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Epigenetic Readers of Lysine Acetylation Regulate Cocaine-Induced Plasticity

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Epigenetic processes that regulate histone acetylation play an essential role in behavioral and molecular responses to cocaine. To date, however, only a small fraction of the mechanisms involved in the addiction-associated acetylome have been investigated. Members of the bromodomain and extraterminal (BET) family of epigenetic "reader" proteins (BRD2, BRD3, BRD4, and BRDT) bind acetylated histones and serve as a scaffold for the recruitment of macromolecular complexes to modify chromatin accessibility and transcriptional activity. The role of BET proteins in cocaine-induced plasticity, however, remains elusive. Here, we used behavioral, pharmacological, and molecular techniques to examine the involvement of BET bromodomains in cocaine reward. Of the BET proteins, BRD4, but not BRD2 or BRD3, was significantly elevated in the nucleus accumbens (NAc) of mice and rats following repeated cocaine injections and self-administration. Systemic and intra-accumbal inhibition of BRD4 with the BET inhibitor, JQ1, attenuated the rewarding effects of cocaine in a conditioned place preference procedure but did not affect conditioned place aversion, nor did JQ1 alone induce conditioned aversion or preference. Investigating the underlying mechanisms, we found that repeated cocaine injections enhanced the binding of BRD4, but not BRD3, to the promoter region of *Bdnf* in the NAc, whereas systemic injection of JQ1 attenuated cocaine-induced expression of *Bdnf* in the NAc. JQ1 and siRNA-mediated knockdown of BRD4 *in vitro* also reduced expression of *Bdnf*. These findings indicate that disrupting the interaction between BET proteins and their acetylated lysine substrates may provide a new therapeutic avenue for the treatment of drug addiction.

Key words: BDNF; BET; BRD4; bromodomain; cocaine; epigenetic

Significance Statement

Proteins involved in the "readout" of lysine acetylation marks, referred to as BET bromodomain proteins (including BRD2, BRD3, BRD4, and BRDT), have been shown to be key regulators of chromatin dynamics and disease, and BET inhibitors are currently being studied in several clinical trials. However, their role in addiction-related phenomena remains unknown. In the current studies, we revealed that BRD4 is elevated in the nucleus accumbens and recruited to promoter regions of addiction-related genes following repeated cocaine administration, and that inhibition of BRD4 attenuates transcriptional and behavioral responses to cocaine. Together, these studies reveal that BET inhibitors may have therapeutic utility in the treatment of cocaine addiction.

Introduction

Dysfunction in histone acetylation has been linked with the pathophysiology of a wide range of psychiatric disorders (Tremolizzo et al., 2002; Kumar et al., 2005; Renthal et al., 2007; Malvaez et al., 2010; Kurita et al., 2012; Stafford et al., 2012; Moonat et al., 2013). In drug addiction, for example, repeated cocaine administration elevates global histone acetylation levels in reward-related brain regions, such as the nucleus accumbens (NAc) (Renthal et al., 2009). Furthermore, manipulation of histone acetyltransferases (HATs) and histone deacetylases (HDACs) by pharmacological inhibition, viral-mediated gene transfer, and/or knock-out models has confirmed that histone acetylation is critically involved in behavioral and molecular responses to cocaine (Kumar et al., 2005; Renthal et al., 2007, 2009; Sun et al., 2008; Im et al., 2010; Malvaez et al., 2010, 2011, 2013; Wang et al., 2010; Kennedy et al., 2013; Rogge et al., 2013). As a consequence, HAT and HDAC inhibitors have emerged as attractive targets for the treatment of drug addiction; however, additional reg-

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ulators of histone lysine acetylation have recently come to light. For instance, proteins involved in the "readout" of lysine acetylation marks, referred to as bromodomain-containing proteins (BRDs), have been shown to be key regulators of chromatin dynamics and the disease-associated acetylome (for review, see Prinjha et al., 2012; Filippakopoulos and Knapp, 2014), but their involvement in addiction is currently unknown.

The bromodomain and extraterminal domain (BET) family of proteins (BRD2, BRD3, BRD4, and BRDT) contain two bromodomains, which bind acetylated histone tails and are involved in transcriptional coactivation and elongation (Dhalluin et al., 1999; Winston and Allis, 1999; Owen et al., 2000; Patel et al., 2013). In particular, BET proteins have high affinity for clustered polyacetylated histone lysine sites but also bind to monoacetylated lysines with modest affinity (Dey et al., 2003; Morinière et al., 2009). Genome-wide analysis studies have revealed that BET-bound nucleosomes are enriched in histone post-translational modifications that regulate actively transcribed euchromatin, and BRD4-bound sites are highly correlated with increased gene expression (LeRoy et al., 2012) and play a role in super-enhancer function (Zhang et al., 2012; Brown et al., 2014). With the development of selective, small-molecule inhibitors of BET bromodomains (Filippakopoulos et al., 2010; Chung et al., 2011), there has been increasing interest in their therapeutic utility (Delmore et al., 2011; Zuber et al., 2011; Belkina and Denis, 2012; Barrett et al., 2014), and inhibitors of these proteins are currently being tested in clinical trials for treatment of several diseases (www.Clinicaltrials.gov; IDs NCT01713582, NCT01949883, NCT02157636, NCT01987362, and NCT01587703). However, it is unclear whether BET proteins regulate molecular and behavioral aspects of addiction. Given that histone acetylation mechanisms regulate cocaine-induced neuroadaptations and behaviors (LaPlant and Nestler, 2011), we hypothesized that BET proteins may also play a vital role in addiction-related phenomena. Using behavioral, pharmacological, and molecular techniques, we show that BET proteins are upregulated and recruited to promoter region of *Bdnf* in the NAc following repeated cocaine administration and that inhibition of these proteins attenuates transcriptional and behavioral responses to cocaine. Together, these studies indicate that the displacement of BET proteins from chromatin may have therapeutic efficacy in addiction-related behaviors.

Materials and Methods

Animals

Male C57BL/6 mice (8–10 weeks old) and Sprague Dawley rats (initial weight ~300–325 g, Charles River Laboratories) were housed 2–4 animals per cage under a regular 12 h/12 h light/dark cycle and had *ad libitum* access to food and water. Rats that received cannula or catheter surgeries were single-housed following surgery to prevent cage-mates from tampering with catheter/cannula implants. Mice and rats were housed in a humidity and temperature-controlled, Association for Assessment and Accreditation of Laboratory Animal Care-accredited, animal facility at the University of Miami Miller School of Medicine. All experiments were approved by the Institutional Animal Care and Use Committee before experimentation and conducted according to specifications of the National Institutes of Health as outlined in the *Guide for the Care and Use of Laboratory Animals*.

Drug treatments

Cocaine HCl (National Institute on Drug Abuse) was dissolved in 0.9% sterile saline. Mice and rats received one injection (intraperitoneally) of saline or cocaine (10 mg/kg for rats and 20 mg/kg for mice) per day for 10 d, and were killed at multiple time points following the last injection. The BET inhibitor, JQ1 or iBET-151 (Tocris Bioscience), was dissolved in 10% DMSO and 10% 2-hydroxypropyl-β-cyclodextrin in sterile PBS; 10–50 mg/kg was given in a volume of 0.08–0.12 ml before the cocaine conditioning session. Vehicle was delivered at the same volume as the JQ1/iBET-151 solution. For intracranial injections, JQ1 (10 μM) was dissolved in aCSF (Tocris Bioscience).

Cell culture

Neuro-2a (N2a) and HEK293 cell lines (ATCC) were maintained in DMEM supplemented with 10% FBS and 1% primocin. In a 6 well plate, ~400,000 cells were plated and treated 24 h later with DMSO (vehicle) or JQ1 (100 nM or 1 μM) for 48 h. In HEK293 cells, gene silencing was achieved by reverse transfection of scrambled control, BRD2-, BRD3-, or BRD4-siRNA (20 nM for 48 h) in lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. These previously validated BET siRNAs (Pastori et al., 2015) were purchased from Ambion (siBRD4-s23901, siBRD3-s15545, and siBRD2-s12070), and the control siRNA was purchased from QIAGEN (SI03650318). Cells were harvested for RNA and/or protein analysis as described below.

Surgeries

Intravenous catheter implantation. Rats were anesthetized with an intraperitoneal injection of a ketamine/xylazine mixture (60–80 mg/kg/10 mg/kg). In cocaine self-administration studies, rats were implanted with chronic indwelling intravenous catheters where sterile Silastic tubing was inserted into the jugular vein and the other end of the tubing passed subcutaneously over the shoulder blades to a cannula mounted on the back. Intravenous catheters were flushed once a day with the antibiotic cefazolin (10 mg, i.v.) and heparin (10 U, i.v.). After 5–7 d of recovery, rats received self-administration training.

Guide cannula implantation. Anesthetized rats were stereotaxically implanted with bilateral guide cannulae (22 gauge, Plastics One) aimed 2 mm above the nucleus accumbens (from skull surface: anteroposterior 1.8, mediolateral ±0.8, dorsoventral −5.5) (Paxinos and Watson, 1998), and guide cannulae were permanently fastened to the skull using acrylic cement and jeweler's screws. To prevent blockage, obturators were inserted into the guide cannulae and removed before injections. For intra-accumbal microinfusions of aCSF or JQ1, injection cannulae (28 gauge) were lowered through the guide, and infusions occurred over 1 min using a syringe and pump (10 μM, 0.3 μl per side). Injection cannulae were kept in place for 1 min after infusion to limit backflow.

Histological verification of cannula sites

To confirm cannula placements and minimal tissue damage for rats receiving intra-NAc infusions, pontamine sky blue (2% in 0.5 M sodium acetate, 300 nl) was injected through the cannulae before tissue collection, and sections were mounted directly on slides and counterstained with cresyl violet to localize cannula placements and to confirm the absence of gliosis or tissue damage.

Conditioned place preference (CPP)/conditioned place aversion (CPA)

In the CPP experiments, we used similar methods previously used (Sartor and Aston-Jones, 2012). Briefly, the CPP apparatus consisted of two distinct compartments that were separated by a removable divider. In a pretest acclimation session, mice and rats were allowed to freely explore both compartments for 15 min via an opening in the partition. The time spent on each side of the CPP chamber was recorded via EthoVision tracking software. Groups were organized such that mean baseline pretest scores were not different between treatments. Mice or rats that spent >65% of the time on one side of the chamber during the pretest were excluded. Next, mice and rats were conditioned for 3 d. During conditioning, animals were injected with cocaine (10 mg/kg for rats and 15 mg/kg for mice, i.p.) and restricted to one side of the chamber by a solid divider for 30 min, or injected with saline and confined to the other side of the chamber for 30 min. Injections were administered on both sides of the apparatus for each animal in a balanced fashion in morning and afternoon sessions (at least 4 h apart). Vehicle, JQ1, or iBET-151 was injected systemically (10, 25, or 50 mg/kg, i.p.) or intracranially (JQ1, 10 μM) 5 min before each cocaine conditioning session. Intracranial injections of JQ1 outside but near the NAc were used as anatomical controls (bilateral or unilateral misses). To test rewarding or aversive properties of

JQ1, a different group of mice were conditioned with JQ1 (50 mg/kg) on one side of the chamber and vehicle on the other side (JQ1/Veh group) or vehicle on both sides of the chamber (Veh/Veh group) for 3 d and were tested for CPP, as described above. After conditioning, mice and rats were given a 15 min (drug-free) preference test.

To determine whether JQ1 affects other types of contextual learning, JQ1 was administered during the acquisition of lithium chloride-induced CPA. First, mice received a 15 min pretest. Next, mice were conditioned for 3 d in a counterbalanced fashion, as described above. During conditioning, JQ1 (50 mg/kg, i.p.) or vehicle was administered 5 min before lithium chloride injection (125 mg/kg, i.p.), and mice were then confined in one side of the CPP chamber for 30 min. The other side of the chamber was paired with a saline injection. The next day, mice were given a 15 min preference test (drug-free). CPP/CPA scores were calculated by measuring the time spent in the drug conditioned side (cocaine, LiCl, or JQ1) during the post-test minus the time spent in the same side during the pretest.

Locomotor activity

Distance traveled was measured in a 27 × 27 cm open field chamber using EthoVision tracking software. Baseline locomotor behavior (distance traveled) was measured in a 30 min habituated test. Mice were grouped such that baseline locomotor activity did not differ between groups. Next, mice were injected with JQ1 (25 mg/kg) or vehicle and placed in the chamber, and distance traveled was measured for 30 min after injection.

Cocaine and sucrose self-administration

Rats were trained daily (2 h/d) to press the active lever in an operant chamber for intravenous delivery of cocaine (0.2 mg/infusion, paired with light cue) under fixed-ratio 1 schedule of reinforcement (FR1 with 30 s timeout after an infusion). Responding at the inactive lever had no scheduled consequences, but inactive presses were recorded. After acquiring self-administration behavior criteria (2 consecutive days with at least 12 infusions earned per day), rats received 10 additional days of cocaine self-administration. For sucrose self-administration studies, rats were trained to press the active lever for 45 mg sucrose pellets (Bio-Serv) under the FR1 schedule of reinforcement, as in cocaine self-administration studies described above. Rats were killed, and brain tissue was collected 24 h after the last session. Control rats were handled daily but did not receive self-administration training.

Western blot

Mice and rats were rapidly decapitated, and the NAc, dorsal striatum and prefrontal cortex (PFC) were dissected over ice and then flash frozen in liquid nitrogen. Samples were homogenized in M-PER lysis buffer (Pierce) with HALT proteinase inhibitors (Pierce). Protein concentrations were determined using a bicinchoninic acid assay kit (Pierce), and 20–30 μg of protein was loaded into precast Tris-HCl polyacrylamide gels (Bio-Rad) for electrophoresis. Protein samples were then transferred to a nitrocellulose membrane and blocked for 30 min in 5% blocking milk (Bio-Rad). Membranes were then incubated overnight at 4°C in blocking milk with the following primary antibodies at a 1:1000 dilution: anti-BRD2 (Cell Signaling Technology, #5848, lot 2), anti-BRD3 (Pierce, #30263, lot PE1859052D), and anti-BRD4 (Santa Cruz Biotechnology, #sc-48772, lot J1310). After multiple washes in 1× TBS, membranes were incubated in their respective HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, 1:1000 to 1:2000) in 5% blocking milk. All protein samples were normalized to GAPDH (Cell Signaling Technology, #2118S, 1:1000). The membranes were imaged with the ProteinSimple Flurochem, and densitometry of protein bands at the appropriate molecular weight was measured using ImageJ.

BDNF ELISA

Approximately 10 million N2a cells were treated with DMSO (vehicle) or JQ1 (100 nM or 1 μM, 48 h) in a 75 mm^2 flask. Cells were collected, pelleted, and lysed with M-PER supplemented with HALT proteinase inhibitors. BDNF proteins were measured with the ELISA procedure using the BDNF E_{max} ImmunoAssay System kit (Promega) according to the manufacturer's instructions. BDNF was normalized to the total protein concentration of each sample using bicinchoninic acid assay kit (Pierce).

Chromatin immunoprecipitation (ChIP)

In mice receiving repeated cocaine or saline injections, NAc tissue was collected, minced, pooled (bilateral NAc tissue from 3 mice per sample), and cross-linked with 1% formaldehyde for 15 min. The reaction was quenched with 1.25 M glycine and then washed three times in cold PBS. Samples were homogenized with 25 strokes in a Dounce homogenizer in lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 0.2% NP-40, 1× Halt protease inhibitor mixture). The samples were pelleted, and the supernatant was transferred to new tubes; 450 μl nuclear lysis buffer (50 mM Tris, pH 8.0, 10 mM 500 mM EDTA, 1% SDS, 1× protease inhibitor mixture) was added, and each sample was sonicated with a probe sonicator (Misonix, QSonica) for 2 cycles of 20 s on/off 4 times per cycle to achieve shearing of ~100–500 bps. Cellular debris were pelleted, and the supernatants were transferred to new tubes; 50 μl of each sample was saved to run on a 2% agarose gel to ensure proper DNA shearing and concentration. Next, 5 μg of ChIP-validated rabbit anti-BRD4 (Active Motif, #39909, lot 13912002) or anti-BRD3 (Active Motif, #61489, lot 30113001), rabbit IgG (Cell Signaling Technology, #2729S, lot 6), or rabbit anti-polymerase II antibody (Santa Cruz Biotechnology, #SC899X, lot H1114) was conjugated to magnetic Protein G Dynabeads (Novex, Invitrogen). Beaded antibodies were incubated with the sheared chromatin overnight (~16 h) at 4°C on rotation and then washed with 700 μl of the following solutions (in order): low salt (twice; 1× PBS, 0.1% SDS, 0.5% NP-40), high salt (once; 5× PBS, 0.1% SDS, 0.5% NP-40), LiCl wash buffer (once; 0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and TE buffer (twice; 10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The samples were eluted, and cross-links were reversed by heating the solution overnight at 65°C shaking at 1400 rpm; 40 μg of Proteinase K (Roche Diagnostics) was added to each sample and heated at 42°C for 2 h while shaking at 1400 rpm. Proteinase K was inactivated by heating the samples at 95°C for 10 min. DNA from each sample was extracted using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions.

qRT-PCR

Mice received systemic injections of the following: vehicle, JQ1 (25 mg/kg), JQ1 (50 mg/kg), vehicle/cocaine (20 mg/kg), JQ1 (25 mg/kg)/cocaine (20 mg/kg), or JQ1 (50 mg/kg)/cocaine (20 mg/kg). Vehicle or JQ1 was injected 10 min before the cocaine injection. Two hours after the initial injection, the NAc was collected and RNA was isolated using a Trizol and RNA extraction kit as described by the manufacturer (QIAGEN). In cell culture experiments, the media was aspirated, Trizol was added to each well, and RNA was extracted using the kit described above. RNA was reverse transcribed using a cDNA reverse transcription kit (Applied Biosystems). Using off-the-shelf TaqMan primer probes for Brain-derived neurotrophic factor (*Bdnf*), Activity-regulated cytoskeleton-associated protein (*Arc*), Cyclin-Dependent Kinase 5 (*Cdk5*), FBJ Murine Osteosarcoma Viral Oncogene Homolog (c-*Fos*), FBJ Murine Osteosarcoma Viral Oncogene Homolog B (*Fosb*), Interleukin 6 (*Il-6*), Interleukin 1β (*Il-1b*), and Tumor Necrosis Factor α (*Tnf-a*) (Invitrogen), qRT-PCR was run in triplicates and analyzed using the $2^{-\Delta\Delta CT}$ method with β-actin as the normalization control. No differences in β-actin mRNA expression were observed between groups.

For ChIP samples, previously validated primers were used to amplify the promoter regions of *Bdnf, Tnf-a, Il-1b*, and tubulin (Kumar et al., 2005; Su et al., 2009; Belkina et al., 2013). SYBR Green qRT-PCR was run in triplicate and analyzed using the $2^{-\Delta\Delta CT}$ method. The following primer sequences were used: *Bdnf* forward, 5′-TGAGGATAGGGG TGGAGTTG-3′, and reverse, 5′-GCAGCAGGAGGAAAAGGTTA-3′; *Tnf-a* forward, 5′-AGCGAGGACAGCAAGGGA-3′, and reverse, 5′-T CTTTTCTGGAGGGAGTGTGG-3′; *Il-1b* forward, 5′-TCTATTTCCC TTCAGTGCTG-3′, and reverse, 5′-TTCATGAGCACAGTCCATCT-3′; β-tubulin forward, 5′-TAGAACCTTCCTGCGGTCGT-3′, and reverse 5′-TTTTCTTCTGGGCTGGTCTC-3′.

Data analysis

GraphPad Prism software was used for graph preparation and statistical analysis. CPP/CPA scores were analyzed by calculating the time spent in

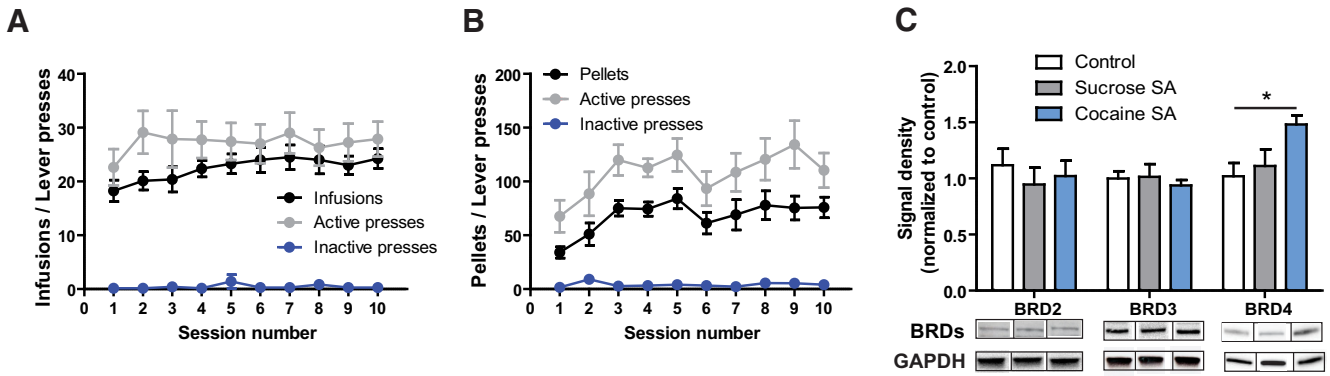

Figure 1. Increased BRD4 protein expression in the NAc following cocaine self-administration. Behavioral data from (***A***) cocaine and (***B***) sucrose SA rats showing the average number of infusions/pellets, active lever presses, and inactive lever presses per session. ***C***, BRD2, BRD3, and BRD4 protein levels in the NAc 24 h after the last cocaine or sucrose self-administration session compared with control naive rats. Representative blot images of BRDs and GAPDH are located below each bar graph. $*p < 0.05$, significant difference (Bonferroni *post hoc* test, $n = 6–8$ per group). Data are mean ± SEM.

the drug-paired side (cocaine, LiCl, or JQ1) on post-test minus the time spent in the same side during pretest. Mean values from CPP/CPA scores, densitometric data from Western blots, and Rq values from qPCR experiments (normalized to controls) were compared between groups using Student's *t* test or ANOVA. When a significant *F* value was obtained, comparisons were performed using *post hoc* analysis. Data are mean ± SEM, and the level of significance was set to 0.05.

Results

Repeated cocaine injections and self-administration increase BRD4 protein expression in the NAc

To determine whether cocaine regulates BET protein expression, rats received 10 d of cocaine or sucrose self-administration (SA). The average number of active lever presses, inactive lever presses, infusions, and pellets per session are shown in Figure 1, *A* and *B*. Using BRD proteins (BRD2, BRD3, and BRD4) as the within-subjects factor and group (naive, sucrose SA, and cocaine SA) as the between-subjects factors, our analysis showed a significant main effect for BRD ($F_{(2,49)} = 3.2$, $p < 0.05$), but no main effect for group or interaction between these factors. Bonferroni *post hoc* analysis revealed a significant difference in BRD4 expression ($p < 0.05$ for control vs cocaine SA and sucrose SA vs cocaine SA, $n = 6–8$) (Fig. 1*C*). No significant difference in BRD2 and BRD3 protein expression was observed between groups.

To determine whether experimenter-delivered cocaine also regulates BET protein expression, mice and rats received daily injections of cocaine (10–20 mg/kg, $n = 5–8$ per group) for 10 consecutive days and were killed 4 h, 24 h, or 7 d following the last injection (Fig. 2). In mouse NAc tissue, our analysis using BRD proteins (BRD2, BRD3, and BRD4) as the within-subjects factor and group (saline vs cocaine) as the between-subjects factor showed significant main effects of group ($F_{(1,30)} = 9.7$, $p < 0.0.1$) and BRDs ($F_{(2,30)} = 3.9$, $p < 0.05$) and an interaction between the two factors ($F_{(2,30)} = 3.8$, $p < 0.05$) at 4 h (Fig. 2*A*), and Bonferroni *post hoc* analysis revealed a significant difference in BRD4 expression ($p < 0.01$). No significant main effect was observed in the mouse NAc at 24 h and 7 d following repeated cocaine injections (Fig. 2*B*,*C*). In rat NAc tissue, a two-way ANOVA showed significant main effects of group ($F_{(1,34)} = 7.2$, $p < 0.05$) and BRDs ($F_{(2,34)} = 3.3$, $p < 0.05$), but no interaction ($F_{(2,34)} = 2.7$, $p > 0.05$) at 4 h. Bonferroni *post hoc* tests revealed a significant difference in BRD4 expression in saline versus cocaine-treated rats ($p < 0.05$) (Fig. 2*D*). No significant main effect was observed in the rat NAc at 24 h and 7 d following repeated cocaine injections (Fig. 2*E*,*F*) (a nonsignificant trend, however, was observed after 24 h in rats, $p = 0.06$, Fig. 2*E*). Acute administration of cocaine did not alter BRD4 protein expression 4 h after a single injection (normalized fold change: saline = 1.0 ± 0.12; cocaine = 1.0 ± 0.15, $p > 0.05$), indicating that the increase in BRD4 expression only occurs following repeated drug exposure. In the dorsal striatum and PFC, BRD4 protein levels were not altered 4 h after the last repeated cocaine injection, indicating that elevation of BRD4 following experimenter-delivered cocaine does not occur in all brain regions (normalized fold change in DS: saline = 1.0 ± 0.12; cocaine = 1.1 ± 0.13; $p > 0.05$. normalized fold change in PFC: saline = 1.0 ± 0.05; cocaine = 0.95 ± 0.21; $p > 0.05$, $n = 5$ or 6). At all time points measured, BRD2 and BRD3 protein levels in the NAc were not significantly changed following repeated cocaine injections. BRDT, a member of the BET family of bromodomains, was not measured in these studies, as it is not expressed in the brain (Shang et al., 2004).

Inhibition of BET proteins attenuates rewarding effects of cocaine

Using the CPP procedure, a measurement of conditioned reward, we found that systemic administration of the BET inhibitor, JQ1, before each cocaine conditioning session dose-dependently reduced cocaine CPP (25 and 50 but not 10 mg/kg, $n = 7–10$ per group) in mice (one-way ANOVA main effect of group: $F_{(4,36)} = 4.2$, $p < 0.01$ followed by Newman–Keuls test, $p < 0.01$ for Veh vs JQ1 25 mg/kg and $p < 0.05$ for Veh vs JQ1 50 mg/kg). A similar BET antagonist that does not measurably cross the blood–brain barrier (i-BET 151, 50 mg/kg) had no significant effect on cocaine CPP ($p > 0.05$ in Newman–Keuls test) (Fig. 3*A*). JQ1 (50 mg/kg, Veh/JQ1 group, $n = 8$) was not rewarding or aversive compared with vehicle (Veh/Veh group, $n = 7$), as it did not induce a CPP or CPA (Fig. 3*B*), whereas cocaine-conditioned mice (Coc/Sal) showed a strong preference for the cocaine-paired side (one-way ANOVA main effect of group: $F_{(2,20)} = 8.1$, $p < 0.01$ followed by Newman–Keuls test: $p < 0.01$ for Coc/Sal vs Veh/Veh and Coc/Sal vs JQ1/Veh; $p > 0.05$ for Veh/Veh vs JQ1/Veh). Additionally, JQ1 (50 mg/kg, $n = 8$) did not attenuate LiCl-induced CPA ($t_{(16)} = 0.03$, $p > 0.05$) compared with vehicle ($n = 10$), revealing that JQ1 does not affect all types of contextual learning (Fig. 3*C*). In addition, locomotor activity (distance traveled) was not altered by JQ1 ($n = 7$) compared with vehicle ($n = 7$) treatment ($t_{(12)} = 0.9$, $p > 0.05$) (Fig. 3*D*). When injected directly into the NAc of rats during acquisition of cocaine CPP, JQ1 (10 μM, $n = 8$) blocked cocaine preference compared with vehicle injec-

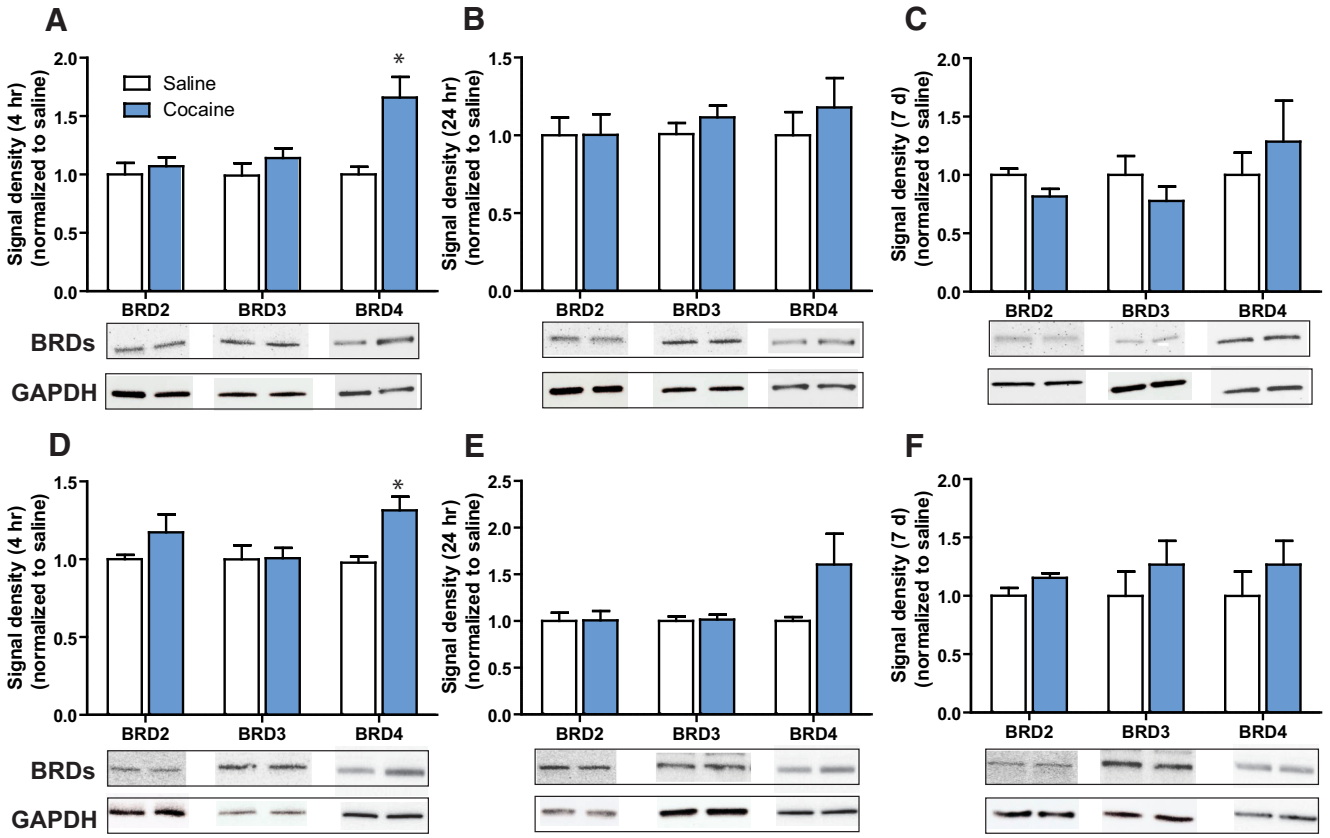

Figure 2. Increased BRD4 expression in the NAc following repeated cocaine injections. BRD2, BRD3, and BRD4 protein levels in the NAc of mice (***A–C***) and rats (***D–F***) at 4 h, 24 h, and 7 d following the last cocaine injection (1 injection per day for 10 d). *$p < 0.05$, significant difference from saline (Bonferroni *post hoc* test, $n = 5–8$ per group). Data are mean ± SEM.

tions into the NAc (Veh, $n = 11$) or injections of JQ1 nearby but outside of the NAc (unilateral and/or bilateral misses, $n = 5$) (one-way ANOVA main effect of group: $F_{(2,21)} = 6.3$, $p < 0.01$ followed by Newman–Keuls test, $p < 0.01$ for JQ1 vs Veh and $p < 0.05$ for JQ1 vs JQ1 miss) (Fig. 3*E*,*F*).

Inhibition of BET proteins alters cocaine-induced gene expression in the NAc

To access potential mechanisms by which BET proteins regulate gene expression in the presence or absence of cocaine, mice received the following intraperitoneal injections: vehicle, JQ1 (25 mg/kg), JQ1 (50 mg/kg), vehicle/cocaine (20 mg/kg), JQ1 (25 mg/kg)/cocaine (20 mg/kg), or JQ1 (50 mg/kg)/cocaine (20 mg/kg). We then examined NAc genes that are known to be involved in cocaine-induced plasticity (*Bdnf*, *c-fos*, *Fosb*, *Arc*, and *Cdk5*) and other genes that have previously been shown to be affected by BET inhibitors (*Il-6*, *Il-1b*, and *Tnf-a*) (Meng et al., 2014) (Fig. 4). Compared with the vehicle treatment, *Bdnf* expression was significantly increased in the NAc following Veh/Coc injections, and pretreatment with JQ1 attenuated cocaine-induced enhancement of *Bdnf* expression (Fig. 4*A*; one-way ANOVA main effect of group: $F_{(5,45)} = 7.6$ followed by Newman–Keuls test, $p < 0.01$ for Veh vs Veh/Coc, $p < 0.001$ for Veh/Coc vs JQ1 (25 mg/kg)/Coc and $p < 0.001$ for Veh/Coc vs JQ1 (50 mg/kg)/Coc). Interestingly, *c-Fos* and *FosB* were significantly elevated by 25 mg/kg and 50 mg/kg JQ1 treatments, respectively (Fig. 4*B*,*C*; one-way ANOVA main effect of group for *c-Fos*: $F_{(5,45)} = 2.6$, $p < 0.05$ followed by Newman–Keuls test, $p < 0.05$ Veh vs JQ1 25 mg/kg; one-way ANOVA main effect of group for *FosB*: $F_{(5,45)} = 3.2$, $p < 0.05$ followed by Newman–Keuls test, $p < 0.05$ Veh vs JQ1 50 mg/kg). *Il-1b* was increased in the Veh/Coc group compared with vehicle, whereas *Tnf-a* was elevated in all the cocaine-treated groups compared with vehicle (Fig. 4*G*,*H*; one-way ANOVA main effect of group for *Il-1b*: $F_{(5,45)} = 7.0$ followed by Newman–Keuls test, $p < 0.05$ for Veh vs Veh/Coc; one-way ANOVA main effect of group for *Tnf-a*: $F_{(5,45)} = 15.2$ followed by Newman–Keuls test, $p < 0.05$ for Veh vs Veh/Coc, $p < 0.001$ for Veh vs JQ1 (25 mg/kg)/Coc and Veh vs JQ1 (50 mg/kg)/Coc). One-way ANOVA analysis did not reveal a significant main effect in *Arc*, *Cdk5*, and *Il-6* gene expression (Fig. 4*D–F*).

JQ1 and BRD4-siRNA attenuate *Bdnf* expression *in vitro*

To determine whether direct application of JQ1 alters *Bdnf* in a similar manner, HEK293 and N2a cells were treated with vehicle or JQ1 (100 nM or 1 μM) for 48 h. JQ1 dose-dependently decreased expression of *Bdnf* mRNA in both cell types (Fig. 5*A*; one-way ANOVA main effect of group for HEK293 RNA: $F_{(2,15)} = 12.8$, $p < 0.001$ followed by Newman–Keuls test, $p < 0.01$ for Veh vs JQ1 100 nM and $p < 0.001$ for Veh vs JQ1 1 μM; one-way ANOVA main effect of group for N2a RNA: $F_{(2,15)} = 106.1$, $p < 0.0001$ followed by Newman–Keuls test, $p < 0.001$ Veh vs JQ1 100 nM and Veh vs JQ1 1 μM) and protein in N2a cells (one-way ANOVA main effect of group for N2a protein: $F_{(2,6)} = 125.4$, $p < 0.0001$ followed by Newman–Keuls test, $p < 0.001$ for Veh vs JQ1 100 nM and Veh vs JQ1 1 μM; $p < 0.05$ JQ1 100 nM vs JQ1 1 μM). These data are consistent with our previous results showing that BET inhibition attenuates *Bdnf* expression in cortical neurons (Zeier et al., 2015). Because JQ1 inhibits all BET bromodomains (BRD2, BRD3, and BRD4), the specific BET protein(s) that regulates *Bdnf* expression was unclear. Using siRNA-

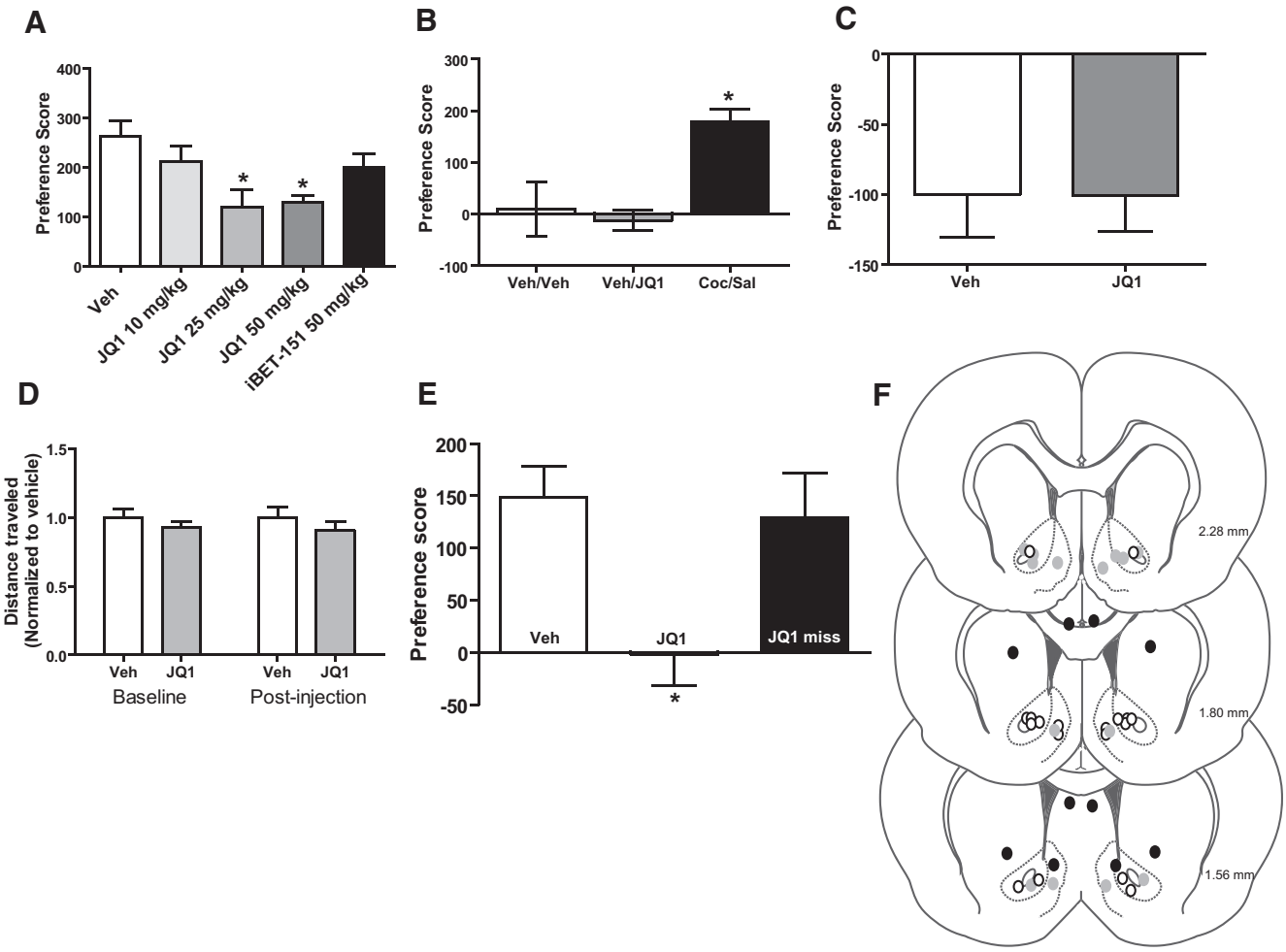

Figure 3. BET inhibition reduces behavioral responses to cocaine. ***A***, Acquisition of cocaine CPP was attenuated by JQ1 (25 and 50 mg/kg, but not 10 mg/kg) compared with vehicle. iBET-151, a similar BET inhibitor that does not cross the blood–brain barrier, did not significantly affect cocaine preference compared with vehicle. ***B***, JQ1 (Veh/JQ1) did not condition an aversion (or a preference) to the treatment-associated chamber, as there was no significant change in the amount time spent in the treatment chamber compared with vehicle ($n = 7$–10)-conditioned mice (Veh/Veh), whereas cocaine (Coc/Sal) induced a robust CPP ($n = 7$–8). ***C***, Acquisition of lithium chloride-induced CPA was not altered by JQ1 ($n = 8$–10), (***D***) nor was locomotor activity ($n = 7$). ***E***, Injections of JQ1 in the NAc of rats blocked acquisition of cocaine CPP compared with vehicle and anatomical control (JQ1 miss) injections. ***F***, Bilateral cannula placements of JQ1 (gray circles, $n = 8$) and vehicle (white circles, $n = 11$) within the NAc and unilateral and/or bilateral injections outside of the NAc (JQ1 miss, black circles, $n = 5$). *$p < 0.05$, significant difference from vehicle (Newman–Keuls *post hoc* test). Data are mean ± SEM.

mediated knockdown, we systemically depleted BRD2, BRD3, or BRD4 by >75% in HEK293 cells (Fig. 4*B*) and found that only BRD4-siRNA significantly attenuated *Bdnf* gene expression, indicating that BRD4 is a key regulator of *Bdnf* (Fig. 4*C*; one-way ANOVA main effect of group: $F_{(3,26)} = 3.9$, $p < 0.05$ followed by Newman–Keuls test, $p < 0.05$ for siControl vs siBRD4, siBRD2 vs siBRD4, and siBRD3 vs siBRD4).

BRD4 is recruited to the *Bdnf* promoter in the NAc following repeated cocaine injections

To better understand the molecular actions of BET-mediated gene regulation, we used ChIP assays to determine whether BRD4 and/or BRD3 shows enhanced binding to the promoter regions of *Bdnf*, *Il-1b*, and *Tnf-a* (genes that were reduced by JQ1) in the mouse NAc following repeated cocaine injections. Our analysis using gene (*Bdnf*, *Il-1b*, and *Tnf-a*) as the within-subjects factor and group (saline vs cocaine) as the between-subjects factor showed significant main effects of group ($F_{(1,38)} = 7.8$, $p < 0.01$) and gene ($F_{(2,38)} = 6.7$, $p < 0.01$) and an interaction between the factors ($F_{(2,38)} = 6.8$, $p < 0.01$) in the BRD4 ChIP (Fig. 6*A*). Bonferroni *post hoc* analysis revealed a significant difference in *Bdnf* expression ($p < 0.001$) in cocaine versus saline-treated mice. These findings are consistent with previously published results showing elevated H3 acteylation at the same promoter region of *Bdnf* following repeated cocaine administration (Kumar et al., 2005) and our *in vitro* data showing that only siRNA-mediated knockdown of BRD4 reduces *Bdnf* gene expression. Binding of BRD3 to the promoters of *Bdnf*, *Tnf-a*, and *Il-1b* in the NAc were not significantly altered following repeated cocaine injections (Fig. 6*B*).

Discussion

A novel role for BET proteins in cocaine reward

This study reveals a novel role for epigenetic reader proteins in cocaine-mediated behavioral and molecular adaptations. First, we found that the expression of BRD4, but not BRD2 or BRD3, protein was significantly increased in the NAc of mice and rats following repeated, but not acute, cocaine exposure and in the NAc of rats following cocaine self-administration. Although BRD4 expression was only increased 4–24 h after the last drug exposure, the downstream effects of BRD4 may have long-term consequences on unique gene programs that contribute to the addictive state. Fur-

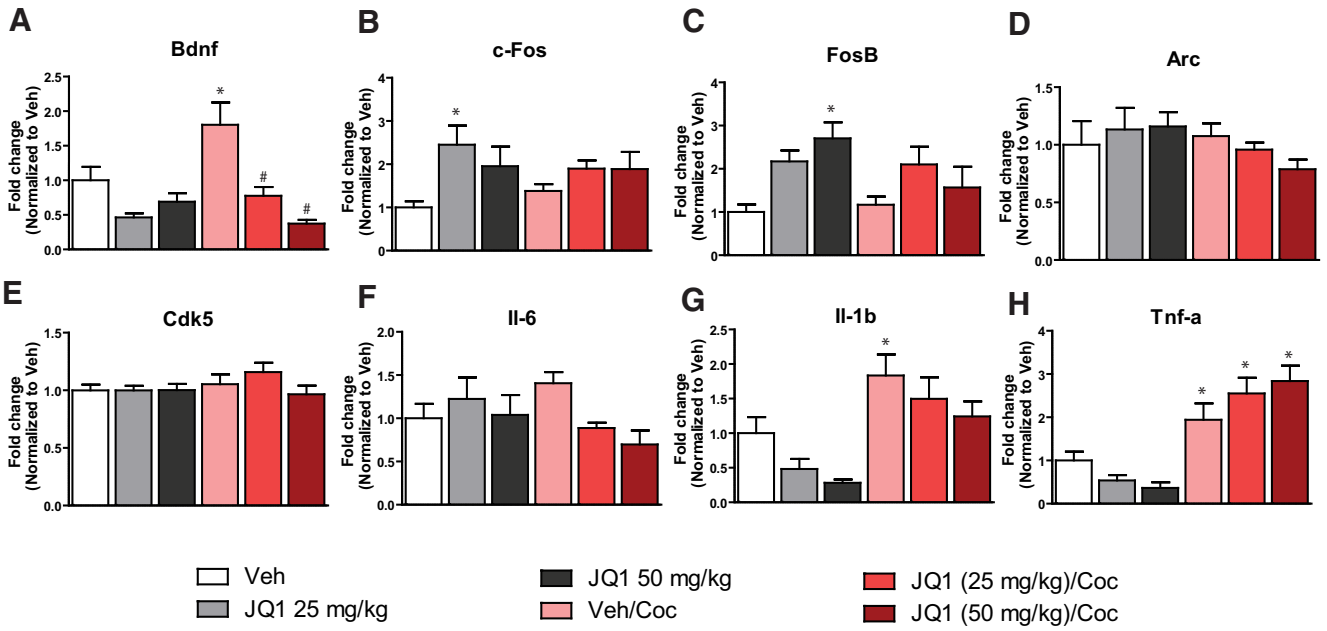

Figure 4. JQ1 alters cocaine-induced gene expression. ***A–H***, Mice received an intraperitoneal injection(s) of vehicle, JQ1 (25 mg/kg), JQ1 (50 mg/kg), vehicle/cocaine (20 mg/kg), JQ1 (25 mg/kg)/cocaine (20 mg/kg), or JQ1 (50 mg/kg)/cocaine (20 mg/kg). *Bdnf*, *c-Fos*, *FosB*, *Arc*, *Cdk5*, *Il-6*, *Il-1b*, and *Tnf-a* mRNA expression in the NAc was measured 2 h later. $^{*}p < 0.05$, significant difference from vehicle (one-way ANOVA followed by Newman–Keuls *post hoc* test, $n = 7–11$ per group). $^{\#}p < 0.001$, significant difference from Veh/Coc (one-way ANOVA followed by Newman–Keuls *post hoc* test, $n = 7–11$ per group). Data are mean ± SEM.

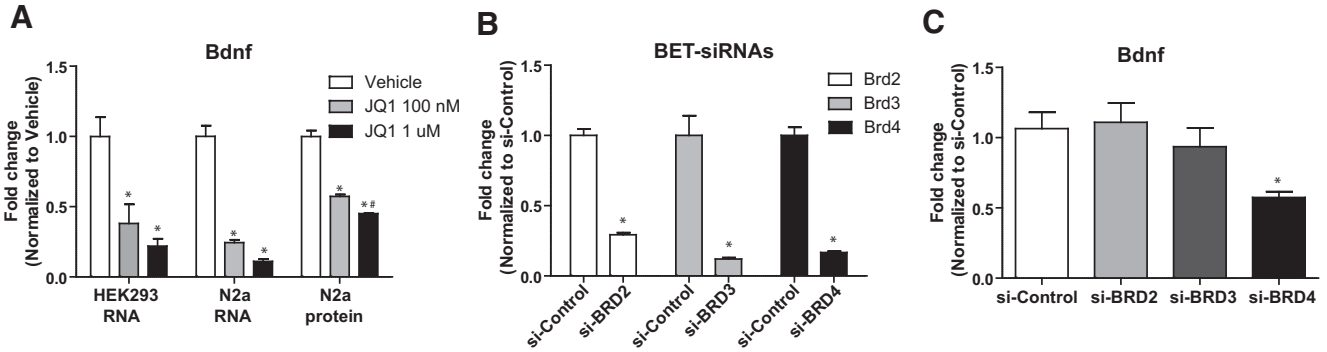

Figure 5. BRD4 regulates *Bdnf* expression *in vitro*. ***A***, JQ1 dose-dependently decreased *Bdnf* mRNA in HEK293 and N2a cells ($n = 6$) and protein in N2a cells ($n = 3$). ***B***, Validation of BR2-, BRD3-, and BRD4-siRNAs. HEK293 cells were transfected with siRNAs (20 nM) targeting BRD2, BRD3, BRD4, and control siRNA, and BRD2, BRD3, and BRD4 mRNA was measured 48 h later ($n = 6–9$). ***C***, BRD4-siRNA, but not BRD2- or BRD3-siRNA, decreased *Bdnf* mRNA expression in HEK293 cells ($n = 7–9$). $^{*}p < 0.05$, significant difference from vehicle or si-Control (one-way ANOVA followed by Newman–Keuls *post hoc* test). $^{\#}p < 0.05$, significant difference from JQ1 100 nM (one-way ANOVA followed by Newman–Keuls *post hoc* test). Data are mean ± SEM.

thermore, BRD4 may play a role in multiple psychostimulant-induced neuroadaptations, as one previous study revealed that *Brd4* mRNA is upregulated in mesotelencephalic neurons following repeated nicotine injections, although no follow-up experiments were performed (Saito et al., 2005). Second, we revealed that pharmacological inhibition of BET proteins with JQ1 attenuated cocaine CPP in a dose-dependent manner. However, iBET-151, a similar BET inhibitor that does not cross the blood–brain barrier, did not significantly affect cocaine CPP, indicating that the results obtained were due to the effects of JQ1 within the CNS. Third, we confirmed that BET activity in the NAc is essential for cocaine CPP, as injections of JQ1 directly into this region blocked cocaine CPP, whereas injections nearby but outside of the NAc had no effect. Additional behavioral studies revealed that JQ1 did not alter CPA, indicating that not all types of contextual learning behaviors are reduced by JQ1. Administration of JQ1 alone did not induce conditioned aversion or preference to the treatment-associated chamber, nor did JQ1 alter locomotor activity at doses found to affect cocaine CPP. Exploring the underlying mechanisms, we found that JQ1 blocked cocaine-induced elevation in *Bdnf* in the mouse NAc; and in siRNA studies, we showed that BRD4, but not BRD2 or BRD3, knockdown attenuated the expression of *Bdnf in vitro*. Further investigation, using ChIP followed by qPCR, revealed that BRD4, but not BRD3, is recruited to promoter region of *Bdnf* in the mouse NAc following repeated cocaine injections. Together, these findings establish BET proteins as key mediators in the epigenetic response to cocaine and offer new therapeutic avenues for the treatment of cocaine addiction.

Potential mechanisms of BET proteins in cocaine-induced plasticity

Growing evidence indicates that the dynamic induction and release of BDNF from mesocorticolimbic neurons during cocaine use facilitate the development and maintenance of addictive be-

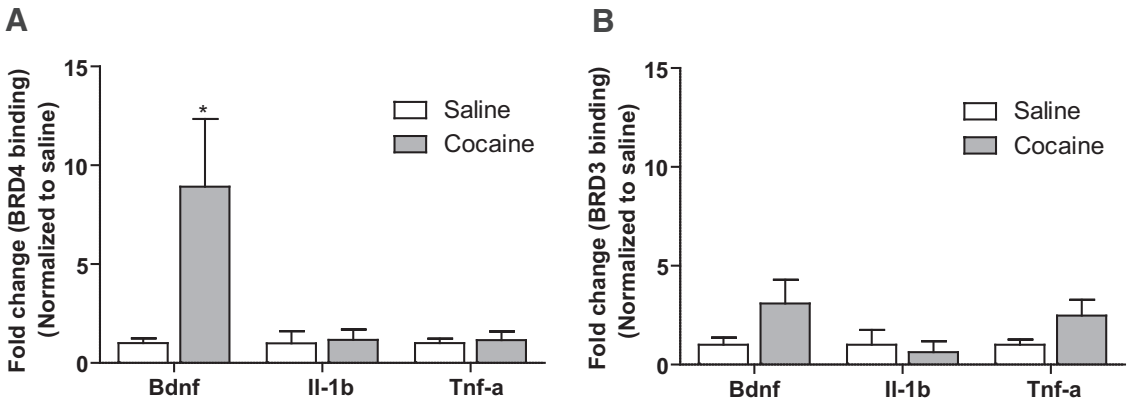

Figure 6. Cocaine-induced changes in BRD4 binding in the NAc. ***A***, Following repeated cocaine injections, BRD4 binding to the *Bdnf* promoter was significantly elevated in the NAc, whereas no increase in binding of BRD4 was observed in the promoter regions of *Il-1b* and *Tnf-a* ($n = 6–8$). ***B***, No significant enrichment of BRD3 was observed at the promoters of *Bdnf*, *Il-1b*, or *Tnf-a* following repeated cocaine treatment ($n = 6–8$). *$p < 0.05$, significant difference from saline (Bonferroni *post hoc* test). Data are mean ± SEM.

haviors. In the PFC, NAc, and ventral tegmental area (VTA), *Bdnf* mRNA and protein are differently regulated following acute or extended administration of cocaine or other drugs of abuse. For example, acute exposure to alcohol, amphetamine, or cocaine transiently increases the expression of *Bdnf* mRNA and/or protein in these regions up to 4 h after drug delivery (Kerns et al., 2005; Le Foll et al., 2005; Graham et al., 2007; Saylor and McGinty, 2008; Fumagalli et al., 2009). These initial changes in *Bdnf* synthesis may represent the early stages of plasticity that contribute to ensuing drug-induced neuroadaptations and behavior. During withdrawal from long-access cocaine self-administration, however, time-dependent increases in BDNF have been observed up to 90 d in the NAc, VTA, and amygdala, an adaptation that may play a role in incubation of drug craving (Grimm et al., 2003). In behavioral studies, inhibiting the dynamic regulation of endogenous BDNF in the NAc or dorsal striatum with anti-BDNF antibodies or viral-mediated gene knockdown attenuates several addiction-related behaviors (Narita et al., 2003; Graham et al., 2007, 2009; Jeanblanc et al., 2009; Im et al., 2010), whereas BDNF infusion in these and other regions enhances behavioral responses to abused drugs (Martin-Iverson et al., 1994; Horger et al., 1999; Lu et al., 2004; Graham et al., 2007, 2009; Bahi et al., 2008). Although the primary source of BDNF protein in the NAc originates from cortical and ventral midbrain neurons (Altar et al., 1997), BDNF production and release from NAc and striatal neurons do occur (Elmér et al., 1998; Filip et al., 2006; Liu et al., 2006) and are necessary for the development and maintenance of cocaine-seeking behaviors (Graham et al., 2007).

The dynamic change in *Bdnf* expression following cocaine use is accompanied by corresponding epigenetic modifications to the *Bdnf* gene in specific brain regions. For example, repressive marks and enzymes (H3K9me2, G9a, and MeCP2) are decreased at the *Bdnf* promoter in the NAc and/or mPFC following cocaine use (Maze et al., 2010; Sadri-Vakili et al., 2010). Conversely, epigenetic marks associated with active transcription, such as H3 acetylation, are increased at the *Bdnf* promoter in the NAc, mPFC, and VTA following cocaine administration (Kumar et al., 2005; Sadri-Vakili et al., 2010; Schmidt et al., 2012), and in the PFC of male offspring of cocaine-experienced sires (Vassoler et al., 2013). The data presented here add another layer of complexity to the epigenetic regulation of *Bdnf* in response to repeated cocaine exposure. We show that pharmacological inhibition of BET proteins reduced cocaine-mediated induction of *Bdnf* expression in the NAc and behavioral response to cocaine, and repeated cocaine injections enhanced BRD4 protein expression and BRD4 binding to the *Bdnf* promoter in the NAc (the same promoter region that was previously shown to have increased histone acetylation) (Kumar et al., 2005). However, increased BRD4 expression is not necessary for the acute effects of cocaine in the NAc, as inhibition of BRD4 blocked acute cocaine-induction of *Bdnf*, but acute cocaine did not increase BRD4.

Increasing evidence suggests that various inflammation factors in the brain are also involved in the behavioral effects of abused drugs (Niwa et al., 2007; Cearley et al., 2011), and BET inhibitors are known to attenuate the expression of many of these inflammation-related genes *in vitro* (Belkina et al., 2013; Meng et al., 2014). Although not statistically significant when comparing all groups, a decreasing trend appears in *Il-6* expression following JQ1/cocaine injections versus Veh/cocaine and in *Il-1b* and *Tnf-a* following JQ1 versus vehicle injection (Fig. 4). However, we did not observe changes in BRD3 or BRD4 binding to the promoters of these genes in the NAc following repeated cocaine exposure. Timing of tissue collection, cell type-specific mechanisms, secondary effects of JQ1, location of BRD binding (promoter, gene body, etc.), and BRD2-mediated mechanisms (BRD2 ChIP was not performed due to lack of validated antibodies) may explain why we did not observe changes in binding of BRD3 or BRD4 to promoter of these genes, even though JQ1 altered their expression in the mouse NAc. Interestingly, *Fosb* was increased in the NAc following a single injection of JQ1, which is consistent with previous reports showing that BET inhibition elevates *Fosb* expression in other cell types (Nicodeme et al., 2010). Because *Fosb* is associated with cocaine-induced plasticity (Hiroi et al., 1997; Harris et al., 2007), additional studies are needed to identify what regulatory role, if any, BET proteins have in *Fosb* expression and whether acute or chronic JQ1 exposure leads to alterations in protein levels in the NAc. In addition, genome-wide ChIP analysis of specific BET proteins (e.g., BRD4 ChIP-seq) following repeated cocaine injections and/or self-administration, and behavioral/molecular studies that selectively inhibit individual BET proteins in specific brain regions are needed to better understand the long-term, cocaine-mediated adaptations associated with BET proteins.

BET inhibitors as potential therapeutics for the treatment of addiction

Identifying novel therapeutics for the treatment of addiction is an area of intensive investigation. To date, only a limited number of

epigenetic-related targets have been studied in the realm of addiction, and the underlying mechanisms of many of these proteins in cocaine-seeking are unclear. As mentioned previously, HDAC inhibitors have been shown to be effective in multiple addiction-related phenomena, and there has been increasing interest in their use for drug abuse treatment. However, some adverse side effects have been reported in clinical studies using HDAC inhibitors (Subramanian et al., 2010), suggesting that these drugs may not be well tolerated by all patients. In the current studies, we report that BET inhibition attenuates behavioral responses to cocaine but does not affect other types of contextual learning, nor were BET inhibitors rewarding or aversive. Korb et al. (2015) recently showed that chronic JQ1 reduced novel object recognition memory, but not acquisition of fear conditioning, indicating that some additional memories may be affected by chronic BET inhibition. Other preclinical studies, however, have shown that animals can be treated chronically with JQ1 (50 mg/kg a day for 1 month) without major systemic toxicity (Matzuk et al., 2012); and in the multiple ongoing Phase I/II clinical trials, no serious side effects of BET inhibitors have been reported so far (www.Clinicaltrials.gov). Thus, our data, along with other preclinical and clinical data, indicate that BET inhibitors may have therapeutic utility in a number of different pathologies, including addiction, and appear to be generally safe for clinical use, but additional studies are needed to thoroughly investigate potential side effects of chronic BET inhibition.

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