects of the drug. When triggered, these maladaptive memories can recall the pleasurable effects of the drug, stimulate cocaine craving and precipitate relapse. However, the cellular and molecular adaptations that enable the maintenance of these memories during abstinence remain enigmatic.

As outlined in Chapter 2, comparatively stable epigenetic modifications, such as DNA methylation, may function as a conserved means of perpetuating memory in the face of rapid transcriptional and proteomic turnover and degradation. Learning-induced modifications of DNA methylation have been implicated in the maintenance of contextual fear memories and may therefore also underpin the maintenance of cocaine-related memories. To investigate this possibility, we first established a novel next-generation sequencing technique (MBD Ultra-Seq) to probe genome-wide region- and cell type-specific changes in DNA methylation in individual animals (outlined in Chapter 3). I then applied this technique to identify genome-wide changes in DNA methylation in mice following chronic cocaine self-administration and passive (yoked) cocaine exposure, after 1 or 21 days of forced abstinence (Chapter 4). Modifications of DNA methylation that regulate the maintenance of cocaine-related memories must arise from learned cocaine-seeking and therefore be unique to the cocaine self-administration paradigm, in addition to being persistent, in order to be congruent with the enduring nature of memory. Overall, I identified 29 genomic regions that became persistently differentially methylated during cocaine self-administration and 28 regions that became selectively differentially methylated during abstinence, all of which may contribute to the maintenance of cocaine-related memories.

Functionally, persistent learning-induced changes in DNA methylation are thought to produce enduring modifications of gene expression, thereby altering the physiology of activated neurons and perpetuating memory. However, as posited in Chapter 2, experience-dependent variations in DNA methylation might also represent a form of genomic metaplasticity that is transcriptionally quiescent during memory storage and instead primes the transcriptional response upon subsequent neuronal or memory re-

activation. As preliminary *in vivo* evidence of this hypothesis, I examined how the relationship between altered DNA methylation and the transcription of overlapped or proximal genes is regulated in response to memory reactivation (Chapter 4). In some cases cocaine self-administration induced changes in DNA methylation that had lasting transcriptional consequences, while in others a relationship between altered DNA methylation and the transcription of proximal genes was only evident following the explicit reactivation of cocaine-associated memories. This is the first evidence to suggest that the reactivation state of a memory may govern the relationship between learning-induced changes in DNA methylation and transcription.

Taken together, these data constitute the first *in vivo* neuron-specific genome-wide profile of variations in DNA methylation associated with learned cocaine seeking and not simple drug exposure, where the former is more relevant to the development and persistence of addiction. Moreover, we demonstrate that the relationship between learning-induced modifications of DNA methylation and transcription is complex and mediated not only by the genomic location of DNA methylation, but also by the reactivation state of the relevant memory.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Danay Baker-Andresen

Publications during candidature

Refereed papers

Li, X., Wei, W., Zhao, Q.Y., Widagdo, J., Baker-Andresen, D., Flavell, C.R., D'Alessio, A., Zhang, Y., Bredy, T.W. (2014) Neocortical Tet3-mediated accumulation of 5-hydroxymethylcytosine promotes rapid behavioural adaptation. *PNAS*. 111(19), 7120-5

Li, X.*, Baker-Andresen, D.*, Zhao, Q., Marshall, V. and Bredy, T.W, (2014) MBD Ultra-Sequencing: a novel method for identifying inter-individual and cell-type-specific variation in DNA methylation, *Genes, Brain, Behav.* 13(7), 721-3

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Contributor	Statement of contribution
Danay Baker-Andresen (Candidate)	Proposed idea of metaplastic priming (80%) Wrote manuscript & response to reviewers Designed figures
Vikram Ratnu	Contributed to the section on active DNA demethylation (50%)
Timothy Bredy	Proposed idea of metaplastic priming (20%) Minor editing of manuscript

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Contributor	Statement of contribution
Xiang Li	Conceived of and optimised library preparation, prepared libraries (70%) Wrote manuscript (10%)
Danay Baker-Andresen (Candidate)	Developed method for isolating neuronal nuclei (100%) Optimised library preparation and prepared libraries (15%) Selected and validated candidates (100%) Wrote manuscript (80%) and responded to reviewers (100%)
Qiongyi Zhao	Designed and applied custom bioinformatics pipelines to identify differential methylation (100%) Wrote manuscript (5%)
All others (except last)	Minor contributions

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Contributions by others to the thesis

Dr. Timothy Bredy contributed to the design of experiments, interpretation of data and to the revision of all writing. Prof. Andrew Lawrence developed the mouse operant cocaine self-administration paradigm, performed many of the jugular cannulations and aided in the interpretation of data. Dr. Bianca Jupp, Dr. Robyn Brown, Rose Chesworth and Nicola Chen helped with aspects of the cocaine self-administration paradigm and the sacrifice of animals. Virginia Nink was instrumental in the development of the FACS technique for isolating neurons. Vikki Marshall assisted in the preparation of the sequencing library for the identification of cocaine self-administration induced changes in DNA methylation. Dr. Qiongyi Zhao, whose contribution cannot be emphasised enough, analysed the results from next- generation sequencing to identify regions of methylation enrichment and differential methylation in response to cocaine self-administration (Chapter 4). He has further generated the heatmap of 5mC enrichment and the plots of the enrichment of reads at each differentially methylated region.

Statement of parts of the thesis submitted to qualify for the award of another degree

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Keywords

DNA methylation, cocaine, neuron, next-generation sequencing, genomic metaplasticity, learning, memory, self-administration

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List of Figures & Tables

*Figures and tables that are part of the publications in Chapter 2 and 3 are not listed

Figures

Tables

List of Abbreviations

Please note: Abbreviations included in publications are not listed.

Abbreviation	Definition
5-hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ADHD	Attention deficit hyperactivity disorder
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Cas9	CRISPR-associated 9
CDH13	Cadherin 13
CDKL5	Cyclin-dependent kinase-like 5
CGI	CpG-rich island
Chr	Chromosome
CP-AMPAR	Calcium-permeable AMPA receptor
CPEB4	Cytoplasmic polyadenylation element binding protein 4
CPP	Conditioned place preference
CRISPR	Clustered regularly interspaced short palindromic repeats
CS	Conditioned stimulus
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
E-SARE	Enhanced synaptic activity–responsive element
FACS	Fluorescence-activated cell sorting
FDR	False discovery rate
FR1	Fixed ratio 1
GABA	Gamma aminobutyric acid
gDNA	Genomic DNA
GFP	Green fluorescent protein
GLRa1	Glycine receptor, alpha 1 subunit
GluN	Glutamate receptor ionotropic, NMDA
GOLGB1	Golgin B1
HS	Hypersensitivity
IVSA	Intravenous self-administration

IVSA 21 NR	Self-administering animals scarified after 21 days of abstinence without a relapse test
IVSA 21 R	Self-administering animals scarified after 21 days of abstinence and a relapse test
KCTD16	Potassium channel tetramerization domain containing 16
L-LTP	Late long-term potentiation
LAD	Nuclear lamina-associated domain
lincRNA	Long non-coding RNA
LTP	Long-term potentiation
MBD	Methyl binding domain
MBD Ultra-Seq	Methyl CpG binding domain ultra-sequencing
MCTP1	Multiple C2 domains, transmembrane 1
MeCP2	Methyl CpG binding protein 2
mGluR	Metabotropic glutamate receptor
miRNA	MicroRNA
mPFC	Medial prefrontal cortex
mRNA	Messenger ribonucleic acid
NeuN	Neuronal nuclei (marker)
NKAIN3	Na+/K+ transporting ATPase interacting 3
NMDA	N-methyl-D-aspartate
PAM	Protospacer adjacent motif
PGK	Phosphoglycerate kinase
PKM-zeta	Protein kinase M-zeta
PP1c	Protein phosphatase 1 catalytic subunit
PP1cβ	Protein phosphatase 1 catalytic subunit beta
PPIA	Peptidylprolyl isomerase A (cyclophilin A)
qPCR	Quantitative polymerase chain reaction
RME	Region of methylation enrichment
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SAM	S-adenosyl methionine
SMRT	Single molecule real time (sequencing)
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA

SNW1	SNW domain-containing protein 1
SOX10	Sex-determining region Y-box 10
TALE	Transcription activator-like effector
TARC3	Thymus and activation-regulated chemokine 3
TE buffer	Tris- ethylenediaminetetraacetic acid buffer
TET1	Ten-eleven translocation methylcytosine dioxygenase 1
TSS	Transcription start site
vmPFC	Ventromedial prefrontal cortex
VTA	Ventral tegmental area

CHAPTER 1

Introduction

1.1 Cocaine addiction as a pathology of reward-related learning and memory

Dependence and withdrawal were once deemed the dominant features of addiction, however neither can explain its most baffling aspect: a life-long vulnerability to relapse that persists long after the physiological symptoms of withdrawal have subsided (O'brien *et al.*, 1998). Instead, converging lines of evidence indicate that addiction arises from a pathological appropriation of the neural pathways normally engaged by reward-related learning and memory (Hyman, 2005, Hyman *et al.*, 2006, Kelley, 2004). During cocaine use, environmental cues (such as drug-related paraphernalia or a particular social milieu) become associated with the rewarding effects of the drug; these associations can be subsequently encoded as unusually salient and persistent cocaine-related memories. Re-exposure to previously cocaine-paired cues or contexts during abstinence can precipitate relapse (Childress *et al.*, 1999, Gawin & Kleber, 1986, O'brien *et al.*, 1998), likely by promoting the retrieval of cocaine-related memories and prompting intractable cravings for cocaine (O'brien *et al.*, 1998, Semenova & Markou, 2003). In animal models, pharmacologically disrupting the maintenance of memories for cocaine-cue associations decreases the reinstatement of cocaine seeking during abstinence (Lee *et al.*, 2006) suggesting that cocaine-related memories are key motivators of continued drug seeking during abstinence. Moreover, unlike the transient neural plasticity associated with withdrawal and dependence, the neural adaptations underpinning cocaine-associated memories can last a lifetime and beget a life-long vulnerability to relapse. Thus, there is strong evidence to support the hypothesis that addiction arises from a pathology of reward-related learning and memory (Hyman *et al.*, 2006, Kelley, 2004, Volkow *et al.*, 2004); however, the molecular features that support the maintenance of cocaine-related memories remain equivocal.

1.1.1 Overlearning and the encoding of pathologically persistent cocaine-associated memories

At the cellular level, memory is embodied in facilitated communication between select neuronal synapses, a phenomenon termed long-term potentiation (LTP). LTP is broadly contingent on two forms of cellular adaptation following neuronal activation: enhanced neurotransmitter release by the pre-synaptic neuron in response to subsequent neuronal stimulation and facilitated reception of these neurotransmitters by the post-synaptic neuron, due to altered densities of post-synaptic receptors. The actions of many neurotransmitters modulate LTP, although glutamate is widely recognised as being central to the induction of LTP at the majority of synapses (Lynch, 2004). Glutamate is an

excitatory neurotransmitter that activates both AMPA receptors (which mediate fast synaptic responses to glutamate) and NMDA receptors (which control slow responses to glutamate following sufficient AMPAR-mediate depolarisation), prompting neuronal depolaristation. However, the likelihood of LTP induction by glutamatergic transmission and its persistence are heavily influenced by the presence of other neurotransmitters, such as dopamine (Otani *et al.*, 2003). Finally, the persistence of LTP is dependent on signal transduction and the production of gene products that encode for various components of the cellular machinery (eg. post-synaptic receptors) (Lynch, 2004).

In addition, the cellular adaptations associated with learning and memory are localised to neurons of specific brain regions, which vary depending on the behaviour examined. In the context of reward-related learning, the mesocorticolimbic circuit (the ventral tegmental area (VTA), limbic areas (amygdala and the nucleus accumbens, Nacc), as well as diverse regions of the prefrontal cortex) features prominently. Unexpected reward (eg. cocaine) triggers activation of the VTA (D'ardenne *et al.*, 2008), whose dopaminergic neurons project to the cortex and various limbic regions to ultimately establish and reinforce drug-seeking behaviour. Less well characterised, though equally important are glutamatergic projections from the prefrontal cortex to the amygdala, Nacc and VTA, which are implicated in establishing learned associations between cues, behaviour and rewarding stimuli (Tzschentke & Schmidt, 2003), such as drugs of abuse. Though each structure plays an important role in reward-related learning and memory, the medial prefrontal cortex (mPFC) may be of particular importance in the context of long-lasting reward-related memories. In cocaine users, as well as self-administering animals, this region is hyperactive upon exposure to cocaine-paired cues (Childress *et al.*, 1999, Ciccocioppo *et al.*, 2001), suggesting that it plays a key role in long-lasting cocaine-related memories. Moreover, the enduring contribution of the mPFC to drug-seeking behaviour is echoed in steadfast neuroadaptive changes in gene expression and the neuroproteome that persist for over 100 days of enforced abstinence in self-administering rats (Freeman *et al.*, 2010a, Freeman *et al.*, 2008, Lull *et al.*, 2009). Finally, repeated exposure to cocaine induces lasting changes in the membrane excitability of prefrontal pyramidal neurons (Dong *et al.*, 2005), where changes in membrane excitability constitute a well-characterized measure of experience-dependent plasticity seen in learning (Zhang & Linden, 2003). Several investigations (Jasinska *et al.*, 2015, Koya *et al.*, 2009) have indicated that subregions of the mPFC, such as the ventral mPFC and dorsal mPFC, may mediate distinct aspects of cocaine-seeking behaviour. However, the plethora of contradicting results emerging from

these lines of investigation indicate that involvement of these regions in cocaine-seeking behaviour is quite nuanced and may instead reflect the actions of distinct sub-circuits within each region, rendering the absolute distinction between both unwise (Moorman *et al.*, 2014).

Notably, the pharmacological actions of cocaine are thought to over amplify many of aforementioned cellular changes to produce tenacious and compelling memories of the association between cocaine, its effects and cues that predict its availability (Hyman *et al.*, 2006, Kalivas & Mcfarland, 2003). Although cocaine and natural rewards act on the same mnemonic pathways, the self-administration of cocaine begets longer-lasting LTP in the VTA (Chen *et al.*, 2008) and normally transient increases in neurotransmission become persistent in the presence of cocaine *in vitro* (Fole *et al.*, 2013). Cocaine achieves a competitive advantage over natural rewards by directly altering the concentration of learning-related neurotransmitters in the synaptic cleft, which heightens neurotransmission (Hyman, 2005). Most prominently, cocaine produces an unusually prolonged and unregulated increase in synaptic dopamine by stimulating the exocytosis of reserve pools of dopamine-containing synaptic vesicles from dopaminergic neurons (Venton *et al.*, 2006) and by inhibiting the reuptake of dopamine (Ritz *et al.*, 1987). Originally it was posited that the overabundance of dopamine motivated cocaine seeking by creating an unprecedented hyper-hedonic state (Koob & Le Moal, 2001) yet, perplexingly, dopamine depletion does not influence how much an animal “likes” a reward (Berridge & Robinson, 1998, Robinson *et al.*, 2005). Outside of drug abuse, dopamine release is particularly pronounced when learning about the cues that predict reward availability is extremely important, such as when food is presented following food deprivation (Wilson *et al.*, 1995) or when a reward is delivered unexpectedly. The dopamine release provoked by rewards under such conditions potentiates on going learning and cements the association between the cues and reward availability, enabling cues to gain motivational salience (Berridge & Robinson, 1998) and stimulate goal directed behaviour when encountered in the future. However, the atypical increase in extracellular dopamine prompts the brain to overlearn the association between cocaine-paired cues and drug availability and attribute excessive importance to such cues (Hyman *et al.*, 2006), such that they are attended to at the expense of other stimuli. Moreover, dopamine is a powerful modulator of the cellular processes underlying memory formation and maintenance (Gonzalez *et al.*, 2014, Gurden *et al.*, 2000, Huang *et al.*, 2004, O'carroll *et al.*, 2006), which may further consolidate drug-seeking repertoires and accelerate the development of habitual cocaine seeking (Berke, 2003). The

exaggerated synaptic dopamine present following cocaine exposure may therefore produce exceptionally persistent memories for cocaine-cue associations leading to continued cocaine seeking during abstinence.

Nevertheless, dopaminergic neurons project to a wide area and cannot account for a specific enhancement of the encoding of cocaine-related memories. Instead dopamine may consolidate ongoing learning-induced changes in glutamatergic neurotransmission (Otani *et al.*, 2003, Sun *et al.*, 2005). Within the PFC, dopamine- and glutamate-releasing terminals converge on the spines of pyramidal neurons, forming “synaptic triads” (Carr & Sesack, 2000). Within the dendritic spines of these neurons, dopamine (D1) receptors and N-methyl-D-aspartate (NMDA) receptors physically interact and D1 receptor activation potentiates NMDA receptor activity (Kruse *et al.*, 2009), suggesting that dopaminergic and glutamatergic pathways directly interact to facilitate the encoding of cocaine-related memories. Moreover, within neurons of the PFC, D1 receptor activation facilitates the insertion of synaptic AMPA receptors, providing yet another mechanism by which dopamine may potentiate LTP induction and memory formation in the PFC (Sun *et al.*, 2005). Additionally, within the cortex, the application of D1 agonists facilitates the maintenance of NMDA-dependent LTP (Huang *et al.*, 2004). Therefore, within the PFC, the combined actions of altered dopaminergic and glutamatergic signaling may be necessary to produce persistent cocaine-related memories. Increasingly, it is hypothesized that dopamine release, either in response to cocaine or to the presentation of a previously cocaine-paired cue, renders neurons of the PFC excitable (Lewis & O'donnell, 2000), and that subsequent excitation of these afferent neurons (projecting to the Nacc and amygdala) prompts drug-seeking behaviour (Kalivas *et al.*, 2005).

Altered glutamatergic neurotransmission is in and of itself required for the acquisition and expression of cocaine-seeking behaviour, as rats treated systemically with NMDA receptor antagonists fail to self-administer cocaine (Schenk *et al.*, 1993). A particularly prominent site of glutamatergic plasticity is the PFC-accumbal pathway. Exposure to previously cocaine-paired cues activates glutamatergic PFC neurons that project to the Nacc and motivate relapse; such pronounced potentiation of this pathway is not seen when animals are exposed to a cue previously paired with a natural reward (Gipson *et al.*, 2013). Interestingly, cocaine-induced dysregulation of glutamatergic neurotransmission is particularly prevalent in paradigms where animals learn to seek cocaine, such as the cocaine self-administration paradigm (Mcfarland *et al.*, 2003). As opposed to passive

cocaine exposure, learned cocaine seeking induces the specific up-regulation of particular subunits of the glutamatergic NMDA receptor within the PFC (Pomierny-Chamiolo *et al.*, 2014), as well an increase in the ratio of AMPA/NMDA receptor expression in reward-related brain regions, such as the VTA (Chen *et al.*, 2008) and the nucleus accumbens (Conrad *et al.*, 2008) which is indicative of enhanced synaptic transmission. Moreover, glutamatergic communication within these regions remains potentiated during abstinence (Conrad *et al.*, 2008) and following the extinction of cocaine seeking behaviour (Chen *et al.*, 2008) which may both enable the maintenance of cocaine-related memories and underlie the prolonged increased reactivity to cocaine paired cues that is observed in former users (Goldstein & Volkow, 2011).

Increasingly the focus of altered glutamatergic transmission in response to cocaine has shifted to G protein-coupled metabotropic glutamate receptors (mGluRs), which modify neuronal activity by regulating NMDA receptor function (Pomierny-Chamiolo *et al.*, 2014) and ionotropic receptor-dependent glutamate transmission (Ferraguti & Shigemoto, 2006, Schoepp, 2001). MGluR 2/3 receptors negatively regulate glutamatergic transmission and are downregulated following chronic cocaine exposure (Baker *et al.*, 2003), which may remove the brake on glutamate neurotransmission and heighten reactivity to cocaine-paired cues during abstinence. Indeed, restoring mGluR2/3 function in the Nacc prevents the cue-induced reinstatement of cocaine seeking behaviour (Baptista *et al.*, 2004). In contrast, mGluR5 receptors positively modulate glutamatergic neurotransmission (Schoepp, 2001) and are required for the acquisition of cocaine self-administration (Chiamulera *et al.*, 2001, Kenny *et al.*, 2003). Correspondingly, mGluR5 activity is required for the cue-induced reinstatement of cocaine seeking (Kumaresan *et al.*, 2009). Remarkably, the disruption of mGluR5 function has no effect on natural reward seeking (Chiamulera *et al.*, 2001) or the maintenance of sucrose self-administration (Chesworth *et al.*, 2013), which may indicate unique dysregulation of glutamatergic transmission in response to drugs of abuse.

Lastly, chronic cocaine exposure affects future learning by promoting the formation of ‘silent’ glutamatergic synapses where long-lasting changes in plasticity are more easily induced and maintained (Lee & Dong, 2011). AMPA receptors are absent or highly unstable at these synapses, which normally decline as the brain matures and are thought to contribute to the superior learning ability often seen in juvenile organisms (Groc *et al.*, 2006). During withdrawal from cocaine, ‘silent’ synapses are highly upregulated in both the

Nacc and PFC (Huang *et al.*, 2009, Ma *et al.*, 2014) and this may prime learning about cocaine-predictive cues upon repeat cocaine exposure, creating even more persistent cocaine-cue memories. Moreover, the disruption of inhibitory GABAergic tone by cocaine (Liu *et al.*, 2005) may also prime the encoding of cocaine-related memories (Lee & Dong, 2011).

In sum, the pharmacological actions of cocaine prime the organism for learning and over amplify normal reward-related learning signals, which may underpin the enhanced encoding and persistence of cocaine-related memories.

1.1.2 Not all roads lead to Rome: the importance of self-administration in establishing cocaine-related memories

In examining the effects of cocaine in animals, many modes of administration may be used. The first is simple acute or chronic injections performed by an experimenter, during which the animal is not required to perform a specific behaviour to receive the drug. A second popular model is conditioned place preference (CPP), where animals are repeatedly administered cocaine and exposed to one of two contexts. When given the choice of exploring either of the two contexts, animals tend to prefer the cocaine-paired context. This paradigm provides a measure of drug-seeking behaviour, indicated by the amount of time an animal spends in the cocaine-paired context. A third popular model is cocaine self-administration, where animals learn to perform an operant response (typically a lever press) to receive an infusion of cocaine through an indwelling jugular catheter. The delivery of cocaine is often paired with an explicit cocaine-paired cue (such as a light). The latter, though technically more challenging than CPP, is considered to be a more ethologically valid model of cocaine seeking and taking. The self-administration paradigm produces similar neurobiological changes in the reward related neurocircuitry, though it does not necessarily lead to ‘addiction’ which is more accurately defined by continued drug-seeking and drug-taking despite negative consequences.

The neurobiology of cocaine addiction is complex and the neural adaptions that arise during the transition to compulsive cocaine-seeking correspond to two distinct, but interacting pathways: the first is the neurobiological effects of the drug itself and the second is the adaptations associated with cocaine-related learning and memory formation. A substantial portion of research is devoted to studying the consequences of chronic non-contingent cocaine exposure, though it is clear that the neurobiological consequences of

involuntary cocaine exposure do not faithfully parallel those produced by voluntary cocaine seeking. Motivated drug seeking and taking (seen in the CPP and self-administration paradigms) is key to the development addiction (Koob *et al.*, 2004) whereas unpredictable involuntary exposure cocaine can be aversive and can retard the development of drug seeking (Twining *et al.*, 2009). Moreover, the neural adaptations incurred during passive cocaine exposure may fail to produce the persistent cocaine-related memories that motivate continued cocaine seeking. Relative to self-administration, passive non-contingent exposure to cocaine fails to induce an equivalent change in long-term potentiation (LTP) in the ventral tegmental area (VTA) (Chen *et al.*, 2008, Martin *et al.*, 2006), or the same magnitude of increase in dopamine (Hemby *et al.*, 1997) or acetylcholine concentration (Hemby *et al.*, 1997, Mark *et al.*, 1999). Furthermore, withdrawal from cocaine self-administration is associated with distinct neural adaptations, including the upregulation of calcium-permeable AMPA receptors (CP-APMARs) and an increase in the expression of brain-derived neurotrophic factor (BDNF), which may facilitate increased excitatory neurotransmission in response to cocaine-paired cues and contribute to the renewal of cocaine seeking during abstinence (Lu *et al.*, 2010, Mccutcheon *et al.*, 2011).

The conditioned place preference (CPP) paradigm is often used to examine neural adaptations induced by cocaine and cocaine seeking as it is does not require extensive operant training, the surgical implantation of a jugular catheter and eliminates the differences attributable to variations in the rate and total intake of cocaine. While CPP has been widely adopted as a measure of drug seeking and is similar to cocaine self-administration, there are some disparities that impart increased ethological validity to the cocaine self-administration paradigm, particularly when studying cocaine-related learning and memory. Firstly, the CPP model of cocaine seeking produces less robust cocaine-associated memories than voluntary cocaine self-administration; higher doses of pharmacological agents are needed to promote the extinction of cocaine-seeking behaviour in self-administering animals (Thanos *et al.*, 2011) and cocaine-related memories produced by self-administration are less prone to destabilisation following memory retrieval (Brown *et al.*, 2008). Furthermore, in the self-administration paradigm, animals voluntarily develop extensive drug-seeking behavioural repertoires and learn about the cues that predict drug availability, in contrast to CPP, where the subjective effects of the drug are already present and initial administration of cocaine is involuntary. Therefore, the cocaine self-administration paradigm more faithfully replicates learned

cocaine self-administration in the natural environment and may provide a more accurate picture neural adaptations that support the formation and maintenance of cocaine-related memories.

1.2 How are cocaine-associated memories maintained over time?

Despite the ubiquity of long-term memory, little is known about the mechanisms that enable its persistence. The formation of memory requires precisely regulated programs of altered gene expression (Agranoff *et al.*, 1967) and protein synthesis (Flexner *et al.*, 1963) and several hundred molecules are known to be transcribed, translated or activated during memory formation (Sanes & Lichtman, 1999). However, these molecules undergo rapid turnover and have restricted half-lives and therefore cannot be the master modulators of memory. Even PKM-zeta, a constituently active protein kinase that is necessary and sufficient for the maintenance of several forms of long-term memory (see (Sacktor, 2012)), has a half-life of approximately 11 days (Sacktor, 2010) and therefore cannot maintain long-term memory on its own. Instead, neuroscience has long ignored the most obvious candidate for the repository of memory: the genome.

DNA is the one cellular component that is present and largely intact for the lifetime of an organism and its long-term memories and was hypothesized to be the site of the self-perpetuating modifications that support long-term memory several decades ago (Griffith & Mahler, 1969). Griffith and Mahler posited that the enzymatic modifications of nucleotides following learning could direct the changes in transcription and translation necessary for memory formation and maintenance. Furthermore, unlike mutations in the genome, these enzymatic modifications would likely be reversible, offering a degree of plasticity that is congruent with the dynamic nature of memory. Today, the collection of enzymatic modifications of genomic DNA forms the ‘epigenome’, where the prefix “epi” indicates that these modifications are above, outside of, or around the genome and do not alter the underlying genetic sequence.

1.3 Epigenetics

Traditionally, ‘epigenetics’ referred to heritable changes in gene function that were not produced by changes in genetic sequence (Bird, 2002). More recently, the requirement of heritability has been rescinded and epigenetics refers to any regulatory process, outside of genetic sequence variation, that serves to “register, signal or perpetuate altered transcriptional states” (Bird, 2007). Epigenetic processes include the modification of nucleotides (ie. DNA methylation and hydroxymethylation), histone modifications,

nucleosome remodelling and non-coding RNA among others. Epigenetic modifications regulate gene expression by controlling the conformation of DNA and chromatin or by recruiting further enzymatic modifiers or components of the transcriptional machinery. While epigenetic regulation was traditionally used to explain the development of stable differentiated cellular phenotypes from virtually identical genetic code, it is now clear that the epigenome is dynamically regulated across the lifespan in response to environmental stimuli. Moreover, the number of possible epigenetic modifications of DNA and histone proteins exceeds 100 (Hurd, 2010) and the summation and interaction of these enables an unprecedented level of transcriptional regulation in response to environmental stimuli.

1.3.1 DNA methylation

DNA methylation involves the addition of a methyl group to one or more nucleotides of DNA, typically a cytosine, to form 5-methylcytosine, although methylation of other bases has been observed in bacteria, plants and insects (Ratel *et al.*, 2006). Amongst epigenetic modifications DNA methylation is comparatively stable as the methyl group is covalently bonded to the 5^{th} carbon of the cytosine ring. Correspondingly, DNA methylation has been widely implicated in establishing and perpetuating stable transcriptional changes during genomic imprinting, cell differentiation and X-chromosome inactivation as well as in the stable repression of transposable elements (Ndlovu *et al.*, 2011).

1.3.1.1 Distribution of DNA methylation: hints at function

Cytosine methylation occurs predominantly in the palindromic CpG dinucleotide context (where guanine follows a cytosine) (Lister *et al.*, 2013); 46-90% of all 5-methylcytosines within the genome occur within this context (Bird, 1986, Guo *et al.*, 2013, Lister *et al.*, 2013). The symmetric nature of the CpG dinucleotide (ie. 5'CpG/GpC5') facilitates the propagation of DNA methylation during DNA replication and may have favoured the evolution of enzymes that preferentially methylated cytosine in the CpG dinucleotide context, which would explain why methylation is predominantly found in this context (Bird, 2002). Nevertheless, CpG dinucleotides are conspicuously depleted from the genome, likely because methylcytosine is prone to spontaneous deamination to thymine, producing at T:G mismatch that is not efficiently recognised for repair (Kondrashov, 2003). Therefore, by examining the distribution of CpG dinucleotides and methylation throughout the genome we can identify regions where they might continue to play important regulatory roles.

Unmethylated CpG dinucleotides tend to cluster in CpG-rich islands (CGI) proximal to transcription start sites (TSS) of constitutively expressed genes (Bird, 1986, Edwards *et al.*, 2010). About 70% of promoters co-localise with CGI (Larsen *et al.*, 1992, Weber *et al.*, 2007) and methylation of these islands tends to be associated with the repression of the corresponding genes. As early studies of DNA methylation concentrated on promoter regions, a dogmatic notion that DNA methylation is associated with the stable repression of gene transcription arose (Jones, 2012). This dogma was reinforced by evidence that methylation of intragenic CGIs also represses transcription of the associated genes (Maunakea *et al.*, 2010) in addition to the known function of DNA methylation in repressing retrotransposition (Rollins *et al.*, 2006).

The advent of powerful genome-wide sequencing technologies has given much greater resolution of the distribution of CpGs throughout the genome and revealed roles for DNA methylation that extend beyond its classical function in transcriptional repression. For example, CpG sites are concentrated at the 5' and 3' ends of internal exons (Edwards *et al.*, 2010), which suggests that CpG methylation might regulate alternative splicing. Indeed, increased CpG methylation is observed at alternatively spliced sites, putative exon splicing enhancers and alternate exons (Anastasiadou *et al.*, 2011, Choi, 2010, Maunakea *et al.*, 2010), though interestingly it mediates exon inclusion (Maunakea *et al.*, 2013), potentially by recruiting specialised methyl-binding proteins such as MeCP2 (Maunakea *et al.*, 2013) or by altering the kinetics of transcriptional elongation (Lorincz *et al.*, 2004). Likewise, gene body methylation is paradoxically a hallmark of actively transcribed genes (Feng *et al.*, 2010b, Hellman & Chess, 2007). Finally, in regions of low CpG density, such as enhancers and gene bodies, DNA methylation is more prone to dynamic regulation (Schmidl *et al.*, 2009, Wiench *et al.*, 2011) and this may be a site of plasticity in the methylome following exposure to important environmental stimuli. Consequently, the relationship between DNA methylation and gene transcription has gained complexity, as the effects of DNA methylation on transcription are highly dependent on the context in which it occurs.

1.3.1.2 Establishing *de novo* DNA methylation

Establishing *de novo* methylation is a complex process mediated by an ever-growing number of factors. Largely, these factors belong to two categories: the enzymes that catalyse the addition of the methyl group to DNA and the factors that direct these enzymes to specific sites in the genome.

1.3.1.2.1 The enzymes: DNA methyltransferases

In mammals, DNA methylation is established and maintained by DNA methyltransferases (DNMTs) that catalyse the addition of a methyl group to a nucleotide from a donor, typically S-adenosyl methionine (SAM). Until recently it was believed that the DNMT3 family (DNMT3a and 3b and co-factor DNMT3L) conferred *de novo* methylation, whereas DNMT1 was thought to mediate the maintenance of DNA methylation, particularly during DNA replication (Auclair & Weber, 2012). However, there is now evidence that DNMT 1 and 3a co-operate to mediate *de novo* methylation (Fatemi *et al.*, 2002, Lin *et al.*, 2002) and conversely that DNTM3a is necessary for the maintenance of DNA methylation (Feng *et al.*, 2010a). More surprisingly, DNMT3a acts as a demethylase when SAM levels are low (Metivier *et al.*, 2008). Therefore, although the DNMTs have a long been implicated in the regulation of DNA methylation, their functions are far from being well defined.

1.3.1.2.2 Targeting DNMTs

A second evolving area of enquiry is understanding how regions are marked for *de novo* methylation or demethylation by DNMTs and DNA demethylases. This is a complex question for several reasons: first, it is unclear if DNA methylation and demethylation are the cause or consequence of changes in transcription, and indeed they may be both. Should altered DNA methylation be the consequence of transcription, the transcripts initially produced by a specific locus could feedback to direct *de novo* methylation or demethylation, yet this does not explain how transcription arises from heavily repressed genes in the first instance. Second, there is the problem of specificity; how is the DNA methylation or demethylation machinery targeted to one CpG but not another? We have only begun to appreciate the dynamic regulation of DNA methylation in differentiated cells, but information derived from genome-wide sequencing suggests that several pathways bidirectionally influence DNA methylation, the most prominent of which are post-translational modifications of histones and a myriad of non-coding RNAs.

Post-translational modification of histones

DNA methylation frequently co-localises with more transient modifications of histone tails, particularly certain forms of histone lysine methylation (Meissner *et al.*, 2008). DNMTs may be recruited directly with histone lysine methyltransferases (Vire *et al.*, 2006) and histone methylation at distinct sites can act to attract or repel DNMTs (Jin *et al.*, 2011). Histone argine methylation can equally recruit DNMTs (Zhao *et al.*, 2009). However, the interplay between histone modifications and DNA methylation is far from a one-way street as DNA

methylation may dictate the deposition of new histone modifications by recruiting histone methyltransferases and histone deacetylases (Jin *et al.*, 2011). Nevertheless, most favour the hypothesis that histone modifications precede DNA methylation/demethylation (Cedar & Bergman, 2009), which serves as a more stable mark for transcriptional repression or activation. Regardless, this cannot explain how the epigenetic machinery is targeted to a specific locus in the genome; this role may fall to non-coding RNAs.

Non-coding RNA

One of the manners in which transcription might direct DNA methylation is through the production of non-coding RNAs that feedback to direct DNA methylation both locally and throughout the genome. Non-coding RNAs can bind to genomic loci proximal to the region from which they produced and form stem-loop structures that bait DNMTs and sequester them, shielding the locus from *de novo* methylation (Di Ruscio *et al.*, 2013). Other classes of small non-coding RNAs, such as PIWI-interacting RNAs, may also be important for directing DNA methylation (Watanabe *et al.*, 2011). While our understanding of RNA-directed methylation is evolving, non-coding RNAs clearly offer a degree of specificity that is advantageous in promoting methylation or demethylation at specific loci.

1.3.2 The dynamic nature of DNA methylation

DNA methylation was once considered to be a relatively static epigenetic modification, renowned for its role in the long-term regulation of genomic imprinting, transposon silencing and X-chromosome inactivation, as well in cellular differentiation. Though DNA demethylation was observed in the early stages of embryonic development (Monk *et al.*, 1987) it was hypothesized that this stemmed from a passive, DNA replication-dependent process whereby newly synthesized daughter strands of DNA fail to acquire methylation at the appropriate sites. Despite the discovery of several DNA synthesis-independent demethylases (Gjerset & Martin, 1982, Ramchandani *et al.*, 1999, Weiss *et al.*, 1996), the possibility of active, replication-independent DNA demethylation remained controversial until recently, as the proposed reactions are thermodynamically unfavorable and the enzymes thought to mediate demethylation were inefficient at doing so (Cortazar *et al.*, 2007). However, rapid replication-independent demethylation was observed following fertilization (Oswald *et al.*, 2000) and in non-dividing neurons (Martinowich *et al.*, 2003) indicating that active DNA demethylation must be possible. Today we know that *de novo* DNA methylation and demethylation occur throughout the genome in response to environmental input, including neuronal activation (Guo *et al.*, 2011a, Guo *et al.*, 2011b,

Ma *et al.*, 2009) and that this process is mediated by several enzymatic pathways (Li *et al.*, 2013).

1.3.3 DNA methylation and neurons

Though the epigenomes of all cells in the brain may be modified in response to learning and environmental input, neurons are conspicuously well suited to be the repository of long-lasting epigenetic modifications that perpetuate long-term memory. Unlike many other somatic cells, mature neurons do not undergo mitosis, which might prevent the disturbance of epigenetic marks that have been acquired during neuronal activity and learning (Griffith & Mahler, 1969). Furthermore, although glial cells contribute to synaptic remodeling (Perea & Araque, 2010, Todd *et al.*, 2006), the cellular manifestation of memory is widely recognized as long-lasting changes in the transmission of signals across neuronal synapses.

Though many were surprised to find that DNMT3a is expressed in mature neurons in 1994 (Goto *et al.*, 1994, Yu *et al.*, 2011), seminal evidence of learning- and activity- induced changes in the neuronal methylome actually emerged two decades earlier. Shortly after Griffith & Mahler (1969) hypothesized that the enzymatic modification of DNA could form the physical basis of memory, Boris Vanyushin demonstrated that DNA methylation was modified in neurons following learning (Guskova Lv, 1977, Vanyushin, 1974). Unfortunately, this work received little recognition and it was not until 2003 that further evidence for the regulation of DNA methylation in response to neuronal activity emerged (Martinowich *et al.*, 2003). In the interim, several others capitalized and spruiked the idea that DNA methylation could support memory as their own (Crick, 1984, Holliday, 1999).

DNA methylation in neurons is unique in several respects. Firstly, in most cells, intergenic regions are heavily methylated (Consortium, 2012), yet in neurons small regions of intergenic hypomethylation appear frequently (Hon *et al.*, 2013). These regions might correspond to the distal regulatory elements that are the predominant site of neuronal activity-induced changes in DNA methylation (Guo *et al.*, 2011a). Furthermore, the neuronal methylome appears to be inherently more plastic than that of other cell types; relative to glial cells, neurons have increased inter-individual variability in DNA methylation, which implies an enhanced propensity for plasticity (Iwamoto *et al.*, 2011). An increased presence of non-CpG methylation (cytosine methylation outside of palindromic CpG dinucleotides) (Lister *et al.*, 2013, Xie *et al.*, 2012), and 5-hydroxymethylcytosine (5-

hmC) in neurons may facilitate demethylation (Feng *et al.*, 2010a, Guo *et al.*, 2013) and the dynamic regulation of the neuronal methylome. Therefore, the unique features of the neuronal methylome leave it well equipped to responded to environmental stimuli and orchestrate the long-lasting changes in transcription and translation necessary for long-term memory formation and maintenance.

1.3.4 DNA methylation and long-term potentiation (LTP)

Late long-term potentiation (L-LTP) is an increase in synaptic strength that gives rise to a long-lasting facilitation of neurotransmission across the synapse, and it is widely regarded as the cellular manifestation of memory (Lynch, 2004). L-LTP arises when stimulation from the presynaptic neuron is sufficiently strong or occurs repeatedly. Under these conditions the response of the postsynaptic neuron becomes potentiated and it is more sensitive to future stimulation from the presynaptic neuron, which facilitates communication across the synapse. L-LTP requires a multitude of changes in the synapse, including a persistent increase in the number of glutamate-sensitive AMPARs on the postsynaptic membrane, increased exocytosis of neurotransmitters from the presynaptic neuron and host of changes in the architecture of the synapse. The persistence of these changes and L-LTP necessitates *de novo* transcription and protein synthesis (Abraham & Williams, 2003, Krug *et al.*, 1984), though these must be orchestrated by a more enduring and self-perpetuating mark, such as neuronal activation-induced changes in DNA methylation.

Early experiments demonstrated that DNA methylation is altered within the promoter of *Bdnf,* (an important regulator of several memory-related processes) during the induction of LTP (Martinowich *et al.*, 2003, Zhao *et al.*, 2003) and that components of the DNA methylation machinery are critical for the induction of LTP (Zhao *et al.*, 2003). However, it was Levenson and colleagues (2006) who first established that *de novo* DNA methylation is required for the induction of LTP *in vitro* by inhibiting DNMTs (Levenson *et al.*, 2006). Originally these findings were restricted to the hippocampus, however, evidence that DNA methylation contributes to synaptic plasticity now extends outside the hippocampus; the induction of LTP in the mPFC and the lateral amygdala is impaired by the local application of DNMT inhibitors (Monsey *et al.*, 2011, Sui *et al.*, 2012). Moreover, this impairment cannot be attributed to the potentially toxic effects of DNMT inhibitors used in initial experiments, as mice lacking DNMT1 and DNMT3a in forebrain neurons have similar deficits in LTP induction (Feng *et al.*, 2010a). Finally, appropriately regulated DNA

methylation is essential for the maintenance of altered neuronal network excitability following depolarization (Nelson *et al.*, 2008), which hints at a role for DNA methylation in the long-term regulation of synaptic plasticity.

1.3.5 DNA methylation and memory maintenance

Although several persistent learning-induced changes in DNA methylation have been reported following contextual fear conditioning (Miller *et al.*, 2010, Mizuno *et al.*, 2012), there is limited causal evidence to implicate DNA methylation in the maintenance of memory once consolidation is complete or following the induction of LTP. In 2010, Miller & Sweatt released a paper titled “Cortical DNA methylation maintains remote memory”, which demonstrated that the application of DNMT inhibitors during memory maintenance disrupts the retrieval of remote fear memories (Miller *et al.*, 2010). However, the DNMT inhibitors were applied within close proximity of the memory test (3x within the 24 hours period leading up to the remote memory test, including once at 1 hour prior to test) and therefore may have interfered with the retrieval of the memory trace rather than its maintenance. Disconcertingly, DNMT inhibitors interfere with the retrieval of other forms of memory (Han *et al.*, 2010). Nevertheless, learning does produce persistent changes in DNA methylation, which is in turn perfectly poised to mediate the long-term changes in transcription and protein levels that are necessary for the maintenance of memory. The development of new tools that can directly manipulate learning-induced DNA methylation and examine its relevance to long-term memory maintenance will be key in advancing our understanding of how DNA methylation contributes to memory maintenance.

Memory maintenance, however, is predicated not just on initial cellular modifications that are induced upon learning, but also by on-going modifications that arise from memory retrieval and reconsolidation. Following retrieval, memories can enter a labile state that permits the incorporation of new information and updating of the memory trace. The memory is returned to long-term storage through reconsolidation. Memory reconsolidation is thought to strengthen memory traces by increasing the persistence and specificity of the memory trace (Forcato *et al.*, 2014), explaining why repeat experiences yield more persistent memories. There is strong evidence that DNMT activity is necessary for the reconsolidation of memory; infusion of DNMT inhibitors into the lateral amygdala (a region key to auditory fear memory) after the reactivation of an auditory fear memory disrupts the expression of reconsolidation-dependent long-term memory but not reconsolidation-independent short-term memory (Maddox & Schafe, 2011). Therefore, *de novo*

methylation following memory retrieval may contribute to the maintenance long-term memory, which is of great relevance to addiction where cocaine-related memories are repeatedly reactivated and strengthened by re-exposure to external and internal cocaine-paired cues.

A further consideration is establishing in which cell-types learning and memory-related changes in DNA methylation might arise. Examining cell-type specific epigenetic modifications is extremely important as cell-type heterogeneity can confound epigenetic profiling attempts by producing spurious results following changes in cellular composition of the region (see Chapter 3). Additionally, it is likely that the epigenetic changes associated with a memory exist only within a discrete subset of neurons of the brain and these changes may be rendered imperceptible by examining heterogeneous regions. It would be desirable to examine changes in DNA methylation uniquely within neurons that are engaged by a memory trace or within a distinct subtype of neuron (ie. glutamatergic neurons), but at this time it is not possible to isolate such a population from a distinct region of an adult mouse brain and obtain sufficient DNA for analysis.

Overall, the evidence of the distinct contribution of DNA methylation to memory maintenance is preliminary, but the presence of persistent changes in DNA methylation in the neuronal genome suggests that learning-induced changes in DNA methylation contribute to memory maintenance. The application of tools that can directly manipulate learning-induced changes in DNA methylation, such as transcription-activator like effector (TALE) – DNA demethylatase fusion proteins (Maeder *et al.*, 2013b), will more convincingly elucidate the role of persistent changes in DNA methylation in long-term memory maintenance.

1.4 DNA methylation and cocaine

As learning-induced changes in DNA methylation are associated with the maintenance of other forms of memory, cocaine-induced changes in DNA methylation may equally underlie the maintenance of cocaine-related memories. A growing body of evidence suggests that exposure to cocaine gives rise to brain region-specific changes in DNA methylation. DNMT3a mRNA expression is altered in a region and time-dependent manner following cocaine exposure, self-administration and withdrawal (Anier *et al.*, 2010, Laplant *et al.*, 2010, Pol Bodetto *et al.*, 2013, Tian *et al.*, 2012), as are the levels of methyl binding proteins MeCP2 and MBD1 (Carouge *et al.*, 2010, Cassel *et al.*, 2006, Host *et al.*,

2011, Im *et al.*, 2010). Likewise, local and systemic manipulations of DNMT activity (Laplant *et al.*, 2010) and methyl donor availability (Laplant *et al.*, 2010, Tian *et al.*, 2012) alter the expression of cocaine seeking in the cocaine conditioned place preference paradigm. Furthermore, several genes (CDKL5, PP1c, TACR3 and PP1Cβ) are differentially methylated following cocaine exposure (Anier *et al.*, 2010, Barros *et al.*, 2013, Carouge *et al.*, 2010) and several of these changes are attenuated by the concurrent administration of DMNT inhibitors (Anier *et al.*, 2010, Pol Bodetto *et al.*, 2013). However, while these experiments provide a surfeit of evidence of dynamic regulation of the methylome in response to cocaine exposure, they fail to distinguish changes in DNA methylation that are attributable to learned cocaine-seeking from those that arise from simple drug exposure, where the former may be key to the development and persistence of cocaine-related memories and addiction. Chronic cocaine exposure and conditioned place preference do not require active cocaine seeking and/or repeated voluntary self-administration of the drug, which are key to the malignant potentiation of the reward-related neural circuitry. A single study has identified that forced abstinence from cocaine self-administration is associated with methylation of the SOX10 promoter in the corpus callosum of rats (Nielsen *et al.*, 2012a) and one other suggests that DNMT3a mRNA expression is altered during withdrawal from cocaine self-administration (Laplant *et al.*, 2010), however this hardly sheds light on the changes in DNA methylation may be related to the maintenance of cocaine-related memories. Therefore, while there is sufficient evidence to suggest that DNA methylation is dynamically regulated in response to cocaine exposure, we must extend this level of profiling to identify changes in DNA methylation incurred by voluntary cocaine self-administration.

1.5 Projects aims and rationale

The overarching hypothesis of this project was that cocaine self-administration produces unique changes in DNA methylation that both persist and arise during forced abstinence, and which are associated with distinct changes in gene expression. Overall, the aim of this project is to identify changes in DNA methylation that may be associated with the maintenance of cocaine-related memories. While epigenetic remodelling in response to chronic cocaine exposure and cocaine place preference conditioning has been reasonably well documented (Anier *et al.*, 2010, Barros *et al.*, 2013, Carouge *et al.*, 2010, Chao *et al.*, 2014, Fragou *et al.*, 2013, Han *et al.*, 2010, Laplant *et al.*, 2010, Nielsen *et al.*, 2012b, Pol Bodetto *et al.*, 2013, Pol Bodetto *et al.*, 2014, Tian *et al.*, 2012), changes in DNA methylation following voluntary cocaine self-administration have not. To identify changes

in DNA methylation associated with the maintenance of cocaine-related memories, it is necessary to distinguish those changes that are associated with learned cocaine seeking from those that arise as a result of simple drug exposure. Moreover, such changes must persist over time to be congruent with the enduring nature of memory. To address these and other issues, I have employed a mouse model of cocaine self-administration and yoked (simple) cocaine exposure to identify changes in DNA methylation that are unique to learned cocaine seeking. Additionally, I examined the persistence of identified learning-associated modifications of DNA methylation during abstinence, to identify those changes that might underwrite the maintenance and reconsolidation of cocaine-related memories. Finally, I probed the relationship between altered DNA methylation and gene expression and questioned how this association is influenced by the retrieval of cocaine-related memories.

To examine the overarching hypothesis, several aims had to be addressed:

Aim 1: To determine if mice persistently seek cocaine after a period of prolonged abstinence. It is anticipated that cocaine self-administration training will lead to persistent cocaine seeking, as examined after 21 days of withdrawal. This is a common phenomenon within most animals trained to self-administer cocaine (Fuchs *et al.*, 2008), however, it has not been established that mice continue to seek cocaine in our paradigm. Continued cocaine seeking (as indicated by a sustained preference for the previously cocaine paired lever) is indicative of the presence of enduring cocaine-related memories, which in turn require long lasting cellular and molecular adaptations in the brain.

Aim 2: To develop a genome-wide sequencing method that can be used to identify changes in DNA methylation in a small amount of DNA derived from neurons of individual animals. As previously mentioned, examining epigenetic modifications in a region and cell-type specific manner may be key to their identification. Moreover, it is likely that animals vary in their preference for cocaine and this approach could be used to examine both generic changes in DNA methylation that arise from cocaine self-administration, but also differences in methylation that contribute to variability in cocaine self-administration.

Aim 3: To characterize DNA methylation in neurons of the mPFC of mice that have actively self-administered cocaine or been passively exposed to it. The mPFC is a

site of long-lasting changes in gene and protein expression following cocaine self-administration and is necessary for the reinstatement of cocaine seeking following abstinence, which suggests that it plays a central role in enduring cocaine seeking behaviour and may harbor long-lasting epigenetic changes that drive cocaine-seeking behaviour. It is hypothesized that cocaine self-administration will induce changes in DNA methylation that are distinct from those induced by simple exposure to the drug, and equally that these changes in DNA methylation will vary over time.

Aim 4: To characterize the transcriptional changes associated with altered DNA methylation.

To exert an influence on behaviour, it is first necessary for altered DNA methylation to direct changes in gene transcription, that in turn give rise to the changes in cell physiology that direct behaviour. It is hypothesized that where the changes in DNA methylation are associated with known genes, the transcription of these genes will be altered. However, this is likely a complex relationship, which (as outlined in Chapter 2) may be mediated by the reactivation state of the cocaine-associated memories.

Outcomes and significance

In this thesis, I provide the first genome-wide profile of altered DNA methylation arising from voluntary cocaine-self administration and contrast these changes with those that are associated with passive cocaine exposure. Moreover, to identify these changes, I developed a genome-wide sequencing protocol that was recently published and which can be used to profile DNA methylation in a region- and cell type- specific manner (see Chapter 3). Furthermore, in considering how modified DNA methylation might contribute to memory maintenance, I recently posited that persistent modifications of DNA methylation might, in some cases, become functionally silent during memory storage and prime transcription when the corresponding memory is reactivated (see Chapter 2). Overall, cocaine self-administration yields unique persistent changes in DNA methylation in addition to a host of modifications of DNA methylation that arise during abstinence. Further, I find preliminary evidence of transcriptional priming by long-lasting changes in DNA methylation, though this remains an avenue for more rigorous investigation.

CHAPTER 2

Dynamic DNA methylation: a prime candidate for genomic metaplasticity and behavioral adaptation

2.1 Preamble

Prior to examining how cocaine IVSA-induced changes in DNA methylation affect the transcription of proximal genes (Aim 4), I first considered how DNA methylation and gene expression interact more generally in the context of long-term memory maintenance. Learning-induced modifications of DNA methylation are thought to propagate long-term memory by perpetuating long-lasting changes in transcription that in turn alter the synaptic physiology of affected neurons and give rise to 'memory', or a potentiation of communication between neurons. However, if all learning-induced modifications of DNA methylation induced enduring transcriptional changes, neurons might be pushed too far from homeostasis and be rendered aplastic and unable to respond to further stimulation. I therefore proposed that experience-induced modifications of DNA methylation might act as transcriptionally quiescent signatures of previous experience while memory is in storage and instead direct transcriptional responses to further neuronal stimulation or memory reactivation. This hypothesis was recently published as an opinion paper in Trends in Neurosciences and forms Chapter 2 and informed the selection of the experimental groups selected in Chapter 4.

2.2 Dynamic DNA methylation: a prime candidate for genomic metaplasticity and behavioural adaptation

Opinion

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Dynamic DNA methylation: a prime candidate for genomic metaplasticity and behavioral adaptation

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DNA methylation was once considered to be a static epigenetic modification whose primary function was restricted to directing the development of cellular phenotype. However, it is now evident that the methylome is dynamically regulated across the lifespan: during development as a putative mechanism by which early experience leaves a lasting signature on the genome and during adulthood as a function of behavioral adaptation. Here, we propose that experience-dependent variations in DNA methylation, particularly within the context of learning and memory, represent a form of genomic metaplasticity that serves to prime the transcriptional response to later learning-related stimuli and neuronal reactivation.

Introduction

A range of epigenetic modifications, including the covalent modification of DNA by cytosine methylation, confers the transcriptional activity of a given gene. DNA methylation was once considered to be a relatively static epigenetic modification, with its primary function restricted to the regulation of transcriptional programming during early cellular development. However, a surge of recent studies point to a continued role for DNA methylation across the lifespan, particularly with respect to alterations in neuronal gene expression that directly impact behavior [1–8]. Drawing from a conservative developmental perspective, investigations into the function of DNA methylation in the adult brain have predominantly explored instances where learning- or activity-induced changes in methylation within gene promoters correlate with changes in gene expression. Instances where basal levels of gene expression remain unaltered following a change in DNA methylation within the corresponding gene [9,10] have been largely overlooked, which has led to a limited appreciation of the functional variations in DNA methylation, both within gene promoters and elsewhere in the genome. However, recent advances in next-generation sequencing indicate that the relation between DNA methylation and transcriptional activity is more complex than previously realized. In the adult brain, neuronal activity-induced changes in DNA methylation frequently occur outside gene promoters [2], and 5-hydroxymethylcytosine, a newly discovered base derived from 5-methylcytosine that represents a functional intermediary in the active demethylation process [11], accounts for almost half of DNA methylation detected in the brain [12]. Furthermore, DNA methylation can interact with other epigenetic marks to jointly regulate transcription [13,14]. However, the relevance of this expanded repertoire of epigenomic modifications, particularly within the context of behavioral adaptation across the lifespan, remains to be determined.

One of the most remarkable features of the adult brain is its plasticity in response to experience. To have a lasting impact on behavior, learning-induced neuronal activity must be accompanied by a functional reprogramming of gene expression with corresponding modifications of protein synthesis and synaptic connectivity [15]. However, sustained changes in gene expression could severely constrain plasticity and jeopardize the ability of a neuron to respond to later stimuli. Instead, similar to the dormancy of memory until recall, learning-related reprogramming of gene expression may be encoded in the genome and reflected in changes in gene expression only when required, such as during neuronal reactivation. This form of latent responsivity, termed 'metaplasticity', or the plasticity of synaptic plasticity, is a fundamental mechanism of behavioral adaptation [16–18]. Experience-dependent metaplasticity allows prior learning to register a signature that directs later plasticity without disrupting cell homeostasis. For example, reward-seeking behavior is governed by the induction of 'silent' synapses, which do not influence the basal efficacy of synaptic transmission but are prominent mediators of plasticity in response to later stimulation, the result of which is enhanced behavioral sensitivity to subsequent exposure to cues related to prior learning [18].

Although the existence of metaplasticity has been recognized for some time, the molecular mechanisms underpinning this adaptation are largely unknown. We propose that activity-induced variations in DNA methylation, particularly within the context of learning and memory, represent a form of genomic metaplasticity that serves to prime the transcriptional response to later neuronal activation. In collaboration with other epigenetic marks, experience-dependent changes in DNA methylation would direct later transcription and plasticity in several ways, including the regulation of alternative splicing [19] and transposable elements [20], the development of bivalent chromatin marks that render genes poised for transcriptional activity [21],

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or by directing nucleosome repositioning to bookmark recently activated genes [22]. DNA methylation is intimately related to the functional capacity of the genome and may therefore contribute substantially to behavioral adaptation across the lifespan through its direct effects on neural plasticity and cognition.

Mechanisms of dynamic DNA methylation

The activity of three DNA methyltransferases, DNMT1, DNMT3a, and DNMT3b, regulate cytosine methylation in mammals. DNMT1 is considered to be a maintenance methyltransferase, whereas DNMT3a and DNMT3b mediate *de novo* methylation. Although each of these enzymes plays a crucial role in establishing genomic methylation patterns during early neurodevelopment, only DNMT1 and DNMT3a are expressed in mature neurons, where they appear to play a complementary role in regulating synaptic plasticity [23] (Figure 1).

Active DNA demethylation permits the dynamic regulation of the methylome in response to neuronal activity [2,24,25] and learning [26]. Active demethylation involves enzymatic removal of 5-methylcytosine and occurs via several mechanisms, including: (i) deamination of 5-methylcytosine to thymine by activation-induced cytidine deaminase (AID) [27] and subsequent removal of a T–G mismatch by the base excision repair pathway (Figure 2a); (ii) conversion of 5-methylcytosine to 5-hydroxymethylcytosine by the ten-eleven translocation 1-3 (Tet1-3) family of DNA hydroxylases followed by base excision repair [24] (Figure 2b); or (iii) nucleotide excision repair, which involves Gadd45a [28] (Figure 2c). Moreover, it is likely that these mechanisms act in conjunction with each other to dynamically regulate DNA demethylation and the transcriptional activity at a specific genomic locus.

DNA methylation and cellular differentiation

A tightly timed interplay between DNA methylation, hydroxymethylation, and active demethylation regulates gene expression and cellular differentiation in the developing nervous system [29–31]. Pluripotency is strongly associated with high levels of 5-hydroxymethylcytosine in embryonic stem cells [32,33] and the loss of 5-hydroxymethylcytosine and subsequent accumulation of 5-methylcytosine corresponds with lineage commitment [32]. Critical developmental stage-specific patterns of DNMT expression further reflect the importance of DNA methylation in directing early neurodevelopment [34]. For example, active demethylation within the promoters of several astrocytic markers [35,36] directs astrocyte lineage commitment from pluripotent neural precursor cells. Furthermore, epigenetic reprogramming via DNA methylation is required for the development of neural precursor cells [37] and is associated with neuronal differentiation [38]. Based on these observations, it is widely believed that the induction, or loss, of DNA methylation during early development drives unidirectional and sustained changes in gene expression, which ultimately give rise to cellular identity [30]. However, differentiating neurons retain a comparatively high level of 5-hydroxymethylcytosine, indicating that perhaps the neuronal methylome retains a greater degree of plasticity throughout development [33]. In humans, neurons show significantly greater interindividual variation compared with non-neuronal cells of the brain, supporting the idea that the neuronal methylome may have an enhanced propensity for plasticity in response to environmental cues [39].

The relation between DNA methylation and gene expression in development is also more complex than previously appreciated. For example, although promoter methylation appears to be a key regulator of cell type-specific programming [29,40], recent evidence suggests that non-promoter DNA methylation also coordinates the expression of neurogenic genes [41]. Moreover, the association between promoter methylation and gene expression appears to be contingent on CpG density [42], and there are instances where altered DNA methylation fails to

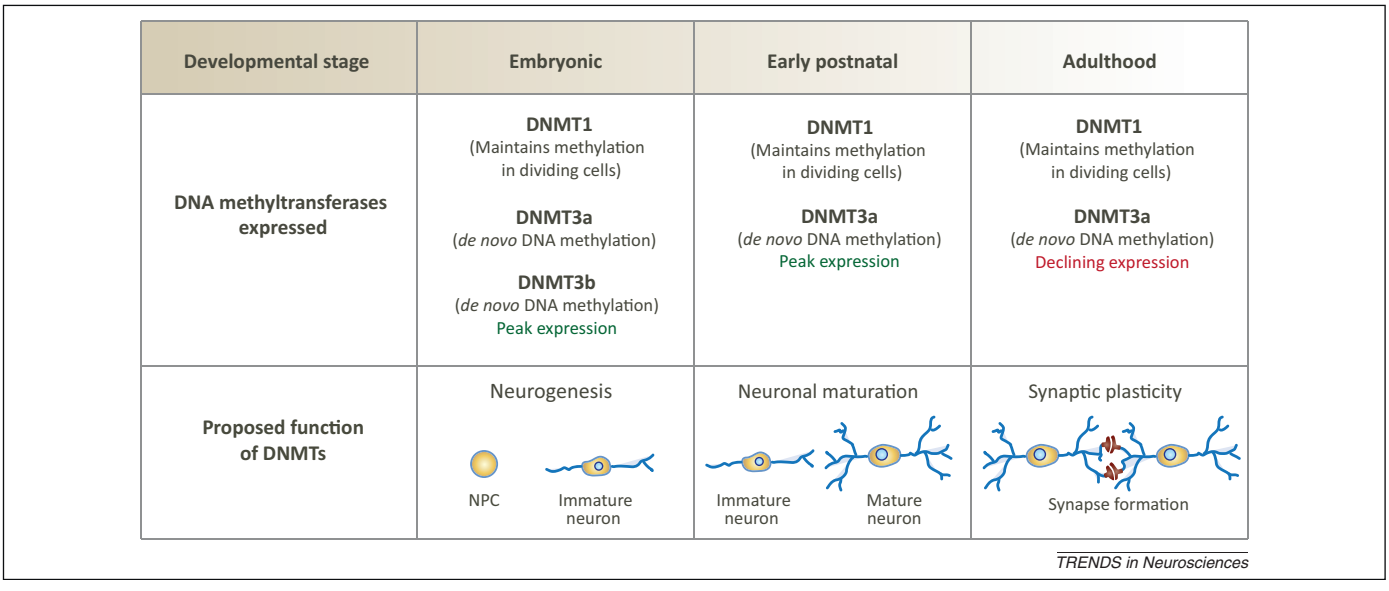

Figure 1. DNA methylation across the lifespan. DNA methylation is mediated by two *de novo* DNA methyltransferases, DNMT3a and DNMT3b, and one maintenance methyltransferase, DNMT1. The expression of these DNMTs varies across the lifespan: the expression of DNMT3b is restricted to embryonic development and corresponds to a period of early neurogenesis, whereas an increase in DNMT3a expression coincides with early postnatal neuronal maturation [34]. DNMT3a and DNMT1 are expressed in the CNS throughout the lifespan and may be important for synaptic plasticity [5,34]. Abbreviation: NPC, neural progenitor cell.

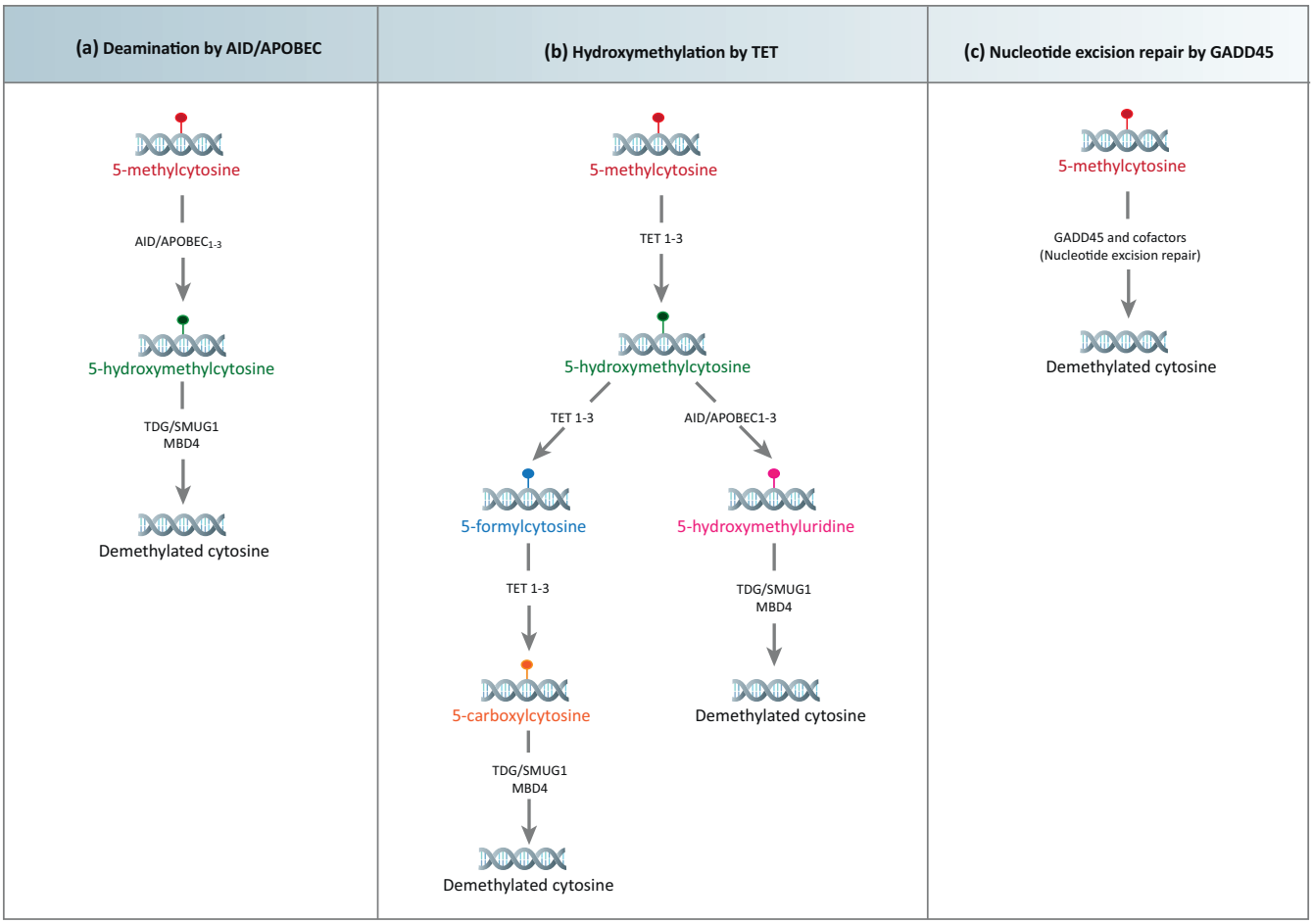

Figure 2. Mechanisms of active DNA demethylation. Active demethylation occurs by several different mechanisms, including base excision repair (BER) pathways **(a,b)** and nucleotide excision repair (NER) **(c)**. (a) Deamination of 5-methylcytosine by AID/APOBEC yields thymine, which is excised by mammalian T–G-specific glycosylases (TDG, SMUG1, or MBD4) through BER [27]. (b) 5-Methylcytosine can also be oxidized by the ten-eleven translocation (TET) family of enzymes to form 5-hydroxymethylcytosine [24]. 5-Hydroxymethylcytosine can then be further oxidized to 5-formylcytosine and 5-carboxylcytosine, or converted to 5-hydroxymethyluridine by AID/APOBEC. Excision of these intermediates is initiated by mammalian T–G specific glycosylases (TDG, SMUG1, or MBD4), resulting in replacement with an unmethylated cytosine. (c) GADD45 and cofactors can also remove 5-methylcytosine by NER [28]. Abbreviations: AID, activation-induced cytidine deaminase; $APOBEC_{1–3}$, apolipoprotein B mRNA editing enzyme-catalytic polypeptides 1–3; GADD45, growth arrest and DNA damage-inducible protein 45; MBD4, methyl-CpG-binding domain protein 4; SMUG1, single-strand-selective monofunctional uracil-DNA glycosylase 1; TDG, thymine DNA glycosylase.

coincide with gene expression [43]. This has prompted the suggestion that DNA methylation does not directly regulate transcription but rather serves as a signal for the long-term maintenance of gene silencing [44]. Taken together, these observations indicate the existence of context-specific variations in DNA methylation and associated epigenetic marks, the functional relevance of which has yet to be fully revealed.

DNA methylation and early development

Stimuli in the pre- and postnatal environments have significant effects on gene expression, which persist long after the initial stimulus has dissipated. Dynamic regulation of the methylome provides an underlying epigenetic signature of early-life experience that could support these sustained changes in gene expression. In rats and mice, prenatal stress [45], maternal cocaine exposure [46], and parental enrichment [47] give rise to changes in promoter methylation within a subset of genes in offspring, which correlate with lasting changes in transcriptional activity. Similarly, postnatal developmental perturbation, such as infant maltreatment [48] or maternal deprivation [49–52], results in corresponding changes in DNA methylation and gene expression that persist into adulthood and are accompanied by enduring behavioral phenotypes. However, variations in 5-methylcytosine levels incurred as a function of early-life experience can be functionally reversed in rats through methyl supplementation at later time points [52,53]. Therefore, the evidence suggests that, in contrast to the proposed role of static DNA methylation in determining cell fate, postnatal epigenetic reprogramming via DNA methylation is an active process that is dynamically regulated across the lifespan.

DNA methylation and behavioral adaptation in adulthood

It has been proposed that long-term memory is contingent on transcriptional regulation that is both stable and self-perpetuating, two characteristics traditionally ascribed to DNA methylation. Less than a decade ago, evidence

Table 1. Examples of the dynamic regulation of DNA methylation in the adult rodent brain that have been associated with altered behaviors[a]

Behavior	Impaired and/or enhanced	Experimental manipulation	Gene	Locus	5-mC	Brain region	Refs
Fear-related learning and memory	↑	WT	*PP1*	Promoter	↑	HPC	[8]
			Reelin	Promoter	↓	HPC	
			CaN	Promoter	↑	ACC	[7]
			BDNF	Exon	↓	HPC	[57]
			BDNF	Promoter	↑/↓	HPC	[26]
	↑	DNMT3A overexpression	N/a	Euchromatin	↑	HPC	[103]
	↓	DNMT inhibition	N/a	N/a	N/a	AMG	[59]
			N/a	N/a	↓	HPC	[3]
	N/a	Fear conditioning	Zif268	Promoter	↑	HPC	[14]
Drug-seeking and/or preference	↓	DNMT inhibition	N/a	N/a	↓	NAc	[74]
Stress reactivity and/or anxiety	↑	Maternal deprivation	*Crh*	Promoter	↓	PVN	[9]
			Avp	Enhancer	↓	PVN	[50]
	↑	Maternal care	$Gr1_7$	Promoter	↑	HPC	[51]
	↑/↓	Methionine and/or TSA	$Gr1_7$	Promoter	↑/↓	HPC	[52]

[a]Abbreviations: ACC, anterior cingulate cortex; AMG, amygdala; Avp, arginine vasopressin; BDNF, brain-derived neurotrophic factor; CaN, calcineurin; DG, dentate gyrus; Erα1β; estrogen receptor alpha 1-beta; $Gr1_7$, exon 1(7) glucocorticoid receptor; HPC, hippocampus; MPA, medial pre-optic area; N/a, not applicable; NAc, nucleus accumbens; PVN, paraventricular nucleus; PP1, protein phosphatase 1; TSA, trichostatin A; WT, wild type.

emerged contradicting the prevailing model for an exclusive role of DNA methylation in development: the expression of *de novo* DNMTs remained unexpectedly high in post-mitotic neurons [34], early maternal care generated persistent gene-specific changes in DNA methylation that were associated with stress vulnerability in adulthood [51], and neuronal activity-induced demethylation was observed both *in vitro* and in the adult brain [54]. Together, these findings indicated that the molecular machinery driving variations in DNA methylation is present in the postnatal brain and is responsive to experience and may therefore be enlisted to perpetuate the learning-induced changes in gene expression that underlie long-term memory. As summarized in Table 1, this possibility has become the focal point of investigations into learning-related changes in DNA methylation [7,8,23,55–57]. For example, acute regulation of DNA methylation occurs as a function of learning [8,26] and following the induction of long-term potentiation (LTP) [58], the accepted cellular analog of memory-related plasticity. DNMT3a expression is upregulated in many learning paradigms [5,59], as well as following the artificial induction of synaptic plasticity [57]. Furthermore, although transient learning-induced modifications of DNA methylation occur in the hippocampus following contextual fear conditioning [8], persistent alterations develop within the prefrontal cortex following an associative learning task [7]. Local inhibition of the maintenance of altered methylation incurs memory deficits, suggesting that cortical DNA methylation is enlisted by the brain to preserve remote memories [7].

As a caveat, the role of DNA methylation in maintaining memory may be structure and locus specific; within the hippocampus both relatively persistent [57] and transient [8] learning-induced changes in DNA methylation occur, suggesting that this modification performs dissociable roles in the formation and maintenance of memory. In addition, DNA methylation is one component of the chromatin environment and can interact with other epigenetic modifications to regulate transcription [13]. Furthermore, concomittant changes in activating or repressing pathways can obscure the relation between DNA methylation and gene expression. Nevertheless, a primary consideration within the context of memory maintenance is how a single neuron, regardless of anatomical region, would cope with enduring changes in the methylome that manifest in lasting cell-wide transcriptional consequences. We propose that DNA methylation constitutes a mark of prior neuronal and transcriptional activity that contributes to memory maintenance by altering genomic responsivity to later neuronal activation.

Dynamic DNA methylation as a mechanism for genomic metaplasticity

Many theories of memory storage suggest that the same network of neurons encodes multiple memories, which retain their independence by enlisting distinct synapses of each neuron. Consequently, cell-wide changes, such as the persistent changes in gene transcription arising from learning-induced changes in DNA methylation [7], would be likely to perturb the maintenance of all memory traces encoded by a given neuron, including those encoded during prior learning. One possibility is that, rather than contributing to the maintenance of a unique memory, persistent changes in DNA methylation alter the ability of a neuron to respond to later stimuli, presumably through lasting transcriptional changes [7,54,60]. According to this hypothesis, enduring epigenetic marks would render a neuron aplastic and stabilize synaptic weights through enduring changes in the transcriptome, conferring responsivity to selective inputs by modulating the degree of plasticity at all other synapses [55]. However, this would both severely restrict the storage capacity of the brain and entail a substantial disruption of cell homeostasis. Instead, we propose that experience-dependent variations in DNA methylation represent a form of metaplasticity that primes the genome for response to later events by regulating transcriptional efficacy in response to incoming inputs, rather than by mediating enduring changes in gene expression (Figure 3).

Priming of the transcriptional response by active DNA demethylation has been clearly demonstrated in mammalian systems [61,62]. For example, in rat hepatic cells, glucocorticoid stimulation can initiate the expression

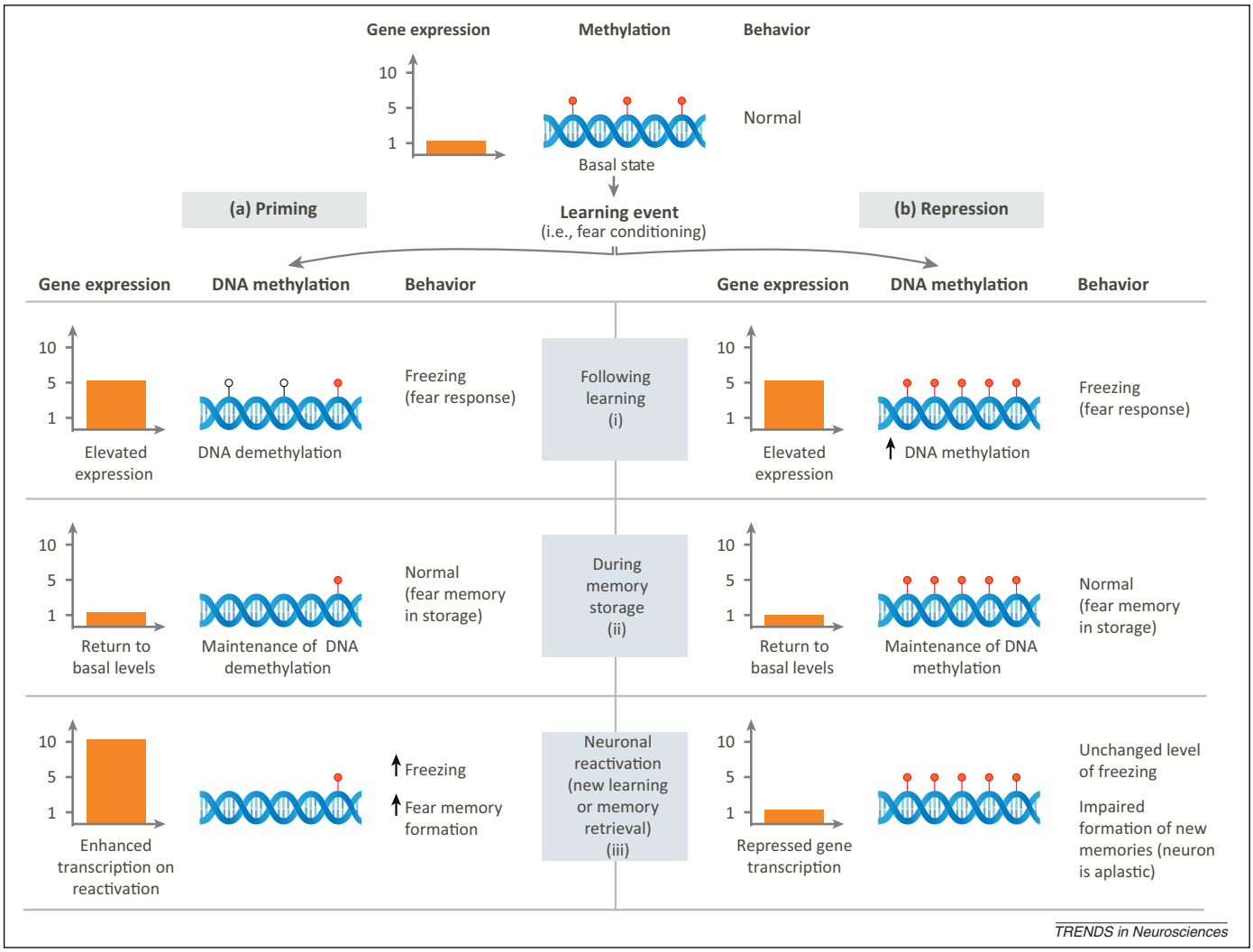

Figure 3. Hypothetical model of the metaplastic priming and repression of gene transcription by changes in DNA methylation. **(a)** Priming: following a learning event (i) or neuronal activation, the expression of a gene is elevated and learning is acquired. More transient epigenetic modifications, such as histone acetylation, may be responsible for the initial burst in expression and DNA demethylation may occur subsequent to transcription. In contrast to current models, the expression of the demethylated gene could return to baseline in the absence of the initial activating stimulus or when the memory is stored (ii). However, persistent DNA demethylation may prime gene transcription upon reactivation of the neuron by memory retrieval or by new learning (iii), potentially enhancing expression relative to the response elicited by initial activation and learning. The priming of a transcriptional response by active DNA demethylation has been clearly demonstrated in other mammalian systems [61,62] and in the nervous system [9]. In the case of memory-enhancing genes, metaplastic priming could facilitate the encoding of new memories, although the priming of memory suppressors could impair the formation of new associations by previously activated neurons. **(b)** Repression: (i) as in the case of priming, increased DNA methylation could occur subsequent to learning- or activation-induced changes in gene transcription, driven by more rapid modifications, such as the binding of transcription factors or histone modifications, which are known to work in concert with DNA methylation to regulate transcription [13]. Alternatively, rapid DNA methylation could promote transcription by preventing the binding of a repressor protein. However, according to our hypothesis, while the memory is dormant and the neuron inactive (ii), a methylation-mediated change in the transcriptional response is not evident and the expression of the inhibited gene returns to baseline. Nevertheless, in response to further stimulation (iii), the transcriptional activity of the gene is suppressed by persistent learning-induced augmentation of DNA methylation, rendering the neuron aplastic and unable to encode new associations. The methylation-induced blunting of the transcriptional response to subsequent stimulation has been observed in the hypothalamic proopiomelanocortin (POMC) promoter, where overfeeding leads to hypermethylation and prevents the increase in transcription in response to high insulin levels [101]. As a caveat of our hypothesis, the effect of DNA methylation or demethylation on the responsivity of the neuron would be locus and gene specific; for instance, by giving rise to alternative splice variants, *de novo* DNA methylation could prime neuronal activity. Furthermore, DNA methylation may enhance transcription by preventing the binding of a repressor protein. However, the primary difference with current models is that DNA methylation or demethylation does not result in persistent changes in gene expression, but rather these changes in expression manifest at the time of neuronal reactivation and affect the ability of the neuron to encode new associations.

and demethylation of an enhancer region proximal to the gene encoding liver-specific tyrosine aminotransferase (*Tat*). Although this modification is stable for up to 3 months, the expression of *Tat* returns to basal levels upon withdrawal of glucocorticoid stimulation. Nevertheless, the capacity for transcriptional activity is primed by DNA demethylation and, upon subsequent glucocorticoid stimulation, the expression of *Tat* is three- to fivefold greater than in previously unstimulated cells [61]. Therefore, there is direct evidence of genomic metaplasticity by active DNA demethylation. Demethylation-dependent transcriptional priming is also evident in the nervous system. In rats, maternal deprivation induces demethylation of corticotropin-releasing hormone (Crh) promoter. However, this change in methylation is not reflected in altered levels of Crh expression until the animals are subject to acute stress, upon which Crh is significantly upregulated [9]. As described below, DNA methylation could contribute to genomic metaplasticity in a variety of ways.

Regulation of alternative splicing

Alternative splicing contributes to the formation and maintenance of memory by fine-tuning receptor composition and ion channel properties following neuronal depolarization [63,64], providing a subtle mechanism for regulating synaptic strength. Moreover, the induction of alternative splicing in response to prior events, such as exposure to stress, modulates the acquisition of new learning and the maintenance of memory by regulating LTP [65], providing a clear example of metaplasticity. Unsurprisingly, a significant percentage of neuronal activity-induced changes in DNA methylation occur in intragenic regions of the genome [2], which is consistent with a conserved role for intragenic methylation in regulating alternative splicing [66].

DNA methylation directs alternative splicing by manipulating the kinetics of RNA polymerase II, an enzyme that catalyzes gene transcription (Figure 4a). In the context of neuronal plasticity, one possibility is that learning-induced changes in DNA methylation persistently up- or downregulate the expression of alternative splice variants to ultimately determine the responsivity of a neuron to new stimulation. An important caveat of this is that enduring changes in DNA methylation may not be reflected in an overall change in the expression of a given gene, but instead appear as an altered ratio of splice variant expression [26]. A second possibility, in agreement with the idea of quiescent metaplastic modifications, is that DNA methylation could regulate the expression of various splice variants at specific points in time, such as when the gene is retranscribed following memory retrieval or during the formation of new memories. The presence of different learning-induced splice variants could determine whether novel information is retained [65] by promoting or inhibiting the changes in synaptic strength that underlie memory maintenance. Accordingly, by regulating alternative splicing, activity-induced modifications of DNA methylation provide an example of genomic metaplasticity that ultimately determines the responsivity of a neuron to future stimuli.

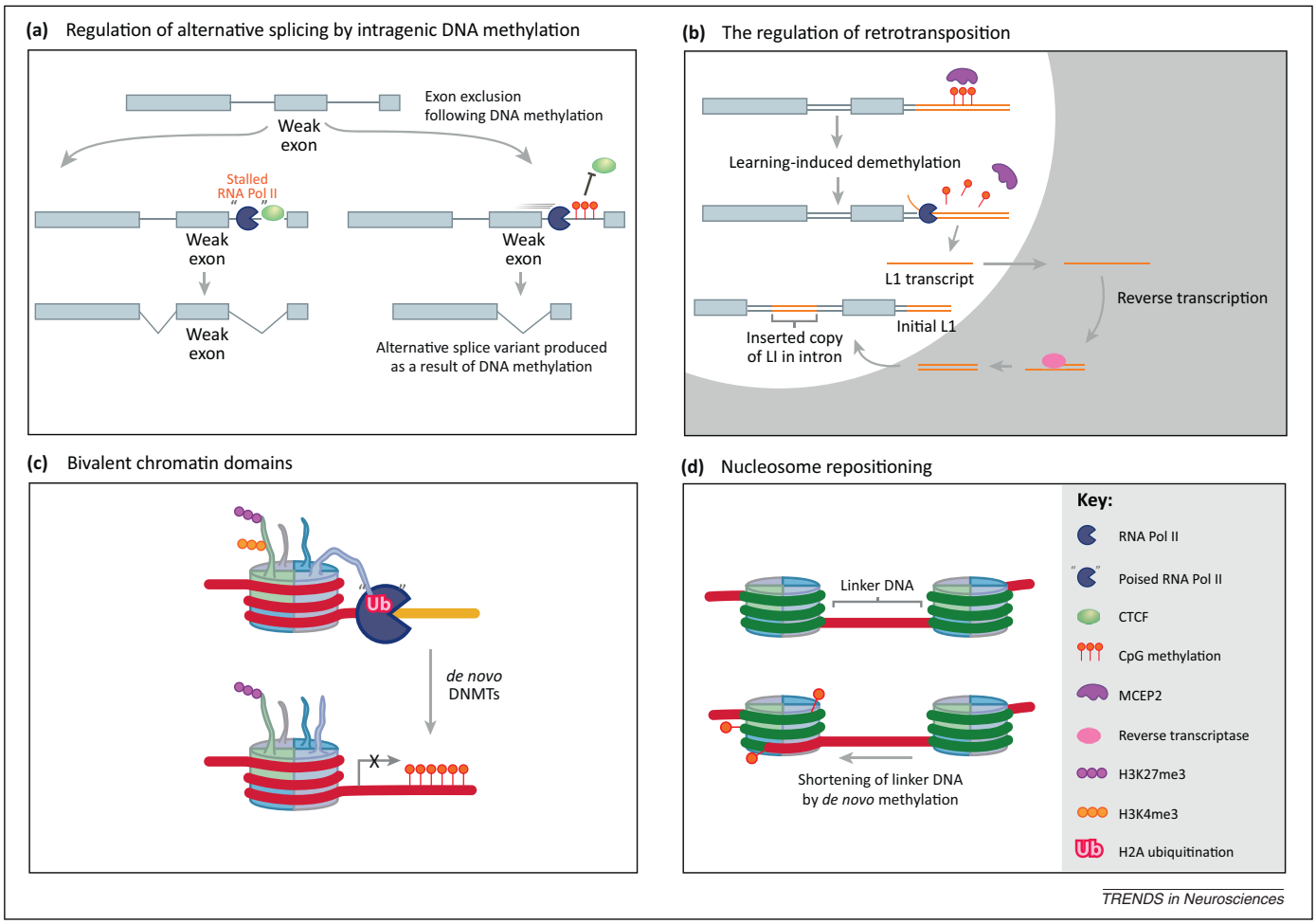

Figure 4. Select mechanisms by which learning-induced variations in DNA methylation can direct genomic metaplasticity. Experience-dependent modifications of DNA methylation could prime future transcription in several ways. **(a)** Alternative splicing: DNA methylation prompts the formation of alternative splice variants by manipulating the kinetics of RNA polymerase II (RNA Pol II). *De novo* DNA methylation prevents CTCF binding and the CTCF-contingent pausing of RNA Pol II. In the absence of CTCF-mediated RNA Pol II stalling, transcriptional elongation is impaired and the subsequent transcription of weak exons is inhibited [19]. Therefore, by preventing CTCF binding, DNA methylation can yield alternative splice variants. **(b)** The regulation of retrotransposition: DNA demethylation may permit the transcription and insertion of L1 retrotransposons into various regions of the genome. Insertion of L1 copies affect gene length, where increased gene length is associated with reduced transcription [70]. **(c)** The development of bivalent domains: *De novo* DNA methylation resolves poised bivalent chromatin domains at promoter regions, leading to gene repression [32]. Bivalent domains are characterized by an activating histone mark (H3K4me3) and a repressive histone mark (H3K27me3), with RNA Pol II tethered at the domain by H2A ubiquitination [102]. **(d)** Nucleosome repositioning: DNA methylation can induce the shortening of linker DNA, thereby increasing internucleosomal interactions and priming clusters in interrelated genes. The addition of a methyl group at the 5′ position of the cytosine decreases the flexibility of DNA, which prompts more DNA to be trapped within the nucleosome, thereby shortening the linker region [90]. Abbreviations: CTCF, CCCTC-binding factor; DNMT, DNA methyltransferase; H3K4me3, histone H3 lysine 4 trimethylation; H3K27me3, histone H3 lysine 27 trimethylation; L1, long interspersed nuclear element 1; MCEP2, methyl CpG binding protein 2.

Regulation of transposable elements

Another example of experience-induced genomic metaplasticity is the movement of transposable elements. The insertion or deletion of a single transposable element can influence gene expression by introducing novel alternative promoter regions, enhancer elements, transcription factor binding sites, premature polyadenylation [67], or by promoting the formation of heterochromatin [68]. Equally, the insertion of several transposable elements can affect transcriptional efficacy by altering gene and/or intron length [69] (Figure 4b), whereby reduced gene length is associated with more highly expressed genes [70]. Although it was previously thought that retrotransposition occurred primarily during early embryogenesis [71], it has now been demonstrated that the expression of a retrotransposon termed 'long interspersed nuclear element 1' (L1) continues during adulthood and is elevated in the brain [20,72,73]. L1 retrotransposition occurs in response to a range of environmental stimuli, including voluntary exercise and chronic cocaine exposure [74,75], and it has recently been proposed to generate the unique experience-dependent transcriptome profile of individual neurons [76]. L1 transcription is repressed by region-specific DNA methylation in the 5′ untranscribed region (UTR) [77] and, accordingly, in neural precursors and differentiated neurons, by the expression of methyl CpG binding protein 2 (MeCP2) [20,78]. Furthermore, L1 elements undergo an age-related depletion of 5-hydroxymethylcytosine in the hippocampus [12], which may reflect reduced plasticity.

Perhaps the most interesting implication of the regulation of transposable elements by DNA methylation is that, to have an enduring and pervasive effect on gene transcription, methylation or demethylation need only occur transiently and allow the movement of transposable elements. In directing the movement of transposable elements, learning-related changes in DNA methylation provide an excellent illustration of genomic metaplasticity: the accumulation of retrotransposons may generate a silent signature of prior neuronal activity that affects memory maintenance and later plasticity. The metaplastic modulation of memory-related genes could be reflected by enduring up- or down-regulation of the affected gene, or be revealed upon future transcription, through alternative splicing (alternative promoter insertion) and enhanced or weakened transcription (insertion of enhancer or insulator elements, or decreased and/or increased gene length), all of which would have lasting consequences for memory maintenance.

Regulation of bivalent chromatin domains

Bivalent chromatin domains are characterized by the presence of a repressive histone modification [trimethylation of histone H3 lysine 27 (H3K27me3)] interspersed with active histone marks [trimethylation of histone H3 lysine 4 (H3K4me3)], which produce a silent but transcriptionally poised state [21] that is characteristic of metaplasticity (Figure 4c). Bivalent promoters are further distinguished by the accumulation of 5-hydroxymethylcytosine and a corresponding depletion of 5-methylcytosine [79]. Traditionally, bivalent states occur within developmental genes that are primed to respond to regulatory cues and, during early development, de *novo* DNA methylation resolves bivalent states to silence genes over time [30]. However, approximately 40% of bivalent domains are retained in terminally differentiated neurons [30], which suggests a continued propensity for this form of plasticity in post-mitotic neurons. The greater degree of 5-hydroxymethylcytosine retained in neurons [33] may protect bivalent domains from becoming permenantly silenced by *de novo* methylation, as it does in other cell types [80]. Moreover, activity-dependent demethylation could permit the further reinstatement of bivalent chromatin domains, rendering genes poised for future activation. In several model cellular systems, pharmacologically induced demethylation by 5-aza-2′ deoxycytidine, a DNMT inhibitor, allows formerly hypermethylated genes to regain a bivalent state by increasing the presence of H3K4me3 [81–83], although this remains to be demonstrated in non-dividing cells. These data further suggest that the processes regulating DNA methylation function synergistically with post-translational modification of histones to promote genomic plasticity, an idea that has been echoed in the context of learning and memory [13,14]. Together with evidence for an age-dependent change in DNA methylation at bivalent domains [84], it appears that the experience-dependent development of bivalent chromatin states, which occurs as a function of active variations in DNA methylation, may perform a metaplastic function by priming a gene for activation without necessarily influencing basal levels of gene expression.

Regulation of nucleosome positioning

Nucleosome repositioning is another mechanism by which altered DNA methylation may prime a gene for transcription. DNA coils around an octamer of condensed histone proteins to form a nucleosome, with each nucleosome separated by a 20–50-base pair (bp) linker region of DNA. Nucleosomal arrangement within neurons is unique in that the distance between nucleosomes is appreciably shorter than that in other cells of the brain [85]. This characteristic emerges at the point of neuronal maturation [86] and may poise the neuronal genome for enhanced plasticity, because reduced linker DNA length facilitates internucleosomal interactions [87]. Consequently, a change in the relative position of one nucleosome impacts the positioning of neighboring nucleosomes to a greater extent [87], potentially providing a mode of transcriptionally priming or repressing clusters of interrelated genes.

In the aging rat brain, an increase in nucleosome repeat length [88] coincides with a loss of DNA methylation [89], which suggests a potential relation. Mechanistically, there are multiple levels at which DNA methylation can influence nucleosome positioning. DNA methylation can decrease the flexibility of DNA to interfere with the exaggerated bending of DNA required to form nucleosomes [90], resulting in a further shortening of the regions of linker DNA [91] (Figure 4d) that can alter the conformational space of a gene [92]. Moreover, activity-dependent demethylation could regulate the relative position of a recently transcribed gene within the nucleus. DNA demethylation can facilitate the incorporation of histone variants, such as H2A.Z [22], which directly oppose DNA

methylation [93] and also coordinate the repositioning of nucleosomes associated with recently activated exons to the nuclear periphery [94,95]. Although the regulation of nucleosome repositioning by DNA methylation or demethylation has yet to be demonstrated *in vivo*, the relocation of genes to a location within the nucleus could serve to either prime or repression expression [96] upon subsequent stimulation of the neuron. These observations point to a potential role for DNA methylation in the regulation of nucleosome repositioning and experience-dependent genomic plasticity, as a result of interactions with ATP-dependent chromatin remodeling and the deposition of non-canonical histone variants.

Genomic metaplasticity: the epigenome and beyond

The neuronal methylome is embedded within a complex epigenetic environment, comprising many modifications, including histone acetylation, methylation, and a myriad other epigenetic marks. DNA methylation can act synergistically with numerous epigenetic modifications [14,56] to form an 'epigenetic code' that can regulate synaptic plasticity [60]. Consequently, a learning-induced transcriptional event would be encoded by the comprehensive epigenetic environment surrounding a given gene [97], rather than by the covalent modification of DNA in isolation. However, to support genomic metaplasticity, an epigenetic modification must be relatively enduring yet possess the potential for plasticity; two key characteristics of DNA methylation, the latter of which has only recently come to light in the context of the nervous system [2,24,25,54]. These recent discoveries suggest that the neuronal methylome is a prime candidate for investigations into the molecular underpinnings of metaplasticity; however, the study of this adaptation should also include its relation with other epigenetic mechanisms, as described in Box 1.

Box 1. Outstanding questions

- **How do other epigenetic modifications interact with DNA methylation to regulate genomic metaplasticity at a given locus?** Particular attention should be directed to enduring epigenetic modifications, such as histone methylation, which is one of the few histone modifications that has been shown to be modified in an enduring fashion following learning [14,104]. Histone methylation may both direct DNA methylation and be reinforced by DNA methylation by way of a positive feedback loop [105], and therefore may jointly regulate long-term changes in gene transcription. Noncoding RNAs may further contribute to the maintenance of DNA methylation and demethylation [106].

  Improved genome-wide sequencing techniques have become available to elucidate the contribution of DNA methylation to experience-dependent genomic metaplasticity. These include approaches such as oxidative bisulfite-seq for the detection of 5-hydroxymethylcytosine [107], RNA-capture approaches for assessing retrotransposition events [108], bisulfite-seq on immunoprecipitated DNA to determine the contribution of DNA methylation toward the development of bivalent chromatin domains [109], as well as Nucleosome Occupancy Methylome sequencing (NOMe-seq) to explore methylation-mediated nucleosome repositioning [110]. Furthermore, some of these techniques are compatible with the analysis of small amounts of DNA or RNA, which could reveal the epigenetic signature of learning-induced neuronal activation *in vivo* and yield better resolution of the alterations in DNA methylation that support discrete memory traces
- **Is genomic metaplasticity possible at all genes, throughout all periods of development or do certain genes have an increased propensity for plasticity at key periods in time?**
- **Is genomic metaplasticity dysregulated in neuropsychiatric diseases (such as addiction) that are marked by decreased cognitive flexibility? If so, is it possible to re-initiate plasticity to alleviate symptoms?**
  Recent work has shown that cocaine exposure epigenetically primes the expression of ΔFosB in response to subsequent cocaine challenge [98], although the mechanisms supporting this response require further investigation. Lasting gene-specific changes in DNA methylation incurred during early development have also been shown to prime gene expression and responsivity to stress in adulthood in rodents [9].
- **How is DNA methylation and/or demethylation directed to specific sites in the genome?**
  Noncoding RNAs proximal to the recently transcribed genes could direct DNA methylation or demethylation [106], although this remains to be elucidated.

Concluding remarks

Investigations into the functional relevance of DNA methylation continue to reveal a role for dynamic regulation of the methylome across the lifespan. However, the guiding principles of these early studies have been based on a developmental perspective, where DNA methylation is thought to restrict plasticity and stabilize changes in gene expression to give rise to cellular identity. By contrast, a critical feature of the adult brain is continued plasticity, which is predicated by an enduring capacity for dynamic regulation in response to environmental stimuli. To date, understanding of the adaptive significance of learning-related changes in DNA methylation has been restricted to the study of candidate genes that demonstrate concomitant changes in DNA methylation and gene expression. However, this approach affords limited insight into the true plasticity of the methylome. Genome-wide sequencing has revealed that a host of activity-modified CpGs occur within regions of the genome that may not engender persistent, cell-wide changes in transcription, but rather prime the genome to respond to future stimuli.

Although direct demonstrations of DNA methylation-mediated genomic metaplasticity within the context of learning and memory are limited, emerging evidence suggests that the priming of genomic capacity by epigenetic modifications accompanies the development of certain psychiatric disorders, such as drug addiction [98–100]. A deeper understanding of the dynamic regulation of DNA methylation and its associated epigenetic marks across the lifespan is on the horizon, which will eventually lead to a clearer picture of gene–epigenome–environmental interactions and behavioral adaptation across the lifespan. It is evident that this epigenetic mechanism has many fundamental biological and functional roles yet to be explained and it may be within cognition, memory, and the fine-tuning of genomic metaplasticity where the influence of dynamic DNA methylation will be most significant.

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CHAPTER 3

Methyl CpG binding domain ultra-sequencing: a novel method for identifying inter-individual and cell-type-specific variation in DNA methylation

3.1 Preamble

As modifications of DNA methylation associated with cocaine self-administration (IVSA) likely arise within a distinct subset of neurons located in specific regions of the brain, performing whole genome analysis of DNA methylation on whole brain or whole region homogenates might prevent the detection of such discrete changes in DNA methylation. To facilitate the detection of IVSA-associated changes, I chose to examine modifications of DNA methylation within neurons of the mPFC. Unfortunately, I was first unable to achieve satisfactory labelling and sorting of neurons and I therefore drew from several methods to create a superior and easier method of labelling and sorting neuronal nuclei by fluorescent activated cell sorting (FACS). Secondly, the amount of DNA retrieved from the mPFC neurons of individual animals was insufficient for the application of existing genome-wide methods of identifying changes in DNA methylation. To apply existing techniques would have required the pooling of DNA from multiple animals and result in the loss of DNA methylation detection within individual animals. To circumvent this problem, myself and another student modified a DNA-barcoding technique, which assigns unique identifiers to strands of DNA from specific animals. Using this technique, we could pool DNA from multiple animals and use a methyl binding domain-based enrichment approach to isolate fragments of DNA possessing a moderate amount of DNA methylation. These fragments are then sequenced and the level of 5-methylcytosine enrichment is compared between groups, which identifies genomic loci that have been differentially methylated between conditions. The entire protocol, from the isolation of neuronal nuclei to the validation of sequencing results was recently published as a methods paper in Genes, Brain and Behaviour and is included as Chapter 3. MBD Ultra-Seq was subsequently used for the identification of IVSA-associated changes in DNA methylation in Chapter 4.

3.2 MBD Ultra-Seq

Methyl CpG Binding Domain Ultra-Sequencing: a novel method for identifying inter-individual and cell-type-specific variation in DNA methylation

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Experience-dependent changes in DNA methylation can exert profound effects on neuronal function and behaviour. A single learning event can induce a variety of DNA modifications within the neuronal genome, some of which may be common to all individuals experiencing the event, whereas others may occur in a subset of individuals. Variations in experience-induced DNA methylation may subsequently confer increased vulnerability or resilience to the development of neuropsychiatric disorders. However, the detection of experience-dependent changes in DNA methylation in the brain has been hindered by the interrogation of heterogeneous cell populations, regional differences in epigenetic states and the use of pooled tissue obtained from multiple individuals. Methyl CpG Binding Domain Ultra-Sequencing (MBD Ultra-Seq) overcomes current limitations on genome-wide epigenetic profiling by incorporating fluorescence-activated cell sorting and sample-specific barcoding to examine cell-type-specific CpG methylation in discrete brain regions of individuals. We demonstrate the value of this method by characterizing differences in 5-methylcytosine (5mC) in neurons and non-neurons of the ventromedial prefrontal cortex of individual adult C57BL/6 mice, using as little as 50 ng of genomic DNA per sample. We find that the neuronal methylome is characterized by greater CpG methylation as well as the enrichment of 5mC within intergenic loci. In conclusion, MBD Ultra-Seq is a robust method for detecting DNA methylation in neurons derived from discrete brain regions of individual animals. This protocol will facilitate the detection of experience-dependent changes in DNA methylation in a variety of behavioural paradigms and help identify aberrant experience-induced DNA methylation that may underlie risk and resiliency to neuropsychiatric disease.

Keywords: DNA methylation, genome-wide, MBD, NeuN, neuron, next-generation sequencing

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Experience-induced changes in DNA methylation are associated with the formation and maintenance of memory (Miller & Sweatt 2007; Miller *et al.* 2010; Vanyushi *et al.* 1974), vulnerability to neuropsychiatric disorders following adverse early life experiences (Chen *et al.* 2012; Labonte *et al.* 2012; Murgatroyd *et al.* 2009; Weaver *et al.* 2004), as well as the development of addiction (Anier *et al.* 2010; Day *et al.* 2013; Muschler *et al.* 2010; Tian *et al.* 2012) and fear-related anxiety disorders (Kang *et al.* 2013; Labonte *et al.* 2013). Accordingly, epigenome-wide association studies stand to reveal a host of novel biomarkers of susceptibility to psychiatric illness and to shed light on common mechanisms of memory formation and maintenance. However, because of technical limitations, genome-wide investigations of experience-dependent changes in DNA methylation *in vivo* have predominantly examined heterogeneous brain tissues (Day *et al.* 2013; Grayson *et al.* 2005; Kang *et al.* 2013; Laufer *et al.* 2013; Mill *et al.* 2008; Mizuno *et al.* 2012; Sabunciyan *et al.* 2012; Simmons *et al.* 2012; Tian *et al.* 2012), which impedes the discovery of disease- and learning-related changes in DNA methylation (Guintivano *et al.* 2013).

Cellular heterogeneity confounds epigenetic profiling in two ways (Guintivano *et al.* 2013; Heijmans & Mill 2012; Michels *et al.* 2013). First, changes in cellular composition within a region can produce spurious discoveries of changes in DNA methylation (Guintivano *et al.* 2013). For example, chronic stress induces microglial proliferation in the prefrontal cortex (Hinwood *et al.* 2012), which could be reflected by global changes in DNA methylation if the entire cortex

was examined, despite the absence of genuine intracellular changes in methylation. Secondly, if the change in DNA methylation occurs within a distinct cell population, such as recently activated neurons, even prominent changes in 5-methylcytosine (5mC) may be imperceptible because of the relative underabundance of these cells in the regional mosaic (Guintivano *et al.* 2013). Fortunately, several methods exist for isolating cells of interest from tissue, including fluorescence-activated cell sorting (FACS) (Guez-Barber *et al.* 2012; Iwamoto *et al.* 2011; Jiang *et al.* 2008; Kozlenkov *et al.* 2013; Okada *et al.* 2011; Saxena *et al.* 2012), magnet affinity cell sorting, laser-capture microdissection (Vincent *et al.* 2002) and statistical correction for varying cellular composition (Guintivano *et al.* 2013). However, the amount of DNA retrieved following the application of these techniques is often insufficient for the preparation of next-generation sequencing libraries that currently call for at least 1 μg of DNA.

To sequence small amounts of DNA, tissue from multiple biological replicates may be pooled or some form of whole genome amplification employed. However, pooling samples obscures inter-individual variation in methylation that could govern differences in susceptibility to developing neuropsychiatric disorders, and whole genome amplification has the potential to introduce bias and artefacts (Aird *et al.* 2011; Warnecke *et al.* 1997). Furthermore, many protocols become exceedingly labour-intensive and expensive if applied to the large number of samples used in behavioural paradigms. Here we describe a novel approach to determine genome-wide CpG methylation in neurons derived from discrete brain regions of individual animals by pairing FACS with a modified MBD-Seq protocol. We have applied this technique using as little as 50 ng of DNA per animal to identify differentially methylated regions (DMRs) within neurons and non-neurons of the ventromedial prefrontal cortex (vmPFC) of individual adult C57BL/6 mice. We find DMRs around several neuron-specific genes and emphasize the importance of using a cell-type-specific technique, as few of the regions of 5mC enrichment (RME) identified in neurons of the vmPFC were also detected in a heterogeneous population of cells derived from the vmPFC. We conclude that MBD Ultra-Seq is a robust and cost-effective method for determining CpG methylation on a genome-wide level in neurons and other cell populations in individual animals. This technique will be invaluable for identifying DMRs associated with learning and memory, as well as for interrogating the neuronal epigenome in neuropsychiatric disorders.

Materials and methods

Mice

Adult, male C57BL/6 mice (9-week-old, 20–25 g) were used for all experiments. Mice were housed four per cage on a 12 h light:dark cycle (lights on 0800 h) in a humidity- and temperature-controlled (22°C) vivarium, with rodent chow and water provided *ad libitum*. Mice were culled by cervical dislocation and whole brains were snap frozen on liquid nitrogen and transferred to –80°C storage. All procedures were conducted according to protocols and guidelines approved by the University of Queensland Animal Ethics Committee.

Dissection of the vmPFC

Ventromedial prefrontal cortices were microdissected by Palkovits punch (Palkovits 1973). Briefly, whole brains were imbedded in Tissue-Tek® O.C.T. compound (Sakura Finetek, Mount Waverley, Australia) and 300 μm coronal sections were serially cryosectioned from 2.96 to 0.26 mm anterior to bregma. The vmPFC was retrieved using a chilled brain punch (Stoelting, Wood Dale, IL, USA), with punches of diameters 0.75–1.5 mm, preselected according to the dimensions of the vmPFC in each slice (Franklin & Paxinos 2007). Isolated regions were placed immediately on dry ice and stored at –80°C until further use.

Isolation of neuronal and non-neuronal nuclei

Individual vmPFCs were dounce-homogenized on ice (Kimble Chase Kontes, A pestle, K885300-0007, Murarie, Australia) in 1.5 ml of chilled nuclear extraction buffer (Jiang *et al.* 2008) [0.32 M sucrose, 5 mM $CaCl_2$, 3 mM $Mg(Ac)_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris–HCl (pH 8), 1 mM dithiothreitol (DTT), 1× EDTA-free protease inhibitor cocktail, 0.3% Triton-X-100]. Nuclear lysates were filtered through a 40-μM cell strainer (BD Biosciences, North Ryde, Australia) to remove clumps and centrifuged at 4°C for 7 min at 700 ***g***. The supernatant was aspirated and nuclei were gently resuspended in 550 μl of ice-cold 1× phosphate-buffered saline (PBS). The blocking solution (5% bovine serum albumin, 10% normal goat serum, 1× PBS) was prepared. The anti-NeuN antibody (1:1200, Millipore, Kilsyth, Australia) was coincubated with the fluorescent secondary antibody (1:1400 Alexa Fluor® 488, Invitrogen, Mount Waverley, Australia) (Table 1, scale for number of samples) for 10 min at 4°C on a rotating shaker; 50 μl was retained from each sample and pooled for a secondary antibody-only control. Respective stains were added to the nuclear solution and incubated for 1 h at 4°C in a dark room (see Table 1 for staining proportions). Prior to FACS, the immunolabelled nuclei were centrifuged at 4°C for 10 min at 700 ***g*** and gently resuspended on ice in ice-cold 1× PBS; 1 μl of DAPI (Invitrogen) was added to the secondary-only control to identify nuclei vs. debris. A BD FACSAria II (BD Biosciences) was used to sort nuclei. Prior to sorting, 10 000 events from the secondary-only control were used to gate events on the basis of their size, granularity and DAPI fluorescence (FITC) to isolate nuclei from debris (Fig. 1a). From the selected nuclear population, we also established non-specific labelling by Alexa488 (Fig. 1b). Prior to the sorting of each NeuN-stained sample, 10 000 events were examined to verify the position of the gates, as a distinct population of NeuN+ events is easily recognizable (Fig. 1c). On average, the NeuN+ population accounted for approximately 60% of the population, or approximately 80 000 events (Fig. 1).

DNA extraction

Nuclei were lysed overnight at 55°C in lysis buffer (100 mM Tris–HCl, pH 8.0, 5 mM EDTA, 0.2% sodium dodecyl sulphate (SDS), 200 mM NaCl, 300 μg/ml proteinase K) (Gu *et al.* 2011). An equal volume of phenol:cholorform:isoamyl alcohol (25:24:1) was added to each sample and mixed by vortexing for 2 min. Samples were centrifuged at 14 000 ***g*** for 5 min at room temperature and the supernatant was carefully isolated and transferred to a new tube; 2.5 sample volumes of ice-cold 100% ethanol, 1 μl of glycogen (20 mg/ml) and NaCl (final concentration 250 mM) were added to each sample. DNA was precipitated for 4 h at –30°C. Following precipitation, DNA was centrifuged at 15000 ***g*** for 30 min at 4°C. Pellets were washed with ice-cold 70% ethanol and subject to further centrifugation (10 min, 15 000 ***g***, 4°C). Ethanol was removed and the pellets were dried by vacuum centrifugation at 45°C, then resuspended in 75 μl of ultrapure H_2O overnight. To maximize recovery, we recommend heating the solution to 37°C in a thermomixer for at least 30 min to overnight. Quantification of DNA was performed by Qubit dsDNA HS assay (Invitrogen). We have found that spectrophotometry (i.e. Nanodrop; Thermo Fisher Scientific, Waltham, MA, USA) is unreliable with low amounts of DNA and can result in as much as a sevenfold overestimation of the quantity of DNA. Qubit is robust in the presence of phenol and salt contamination and provides reliable quantification; 70 000 events yield approximately 400 ng of genomic DNA (gDNA).

Table 1: Nuclear staining of neuronal and non-neuronal samples

Reagent	Anti-NeuN (μl)	Alexa488 (μl)	Blocking buffer (μl)	1× PBS (μl)	Nuclear solution (μl)
Staining solution	0.6 (1:1200)	0.5 (1:1400)	50	150	500
Secondary control	–	0.5	50	150	500

Staining solution in the proportion that is added to 500 μl of nuclei to identify NeuN+ and NeuN– nuclei. A secondary-only control is used to account for background fluorescence.

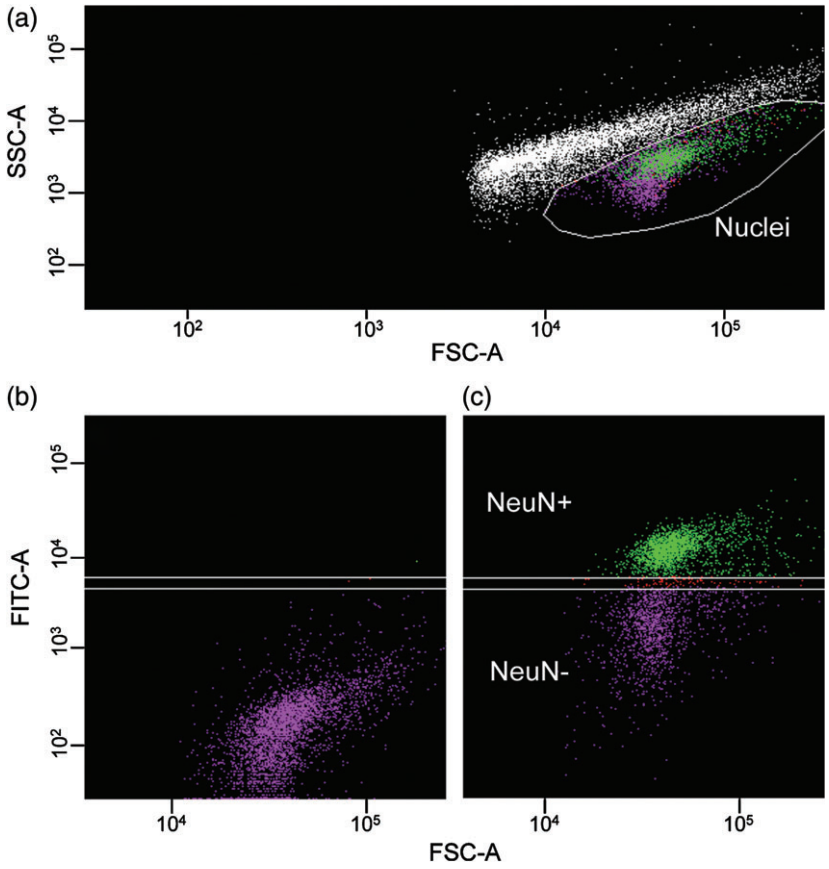


Figure 1: Isolation neuronal and non-neuronal nuclei by FACS. (a) The population of nuclei (green and purple) was gated based on size (FCS-A), cell granularity (SSC-A) and fluorescence (DAPI, not shown). (b) A secondary-only control was used to gate NeuN– samples. (c) Neurons (green) were identified using anti-NeuN preconjugated to a fluorescent secondary antibody (Alexa488). Approximately 80 000 events were identified as NeuN-positive, constituting approximately 60% of all events.

Library preparation

DNA fragmentation and concentration

DNA was randomly sheared by sonication (Covaris S2, North Melbourne, Australia) to create fragments of approximately 300 bp in length. For each sample, 50 ng of DNA was resuspended in 130 μl of ultrapure H_2O (Invitrogen), transferred to microTUBEs (Covaris) and sonicated with the following settings: bath temperature: 4°C, duty: 10%, intensity: 6, cycle/burst: 100, time: 180 seconds. Fragment size was verified by Agilent 2100 Bioanalyzer DNA High Sensitivity Assay (Fig. 2). Although generally robust for varying quantities of DNA, sonication of low amounts of DNA requires optimization on a per lab basis. Following sonication, samples were placed in a new microcentrifuge tube and DNA was vacuum concentrated (Eppendorf concentrator plus, Eppendorf, Hauppauge, NY, USA, V-AQ, 45°C) to a final volume 50 μl.

End repair

Sonication generates fragments with varying 3′ and 5′ overhangs that may also lack a phosphate group at the 5′ end, which renders them incompatible with A-tailing and adapter ligation. End repair uses a combination of polymerases with 3′ to 5′ exonuclease activity and 5′ to 3′ polymerase activity to remove 3′ overhangs and fill in 5′ overhangs respectively, as well as a polynucleotide kinase to add the 5′ phosphate group. All of these are contained in the End Repair Mix (Illumina TruSeq™ DNA V2, San Diego, CA, USA).

The Resuspension Buffer and End Repair Mix were thawed on ice. Samples were transferred to 200 μl thin-walled DNAse/RNAse-free PCR tubes (Axygen, Wembley, Australia), and 10 μl of Resuspension Buffer and 40 μl of End Repair Mix were added to each sample of fragmented DNA. Samples were mixed thoroughly by gentle pipetting and incubated in a thermal cycler (Bio-Rad, Hercules, CA, USA) for 30 min at 30°C (lid 100°C). After incubation, all samples were spun briefly and transferred to 1.7 ml low-bind microcentrifuge tubes (Maxymum Recovery®; Axygen); 160 μl of AMPure XP beads (Beckman Coulter, Brea, CA, USA) (1.6:1 beads:sample ratio) was added to each sample and DNA was purified as prescribed in the Illumina® V2 TruSeq sample preparation guide. We recommend using 1.7 ml microcentrifuge tubes and a DynaMag (Invitrogen or similar strength magnet) to facilitate handling. Samples were eluted in 17.5 μl of Resuspension Buffer (vortex 15 sec, incubate at room temperature for 30 min, place on magnetic stand for 5 min) and 15 μl of supernatant from each sample was transferred to new DNAse/RNAse-free PCR tubes for A-tailing.

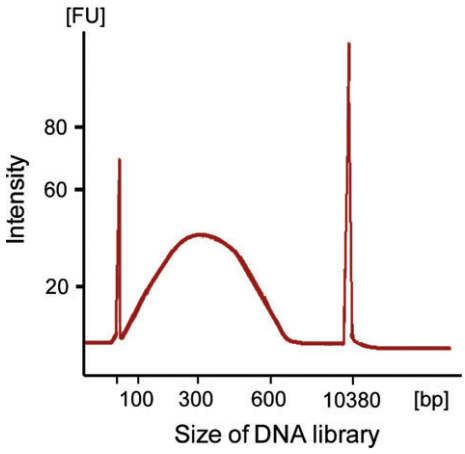


Figure 2: Quantification of the size of DNA fragments following sonication. After sonication, fragment size was verified using using a Bioanalyzer with a High Sensitivity DNA chip (Agilent Technologies). The majority of fragments were approximately 300 bp in length.

A-tailing

The addition of an overhanging dA allows the ligation of the adapters, which carry a complementary overhanging dT; 15 µl of end-repaired DNA was mixed with 2.5 µl of Resuspension Buffer and 12.5 µl of A-tailing Mix by gentle pipetting and incubated at 37°C for 30 min in a preheated thermal cycler (lid 100°C, temperature 37°C). *Troubleshooting note:* poor A-tailing may cause inefficient adapter ligation. The addition of fresh dATP may be helpful in resolving this issue.

Adapter ligation

The addition of adapters with sample-specific barcodes prior to MBD pull-down allows multiple samples to be pooled for a robust pull-down, while retaining sequence information pertaining to individual animals. The dilution of adapters for low-input samples is key to avoiding adapter dimers that would be sequenced and reduce the coverage of the samples being sequenced.

Adapters were thawed and diluted (1:40) in Resuspension Buffer (we recommend optimizing this dilution for specific quantities of DNA and confirming the presence or absence of adapter dimers by Bioanalyzer); 2.5 µl of Resuspension Buffer, 2.5 µl of ligation buffer and 2.5 µl of the designated diluted adapter were added to individual samples and mixed thoroughly by gentle pipetting. All samples were incubated at 30°C for 10 min in a preheated thermal cycler. After incubation, 5 µl of Stop Ligation Buffer was added to each sample and mixed by pipetting. After ligation, samples were purified using AMPure XP beads in a 1:1 v/v ratio according to TruSeq V2 guidelines. Ligated DNA was eluted in 67 µl of ultrapure H_2O. Please note that Illumina has discontinued the V2 kit, and we recommend the adapters used in the latest HT kit or independent synthesis as an alternative.

We detected some adapter dimers by Bioanalyzer and therefore repeated purification with AMPure XP beads in a 1:1 ratio, eluting in 67 µl of ultrapure H_2O and retaining 65 µl of eluate in a new DNA low-bind tube. We confirmed by Bioanalyzer that the amount of adapter dimers was negligible in all samples. A 1:1 beads:sample ratio removes a greater number of small fragments (size is less than 100bp) compared to the standard 1.6:1 ratio and this eliminates the need for gel-based size selection.

Checking ligation efficiency

We determined that the percentage of remaining DNA that was well-ligated by employing the Qubit dsDNA HS assay for total DNA quantification, and the SYBR Fast Illumina Library Quantification Kit (Kapa Biosystems, Thebarton, Australia) to determine the amount of well-ligated DNA in each sample. Each sample was diluted 1:1000 in Library Dilution Buffer prior to use of the Library Quantification Kit, according to the manufacturer's instructions.

Once the size-adjusted concentration of ligated sample in pM was determined, we converted to ng/ml using the following formula:

$$\text{ng/ml} = (\text{concentration in pM} \times \text{molecular weight}) / 10^6$$

$$\text{Molecular weight} = (\text{average length of fragment} + \text{adapter} \times 607.4) + 157.9$$

We then determined how many ng of well-ligated DNA was present in each sample as a percentage of the amount (ng) from Qubit. Typically 50–60% of fragments were well ligated.

Pooling and MBD pull-down

Within each group (vmPFC, NeuN+ cells, NeuN−cells) we pooled 12.5 ng of well-ligated DNA from each biological replicate ($n = 8$) for a total of 100 ng in a final volume of 10 µl. It is preferable to pool an equal number of animals from each group in each pull-down, to account for differences in MBD pull-down efficiency. To facilitate handling, we recommend using sterile 1.7 ml microcentrifuge tubes and the DynaMag magnetic stand (Invitrogen) in lieu of the items provided. In cases of prolonged storage, it is often best to validate the kit using the included controls prior to use. Methyl CpG binding domain pull-downs (Methyl-collector Ultra; Active Motif, Karrinyup,, Australia) were performed as per the manufacturer's directions, under high salt (high stringency) binding conditions. The captured methylated DNA was eluted in 100 µl of complete elution buffer. The methyl-enriched fraction was then purified using AMPure XP beads (1.6:1 ratio, beads:sample) and the enriched sample was eluted in 20 µl of Resuspension Buffer.

Amplification

Following MBD pull-down, 10 cycles of polymerase chain reaction (PCR) were used to amplify well-ligated methylated DNA. The primers provided anneal to a sequence common to all adapters and preserve the sample-specific barcodes during amplification. Each pool was amplified independently; 20 µl of each sample was transferred into DNAse/RNAse-free PCR tubes and 5 µl of primer cocktail and 25 µl of PCR master mix was added to the sample. PCR was performed by initially denaturing for 30 seconds at 98°C, followed by 10 cycles of denaturation at 98°C for 10 seconds, 30 seconds of annealing at 60°C and 30 seconds of elongating at 72°C, then a final 5 min at 72°C, with the lid at 100°C for the duration of thermocycling. PCR products were purified using a 1:1 ratio of AMPure XP beads as previously described, and eluted in 32.5 µl of Resuspension Buffer; 30 µl of eluate was transferred to 0.6 ml DNA low-bind tubes (Maxymum Recovery; Axygen). If there is primer dimer contamination, another AMpure XP beads purification step is required.

Final QC and sequencing

The final concentration of the PCR-enriched libraries was determined by quantitative polymerase chain reaction (qPCR) against a reference library of known cluster density using the Library Quantification Kit (Kapa Biosystems) and Stratagene Mx3000P qPCR instrument (Agilent Technologies, Mulgrave, Australia). The distribution of library fragment size was determined using a Bioanalyzer with a High Sensitivity DNA chip (Agilent Technologies). Each final library was adjusted to 8 µM and loaded into one lane of a HiSeq v.3 flow cell (Illumina) following cluster generation on the Illumina c-Bot instrument and paired-end (101 bp) sequencing was performed using a HiSeq 2000 (Illumina) according to standard manufacturer's instructions.

Bioinformatics

High-throughput DNA sequencing

Paired-end (PE) libraries were sequenced using the Illumina HiSeq2000 sequencing platform with the read length of 101 bp*2, image processing was performed using the standard Illumina Genome Analyzer software and pipelines developed in house. CASAVA software (v1.8.2) was used to demultiplex the samples.

Aligning short reads to reference genome

PE reads were aligned to the reference genome of mouse (mm9) using BWA (v0.6.2) (Li & Durbin 2009). Samtools (v0.1.17) (Li *et al.* 2009) was then used to convert '.sam' files to '.bam' files, sort and index the '.bam' files and remove duplicate reads. If the same library was sequenced in different Illumina runs or lanes, we merged these '.bam' files using Samtools before the step in which duplicates were removed. Reads with low mapping quality ($Q < 20$) or that did not align to the reference genome were excluded from the downstream peak calling analysis.

Calling peaks

Model-based analysis of Chip-Seq (MACS; v1.4.2) (Zhang *et al.* 2008) was used to call peaks for each sample with the parameter setting '-f BAM –keep-dup = all –nomodel –shiftsize 100 -g mm -p 1e-5–bdg'. MACS is a popular peak calling software program used to identify areas in a genome that have been enriched with aligned reads (or peaks) as a consequence of performing next-generation sequencing. In the case of MBD-Seq, these peaks correspond to regions with increased methylation. MACS uses a dynamic Poisson distribution to capture local biases effectively in the genome sequence and evaluates the significance of enriched regions. '–keep-dup = all' was used because duplicate PE reads had been previously removed using Samtools. '-p 1e-5/ was set as a default value of the *P* value cut-off for peak detection relative to background. '–nomodel –shiftsize 100' was used to avoid model-based estimation of fragment size and instead shift forward and reverse tags by 100 bp to identify the midpoint of the region of enrichment. '-g mm' was set for mouse reference and '-bdg' was set to save extended fragment pileup at every position into a bedGraph file, which is helpful for plotting peaks.

All peak summits identified by MACS were then collected to get a full list of potential methylation sites. Custom PERL script was applied to parse the number of fragments (hereafter referred to as counts) that covered the peak summit in each sample. Here fragments refer to DNA fragments in the library that was used for sequencing. Thus, each pair of properly aligned PE reads represents one fragment. The total counts in each sample were normalized to 10 million, after which the normalized counts for each summit were compared between conditions using two-sided Student's *t*-test. Peaks with $P < 0.05$ and the mean of normalized counts in at least one group >5 were considered as differentially methylated peaks between conditions. Peak summits located within 600 bp were grouped together and treated as the same peak using a custom PERL script. The analysis pipeline 'Differentially Methylated Sites Analyzer (DMSA)' is shown in Appendix S5, Supporting Information and all PERL scripts used in DMSA can be downloaded from https://github.com/Qiongyi/DMSA/.

Candidate DMR selection

We did not apply multiple testing corrections, choosing instead to attempt validation of a number of DMRs with varying characteristics in order to determine a criterion of real peaks vs. false positives as others have done (Li *et al.* 2013). We took note of the *P* value and fold change between groups as well as the read coverage, as others recommend 5× coverage for identifying true DMRs (Trimarchi *et al.* 2012).

A 300 bp region surrounding the peak summit of each candidate DMR ($n = 16$) was retrieved using UCSC's genome browser (mm9), and primers (Appendix S6) were designed (Primer3) to amplify a 120–200 bp amplicons overlapping the peak summit. Primer specificity was tested using the NCBI blastn suite and optimized using 1 ng/μl sonicated gDNA, in 10 μl reactions [5 μl 2×Sybr, 1 μl primers (forward + reverse, 10 μM), 2 μl gDNA, 1 μl upH20].

Validation by MBD-qPCR

To provide both biological and technical validation, we generated another cohort of neuronal and non-neuronal gDNA and performed MBD pull-downs (Methylcollector Ultra; Active Motif) on individual animals according to the manufacturer's directions. Briefly we fragmented 300 ng of gDNA by sonication and retained 14 μl as the input DNA and to verify the fragment size using the Bioanalyzer. The remaining DNA was vacuum concentrated to a volume of 10 μl prior to MBD pull-down (high salt conditions). The methyl binding reaction was incubated overnight at 4°C on an end to end rotator. DNA was purified by phenol chloroform extraction and EtOH precipitation for 4 h at –30°C. Methyl-enriched DNA was resuspended in 60 μl of ultrapure H_2O and incubated at 37°C for 3 h to maximize dissolution. Both input (in a final volume of 80 μl) and methyl-enriched DNA (in 60 μl) were stored at –30°C in DNA low-bind tubes (Maxymum recovery; Axygen). Quantitative polymerase chain reaction was performed in duplicate using a Rotor Gene Q (Qiagen, Chadstone Centre, Australia), and replicated if there was any uncertainty. Relative enrichment was calculated by normalizing to input, then using the delta-delta ct method with the NeuN– group set as the control.

Results

Isolation of neuronal and non-neuronal nuclei from the vmPFC

Immunolabelled neuronal (NeuN+) and non-neuronal (NeuN–) nuclei were purified from whole vmPFCs by FACS (Fig. 1, a–c). Approximately 60% of nuclei were neuronal (or positive for NeuN); the remaining 40% was designated as non-neuronal (or NeuN–) (Fig. 1c).

Neurons exhibit greater 5mC enrichment than non-neurons

Neurons derived from the vmPFC have a distinct CpG methylation profile compared to non-neurons from the same region or whole vmPFC (Fig. 3). On average, in each individual, we identified significantly more RME (enriched relative to background) in neurons (178 ± 28 RME) than in non-neurons (121 ± 25 RME) or total vmPFC (128 ± 48 RME) ($F_{2,18} = 7.72$, $P < 0.01$, Tukey's *post-hoc* test, neurons vs. non-neurons, $P < 0.01$, neurons vs. vmPFC, $P < 0.01$, Fig. 4). Furthermore, there were significantly more properly paired reads aligned in neuronal samples (mean: 20083228 reads) than non-neurons (mean: 10980756 reads) ($t_{11} = 2.74$, $P < 0.05$), which suggests that this is because of more CpG methylation and not a facilitated discovery of peaks in a homogeneous neuronal population. However, only 43 of 113 significant DMRs (as identified by Student's *t*-test, Appendix S1) were hypermethylated in neurons. This is consistent with a previous report of global hypomethylation in neurons (Iwamoto *et al.* 2011). We reconcile these observations by proposing that neurons have more CpG methylation overall (Kozlenkov *et al.* 2013; Li *et al.* 2013; Lister *et al.* 2013) but are hypomethylated in CpG-rich regions that are preferentially examined by the LUminometric Methylation Assay (LUMA) assay (Iwamoto *et al.* 2011) and the high-stringency MBD pull-down we employed (Karimi *et al.* 2006; Nair *et al.* 2011).

CpG DNA methylation is enriched in the 3′ UTRs of neurons

As expected, most regions of differential methylation (in neurons vs. non-neurons) were located within intergenic regions (Fig. 5a); collectively, these regions comprise the majority of the genome. However, after normalizing for the cumulative length of each region, we found an unexpected enrichment of CpG methylation in the 3′ untranslated region (UTR) of neurons (Fig. 5b). In contrast, there was significant enrichment of

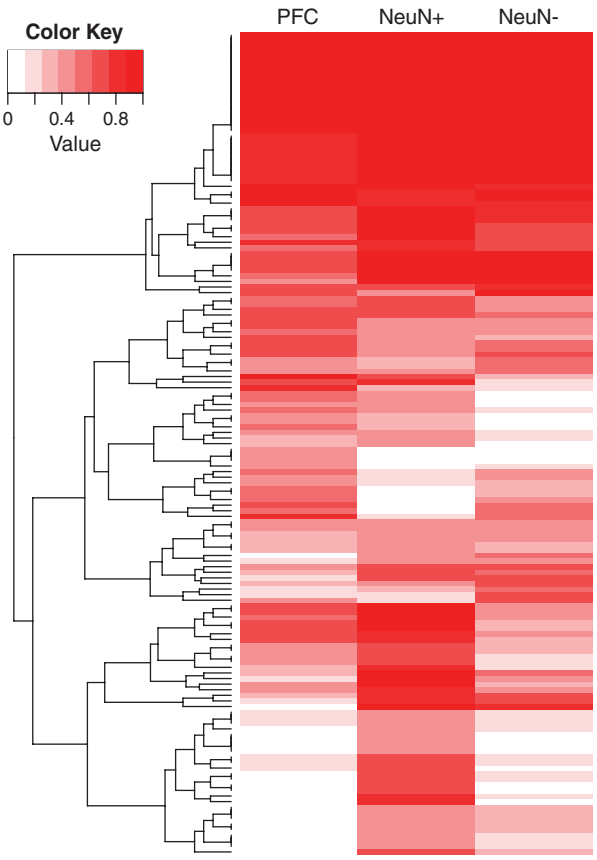


Figure 3: Neurons derived from the vmPFC have distinct CpG DNA methylation compared to non-neurons or bulk vmPFC. For each grouped differentially methylated region (see Fig. 5), the number of biological replicates displaying significant 5mC enrichment (as detected by MACS) was considered. White indicates that none of the samples ($n = 6–8$/group) showed significant enrichment; red indicates that all samples had significant enrichment (displayed as ratio of all samples in the group, referred to as 'Value' in this figure).

5mC in the 5′UTRs of non-neurons (Fig. 5b). There is extensive lengthening of 3′UTRs in the mammalian brain, particularly in neurons (Miura *et al.* 2013), which may be regulated by differential DNA methylation. For example, methylation of CpG islands in the mouse *H13* and *Herc3/Nap1l5* genes promotes the use of downstream alternative polyadenylation sites that result in the production of longer transcripts for these genes (Cowley *et al.* 2012; Wood *et al.* 2008). Therefore, hypermethylation of the 3′UTR in neurons may regulate the lengthening of 3′UTRs in the mammalian brain, which could in turn affect the stability of these transcripts (Miura *et al.* 2013). It should be noted that each identified peak was used for downstream analysis (for illustration, see Fig. 6), rather than grouping peaks in close proximity, hence the discrepancy between the average number of regions of 5mC enrichment identified in individuals and the total number of cell-type-specific DMRs (Appendix S2).

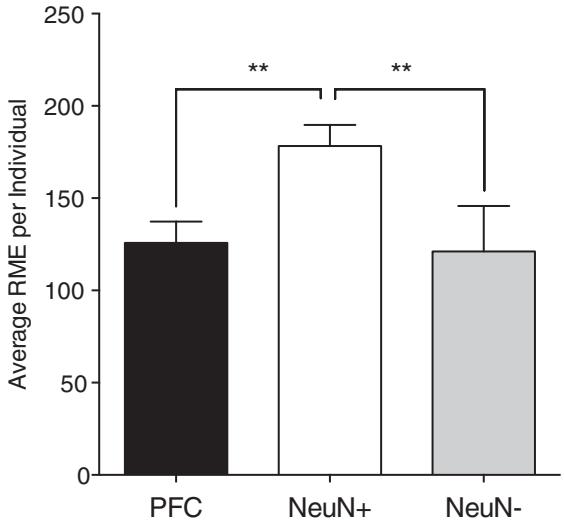


Figure 4: Neurons display greater CpG methylation than non-neurons or bulk vmPFC. On average more regions of 5mC enrichment are identified in neurons derived from the vmPFC (178 ± 28) than in non-neurons from the same region (121 ± 25) or bulk vmPFC (128 ± 48), $F_{2,18} = 7.72$, $P < 0.01$, Tukey's *post-hoc* test, neurons vs. non-neurons, $^{**}P < 0.01$, neurons vs. vmPFC, $^{**}P < 0.01$.

Validation of cell-type-specific methylation

A total of 16 DMRs were selected for confirmatory analysis by MBD-qPCR (Appendix S3, Appendix S4). Candidate loci varied in statistical significance, in fold difference in methylation between cell types, in coverage of the region and in the number of samples that were initially identified as having the RME. A wide range of genomic loci were selected in an attempt to identify the characteristics of DMRs that reliably validate. We found that the DMRs identified by sequencing must be covered by at least a mean of PE 5 reads (herein referred to as 5× coverage, following normalization to 10 million reads) in at least one cell type in order to validate reliably by qPCR. Of the DMRs with 5× coverage in at least one group, 10 of 12 candidates were validated by MBD-qPCR (see Fig. 7 for select examples). As expected, we were unable to validate similar DMRs with <5× coverage (four candidates, see Appendix S3). These DMRs were marked by substantial inter-individual variability, with regions of enrichment identified in only one or two biological replicates. While these may reflect individual differences in methylation, they are not reflected in consistent group differences. Correspondingly, DMRs must be initially identified in at least half (3/6) of the biological replicates in order to be validated as reliable group differences by MBD-qPCR. We further caution that at this low level of coverage (<5×) these DMRs may be misalignments or artefacts of normalization. On the basis of our experience, we recommend selecting candidates of the following characteristics: $P < 0.02$, fold difference >1.2, mean reads >5 and present in at least half of all biological replicates. A minimum of 5× coverage is key, as the ability to identify differences between groups is strongly affected by enrichment levels

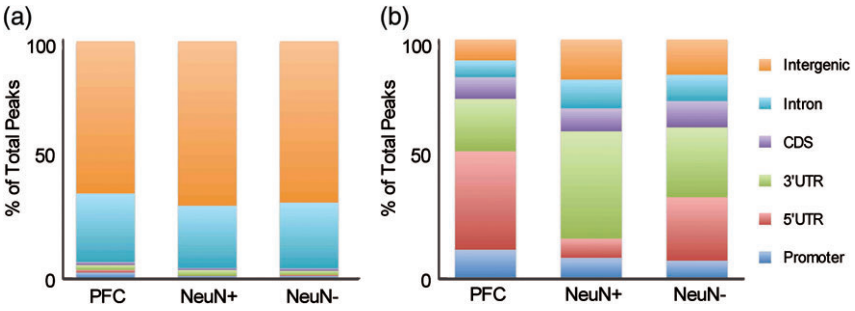


Figure 5: Distribution of the genomic locations of the regions of 5mC enrichment. (a) When the number of peaks is not normalized for the length of the genomic region over 50% of regions of 5mC enrichment are found in the intergenic region, as this region is substantially longer than all others. (b) When the number of peaks is normalized for the relative length of the region, the neuronal genome has a pronounced enrichment of 5mC in 3′UTRs of genes.

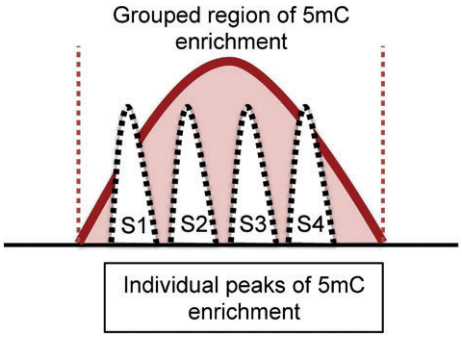


Figure 6: Individual regions of enrichment (S1–S4) vs. a grouped region of 5mC enrichment. All individual regions of 5mC enrichment (S1–S4) were used in downstream analysis, although these may occur within one broad region of 5mC enrichment (grouped region of 5mC enrichment). Using all regions for analysis is preferable as it avoids equally weighting DMRs that are only defined by one or two biological replicates.

surrounding the locus, because of the fact that greater enrichment results in greater depth of sequencing (Robinson *et al.* 2010; Trimarchi *et al.* 2012).

Discussion

To date, investigation of neuronal DNA methylation has been restricted to candidate loci (Labonte *et al.* 2012; Matrisciano *et al.* 2013; Nishioka *et al.* 2013; Schor *et al.* 2013), reduced representations of the genome (Guo *et al.* 2011, Oh *et al.* 2013), or a meagre number of samples pooled from a number of individuals (Guo *et al.* 2013; Lister *et al.* 2013). These methods are not amenable to the analysis of changes in DNA methylation in response to learning and other environmental experiences, where experiments necessitate the use of discrete brain regions and cell types (i.e. neurons), as well as numerous samples. MBD Ultra-Sequencing is well-suited for such experiments, and the increase in resolution afforded by the use of neurons and individuals, as well as the ability to use as little as 50 ng of input DNA, will facilitate the discovery of changes in DNA methylation related to memory formation and maintenance.

The strength of MBD Ultra-Seq derives from several improvements to existing protocols. First, we have increased the yield of nuclei from tissue by eliminating the use of a sucrose cushion when isolating nuclei (Jiang *et al.* 2008). Moreover, the FACS procedure is applicable to other cell types with distinct nuclear markers (i.e. TBR1 for pyramidal neurons, unpublished observations) or to transgenic mice with nuclear reporters. Barcoding individual samples using sample-specific indices (Illumina Truseq V2) prior to MBD pull-down also enabled the detection of inter-individual variability in DNA methylation from 'pooled' samples. Pooling samples during MBD pull-down reduces the variability attributed to differences in handling and pull-down efficiency, particularly where low amounts of DNA are considered. Sample-specific barcoding was also recently used in the construction of reduce-representation bisulphite sequencing (RRBS) libraries (Boyle *et al.* 2012).

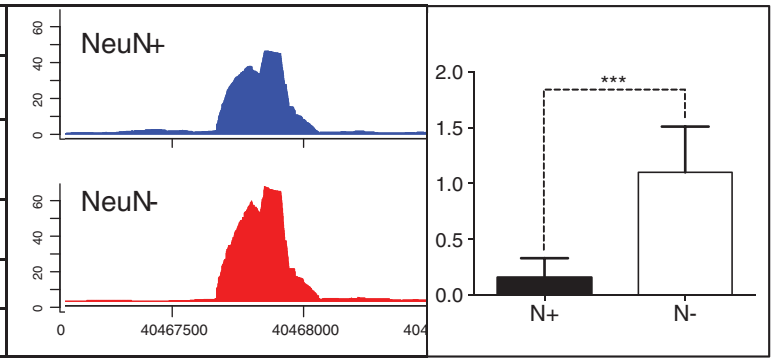
When the size of the genomic region is not considered, we find that the majority of differences in DNA methylation between neurons and non-neurons occur within intergenic regions. As most neuronal activity-induced changes in DNA methylation are likewise located intergenically (Guo *et al.* 2011), the use of MBD-based enrichment approaches is preferable to RRBS, which does not provide coverage of intergenic regions or 3′UTRs outside CpG islands (Wang *et al.* 2013).

Limitations

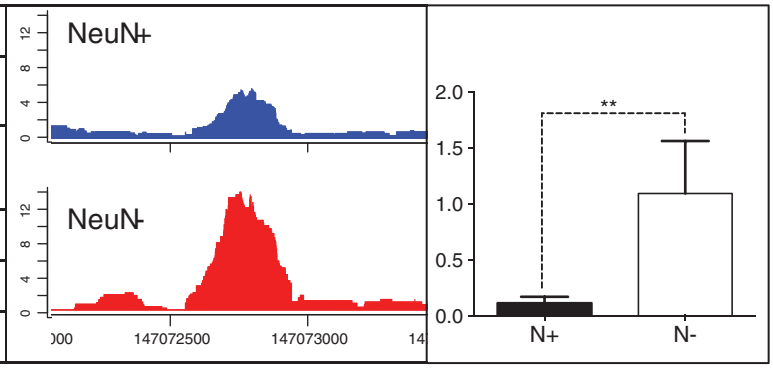
MBD Ultra-Seq provides one of the few methods for profiling genome-wide methylation in a large number of samples; however, it is subject to certain limitations. Firstly, messenger RNA (mRNA) cannot be extracted simultaneously from neurons, and consequently the direct relationship between altered DNA methylation and gene expression cannot be explored. There are a handful of protocols for isolating whole neurons from adult brains (Guez-Barber *et al.* 2012; Lobo *et al.* 2006; Saxena *et al.* 2012); however these suffer from reduced yield (120 000 NeuN+ events from an entire mouse frontal cortex) and the RNA obtained may be degraded (unpublished observations).

Secondly, unlike whole-genome bisulphite sequencing (WGBS) (Guo *et al.* 2013; Lister *et al.* 2013), MBD Ultra-Seq cannot be used to profile non-CpG methylation as MBD 2B/3 L binds exclusively to methylated cytosines in the CpG dinucleotide context. However, in neurons, CpG methylation accounts for 46–75% of all methylated cytosines (Guo *et al.* 2013; Lister *et al.* 2013) and exhibits greater inter-individual variability than non-CpG methylation (Lister *et al.* 2013), which may be indicative of an increased

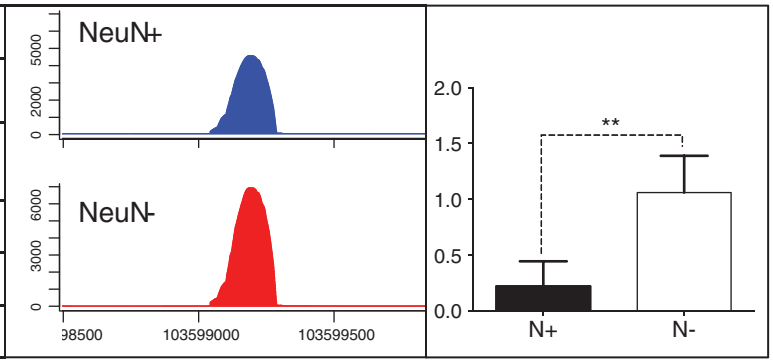
Gene symbol	Kctd16
Peak summit	Ch18: 40467865
Fold difference (N-/N+)	1.39
P-value	0.009
Mean NeuN+	47
Mean NeuN-	66



Gene symbol	Cdk8
Peak summit	Ch5: 147072760
Fold difference (N-/N+)	2.63
P-value	0.003
Mean NeuN+	5.13
Mean NeuN-	13.44



Gene symbol	NCAM
Peak summit	Ch6: 103599191
Fold difference (N-/N+)	1.37
P-value	0.014
Mean NeuN+	5028
Mean NeuN-	6692



Gene symbol	N/A
Peak summit	Ch6: 15137969
Fold difference (N+/N-)	9.87
P-value	0.008
Mean NeuN+	0.3
Mean NeuN-	2.96

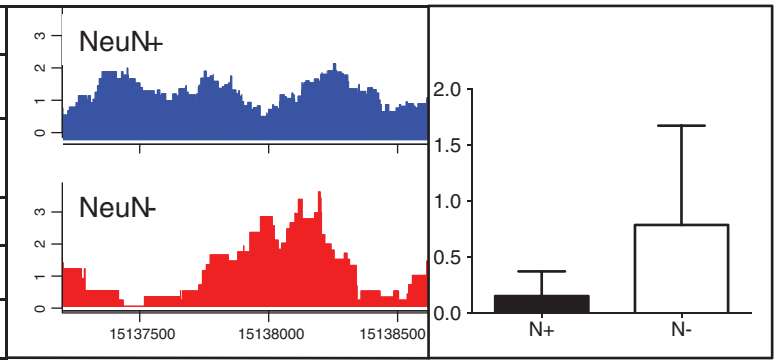


Figure 7: Candidate DMRs. Sequencing results (table) are derived from the average of all individual peaks within the grouped DMR. The plot is indicative of the normalized reads surrounding the locus, whereas the graph shows the relative enrichment as determined by MBD-qPCR.

propensity for experience-induced changes in methylation. Moreover, to identify differential methylation, WGBS requires 15- to 30-fold more coverage of the genome than MBD-Seq (Stevens *et al.* 2013) and is consequently unsuited for behavioural paradigms that require a large number of animals, and for which individual animal resolution may be required.

Alternatively, MeDIP-Seq can be used to assay non-CpG methylation, and protocols optimized for low amounts of DNA and numerous samples have been developed (Taiwo *et al.* 2012). However, within the CpG dinucleotide context, methylated DNA immunopreciptation (MeDIP) suffers from reduced sensitivity and specificity compared to MBD, generating a high degree of background 'noise' (De Meyer *et al.* 2013) that may impede the detection of DMRs. Nevertheless, although MeDIP-Seq suffers from additional technical limitations, such as the use of single-stranded DNA, it is a viable alternative for those wishing to explore non-CpG methylation. As a final limitation, we find that, although MBD 2B/3L is highly efficient at enriching for fragments with seven or more methylated CpGs (De Meyer *et al.* 2013), CpG-rich regions receive greater coverage, which facilitates the discovery of DMRs in these areas.

Conclusion

MBD Ultra-Seq is a novel approach for identifying DNA methylation in neurons and non-neuronal populations derived from discrete brain regions of individuals, using as little as 50 ng of DNA. We find that the neuronal methylome is unique and that CpG-rich regions are hypomethylated. Differences in DNA methylation between neurons and non-neurons are predominantly localized to intergenic regions, which emphasizes the importance of using genome-wide methods to examine this region of the genome. However, when the size of the genomic region is accounted for, the greatest proportionate difference in methylation between neurons and non-neurons is in the 3′UTR, which may be related to the lengthening of 3′UTRs in the neuronal transcriptome of the mammalian brain. We find that DMRs with >5× coverage validate reliably, demonstrating the utility of this method. Overall, MBD Ultra-Seq provides a tool that can be used to examine and detect neuronal DNA methylation with individual animal resolution in a large number of biological replicates, which should greatly facilitate the detection of differential methylation related to learning and neuropsychiatric disorders.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Appendix S1: The 'summits' of regions of 5mC enrichment in individuals (S1–S4, Fig. 6) are often separated by a small distance. Regions of 5mC enrichment identified in individuals that were within 600 bp of each other were grouped together to explore the overall degree of methylation of the vmPFC, neurons and non-neurons and the sequencing results are available here.

Appendix S2: Regions of differential DNA methylation between neurons and non-neurons (individual). To avoid failing to detect any cell-type-specific differences in DNA methylation, we compared methylation in all biological replicates at each region of 5mC enrichment (i.e. S1–S4 in Fig. 6) rather than at grouped DMRs. Significant individual regions of 5mC enrichment are listed here.

Appendix S3: Candidate DMRs for validation by MBD-qPCR. MBD Ultra-Seq information for candidate differentially methylated loci for validation (16). Position of the peak summit is the median value for the grouped peak summit derived from all individual peaks surrounding the loci. The number of samples with peak refers to the number of samples initially identified as having the peak by MACS, which remained statistically significant ($P < 0.05$) after a Student's *t*-test. For example, a region of 5mC enrichment may have been identified by MACS in all NeuN– samples ($n = 7$) for Chr 6 ~ 15137969; however the enrichment was only significant at one of the seven peaks identified.

Appendix S4: MBD Ultra-Seq data for candidate cell-type-specific RME. The original data are available here.

Appendix S5: Differentially Methylated Sites Analyzer (DMSA) analysis pipeline.

Appendix S6: Primers used for validation of candidate DMRs by MBD-qPCR. List of primers used for the validation of candidate DMRs by MBD-qPCR.

Additional file 3. Candidate regions that are differentially methylated in neurons and non-neurons (MBD qPCR validation)

Chr	Peak summit	Genomic location	# of samples	NeuN+ mean	NeuN- mean	P-value	Fold difference	Overlapped gene	Right gene (distance)	Left gene (distance)
5	33080729	CDS	6	10.58	20.67	0.01	1.96	Pisd	Yes1	C330019G07 Rik
5	147072761	Intron	3	5.13	13.44	0.003	2.63	Cdk8	Rnf6	Wasf3
6	49186533	5'UTR	2	4.67	10.43	0.02	2.25	N/A	Igf2bp3 (22065 bp)	Tra2a (8211 bp)
6	103599191	Intron	6	5028	6892	0.01	1.37	NCAM	Cntn3	Cntn6
8	14306933	Intron	2	6.41	12.03	0.02	1.88	Dlgap2	Erich1	Cln8
8	56208439	Intergenic	4	10.42	22.33	0.002	2.15	N/A	Gpm6a (64157 bp)	Adam29 (2141514 bp)
11	108873349	Intergenic	4	6.74	19.54	0.007	2.90	N/A	Axin2 (62640 bp)	Rgs9 (213653 bp)
13	3372785	Intergenic	6	59.22	84.89	.0007	1.43	N/A	N/A	Gdi2 (164760 bp)
15	74917258	Intergenic	6	223	360	.00006	1.61	N/A	Ly6c1 (38319 bp)	Ly6c2 (21855 bp)
17	39984652	Intergenic	6	43.38	81.98	.002	1.69	N/A	Esp23 (772314 bp)	Pgk2 (359752 bp)
18	40467865	Intron	6	47.53	65.91	0.01	1.39	Kctd16	Yipf5	Prelid2
19	61275693	Intergenic	4	5.14	21.01	0.02	4.09	N/A	Gm7102 (23828 bp)	Csf2ra (25344 bp)
2	4496971	Intergenic	1	2.04	0.2	0.0002	10	-	Gm 13476 (1114 bp)	Acvr2a (3700501 bp)
5	24309239	CDS	1	1.83	0	0.01	NC	N/A	Crygn (45580 bp)	Rheb (245 bp)
6	5055404	Intron	1	0	2.94	0.02	1.67	Ppp1r9a	Peg10	Pon1
6	15137969	5'UTR	1	0.3	2.96	0.008	9.87	N/A	Ppp1r3a (43272 bp)	Foxp2 (8992 bp)

Additional file 6. Primers used for validation of candidate DMRs by MBD qPCR

Peak summit of DMR	Left primer	Right primer
Chr 2: 4496971	CCACTTCCCAGGGGATTTT	GCATCCTCAAATTTGGCTGT
Chr 5: 33080729	GGATACGGATAGAGCCCACA	GGCTCTGAGCAGAAAGTAGCA
Chr 5: 24309239	GCCGATAAAATTCTTGGTCA	TGAAAGATGTCATTTGGGTCAG
Chr 5: 147072761	CTCTTCCTGGAGTCGGATTG	TTCACGCCCTCTTGAACTCT
Chr 6: 5055404	CTCAGGCCGTAGCTCAGAGT	TGCATGGTTAGCTTCATTGC
Chr 6: 15137969	CCCTGGCAATTTTCTGTTTT	CCCAATTCTGCTAATGAAGAGTG
Chr 6: 49186533	CCAACCCCAAGAGTTATCCA	TGTCAGGGAGGACAGAAACC
Chr 6: 103599191	AATTCCCGTTTCCAACGAAT	CAAGCAATCTTTCAAGCAAGG
Chr 8: 14306933	AAAATGAGAAGGCTCCATCC	TCTGTGAACCTCACAATGCAC
Chr 8: 56208439	CAGGACTTTGGCTTGGAGAA	ATTTTCGGCTCTGGCTTAGG
Chr 11: 108873349	CTCTGGATTTGGGGATCTGA	GAACTTTGAAGGCCGAAGTG
Chr 13: 3372785	TTGTTTTCCTGGAGGTGTGG	CCCGACAGGTGAGGATGTAG
Chr 15: 74917258	CCAAAGCGACCTGAAACAAT	GCACATTGACCTCACCAAGA
Chr 17: 39984652	CTTGTAAGCGTCGAGGTGCT	CTCAGACACAAACGGGAAGG
Chr 18: 40467865	TGGAGCCTTCCTCTTTCTGT	GCCAGAAGTCCGTGAGTGAT
Chr 19: 6127569	CACTCACGCACACATGACAG	TGATTTCGCCTGTTTTCACA

CHAPTER 4

Epigenetic and transcriptional consequences of cocaine self-administration

4.1 Abstract

To date, most investigations have focussed on the epigenetic consequences of repeated involuntary cocaine exposure. However, the neural adaptations arising from forced cocaine exposure differ significantly from those incurred by voluntary cocaine self-administration and may not be representative of the plasticity produced by cocaine consumption in the natural environment. A fundamental difference is that cocaine self-administration produces memories for the association between cocaine-paired cues, drug availability and the rewarding effects of the drug; when reactivated during abstinence, these cocaine-related memories can incite cocaine craving and precipitate relapse. The enduring presence of cocaine-related memories is one of the primary reasons that the treatment of cocaine addiction is largely unsuccessful, and therefore, in identifying the cellular adaptations that mediate the maintenance of cocaine-related memories, we may provide valuable targets for treatment. One possibility is that long-lasting modifications of DNA methylation are produced by cocaine self-administration and underpin the maintenance of cocaine-related memories, as enduring changes in DNA methylation beget the maintenance of memory in other paradigms. To this end, we used MBD Ultra-Seq, a technique developed to detect region- and cell type- specific modifications of DNA methylation in individual animals, to identify changes in DNA methylation in neurons of the mPFC that occur as a result of cocaine self-administration. We identified 29 genomic regions that were persistently differentially methylated following cocaine self-administration but not passive cocaine exposure, and 28 regions that became differentially methylated during abstinence, all of which may contribute to the maintenance of cocaine-related memories. These differentially methylated regions (DMRs) were predominantly located within or proximal to annotated genes (coding and non-coding). Gene-associated DMRs primarily arose within introns or non-coding loci. Abstinence-associated DMRs were curiously enriched within nuclear lamina-associated domains. Functionally, gene-associated DMRs regulate the expression of co-localising genes in an isoform-specific manner. Finally, preliminary evidence suggests that the reactivation state of cocaine-associated memories further modulates the relationship between differential methylation and gene expression. Together this body of work provides the first *in vivo* profile of neuron-specific changes in DNA methylation that are uniquely produced by voluntary cocaine self-administration rather than simple drug exposure. Moreover, it yields cursory examples of the metaplastic priming of gene expression by long-lasting learning-induced changes in DNA methylation, as described in Chapter 2.

4.2 Introduction

A key feature of cocaine addiction is the pathological overlearning of the association between cues in the environment and the rewarding effects of the drug. Memories of these associations are extraordinarily persistent and, when reactivated, can trigger overpowering cocaine craving and relapse despite prolonged abstinence (Childress *et al.*, 1999). However, how cocaine-associated memories are perpetuated in the brain in the face of rapid transcriptional and proteomic degradation remains enigmatic.

Accumulating evidence suggests that persistent learning-induced epigenetic modifications may support the maintenance of long-term memories (Miller *et al.*, 2010). In particular, relatively stable epigenetic modifications, such as DNA methylation, have been implicated in the maintenance of contextual fear memories (Miller *et al.*, 2010) and arise in response to neuronal activity (Vanyushin, 1974) and the induction of synaptic plasticity (Levenson *et al.*, 2006). Moreover, dysregulated DNA methylation is associated with several disorders of memory formation and maintenance (Matijevic *et al.*, 2009, Yu *et al.*, 2015). Therefore, enduring learning-induced variations in DNA methylation may function as a conserved mechanism of memory maintenance and underlie the preservation of cocaine-related memories. In support of this hypothesis, recent investigations indicate that cocaine self-administration results in long-lasting changes in DNA methylation in the nucleus accumbens and that local inhibition of DNMTs within this region abolishes cue-induced cocaine-seeking (Massart *et al.*, 2015).

To maximize the probability of detecting and correctly identifying learning-induced changes in DNA methylation it is first necessary to determine where these changes are prone to develop. Firstly, the brain region(s) that mediate the maintenance of cocaine-related memories are likely the regions in which persistent memory-associated modifications of DNA methylation reside. The medial prefrontal cortex (mPFC) is required for the reinstatement of cocaine seeking during abstinence (Capriles *et al.*, 2003, Di Pietro *et al.*, 2006, Fuchs *et al.*, 2007, Mcfarland & Kalivas, 2001, Mclaughlin & See, 2003), which suggests that it is necessary for the maintenance of cocaine-related memories. Befittingly, steadfast modifications of gene expression, histone acetylation and the neuroproteome occur in the mPFC following intravenous cocaine self-administration (IVSA) and persist for up to 100 days of enforced abstinence (Freeman *et al.*, 2010b, Freeman *et al.*, 2008, Lull *et al.*, 2009). However, these molecules and modifications are subject to rapid degradation and unable to support the maintenance of cocaine-related

memories. Instead, IVSA-induced modifications of DNA methylation within the mPFC may sustain enduring cocaine-related memories.

Cell type-specific profiling is equally vital to the identification of IVSA-associated changes in DNA methylation. Discrete, self-administration-induced changes in DNA methylation will occur in a select population of cells or neurons that are engaged by this behaviour and these limited changes may be indiscernible if methylation is analysed at the whole brain or whole region level. Furthermore, regional changes in cellular composition may occur following cocaine exposure (Bowers & Kalivas, 2003, Ciccarelli *et al.*, 1997) and could produce artefactual changes in DNA methylation, as outlined in Chapter 3. To avoid these confounds, I have explored cocaine-induced modifications of DNA methylation in mature NeuN+ neurons. NeuN recognizes FOX-3, a neuron-specific splicing factor present in the nuclei of most neuronal subtypes of the cortex (Kim *et al.*, 2009) Experience-induced variations in neuronal DNA methylation are particularly relevant to the maintenance of long-term memory, as neurons are fundamental to learning and have the peculiar property of not dividing, which might prevent the disturbance of acquired information stored in their DNA (Griffith & Mahler, 1969). Therefore, the maintenance of cocaine-associated memories might be predicated on experience-induced changes in DNA methylation in neurons of the mPFC. Repeated cocaine exposure induces LTP at excitatory synapses of layer V neurons of the mPFC (Huang *et al.*, 2007), which suggests that neurons in this region contribute to the maintenance of cocaine-related memories. In addition, withdrawal from cocaine self-administration produces morphological abnormalities in layer V pyramidal neurons of the mPFC (Rasakham *et al.*, 2014). It is further possible that distinct epigenetic changes arise in the subtypes of neurons within the mPFC (glutamatergic, GABAergic, dopaminergic etc.) however these cannot be isolated in sufficient number and quality by FACS, due to the lack of nuclear markers.

Finally, one must consider where in the genome experience-induced changes in DNA methylation are likely to occur, as this determines which methods are suitable for their detection. Traditionally, investigations have concentrated on CpG-rich promoter regions, however these regions are relatively resistant to experience- and cocaine- induced changes in DNA methylation (Guo *et al.*, 2011a, Laplant, 2010). Therefore, I elected to explore genome-wide changes in DNA methylation, using MBD Ultra-Seq (see Chapter 3). While previous investigations determined that components of the DNA methylation machinery and DNA methylation itself are regulated in response to cocaine exposure

(Anier *et al.*, 2010, Barros *et al.*, 2011, Carouge *et al.*, 2010) and mediate the rewarding effects of the drug (Laplant, 2010, Massart *et al.*, 2015, Tian *et al.*, 2012), they fail to distinguish the changes in DNA methylation that arise from simple cocaine exposure from those that are associated with voluntary cocaine self-administration. This distinction is important because passive involuntary cocaine exposure fails to produce the persistent changes in long-term potentiation (Chen *et al.*, 2008, Martin *et al.*, 2006) and sharp rise in extracellular dopamine (Hemby *et al.*, 1997) and acetylcholine (Mark *et al.*, 1999) that may underlie overlearning and the extreme persistence of cocaine-associated memories, as well as the development of addiction. In addition, involuntary exposure to cocaine is strongly aversive and retards the acquisition of cocaine self-administration (Twining *et al.*, 2009). We therefore employed a mouse model of intravenous cocaine self-administration (IVSA) that contrasts learned cocaine self-administration with passive yoked cocaine exposure to identify modifications in DNA methylation that are uniquely associated with learned cocaine-seeking and enduring cocaine-seeking during abstinence.

Likewise, little is known about the persistence of changes in DNA methylation that arise from cocaine self-administration (Nielsen *et al.*, 2012a), though persistence is a critical indicator of an epigenetic modification's ability to contribute to memory maintenance. Therefore, the aim of this body of research was to identify modifications of DNA methylation that are exclusively produced by voluntary cocaine self-administration and to examine the persistence of these modifications over time, in order to identify changes in DNA methylation that may contribute to the maintenance of cocaine-related memories.

I sought to identify persistent and abstinence-associated modifications of DNA methylation that arise in neurons of the mPFC following cocaine self-administration. Age-matched yoked controls accounted for the changes in DNA methylation produced by simple passive cocaine exposure. Genome-wide modifications of DNA methylation were identified by MBD Ultra-Seq, a next-generation sequencing technique outlined in Chapter 3. Following the statistical selection of DMRs, differences in DNA methylation between naïve and self-administering animals were confirmed by MBD qPCR in an independent biological cohort.

A common functional output of altered DNA methylation is a change in the expression of proximal or co-localising genes. Persistent learning-induced changes in DNA methylation may direct enduring modifications of gene expression, or, as I hypothesize in Chapter 2, provide a quiescent signature of memory and prime transcription upon subsequent

memory or neuronal reactivation. To provide *in vivo* evidence of the latter possibility, I examined the relationship between persistent differential methylation and gene expression in animals that underwent a brief relapse test after 21 days of abstinence and those that were simply sacrificed. Re-exposure to the self-administration chamber and cocaine-paired cues during the relapse test reactivates cocaine-related memories (as evidenced by continued cocaine seeking) whereas cocaine-related memories likely remain dormant in mice that have been simply sacrificed after prolonged abstinence. In the absence of differences in DNA methylation between relapsed and non-relapsed mice, altered gene expression unique to either group indicates that the reactivation state of memory modulates the relationship between IVSA-associated modifications of DNA methylation and gene expression. Moreover, a change in the expression of proximal genes provides preliminary evidence of a functional role for cocaine IVSA-associated changes in DNA methylation.

Collectively, this body of work yields the first *in vivo* neuron-specific genome-wide profile of changes in DNA methylation associated with learned cocaine seeking and not simple drug exposure, where the former may be more relevant to the development and persistence of addiction. Moreover, it provides the first demonstration of the modulation of the relationship between learning-induced modifications of DNA methylation and transcription by the reactivation state of a memory, suggesting that the function of persistent modifications of DNA methylation extends beyond the enduring regulation of transcription.

4.3 Methods

4.3.1 Operant cocaine self-administration in mice

4.3.1.1 Animals

Adult male C57BL/6 mice (8-9 weeks of age at the start of experiments, 20-24 g) were singly housed under a 12 hr reverse light-dark cycle (lights off 7 am) under standard housing conditions (*ad libitum* standard rodent chow and water, upon cat litter with tissue nesting material) unless otherwise specified.

4.3.1.2 Ethics statement

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act (1986), under the guidelines of the National Health and Medical Research Council

Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia (Florey Animal Ethics Committee approval number: 10-079).

4.3.1.3 Intravenous self-administration (IVSA)

Operant self-administration training was conducted in mouse operant chambers (model ENV-370W, Med Associates) housed in individual ventilated sound- and light- attenuating cubicles. Two retractable levers (ENV-312M, Med Associates), one paired with reward delivery (active lever) and the other with no programmed consequence (inactive lever), were present either side of a fluid receptacle (ENV-303LP, Med Associates) outfitted with a contact lickometer (ENV-250, Med Associates). A stimulus light (conditioned stimulus, CS) was located over the active lever and illuminated for 5 sec concomitant to reward delivery. A vanilla-scented olfactory cue (discriminative cue) placed beneath the reward-paired lever. Responses were registered in a separate room on a computer running Med-PC IV software (Med Associates).

Self-administration training was conducted in the dark photoperiod. Animals were acclimated to single housing and the reverse light dark cycle for 7 days, after which point access to standard laboratory chow was restricted (7g/day, unless body weight fluctuated more than 2g). The instrumental response (lever press) and discrimination between the active and inactive levers was established using 10% w/v oral sucrose (Coles) under a FR1 schedule. Each active lever press resulted in the presentation of a 5-sec light cue and the concurrent delivery of 5 µl of sucrose to the fluid receptacle over a 1.7 s period. The instrumental response was considered to be acquired if animals performed >100 lever presses per 2 hr session with >75% discrimination between the reward-paired and inactive levers over the last 3 days of training. Licking events were recorded by a contact lickometer to ensure that lever pressing during cocaine self-administration was not due to continued sucrose seeking. Yoked control animals received equivalent amounts of sucrose, under the control of their paired self-administering counterparts.

Following sucrose training, all mice were anaesthetized with isofluorane (5% induction, 1.5-1.8% maintenance in air, Rhodia Organic Fine Ltd.) and implanted with a custom built indwelling jugular catheter (Brown *et al.*, 2009). Mice received analgesic (meloxicam, 3 mg/kg, i.p., Boehringer Ingelheim) and were allowed to recover for two days prior to cocaine self-administration training. During the recovery period, catheters were flushed with 0.03 ml of 0.9% physiological saline containing heparin (90 U, CSL) and neomycin

sulphate (4 mg/ml, Delta Veterinary Laboratories). During IVSA training catheters were flushed with 0.03 ml of 10 U heparinized saline with antibiotic prior to each session and 0.03 ml of 90 U heparinized saline after each training session.

Mice self-administered cocaine intravenously under an FR1 schedule over 12 daily, 2-hour sessions in the first half of the dark phase of the photoperiod. Responses on the reward-paired lever resulted in the infusion of 0.5mg/kg of cocaine hydrochloride (Sigma Aldrich, in 0.9% physiological saline) over 1.7 s (infusion volume of 19 µl) and the concurrent presentation of a 5-sec light cue. To minimize the risk of overdose, cocaine was unavailable during the presentation of the light cue and a within session maximum of 80 cocaine-paired lever presses was applied. Lever presses during the timeout period were recorded. Yoked animals received cocaine contingent on the response of a paired self-administering animal. Subsequent to self-administration training, animals were assigned to groups (1 day of abstinence + relapse test; IVSA 1, 21 days of abstinence + relapse test; IVSA 21 or IVSA 21 R, 21 days of abstinence + no relapse test; IVSA 21 NR) counterbalanced for cocaine-seeking behaviour (determined by the average number of active lever presses over the final 3 days of cocaine self-administration).

Group	Conditions	Experiments
Naïve	Caged during experiments Sacrificed at same time point as IVSA 1 animals	Two cohorts - Next-gen sequencing - MBD qPCR, mRNA analysis
IVSA Day 1 (IVSA 1)	12 days cocaine IVSA 1 day forced abstinence 1 hour relapse test	Two cohorts - Next-gen sequencing - MBD qPCR, mRNA analysis
IVSA Day 21 (IVSA 21/IVSA 21 R)	12 days cocaine IVSA 21 days forced abstinence 1 hour relapse test	Two cohorts - Next-gen sequencing - MBD qPCR, mRNA analysis
IVSA Day 21 NR (IVSA 21 NR)	12 days cocaine IVSA 21 days forced abstinence NO relapse test	One cohort - MBD qPCR, mRNA analysis
Yoked 1	12 days yoked cocaine 1 day forced abstinence 1 hour context re-exposure	One cohort - Next-gen sequencing
Yoked 21	12 days yoked cocaine 21 days forced abstinence 1 hour context re-exposure	One cohort - Next-gen sequencing

Table 4.1 Behavioural conditions

4.3.1.4 Abstinence

During abstinence, all mice were returned to their home cages. Periods of abstinence were either short (1 day) or long (21 days). In rats, self-administration of cocaine or natural rewards (food, sucrose) potentiates glutamatergic signalling in the VTA, however only following cocaine self-administration does this potentiation persist beyond 21 days (Chen *et al.*, 2008), which suggests that this time point may be used to identify long-lasting persistent epigenetic changes that are unique to cocaine self-administration.

4.3.1.5 Continued cocaine seeking during abstinence

With the exception of IVSA 21 NR mice, self-administering animals were subject to a 1-hour relapse test in the absence of cocaine after either 1 or 21 days of forced abstinence to examine the persistence of cocaine-seeking behaviour. Active lever presses resulted in the presentation of the previously cocaine-paired light cue and the activation of the syringe

pump. Cocaine-seeking behaviour was measured as the sum of all active lever presses over the course of the hour, including those performed during the timeout period. Yoked animals underwent simple contextual re-exposure for 1 hour. Animals used for next-generation sequencing were sacrificed by cervical dislocation immediately after relapse test, whereas those employed for the validation of the differentially methylated regions and gene expression analysis were sacrificed 2 hours after the end of the relapse test. Brains were removed following cervical dislocation, snap-frozen on liquid nitrogen and stored at -80C.

4.3.2 Identification of genome-wide changes in DNA methylation by MBD Ultra-Seq

Neuronal genomic DNA was isolated from the mPFC of individual animals, as described in Chapter 3. Next-generation sequencing was performed by MBD Ultra-Seq (Chapter 3), with two minor modifications: 75 ng of gDNA from each animal was used for library preparation and the final pooled, amplified, methyl-enriched libraries for each treatment group was loaded into two lanes, to prevent the production of artificial differences by inter-lane discrepancies. Regions of methylation enrichment (RMEs, or peaks) were identified as per Chapter 3.

4.3.3 Candidate DMR selection

To identify differentially methylated regions, RMEs covered by a minimum of 5 normalised reads in at least one of the treatment groups were selected. From this selection, RMEs within 600 bp of each other were grouped and defined as a single RME. Student's t-tests were performed to compare the relative level of 5mC enrichment (as measured by the normalised read counts) between naïve and cocaine-treated animals (IVSA 1, IVSA 21, Yoked 1, Yoked 21) at each grouped RME. The Benjamini-Hochberg false discovery rate multiple testing correction was used to calculate adjusted p-values, allowing for a false discovery rate of 10%. The level of 5mC enrichment was considered significantly different between groups if the FDR-adjusted p-value was less than 0.1 (Ellis *et al.*, 2012, Non *et al.*, 2014). Persistently differentially methylated regions (persistent DMRs) arising from cocaine self-administration are those that are significantly different in the IVSA 1 and IVSA 21 groups, but not in the yoked cocaine controls relative to naïve animals. Abstinence-associated DMRs are significantly different to naïve animals after 21 days of abstinence from cocaine self-administration. As the MBD2b/3L1 complex captures DNA fragments containing a minimum of 5 methylated CpGs (Active Motif), I ensured that the genomic region (RME summit +/- 150 bp) surrounding each DMR of interest contained at least 5

CpG dinucleotides. Finally, I confirmed the 300 bp region overlapping each DMR corresponded to a unique location in the mouse reference genome (mm9) to avoid artefacts produced by poor mapping of repetitive regions.

4.3.4 MBD quantitative PCR (MBD qPCR)

MBD pull downs were performed using 150 ng of neuronal DNA derived from the mPFCs of individual animals from a second cohort of animals. Genomic DNA was extracted as described in Chapter 3, except that two 250 µl aliquots were retained for the analysis of gene expression following the disruption of the tissue in the douncing buffer. Genomic DNA was sheared by sonication in 130 µl of ultrapure H_20 (Covaris S2, bath temperature: 4°C, duty: 10%, intensity: 6, cycle/burst: 100, time: 180 seconds) to fragments of ~300bp in length, as verified by Bioanalyzer HS (Agilent) and 13 µl of fragmented DNA was diluted in a total volume of 80 µl of DNAse/RNase free TE buffer (pH 8) and retained as input to control for slight variations in the amount of DNA used in each pull down. MBD pull downs were performed according to manufacturer's protocol (Methylcollector Ultra, Active Motif). Captured methylated fragments were eluted in 60 µl of TE buffer (pH 8).

A 300 bp region surrounding each candidate DMR was retrieved (UCSC genome browser, mm9) and primers (**Table 4.2**) were designed using Primer3 (Untergasser *et al.*, 2012) to amplify 120-300 bp regions overlapping the peak summit of select candidate DMRs. qPCR was performed in duplicate using a Rotor gene Q (Qiagen) in 10 µl reactions (5 µl 2X Sybr, 1 µl 10 µM primer (F+R), 3 µl H_2O, 1 µl MBD DNA/input DNA). Ct values for methyl-enriched DNA were normalised to input and the relative enrichment between groups was calculated using the delta-delta ct method.

DMR	Forward primer	Reverse Primer
KCTD16-associated	AGGGCGAGGCTTCTAGTGA	TCCTTTCAGGTCCACCTTTG
GOLGB1-associated	GCAGTCGTACGAGAACGTGA	ATCCTGCAGCTGCTTCTCTC
SNW1-proximal	ACTTTTTCCCCTCCATCGAC	ACTACTGAGAGCCCCGGAAT
GLRa1-associated	GAGCAGCAGGTGAGTGACAG	AGAGAGATGGAGGAGGTGAGG
CDH13-assocatied	TGCCTACCTGTGCGTATGAG	TTTCCAGGCTCCTGTCCTAA
Gm10375-proximal	TGGAACCCAGTGGAAGTCTC	CCCTCCTCAGCGATTACAAA

Table 4.2 Primers used for the validation of DMRs by MBD qPCR

4.3.5 Analysis of gene expression

4.3.5.1 RNA extraction and cDNA synthesis

250 µl of whole mPFC homogenate was retained from each animal in the second biological cohort following homogenisation of the brain punches in nuclear lysis buffer. RNA was extracted by Trizol LS (Invitrogen) according to the manufacturers protocol and quantified by spectrophotometry (Nanodrop). 250 ng of RNA was reverse transcribed (QuantiTect Reverse Transcription, Qiagen). cDNA was diluted to approximately 100 ng/ul prior to qPCR (Nanodrop). Whole mPFC homogenate was used because it is not possible to obtain neuronal mRNA from neuronal nuclei (mRNA resides in the cytoplasm and attempts at sorting whole neurons have been unsuccessful).

4.3.5.2 Quantitative PCR (qPCR)

Primers (IDT) for gene expression quantification were designed using Primer3 (Untergasser *et al.*, 2012), AutoPrime (Wrobel *et al.*, 2004) or obtained from PrimerBank (Wang *et al.*, 2012) (**Table 4.3**). In select cases, the expression of multiple isoforms of the gene of interest was examined. qPCR was performed in triplicate using a Rotor gene Q (Qiagen) in 10 µl reactions (5 µl of 2X Sybr, 2 µl of 5 µM primer (Foward+ Reverse), 2 µl H_2O, 1 µl 100 ng/ul cDNA). Relative expression was quantified using the delta-delta Ct method, normalised to dynein expression, which was the most consistent of 5 housekeeping genes tested (B-actin, PPIA, PGK, 18S and dynein).

Gene (isoform)	Forward primer	Reverse primer
Cdh13 (all)	CCTGTCCTAAACTTGACC	GAGTTCTGCCATGTCTTC
Cdh13 (001, 003-006	CTGCTGTCCCAGGTGCTC	TGAAGGTCAAGTTTAGGACAGG
Cpeb4 (all)	AGGATAAACCAGTGCAGATCC	GAGCCATCCATCACAAAGTC
Cpeb4 (201,203, 002)	GCGAAGGAGAGGTCAGTC	CTGGTGAGTGAAGCAGTGAG
Glra1 (all)	CGGAATGGCAATGTCCTCTAC	GAGTTGCATGATACACGTCTGT
Golgb1 (all)	CTTCCTCAGACGCTGACTC	CAGCTCCACCACTAACTTCT
Kctd16 (all)	TCTTCTATCGTGAGCCTTCC	CAGGTCACTTTTCCGCCTCAT
Mctp1 (all)	CCACAAGAACCTAAATCCTGTGT	AAAGGCTGAGCCCATAAAGTC
Mctp1 (001)	CAGGCTCTGCAGAAGGACAT	CTGGTACATTCCGGGATCAG
Nkain3 (all)	TGCTCGCTGGTCTGTCTCT	ACACCATGATGTAACGTGGTCTA
Snw1 (all)	GCTCACCAGCTTTTTACCTGC	GCTCCCTTCGAGAGGAGAC
Dynenin	GGACATTGCTGCCTATATCAAGAAG	CGTGTGTGACATAGCTGCCAA

Table 4.3 Primers used for quantification of mRNA expression by qPCR.

Primers were designed by Primer3, AutoPrime or retrieved from PrimerBank. In the case of isoforms, “all” refers to the fact that the primer amplifies all the isoforms of the gene overlapped by the corresponding DMR, but not necessarily all isoforms of the gene itself. Isoforms are numbered according to Ensembl Build 37 of the mouse reference genome.

4.3.6 Statistical analysis

4.3.6.1 Intravenous cocaine self-administration

A two-way analysis of variance (ANOVA) was performed with factors ‘lever’ (cocaine-paired or inactive) and ‘days of abstinence’ (1 or 21 days) in Prism 6.0. A Holm-Sidak posthoc test was used to examine the significant main effect of ‘lever’. A Kruskal-Wallis test followed by Dunn’s multiple comparison post hoc tests was used to examine differences the number of lickometer contacts, as this data was non-parametric. Lickometer contacts indicated as mean contacts ± standard error of mean.

4.3.6.2 Heatmap

All regions of methylation (RMEs) were identified by MACS, as described in Chapter 3 (calling peaks). If enrichment for methylation was identified in greater than 50% of biological replicates in either the Naïve, IVSA 1 or IVSA 21 groups, that RME was retained and illustrated in the heatmap for all groups. The colour of each tile indicates the number of biological replicates within each group that displayed enrichment at a given RME (red = all biological replicates, white = no biological replicates).

4.3.6.3 Quantitative PCR (MBD qPCR and gene expression analysis)

For each candidate DMR or gene, a one-way ANOVA was used to examine variance, followed by Holm-Sidak multiple comparison post hoc tests if the overall ANOVA was significant. In instances where the homogeneity of variance assumption was violated, Welch's one-way ANOVA test was used, followed by Games-Howell post hoc tests if the overall ANOVA was significant. Where data was non-parametrice, a Kruskal-Wallis test was used, followed by Dunn's multiple comparison post hoc tests. For all tests, the significance confidence limit was set at 95%. Statistical analysis was performed using GraphPad Prism version 6.0.

4.4 Results

4.4.1 Mice persistently seek cocaine during protracted abstinence

Enduring cocaine-related memories are a prerequisite for continued cocaine seeking during abstinence; conversely, this behaviour indicates the presence of such memories. We therefore sought to establish that our paradigm produces continued cocaine seeking during abstinence. Cocaine-seeking behaviour manifests as a sustained and significant preference for the previously cocaine-paired lever relative to the inactive lever. In our paradigm, mice trained to self-administer cocaine continue to seek cocaine during abstinence, pressing the previously cocaine-paired lever significantly more than the inactive lever after 1 and 21 days of abstinence, ($F_{1,62}$ = 84.35, p<0.0001, **Figure 4.1**). The number of responses on the previously cocaine-paired lever did not differ across the period of enforced abstinence mice do not press the previously cocaine-paired lever significantly more following 21 days of abstinence, as compared to 1 day of forced abstinence (Holm-Sidak's post hoc, *ns***, Figure 4.1**). Moreover, it is unlikely that continued lever pressing is due to continue sucrose-seeking (following the use of sucrose self-administration in establishing the instrumental response) as the mean number of contacts with the lickometer was significantly less during cocaine self-administration (1.56 ± 0.69

contacts), the relapse test after 1 day of abstinence (0.43 ± 0.43 contacts) and during the relapse test after 21 days of abstinence (10 ± 4.55 contacts) than during sucrose self-administration (4185 ± 282 contacts), ($H_{4,57}$ =42.82, p<0.0001, Dunn's post hoc tests: cocaine IVSA vs. sucrose SA, p<0.0001, IVSA 1 vs. sucrose SA, p<0.001, IVSA 21 vs. sucrose SA, p<0.05). Importantly, continued cocaine seeking during abstinence suggests that mice 'remember' the cues, stimuli, context and behaviours associated with cocaine availability and denotes the presence of long-lasting cocaine-related memories. This model of cocaine self-administration is therefore appropriate for the examination of the neural adaptations that underlie the maintenance of cocaine-related memories during abstinence.

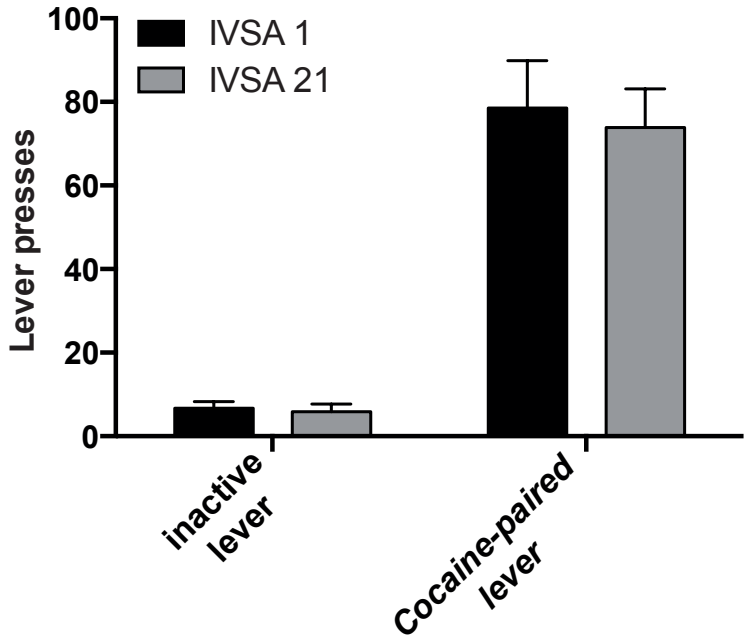


Figure 4.1 Mice persistently seek cocaine during protracted abstinence.

Mice pressed the previously cocaine-paired lever significantly more than the inactive lever after 1 and 21 days of forced abstinence, $F_{1,62}$ = 84.35, p<0.0001 (n=18 and 15, respectively). There was no difference in the number of cocaine-paired lever presses performed after 1 or 21 days of forced abstinence (Holm-Sidak's post hoc, *ns*). Data displayed as mean lever presses ± SEMs.

4.4.2 Altered DNA methylation following cocaine self-administration

DNA methylation was identified by MBD Ultra-Seq (Li *et al.*, 2014) using neuronal DNA derived from the mPFCs of naïve animals (n=5), cocaine self-administering mice sacrificed after either 1 or 21 days of abstinence (n= 7 and 6 respectively) and yoked cocaine controls sacrificed at the same time points (n=3 and n=4). The mean total intake of cocaine across self-administration (IVSA 1: 40.85 ± 1.73 infusions, IVSA 21: 43.01± 2.14 infusions) or yoked cocaine exposure (Yoked 1: 39.64 ± 3.32 infusions, Yoked 21: 45.69 ± 2.87 infusions) did not differ between groups ($F_{3,44}$ = 1.05, *ns*). MACS (Zhang *et al.*, 2008) identified 46,464 regions of methylation enrichment (RMEs) across the genome. Preliminary inspection of a heatmap of RMEs identified in at least half the biological replicates of either the naïve, IVSA 1 or IVSA 21 groups (**Figure 4.2**), reveals that both cocaine exposure and cocaine self-administration produce distinct patterns of 5mC enrichment.

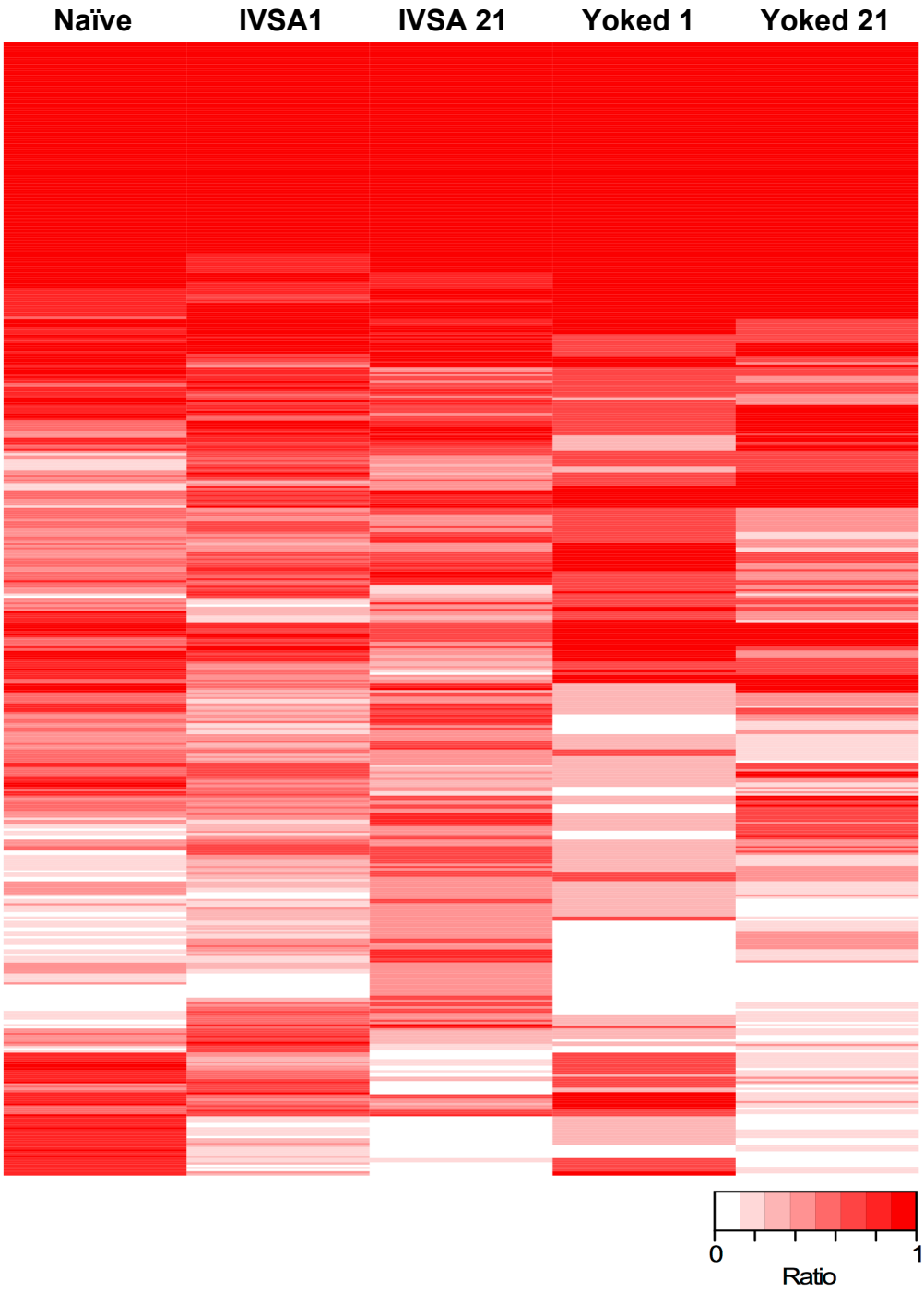


Figure 4.2 Representative heatmap of 5mC enrichment

All RMEs that were supported by enrichment (relative to background genomic coverage) in at least 50% of biological replicates in naïve (n=6), IVSA 1 (n=7) or IVSA 21 (n=6) animals are plotted. White indicates that no biological replicates displayed enrichment for 5mC, while dark red indicates that all biological replicates had enrichment for 5mC at the given genomic locus.

4.4.3 Persistent modifications of DNA methylation following cocaine self-administration

Modifications of DNA methylation that arise during cocaine self-administration and persist over time are consistent with the enduring nature of cocaine-related memories and consequently more prone to be implicated in their maintenance. 29 genomic regions were persistently significantly differentially methylated (relative to naïve animals) following the self-administration of cocaine but not passive cocaine exposure ($p<0.1$ in IVSA 1 and IVSA 21 animals, $p>0.1$ in Yoked 1 and Yoked 21 animals, FDR-adjusted p-values relative to naïve animals following Student's t-tests). The genomic coordinates of the persistent DMRs and associated or proximal genes are listed in **Table 4.4.** Five persistent DMRs were demethylated following cocaine self-administration; the remainder were subject to increased methylation.

The genomic distribution of persistent IVSA-associated DMRs was examined using EpiExplorer (Halachev *et al.*, 2012), a web-based tool that identifies genes and regulatory features overlapped by user-defined genomic regions. 15 of 29 DMRs overlap genes and a further 5 are located within less than 10 kB of a gene (NCBI Ensembl Build 37). Therefore, a chief function of persistent IVSA-associated changes in DNA methylation may be the regulation of the transcription of proximal genes, both coding and non-coding. Interestingly, gene-associated DMRs are predominantly located with intronic regions or non-coding loci; only 3 of these DMRs overlap principal promoter regions (-5kb to 1kb from TSS) or exons. The most common regulatory feature associated with persistent DMRs is repetitive elements (19 of 29 DMRs overlap repetitive elements identified by RepeatMasker). However, repetitive elements tend to be heavily methylated and it is easier to discern changes in DNA methylation at these loci (Hardcastle, 2013). Nevertheless, as cocaine exposure leads to repetitive element unsilencing (Maze *et al.*, 2011), it is possible that persistent changes in DNA methylation following cocaine self-administration oversee the regulation of repetitive elements. Persistent DMRs further consort with DNase I hypersensitive sites (13 of 29 DMRs overlap DNase HS sites). DNase I hypersensitivity is characteristic of open chromatin and classically associated with transcriptional activity (Weintraub & Groudine, 1976), which further advocates for a role of persistent IVSA-associated changes in DNA methylation in the regulation of the transcription of proximal genes. Additionally, 5 persistent DMRs overlap active enhancer regions (defined by an enrichment of histone H3 lysine 4 methylation and histone H3 lysine 27 acetylation (Creyghton *et al.*, 2010)). Finally, 5 persistent DMRs are located

within regions of the genome that are ordinarily anchored to the nuclear lamina (LADs). LADs regulate chromosomal architecture and comprise approximately 35% of the genome (Meuleman *et al.*, 2013). In sum, cocaine self-administration leads to distinct persistent modifications of the neuronal methylome and a primary function of these changes in DNA methylation is likely the regulation of the transcription of proximal genes, possibly through the modulation of several genomic features.

Chr	Location	Overlapped gene	Left gene (distance)	Right gene (distance)	Genomic region
4	3010811	None	N/A (centromere)	Vmn1r2-ps2 (20095 bp)	Intergenic
4	46790936	Gabbr2	Tbc1d2 (127855 bp)	Gm568 (230252 bp)	Intronic
4	141306461	Agmat	Ddi2 (27127 bp)	Dnajc16 (9643 bp)	Intronic
4	145428809	None	Gm13238 (908 bp)	Gm13246-001 (5498 bp)	Intergenic
4	146141283	Zfp600 Gm13165 Gm17452	Gm13051 (47261 bp)	Gm13170 (4242 bp)	Intronic Non-coding
4	146599023	Gm16889 Gm17317	Gm13163 (29703 bp)	Gm13147 (5370 bp)	Non-coding
4	146711210	Gm16889 Gm17317	Gm13151 (3367 bp)	Gm13145 (22407 bp)	Non-coding
5	33068902	Gm2420	Yes1 (79472 bp)	Pisd (10047 bp)	Non-coding
6	47698472	None	Y-RNA (11254 bp)	AC166252.1 (24856 bp)	Intergenic
6	103264621	None	U6.880 (452125 bp)	Chl1 (196249 bp)	Intergenic
7	23614005	None	Gm4207 (21673 bp)	Vmn1r159 (13072)	Intergenic
7	46628130	None	U6.424 (88627 bp)	Gm5114 (34534)	Intergenic
7	148824812	Muc6	Ap2a2 (5902 bp)	Muc2 (51456)	Exonic
8	14306925	Dlgap2	SNORA17.403 (33614 bp)	C030037F17Rik (438404 bp)	Intronic
8	19929891	Gm172379 2610005L07Rik	6820431F20Rik (36881 bp)	Gm15319 (3351 bp)	Non coding
8	73672924	None	Gm17576 (105985 bp)	Haus8 (102100 bp)	Intergenic
8	74324225	None	Fcho1 (74610 bp)	Gm5373 (52846 bp)	Intergenic
10	7233614	None	9230019H11Rik (6912 bp)	Lrp11 (76001 bp)	Intergenic
11	31822731	Cpeb4	Gm12107 (84517 bp)	4930524B15Rik (42861 bp)	Intronic
11	53954092	None	P4ha2 (8938 bp)	Gm12221 (18336 bp)	Intergenic
12	88821123	None	Snw1 (7874 bp)	Gm2022 (12151 bp)	Intergenic
13	3372770	None	Speer6d-ps1 (184207 bp)	2810429I04Rik (104779 bp)	Intergenic
13	76730298	Mctp1	Gm10760 (207822 bp)	SNORA17.278 (13782 bp)	Intronic
16	36915248	Golgb1	4930565N06Rik * (17973 bp)	Hcls1 (19821 bp)	Exonic

18	3005873	None	None (centromere)	Vmn1r-ps151 (21082 bp)	Intergenic
18	40467890	Kctd16	2900055J20Rik (50552 bp)	Rps19-ps13 (418003 bp)	Intronic
19	39341696	Cyp2c53 Cyp2c29	Cyp2c66 (80134 bp)	Cyp2c38 (122350 bp)	Intronic Non-coding
X	131588748	3632454L22Rik	Zmat1 (28917 bp)	Gm6207 (65752 bp)	Non coding
X	166505941	None	Mid1 (62273 bp)	Gm15069 (9891 bp)	Intergenic

Table 4.4 Persistent DMRs unique to cocaine self-administration.

Chromosome (Chr) and location are derived from Ensembl Build 37 (NCBI37) with location defined as the middle of the DMR.

Black: protein coding

Blue: processed transcript

Purple: Linc RNA

Grey: transcribed processed pseudogene

Orange: transcribed unprocessed pseudogene

Pink: Unprocessed pseudogene

Red: Miscellaneous RNA

Green: miRNA

Turquoise: snRNA

Indigo: SNORA

*: antisense

4.4.4 Abstinence-associated modifications of DNA methylation following cocaine self-administration

28 genomic regions became differentially methylated during abstinence from cocaine self-administration but not passive cocaine exposure ($p<0.1$ in IVSA 21 animals, $p>0.1$ in IVSA 1, Yoked 1 and Yoked 21 animals, FDR-adjusted p-values relative to naïve animals, **Table 4.5**). Moreover, 5mC enrichment at abstinence-associated DMRs is significantly different in IVSA 21 animals relative to IVSA 1 or Yoked 21 animals (follow-up Student's t-tests, $p<0.05$). 8 abstinence-associated DMRs became demethylated during prolonged abstinence, while the rest became methylated. 13 of 28 DMRs overlap genes and 3 more are located within <10 kB of a gene. Again, gene-associated DMRs predominantly occur within introns or non-coding loci; 5 of 19 gene-associated DMRs are located within exons or promoter regions. A greater percentage of abstinence-associated DMRs occur within 10-100 kB of annotated genes (39% of abstinence-associated DMRs as opposed to 14% of persistent DMRs) which may indicate a more pronounced role for abstinence-associated DMRs in the regulation of enhancer or insulator regions. Once more, abstinence-associated DMRs frequently co-localise with repetitive elements (16 of 28 DMRs overlap repetitive elements). Astonishingly, 24 of 28 abstinence-associated DMRs overlap or are located proximal to nuclear lamina domains (13 overlap and 11 are located within < 1 kB of nuclear lamina domains). The unusual association of abstinence-associated DMRs with lamina domains suggests extensive repositioning of the genome during abstinence, potentially with profound transcriptional consequences.

Chr	Peak summit	Overlapped gene	Left gene (distance)	Right gene (distance)	Genomic feature
1	26744426	None	4931408C20Rik (121 bp)	n-R5s209 (271552 bp)	promoter
1	85243851	C130026I21Rik Gm16028*	Gm7069 (33490 bp)	Gm16026 (19422 bp)	Intronic Non-coding
3	22163381	None	RNaseP_nuc.3 (12796 bp)	SNORA17.487 (145464 bp)	Intergenic
4	20119038	Nkain3	Ggh (125141 bp)	Gm11872* (151815 bp)	Intronic
4	60276939	None	Mup-ps3 (20240 bp)	Gm14311** (97572 bp)	Intergenic
4	145029937	Gm13277	Gm13224** (35635 bp)	Gm13230 (6706 bp)	Non-coding
chr4	145761171	None	Gm13251 (12155 bp)	Rex2 (120144 bp)	Intergenic
4	146593852	Gm16889 Gm17317	Gm13163 (24532 bp)	Gm13147 (10541 bp)	Non-coding
4	146624923	Gm16889 Gm17317 Gm13147	Gm13163 (55603bp)	Gm13158 (16599 bp)	Non-coding
4	146847085	Gm16889	Gm17317 (15957 bp)	Gm13152 (19441 bp)	Non-coding
4	147029598	2610305D13Rik	Gm13155** (30604 bp)	Gm16211 (13029bp)	Intronic
5	15195434	Speer7d-ps1	4930572O03Rik (32557 bp)	Speer-4d (19855 bp)	Non-coding
5	26425785	5031410I06Rik	Gm10471 (9954 bp)	Gm10220 (16472 bp)	3' UTR
5	95153090	None	Gm6025 (37712 bp)	Gm3176 (227009 bp)	Intergenic (island)
7	12595989	None	Vmn1r-ps60 (4430 bp)	Vmn1r77 (30659 bp)	Intergenic
8	20036646	None	2610005L07Rik (16254 bp)	None	Intergenic
9	120196465	None	Mobp (105863 bp)	Myrip (16589 bp)	Intergenic
10	7229820	None	9230019H11Rik (3118 bp)	Lrp11 (79778 bp)	Intergenic
10	7233650	None	9230019H11Rik (6948 bp)	Lrp11 (75948 bp)	Intergenic
11	55343509	Glra1	Gm12236* (2292 bp)	Gm12237 (24933 bp)	Intronic
14	43641447	None	Gm8068 (17965 bp)	Gm3102 (14108 bp)	Intergenic
14	43981432	Gm9732	Gm8127 (9086 bp)	Gm17155 (15276 bp)	Intronic
15	75934238	None	Nrbp2 (13795 bp)	BC024139 (15709 bp)	Intergenic
17	3082304	Pisd-ps2	Pisd-ps2	Scaf8	Non-coding

			(192 bp)	(32668 bp)	
17	29240998	None	Cdkn1a (3331 bp)	Gm16194* (10092 bp)	Intergenic
18	82390610	None	MiR5127 (201791 bp)	Gm17383** (117556 bp)	Intergenic
X	32099759	None	Gm2940 (145178 bp)	Gm2946 bp (30027 bp)	Intergenic
X	166584479	None	Gm15069 (68276 bp)	Gm15068 (54108 bp)	Intergenic

Table 1.5 Abstinence-associated DMRs arising from cocaine self-administration

Black: protein coding

Blue: processed transcript

Purple: Linc RNA

Grey: transcribed processed pseudogene

Orange: transcribed unprocessed pseudogene

Pink: Unprocessed pseudogene

Red: Miscellaneous RNA

Green: miRNA

Turquoise: snRNA

Indigo: SNORA

Light green: ribosomal (rRNA)

*: antisense

** known pseudogene

4.4.5 Verification of differential methylation by MBD qPCR

A selection of the observed differences in methylation between naïve and self-administering animals (Day 1 and 21) were selected for validation by MBD qPCR in an independent cohort of animals. An additional group that did not undergo relapse testing after 21 days of abstinence (IVSA 21 NR) was included to ensure that abstinence-associated changes in DNA methylation were not simply the product of the relapse test. The mean cocaine intake did not differ between Day 1 (56 ± 1.8 infusions), Day 21 R (57 ± 0.7 infusions) and Day 21 NR (55 ± 1.5 infusions) ($F_{2,33} = 0.51$, *ns*). Select DMRs included three regions that were persistently differentially methylated following cocaine self-administration and one that became differentially methylated during abstinence. Two of the persistent DMRs overlapped coding genes (KCTD16 and GOLGB1) and one was located proximal to SNW1, while the abstinence-associated DMR was located within an intron of GLRa1. Graphs of the normalised read distributions at these DMRs are provided in **Figure 4.3.** All candidate DMRs were successfully validated by MBD qPCR **(Figure 4.4)**, indicating that MBD Ultra-Seq and the statistical limitations employed reliably identify changes in DNA methylation induced by cocaine self-administration.

While DMRs identified by the statistical means above validate reliably, DMRs covered by less than 5 mean normalised reads or with more stereotypical p-values ($p<0.02$-0.05 prior to FDR correction) show marked variation between individuals. Nevertheless, two DMRs within the latter category were validated by MBD qPCR (**Figure 4.5**) and merit inclusion. The first was located within Cadherin 13 (CDH13), a gene that is heavily implicated in addiction to multiple substances (Hart *et al.*, 2012) (**Figure 4.5a**). The second DMR was located upstream of gm10375 (**Figure 4.5b**) and illustrates the rapid and dynamic regulation of DNA methylation in the hours that follow relapse and memory retrieval, indicating that the retrieval of cocaine-related memories during abstinence may induce modifications of DNA methylation.

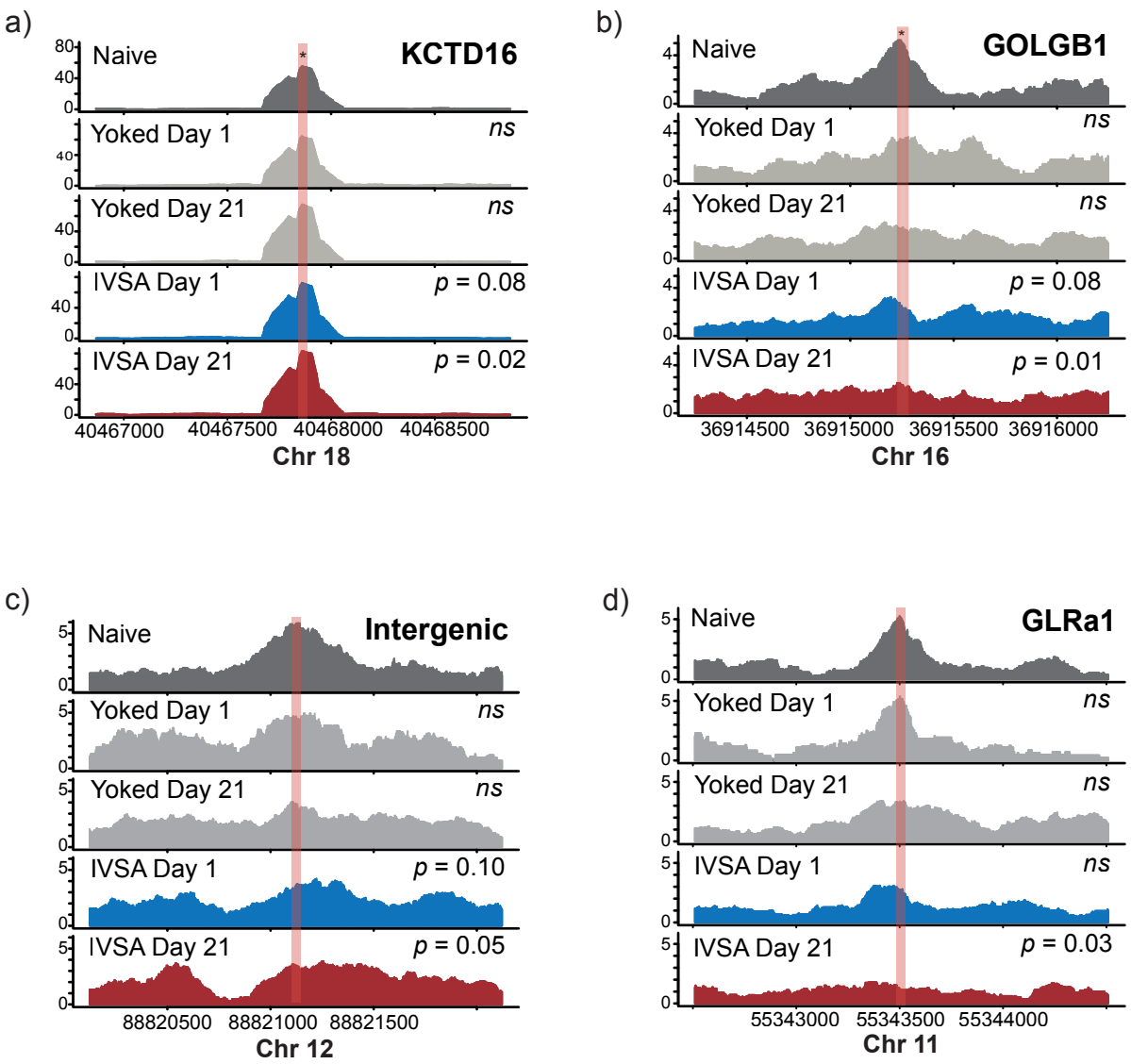

a)
Naive
KCTD16
Yoked Day 1
ns
Yoked Day 21
ns
IVSA Day 1
p = 0.08
IVSA Day 21
p = 0.02
40467000 40467500 40468000 40468500
Chr 18
b)
Naive
GOLGB1
Yoked Day 1
ns
Yoked Day 21
ns
IVSA Day 1
p = 0.08
IVSA Day 21
p = 0.01
36914500 36915000 36915500 36916000
Chr 16
c)
Naive
Intergenic
Yoked Day 1
ns
Yoked Day 21
ns
IVSA Day 1
p = 0.10
IVSA Day 21
p = 0.05
88820500 88821000 88821500
Chr 12
d)
Naive
GLRa1
Yoked Day 1
ns
Yoked Day 21
ns
IVSA Day 1
ns
IVSA Day 21
p = 0.03
55343000 55343500 55344000
Chr 11

Figure 4.3 Mean normalised reads surrounding validated DMRs

Plots of mean normalised read counts from sequencing surrounding DMRs that have been validated by sequencing. (a) A plot of the normalised mean read counts surrounding a persistently methylated DMR located within an intron of KCTD16 (potassium channel tetramerisation domain containing 16), which was centred at chr 18: 4046790 (mm9 reference genome) (b) A plot of the normalised mean read counts surrounding a persistently demethylated DMR located within an exon of GOLGB1 (Golgin b1) and centred at chr 16: 36915248 (c) A plot of the normalised mean read counts surrounding a persistently demethylated intergenic DMR located proximal to SNW1 (snw1 domain containing gene) and centred at chr 12: 88821123 (d) A plot of the normalised mean read counts surrounding an abstinence-associated DMR that was located within an intron of GLRa1 (glycine receptor subunit alpha 1) and demethylated during abstinence. P-values are relative to naïve animals and are FDR-corrected p-values derived from a Benjamini-Hochberg correction following Student's t-tests. Y-axis values are the mean number of aligned reads for each treatment group, X-axis values are location within the chromosome.

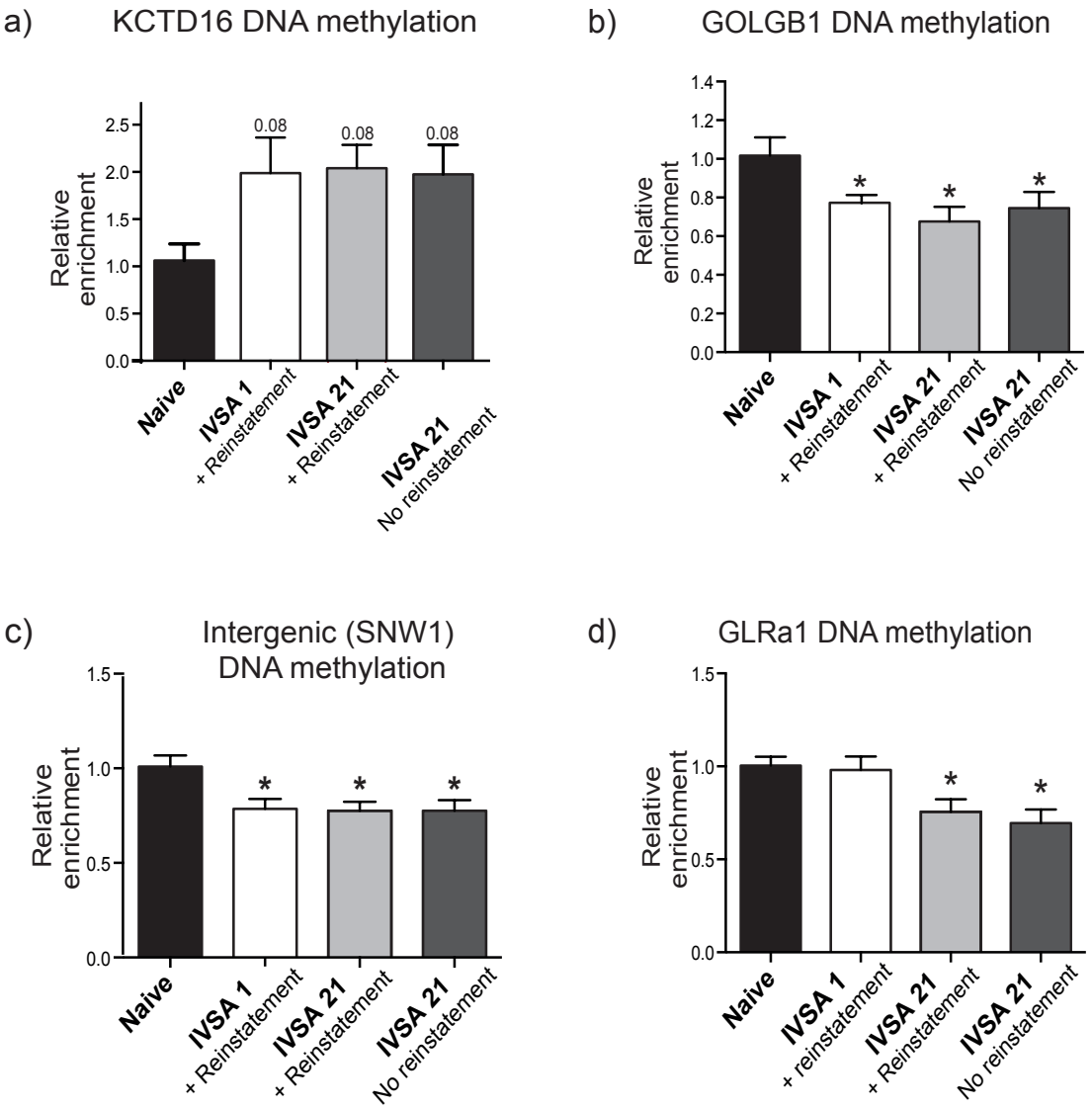
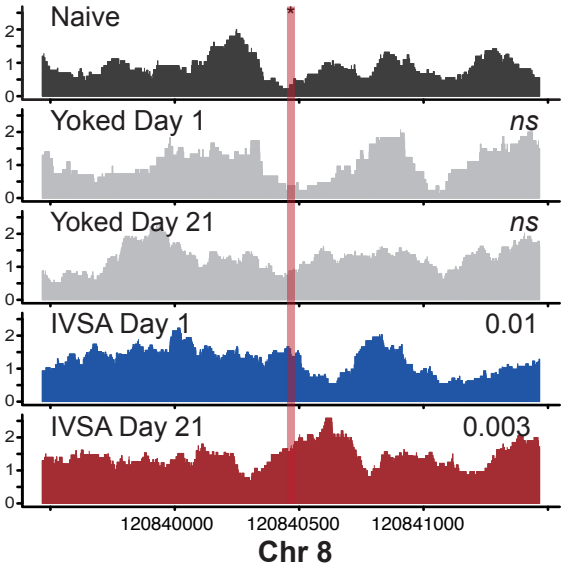
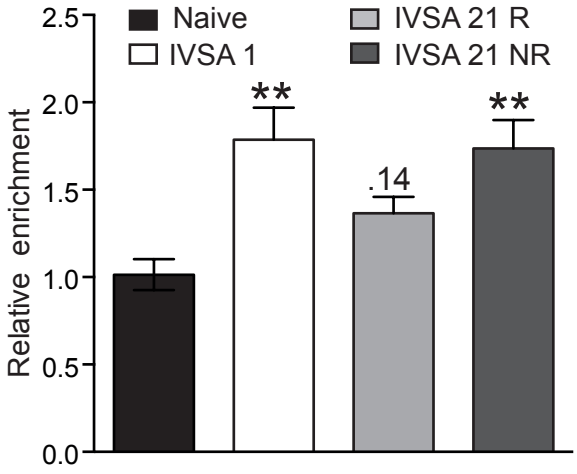


Figure 4.4 Validation of select DMRs by MBD qPCR

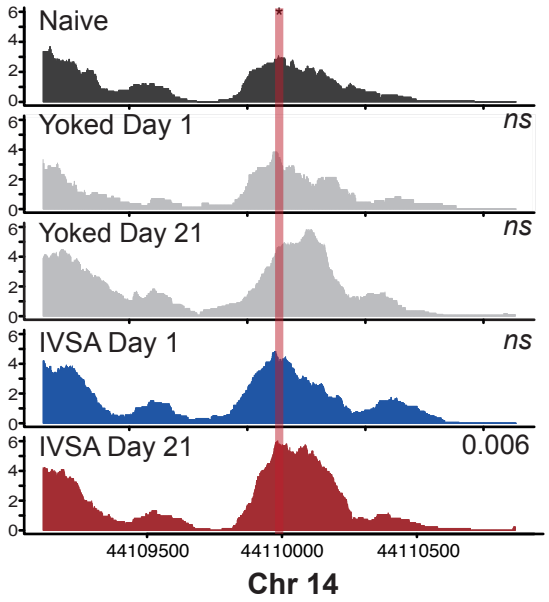
In an independent cohort of animals, qPCR was performed to assess the relative levels of 5mC enrichment at DMRs identified in Figure 4.4. (a) In accordance with sequencing results, the DMR located within KCTD16 was persistently methylated following cocaine self-administration, $F_{3,16}$ = 2.73, p=0.07. (b) Similarly, the locus within GOLGB1 was demethylated following cocaine self-administration, $F_{3,17}$= 3.57, p<0.05. (c) Demethylation was also replicated at the intergenic locus located proximal to SNW1, $F_{3,17}$= 4.35, p<0.05. (d) Finally, demethylation of the GLRa1-associated DMR was reproduced following 21 days of abstinence, $F_{3,17}$ = 5.59, p<0.01. All values displayed as means ± SEM, p-values derived from Holm-Sidak post hoc tests relative to naïve, * = p<0.05.

a) DMR associated with Cadherin 13





b) Intergenic abstinence-associated DMR upstream of gm10375



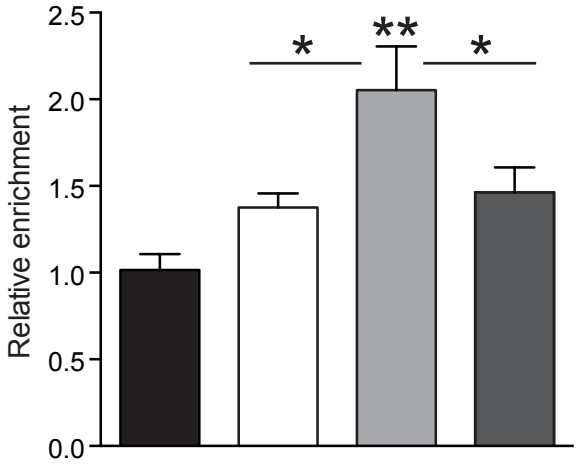


Figure 4.5 Validation of DMRs that did not pass FDR-based statistical thresholds

Two DMRs that did not pass initial FDR-based statistical thresholds were nevertheless replicated in a second cohort by MBD qPCR. Left panels display the mean normalised sequencing reads surrounding the DMRs, right panels illustrate the MBD qPCR results (a) Cocaine self-administration led to an increase in methylation at the locus located within CDH13 (Cadherin 13), $F_{3,16}$ = 6.13, p<0.01 (right panel). (b) CpG methylation was increased at a locus upstream of gm10375 after 21 days of abstinence and relapse testing, $F_{3,17}$ = 7.17, p<0.01 (right panel). This change in methylation was the product of epigenetic remodelling during the relapse test, as there was a significant difference between the degree of methylation in the animals subject to relapse testing and those that were simply sacrificed after prolonged abstinence (right panel). * = p<0.05, ** = p<0.01, Holm-Sidak post hoc values.

4.4.6 Cocaine self-administration produces enduring changes in DNA methylation

One might predict that most modifications of DNA methylation induced by self-administration would be transient, with a handful persisting during abstinence. However, only one locus (centred at chr 6: 3133868, not shown) was transiently differentially methylated in response to cocaine self-administration, but not passive cocaine exposure ($p<0.1$ in IVSA 1, $p>0.1$ in IVSA 21, Yoked 1 and Yoked 21 animals relative to naïve, FDR-adjusted p-values, follow up Student's t-tests IVSA 1 vs. IVSA 21, $p<0.05$, IVSA 1 vs. Yoked 1, $p<0.05$). The dearth of transient changes in DNA methylation may be due to several factors: 1) most parsimoniously, animals underwent 12 days of self-administration and transient changes in DNA methylation may have long dissipated, 2) most changes in DNA methylation incurred by cocaine self-administration are persistent and short-term changes in gene expression are orchestrated by ephemeral epigenetic modifications, such as histone methylation and acetylation or 3) transient changes in DNA methylation occur in other regions of the brain following cocaine self-administration, such as the hippocampus, as was observed following fear conditioning (Miller *et al.*, 2010). Nevertheless, the lack of transient IVSA-associated changes in DNA methylation is intriguing.

4.4.7 Transcriptional consequences of cocaine-induced changes in DNA methylation

Modifications of DNA methylation may exert a number of effects, but a primary function of altered DNA methylation remains the regulation of gene expression. In addition to propagating enduring changes in gene transcription, persistent experience-induced modifications of DNA methylation might prime the transcription of the affected locus upon subsequent neuronal (or memory) reactivation (see Chapter 2). According to this hypothesis, some persistent or abstinence-associated DMRs arising from cocaine self-administration would have no transcriptional consequence until the cocaine-related memories are in a reactivated state. To test this hypothesis, half of the animals that underwent prolonged abstinence were subject to a relapse test after 21 days of abstinence, while the remainder were simply sacrificed at the same time point. The relapse test (and exposure to previously cocaine-paired cues and context) should reactivate cocaine-associated memories, while in the latter instance these memories are likely to remain dormant. The DMRs co-localising with GOLGB1 **(Figure 4.6)** and GLRa1 **(Figure 4.7)** were accompanied by decreased gene expression, regardless of whether or not relapse testing occurred (GOLGB1: $F_{3,26} = 5.42$, $p<0.01$, Holm-Sidak's post hoc, naïve vs. IVSA 1, IVSA 21 R and IVSA 21 NR all $p<0.01$, GLRa1: Welch's $F_{3,13.36} = 7.52$, $p<0.01$,

Games-Howell post hoc, naïve vs IVSA 21 R and IVSA 21 NR, $p<0.1$). Conversely, despite increased DNA methylation in all groups, the expression of KCTD16 was altered only in animals that underwent a relapse test, $F_{3,26} = 6.55$, $p<0.01$, Holm-Sidak post hoc, naïve vs. IVSA 21 R, $p<0.1$, IVSA 21 R vs. IVSA 21 NR, $p<0.05$ **(Figure 4.8)**, providing preliminary evidence of the priming of gene expression by learning-induced changes in DNA methylation. Unsurprisingly, the intergenic DMR located proximal to SNW1 had no effect on its expression, $F_{3,26} = 0.07$, *ns* **(Figure 4.9)**. Finally, differential methylation within CDH13 was associated with an enduring change in the transcription of this gene, $F_{3,26} = 2.53$, $p=0.07$, Holm-Sidak post hoc, naïve vs. IVSA 1, IVSA 21 R, IVSA 21 NR, $p<0.1$ **(Figure 4.10).**

A number of significant DMRs were unsuited to validation by MBD qPCR, as they were located within lengthy repetitive elements for which region-specific primers could not be designed. Nevertheless, several of these DMRs consort with genes that have been implicated in addiction, including MCTP1 (multiple C2 domains, transmembrane 1), CPEB4 (cytoplasmic polyadenylation element binding protein 4) and NKAIN3 (Na+/K+ transporting ATPase interacting 3). MCTP1 and CPEB4-associated DMRs were persistently methylated following cocaine self-administration **(Figure 4.11a** and **b**, left panels**)**, while the DMR within NKAIN3 was methylated following prolonged abstinence from cocaine self-administration **(Figure 4.11c,** left panel**).** The persistent increase in DNA methylation within MCTP1 was associated with an enduring and significant decrease in the expression of MCTP1 (-001 isoform), $F_{3,26} = 4.07$, $p<0.05$, Holm-Sidak post hoc, naïve vs. IVSA 1, IVSA 21 R and IVSA 21 NR all $p<0.05$, while a similar increase in DNA methylation within CPEB4 was associated with a significant increase in the expression of this gene (-001 isoform), $F_{3,26} = 5.96$, $p<0.01$, Holm-Sidak post hoc, naïve vs. IVSA 1 and 21 NR, $p<0.01$, naïve vs. IVSA 21, $p<0.05$ **(Figure 4.11a** and **b,** right panel**)**. Interestingly, the expression of NKAIN3 was exclusively decreased in mice have undergone relapse testing after 21 days of abstinence, $F_{3,26} = 4.56$, $p<0.05$, Holm-Sidak post hoc, naïve vs. IVSA 21 R, $p<0.05$, IVSA 21 R vs. IVSA 21 NR, $p<0.01$ **(Figure 4.11c**, right panel**),** which provides further preliminary evidence of a complex relationship between learning-induced changes in DNA methylation, gene expression and the reactivation state of the memory.

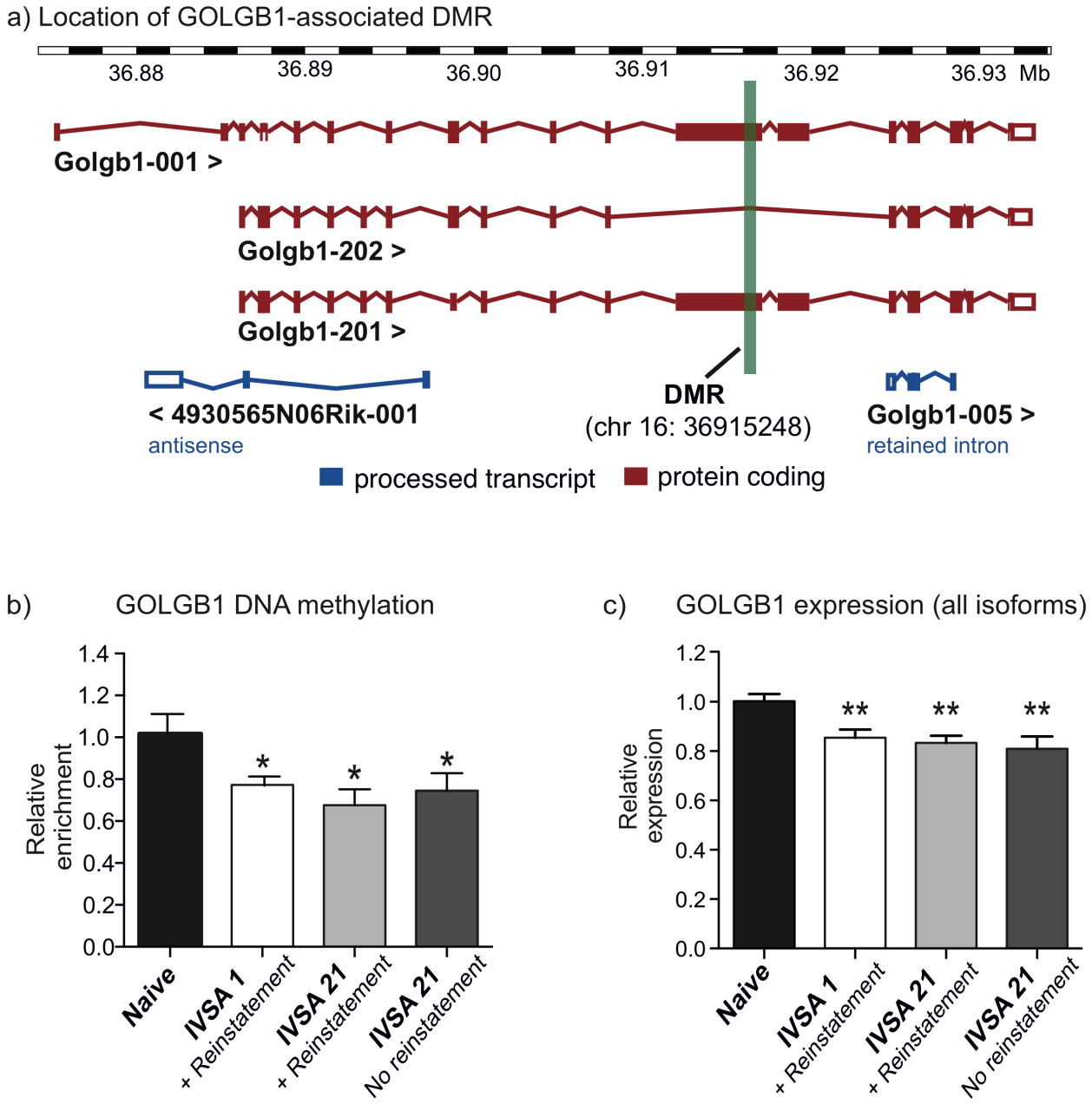


Figure 4.6 Differential DNA methylation and expression of GOLGB1

(a) The GOLGB1-associated DMR was located intragenically and overlapped an exon of this gene. (b) As previously shown (Figure 4.5), the DMR was persistently demethylated following cocaine self-administration c) Decreased DNA methylation was correlated with a significant reduction in the expression of GOLGB1 (all isoforms), $F_{3,26}$ = 5.42, p<0.01. Holm-Sidak post hoc, * = p<0.05, ** = p<0.01, data displayed as means ± SEM.

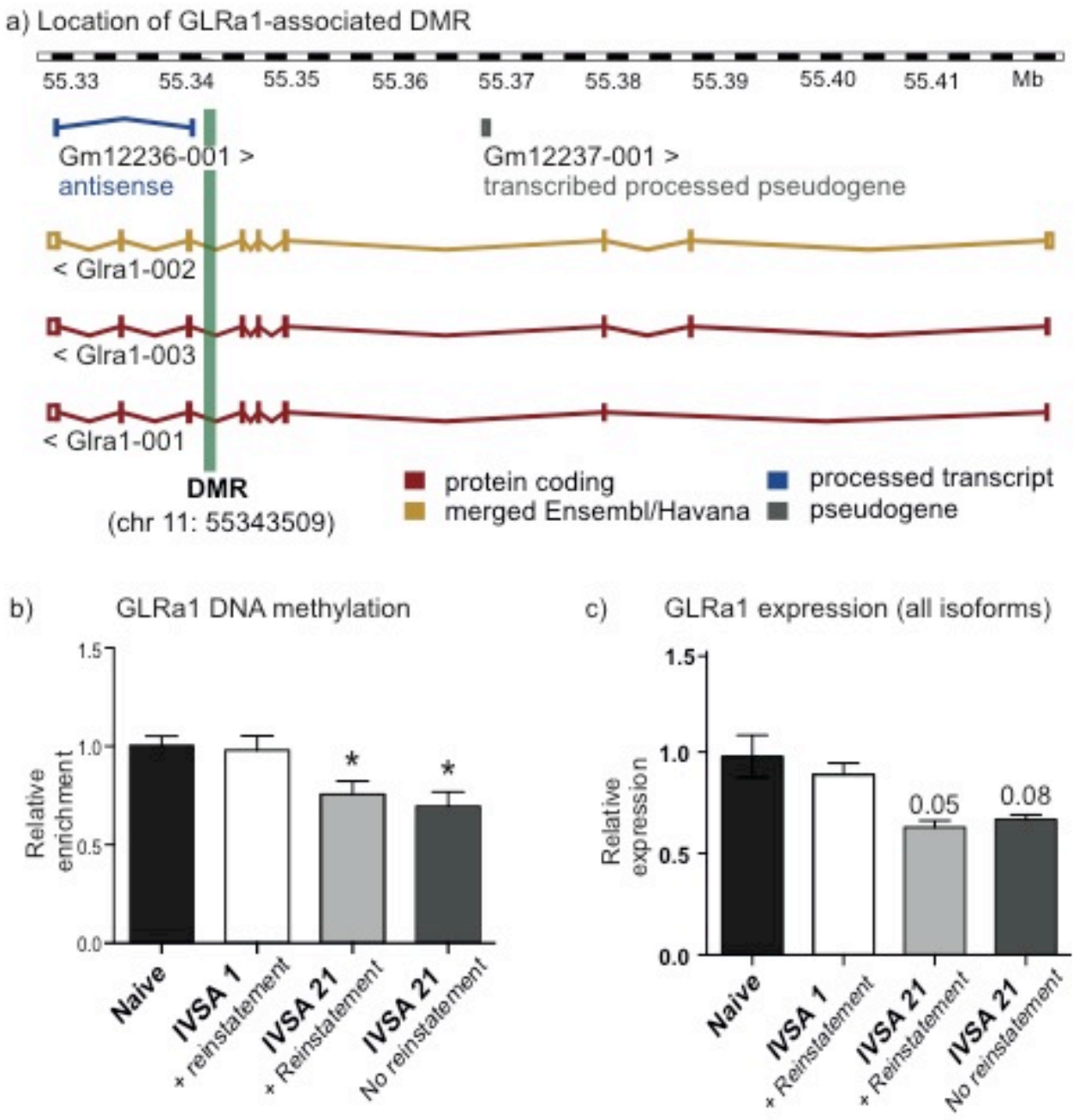


Figure 4.7 Differential methylation and expression of GLRa1

(a) The DMR associated with GLRa1 was located within an intron of this gene. (b) The DMR was significantly demethylated relative to naïve animals after 21 days of abstinence, as previously described in Figure 4.5 (c) Demethylation of the GLRa1-associated DMR was associated with a trend towards a significant reduction in the expression of GLRa1 (all isoforms) after 21 days of abstinence, Welch's $F_{3,13.36} = 7.52$, $p<0.01$, Games-Howell post hoc. * = $p<0.05$, data displayed as means ± SEM.

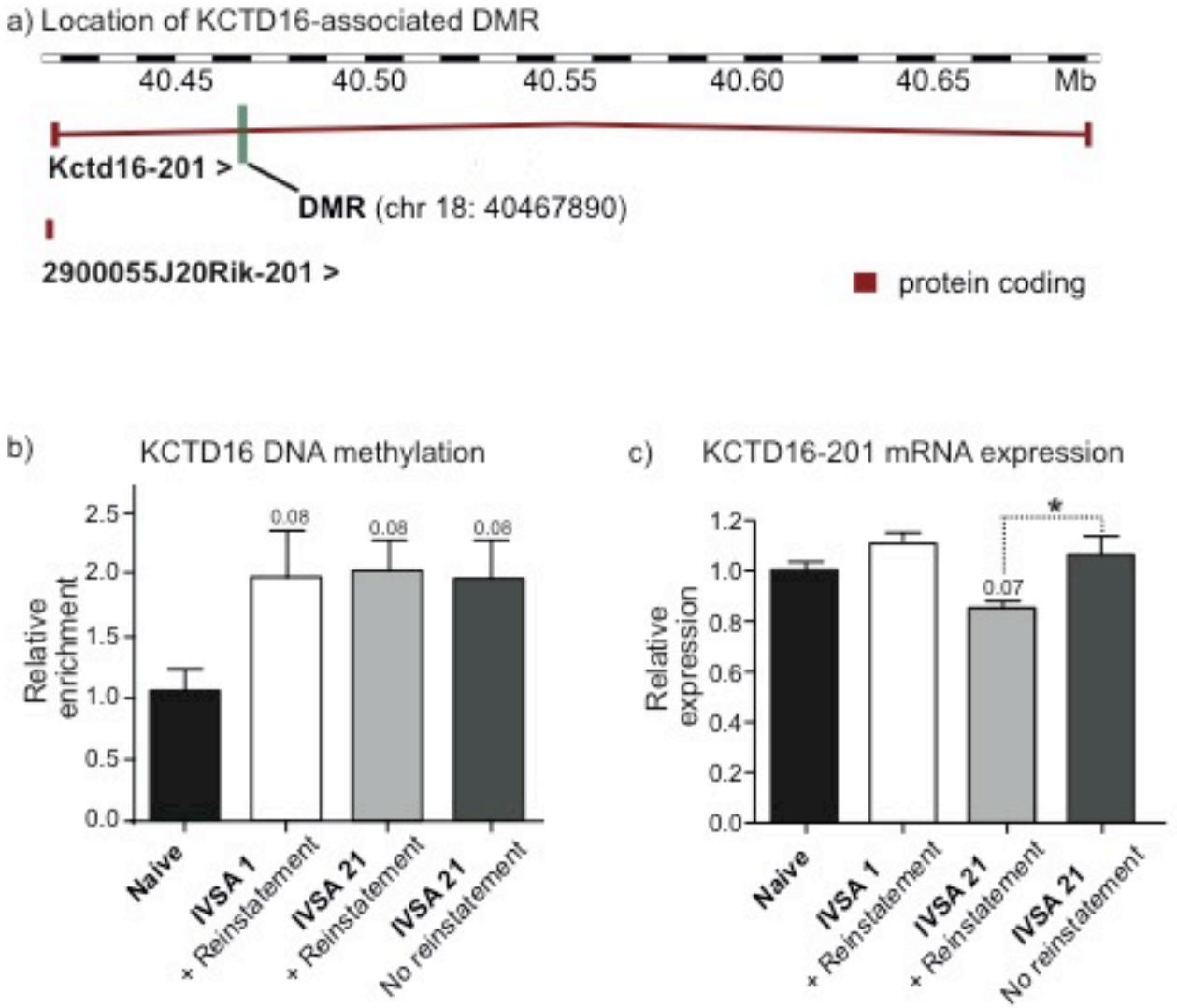


Figure 4.8 Differential DNA methylation and expression of KCTD16

(a) The DMR associated with KCTD16 was located within the sole intron of KCTD16. (b) As seen in Figure 4.5, cocaine self-administration resulted in a persistent increase in DNA methylation at the locus highlighted in (a) (c) A trend towards a significant decrease in gene expression was observed after 21 days of abstinence, $F_{3,26} = 6.55$, $p<0.01$. After 21 days of abstinence, KCTD16 expression was decreased in animals that underwent a relapse test (IVSA 21 R) compared to those that were simply sacrificed (IVSA 21 NR), $p<0.05$. Holm-Sidak post hoc, * = $p<0.05$, data displayed as means ± SEM.

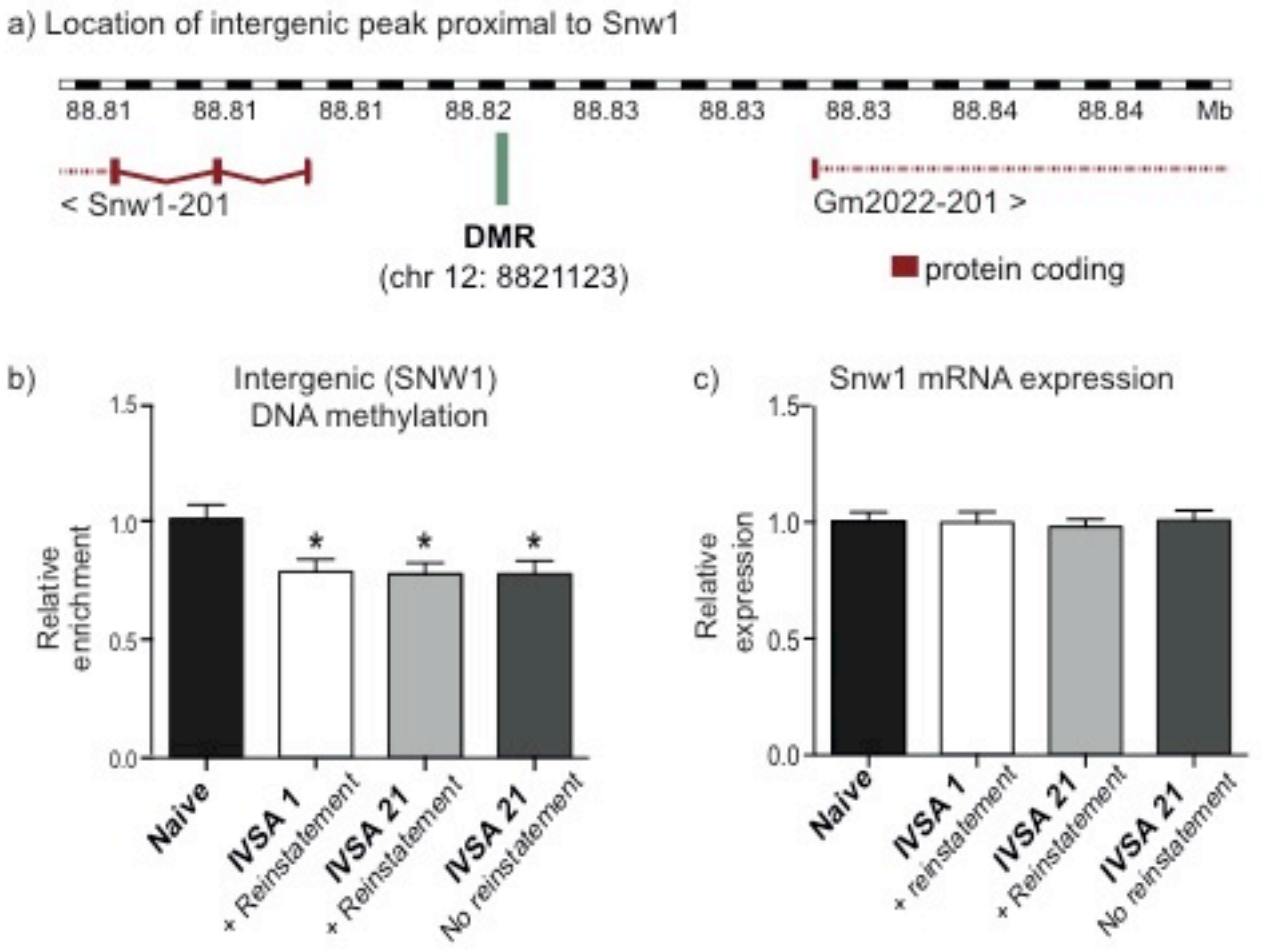


Figure 4.9 Differential intergenic DNA methylation and SNW1 expression

(a) The intergenic DMR was located proximal to SNW1. (b) At this locus, cocaine self-administration decreased DNA methylation, as previously seen in Figure 4.5. (c) Decreased DNA methylation was not associated with a significant change in the expression of SNW1, $F_{3,26} = 0.07$, *ns*. Holm-Sidak post hoc, * = $p<0.05$, data displayed as means ± SEM.

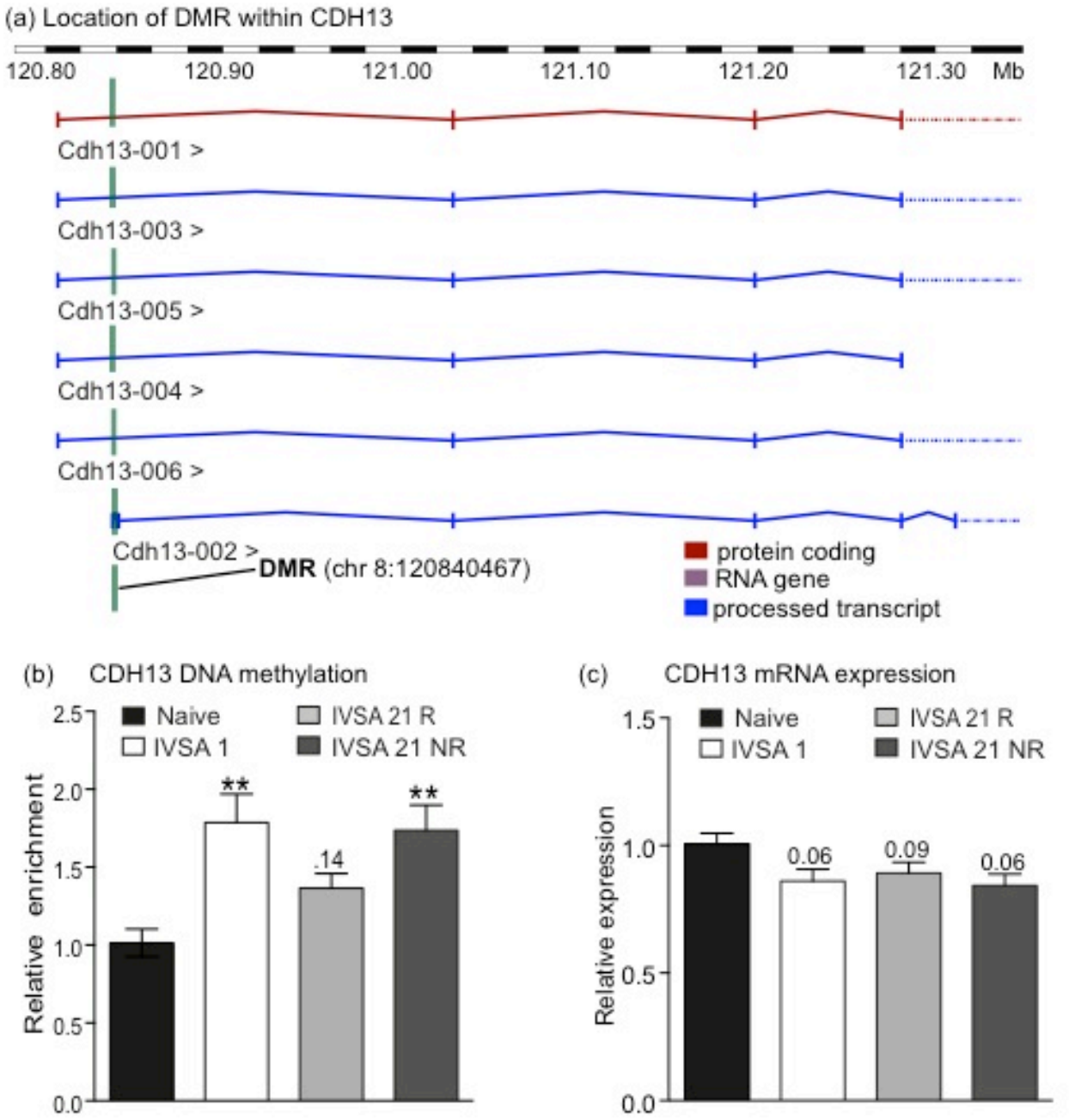
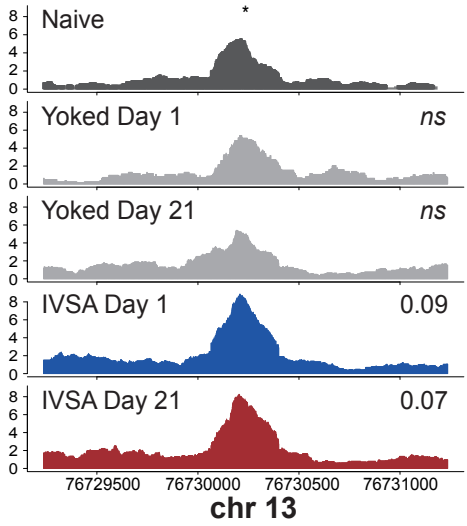
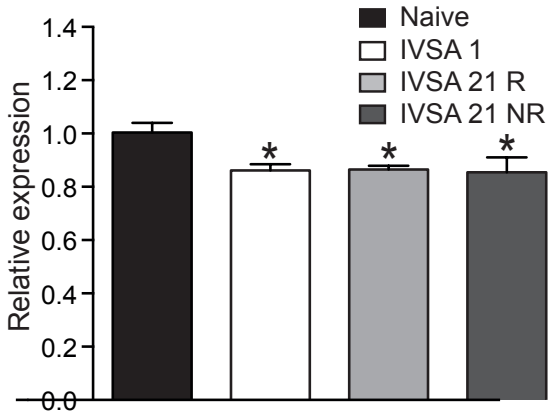


Figure 4.10 Differential DNA methylation and expression of CDH13

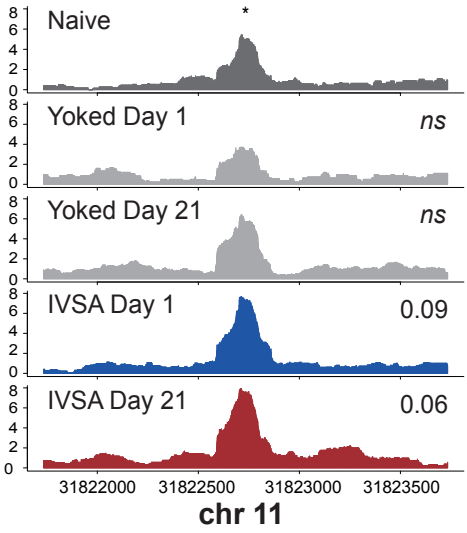
(a) Persistent DNA methylation following cocaine self-administration was located within an intron of CDH13-001, but overlapped the first exon of CDH13-002. (b) The DMR located within CDH13 was persistently methylated following cocaine self-administration, as previously seen in Figure 4.6 (c) There was a trend towards the reduction of CDH13 expression, $F_{3,26}$ =2.53, p=0.07, Holm-Sidak post hoc, ** = p<0.01, data displayed as means ± SEM.

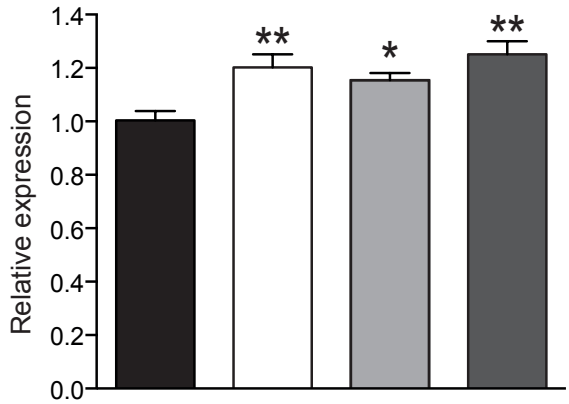
a) Differential DNA methylation and expression of MCTP1



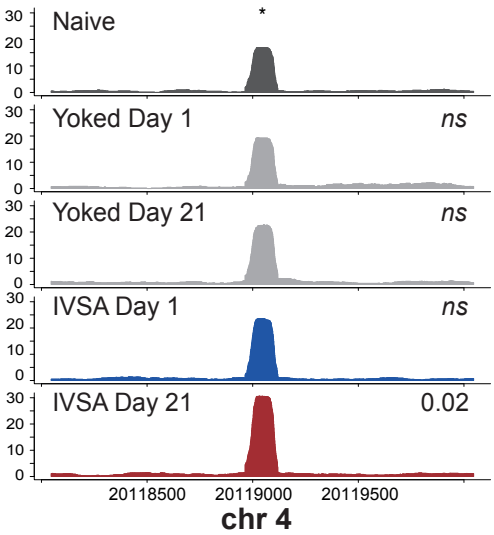


b) Differential DNA methylation and expression of CPEB4





c) Differential DNA methylation and expression of Nkain3



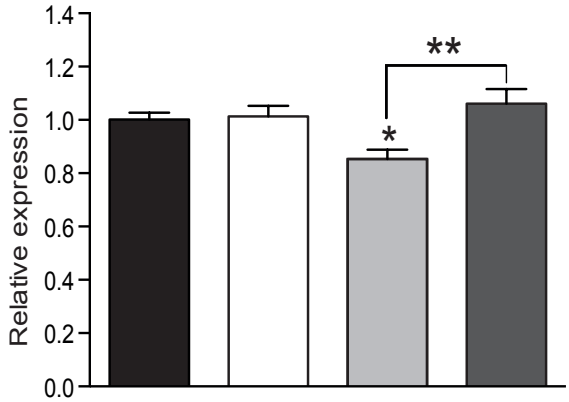


Figure 4.11 Expression of genes co-localising with DMRs that cannot be validated

(a) Cocaine self-administration produced a persistent increase in DNA methylation at a locus within MCTP1 (left panel). Increased DNA methylation was correlated with a concomitant decrease in the expression of MCTP1 (-001 isoform), $F_{3,26}$ = 4.07, p<0.05, Holm-Sidak post hoc (right panel). (b) Cocaine self-administration resulted in a persistent increase in DNA methylation within an intragenic region of CPEB4 (left panel). Increased DNA methylation was associated with a long-lasting upregulation of CPEB4 (001/201 isoform) expression, $F_{3,26}$ = 5.96, p<0.01, Holm-Sidak post hoc (right panel). (c) A significant increase in DNA methylation within an intron of NKAIN3 occurred after 21 days of abstinence (left panel). The increase in DNA methylation was associated with a decrease in the expression of NKAIN3 (all isoforms) after 21 days of abstinence and relapse testing, $F_{3,26}$ =4.56, p<0.05, naïve vs. IVSA 21 R, p<0.05, IVSA 21 R vs. IVSA 21 NR, p<0.01, Holm-Sidak post hoc. The expression of NKAIN3 was unaltered when mice were simply sacrificed after 21 days of abstinence. ** = p<0.01, * = p<0.05, data displayed as means ± SEM.

4.4.8 Regulation of alternative splicing by intragenic DNA methylation

A chief function of intragenic methylation is the regulation of alternative splicing. In our paradigm, DMRs within CPEB4, CDH13 and MCTP1 were associated with the regulation of specific isoforms of the co-localising genes (**Figure 4.12**). DNA methylation within CDH13 correlated with the overall downregulation of the expression of this gene, $F_{3,26}$ = 2.53, p=0.07 (**Figure 4.12a**), however this appears to stem from the regulation of non-coding isoforms as the expression of the protein coding isoform, CDH13-001 (Ensembl 37) was unaltered, $F_{3,26}$ = 1.35, *ns,* following cocaine self-administration (**Figure 4.12b**). Similarly, the overall expression of CPEB4 increased following cocaine self-administration, $F_{3,26}$ = 5.96, p<0.01 (**Figure 4.12c**), but the most common variant (CPEB4-001, Ensembl 37) exhibited a different pattern of expression, $F_{3,26}$ = 4.32, p<0.01; CPEB4-001 expression was unchanged relative to naïve animals in all groups, yet was differentially expressed in animals subject to a relapse test after 21 days of abstinence compared to those that were simply sacrificed (Holm-Sidak post hoc, p<0.01, **Figure 4.12d**). The splice variants that gave rise to the overall increase in the expression of CPEB4 remain to be elucidated. Finally, the persistent increase in methylation within MCTP1 was associated with an enduring decrease in the expression of MCTP-001, $F_{3,26}$ = 4.07, p<0.05 (**Figure 4.12f**), but the overall expression of all overlapped protein-coding transcripts of this gene was only significantly different in animals that underwent a relapse test after 21 days of abstinence compared to those that did not, $F_{3,26}$ = 3.62, p<0.05, Holm-Sidak post hoc, IVSA 21R vs IVSA 21 NR: p<0.05 (**Figure 4.12e**). Together, these data suggest that the intragenic modifications of DNA methylation produced as a result of cocaine self-administration regulate the expression of specific isoforms of associated genes.

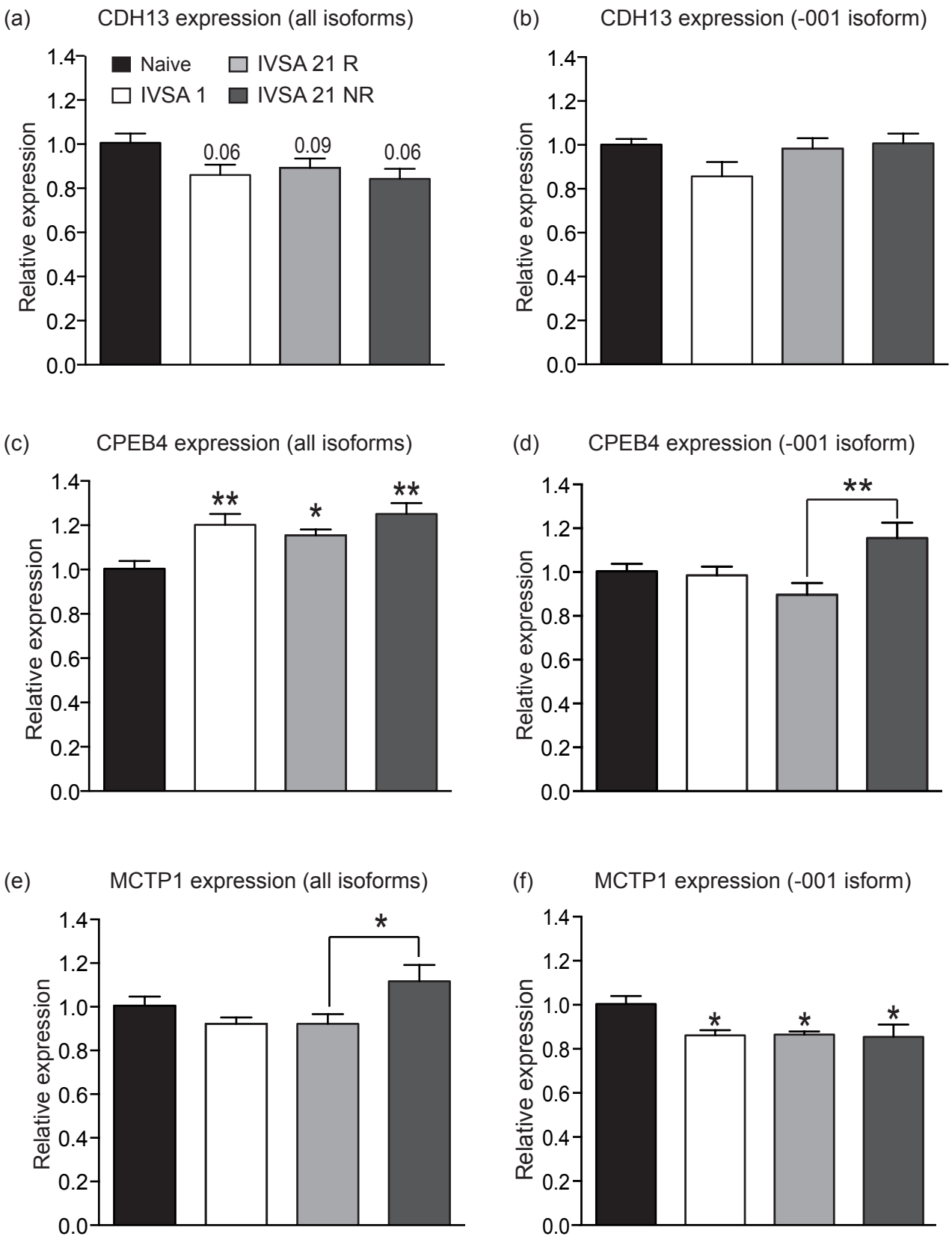

(a)
CDH13 expression (all isoforms)
Naive
IVSA 1
IVSA 21 R
IVSA 21 NR
Relative expression
0.06
0.09
0.06
(b)
CDH13 expression (-001 isoform)
Relative expression
(c)
CPEB4 expression (all isoforms)
Relative expression
**
*
**
(d)
CPEB4 expression (-001 isoform)
Relative expression
**
(e)
MCTP1 expression (all isoforms)
Relative expression
*
(f)
MCTP1 expression (-001 isform)
Relative expression
*
*
*

Figure 4.12 Regulation of alternative splicing by intragenic DNA methylation

Intragenic modifications of DNA methylation arising during cocaine self-administration may contribute to the differential regulation of select splice variants (a) There was a trend towards the overall decreased expression of CDH13, $F_{3,26}$ =2.53, p=0.07. However, this was likely due to the regulation of non-coding transcripts of CDH13 (see Figure 4.11) as the expression of the protein-coding transcript was not significantly altered following cocaine self-administration, $F_{3,26}$ = 1.35, *ns* (see b). (c) Subsequent to self-administration, CPEB4 expression was increased, $F_{3,26}$ = 5.96, p<0.01. (d) However, there was no significant change in the expression of the common isoform, CPEB4-001, relative to naïve animals, $F_{3,26}$ = 4.32, p<0.01, though there was a significant difference in the expression of this isoform in animals subject to a relapse test at 21 days of abstinence compared to those that were not (p<0.01, Holm-Sidak). (e) When the collective expression of all protein-coding isoforms of MCTP1 was explored, the sole significant difference was again between animals subject to a relapse test at 21 days and those that were not, $F_{3,26}$ = 3.62, p<0.05, Holm-Sidak post hoc, IVSA 21 R vs. IVSA 21 NR, p<0.05. (f) A persistent increase DNA methylation within an intron of MCTP1 was associated with a persistent decrease in the expression of MCTP1-001 (Ensembl 37), $F_{3,26}$= 4.07, p<0.05.

4.5 Discussion

In summary, voluntary cocaine self-administration (IVSA) induces modifications of the neuronal methylome that are distinct from those incurred by passive cocaine exposure. IVSA-associated changes in DNA methylation are predominantly persistent or arise over the course of abstinence and are therefore congruous with the enduring nature of memory. Moreover, differential methylation within genes is associated with concomitant changes in their expression. Surprisingly, persistent modifications of DNA methylation do not invariably yield enduring changes in the transcription of corresponding genes; in select cases altered transcription is exclusively evident following re-exposure to previously cocaine-paired cues and the self-administration context. Therefore, in some cases, learning-induced modifications of DNA methylation may remain transcriptionally silent and prime the transcriptional response to subsequent neuronal or memory reactivation, rather than ceaselessly modifying the expression of a gene.

A significant advance of this body of work is the identification of modifications of DNA methylation that are uniquely attributable to voluntary cocaine-self administration, rather than simple cocaine exposure. To date, many investigations have explored the epigenetic consequences of repeated involuntary cocaine exposure (Feng *et al.*, 2014, Maze *et al.*, 2011, Renthal *et al.*, 2009), a paradigm that is disturbingly unrelated to the consumption of cocaine under native conditions, and in which cocaine exposure is actually aversive (Twining *et al.*, 2009). In demonstrating that the modifications of DNA methylation associated with self-administration are unique, we hope to spur the adoption of a more ethologically valid model of cocaine-seeking in future epigenetic studies.

While the self-administration model of cocaine consumption is appropriate to the study of learned drug seeking and cocaine-related memories, the simple self-administration of cocaine is not a reliable indicator of addiction. Several of the identified DMRs may contribute to addiction, but in order to establish this it will be necessary to demonstrate that these DMRs are equally specific to animals that continue to self-administer cocaine in the face of adverse consequences, perhaps by using the conflict model of cocaine-self administration (see Chapter 5, (Cooper *et al.*, 2007). In addition, extended access to cocaine (ie. 6+ hrs of cocaine access) may result in further epigenetic modifications that are not observed following restricted (1-2 hr) access to cocaine (Ploense *et al.,* 2015). Finally, further investigations will determine if these changes are common to other reward-related learning paradigms.

IVSA-associated changes in DNA methylation likely stem from learned cocaine seeking and may therefore underpin the maintenance cocaine-related memories. Fittingly, the majority of these modifications of DNA methylation are persistent or develop as a consequence of forced abstinence; both types of modification may direct the maintenance of cocaine-related memories. Persistent IVSA-associated changes in DNA methylation may govern the maintenance of cocaine-related memories by orchestrating long lasting changes in gene transcription or by regulating transcriptional responses to incoming stimuli. Abstinence-associated changes in DNA methylation likely originate from the retrieval and reconsolidation of cocaine-related memories and beget their unusual persistence. Reconsolidation is the process by which memories are returned to long-term storage following retrieval and is dependent on *de novo* gene transcription and DNA methylation (Maddox & Schafe, 2011). Cocaine-related memories are readily retrieved by re-exposure to external cocaine-paired cues (discrete or contextual) and interoceptive cues (such as stress) (Saunders & Robinson, 2011) and may repeatedly undergo reconsolidation, producing *de novo* modifications of DNA methylation during abstinence. Befittingly, undergoing reconsolidation can increase the strength of memory trace (Tronson & Taylor, 2013) and consequently variations in DNA methylation produced by the reconsolidation of cocaine-related memories may contribute to their outstanding persistence. Moreover, DNA methylation is altered following memory retrieval at the DMR upstream of gm10375, providing tangible evidence of a retrieval/reconsolidation-induced change in DNA methylation.

It is possible that the IVSA-associated modifications of DNA methylation do not perpetuate the initial transcriptional signature of cocaine-related learning but instead constitute homeostatic responses to the wide range of changes in gene transcription that occur as a result of cocaine self-administration and exposure. Homeostatic *de novo* methylation could oppose transcriptional changes induced by cocaine, either by blunting the expression of up regulated genes or by forcing the expression of those that are down regulated. However, homeostatic responses in DNA methylation may nevertheless result in long-lasting changes in gene expression once the initial stimulus (ie. cocaine) is removed and potentially alter neuronal excitability. In some respects, memory is not only the perpetuation of an initial learning signal but also the conservation of the cell's response that to that initial signal, including homeostatic responses.

The majority of IVSA-related modifications of DNA methylation are situated within or proximal to genes (both coding and non-coding) and predominantly within introns. As the bulk of neuronal activity-induced changes in DNA methylation also occur within introns (Guo *et al.*, 2011a), this is relatively unsurprising. Equally expected is the relative enrichment of DMRs within repetitive elements. Approximately 42% of the mouse genome corresponds to repetitive elements (Church *et al.*, 2009) and these are often heavily methylated, which facilitates the detection of differences by enrichment-based methods such as MBD Ultra-Seq. However, the abundance of abstinence-associated DMRs within or proximal to nuclear lamina-associated domains was surprising. A ring of heterochromatin is normally found beneath the nuclear envelope and the tethering of a genomic region to the nuclear lamina inhibits the transcription of genes within the region (Peric-Hupkes & Van Steensel, 2010). DNA methylation directly influences the association between genomic regions and the nuclear lamina as the methyl-CpG binding protein MeCP2 interacts with inner nuclear lamina-associated proteins (Guarda *et al.*, 2009). Furthermore, neuronal activity influences the association of genes with the nuclear lamina (Walczak *et al.*, 2013) and inappropriate regulation of genome-lamina associations has been implicated the development of neuropsychiatric disorders (Ito *et al.*, 2014, Wilczynski, 2014). Repeated cocaine exposure results in a significant decrease in heterochromatin (Maze *et al.*, 2011). Therefore, abstinence-associated changes in DNA methylation may alter genome-lamina associations and heterochromatin stability, though an explanation for the specific enrichment of abstinence-associated DMRs within lamina-associated domains remains elusive.

A number of gene-associated DMRs were located within genes that have been previously implicated in learning, memory and addiction and their potential biological functions merit elaboration. KCTD16 functions as an auxiliary subunit of the $GABA_B$ receptor, enhancing its sensitivity and accelerating responses to agonists (Schwenk *et al.*, 2010) but paradoxically causing $GABA_B$ receptor activation to produce non-desensitizing responses in neurons (Gassmann & Bettler, 2012). Activation of the $GABA_B$ receptor, which is equally subject to persistent changes in methylation following cocaine self-administration, is associated with a reduction in cocaine self-administration (Roberts & Brebner, 2000) and therefore by altering the kinetics and sensitivity of this receptor, the differential methylation of both genes may modulate cocaine seeking. MCTP1 polymorphisms are associated with bipolar disorder (Scott *et al.*, 2009) and MCTP1's expression is altered during abstinence from several drugs of abuse (Le Merrer *et al.*, 2012). CDH13 is a particularly striking

candidate as polymorphisms within this gene have been implicated in methamphetamine dependence (Uhl *et al.*, 2008), alcohol dependence (Treutlein *et al.*, 2009), nicotine dependence (Uhl *et al.*, 2010), vulnerability to addiction (Johnson *et al.*, 2011) and disorders of impulse control including ADHD (Arias-Vasquez *et al.*, 2011), though no studies have explored the epigenetic regulation of this gene in cocaine seeking and addiction. Together with the other DMRs identified, these changes in DNA methylation have a clear potential to contribute to development of addiction and thanks to the malleable nature of epigenetic modifications, are promising targets for pharmacological interventions in the treatment of addiction. Nevertheless, as previously mentioned, it is first necessary to establish that these DMRs regulate addiction and not simple cocaine self-administration. Moreover, many DMRs are located within repetitive regions and will require the application of new techniques for their verification. One potential technique for the validation of DMRs within repetitive regions is SMRT sequencing (PacBio), which can be used to sequence single molecules of DNA of up to 40,000 bp in length without amplification. However, this and other viable techniques remain in development and will necessitate further optimisation.

Repeated exposure to cocaine generates 'silent' glutamatergic synapses that do not influence the basal efficacy of synaptic transmission but are pronounced sites of plasticity in response to subsequent stimulation (Lee & Dong, 2011). An analogous phenomenon may occur in the context of learning-induced changes in DNA methylation, whereby learning (or IVSA)-induced changes in DNA methylation do not necessarily produce long-lasting changes in the transcription of co-localising genes, but instead prime their transcription in response to subsequent neuronal and memory reactivation. To this effect, in a subset of genes (KCTD16, CPEB4 and NKAIN3), the association between differential methylation and altered gene transcription was modulated by the re-activation of cocaine-related memories through a relapse test, which suggests that the function of some changes in DNA methylation is indeed to prime transcription upon memory reactivation. KCTD16-associated data is particularly convincing as there is no difference in methylation in animals that underwent a relapse test after 21 days of abstinence and compared to those that were simply sacrificed; caution must be used when interpreting the NKAIN3- and CPEB4- related data as the possibility that DNA methylation is altered by the relapse test cannot be excluded. Further experiments will extend these correlative findings and conclusively demonstrate that the observed metaplastic priming of gene transcription is a direct consequence of memory reactivation and DNA methylation at a specific locus. It is

possible to establish that memory re-activation mediates the association between IVSA-associated DNA methylation and gene expression by subjecting mice to a relapse test in the presence of pharmacological agents that prevent the retrieval of memory (such as propranolol, which prevents the retrieval of cocaine-associated memories in CPP (Otis & Mueller, 2011)). Moreover, by artificially reversing the IVSA-associated changes in methylation at a given DMR, one can establish its role in regulating transcription and cocaine seeking behaviour. Targeted DNA methylation or demethylation can be produced by engineered transcription activator–like effectors (TALEs), which bind to specific sequences of DNA and can be fused to demethylating enzymes such as TET1 (Maeder *et al.*, 2013a) or methylating enzymes such as DNMT3a. The concurrent application of both techniques will demonstrate the importance of learning-induced modifications of DNA methylation in the metaplastic priming of gene expression, a phenomenon for which this body of work has provided preliminary evidence.

Finally, IVSA-associated changes in intragenic DNA methylation are associated with the regulation of alternative splicing. Importantly, we demonstrated that the absence of an overall change in the expression of a gene does not exclude the possibility that individual splice variants are differentially regulated. The overall expression of MCTP1 is unchanged, yet the expression of a single isoform (MCTP-001) was differentially regulated. Opposing changes in the expression of specific isoforms of genes could obscure global changes in the expression of a given gene. Evidently, each isoform of a gene may produce proteins with vastly different functional capabilities and therefore it is critical that the expression of specific splice variants is determined. At the level of individual DMRs, exploring the expression of each splice variant of co-localising genes may be too time-consuming and expensive; it may be preferable simultaneously perform whole-transcriptome sequencing to broadly explore the regulation of alternative splice variants.

Together, this body of work has identified persistent and abstinence-associated changes in DNA methylation in neurons of the mPFC that are unique to the voluntary cocaine self-administration and which may underlie the maintenance of cocaine-related memories. Future experiments will functionally establish the role of these specific changes in DNA methylation in cocaine-seeking behaviour and memory maintenance and explore their function in the regulation of transcription.

CHAPTER 5

Conclusions & Outlook

5.1 Summary of findings

As hypothesized (Aim 1), mice persistently sought cocaine after 21 days of abstinence, indicating the presence of long-lasting cocaine-related memories on which this behaviour is contingent. IVSA-associated modifications of DNA methylation were identified by MBD Ultra-Seq, a next generation sequencing technique we developed to pinpoint genome-wide region- and cell type- specific DNA methylation states in individual animals (Aim 2). As hypothesized (Aim 3), in neurons of the mPFC, the modifications of DNA modifications arising from cocaine self-administration are distinct from those that are incurred by passive cocaine exposure. Variations in DNA methylation produced by cocaine self-administration are surprisingly persistent, which is consistent with the hypothesis that these variations facilitate the maintenance of cocaine-related memories and enduring cocaine-seeking behaviour. Abstinence-associated changes in DNA methylation arise with curious frequency in nuclear lamina-associated domains, which is indicative of extensive re-organisation of chromatin during abstinence. Both persistent and abstinence-associated DNA modifications preferentially co-localise with transcribed regions and may therefore function primarily in the regulation of transcription. However, as outlined in Chapter 2 and hypothesized in Aim 4, some IVSA-associated DNA modifications prime gene expression in response to the reactivation of cocaine-related memories rather than perpetuating changes in transcription, providing an *in vivo* example of genomic metaplasticity.

5.2 Future directions

The overarching objective of future experiments is to conclusively establish that IVSA-induced modifications of DNA methylation mediate the maintenance of persistent cocaine-related memories and cocaine-seeking behaviour. Broadly, this objective can be dissected into 3 tasks. First, we will identify DMRs associated with the maintenance of cocaine-related memories by examining methylation within neurons selectively engaged by the memory trace. Moreover, we will manipulate DNA methylation at specified DMRs to functionally establish their role in continued cocaine-seeking behaviour during abstinence. Lastly, we will unambiguously demonstrate that enduring neuronal activity- or learning-induced modifications of DNA methylation do not necessarily prompt lasting changes in gene expression, but may instead represent a form of genomic metaplasticity that primes the transcriptional response to subsequent neuronal or memory reactivation.

5.2.1 Honing in on memory-related DNA methylation and gene transcription

During cocaine-self administration, animals learn to associate behaviours, cues and contextual stimuli with cocaine availability, which gives rise to enduring cocaine-related memories that encode these learned associations and motivate cocaine-seeking behaviour. In contrast, passive cocaine exposure generates memories for the experience of cocaine, which are incapable of driving cocaine seeking. In identifying the modifications of DNA methylation that are uniquely produced by cocaine self-administration, we may have indirectly characterised the changes in DNA methylation that mediate the maintenance of cocaine-related memories. However, by examining the IVSA-associated changes in DNA methylation and gene expression in neurons selectively engaged by cocaine-related memories (ie. activated upon the retrieval of cocaine-related memories by self-administration or relapse testing), we can directly identify the modifications of DNA methylation that beget the maintenance of cocaine-related memories. In addition, future animals will be directly trained to self-administer cocaine, eliminating the possibility that any behavioural/epigenetic changes are due to the initial instrumental training with oral sucrose.

Fluorescence-activated cell sorting (FACS) can be coupled to transgenic or viral vector-mediated approaches to identify and capture recently activated neurons. In transgenic mice, recently activated neurons are labelled by a reporter protein that is expressed under the control of an endogenous promoter of neuronal activity-regulated genes (ie. *c-fos*, *egr-1* or *Arc*) (Eguchi & Yamaguchi, 2009, Smeyne *et al.*, 1992). After memory reactivation FACS is subsequently used to isolate neurons expressing the reporter protein. However, within certain neuronal subtypes (ie. inhibitory GABA neurons) the endogenous promoters are differentially regulated in response to neuronal activity (Kawashima *et al.*, 2014) and therefore this method may only capture recently activated neurons of a certain variety. A synthetic, virally introduced, activity-dependent promoter termed E-SARE (enhanced synaptic activity reactive element) can be used to overcome this bias and drive the expression of reporter proteins in all recently activated and transfected neurons (Kawashima *et al.*, 2013). Expressing reporter proteins under the control of the E-SARE promoter is perhaps preferable as it greatly reduces region- and cell type- biases in the expression of the reporter protein. Future experiments will use an E-SARE-driven destabilised green fluorescent protein (GFP) and FACS to isolate neurons that are activated in response to the retrieval cocaine-related memories.

Though it is possible to isolate recently activated neurons from the mPFC, the quantity of DNA obtained from this limited population may restrict the methods that can be applied to identify IVSA-associated changes in DNA methylation. The present IVSA-associated modifications of DNA methylation were identified using gDNA derived from all NeuN+ neuronal nuclei of the mPFC (about 80,000 nuclei per animal). However, based on the number of neurons that mediate drug-seeking behaviour in other structures and drug-taking paradigms (Bossert *et al.*, 2011, Cruz *et al.*, 2014), enduring cocaine-seeking behaviour is likely directed by 3-6% of neurons within the mPFC (or a total of 2400-4800 neurons). Diploid neurons contain approximately 6 pg of DNA (Moroz & Kohn, 2013); unreasonably assuming the perfect extraction of all activated neurons and their gDNA from the mPFC, we would recover approximately 28 ng of DNA per animal. With further optimisation, MBD Ultra-Seq may be applicable, but alternative methods of sequencing must be considered. A promising alternative is the direct detection of DNA methylation by single-molecule real-time (SMRT) sequencing (Flusberg *et al.*, 2010). Similar to Illumina's HiSeq, SMRT sequencing uses parallelised DNA sequencing by synthesis, where DNA polymerases catalyze the incorporation of complementary fluorescent nucleotides to single stranded DNA. As each nucleotide is added, the fluorescent tag is cleaved to produce a fluorescent signal that indicates which nucleotide was incorporated. The kinetics of DNA polymerase are altered by the presence of epigenetic modifications, which results in differences in the duration and arrival times of the fluorescent pulses produced by nucleotide addition and reveals the presence of N6-methyladenosine, 5-methylcytosine, and 5-hydroxymethylcytosine. SMRT sequencing overcomes the need for PCR-based whole genome amplification of DNA prior to sequencing, which can introduce bias (Benjamini & Speed, 2012). However, it is not practical to use SMRT sequencing for the genome-wide discovery of 5mC in the 2.5 GB mouse genome, as it generates a maximum of 40,000 bp reads and a minimum of 25X coverage is recommended for the identification of 5mC (PacBio). Nevertheless, the 40,000 bp reads permit the identification of DNA methylation within repetitive regions and perhaps this technique could be coupled to MBD Ultra-Seq for the validation of locus-specific changes in DNA methylation within repetitive regions.

One limitation of the current data is that gene expression was explored in mRNA derived from the entire mPFC. In future experiments, whole neurons will be isolated from the mPFC and gene expression will be analysed in the neurons engaged by cocaine-related memories. Several protocols have optimised the isolation of whole neurons and quality

RNA from adult brains (Fanous *et al.*, 2013, Guez-Barber *et al.*, 2012, Saxena *et al.*, 2012), but the simultaneous extraction of RNA and DNA from a small sample will require optimisation. Nevertheless, with further optimisation, it will be possible to perform genome-wide DNA methylation profiling and whole-transcriptome sequencing on neurons selectively engaged by cocaine-related memories. Together, these novel techniques will more rigorously identify the variations in DNA methylation states and gene expression that support enduring cocaine- seeking behaviour and long-lasting cocaine-related memories.

5.2.2 Establishing the function of specific DMRs in behaviour

Overall, the appropriate regulation of DNA methylation is necessary for the expression of cocaine-seeking behaviour (Laplant *et al.*, 2010, Tian *et al.*, 2012) and memory maintenance (Miller *et al.*, 2010). However, manipulating global levels of DNA methylation to demonstrate function is almost inconsequential after the identification of such specific changes in DNA methylation. Future experiments will establish the function of discrete IVSA-associated DMRs in the regulation of cocaine-seeking behaviour and memory.

5.2.2.1 Targeting specific DMRs

To demonstrate the function of specific DMRs in cocaine-seeking behaviour it is necessary to exclusively manipulate their persistence in neurons that are engaged by this behaviour. Two evolving technologies may enable the manipulation of specific epigenetic modifications at discrete genomic loci. The first is transcription activator-like effector (TALE) proteins, which can be engineered to bind precise locations in the genome by fusing several TALE repeat domains together. TALE repeat domains are approximately 34 amino acids in length and vary by two amino acid residues, which causes them to bind specific DNA nucleotides. By fusing several TALE repeat domains that bind specific nucleotides with a defined distance between each binding site, one can create a TALE protein that binds to a single site in the genome. Moreover, TALE proteins can be fused to the catalytic domains of methylating or demethylating enzymes to force DNA methylation or demethylation at nucleotides proximal to their binding sites (Maeder *et al.*, 2013a). Furthermore, as TALE proteins are expressed from viral vectors, it is possible to design an inducible TALE protein whose expression is restricted to recently activated neurons, by placing the vector under the control of an E-SARE promoter. A relapse test at an intermediate time point (ie. 10 days) would induce the expression of the TALE protein in the neurons engaged by cocaine seeking and TALE fusion protein(s) would subsequently force methylation/demethylation at the desired DMRs within these select neurons. The

effects of the methylation manipulations on cocaine-seeking behaviour and the expression of co-localising genes could be examined upon a second relapse test, thereby establishing the role of specific DMRs in cocaine-seeking behaviour. Nevertheless, designing TALE proteins remains very challenging. Moreover, there remains the question of whether one DMR contributes substantially to the regulation of behaviour. It appears unlikely that one DMR, within a handful of neurons, would significantly contribute to the maintenance of memory (and regulation of behaviour), as this would render memories incredibly sensitive to disruption. Instead, a network with regional and epigenomic redundancies may maintain memory and compensate for the inadvertent loss of discrete epigenetic changes. Therefore, it be necessary to simultaneously manipulate several DMRs to observe a behavioural effect.

A second approach to artificial epigenetic editing is the CRISPR/Cas9 system, which has been previously used for genomic editing (Cho *et al.*, 2013). Here, CRISPR RNAs (cRNAs) are expressed and bind the endonuclease Cas9, changing its conformational state and causing it to scan the genome for endogenous protospacer adjacent motif (PAM) sites (5'-NGG). Upon binding to a PAM site, the cRNA/Cas9 complex prompts the unwinding of the DNA helix. To remain tethered to the DNA, a designated sequence-specific region within the cRNA must be complementary to the DNA sequence proximal to the PAM site. If complementarity is present, the cRNA binds the DNA and the CRISPR/Cas9 complex causes double-stranded break in DNA at the complementary region. However, the substitution of two amino acids within Cas9 can inactivate its nuclease domains to form dCas9, which retains its function as a DNA-binding scaffold (Jinek *et al.*, 2012). As with TALEs, it may be possible to create a fusion protein of dCas9 and the catalytic domains of methylating or demethylating enzymes and force site-specific methylation or demethylation. Unlike TALE proteins, the CRISPR/Cas9 system does not necessitate the design of new proteins for each target and the expression of multiple cRNAs by distinct (Swiech *et al.*, 2015) or a single (Kabadi *et al.*, 2014) viral vector can target dCas9 to multiple genomic loci. However, compared to TALEs, cRNA-guided dCas9 binds relatively promiscuously throughout the genome (Wu *et al.*, 2014) and may cause undesirable *de novo* epigenetic modifications at non-targeted loci. Further, the expression multiple cRNAs, dCas9 and the catalytic domain of a methylating/demethylating enzyme will require co-transfection by multiple plasmids. However, differences in transfection rates amongst the plasmids could result in the unequal expression of each vector and therefore each epigenetic modification may not be targeted to the same extent. Moreover, CpG

methylation and chromatin state may interfere with dCas9 binding (Wu *et al.*, 2014). Nevertheless, as the CRISPR/dCas9 system evolves, its relative ease of use may favour its adoption in the manipulation of site-specific changes in DNA methylation, particularly as it enables the simultaneous manipulation of several DMRs.

In conclusion, TALE and dCas9 fusions proteins under the control of E-SARE promoters may permit the site-specific manipulation of IVSA-associated DMRs within neurons that are selectively engaged by cocaine-seeking behaviour. Future investigations will enlist these approaches to directly examine the importance of specific DMRs in the maintenance of cocaine-related memories and cocaine-seeking behaviour. It is unlikely that a single DMR within a discrete neuronal population significantly regulates a complex behaviour or begets the maintenance of a memory, but it remains possible that by disrupting a single DMR/gene we may exert a 'butterfly' effect, whereby the disruption of one component of an adaptive pathway leads to its complete interruption and consequently exerts a significant effect on behaviour. More promisingly, both the TALE and CRISPR/dCas9 system permit epigenetic remodelling at multiple loci.

5.2.3 IVSA-associated DMRs, addiction and natural reward seeking

Cocaine addiction may arise in part from the pathological usurpation of the neural pathways and adaptions normally enlisted by natural reward learning and memory (Hyman, 2005, Hyman *et al.*, 2006). Therefore, in identifying the changes in DNA methylation associated with enduring cocaine-seeking behaviour and cocaine-related memories, we may have inadvertently distinguished the modifications of DNA methylation that beget addiction. However, the continued self-administration of cocaine in the absence of adverse consequences can hardly be considered indicative of cocaine addiction. A key feature of cocaine addiction in humans is compulsive cocaine seeking despite adverse consequences, which are absent from the standard self-administration paradigm, but present in the 'conflict' model of cocaine self-administration (Cooper *et al.*, 2007). In this model, adverse consequences (such as electrified floor grid proximal to the cocaine-paired lever) are gradually introduced during cocaine self-administration until they completely deter cocaine-seeking behaviour. Cue-, context- or cocaine- induced relapse is examined in the presence of the maximal adverse consequence; animals that continue to seek cocaine despite the presence of adverse consequences are considered addicted. Ultimately, it will be necessary to employ this or a similar model, to identify the modifications of DNA methylation directly associated with addiction; until such time, the

epigenetic modifications identified are simply associated with cocaine seeking behaviour and potentially cocaine-related memories.

A further avenue of investigation will distinguish the modifications of DNA methylation that drive cocaine-seeking behaviour from those that motivate natural reward (food, water) seeking, or the self-administration of other drugs, as an increasing amount of evidence suggests that the epigenetic modifications associated with natural reward consumption differs (Pol Bodetto *et al.*, 2014, Romieu *et al.*, 2008).

Finally, it will be necessary to determine if the changes in DNA methylation associated with cocaine-related memories are region-specific (ie. localised to the mPFC), occur throughout activated neurons with the reward-related circuitry, or arise throughout the brain. A recent investigation (Massart *et al.*, 2015) found persistent changes in DNA methylation in the nucleus accumbens following cocaine self-administration. However, as they used promoter microarrays to identify changes in DNA methylation, it is not possible to determine if candidates from this thesis overlap with those described in the paper.

5.2.4 DNA methylation and the metaplastic priming of gene transcription

This body of work has provided preliminary evidence that the reactivation state of memory influences the relationship between gene expression and DNA methylation. However, a conclusive demonstration of the priming of gene expression by enduring learning-induced changes in DNA methylation remains to be established. Future investigations will first examine the priming of gene expression by long-lasting neuronal activation-induced modifications of DNA methylation in cultured neurons, as this can be done in advance of the optimisation of whole neuron sorting. To begin, we will identify persistent modifications of DNA methylation that arise following neuronal depolarisation. Furthermore, we will pinpoint the persistent modifications of DNA methylation that are only associated with a change in gene transcription upon subsequent neuronal stimulation, by comparing expression within neurons that have not been re-stimulated, those that have, and neurons that have been re-stimulated in the presence of a pharmacological agent that blocks depolarisation. An association between gene expression and DNA methylation that is unique to neurons that have been activated would suggest a role for DNA methylation in genomic metaplasticity. Finally, neurons will be transfected with TALE/CRISPR/Cas9 – DNMT/TET 1 fusion protein to reverse activation-induced changes in DNA methylation at the identified region, with expectation that subsequent stimulation will not produce a

change in the expression of the co-localising gene if the modification of DNA methylation was necessary to prime transcription. Together, these approaches offer a more conclusive demonstration of the metaplastic priming of gene transcription by neuronal activity-induced modifications of DNA methylation. Further, it may be possible to extend these findings *in vivo*, if recently activated whole neurons with good quality RNA can be isolated and learning-induced changes in DNA methylation can manipulated in select cell populations.

5.3 Summary of future directions

In sum, this body of work has yielded a novel hypothesis about how transcriptionally quiescent DNA methylation regulates the maintenance of memory, an innovative technique for the identification of region- and cell type- specific genome-wide changes in DNA methylation in multiple individuals, the first *in vivo* profile of exclusively IVSA-associated changes in DNA methylation and preliminary evidence of the priming of gene expression by long-lasting changes in DNA methylation. Future experiments will identify modifications of DNA methylation and gene expression explicitly associated with cocaine-related memories and demonstrate a functional role for these specific changes in cocaine-seeking behaviour. Finally, the transcriptional consequences of neuronal activation-induced changes in DNA methylation will be explored. Together these experiments present several technical challenges, but are necessary to conclusive demonstrate the role of learning-induced modifications of DNA methylation in the maintenance of cocaine-related memories.

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Appendix

Additional publications arising from candidature

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Brief Communication

Activation of BDNF signaling prevents the return of fear in female mice

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There are significant sex differences in vulnerability to develop fear-related anxiety disorders. Females exhibit twice the rate of post-traumatic stress disorder (PTSD) as males and sex differences have been observed in fear extinction learning in both humans and rodents, with a failure to inhibit fear emerging as a precipitating factor in the development of PTSD. Here we report that female mice are resistant to fear extinction, and exhibit increased DNA methylation of *Bdnf* exon IV and a concomitant decrease in mRNA expression within the medial prefrontal cortex. Activation of BDNF signaling by the trkB agonist 7,8-dihydroxyflavone blocks the return of fear in female mice after extinction training, and thus represents a novel approach to treating fear-related anxiety disorders that are characterized by a resistance to extinction and increased propensity for renewal.

There are significant sex differences in terms of vulnerability to develop fear-related anxiety disorders. Females exhibit twice the rate of post-traumatic stress disorder (PTSD) as males (Olff et al. 2007), which cannot be explained by the severity of the experienced trauma or comorbidity with other affective disorders (Tolin and Foa 2006). Furthermore, sex differences have been observed in fear extinction learning in humans and rodents, with a failure to inhibit fear emerging as a precipitating factor in the development of PTSD (Lebron-Milad et al. 2012; Ter Horst et al. 2012). In accordance with previous observations we have found that there are significant sex differences in the extinction of conditioned fear in mice. Male and naturally cycling female C57BL/6 mice ($n = 15–16$/group, 9 wk of age) were first trained on a cued-fear-conditioning task in which a 70-dB white noise (conditioned stimulus [CS], 2 min) coterminated with mild foot shock (unconditioned stimulus [US] 0.7 mA, 1 sec) on three occasions. Twenty-hours later, mice were extinction trained in a new context (30 nonreinforced, 2-min, 5-sec inter-trial interval, tone (CS) exposures in context B). On Day 3, all mice were returned to context B and tested (two CS presentations) for retention of memory for fear extinction. There was no difference between male and female mice in the acquisition of cued fear (data not shown). However, there was a significant sex difference in memory for the extinction of conditioned fear ($F_{(3,59)} = 11.41$, $P < 0.0001$; Tukey's post hoc test: fear conditioned without extinction training (FC-No EXT) male vs. extinction trained (EXT) male, $P < 0.0001$; EXT male vs. EXT female, $P < 0.05$), with male mice exhibiting significantly lower levels of freezing than females (Fig. 1). These data suggest that female mice are resistant to the extinction of conditioned fear; however, the neural mechanisms underpinning this effect are not known.

Epigenetic mechanisms influence cognition and memory by regulating learning-induced gene expression (Day and Sweatt 2011). One such epigenetic mechanism is DNA methylation, which has been implicated in experience-dependent plasticity and in the formation and maintenance of fear-related memories (Miller et al. 2010; Baker-Andresen et al. 2012). Activity-dependent brain-derived neurotrophic factor (BDNF) signaling enhances neural plasticity and is necessary for the formation of fear-related memories (Peters et al. 2010; Andero and Ressler 2012) with the epigenetic regulation of *Bdnf* expression being shown to be critical for the acquisition and extinction of conditioned fear (Bredy et al. 2007). Furthermore, dysregulation of BDNF has been implicated in many neuropsychiatric disorders (Boulle et al. 2012). Recently, significant sex differences in BDNF signaling have been observed within the prefrontal cortex (Hill and van den Buuse 2011), a region of the brain in which sexual dimorphism in epigenomic function has also been reported (Xu et al. 2008). However, it is not yet known whether there are sex differences in the epigenetic regulation of *Bdnf*, which would subsequently contribute to the observed differences in fear-related learning and memory. To address this issue, we measured the level of DNA methylation surrounding the transcription start site (TSS) of exon IV of the gene encoding BDNF by methylated DNA immunoprecipitation (MeDIP) followed by quantitative PCR (qPCR) (Fig. 2A). Briefly, genomic DNA was isolated from tissue samples encompassing the prefrontal cortex by overnight proteinase K treatment, phenol–chloroform extraction, ethanol precipitation, and RNase digestion. Prior to MeDIP, genomic DNA was randomly fragmented by sonication into fragments of ~500 bp in length, with 1 μg fragmented DNA used for each MeDIP assay. MeDIP was performed using a MeDIP assay kit (Active Motif) as per the manufacturer's instructions. Methylated DNA fragments were recovered by reverse cross-link followed by ethanol precipitation and then quantified by qPCR using MEDIP-qPCR primers for *Bdnf* exon IV (forward, 5′-GTGGACTCCCACCCACTTT-3′; reverse, 5′-TATTACCTCCGCCATGCAAT-3′).

In response to the extinction of conditioned fear, *Bdnf* expression is altered in an isoform-specific manner; *Bdnf* exon IV expression increases in response to fear extinction and is marked by learning-induced epigenetic modifications surrounding its promoter (Bredy et al. 2007). Furthermore, reduced BDNF exon IV expression is associated with deficits in inhibitory neurotransmission in the prefrontal cortex (Sakata et al. 2009), thus adversely affecting the formation and maintenance of extinction memories when occurring in the medial prefrontal cortex (ILPFC) (Akirav et al. 2006), an area of the brain that is critically involved in fear extinction. As opposed to examining the well-characterized promoter region of *Bdnf* exon IV, we selected a locus immediately downstream of TSS due to the presence of several binding motifs for transcription factors, including Sp1, YY1, and CTCF, all of which are known to interact with DNA methylation in regulating

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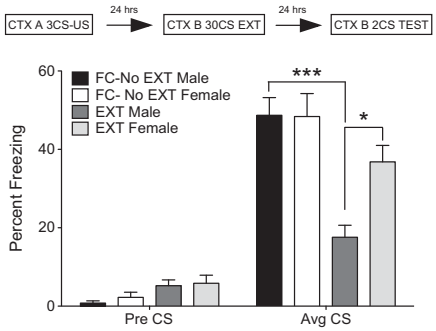

Figure 1. Female mice exhibit impaired retention of fear extinction memory compared to males. Twenty-four hours after a 30 CS extinction session in context B, male mice (EXT male) freeze significantly less than no-extinction controls (FC-No EXT male) when presented with two CS in the extinction context, suggesting retention of the memory for extinction training. While no difference in the retention of fear is observed between sexes (FC-No EXT male vs. FC-No EXT female), female mice (EXT female) display a significant extinction impairment compared to extinguished males. (***) $P < 0.0001$, (*) $P < 0.05$.

transcriptional activity. As an indirect measure of the functional relevance of variations in DNA methylation, we also measured *Bdnf* exon IV mRNA expression within the ILPFC of naive male and female mice. Briefly, RNA was isolated from samples encompassing the ILPFC of naive male and female mice using the Trizol extraction method (Invitrogen). Total RNA (1 μg) was used for cDNA synthesis using the Omniscript RT kit (Qiagen). PCR was then performed using primers for *Bdnf* exon IV (forward, 5′-GCAGCTGCCTTGATGTTTAC-3′; reverse, 5′-CCGTGGACGTTTACTTCTTTC-3′) and for phosphoglycerate kinase (*Pgk*) as an internal control (forward, 5′-TGCACGCTTCAAAAGCGCACG-3′; reverse, 5′-AAGTCCACCCTCATCACGACCC-3′). qPCR was performed using a RotorGeneQ (Qiagen) cycler using SYBR-green (Qiagen). The threshold cycle for each sample was chosen from the linear range and converted to a starting quantity by interpolation from a standard curve run on the same plate for each set of primers. *Bdnf* exon IV mRNA levels were normalized for each well to *Pgk* mRNA using the ΔΔCT method, and each qPCR was run in duplicate for each sample and repeated at least two times. mRNA levels were analyzed by unpaired *t*-tests.

Our results revealed a significant sex difference in DNA methylation and mRNA expression. Naive female mice exhibited significantly greater methylation (unpaired *t*-test, $t = 2.75$, $df = 7$, $P < 0.05$) (Fig. 2C) relative to naive males, which was accompanied by decreased levels of *Bdnf* exon IV mRNA expression (unpaired *t*-test, $t = 2.04$, $df = 6$, $P < 0.05$) (Fig. 2B). Together, these data suggest a potential relationship between sex differences in the epigenetic regulation of *Bdnf* exon IV within the medial prefrontal cortex and fear extinction in mice. Therefore, we next asked whether these differences could collectively be overcome to promote the extinction of conditioned fear in female mice.

A brief reminder CS after fear conditioning renders the memory for that fear temporarily labile and sensitive to modification, requiring re-stabilization or updating of the memory trace through a process known as reconsolidation (Przybyslawski and Sara 1997; Przybyslawski et al. 1999; Nader et al. 2000). Monfils et al. (2009) capitalized on the labile nature of memory to introduce a retrieval-extinction procedure to interfere with reconsolidation and strengthen the formation of fear extinction memory in rats. They observed a significant reduction in the fear response that did not return as a function of spontaneous recovery, renewal, or reinstatement. These findings were subsequently replicated in humans (Schiller et al. 2010), and in mice in which a single nonreinforced CS prior to extinction training diminished the renewal of conditioned fear (Clem and Huganir 2010). However, not all studies have demonstrated beneficial effects using this approach, and an exaggerated return of fear, dependent on the context in which the fear memory is retrieved, has also been reported (Chan et al. 2010). Based on these observations, we reasoned that a retrieval-extinction protocol might be useful in promoting fear extinction in females, which are otherwise resistant when examined in a standard fear extinction training protocol. Moreover, as there are sex differences in the epigenetic regulation of cortical BDNF expression and in basal levels of BDNF expression in other structures of the fear extinction-related circuitry (Zhu et al. 2006), we hypothesized that fear extinction would be facilitated through direct systemic activation of the downstream signaling target of BDNF, the trkB receptor. 7,8-Dihydroxyflavone (7,8-DHF) is a small molecule activator that binds to the trkB receptor (Jang et al. 2010), thereby mimicking the effect of BDNF in the brain. Systemic administration of 7,8-DHF has been shown to activate trkB receptors, enhance learning and memory (Liu et al. 2010; Andero et al. 2011, 2012), and reverse memory deficits in

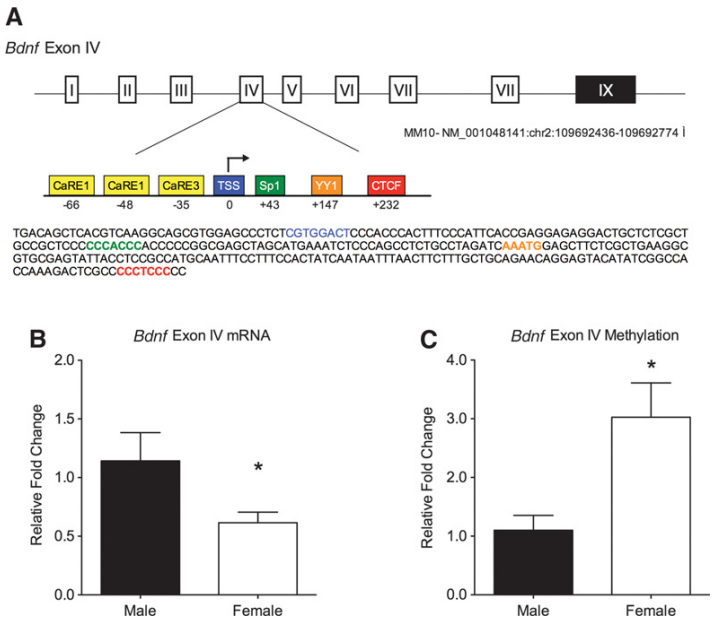

Figure 2. Expression of *Bdnf* exon IV mRNA differs in naive males and female C57BL/6 mice and is accompanied by differences in DNA methylation surrounding TSS. (*A*) DNA methylation levels were measured at the TSS of exon IV of the gene encoding BDNF. A significant increase in methylation was observed in females (*C*), along with a concurrent decrease in *Bdnf* exon IV expression (*B*), compared to males. (*) $P < 0.05$

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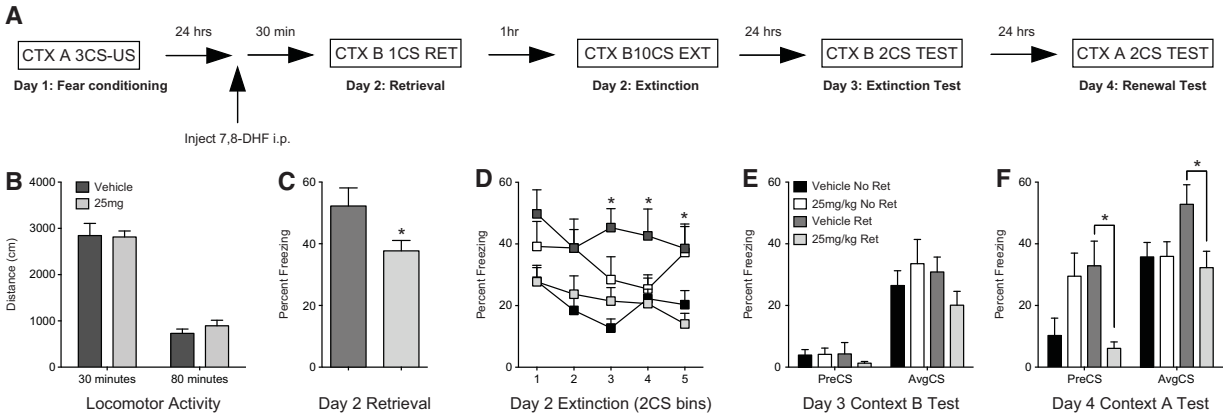

Figure 3. 7,8-Dihydroxyflavone prevents retrieval-induced renewal of fear. (*A*) Schematic representation of experimental protocol. (*B*) Analysis of distance traveled 30 and 80 min post-injection with either vehicle (10% DMSO in PBS) or 25 mg/kg 7,8-DHF reveals no effect of drug on locomotor activity. (*C*) Treatment with 7,8-DHF 30 min prior to retrieval significantly decreased the expression of fear in female mice. (*D*) Vehicle-treated animals subjected to a retrieval cue (Vehicle Ret) demonstrate a resistance to extinction compared to drug-treated (25 mg/kg 7,8-DHF Ret) and nonretrieval groups (Vehicle No Ret and 25 mg/kg 7,8-DHF No Ret). (*E*) No significant differences were observed at test in context B. (*F*) A significant retrieval-induced renewal of fear was observed in the vehicle retrieval group compared to nonretrieval groups, an effect which was blocked in the 7,8-DHF Ret group. (*) $P < 0.05$.

a preclinical model of age-related cognitive decline (Devi and Ohno 2012; Zeng et al. 2012a, b). We therefore investigated whether administration of 7,8-DHF, in combination with a modified version of the retrieval-extinction protocol, could aid in further promoting extinction in female mice.

Female mice ($n = 8$/group) were trained on a cued-fear-conditioning task and 24 h later were exposed to a single retrieval cue prior to extinction training in a new context (10 or 11 nonreinforced tone exposures in context B, balanced for CS exposure on retrieval). All mice were then returned to context B and tested for retention of memory for fear extinction on Day 3 before being tested for renewal of fear memory 24 h later in context A (Fig. 3A). We found that a single exposure to a retrieval cue, prior to fear extinction training, led to significantly higher levels of within-session freezing, and an exaggerated return of fear when the mice were tested in the context in which fear was initially learned (Fig. 3F), similar to the findings of Chan et al. (2010). In contrast, there were no differences when tested in the extinction context B (Fig. 3E). A single injection of 7,8-DHF (25 mg/kg, i.p., dissolved in 10% DMSO in sterile saline), administered systemically 30 min prior to retrieval, led to a significant reduction in freezing upon retrieval (unpaired *t*-test, $t = 2.88$, df = 14, $P < 0.05$) (Fig. 3C) and completely prevented the return of fear in context A ($F_{(3,31)} = 3.03$, $P < 0.05$; Tukey's post hoc test; Vehicle Ret vs. 25 mg/kg 7,8-DHF Ret, $P < 0.05$) (Fig. 3F). This effect on renewal was due in large part to the very strong effect of 7,8-DHF on contextual memory, as indicated by the pre-CS freezing levels in drug-treated mice ($F_{(3,31)} = 4.66$, $P < 0.01$; Tukey's post hoc test; Vehicle Ret vs. 25 mg/kg 7,8-DHF Ret, $P < 0.05$) (Fig. 3F). Furthermore, this reduction in freezing was not due to a generalized increase in activity as there were no significant differences in locomotion (distance traveled) either 30 min or 80 min post-injection (Fig. 3B). Together these data suggest that 7,8-DHF promotes a reduction in fear-related responding during and following extinction training in female mice, consistent with results suggesting that activation of BDNF signaling can induce extinction in the absence of training (Peters et al. 2010).

This study generated three main findings: (1) female mice are resistant to the extinction of conditioned fear; (2) there are sex differences in the epigenetic regulation of *Bdnf* expression in the medial prefrontal cortex; and (3) female mice exhibit a significant return of fear after extinction training in a retrieval-extinction paradigm, effects that are completely blocked by targeted activation of BDNF signaling prior to memory retrieval and extinction training. It is important to note that there is evidence to suggest that sex differences in fear extinction are dependent on circulating gonadal hormone levels (Zeidan et al. 2011; Merz et al. 2012); however, naturally cycling mice were examined in this study to closely mimic what occurs in the general population. Moreover, regardless of cycle, we observed a significant sex difference in fear extinction that is associated with basal differences in epigenetic regulation of *Bdnf* within the medial prefrontal cortex. We cannot exclude the possibility that stage of estrous contributed to these effects (Spencer et al. 2010); however, our data on the return of fear after exposure to a retrieval cue in female mice represent a third replication of this effect, making it unlikely that these differences are strictly due to a hormonal influence on fear extinction learning.

Few studies have considered whether there are sex differences in the epigenetic regulation of gene expression, although this is an emerging concept (McCarthy et al. 2009). Using a mouse model that separates hormonal effects from sex chromosome-linked gene effects, Xu et al. (2008) were the first to examine how genes specifically encoded on sex chromosomes influence the way in which the epigenome exerts an effect on gene expression. They found that, regardless of gonadal phenotype, the expression pattern of the H3K27^{me3} histone demethylase ubiquitously transcribed tetratricopeptide repeat gene on X chromosome (*Utx*) was highest in the cortex of female mice. We have observed a similar pattern of *Utx* expression specifically in the medial prefrontal cortex in male and female mice (data not shown). Given the tight association between H3K27^{me3} and DNA methylation, it is likely that sex differences in the epigenome are broadly distributed and contribute to the function of many genes and related behaviors. Our data on DNA methylation status of *Bdnf* exon IV give but one prototypical example of how sex differences in the epigenetic regulation of gene expression may influence fear-related learning and memory. With respect to the influence of 7,8-DHF on the return of fear, previous studies have indicated beneficial effects of 7,8-DHF on fear extinction in paradigms where diminished capacities for extinction are observed, such as following exposure to an acute stressor (Andero et al. 2011). These

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observations were later extended by the demonstration of a beneficial effect of 7,8-DHF on stress-induced spatial memory impairments (Andero et al. 2012). Thus, exposure to a retrieval cue prior to fear extinction training may represent an acute stressor, which leads to a significant increase in the return of fear in female mice, an effect that can be completely prevented by activation of BDNF signaling.

In summary, female mice are resistant to fear extinction, and exhibit increased DNA methylation of *Bdnf* exon IV and a concomitant decrease in mRNA expression within the medial prefrontal cortex. Together these findings suggest the intriguing possibility that sex differences in epigenetic regulation of gene expression may represent a general distinction between the male and female brain that will impact a variety of behaviors. In the case of BDNF signaling, targeting downstream trkB receptors to block renewal in female mice offers a novel approach to treating fear-related anxiety disorders characterized by a significant return of fear.

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