The *SLC6A4* VNTR genotype determines transcription factor binding and epigenetic variation of this gene in response to cocaine in vitro

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ABSTRACT

We demonstrated that the genotype of the variable number tandem repeats (VNTRs) in the linked polymorphic region (LPR) of the 5′ promoter and in the intron 2 (Stin2) transcriptional regulatory domains of the serotonin transporter *SLC6A4* gene determined its promoter interactions with transcription factors and co-activators in response to cocaine in the JAr cell line. The LPR variants contain 14 (short, *s*) or 16 (long, *l*) copies of a 22–23 bp repeat element, whereas the Stin2 VNTR exists as three variants containing 9, 10 or 12 copies of a 16–17 bp repeat. We observed a differential effect of cocaine on the association of the promoter with the transcription factor CTCF, which bound to both LPR alleles prior to cocaine exposure but only to the *l*-allele following exposure. Significantly, this differential effect of cocaine was correlated with the binding of the transcriptional regulator MeCP2 specifically to the *s*-allele and recruiting the histone deacetylase complex (HDAC). Concurrently, cocaine increased the association of positive histone marks over the *SLC6A4* gene locus. At the Stin2 domain, we lost binding of the transcription factor YB-1, while CTCF remained bound. Our biochemical data are consistent with differential reporter gene activity directed by the individual or dual domains in response to cocaine in an Epstein–Barr virus-based episome model of stable transfections. These observations suggest that exposure of JAr cells to cocaine may result in differential binding of transcription factors and activators based on a specific genotype that might alter epigenetic parameters affecting gene expression after the initial challenge.

Keywords  Cocaine, CTCF, epigenetics, LPR, *SLC6A4*, VNTR.

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INTRODUCTION

A predisposition to addictive behaviour often correlates with specific polymorphisms in candidate genes. Additionally, environmental factors have been considered as potential modifiers of the genetic component (Eley et al. 2004), which is reflected in the fact that clinical conditions are often episodic and require an environmental stress or challenge to exhibit a phenotype. This genetic–environmental interplay has been exemplified by studies of the human serotonin transporter gene *SLC6A4*. A variable number tandem repeat (VNTR) within the 5′-promoter of *SLC6A4* termed the 5′ linked polymorphic region (LPR) has been studied extensively for such gene–environment associations (Caspi et al. 2003; Uher & McGuffin 2008); the sequence of the VNTR repeats is outlined in Fig. S1. The 5′LPR and a second VNTR within intron 2 termed Stin2 have been hypothesized as predisposing genetic factors for several neurological disorders (reviewed in Haddley et al. 2008), although conflicting evidence has been reported in the literature (Lotrich & Pollock 2004). Both the 5′ LPR and Stin2 VNTRs are transcriptional regulatory domains, and therefore, modulation of their function could affect the levels of *SLC6A4* expression in a tissue-specific and stimulus-inducible manner as reviewed in Haddley et al. (2008).

The *SLC6A4* gene is an important modulator of addictive behaviour (Hall et al. 2004), and studies have
demonstrated the role of the serotonergic system in cocaine addiction (Little et al. 1998; Cabrera-Vera et al. 2000; Uhl, Hall & Sora 2002). The study by Little et al. (1998) suggested that SLC6A4 expression could be regulated in a region-specific pattern and that this could be affected by the genotype of the LPR variant. Although performed in post-mortem material, this would be consistent with the action of these domains as tissue-specific or stimulus-induced regulators (Haddley et al. 2008; Ali et al. 2010). That cocaine can alter SLC6A4 expression was also reflected in altered levels of transporter in platelets from individuals with cocaine dependence; however, that variation was not associated with the LPR genotype (Patkar et al. 2004). This conflict could be, in part, due to either the action of the VNTRs as a tissue-restricted regulator or that in these cells the Stin2 or other variants might be having a larger affect. Recent psychiatric research suggests that drugs of abuse and drug-associated cues or stresses, such as cocaine, exert long-lasting changes in gene expression through changes in epigenetic mechanisms, such as regulation of chromatin structure (Tsankova et al. 2007; Renthal & Nestler 2008). Further, it is proposed that repeated exposure to cocaine can lead to cumulative effects that may last for months or years and may be irreversible (Nestler 2004). However, it is still unclear why drug behaviour persists long after drug intake cessation and why gene expression profiles altered during drug intake persist following drug abstinence (Grimm et al. 2003). We therefore hypothesized that cocaine could differentially modulate epigenetic markers on the SLC6A4 gene based on the genotype of the polymorphisms. This could help improve our understanding of the genetic component in the progression to addictive behaviour based on differential gene expression and may explain the role of these polymorphic VNTRs in a number of neurological and addictive conditions. However, it is difficult to analyze biochemically such changes in vivo over the VNTRs as they are human-specific, having changed significantly from those in other primates. We therefore have no rodent or other model in which to address their action in vivo in response to repeated exposure to stimuli such as cocaine. In an initial attempt to gain insight into potential mechanisms that might function over these polymorphic domains in response to cocaine exposure, we have used a tumour cell model in which the endogenous human SLC6A4 gene is expressed and whose transcription is responsive to cocaine. In this model, we have used the human cell line JAr, which is known to constitutively express the serotonin transporter (Keating et al. 2004). Although these clonal cell lines do not have the properties of neurons, they do allow for sufficient cell number and homogeneity of cell type for biochemical analysis and characterization of mechanisms that can, in part, operate to regulate gene function. JAr cells were used in the initial characterization of the SLC6A4 promoter activity and regulation (Heils et al. 1995; Bradley & Blakely 1997), which have the added advantage of being heterozygous for both the LPR and Stin2 VNTRs alleles, central to the manuscript, and this is of tremendous value as internal control in our analysis of potential epigenetic variation on specific genotypes. Here, we describe transcription factor and histone changes over the 5′ LPR and Stin2 VNTRs and the proximal SLC6A4 promoter. Further, we address epigenetic modulation of the promoter and VNTR regions resulting from cocaine exposure as a potential mediator of gene expression. We focus on the transcription factor CTCF, which has been implicated in epigenetic remodelling (Ishihara, Oshimura & Nakao 2006), and which we have previously characterized as a regulator of both VNTRs (Klenova et al. 2004; Roberts et al. 2007; Ali et al. 2009).

MATERIALS AND METHODS

Cloning the SLC6A4 VNTRs into an Epstein–Barr virus (EBV)-based vector

Episomal reporter gene constructs were generated containing a ‘reporter cassette’ constituting the renilla luciferase reporter gene with either the short (s) or the long (l) LPR allele cloned upstream of the minimal SV40 promoter or alternatively, one of the intron 2 VNTR alleles (either Stin2.10 or Stin2.12) located within a synthetic intron upstream of the renilla luciferase coding sequence. These positions were chosen to mimic the endogenous position of these domains in the gene, albeit these two domains are 15.5 kb apart in the human genome. Further VNTR-renilla luciferase reporter cassettes were generated, termed ‘dual-region cassettes’, which contained a combination of either of the 5′ LPR alleles with either of the Stin2 VNTR alleles as described in Ali et al. (2009). The relative positions of the polymorphic sequences were maintained as in the single-region cassettes. These cassettes were digested with SacI and NotI to isolate the fragment containing the appropriate VNTR/s, the SV40 promoter and the renillin reporter gene. These fragments were cloned into a previously modified version of the EBV-based vector pMep.9, which expresses EBNA-1 protein for episomal maintenance (Invitrogen, Paisley, UK). The pMep.9 vector had been modified by removal of the metallothionein promoter. This vector also contains a neomycin gene for selection of transfectants.

Generation of stable cell lines

JAr cells [human placental choriocarcinoma (ATCC HTB-144) American Type Culture Collection, Manassas, VA] were maintained as monolayers in RPMI-1640 medium
(Autogen Bioclear, Calne, UK) supplemented with 10% heat-inactivated fetal calf serum (Hy-Clone, Loughborough, UK), 2 mg/ml glucose, 1 mM sodium pyruvate and 100 units/ml penicillin/100 μg/ml streptomycin (Sigma-Aldrich, Dorset, UK).

2 × 10^6 cells were transfected with 7 μg of plasmid DNA using ExGen500 in vitro transfection reagent following manufacturers’ guidelines (Fermentas, York, UK). Forty-eight hours post-transfection, the cells were replated at no more than 25% confluency and the culture medium was supplemented with 50 ng/ml of the antibiotic geneticin (Gibco, Paisley, UK). The concentration of geneticin was determined as the minimum concentration required to inhibit the growth of non-transfected JAr cells within two to three weeks. The cells were incubated in selective media, which were replenished every three to for days until geneticin-resistant clones were identified and selected.

**Exposure of JAr cells to cocaine**

Prior to treatment with cocaine, for chromatin immunoprecipitation (ChIP) analysis, the JAr cells were incubated in serum-free RPMI-1640 medium overnight. For reporter gene expression analysis, stable cell lines were incubated in geneticin selective serum-free media. The cells were subsequently incubated in serum-free RPMI-1640 medium supplemented with a final concentration of either 1 μM or 10 μM cocaine hydrochloride (Sigma-Aldrich) for one hour. Potential toxicity of 10 μM cocaine on this cell line was addressed using a ‘LIVE/DEAD Cell Viability Assay’ (Molecular Probes/Invitrogen; Cat. No. L-3224) (Supplementary materials and Fig. S2).

**Analysis of reporter gene expression**

The cells were harvested from 24-well plates after exposure to cocaine and assayed using the Dual Luciferase Reporter Assay System according to the manufacturers’ instructions (Promega, Southampton, UK). Luminescence was measured using a Glomax 96 microplate lumino-meter (Promega). Mean and SEM were calculated from the results of three independent experiments performed in triplicate.

**Quantitative real-time polymerase chain reaction (qPCR)**

Total RNA was extracted with Trizol reagent (Invitrogen) from either untreated JAr cells or after acute treatment with cocaine. Genomic DNA was digested with DNase (Promega) at 37°C for 30 minutes. 1 μg of total RNA was reverse-transcribed to single-stranded cDNA using Reverse Transcription System (Promega) and random primers according to the manufacturers’ instructions. A total of 30 ng of cDNA was used per qRT–PCR reaction. qRT–PCR was performed in the iQ5 real-time PCR detection system (Bio-Rad, Hertfordshire, UK) using the iQ SYBR Green supermix (Bio-Rad). β-actin was used as the housekeeping gene. The primer sequences used were as follows: β-actin (forward) 5′-catctccacctgaagttacc-3′ and (reverse) 5′-ataagcaatcgtgcttg-3′; SLC6A4 (forward) 5′-gtagttgctatgctgtgc-3′ and (reverse) 5′-ccgtgagagagatgagtagta-3′ from exons 3 and 5, respectively, amplifying a 304 bp fragment of the SLC6A4 mRNA. Thermal cycling conditions were as follows: 95°C for 10 minutes, followed by 45 cycles of amplification, composed of 94°C for 30 seconds, 60°C for 20 seconds, followed by data acquisition. Results of the qRT–PCR assays were analyzed using MYIQ5 Biorad software.

**ChIP**

DNA/protein complexes from 10⁷ JAr cells were cross-linked with 1% formaldehyde and mixed thoroughly at room temperature for 10 minutes. ChIP was performed using ChIP-IT express (Active Motif, Rixensart, Belgium) according to the manufacturers’ instructions. Briefly, cross-linking was quenched by addition of 125 mM glycine ‘Stop-Fix’ buffer, nuclei were isolated and lysed in lysis buffer supplemented with 1X protease inhibitor cocktail (PIC) and 1 mM PMSF to ensure that the protein/DNA interactions were preserved during chromatin purification and immunoprecipitation. The chromatin was sheared by sonication (10 × 30-second pulses) at 50% power using a Microson sonicator (Misonix, CT, USA). The efficiency of sonication was determined by agarose gel electrophoresis. The size of our sheared chromatin ranged between 500–1500 bp as seen in agarose gel electrophoresis. Following phenol–chloroform extraction, DNA concentration was determined. 6.3 μg of sheared chromatin, 3 μg of antibody: anti-CTCF (BD Transduction, Lexington, KY, USA); anti-H3K4m2; anti-H3K36m3; anti-IgG; as a control (Abcam, Cambridge, UK); anti-H3K9m2; anti-H3K9-ac; anti-histone deacetylase complex 1 (HDAC1) (Upstate, Watford, UK); anti-CREB1 (Novus Biologicals, Suffolk, UK); anti-BRCA1 (Novus Biologicals, Suffolk, UK); anti-YB-1 (Santa Cruz Biotechnology, Heidelberg, Germany); anti-RNA Polymerase II (Active motif cat no. 35097, raised against specific peptide, YSPTspPS) or anti-MeCP2 (Dr Cardoso, Darmstadt) and 25 μl of protein G magnetic beads (Active Motif) were mixed for each immunoprecipitation reaction, and the samples were incubated at 4°C overnight on a rotating wheel. Following immunoprecipitation, the samples were transferred to fresh siliconized tubes to reduce background contamination. Following DNA elution and the reversal of protein/DNA cross-links, the DNA was purified before subsequent PCR analysis. The PCR conditions were one cycle at 96°C (three
minutes), followed by 30 cycles composed of 95°C denaturing step (one minute), 65°C annealing step (one minute), 72°C extension step (one minute), in a P x 2 thermal cycler (Thermo Scientific, Loughborough, UK). Primers used for 5’ LPR amplification were as follows: forward: 5′-gggatccctgtgtctgaaatc-3′ and reverse: 5′-tctagctctcagctctgtgtcag-3′. Those used for amplification of the Stin2 VNTR were as follows: forward: 5′-ggaattcctgctgtctgtgtcag-3′ and reverse: 5′-cctagctctcagctctgtgtcag-3′. Primers for scanning ChIP analysis were as follows: SC-3000_forward: 5′-aaggaacctgtttcctgtccacacaccacacc-3′ and SC-3000_reverse: 5′-ggttcttttcttcttcacctgtgccctgg-3′ (amplifies nucleotides −3156 to −2620 of the proximal promoter); SC-2500_forward: 5′-acgctctgacgctgtcctgtgtgacttcttagcagactgtcag-3′ and SC-2500_reverse: 5′-tctgctctgcctgtctgtctgtcag-3′ (amplifies nucleotides −2868 to −2329); SC-2000_forward: 5′-ccagtgtgtgctgccgactctgcat-3′ and SC-2000_reverse: 5′-acgctctgtctgccgactctgcatcctgca-3′ (amplifies nucleotides −2593 to −2068); SC-1500_forward: 5′-tcagctgcacctggtcatcctgca-3′ and SC-1500_reverse: 5′-tcagctgcacctggtcatcctgca-3′ (amplifies nucleotides −2068 to −1565); SC-1000_forward: 5′-tctgctccacgccagctcaaatc-3′ and SC-1000_reverse: 5′-tagatctggtttctttctgtctgtgctgtctgtg-3′ (amplifies nucleotides −1096 to −581); SC-1000_reverse: 5′-tagatctggtttctttctgtctgtgctgtctgtg-3′ (amplifies nucleotides −773 to −241); SC-500_forward: 5′-ttttgctgacgtggtctttgtggtgtt-3′ and SC-500_reverse: 5′-ttttgctgacgtggtctttgtggtgtt-3′ (amplifies nucleotides −605 to +93). Primers for the promoter region of the luteinizing hormone receptor (LHR) gene were as follows: forward: 5′-actcgccaagtcgagctgtcgcag-3′ and reverse: 5′-catggtgcgggtggaagctgtcgcag-3′.

**RESULTS**

An allele-specific DNA protein interaction in response to cocaine treatment

JAr cells were shown to be heterozygous for the L- and S-alleles of the 5′ LPR of SLC6A4 by PCR analysis (Fig. 1a, input), which facilitated the investigation of the binding of transcription factors to the individual alleles of the 5′ LPR domain and allowed us to assess a potential variation in binding to distinct alleles following cocaine exposure. Potential toxicity of cocaine on the JAr cell line was addressed using a ‘LIVE/DEAD Cell Viability Assay’. No effect of 10 µM cocaine was observed under the growth conditions on cell viability (Fig. S1, supplementary material). At the 5′ LPR, CTCF bound both alleles under normal growth conditions (Fig. 1a). In the presence of cocaine, CTCF was found to bind only to the L-allele (Fig. 1b). Because of lack of CTCF binding to the S-allele following cocaine treatment, we examined the binding of the methyl-binding protein MeCP2, which albeit not exclusive, can bind to methylated DNA. Under basal conditions, MeCP2 did not bind to either allele of the 5′ LPR (Fig. 1c); however, after cocaine treatment, MeCP2 was found to bind only to the S-allele (Fig. 1c). The lack of CTCF binding and the detection of MeCP2 binding to the S-allele following cocaine treatment could not be attributed to methylation as demonstrated by the lack of binding of the methyl-binding protein MBD2 (Fig. 1d). To validate the lack of enrichment for methylated DNA within the 5′ LPR, purified positive and negative control DNA fragments and their respective input fractions were amplified by PCR (Fig. 1e).

MeCP2 can be both an activator or a repressor of transcription and it has been shown to recruit and associate with the co-repressor HDAC (Jones et al. 1998; Nan et al. 1998). More recently, Chahrour et al. have identified an association between the transcription activator CREB and MeCP2, which leads to the activation of the expression of a wide range of genes (Chahrour et al. 2008). Therefore, in order to examine whether MeCP2 was able to recruit and associate with either of these proteins upon binding to the S-allele of the 5′ LPR, sequential ChIP
DNA was initially immunoprecipitated with the MeCP2 antibody, followed by either HDAC1 or CREB antibodies. Under basal conditions, as predicted, MeCP2 was found not to associate with either HDAC or CREB based on the lack of MeCP2 binding under these conditions (Fig. 2); however, after cocaine treatment, HDAC, but not CREB, was found to associate with MeCP2 over the s-allele (Fig. 2).

**Figure 1** Differential binding of CTCF and MeCP2 to the alleles of the 5′ LPR after cocaine treatment. Chromatin immunoprecipitation (ChIP) analysis of CTCF and MeCP2 binding to the alleles of the 5′ LPR of the SLC6A4 gene in JAr cells under basal conditions and following treatment with 10 μM cocaine. ChIP analysis was performed as described in the methods section. IgG was included as a control for non-specific background binding. PCR analysis was carried out using primers specific to the SLC6A4 5′ LPR sequence. (a) Under basal conditions, CTCF was found to bind both alleles of the 5′ LPR. (b) After acute treatment with 10 μM cocaine, CTCF bound merely the l-allele. (c) MeCP2 does not bind either alleles of the 5′ LPR under basal conditions, whereas it binds merely to the s-allele after cocaine treatment. (d) Methylation analysis was performed as described in the methods section; MBD2 did not bind either allele of the 5′ LPR under basal conditions or following cocaine treatment. (e) Positive and negative control binding sites for MBD2. The figure shown is representative of three independent experiments.
As we have recently demonstrated that the LPR and Stin2 VNTRs can act co-operatively in regulating reporter gene expression (Ali et al. 2009), we sought to address the effect of cocaine treatment on CTCF and YB-1 binding to the Stin2 VNTR. JAr cells are heterozygous for the 10- and 12-copy Stin2 VNTR alleles. We have previously identified CTCF and YB-1 as transcription factors that bind to and modulate the expression of the Stin2 VNTRs (Klenova et al. 2004; Roberts et al. 2007).

In this study, we could not demonstrate YB-1 binding to either allele of the 5′ LPR under basal growth conditions or following cocaine treatment (data not shown); however, in the same samples, YB-1 did bind to both of the Stin2 alleles under basal conditions, and this binding was abolished following cocaine treatment (Fig. 3a). Acute cocaine treatment was found to have no effect on CTCF binding to either allele of the Stin2 VNTR (Fig. 3b).

Cocaine enhances the association of positive epigenetic markers over the SLC6A4 gene

We examined the effect of cocaine at either 1 μM (supplementary materials S3–S5) or 10 μM on the association of histone markers with the SLC6A4 proximal promoter and exon2/intron2 region as this region encompasses the Stin2 VNTR. Several histone markers were selected for analysis. In JAr cells, under basal growth conditions, low levels of active chromatin were observed at the SLC6A4 proximal promoter (Fig. 4a). However, following treatment with 10 μM cocaine, the association of this active chromatin marker (H3K4m2) with the SLC6A4 promoter significantly increased (Fig. 4a), suggesting that cocaine treatment may de-condense and unfold the chromatin associated with the SLC6A4 promoter to support transcription. We then performed scanning ChIP analysis to examine whether this increase in association of this active marker is within a specific region of the proximal promoter. Primers were designed to amplify ~500 bp overlapping fragments from nucleotides +93 to +3156 of the SLC6A4 gene (Fig. 5a). It was found that the increase in the association of this active marker (H3K4m2) was predominantly within the region spanning nucleotides −1000 to −2000 (Fig. 5b). Nucleosomes in the region of nucleotides −2500 to −3000 did not demonstrate a similar increase in H3K4m2 binding with little or none seen at the −3000 fragment. Genomic DNA was used as a positive control for PCR to demonstrate that the variability of association was not due to variable primer efficiencies (Fig. 5c).

Figure 2 MeCP2 associates with histone deacetylase complex (HDAC) at the s-allele of the 5′ LPR following acute cocaine treatment. Sequential chromatin immunoprecipitation (SeqChIP) analysis of MeCP2 interaction with HDAC or CREB over the alleles of the 5′ LPR of the SLC6A4 gene in JAr cells under basal conditions and following acute treatment with 10 μM cocaine. SeqChIP analysis detected (a) the interaction of MeCP2 with the co-repressor histone deacetylase complex (HDAC) over the s-allele following cocaine treatment. (b) No association of MeCP2 with CREB was observed over either allele of the 5′ LPR either under basal conditions or following cocaine treatment. SeqChIP analysis was performed as described in the methods section. The primary ChIP was performed with an antibody to MeCP2, and the secondary ChIP was performed with antibodies to either CREB or HDAC1. IgG was included as a control for non-specific background binding. PCR analysis was carried out using primers specific to the SLC6A4 5′ LPR sequence. The figure shown is representative of three independent experiments.
histone marker in JAr cells (Fig. 4c), confirming that the lack of association of this histone marker with SLC6A4 proximal promoter was specifically due to the chromatin state. Low levels of H3K9m2 association were observed over the exon2/intron2 region; this was diminished following 1 μM cocaine treatment and abolished following 10 μM cocaine treatment (Fig. S3, supplementary material).

To explore whether the increase in positive histone modifications associated with the SLC6A4 proximal promoter following cocaine treatment is further reflected elsewhere within the SLC6A4 gene, the association of histone markers with a region of the SLC6A4 gene open reading frame (ORF) (exon2/intron2) was assessed. Tri-methylation at lysine 36 on histone H3 (H3K36m3) is a marker for a positive correlation with transcription rates when found within the ORF of a gene and repressive when found in the promoter region (Kouzarides 2007). When this marker was screened over the promoter region of the SLC6A4 gene, this histone modification was not detected either under basal conditions or following cocaine treatment (data not shown). When we screened the association of this marker downstream of the promoter (exon2/intron2 region), we found that under basal conditions, this modification was below detectable levels; however, after acute treatment with 10 μM of cocaine, an association between H3K36m3 and the SLC6A4 exon2/intron2 region was observed (Fig. 4d). Similar results were obtained when cells were treated with lower cocaine concentration (1 μM) (Fig. S4, supplementary materials).

Accumulating evidence in the literature supports a general model in which histone acetylation associates with transcriptionally active DNA, whereas deacetylation associates with heterochromatin representing repressed DNA (Mizzen & Allis 1998; Struhl 1998; Cheung, Briggs & Allis 2000). In order to establish whether the SLC6A4 gene associates with either modification, we assessed association of the SLC6A4 exon2/intron2 with either acetylation of lysine 9 in histone H3 (H3K9-ac) or deacetylation of lysine 9 in histone H3 (HDAC1) under basal growth conditions and following acute treatment with 10 μM of cocaine. Under basal conditions, acetylation, but not deacetylation, was detected within the SLC6A4 exon2/intron2 region (Fig. 4e). Following cocaine treatment, the acetylation levels associated with the SLC6A4 exon2/intron2 were found to increase; however, low levels of deacetylation were also observed under these conditions (Fig. 4e). Similar results were obtained with 1 μM cocaine treatment (Fig. S5, supplementary materials).

To explore whether the increased association of active chromatin markers with the SLC6A4 gene following cocaine treatment correlated with the binding of the
general transcriptional activation machinery, we examined the binding of RNA polymerase II to the SLC6A4 promoter. Under basal conditions, RNA polymerase II binding could not be detected (Fig. 6a); this may be due to the low expression levels of the SLC6A4 gene in these cells (data not shown). However, following acute cocaine treatment, RNA polymerase II was detected to be bound to the SLC6A4 promoter (Fig. 6a), demonstrating that cocaine treatment results in enhanced RNA polymerase II binding to the promoter region. To confirm that the enhanced binding was specific to the SLC6A4 promoter, RNA polymerase II binding to the promoter of the GAPDH housekeeping gene was tested. Figure 6b demonstrates that there was no difference between RNA polymerase II binding to the GAPDH promoter before or after cocaine treatment. Finally, to determine whether the increase in the association of transcriptionally active chromatin with the SLC6A4 gene was reflected in the transcription rate of the SLC6A4 gene, in addition to the increase in RNA polymerase II binding to the promoter following cocaine treatment, we assessed SLC6A4 mRNA levels by qRT–PCR. Exposure to 10 μM of cocaine increased SLC6A4 mRNA levels by 4.5-fold (Fig. 6c). The housekeeping gene β-actin was used as a control to confirm specificity of the effect of cocaine on SLC6A4 mRNA transcription (data not shown).

Cocaine differentially modulates reporter gene expression directed by the SLC6A4 VNTRs by affecting chromatin structure

The differential effect of cocaine on the binding of CTCF, MeCP2 and YB-1 to the specific alleles of the SLC6A4 VNTRs suggested that these alleles could exhibit a differential transcriptional response to this stimulus. To
investigate this proposition, stably transfected JAr cell clones containing an episomally maintained plasmid expressing renilla luciferase directed by either the s- or l-allele alone upstream of the minimal SV40 promoter or either the Stin2.10 or Stin2.12 allele of the intron 2 VNTR alone located within an intron of the cassette or a combination of either of the 5′LPR alleles with either of the Stin2 VNTR alleles (S10, S12, L10 and L12, respectively) were generated. Previously, we have used non-episomal reporter gene transfected cell lines to demonstrate the response of the Stin2 VNTR to lithium (Roberts et al. 2007). In this study, the EBV episomal model was favoured because the episomes remain as stable (in the context of these experiments), independent genomic units and do not randomly integrate into the host genome thus eliminating positional effects. Furthermore, they are assembled into nucleosomes, thus mimicking the chromatin structure of the VNTRs in vivo (Avolio-Hunter, Lewis & Frappier 2001; Horrocks et al. 2002; Lipps et al. 2003; Biard et al. 2005). Acute cocaine treatment caused a significant increase in renillin activity (fourfold over basal level) when directed by the l-allele. No significant response of the s-allele was observed (Fig. 7a).

A twofold increase in renillin activity directed by the Stin2.12, but not the Stin2.10, allele was observed following cocaine treatment (Fig. 7c). When the combinatorial constructs were tested, only the S12 and L10 constructs showed a significant response to cocaine; specifically, L10 displayed a sixfold increase in renillin activity (Fig. 7b), while S12 displayed a twofold increase over the basal levels seen (Fig. 7d).

**DISCUSSION**

In this study, we demonstrate that cocaine can exert a novel mechanism of regulation on the *SLC6A4* gene in vitro in the JAr cell line model by altering chromatin modifications and DNA-binding activity of selected transcription factors on the 5′LPR and Stin2 VNTR polymorphisms along with the proximal promoter. Specifically, we have shown that exposure of JAr cells to acute cocaine treatment resulted in CTCF binding only to the l-allele of the 5′LPR whereas MeCP2 was observed to bind only to the s-allele and to recruit the HDAC to this allele. These changes correlated with an increase in the association of positive histone marks and RNA polymerase II binding within the *SLC6A4* proximal promoter. However, we were unable to distinguish on which allele of the promoter these histone marks were located; this is
a subject for future study. This regulation of chromatin structure by the known modulators of epigenetic structure, CTCF and MeCP2, suggests that cocaine may exert changes in SLC6A4 expression based, at least partly, on the genotype of the polymorphisms. Initial exposure to cocaine was followed by an increase in SLC6A4 expression, which correlated with the detection of both active chromatin marks and increased RNA polymerase II binding. This cell line model demonstrates one mechanism by which cocaine could alter epigenetic parameters over the SLC6A4 gene in neurons; if validated in neurons, it may represent an early step in the cascade of changes in neurons by repeated exposure to cocaine over time, which might result in stable epigenetic changes. The important point from our study is the potential for the genotype of the VNTR to modulate that epigenetic response. However, extrapolation of this mechanism to human central nervous system (CNS) neurons requires careful consideration as cell lines are not accurate models for post-mitotic neurons. Nevertheless, cell lines...
provide sufficient reproducible material that can be manipulated in vitro to investigate potential molecular and biochemical mechanisms that are difficult to address in either primary cultures (due to cell number and heterogeneity) or in vivo. The latter is especially important in dealing with the potential for human specific polymorphism to be functionally important as is the situation with the VNTRs within the SLC6A4 gene.

We have previously demonstrated that CTCF bound to and regulated the activity of the SLC6A4 5′ LPR and Stin2 VNTRs (Klenova et al. 2004; Roberts et al. 2007; Ali et al. 2009), suggesting it may play a role in regulating the expression of the SLC6A4 gene through these domains. CTCF has various functions in chromatin organization and gene regulation, ranging from chromatin insulator to transcriptional repressor and activator (Phillips & Corces 2009). Although many of these functions rely on CTCF–DNA interaction, evidence is emerging that the function of CTCF may depend on its interaction with other proteins (Wallace & Felsenfeld 2007). We assessed MeCP2 binding

Figure 7 Cocaine differentially modulates reporter gene expression directed by the 5′ LPR and Stin2 VNTRs in stably transfected JAr cells. JAr cells containing stably transfected reporter gene constructs expressing renilla luciferase directed by either the s-allele or l-allele upstream the minimal promoter SV40 or either the Stin2.10 or Stin2.12 allele within a synthetic intron or combination of either of the 5′ LPR alleles with either of the Stin2 VNTR alleles were treated with 10μM cocaine for 1 hour, harvested and assayed for renillin activity as described in methods section. Bars represent the fold change in renillin activity normalized to untreated cells and non-specific backbone effect. Significant increase in renillin activity was observed with the (a) l-allele but not the s-allele (c) Stin 2.12 allele but not the Stin 2.10 allele after acute treatment with 10μM cocaine. Only the (b) S12 and (d) L10 displayed significant increase in renillin activity within the combinatorial constructs. Errors bars indicate S.E.M. Student t-test indicates that the up-regulation was significant ***p = 0.005. The figure shown is representative of three independent experiments
to the alleles of the 5′ LPR after identifying an interaction of CTCF predominantly with the l-allele following cocaine treatment (Fig. 1b) as several studies have shown that CTCF binding sites are sensitive to methylation (Bell & Felsenfeld 2000; Hark et al. 2000; Kanduri et al. 2000; Rand et al. 2004) and that CTCF does not recognize its binding sequence when the site is methylated (Hark et al. 2000; Kanduri et al. 2000). The methyl-binding protein MeCP2 is predominantly described as a transcriptional repressor (Nan, Campoy & Bird 1997). It specifically binds to CpG dinucleotides through its MBD (Nan, Meehan & Bird 1993) and interacts with the Sin3A/HDAC co-repressor complex through its transcription repression domain, resulting in chromatin compaction (Nikitina et al. 2007). The lack of CTCF binding to the s-allele along with the observed binding of MeCP2 to this allele (Fig. 1c) in this study was not due to methylation of this allele as shown by the lack of binding of another methyl-binding protein (MBD2) (Fig. 1e,f). MeCP2 is also capable of binding directly to DNA through its C-terminus irrespective of the methylation state of the DNA (Buschdorf & Strattling 2004), and such a mechanism might be operating at this promoter region. Furthermore, it is simplistic to think of MeCP2 only as a repressor of transcription as it can also act as an activator based on promoter context and the presence of accessory proteins, e.g. MeCP2 via interaction with CREB is an activator of transcription (Chahrour et al. 2008). We therefore analyzed allele-specific DNA–protein interactions through sequential ChIP analysis to determine if MeCP2 interacted with other proteins upon binding to the s-allele of the 5′ LPR. Data presented in Fig. 2 revealed that MeCP2 recruited and associated with the HDAC over the s-allele of the 5′ LPR following cocaine treatment, suggesting that here, MeCP2 acts as a negative transcriptional regulator. Additionally, to the binding of CTCF and MeCP2, we have examined the effect of cocaine on histone modifications associated with the SLC6A4 gene. The increased association between the SLC6A4 proximal promoter and the H3K4m2 histone mark following cocaine treatment (Fig. 4a) suggested that this stimulus may de-condense the chromatin associated with the SLC6A4 promoter, thus enhancing transcriptional rate. This suggests that cocaine may exert its effect on regulation of this gene in part by increasing the association of this histone mark with the promoter region of the SLC6A4 gene and subsequently increasing the binding of RNA polymerase II to the promoter region (Fig. 6). Furthermore, the scanning ChIP analysis confirmed the positional specificity of the H3K4m2 histone mark, which was enriched in the proximal promoter region that encompasses the 5′ LPR (Fig. 5). Many studies suggest that H3K4m2 is a modification that does not affect transcription elongation per se (Pavri et al. 2006) but rather associates within the early transcribed regions of active genes (Pavri et al. 2006; Shilatifard 2006; Kouzarides 2007). We therefore screened for histone modifications within the SLC6A4 gene. Although, both dimethylation and trimethylation of histones occur 3′ of the ORF, only trimethylation displays a positive correlation with transcription rates and associates with the body of transcribed genes (Pokholok et al. 2005; Shilatifard 2006; Kouzarides 2007). For that reason, H3K36m3 was selected as a marker. The increased association of this histone marker with the SLC6A4 exon2/intron2 region after cocaine exposure (Fig. 4d) indicated that cocaine is increasing active histone modifications associated with the SLC6A4 gene. Under basal growth conditions, the SLC6A4 exon2/intron2 region was found to be acetylated at lysine 9 in histone H3 (H3K9-ac), demonstrating an active state of chromatin; however, this dramatically increased following cocaine exposure (Fig. 4e), reflecting a positive effect of cocaine on active histone marks. The presence of both positive (acetylation) and negative (deacetylation) markers within the SLC6A4 exon2/intron2 region may initially seem paradoxical; however, while tri-methylation of lysine 36 in histone H3 is linked to the passage of RNA polymerase II across the ORF, a second function of this modified histone is in recruitment of a deacetylase complex. Deacetylation is commonly associated with repressed DNA (Kuo & Allis 1998; Bulger 2005); however, deacetylation is also required to suppress spurious intragenic transcription, such as transcripts from ‘cryptic’ TATA-like sequences, which is achieved by removing nucleosome-destabilizing acetyl groups to facilitate nucleosome reforming after the passage of RNA polymerase II (Kaplan, Laprade & Winston 2003; Carrozza et al. 2005). Therefore, H3K36 methylation and subsequent involvement in deacetylation activity would result in marks as seen within the SLC6A4 exon2/intron2 region and may be indicative of suppression of spurious intragenic transcription in addition to marking active transcriptional regions (Vakoc et al. 2005; Brinkman et al. 2006). An alternative explanation could be that the region examined by ChIP included the intron 2 VNTR, which is heterozygous (10- and 12-copy alleles) in JAr cells, therefore the presence of both positive and negative markers might be due to differential histone modification of these alleles as one allele might be acetylated and the other deacetylated after cocaine treatment. Unfortunately, ChIP does not allow precise delineation of the specific binding sites within the genome, which are bound by specific proteins, but rather, defines a region of the genome where they are binding. A cartoon of the working hypothesis for these epigenetic changes before and after exposure to cocaine is shown in Fig. S6.

Key future experiments will be to delineate allelic-specific histone changes and allelic-specific expression.
Cell lines that are homozygous for specific alleles are an option for such analysis; however, they lack the inherent ability to address changes in the heterozygous cell at both alleles at the same time. In an attempt to look at the functional interactions of the distinct VNTRs in a nucleosomal structure, we addressed reporter gene expression directed by different VNTR combinations in an EBV-based episome cassette. In this model, the reporter gene levels of the dual VNTR cassette could not be simplistically predicted from the function of the individual VNTRs (Fig. 7). This suggests that similar to our biochemical data, the VNTRs act as quite distinct functional units in response to cocaine. In vivo, this activity could be modulated by other SLC6A4 regulatory domains; however, genotype may be important for the VNTR response to challenge when analyzed both individually (Fig. 7a,c) and in combination (Fig. 7b,d). It will also be important to tease out temporal changes in chromatin structure with the observed expression. Our data could help explain why an individual’s vulnerability to addiction is influenced by their genotype and is moderately to highly heritable (Goldman, Oroszi & Ducci 2005): carriers of the s-allele of the LPR have been reported to be more likely to develop an episode of major depression only following stressful life events (Champoux et al. 2002; Caspi et al. 2003; Barr et al. 2004). One must be wary of extrapolation of the changes observed in the in vitro model to the changes associated with cocaine abuse in the CNS. Although JAr cells do express the serotonin transporter, which could mediate a response to cocaine exposure, they do not contain all the features of neurons nor the complex cellular interactions observed in the CNS. Cell lines and primary culture models only offer a window of the transcriptional plasticity under the conditions in which the cells are grown. The transcriptome is a constantly being modified by all environmental challenges (such as serum concentration, the plastic on which the cells are grown, confluences and other parameters). The neurons in the CNS are under greater and constant transcriptional changes to their environment. Therefore, the action of cocaine to alter transcription will be dependent on other challenges the cell is receiving. Addiction is complex and relates to a plethora of human experience; however, as there is a large genetic component involved in the risk of addiction, we hope our data allow insight into changes in transcription in response to cell line exposure to cocaine that might be similar to those that operate in vivo in post-mitotic CNS neurons.

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Authors Contribution
JPQ and VJB were responsible for the study concept and design. KH and FRA performed all reporter gene analysis. ASV and FRA performed all the biochemistry. All assisted with data analysis and interpretation of findings. JPQ, ASV and FRA drafted the manuscript. KH and VJB provided critical revision of the manuscript for important intellectual content. All authors have critically reviewed content and approved final version for publication.

References


SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 Schematic representation of the LPR and STin2 polymorphisms in the SLC6A4.
Figure S2 Live/dead assay of JAr cells treated with 10 uM cocaine in serum-free conditions.
Figure S3 Cocaine reduces the association of the repressive histone marker (H3K9m2) over the SLC6A4 exon2/intron2 region.
Figure S4 Cocaine significantly increases the association of the active histone marker (H3K36m3) over the SLC6A4 exon2/intron2 region.
Figure S5 Cocaine increases the association of the active histone marker (H3K9-ac) over the SLC6A4 exon2/intron2 region.
Figure S6 A model representing proteins binding to the alleles of the 5′ LPR and epigenetic modifications over the SLC6A4 proximal promoter and intron 2 under basal conditions and following cocaine treatment.

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Figure S1: Schematic representation of the LPR and Stin2 polymorphisms in the SLC6A4 gene. The left-hand panel shows the Linked Polymorphic Region (LPR) that consists of 14-16 repeats for the short and long allele, respectively. The bold underlined italic sequence represents the 44bp deletion that is absent in the short allele. The simplistic CTCF transcription factor binding consensus sequence with the core (CCCTC) appears as large bold font. The single nucleotide polymorphism (a/g SNP) is shown in lowercase. The right-hand panel represents the VNTR in the second intron of SLC6A4, STin2. This consists of 3 allelic variants of 9, 10 or 12 repeats of a 16-17bp sequence. The 12 copy VNTR is shown, the 10 copy is missing those repeats labelled with a closed star and the 9 copy is lacking repeats labelled with a closed star and a closed triangle.

Figure S2: Live/dead assay of JAr cells treated with 10uM cocaine in serum-free conditions. JAr cells at 80% confluency were incubated overnight in serum-free medium and subsequently treated with 10 µM cocaine hydrochloride in serum-free medium for 1 hour and then incubated overnight in full serum containing medium. Green fluorescence is produced by intracellular esterase breakdown of cell permeable calcein AM to calcein and is indicative of live healthy cells. Red fluorescence is produced when non-membrane permeable ethidium homodimer binds to nucleic acids in damaged or dead cells. The above panels are representative of four experiments and indicate that treating the cells using this protocol does not induce any significant cellular damage or death within 24 hours. The left panels are untreated vehicle control and the two right panels are exposed to cocaine. In addition within the same period of time the cells show significant proliferation as evidenced by over-confluence.
Figure S3: Cocaine reduces the association of the repressive histone marker (H3K9m2) over the SLC6A4 exon2/intron2 region.

Chromatin immunoprecipitation (ChIP) analysis of the effect of 1 µM and 10 µM acute cocaine treatment on the association of H3K9m2 with the SLC6A4 exon2/intron2. Acute cocaine treatment reduces the association of H3K9m2 with the SLC6A4 exon2/intron2. Low levels of H3K9m2 were observed within the SLC6A4 exon2/intron2 and this association was reduced after treatment with (a) 1µM cocaine and abolished with (b) 10 µM cocaine treatment. ChIP was performed as described in the methods section. IgG was included as a control for non-specific background binding. PCR analysis was carried out using primers specific to the SLC6A4 exon2/intron2 sequence. The figure shown is representative of three independent experiments.

Figure S4: Cocaine significantly increases the association of the active histone marker (H3K36m3) over the SLC6A4 exon2/intron2 region.

Chromatin immunoprecipitation (ChIP) analysis of the effect of 1 µM and 10 µM acute cocaine treatment on the association of H3K36m3 with the SLC6A4 exon2/intron2 region. Acute cocaine treatment increased association of H3K36m3 with the SLC6A4 exon2/intron2 region. ChIP was performed as described in the methods section. IgG was included as a control for non-specific background binding. PCR analysis was carried out using primers specific to the SLC6A4 exon2/intron2 sequence. The figure shown is representative of three independent experiments.

Figure S5: Cocaine increases the association of the active histone marker (H3K9-ac) over the SLC6A4 exon2/intron2 region.

Chromatin immunoprecipitation (ChIP) analysis of the effect of 1 µM and 10 µM acute cocaine treatment on the association of H3K9-ac and H3K9-deac (HDAC1) with SLC6A4 exon2/intron2 region. Under basal conditions, acetylation of lysine 9 in histone H3 (H3K9-ac)
was detected within the *SLC6A4* exon2/intron2, and this acetylation level increased after 1 µM and 10 µM cocaine treatment. No deacetylation levels were observed under basal conditions, however, low levels of deacetylation were observed within the *SLC6A4* exon2/intron2 after 1 µM and 10 µM cocaine treatment. ChIP was performed as described in the methods section. IgG was included as a control for non-specific background binding. PCR analysis was carried out using primers specific to the *SLC6A4* exon2/intron2 sequence. The figure shown is representative of three independent experiments.

**Figure S6: A model representing proteins binding to the alleles of the 5’ LPR and epigenetic modifications over the *SLC6A4* proximal promoter and intron 2 under basal conditions and following cocaine treatment.**

(a) Under basal conditions, CTCF binds both alleles of the 5’ LPR. CTCF can either act solely or recruit either co-activators or co-repressors (HDAC) upon binding to the 5’ LPR, modulating the transcriptional activity of the *SLC6A4* gene. (b) Following cocaine treatment, CTCF no longer binds the *s*-allele and binds solely the *l*-allele. The methyl-binding protein (MeCP2) binds the *s*-allele and associates with the co-repressor HDAC, which may block the regulatory effect of the *s*-allele on the expression of the *SLC6A4*. (c) Under basal conditions, low levels of the positive histone marker (H3K4m2) were associated with the *SLC6A4* promoter, and low levels of H3K9-ac and H3K36m3 were associated with the intron 2 region. Following cocaine treatment, the association of all these positive histone markers increased (H3K4m2 over the promoter, H3K9-ac and H3K36m3 over the intron 2 region), and this was further reflected in an increase in *SLC6A4* mRNA levels. This suggests that cocaine may de-condense and unfold the chromatin associated with the *SLC6A4* gene locus, enhancing transcriptional rate, which was reflected in RNA polymerase II binding. However, low levels
of H3K9-deac were observed over the intron 2 region following cocaine treatment, this might be due differential modifications of the alleles of the intron 2 VNTR.

**Materials and Methods for Live/Dead Assay:**

Human JAr (placental choriocarcinoma) clonal cells were maintain in glass bottomed 35mm mat tek dishes in RMPI-1640 medium supplemented with 4.5 g/L D-glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 10 % foetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) at 37 °C, 5% CO2 until they reached 70-80% confluency. Medium was changed to supplemented RMPI-1640 without FCS for 24 hours. Media was then removed and cocaine hydrochloride was added to fresh serum free media to give a final concentration of 10µM. Cells were incubated in cocaine-containing medium for 1 hour before subsequent wash off and replacement with supplemented RPMI 1640 containing 10% FCS. Cells were processed 24 hours later for live/dead cytotoxicity and viability assay following manufacturers’ instructions. Briefly, Media was removed from cells and 150 µl of live/dead solution (PBS containing 2µM calcein AM and 4 µM ethidium homodimer) was added directly to the cells. Cells were incubated at room temperature for 30 mins and fresh live/dead solution was applied before images were taken on a Ziess Axiovert 40 microscope. Calcein fluorescence (λ 494/517 nm) was measured using a FITC filter (λ 448-518 nm) and ethidium homodimer fluorescence (λ 525/617 nm) was measured using a texas red filter (λ 488-615 nm).
<table>
<thead>
<tr>
<th>Input</th>
<th>IgG</th>
<th>H3K36m3 Basal</th>
<th>H3K36m3 1 μM Cocaine</th>
<th>H3K36m3 Basal</th>
<th>H3K36m3 10 μM Cocaine</th>
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![Image of Western Blot](image.png)

**SLC6A4 exon2/intron2**
Under basal-growth conditions

CTCF will either recruit co-activator or co-repressors or act solely in modulating how the S- or L-allele regulate the SLC6A4 expression

Following cocaine treatment

MeCP2/HDAC complex will block the cis-regulatory effect of the S-allele on SLC6A4 expression

CTCF will recruit either co-activator or co-repressors or act solely in modulating how the L-allele regulate the SLC6A4 expression

Under basal-growth conditions

Following cocaine treatment