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Gene Profiling the Response to Repeated Cocaine Self-administration in Dorsal Striatum: A Focus on Circadian Genes

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Abstract

Alterations in gene expression in the dorsal striatum caused by chronic cocaine exposure have been implicated in the long-term behavioral changes associated with cocaine addiction. To gain further insight into the molecular alterations that occur as a result of cocaine self-administration, we conducted a microarray analysis of gene expression followed by bioinformatic gene network analysis that allowed us to identify adaptations at the level of gene expression as well as into interconnected networks. Changes in gene expression were examined in the dorsal striatum of rats 1 day after they had self-administered cocaine for 7 days under a 24-hr access, discrete trial paradigm (averaging 98 mg/kg/day). Here we report the regulation of the circadian genes *Clock*, *Bmal1*, *Cryptochrome1*, *Period2*, as well as several genes that are regulated by/associated with the circadian system (i.e., early growth response 1, dynorphin). We also observed regulation of other relevant genes (i.e., *Nur77*, *beta catenin*). These changes were then linked to curated pathways and formulated networks which identified circadian rhythm processes as affected by cocaine self-administration. These data strongly suggest involvement of circadian-associated genes in the brain's response to cocaine and may contribute to an understanding of addictive behavior including disruptions in sleep and circadian rhythmicity.

Classification

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Section

Disease-Related Neuroscience

Keywords

dopamine; glutamate; gene expression; rat; microarray

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Introduction, 2¹

Chronic exposure to drugs of abuse is known to produce neuroadaptations in the dorsal striatum and other mesolimbic brain regions and such changes have been hypothesized to contribute to synaptic plasticity and the behavioral changes that underlie drug addiction (Schwendt et al. 2007; See et al. 2007; Todtenkopf et al. 2004). Cocaine increases extracellular concentrations of dopamine throughout the mesolimbic system leading to stimulation of dopamine receptor signaling and the activation of transcription factors and immediate-early genes (Hyman and Malenka 2001). This sequence of events has been reported to produce a number of changes in gene expression ranging from neurotransmitters to transcription factors to signal transducers (Yuferov et al. 2005a). While this gene set represents a significant step, it is likely that there are other changes that have not yet been identified. Moreover, the molecular adaptations that occur as a consequence of cocaine may result from interactions among genes, proteins, and networks rather than the result of a change in the expression of individual genes, although very little experimental attention has been paid to co-regulation of genes.

Recent work suggests that circadian genes may play a role in modulating cocaine reward. Work with *Drosophila* has identified a family of circadian genes as important in the development of cocaine sensitization including genes such as Period1 (Per1), Period2 (Per2) and Clock (Hirsh 2001). Although many of the genes involved in circadian rhythms are expressed in areas outside the suprachiasmatic nucleus (SCN, the brain's circadian clock), including the dorsal striatum, little is known with regard to their function in these areas. Mice lacking the circadian genes Per1, Per2, and Clock have abnormalities in cocaine-produced locomotor sensitization and conditioned-place preference (Abarca et al. 2002; McClung et al. 2005). Additionally, Egr1 (NGFI-A; Zif268), a circadian associated genes, has been shown to be required for long-lasting association of environmental context with specific behavioral responses after short exposures to cocaine (Valjent et al. 2006). Work with microarray analysis of cocaine-induced changes in the striatum has revealed changes in the circadian gene Per1 following *experimenter* administered cocaine (Yuferov et al. 2003). Few studies have investigated neurochemical adaptations in the striatum following *self-administered* cocaine even though evidence suggests that there are important neurochemical and behavioral differences between contingent- and non-contingent administration of cocaine (Hemby et al. 1997). Thus, the purpose of this study was to obtain further insight into the molecular mechanisms that occur as a result of repeated cocaine self-administration.

A continuous access paradigm was used that allowed rats 24-hr access to cocaine infusions that were available in discrete 10 minute trials (4 trial/hr, 1.5 mg/kg/infusion). We chose this model because access under these conditions results in heavy levels of intake and binge patterns of use--characteristics that are believed to mark the transition to cocaine abuse/dependence in humans (Pottier et al. 1995). Specifically, access to cocaine under the discrete trial procedure has been shown previously to result in high levels of intake (averaging 100 mg/kg/day) with limited signs of toxicity (Roberts et al. 2002). A binge pattern of intake has been described for self-administration under these conditions with rats self-administering cocaine across the light/dark cycle taking every available injection for several hours or even days (Fitch and Roberts 1993; Lynch and Roberts 2004; Roberts et al. 2002). Changes in gene expression were investigated 1 day after the last cocaine self-administration session using a custom microarray chip focused on growth factor signaling molecules, transcription factors and known classes of circadian or timekeeper genes. Each of the cocaine-regulated genes were examined for their involvement in the circadian systems, focusing not only on the primary circadian genes (Arntl/Bmal1, Clock, Per1, Per2, Cry1, Cry2), but also genes that have been shown previously to be

¹List of abbreviations: cryptochrome 1 and 2, Cry1 and 2; N-methyl-D-aspartate, NMDA; Period 1 and 2, Per1 and Per2; suprachiasmatic nucleus, SCN.

regulated by or associated with the circadian system. Changes in gene expression were examined in the dorsal striatum, because like the ventral striatum (nucleus accumbens), which the majority of the studies of cocaine-responsive gene expression have focused on, the dorsal striatum has been implicated in cocaine-taking and -seeking behaviors (See et al. 2007; Ito et al. 2002; Kantak et al. 2002; Vanderschuren et al. 2005; Di Ciano et al. 2007). The role of the dorsal striatum in mediating cocaine-taking and -seeking behavior appears to be enhanced during early abstinence (See et al. 2007) and following chronic cocaine exposure (Porrino et al. 2004; see also Macey et al. 2004). Interestingly, chronic psychostimulant exposure has been reported to produce a disruption in the normal circadian pattern of dopaminergic signaling in the dorsal, but not the ventral striatum (Paulson and Robinson 1996). In order to identify networks that are affected by cocaine self-administration, we used novel functional network mapping software, GeneGo (MetaCore). It was hypothesized that cocaine would induce neuroadaptations in circadian genes as well as genes associated with the circadian system.

Results, 3

Cocaine self-administration

Figure 1 shows the average daily number of infusions obtained across the seven days of access under the discrete trial procedure for the cocaine group and the saline control group. The number of infusions obtained by saline control rats was minimal throughout the 7 day access period. Levels of cocaine intake were maximal during the first 1–2 days followed by a decrease and stabilization of intake at a lower level (days 3–7; significant overall effect of day, $F(6,24) = 5.2$, $p < 0.01$).

The hourly pattern of self-administration is illustrated in Figure 2. Initially, rats took nearly every injection available for a mean of 34.9 hr before taking a one hr break from cocaine (see Figure 2, left panel). After this initial “binge”, responding continued to occur across the light/dark cycle (Figure 2, right panel) with approximately 55% of the total responding occurring in the dark phase.

Cocaine-induced changes in gene expression

The effects of cocaine on striatal gene regulation were examined by comparing cocaine self-administering animals with saline self-administering controls 1 day after the last self-administration session. Five microarrays were completed, representing a gene expression profile from each of the five cocaine self-administering rats and five saline self-administering rats. Table 1A represents the genes identified to be upregulated in the striatum following cocaine self-administration, and down-regulated genes are shown in Table 1B. All genes pass the 0.05 p-value FDR multiple correction. Some of the most common functional groups include: (1) transcription factors (i.e., several zinc finger genes, Fos11, CREB), (2) growth factors/hormones (i.e., Chgb), (3) enzymes/kinases (i.e., Camk1g, Fdft1), (4) receptors/signal transduction proteins (i.e., 5-HT2C; Epac1; NMDA2A), (5) neurotransmitter signaling (i.e., Park2) and (6) other miscellaneous genes (i.e., Csrp2).

Notably, we observed upregulation of 27 genes that are known to have circadian function or to be associated with/regulated by the circadian system (see Table 2A) and downregulation of 2 genes (see Table 2B). Again, the genes identified were from multiple functional classes, including (1) transcription factors (i.e., Arntl/Bmal1, Egr1/NGFI-A/Zif268, Cry1), (2) growth factors/hormones (i.e., Npy, Sst), (3) receptors/signal transduction proteins (i.e., Numbl, Nur77), (4) neurotransmitter signaling (i.e., S100a12/a4, Dyn, Gad2/65), and (6) other miscellaneous genes (i.e., Slc1a1, Eaac1, vGlt1). Based on previous research showing regulation by cocaine (e.g., Freeman et al. 2007; Yuferov et al. 2003), we examined gene expression of three of these circadian associated/regulated genes, Egr1/NGFI-A/Zif268, Arntl/

Bmal1, and Dyn, using quantitative real-time PCR and confirmed that each gene was up-regulated in the cocaine group compared to the saline group (Figure 3). Because we observed so many changes in circadian genes and circadian associated/regulated genes, we further explored known circadian genes that were not represented on the gene array using real-time PCR validation which included Clock, Cry1 and 2 and Per1 and 2. These analyses revealed regulation of Clock, Per2, and Cry1 (Figure 3), but not Per1 or Cry2 (data not shown).

Visualization of Microarray Data on Gene Networks in GeneGo

The same microarray datasets uploaded into Genespring, previously, were evaluated in MetaCore (GeneGo) with the analyze network algorithm. The most statistically significant networks based on the *p* value were generated in all cases. For cocaine self-administration, 139 regulated genes were uploaded, and this gene list was analyzed by the map editor yielding 30 networks of significance. Cry1, BMAL1, CCL2, Bax and BMAL2 were mapped for the following processes (by most statistical significance): circadian rhythm (5.650e-11), rhythmic processes (1.470e-09), response to external stimulus (1.573e-07), inflammatory response to antigenic stimulus (4.582e-07) and response to wounding (3.431e-06). A network was generated from this list (Figure 4).

Discussion, 4

Through the use of a focused cDNA microarray a total of 139 genes were identified as regulated by cocaine and greater than 20% (29 genes total) were subsequently identified as genes associated with/regulated by the circadian system. The use of Metacore allowed for the generation of functional gene networks to identify co-regulation of certain mRNAs, which included cocaine-induced changes in circadian rhythmicity. The combination of gene expression with gene network analysis represents a novel and powerful approach to understand the molecular alterations that occur as a result of repeated cocaine self-administration. A comparison of these findings with previous findings is useful in understanding the potential mechanisms that may be activated following repeated cocaine self-administration.

Circadian genes and genes associated with/regulated by the circadian system

By examining each of the genes that were regulated by cocaine, we identified 29 genes that have previously been reported as circadian genes or genes associated with/regulated by the circadian system. Through the use of RT-PCR, we identified an additional 3 genes as being regulated by cocaine and confirmed the involvement of several other circadian genes (see Figure 3). Regulation of some of these genes, namely Per2 (Yuferov et al. 2003), Egr1/NGFI-A/Zif268 (Yuferov et al. 2003), Npy (Westwood and Hanson 1999), Sst (Aguila-Mansilla et al. 1997), NGFI-B/Nur77 (Yuferov et al. 2003;Freeman et al. 2002a;b;Werme et al. 2000), Dyn (Schlussman et al. 2003;Yuferov et al. 2003), Pomc (Zhou et al. 2004), Arntl/Bmal1 (Uz et al. 2005), Cry1 (Uz et al. 2005), have been reported previously within brain regions associated with cocaine reward (i.e., dorsal striatum, prefrontal cortex, nucleus accumbens) in various studies following both repeated experimenter administered and self-administered cocaine using in situ hybridization, Northern blots, differential display, and microarray technology suggesting that these genes may be non-specifically activated by cocaine. Here we show for the first time the regulation of Slc1a1/Eaac1, Otx2, Pcaf, Pax4 and 2, Fgf7, Epo, Numbl, Kiss1r, S100a12 and 14, Grb14, Lrrn3, Cop1, Itgav, JunD1, and Nos3 in the dorsal striatum following repeated cocaine self-administration. These novel genes may represent specific adaptations that occur in the dorsal striatum as a consequence of self-administered cocaine. Of these findings, MetaCore was able to identify a network of circadian rhythm processing genes as affected by cocaine self-administration including Cry1, BMAL1 and BMAL2 which are key players in maintaining circadian rhythm and Per2 and Cry1 which block

the action of the two positive clock components. Taken together, these findings indicate profound effects of cocaine on circadian system genes in the dorsal striatum.

A possible line of convergence between cocaine-activated systems and the circadian system is the dopamine D1-cAMP signaling pathway (McClung et al. 2005). Repeated cocaine exposure is known to induce the cAMP-dependent pathway and our current findings of upregulation of CREB, Epac1, and Nos3 support this idea. Additionally, research has revealed that the clock genes *Clock* and *Arntl/Bmal1*, also shown to be regulated by cocaine in the current study, have CRE binding sites in their promoters (Manev and Uz, 2006). Notably, previous work has shown that methamphetamine-induced changes in the expression of *Per1* are blocked by dopamine D1 receptor antagonism, but not by D2 receptor antagonism (Nikaido et al. 2001). Results from this same study also showed that treatment with an N-methyl-D-aspartate (NMDA) glutamate receptor antagonist blocked changes in *Per1* gene expression suggesting that the glutamatergic signaling pathway may also mediate adaptations in clock gene expression in the striatum. Here we found not only upregulation of the NMDA2A glutamate receptor gene, but also the glutamate transporter gene, *Slc1a1/Eaac1*, which was identified as interacting with the circadian system (see Table 2).

There is also evidence to suggest that cocaine-induced changes in the ERK/MAPK signaling pathway may have mediated adaptations of clock gene expression (i.e., Zhang et al. 2004). Certainly there are interactions of this signaling pathway and cocaine reward and recent work has implicated markers within this pathway as underlying long-term changes that are associated with the development of cocaine addiction (i.e., *Egr1/NGFI-A/Zif268*; MAPK phosphatases; for review see Lu et al. 2006). Evidence of interaction of the ERK/MAPK signaling pathway with the circadian system also exists (i.e., Hainich et al. 2006), and in fact, our present findings have revealed a number of changes in circadian associated genes within this signaling pathway (i.e., MAPK14/p38, *Egr1/NGFI-A/Zif268*, *NGFI-B/Nur77*, *Lrrn3*, *JunD1*, *Fgf7*, *Kiss1r*), as well as several other genes that do not have documented circadian interaction (i.e., *Pdgfra*, *Znf322*).

It has been speculated that in areas outside the SCN, such as the striatum, clock genes may have a “non-clock” function with evidence showing that expression depends on external inputs to neurons expressing these genes (i.e., schedule of stimulant administration or feeding; Manev and Uz, 2006). It has been hypothesized that it is through these “non-clock” functions that these circadian genes interact with systems involved in the development of cocaine addiction (Manev and Uz 2006; Uz et al. 2003; Yuferov et al. 2005b). For example, it has been reported that stimulant drug treatment produces gene expression changes in *Per* gene expression in the striatum without affecting the rhythmic expression in SCN (Manev and Uz 2006). Here we showed that the *Per2* gene was regulated following repeated cocaine self-administration which is consistent with previous work using a non-contingent repeated dosing protocol (Yuferov et al. 2005a,b). These results are interesting in light of previous work showing that *Per2* knockout mice display an enhanced cocaine-induced place preference (McClung et al. 2005). While these data implicate clock genes in common mechanisms of cocaine addiction-related behaviors, it is also possible that cocaine may be exerting effects in the striatum that are not related to its reinforcing effects. We do know that under these discrete trial cocaine self-administration conditions, like humans, rats self-administer the drug throughout the light/dark cycle with marked disruptions in their diurnal patterns of feeding and drug-taking behavior (Figure 2; Lynch and Roberts 2002; Roberts et al. 2004). Although cocaine was not “on board” at the time the tissue was obtained, the diurnal dysregulation may have persisted at 1 day withdrawal, when the tissue was obtained. Additionally, given that cocaine infusions were paired with a light stimulus which in itself may disrupt the circadian system, we cannot rule out the possibility that regulation of some of these circadian genes were the result of the experimental conditions rather than cocaine reinforcement. As mentioned above, however, human cocaine abusers also

use cocaine in extended binge patterns (Pottierer et al. 1995) that occur throughout the day/night cycle. Together, these findings support the hypothesis that circadian genes play an important role in modulating the response to cocaine, although the mechanism for their involvement is not yet known.

Cocaine-responsive gene expression of non-circadian genes

The results of our study further support previous findings and include several new genes not previously identified as being regulated by cocaine, extending the list of cocaine regulated genes. For example, numerous studies have revealed regulation of Fos11/Fra1 in different brain regions and following both experimenter-administered and self-administered cocaine (i.e., Nye and Nestler 1996). Numerous studies have also revealed adaptations in the NMDA2A receptor following repeated cocaine exposure (i.e., Hemby et al. 2005a,b; Zhang et al. 2007) and recent work suggests that this gene may play an important role in integrating dopamine-glutamate signaling which has been speculated to promote the long-lasting behavioral effects associated with chronic cocaine exposure (Liu et al. 2006). Indeed, pharmacological manipulation of the NMDA receptor modulates cocaine-seeking behavior (Backstrom and Hyttia 2007) and escalation of cocaine consumption (Allen et al., 2007). Notably, one of the novel non-circadian related genes identified here is Park2 which has been shown previously to inhibit amphetamine-induced dopamine release and glutamate neurotransmission (Benavides et al. 2003) suggesting that it may also be a candidate for integrating dopaminergic and glutamatergic signaling.

The current finding of upregulation of the 5-HT2C gene is consistent with results from many previous studies showing that this particular receptor is involved in mediating the behavioral (i.e., Fletcher et al. 2006) and neurochemical (i.e., Broderick et al. 2004) response to cocaine. Beta catenin is another gene that has been routinely reported in microarray studies as regulated by cocaine (i.e., Freeman et al. 2001a,b; Zhang et al. 2002; Novikova et al. 2005) and recent work suggests that the D1 receptor is a critical mediator for cocaine-induced expression of this gene (Zhang et al. 2002). Other genes identified here that have been previously shown to be involved in cocaine reward/regulation by cocaine include, Hive2 (Reynolds et al. 2006), Rxrb (Krezel et al. 1998), and Chgb (Che et al. 2006). Interestingly, the findings of regulation of Fdft1 and Camk1g are consistent with recent work in human cocaine, cannabis, and PCP abusers (Lehrmann et al. 2006) suggesting that these genes may represent common molecular features of drug addiction.

Conclusions

Taken together, these results demonstrated regulation of a number of clock genes and a network of genes interacting with circadian processes as well as a number of other genes in rat dorsal striatum following chronically self-administered cocaine. The circadian rhythm network of genes identified in the present study provides a number of excellent candidates for future hypothesis-driven studies that can be characterized for their role within a single pathway or within a global construct/network. Research focused on manipulating these signaling pathways at different levels using specific inhibitors/activators and assessing the behavioral outcomes would be useful in elucidating the contributions of individual regulated molecules and may provide novel therapeutic avenues for the treatment of cocaine addiction.

Experimental Procedure, 5

Subjects and surgery

Ten male Sprague-Dawley rats (380–410 g; Charles River Laboratories, Portage, ME, USA) were housed in pairs upon arrival at the facility and maintained on a 12-hr light/dark cycle (lights on at 7-am) with free access to food and water. After a 5-day acclimation period, rats were anesthetized with equithesin (4.32 mg/kg) and implanted with a chronic indwelling

cannula into the right jugular vein. After cannulation, rats were housed individually in operant conditioning chambers (ENV-018M; Med Associates, St. Albans, VT), and once grooming and eating behaviors resumed, behavioral testing began (typically after 24-hrs of recovery). Rats were weighed a minimum of once per week. Cannula patency was assessed approximately every 7 days, and if a cannula was not patent a new one was implanted into the left jugular vein and testing resumed a minimum of 24-hrs later. In order to minimize stress during periods of heavy cocaine intake, rats were not handled during the 7-day period in which cocaine was available under the 24-hr/day discrete trial procedure. The health of the animals was monitored daily. The experimental protocol was approved by the Animal Care and Use Committee of Yale University and was conducted in accordance with guidelines set by the National Institutes of Health.

Microarray chip

The focused array employed in this study is an upgraded version of the one previously described (Newton et al. 2003; Hunsberger et al. 2005; see also Supplementary Materials). Briefly, the array contained 1561 genes which included primarily growth factor signaling genes (approximately 500 genes), transcription factors (approximately 500 genes), relevant receptors (approximately 200 genes), and other neurobiologically significant genes. Spotted targets were approximately 300 bp PCR products representing only the coding sequence of arrayed genes, effectively excluding polynucleotide tails and providing consistent length. The 300 bp targets were designed from sequence input of approximately 1000 bp from the 3' end. The melt T_m of the primers was uniformly set to between 63–65°C. Products were printed under denaturing conditions to facilitate optimal hybridization.

Cocaine self-administration

Five rats were trained to self-administer cocaine infusions (1.5 mg/kg) during daily sessions beginning at 12.00 pm. Session onset was signaled by the extension of the left lever into the operant chamber. Each response on this lever produced an infusion of cocaine (i.e., 1 sec/kg body weight delivery in a 1.5 mg/ml solution). The stimulus light above the lever was illuminated for the duration of the infusion. Each rat received one 'priming' infusion of cocaine at the beginning of each training session. Sessions were terminated and the levers retracted once rats obtained all 20 infusions that were available. The right lever (activity lever) was extended into the chamber for the duration of the experiment, and responses on it were recorded but produced no consequence.

Once rats acquired cocaine self-administration under an FR schedule (defined as five consecutive sessions in which rats earned all 20 infusions that were available), cocaine infusions (1.5 mg/kg) were available in discrete 10-min trials 24-hr per day (4 trials/hr). This discrete trial cocaine self-administration procedure has been shown previously to result in high levels of intake (averaging 100 mg/kg/day) with limited signs of toxicity (Freeman et al., 2002b).

Each discrete trial was initiated by the extension of the left lever into the chamber. Each response on this lever during a 10-minute discrete trial produced an infusion of cocaine (1.5 mg/kg/infusion), and the stimulus light above the lever was illuminated for the duration of the infusion (approximately 5 seconds). There were 4 trials/hr with trials initiating every 15-min for a total of 7 days. A discrete trial was terminated and the lever was retracted following a response under a fixed-ratio 1 schedule or after 10-min had elapsed.

Another group of 5 rats were given access to saline infusions using the same training and discrete trial testing conditions that were used for cocaine self-administration. These saline control rats were trained at the same time as the rats given access to cocaine and they were

matched with rats in the cocaine group for the total number of self-administration training sessions prior to testing under the discrete trial procedure. This group was never exposed to cocaine.

Tissue preparation and microarray analysis of gene expression

Twenty-four hrs after the last cocaine or saline self-administration session, animals were sacrificed by rapid decapitation and their brains hemisected. The dorsal striatum was manually dissected from 1-mm-thick coronal brain slices from the bregma 1.70 to 0.20 mm according to Rat Brain in Stereotaxic Coordinates (Paxinos and Watson 1998; See Figure 5). Brain tissue was rapidly frozen on dry ice and stored at -80°C until further processing. One microarray per cocaine/saline pair was completed for a total of five microarrays. Microarray analysis of striatal gene expression was completed essentially as previously described (Newton et al. 2003). Briefly, 5 micrograms of total RNA from each sample was reverse-transcribed into cDNA using oligo dT-capture sequence primers. A two-step hybridization and labeling procedure was used wherein cDNA was hybridized overnight at 46° in formamide buffer, stringently washed, and then post-stained with fluorescent Cy5 and Cy3 dendrimers (Genisphere). Following posthybridization washes, slides were scanned using a GenePix Scanner (Molecular Devices, Union City, CA, USA). Image analysis was performed using GenePix Pro 4.0 software. Resulting files from GenePix 4.0 (Axon Instruments) analysis were imported into Genespring 7.0 (Agilent Technologies, Santa Clara, CA, USA) for data analysis. A gene was considered to be expressed if its signal intensity was a minimum of twice the background in at least four of the five replicates. Per-chip normalization was performed by dividing the expressed genes by the median of the housekeeping control genes (i.e., cyclophilin and beta tubulin) that were not regulated. Upregulated genes were defined as having an average expression ratio of >1.4 , and the downregulated genes were defined as having an average expression ratio of <0.7 . A gene was considered to be significantly regulated if the t-test p-value was <0.05 with the false discovery rate (FDR) as the multiple testing correction. This strategy of using a combination of higher fold regulation plus a nonstringent P cutoff was selected based on recent findings from the Microarray Quality Control project (MAQC Consortium 2006) which indicated that this strategy produces the most interesting biological profile from array data with highly reproducible results (see also Ploski et al. 2006). Each of the genes that met these criteria for regulation by cocaine were screened for circadian activity or an association with the circadian system by performing searches through PubMed, Gene, iHop, and Google with the gene name (using all possible variations) and circadian as the keywords. A gene was designated as associated with/regulated by the circadian system if data from at least one study in mammals showed that the gene regulated a clock gene, formed an association with a clock gene, displayed a circadian rhythm, had a timing function, played a role in entrainment to circadian clocks, or was involved in phase advances in circadian clock.

Quantitative real-time PCR

RNA from each animal (1 μg) was reverse-transcribed into cDNA (Superscript II, Invitrogen), precipitated, and re-suspended in 50 μl nuclease-free water. Real time PCR (Smartcycler, Cepheid, Sunnyvale, CA, USA) analysis was performed using the SYBR Green master mix (Quantitect, Qiagen, Valencia, CA, USA), high melt temperature primers, and 1 μl of input cDNA for each reaction. Cyclophilin, a house keeping gene, was amplified in parallel for each control and experimental sample to facilitate normalization. Melt peak analysis was implemented to confirm specificity of the amplified targets. We focused independent secondary confirmation mostly towards circadian genes in order to further strengthen the microarray results.

Cyclophilin, TGTGCCAGGGTGGTGACTTC and

TGCCATCCAGCCACTCAGTC
Per2, CAGCTACCCGTTTCCACCAG and
 CTGAGCATCGAGGTCCGACT
Cry1, TGCAGTTGCCTGTTTCCTGA and
 CAGCATTGATGCTCCAGTCG
Arntl (Bmal1), AACCCGTGGACCAAGGAAGT
 GTGAGCTGTGGAAGGTTGG
Dyn, CAGGTTTGGCAACGGAAGAG
 CTGTGCGGCTTCATCATTCA
Egr1 (NGFI-A, Zif268), ACCTACCCGTCTCCTGCACA
 CCTGCAGACTGGAAGGTGCT
Clock, CCGCAGAATAGCACCCAGAG
 TGGTACTTGGCACCATGACG

Network Visualizations and Analysis

Visual analysis of cocaine-induced gene networks were performed using MetaCore analytical suite version 2.0 (GeneGo). MetaCore is a web-based computational platform designed for the analysis of high-throughput experimental data in the context of regulatory networks and pathways. It includes a database of protein activation, interactions and metabolism, curated and updated using the most recent literature. The map represents a network display and interactions compiled from a list of cocaine regulated genes. MetaCore also calculated statistical significance of the network based on the P-values of the regulated genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements, 6

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Figure 1. Mean ($\pm$ SE) number of infusions obtained for the cocaine group (n=5) and the saline control group (n=5) for each of the 7 days of access under the discrete trial self-administration procedure.

Figure 2.

Patterns of cocaine self-administration. (Left panel) Representative event record from an animal self-administering cocaine under the discrete trial procedure (4 trials/hr). Each horizontal line represents a 24 hr period. Each symbol represents a trial during which a cocaine infusion was obtained. (Right panel) Mean ($\pm$ SE) hourly cocaine intake across the light/dark cycle averaged across days 3–7 (after the initial 1–2 day 'binge') under the discrete trial procedure.

Figure 3.

Real-time PCR quantification of mRNA levels where each value is expressed as fold regulation determined by cycle number difference. We were able to confirm regulation of Egr1 (NGF-IA, Zif268), Arntl (Bmal1), and Dyn. Clock, Per2, and Cry1 were also shown to be regulated by cocaine (relative to saline). * $p < 0.05$

Figure 4.

Visualization of gene networks obtained from cocaine self administration microarray data. The genes were incorporated into a direct interactions network associated with circadian rhythm processes. Colored symbols (nodes) indicate genes. The pink solid circles correspond to significantly up-regulated cocaine induced genes. The purple stars indicate genes significantly up-regulated and confirmed by qRT-PCR. Highlighted node represents genes associated directly with the circadian rhythm processes. Red interaction lines indicate an inhibitory effect and green interaction lines indicate an activation effect. Interactions are also characterized by their specific biological activity: bonding (B), transcriptional regulation (TR) or covalent modification (CM).

Figure 5. Coronal section (adapted from Paxinos and Watson 1998) of the rat brain at Bregma 1.6 mm illustrating the dorsal region of the striatum that was dissected (shaded region).

Table 1

Genes that were identified as being differentially regulated in the rat striatum following cocaine self-administration. **(A)** Genes identified as upregulated in the striatum following cocaine self-administration using microarray. **(B)** Genes identified as downregulated in the striatum following cocaine self-administration using microarray.

(A) Upregulated Genes		
Gene (abbreviations)	FDR P-Value	Fold Change
<i>Transcription factors</i>		
zinc finger protein 285A (Znf285a)	<0.001	2.6
zinc finger protein 347 (Znf347)	0.0128	2.4
achaete-scute complex homolog-like 1 (drosophila) (Ascl1, Mash1)	0.0155	2.2
fos-like antigen 1 (Fosl1, Fra1)	0.0112	2.1
AT rich interactive domain 3A (BRIGHT-like) (Arid3a, Dril1)	0.0363	1.9
neurogenic differentiation 1 (Neurod1)	0.0116	1.9
Kruppel-like factor 5 (Klf5)	0.0487	1.8
distal-less homeobox 5 (Dlx5)	0.0252	1.9
SIN3 homolog A, transcription regulator (yeast) (Sin3a)	0.0080	1.8
ZNF322A zinc finger protein 322 (Znf322)	0.0194	1.8
RNA and export factor binding protein 1 (Rexbp1)	0.0027	1.8
ets variant gene 4 (E1A enhancer binding protein) (E1af, Etv4)	0.0018	1.8
forkhead box M1 (FoxM1)	<0.001	1.7
achaete-scute complex homolog-like 1 (Drosophila) (Ascl1)	<0.001	1.7
MYC associated factor X (MAX)	0.0161	1.7
oligodendrocyte transcription factor 2 (Olig2)	0.0093	1.7
PHD finger protein 3 (Phf3)	0.0070	1.7
zinc finger protein 267 (Znf267)	0.0309	1.7
fourteen-three-three beta interactant 1 (Fbi1)	0.0120	1.7
Scm-like with four mbt domains 1 (Sfmbt1)	0.0231	1.7
chromosome 20 open reading frame 157 (C20orf157)	0.0023	1.6
nuclear factor, erythroid derived 2 (Nfe2)	<0.001	1.6
tumor protein p53 (Tp53)	0.0140	1.6
orthopedia homolog (Drosophila) (Otp)	0.0318	1.6
transcription factor CP2 (Tfcp2)	0.0299	1.6
zinc finger protein 521 (Znf521, Ehzf)	0.0150	1.6
cAMP responsive element binding protein 3-like 3 (Creb-H)	0.0186	1.6
zinc finger and BTB domain containing 47 (Zbtb47)	0.0016	1.6
Max interacting protein 1 (Mxi1)	<0.001	1.6
msh homeobox 2 (Msx2)	0.0012	1.5
zinc finger protein 14 (Znf14, Hit40, Kox6)	0.0037	1.5
distal-less homeobox 4 (Dlx4)	0.0015	1.5
human immunodeficiency virus type 1 enhancer binding protein 2 (Hivep2)	0.0352	1.5
homeodomain leucine zipper-encoding gene (Homez)	<0.001	1.5
vacuolar protein sorting 72 (yeast) (Vps72, Tcfl1)	0.0055	1.5
caudal type homeobox transcription factor 1 (Cdx1)	<0.001	1.5
single-minded 2 (Sim2)	0.0283	1.5
OG9 homeobox gene (Og9x)	0.0113	1.5
pre-B-cell leukemia homeobox 1 (Pbx1)	0.0187	1.5
pre-B-cell leukemia homeobox 3 (Pbx3)	0.0289	1.5
pre-B-cell leukemia homeobox 4 (Pbx4)	0.0375	1.5
CDC5 cell division cycle 5-like (Cdc5l)	0.0227	1.5
Kruppel-like factor 2 (lung) (Klf2)	0.0253	1.5
zinc finger protein 350 (Znf350)	0.0211	1.5
Kruppel-like factor 9 (Klf9)	<0.001	1.5
<i>Growth factors/hormones</i>		
neurotrophin 5 (Nt5)	0.0103	1.9
chromogranin B (Chgb)	0.0388	1.8
neurotrophin 6, alpha pseudogene (Ntf6a)	0.0403	1.7
<i>Enzymes/Kinases</i>		
Pyruvate carboxylase (Pyc)	0.0487	2.1
dihydropyrimidinase-like 3 (Dpysl3, Tuc-4B)	0.0147	1.8
dihydropyrimidinase-like 2 (Dpysl2, Toad-64)	<0.001	1.8
polymerase (DNA-directed), epsilon 4 (p12 subunit) (Pole4)	0.0066	1.6
farnesyl diphosphate farnesyl transferase 1 (Fdft1)	0.0066	1.6
calcium/calmodulin-dependent protein kinase I gamma (Camk1g)	0.0102	1.5
adrenergic receptor kinase, beta 2 (Adrbk2, Grk3)	0.0236	1.5
<i>Receptors/signal transduction proteins</i>		
Sensory neuron-specific G-protein coupled receptor 2 (Snsr2)	0.0026	3.2
olfactory receptor, family 51, subfamily B, member 2 (Or51b2)	<0.001	2.6
heparin-binding EGF-like growth factor (Hbegf)	0.0145	2.5
olfactory receptor, family 1, subfamily E, member 1 (Or1e1)	0.0307	2.2
olfactory receptor, family 1, subfamily K, member 1 (Or1k1)	<0.001	2.0
G protein-coupled receptor 21 (GPR21)	<0.001	2.0
latrophilin 1 (Lphn1, Lec2)	0.0303	1.9
neuropeptide FF receptor 1 (Npffr1)	0.0172	1.9

(A) Upregulated Genes

Gene (abbreviations)	FDR P-Value	Fold Change
KRIT1, ankyrin repeat containing (Krit1, Ccm1)	<0.001	1.9
fibroblast growth factor binding protein 1 (Fgfbp1)	0.0092	1.8
olfactory receptor, family 51, subfamily E, member 2 (Or51e2)	0.0022	1.8
olfactory receptor, family 1, subfamily D, member 4 (Or1d4)	0.0129	1.8
plexin A2 (Plexin-A2)	0.0163	1.7
protein-coupled receptor 77 (GPR77)	0.0202	1.7
G protein-coupled receptor 62 (GPR62)	0.0044	1.7
leucine-rich repeat-containing G protein-coupled receptor 8 (LGR8)	0.0268	1.7
estrogen related receptor, alpha (Esrra)	0.0097	1.7
formyl peptide receptor-like 1 (Fprl1m)	0.0015	1.7
MAS-related GPR, member X2 (Mrgx2)	0.0105	1.7
syndecan 4 (Sdc4)	0.0217	1.7
olfactory receptor, family 12, subfamily D, member 2 (Or12d2)	<0.001	1.7
dishevelled, dsh homolog 1 (Drosophila) (Dvl1)	0.0063	1.7
receptor (calcitonin) activity modifying protein 3 (Ramp3)	0.0131	1.6
G protein-coupled receptor 20 (GPR20)	0.0096	1.6
Rap1 guanine-nucleotide-exchange factor directly activated by cAMP (Epac1)	0.0423	1.6
glutamate receptor, ionotropic, N-methyl D-aspartate 2A (NMDA2A)	0.0148	1.5
chemokine (C-C motif) receptor 9 (Ccr9)	0.0048	1.5
5-hydroxytryptamine (serotonin) receptor 2C (5-HT2C)	0.0217	1.5
Sensory neuron-specific G-protein coupled receptor 5 (Snsr5)	0.0361	1.5
olfactory receptor, family 1, subfamily Q, member 1 (Or1q1)	0.0425	1.5
G protein-coupled receptor 85 (GPR85)	0.0493	1.5
G protein-coupled receptor 56 (GPR56)	0.0139	1.5
<i>Neurotransmitter signaling</i>		
Angiotensin (Amot)	0.0132	2.1
ankyrin repeat and SOCS box-containing 13 (Asb13)	<0.001	1.9
allograft inflammatory factor 1 (Aif1, Mrf-1)	0.0055	1.7
RAT brain class A (Rba)	0.0021	1.6
parkin (Park2)	0.0059	1.5
<i>Miscellaneous</i>		
procollagen, type IV, alpha 3 (Col4a3)	<0.001	1.9
serine (or cysteine) peptidase inhibitor, clade I, member 1 (Serpini1, Neuroserpin)	0.0116	1.9
thymosin, beta 4 (Tmsb4x)	0.0061	1.9
TBP-like 1 (Tbpl1)	0.0051	1.8
BMP/retinoic acid-inducible neural-specific protein 2 (Brinp2)	<0.001	1.8
suppression of tumorigenicity 13 (St13, Hip)	0.0423	1.6
cysteine and glycine-rich protein 2 (Csrp2)	<0.001	1.6
matrix metalloproteinase 11 (Mmp11)	<0.001	1.6
kelch-like 10 (Drosophila) (Klhl10)	0.0054	1.5
EGF-like repeats and discoidin I-like domains 3 (Edil3)	<0.001	1.5
hairy and enhancer of split 5 (Drosophila) (Hes5)	0.0109	1.5
angiopoietin-like 2 (Angptl2)	<0.001	1.5
WD repeat domain 12 (Wdr12)	<0.001	1.5
lethal giant larvae homolog 1 (Drosophila) (Llglh)	0.0033	1.5
angio-associated, migratory cell protein (Aamp)	0.0067	1.5

(B) Downregulated Genes

<i>Transcription factors</i>		
Beta catenin (Ctnnb1)	0.0061	0.6
<i>Receptor</i>		
platelet derived growth factor receptor, alpha polypeptide (Pdgfra)	0.0051	0.5
<i>Miscellaneous</i>		
Reduced expression protein 3 (Rex3)	0.0231	0.6

Table 2

Circadian genes and genes regulated by/associated with the circadian system that were identified as being differentially regulated in the rat striatum following cocaine self-administration. **(A)** Genes identified as upregulated in the striatum following cocaine self-administration using microarray. **(B)** Genes identified as downregulated in the striatum following cocaine self-administration using microarray.

(A) Upregulated Genes			
Gene (abbreviations)	Evidence for circadian involvement	FDR P-Value	Fold Change
<i>Transcription factors</i>			
orthodenticle homolog 2 (Drosophila) (Otx2)	A positive regulatory loop exists between Clock and Otx2 (Morgan 2002)	0.0243	1.9
aryl hydrocarbon receptor nuclear translocator-like (Bmal1, Arntl)	Forms a heterodimer with Clock (Reppert and Weaver 2001)	0.0011	1.8
cryptochrome 1 (photolyase-like) (CRY1)	Post-translationally regulates the Clock/Bmal1 complex (Reppert and Weaver 2001)	<0.001	1.8
early growth response 1 (Egr1, NGFI-A, Zif268)	Phase advanced expression following diurnal light pulse (Abizaid et al. 2004)	0.0014	1.8
p300/CBP-associated factor (Pcaf)	Associates with Clock and Npas2 to regulate clock gene expression (Curtis et al. 2004)	0.0095	1.6
paired box gene 4 (Pax4)	Diurnally regulated in rat (Martin et al. 1992)	0.0016	1.6
paired box gene 2 (Pax2)	Has a unique timing function (Silverstein et al. 2006)	0.0077	1.5
<i>Growth factors/hormones</i>			
fibroblast growth factor 7 (Fgf7)	Distinct circadian rhythm (Haus et al. 2001)	0.0034	2.4
neuropeptide Y (Npy)	Reduces Per1 mRNA levels induced by the light pulse (Gamble et al. 2006)	<0.001	1.5
somatostatin (Sst)	Transmitter for mediating information of entrainment to circadian clocks (Hamada et al. 1993)	<0.001	1.5
<i>Enzymes/kinases</i>			
ornithine decarboxylase (ODC)	Distinct circadian rhythm (Waldrop et al. 1989)	0.0081	2.6
nitric oxide synthase 3, endothelial cell (Nos3)	Involved in light-induced phase advances of the circadian clock: Glu-Ca2+CaMKII-nNOS-GC-cGMP-cGK (Golombek et al. 2004)	0.0121	1.6
mitogen activated protein kinase 14 (MAPK14, p38)	Regulates oscillation of circadian clock (Hayashi et al. 2003)	0.0048	1.5
<i>Receptors/signal transduction proteins</i>			
erythropoietin (Epo)	Shows a circadian rhythm (i.e., Wide et al. 1989)	0.0034	2.4
numb-like (Numbl)	Rhythmically expressed gene (in Drosophila; Stempfl et al. 2002)	0.0068	2.2
nerve growth factor induced protein I-B (NGFI-B, Nur77)	Expressed on circadian basis (Humphries et al. 2004)	0.0041	1.7
KISS1 receptor (Kiss1r)	Involved in GnRH/LH surge that is activated by a circadian signal from the SCN (Smith et al. 2006)	0.0437	1.7
mineralocorticoid receptor (MCR, Nr3c2)	Maintenance of the circadian ACTH and cortisol rhythm through the modulation of CRH and AVP release (Giordano et al. 2006)	0.0334	1.6
<i>Neurotransmitter signaling</i>			
S100 calcium binding protein A12 (S100a12)	Rhythmically expressed gene (Trasforini et al. 1991)	<0.001	2.1
S100 calcium-binding protein A4 (S100a4)	Rhythmically expressed gene (Trasforini et al. 1991)	0.0126	1.7
prodynorphin (Dyn)	Involved in maintaining day-night patterns of eating and drinking (Reid et al. 1982)	<0.001	1.6
<i>Miscellaneous</i>			
growth factor receptor bound protein 14 (Grb14)	Regulated by circadian clock genes (Menger et al. 2005)	0.0017	2.4
neuronal/epithelial high affinity glutamate transporter (Slc1a1, Eaac1)	Circadian control of the Eaac1 mRNA by the clock intrinsic to the SCN (Cagampang et al. 1996)	0.0042	1.7
leucine rich repeat protein 3, neuronal (Lrrn3)	SCN circadian oscillatory protein is a member of the leucine-rich repeat (LRR)-containing protein family (Shimizu et al. 2003)	0.0055	1.7
similar to constitutive photomorphogenic protein 1 (Cop1)	Represses photomorphogenesis in Arabidopsis (Kim and von Arnim 2006)	0.0093	1.7
BCL2-associated X protein (Bax)	Circadian regulated gene (Granda et al. 2005)	<0.001	1.5
integrin, alpha V (Itgav, Cd51)	Exhibits a circadian rhythm in asthma (Kraft et al. 1998)	0.0050	1.5
(B) Downregulated Genes			
<i>Transcription factors</i>			
Jun proto-oncogene related gene d1 (JunD1)	DeltaFosB/JunD form a complex that c-Fos, Fra-2, FosB and JunB compete for binding after photic stimulation (Schwartz et al. 2000)	0.0365	0.5
<i>Growth factors/hormones</i>			
pro-opiomelanocortin (POMC)	Released in a circadian fashion (Arjona et al. 2004)	0.0394	0.5

(A) Upregulated Genes			
Gene (abbreviations)	Evidence for circadian involvement	FDR P-Value	Fold Change