

REGULAR RESEARCH ARTICLE

Transcriptome Sequencing Reveals Candidate NF- κ B Target Genes Involved in Repeated Cocaine Administration

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Abstract

Background: Drug-induced alterations in gene expression play an important role in the development of addictive behavior. Numerous transcription factors have been implicated in mediating the gene expression changes that occur in drug addiction. Nuclear factor kappa B is an inducible transcription factor complex that is rapidly activated by diverse stimuli.

Methods: We performed next-generation high-throughput sequencing of the prefrontal cortex in a mouse model of repeated cocaine administration combined with pharmacological nuclear factor kappa B inhibition to identify nuclear factor kappa B target genes that participate in the cocaine addiction process.

Results: We found that the nuclear factor kappa B antagonist sodium diethyldithiocarbamate trihydrate significantly reversed the cocaine-induced expression changes of the amphetamine addiction pathway. Genes that demonstrated differential expression in response to cocaine treatment that was also reversed by sodium diethyldithiocarbamate trihydrate were enriched for the axon guidance pathway. Furthermore, the nuclear factor kappa B homo-dimer motif could be mapped to 86 of these sodium diethyldithiocarbamate trihydrate-reversed genes, which were also enriched for axon guidance.

Conclusions: We suggest that nuclear factor kappa B directly modifies the expression of axon guidance pathway members, leading to cocaine sensitization. Our findings reveal the role of prefrontal cortex nuclear factor kappa B activity in addiction and uncover the molecular mechanisms by which nuclear factor kappa B drives changes in the addicted brain.

Keywords: addiction, cocaine, nuclear factor kappa B, prefrontal cortex, sodium diethyldithiocarbamate trihydrate

Introduction

Drug addiction is a global issue that generates extensive harm to the health and socioeconomic status of affected individuals and inflicts further burdens on society. Chronic addictive behavior develops as a consequence of progressive and multifaceted alterations to neural circuits upon repeated administration of drugs of abuse (Chao and Nestler, 2004). Though various drugs

alter the structure and function of numerous and distinct brain regions, at least one of the major constituents of the mesolimbic reward pathway—the ventral tegmental area, the nucleus accumbens, and the prefrontal cortex (PFC)—is modified by all addictive substances (Levy, 2013). In the context of cocaine addiction, it has been demonstrated that the PFC is involved in

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Significance Statement

Despite a growing number of treatments, drug addiction continues to be a source of tremendous physical, psychological, social, and economic harm. Understanding the molecular mechanisms by which the brain is altered during the onset of addiction can lead to the development of more effective therapies. It is widely accepted that chronic drug exposure results in changes in gene expression. Here, we investigate the role of the transcription factor NF- κ B in mediating these gene expression changes in the prefrontal cortex. Our findings demonstrate the role of NF- κ B activity in the prefrontal cortex in the establishment of cocaine addiction and uncover target genes and biological pathways through which this process occurs.

both the primary reinforcing effects of cocaine and long-term sensitization to cocaine. Previous studies have further demonstrated that PFC-dependent processes, such as executive function, explicit learning, and memory, are damaged in animal models of cocaine addiction and in human cocaine abusers (Chao et al., 2014). However, the molecular mechanisms driving altered PFC biology in response to cocaine addiction remain poorly understood, and, as a result, there continues to be a lack of effective therapies for addicted patients.

Though drug-induced changes in the expression of numerous neuronal genes contribute to behavioral abnormalities, the origin and causality of these genetic signatures are unclear (Zhou et al., 2014). Consistent changes in characteristic pathways suggest the action of specific transcription factors, which respond to environmental changes by binding to regulatory regions of target genes to alter their expression (Baldwin, 1996). In the past several decades, numerous transcription factors, such as Fos family protein, cyclic adenosine monophosphate response element binding protein, nuclear factor kappa B (NF- κ B), and myocyte enhancing factor-2, have been implicated in the addiction process (Nestler, 2012).

NF- κ B represents a small family of inducible transcription factors that are rapidly activated by diverse external stimuli and therefore serve as critical mediators of many environmental inputs (Nestler, 2012). The NF- κ B complex is typically held in an inactive form in the cytosol by interaction with members of the inhibitor of NF- κ B ($\text{I}\kappa\text{B}$) family of inhibitory proteins. Upon stimulation by extracellular inducers, $\text{I}\kappa\text{B}$ s are phosphorylated by an $\text{I}\kappa\text{B}$ kinase complex, which leads to ubiquitination and proteasome-mediated degradation of the $\text{I}\kappa\text{B}$ s. Then, active NF- κ B, through classical or noncanonical pathways, translocates to the nucleus, where it binds consensus κB sequences in the promoter and enhancer regions of responsive genes (Widera et al., 2006). NF- κ B binding sites are found in numerous genes that are known to participate in memory formation and drug addiction: brain-derived growth factor, inducible nitric oxide synthase, and various opioid receptors (Simeonidis et al., 1999; Malek et al., 2001). However, these examples represent a small number of NF- κ B targets, and there is no evidence whether these and other genes are regulated by NF- κ B in the PFC during cocaine addiction or whether they play a causal role in addiction onset and persistence.

It had been previously demonstrated that the NF- κ B signaling pathway, including several candidate downstream genes, is activated in the nucleus accumbens of mice exposed to chronic cocaine and inhibition of this pathway by a virally expressed dominant-negative form of $\text{I}\kappa\text{B}$ kinase attenuates the addictive effects of cocaine (Russo et al., 2009). However, in the context of cocaine addiction, the importance of NF- κ B signaling in the PFC has not been investigated, and the great genome-wide diversity of NF- κ B target genes has not been explored.

The major goals of this study were to determine whether NF- κ B signaling in the PFC is important in the development of cocaine addiction and to identify the potential target genes

through which NF- κ B may mediate cocaine-induced cellular and behavioral plasticities. To do so, we examined the effects of an NF- κ B inhibitor on the behavioral response to repeated cocaine administration and combined our model with high-throughput transcriptome sequencing to identify target genes activated by NF- κ B in the PFC upon cocaine treatment. We demonstrate the efficacy of pharmacological NF- κ B inhibition in counteracting the effects of cocaine and, by establishing the genes and pathways modulated by NF- κ B in response to cocaine, we reveal novel targets for further therapeutic intervention.

Materials and Methods

Animals

Male C57BL/6J mice (purchased from the Academy of Military Medical Science) weighing 25 to 30 g (8 weeks old) at the beginning of the experiments were used in this study. Five mice were housed per cage and all mice were given food and water ad libitum. The colony was maintained at $22 \pm 2^\circ\text{C}$ with a standard 12-h-light/dark cycle (lights on at 7:00 AM). The animals were handled daily for 1 week before injection. Testing was performed during the light cycle. All animal protocols were approved by the Review Board of the Institute of Psychology, Chinese Academy of Sciences, and were performed strictly in accordance with the Guideline for Care and Use of Laboratory Animals of the Chinese Academy of Sciences.

Drugs

Cocaine hydrochloride (Qinghai Pharmaceutical Co., Ltd.) and sodium diethyldithiocarbamate trihydrate (DDTC; NF- κ B antagonist, Sigma-Aldrich) were dissolved in sterile 0.9% saline and administered i.p. at 20 and 200 mg/kg, respectively.

Locomotor Sensitization Test

Mice were placed in the center of the field (30 x 30 cm), and total distance travelled by each animal was recorded for 60 minutes and analyzed by a computer-based system (Anilab). After each trial, the apparatus was cleaned with a 30% ethanol solution. The order of testing was balanced by group.

Treatment and Behavioral Testing Procedures

Mice were randomly assigned to 4 treatment groups: (1) saline+saline (SS, $n=10$), (2) DDTC+saline (DS, $n=10$), (3) DDTC+cocaine (DC, $n=10$), and (4) cocaine+saline (CS, $n=10$). The treatment period was 8 days, as shown in Figure 1. On days 1 to 3, baseline locomotor activity of every treatment group was assessed for 1 hour immediately following an i.p. injection of saline. On days 4 to 8, groups (2) and (3) received an injection of saline or cocaine, respectively, 30 minutes after an injection of DDTC, while groups (1) and (4) also received an injection of saline or cocaine, respectively, but 30 minutes following

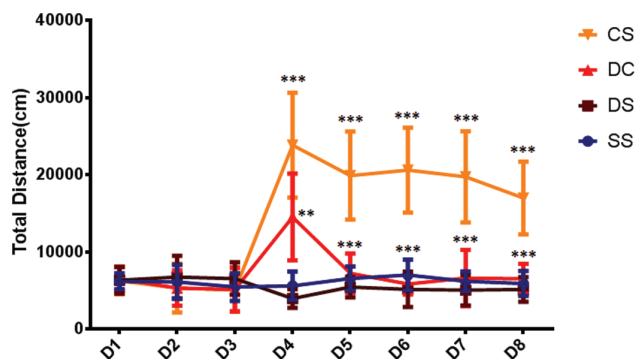


Figure 1. Effect of behavior of sodium diethyldithiocarbamate trihydrate (DDTC; 200 mg/kg) in response to cocaine-addicted mice (20 mg/kg, i.p.). CS, saline+cocaine; DC, DDTC+cocaine; ds, DDTC+saline; SS, saline+saline. All results represent means \pm SD of 10 independent determinations ($n=10$ /group) in the behavioral test experiments. **** $P < .0001$, *** $P < .001$.

an injection of saline. All mice were put into the locomotor box immediately following the second injection and monitored for 1 hour.

RNA Sequencing and Bioinformatics

Two hours after the final (day 8) locomotor activity test, animals were killed and their PFCs were surgically excised. The tissue was stored in liquid nitrogen immediately and then transferred to a -80°C freezer. Total RNA was extracted from the frozen tissues using the TIANamp DNA/RNA Isolation Kit (TIANGEN), including additional treatment with RNase-free DNase I (Ambion) for 30 minutes at 37°C to remove contaminating DNA.

Then $1\ \mu\text{g}$ of RNA from a pool of 5 animals (200 ng each) per experimental condition was used to generate an mRNA sequencing library using the TruSeq RNA Sample Prep Kit (Illumina) following the manufacturer's protocol. Briefly, polyA mRNA was purified and fragmented. Then, first and second strand cDNA synthesis was performed, ends were repaired and adenylated, adapters were ligated, and fragments were enriched with PCR amplification. High library qualities were verified with an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies). Libraries were sequenced using the Illumina HiSeq2K platform (Illumina).

FastQC was used to assess RNA-seq quality. Because of the low quality of the 3' end of raw reads, the last 20 base-pairs were trimmed from each read. Reads were also trimmed sequentially for adapter content. The paired-end reads were aligned to the mouse reference genome (mm9, Jul. 2007) with TopHat 2.0.9 (Trapnell et al., 2012) using default parameters. Aligned bam files were processed by Cufflinks v.2.2.1 (Trapnell et al., 2010) to generate a transcriptome assembly and to estimate the expression level (fragments per kilobase of transcript per million mapped reads [FPKM]) of all detected isoforms. FPKM was calculated as the number of paired-end reads (a single fragment per end-paired reads) mapped to a gene divided by the number of all fragments mapped to the genome (in millions) and the length of the transcript (in kilobases). The tagwise dispersion was estimated and then used for \log_2 fold change calculation. Differentially expressed genes (DEGs) were identified by applying the threshold false discovery rate of 0.2 to adjusted P values following Fisher's exact test (Chen et al., 2011). DEGs induced by cocaine sensitization (\log_2 fold_change [CS/SS]) were plotted against baseline gene expression levels of the SS group (\log_2 FPKM) using ggplot2 in R. KEGG pathway, and Gene Ontology (GO) analyses of DEGs were performed with Metascape software

(Tripathi et al., 2015) with a cutoff of $P < .05$ and a minimum overlap of 3. These raw sequencing data sets were deposited in the Gene Expression Omnibus of the NCBI (<https://www.ncbi.nlm.nih.gov/geo>) under accession number GSE108836.

Real-Time PCR Validation

Validation of DEGs was performed by quantitative reverse-transcription PCR (RT-qPCR) using the Maxima SYBR Green qPCR Master Mix kit (Fermentas), according to the manufacturer's instructions, in an ABI Prism 7500 Sequence Detection System machine (Applied Biosystems Inc). All real-time RT-qPCR data were normalized to SS expression level (see additional data file for primer information).

Motif Search

To discover DEGs with NF- κ B binding sites, we applied the UniProbe Database (<http://thebrain.bwh.harvard.edu/nfkb/>) to promoters (-500 to $+200$ bp relative to the transcription start site) of DEGs. The z-score cutoff was set to the default value of 4.

Data Analysis

Data are expressed as the mean \pm SEM. Statistical analyses were performed with GraphPad Prism 6.0 software. Comparisons of means of behavioral sensitization were analyzed by 2-way repeated-measures ANOVA followed by Tukey's posthoc tests.

Results

Effects of DDTC on Cocaine-Induced Behavior

First, we analyzed the effects of the injection of (1) saline + saline (SS), (2) DDTC+saline (DS), (3) DDTC+cocaine (DC), and (4) cocaine+saline (CS), repeated daily for 5 days, on mouse locomotor activity after 3 days of baseline testing. As shown in Figure 1, 2-way repeated-measures ANOVA of the locomotion test results demonstrated that there were significant differences in the total distance traveled between the 4 treatment groups ($F_{(3,27)}=44.34$, $P < .001$). Posthoc analysis revealed that there was no significant difference between the locomotor activities of each group at baseline (days 1–3). However, total distance traveled increased significantly following cocaine treatment (SS vs CS $P < .001$) and DDTC administration significantly inhibited cocaine-induced activity (CS vs DC $P < .001$). Additionally, there was no significant difference between the locomotor activities of DC and SS groups on Day 5 onwards, suggesting that DDTC can completely inhibit the hyperlocomotion induced by repeated cocaine administration.

GO and KEGG Pathways Induced by Cocaine

To understand the molecular mechanisms underlying the effects of cocaine and to delineate the functional consequences of DDTC treatment in the context of cocaine addiction, we performed mRNA-sequencing of extracted PFCs of mice from each of the 4 treatment groups, immediately following the 8th and final day of treatment and testing. A total of approximately 60 million 100 base-pair paired-end reads were obtained per sample.

Of 266 DEGs, 175 genes were upregulated and 91 genes were downregulated in the CS group compared with the SS group (Figure 2A). We then analyzed whether certain (KEGG) pathways were enriched for in the list of PFC DEGs and discovered 13 significantly enriched pathways upon repeated cocaine

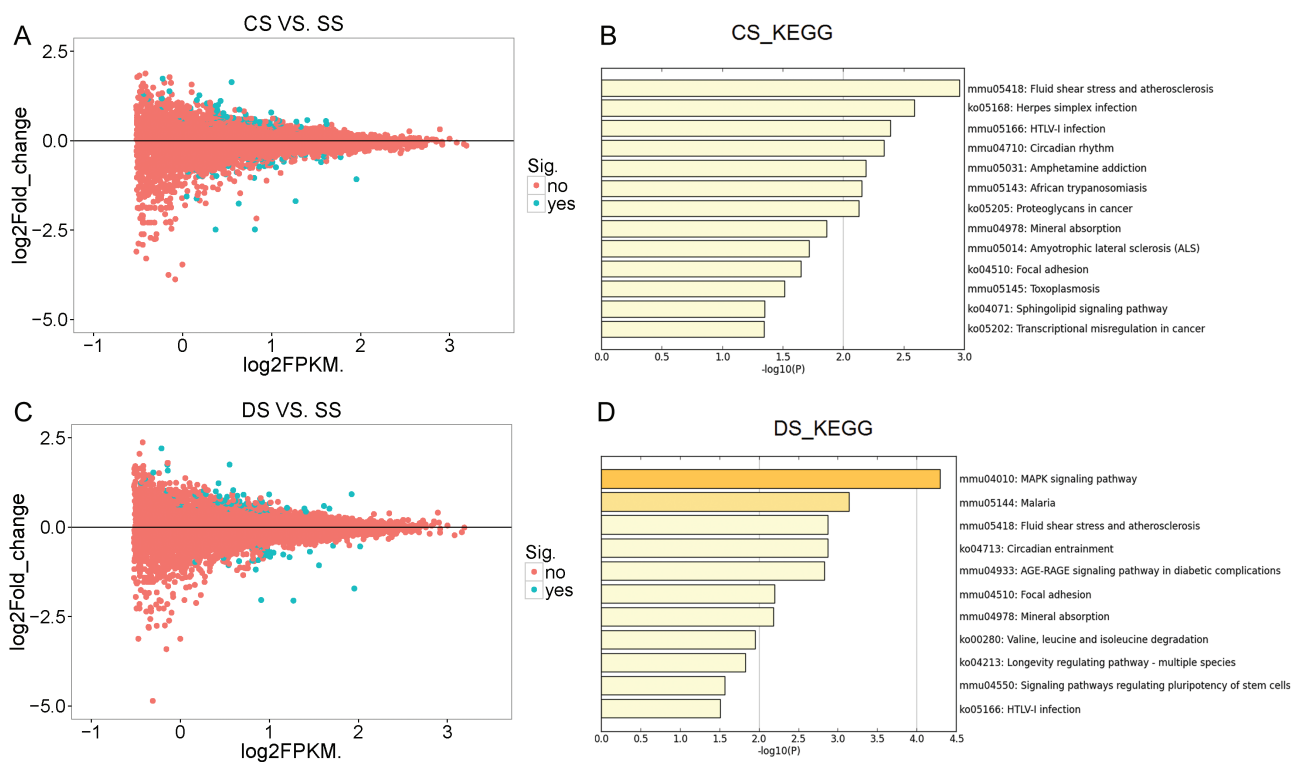


Figure 2. Cocaine and sodium diethyldithiocarbamate trihydrate (DDTC) cause broad changes to the transcriptome. mRNA from prefrontal cortex (PFC) of SS (saline+saline), CS (saline+cocaine), and DS (DDTC+saline) was subjected to RNA sequencing and analysis as described in the methods. (A) MA-plot [M (log ratios); A (mean average)] of differentially expressed genes (DEGs) of cocaine+saline (CS), respectively ($q < 0.2$). DEGs are represented by green dots. Log₂ fold change values for CS vs SS are plotted against average log expression values (fragments per kilobase of transcript per million mapped reads [FPKM]). (B) Chart of enriched pathways of significantly expressed genes in CS ($P < .05$). (C) MA-plot of DEGs of DDTC+saline (DS), respectively ($q < 0.2$). DEGs are represented by green dots. Log₂ fold change values for DS vs SS are plotted against average log expression values (FPKM). (D) Chart of enriched pathways of significantly expressed genes in DS ($P < .05$).

administration. Among these, 3 pathways were directly related to central nervous system function: most notably, the amphetamine addiction pathway, as well as the circadian rhythm pathway and amyotrophic lateral sclerosis (ALS) (Figure 2B). Additional GO analysis revealed multiple terms relevant to neurological function (supplementary Figure 1), including regulation of circadian rhythm (GO:0042752), synaptic vesicles (GO:0008021), and MAPK function (GO:0043408 and GO:0017017), which were reported to be involved in the control of synaptic plasticity (Thomas and Hugarir, 2004).

GO and KEGG Pathways Induced by DDTC

We simultaneously analyzed how DDTC treatment modifies the mouse transcriptome (Figure 2C). A total 143 DEGs were upregulated while 58 DEGs were downregulated in DS compared with SS. KEGG pathway analysis of all DS DEGs revealed a statistically significant enrichment for 11 pathways (Figure 2D). Interestingly, the MAPK signaling pathway, the circadian rhythm (entrainment) pathway, and the focal adhesion pathway, which were significantly enriched in cocaine treatment (CS vs SS), were also affected by DDTC treatment, indicating that cocaine and DDTC modulate several of the same molecular pathways in the PFC. GO analysis again revealed numerous enriched pathways related to neuronal function, including MAPKs (GO:0017017), synapse (GO:0045202), neurotransmitter transport (GO:0006836), central nervous system development (GO:0007417), learning or memory (GO:0007611), and neuron projection development (GO:0031175) (supplementary Figure 2).

GO and KEGG Analyses Reveal the Effects of DDTC on Pathways Altered by Repeated Cocaine Administration

Next, we applied the same analyses to study the effects of combining cocaine with DDTC treatment. As a major goal of this study was to identify the target genes by which NF- κ B mediates the development of cocaine addiction, we sought to find genes whose expression was altered by repeated cocaine treatment with the condition that this differential expression is abolished upon NF- κ B inhibition by DDTC treatment. In the process of identifying these genes, the next step was to perform a differential expression analysis between DC and CS groups to obtain a list of DEGs whose expression is affected by DDTC treatment in the context of cocaine treatment. A total 451 DEGs were upregulated and 410 DEGs were downregulated in DC-treated mice compared with CS-treated mice (Figure 3A). The top 20 significantly enriched pathways showed that DDTC can regulate pathways known to be related to addiction, such as amphetamine, calcium signaling, ABC transporters, gap junctions, glutathione metabolism, ALS, glycolysis, Alzheimer's disease, and neuroactive ligand-receptor interaction (Li et al., 2008), as well as other pathways related to neuronal function such as circadian entrainment, neurotrophin signaling, and axon guidance (Figure 3B). GO analysis (supplementary Figure 3) showed that the terms neuron projection development (GO:0031175), behavior (GO:0007610), calcium channel activity (GO:0005262), synapse (GO:0045202), and axon (GO:0030424) were enriched.

We were then interested in isolating pathways from DEGs that met 3 requirements: (1) are altered by cocaine alone

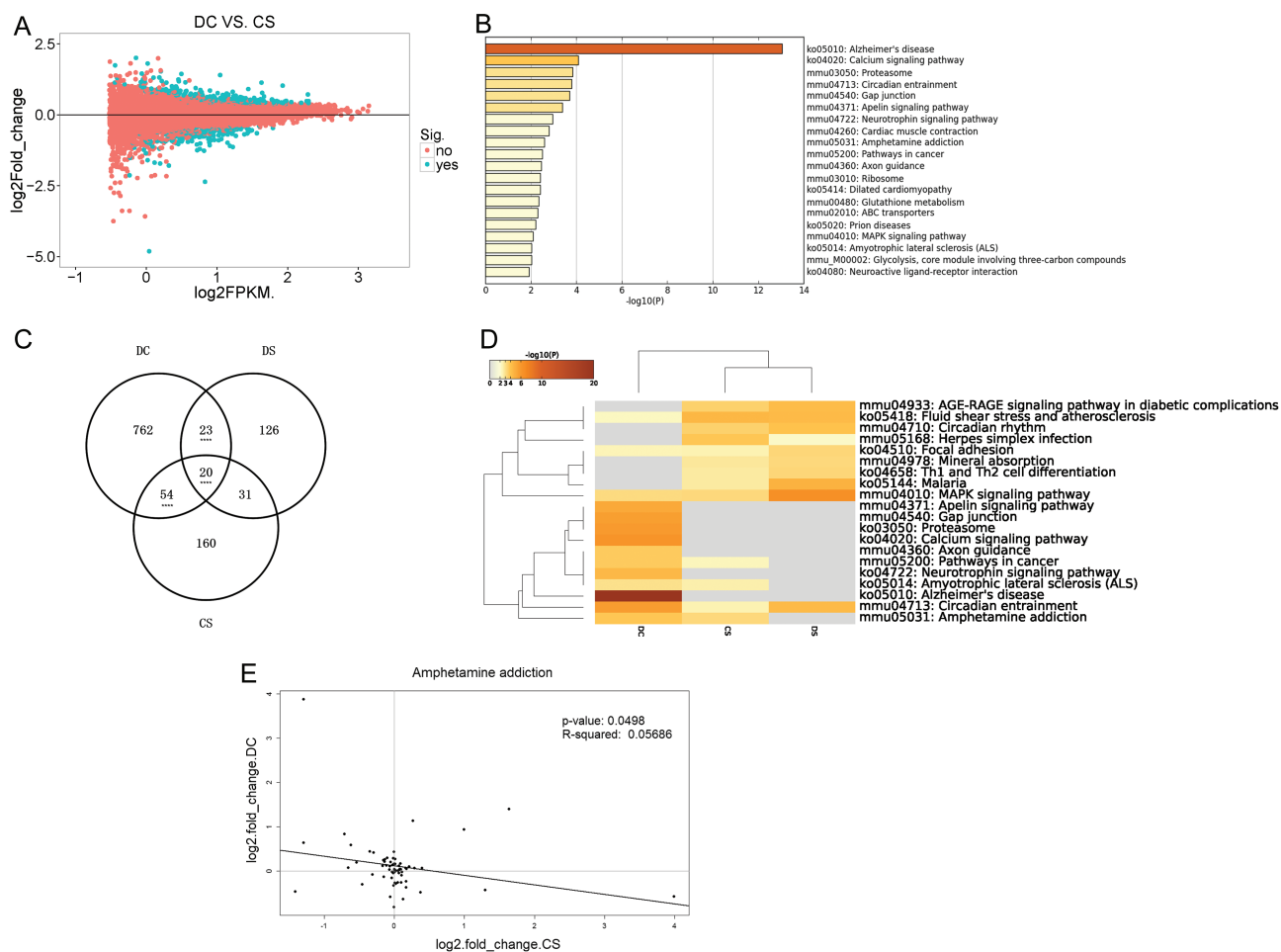


Figure 3. Transcriptome-wide response of sodium diethyldithiocarbamate trihydrate (DDTC) to cocaine addiction. (A) MA-plot of differentially expressed genes (DEGs) of DDTC+cocaine (DC), respectively ($q < 0.2$). DEGs are represented by green dots. \log_2 fold change values for DC vs saline+cocaine (CS) are plotted against average log expression values (fragments per kilobase of transcript per million mapped reads [FPKM]). (B) Chart of enriched pathways of significantly expressed genes in DS ($P < .05$). (C) Venn diagram depicting the overlap of DEGs among DC, DDTC+saline (DS), and CS. (D) Heatmap of comparison of enriched pathways of CS, DS, and DC. (E) Scatter plot of all genes in amphetamine addiction pathway.

(Figure 2A); (2) are modulated by DDTC in cocaine-treated (DC) mice (Figure 3A); and (3) result from DDTC treatment alone (DS) (Figure 2C). These DEGs and pathways would represent changes in cocaine-sensitization that are specifically caused by DDTC in DC-treated mice rather than representing a combined effect of DDTC and cocaine coadministration. The 3 requisite DEG data sets are plotted in a Venn diagram (Figure 3C; <http://bioinfo.gp.cnb.csic.es/tools/venny/>), which interestingly depicts a significant overlap between all 3 data sets, between DC and DS DEGs as well as DC and CS DEGs, but not between CS and DS DEGs, which are not present in the DC group (chi-squared test). Next, we performed a 3-way statistical comparison of enriched pathways and GO terms from DEGs induced in CS, DC, and DS treatments (Figure 3D). The KEGG pathways common to all 3 DEG groups were fluid shear stress and atherosclerosis, focal adhesion, MAPK signaling, and circadian entrainment.

However, common pathways significantly enriched in both cocaine treatment alone (CS) and DDTC+cocaine (DC) treatment DEGs included pathways in cancer, ALS, and amphetamine addiction, and several common GO terms were related to neural signal conduction (supplementary Figure 4), such as calcium channel activity (GO:0005262), neurotransmitter receptor activity (GO:0030594), and presynapse (GO:0098793).

Interestingly, scatter plots (Figure 3E; supplementary Figure 5) showed that DDTC (DC) significantly reversed the direction of cocaine-induced (CS) gene expression changes of the amphetamine addiction pathway ($P = .0498$) but not of the ALS pathway ($P = .078$), indicating that the amphetamine addiction pathway is an important pathway by which DDTC modulates gene expression changes in response to repeated cocaine administration.

DDTC Reverses Cocaine-Induced Changes in Expression of Genes in the Axon Guidance Pathway

While the previous analyses identified common pathways by which DDTC can reverse changes seen upon repeated cocaine treatment, pathways consist of many genes whose products exert contradictory functions on pathway activity, obscuring conclusions as to whether DDTC clearly reversed cocaine-induced behavior of these pathways. Therefore, we wished to determine if DDTC is able to antagonize cocaine-related expression changes of specific genes. We plotted the \log_2 fold change of DEGs from DC, CS, and DS conditions in a heatmap, with which it was immediately visible that many more genes exhibited fold changes in opposite directions in

the DC mice compared with CS and DS mice, an observation that was confirmed by hierarchical clustering (Figure 4A). In fact, the \log_2 fold changes of DEGs in DC and CS displayed a significantly negative relationship ($P=2.994e^{-15}$, $R^2=0.2647$), while those of DS and CS had a significantly positive relationship ($P=2.2e^{-16}$, $R^2=0.4723$) (Figure 4B; supplementary Figure 5). These results indicate that DDTC reverses specific gene expression changes that occur in response to cocaine treatment.

We then performed an additional pathway analysis to determine which pathways were enriched in the list of genes whose cocaine-induced expression changes were specifically reversed by DDTC (i.e., fold change in the opposite direction). There were a total of 142 such genes, 58 of which were upregulated and 84 of which were downregulated by cocaine, while these changes were reversed by DDTC (supplementary Table 2). KEGG analysis revealed that the most significantly enriched pathway was axon guidance (mmu04360, $P=.0088$). Interestingly, when we analyzed the \log_2 fold changes of all DEGs in the axon guidance pathway posthoc, that is, in a less stringent manner, not only were the DEGs with significant cocaine-induced expression changes significantly reversed by DDTC but there was a significantly negative relationship between CS and DC groups ($P=4.211e^{-08}$), thereby further demonstrating that DDTC can reverse the axon guidance behavior that is modified by cocaine treatment (Figure 4C).

Candidate NF- κ B Target Genes Involved in Response to Repeated Cocaine Administration

NF- κ B is a protein complex composed of homo- or heterodimers of 5 different family members: c-Rel (Rel), RelA/p65 (Rela), RelB (Relb), NF- κ B₁/p50/p105 (Nfkb1), and NF- κ B₂/p52/p100 (Nfkb2) (Natoli, 2006). Studies of protein-DNA crystal structures and DNA-binding studies have shown that NF- κ B₁ and NF- κ B₂ recognize the 5 base-pair motif of 5'-GGGRN-3', whereas c-Rel, RelA/p65, and RelB recognize a 4 base-pair 5'-GGRR-3' motif, and the entire κ B family recognizes a consensus motif of 5'-GGGRN(Y)YYCC-3' (Hoffmann et al., 2006; Bracchi-Ricard et al., 2007). Using this information, we applied online tools (see Methods) to scan the promoter regions of DEGs for NF- κ B family binding sites. The NF- κ B motif could be mapped to the promoter regions 86 DEGs (supplementary Table 3). This gene list was also enriched in the axon guidance term (mmu04360, $P=.089$), supporting the hypotheses that NF- κ B directly regulates the axon guidance pathway in cocaine addiction and that DDTC interferes with this activity.

Validation of Genes Reversed by DDTC

The top-ranked DEGs by fold-change expression were EphA8 (Ephrin Type-A Receptor 8), a member of the axon guidance pathway, and Dpf3 (Double PHD Finger 3), which belongs to the neuron-specific chromatin remodeling complex involved in

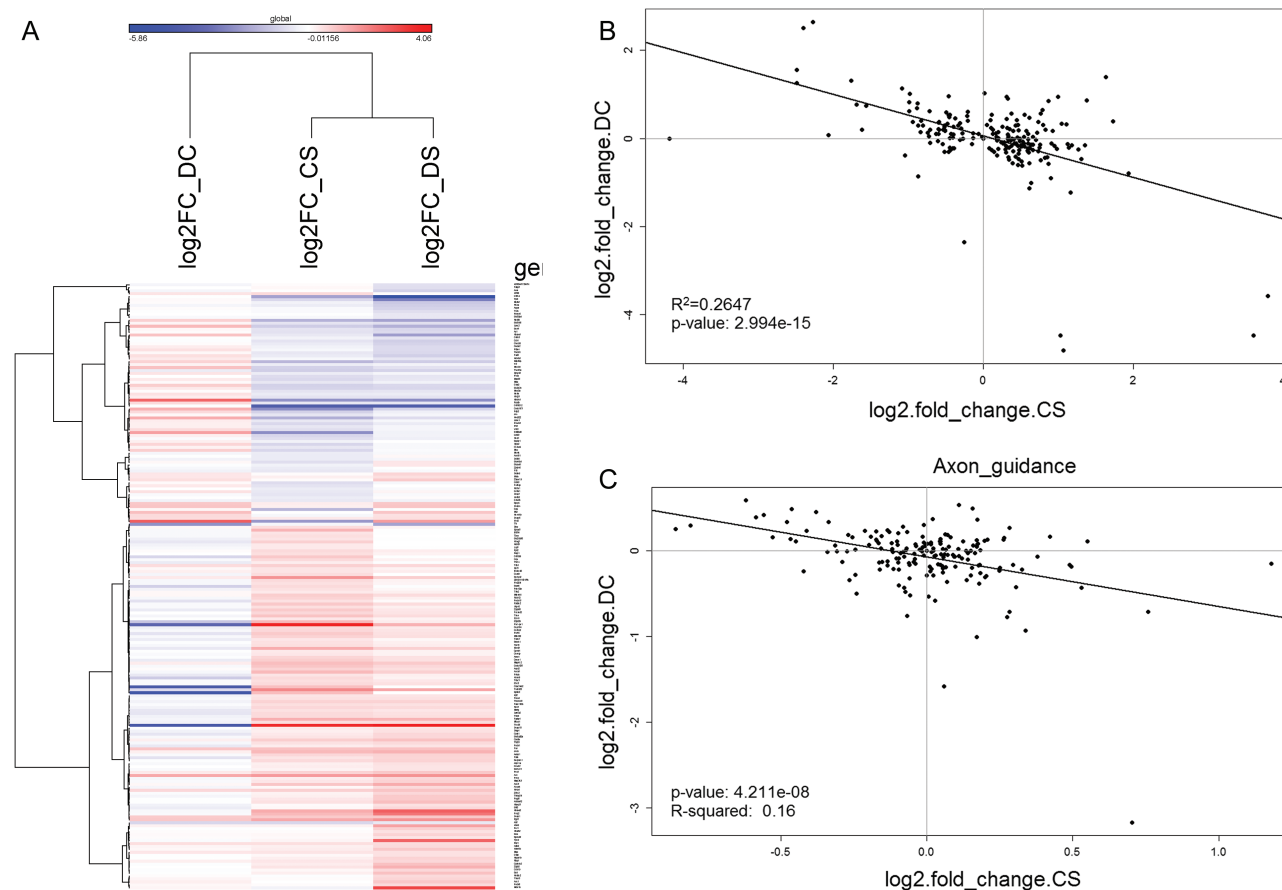


Figure 4. Sodium diethyldithiocarbamate trihydrate (DDTC) reverses genes regulated by cocaine addiction. (A) Heatmap of all significantly differentially expressed genes in each group (saline+cocaine [CS], DDTC+saline [DS], and DDTC+cocaine [DC]). Red, upregulated; blue, downregulated. (B) Scatter plot of all differentially expressed genes of \log_2 fold_change of CS (X axis) and \log_2 fold_change of DC (Y axis). (C) Scatter plot of all genes in axon guidance pathway.

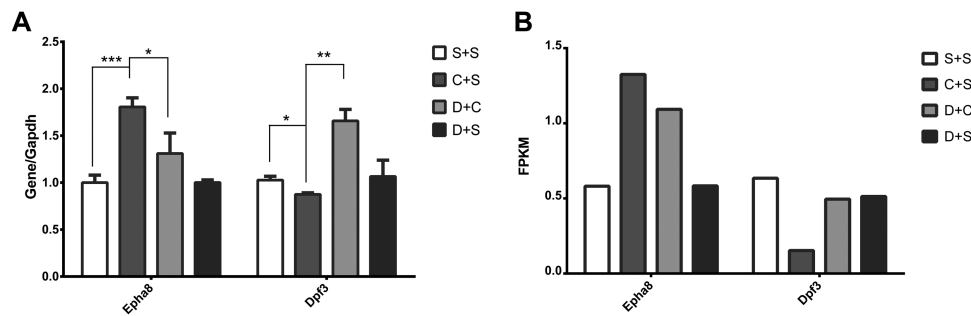


Figure 5. Expression of cocaine and sodium diethyldithiocarbamate trihydrate (DDTC) target genes as determined by qPCR. (A) Normalized mRNA expression quantified by RT-qPCR for genes whose expression is altered by cocaine treatment and reversed by DDTC: EphA8 and Dpf3. Results represent mean \pm SD of 3 determinations in each of the 2 types of experiments; **** $P < .0001$; *** $P < .001$; ** $P < .01$; * $P < .05$. (B) Chart of fragments per kilobase of transcript per million mapped reads (FPKM) of EphA8 and Dpf3 from mRNA-seq.

dendrite growth. An RT-qPCR assay confirmed that expression of these 2 tested genes was significantly altered by cocaine and reversed by DDTC, which coincided with the results obtained from RNA-sequencing (Figure 5).

Discussion

The first major finding of this study was that the NF- κ B inhibitor DDTC, when coadministered with cocaine, is capable of producing statistically significant partial (>50%) rescue of cocaine-induced locomotor stimulation on the first day of treatment, while completely desensitizing this response to cocaine when coadministered over several consecutive days. While virus-mediated gene therapy that results in NF- κ B inhibition has shown success in animal models of cocaine sensitization, it is faced with obvious barriers to clinical implementation. DDTC is a pharmacological inhibitor that has been demonstrated to be safe in clinical trials and is a small lipid-soluble molecule that is capable of crossing the blood-brain barrier (Hersh et al., 1991; Kodama et al., 2005) and as such could be considered for the treatment of cocaine-addicted patients pending more thorough analyses of its effects on the development and persistence of addictive behavior. The major goal of this study, however, was not to evaluate the efficacy of DDTC as a treatment for cocaine addiction but rather to use DDTC to reveal genes and pathways regulated by NF- κ B during repeated cocaine administration.

We found that the MAPK signaling cascade is a key molecular pathway in the PFC that is altered in response to cocaine and that inhibition of NF- κ B by DDTC with or without cocaine treatment also results in gene expression changes of MAPK pathway members. The MAPK signaling cascade involves the activation of extracellular signal-regulated kinases-1 and -2 (ERK1 and ERK2) and is known to play an important role in the control of synaptic plasticity in the adult brain (Thomas and Huganir, 2004). In fact, MAPK/ERK signaling contributes to various behavioral effects of cocaine, such as psychomotor sensitization, conditioned place preference, and reconsolidation of memories for cocaine cues (Lu et al., 2004, 2005, 2006) (Radwanska et al., 2005). Our previous study also demonstrated that cocaine treatment induced long-lasting changes of the ERK signaling pathway in the PFC (Li et al., 2017). Consistent with this (Li et al., 2017) and other studies (Abarca et al., 2002; Lynch et al., 2008; Imbesi et al., 2009), our results also show that the circadian pathway can play a role in cocaine sensitization. However, while both the MAPK and circadian pathways were involved in cocaine response and DDTC also generally affected both pathways, they were not enriched in the list of DEGs reversed by DDTC. On the other hand, DDTC significantly reversed the cocaine-induced changes in the

amphetamine addiction pathway, indicating that this pathway, but not MAPK and circadian pathways, is an important mechanism by which DDTC modifies the effects of cocaine.

Axon guidance is modulated during the development of drug addiction (Bahi and Dreyer, 2005). For example, cocaine exposure alters the expression of axon guidance molecules that regulate the formation of axon-target connections (Bahi et al., 2005), which contribute to the structural adaptations that underlie the long-term effects of prolonged drug exposure (Pasterkamp et al., 2009). We found that DDTC can significantly reverse changes in gene expression of the axon guidance pathway that are induced by repeated cocaine administration, suggesting that it may be able to prevent structural changes associated with chronic cocaine addiction. Though DDTC is known to have other functions, such as zinc chelation and superoxide dismutase inhibition, it is important to note that the NF- κ B homo-dimer motif can be mapped to 86 genes whose expression changes were reversed by DDTC and that these genes are also enriched in axon guidance. Thus, we suggest that NF- κ B responds to cocaine exposure by directly modifying the expression levels of members of the axon guidance pathway, leading to cocaine sensitization. The amphetamine addiction pathway may then be modified indirectly by downstream NF- κ B pathway effectors.

In summary, our results shed light on NF- κ B target genes at a genome-wide level in an effort to understand the mechanisms by which this critical transcription factor complex mediates the functional effects of repeated cocaine administration. Our work provides new insight into the molecular mechanisms underlying the role of NF- κ B in addiction.

Supplementary Material

Supplementary data are available at *International Journal of Neuropsychopharmacology* online.

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Statement of Interest

None.

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