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Direct regulation of diurnal *Drd3* expression and cocaine reward by NPAS2

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Abstract

Background—Circadian gene disruptions are associated with the development of psychiatric disorders, including addiction. However, the mechanisms by which circadian genes regulate drug reward remain poorly understood.

Methods—We used mice with a mutation in *Npas2*, and AAV-shRNA mediated knock-down of *Npas2* and *Clock* in the nucleus accumbens (NAc). We performed conditioned place preference (CPP) assays for cocaine. We utilized cell sorting techniques, qPCR and chromatin immunoprecipitation (ChIP) assays followed by deep sequencing (ChIP-seq).

Results—*Npas2* mutants exhibit decreased sensitivity to cocaine reward which can be recapitulated with a knock-down of NPAS*2* specifically in the NAc, demonstrating the functional importance of NPAS2 in this region. Interestingly, reducing CLOCK (a homologue of NPAS2) expression in the NAc had no effect, suggesting an important distinction in NPAS2 and CLOCK function. Furthermore, we find that NPAS2 expression is restricted to *Drd1* expressing neurons, (i.e. “direct” pathway circuitry) while CLOCK is ubiquitous. Moreover, NPAS2 and CLOCK have distinct temporal patterns of DNA binding, and we identified novel and unique binding sites for each protein. We identified the *Drd3* dopamine receptor as a direct transcriptional target of NPAS2 and find that NPAS2 knock-down in the NAc disrupts its diurnal rhythm in expression.

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Chronic cocaine treatment likewise disrupts the normal rhythm in *Npas2* and *Drd3* expression in the NAc, which may underlie behavioral plasticity in response to cocaine.

Conclusions—Together, these findings identify an important and novel role for the circadian protein, NPAS2, in the NAc in the regulation of dopamine receptor expression and drug reward.

Keywords

Cocaine; Reward; Circadian; Clock; Npas2; Drd3

INTRODUCTION

Disruptions in circadian rhythms are thought to contribute to the pathophysiology of several psychiatric diseases, including drug addiction (1–4). Circadian rhythms are centrally controlled by a transcriptional/translational feedback loop in the suprachiasmatic nucleus (SCN) of the hypothalamus (5). The central molecular clock is controlled by the circadian locomotor output cycles kaput (CLOCK) and brain and muscle Arnt-like protein-1 (BMAL-1) proteins, which heterodimerize and act as transcriptional regulators of the *Period* genes (*Per1*, *Per2*, and *Per3)*, the *Cryptochrome* genes *(Cry1* and *Cry2)*, as well as many other genes by binding to E-box elements (CANNTG) (5). Following translation, PER and CRY proteins undergo post-translational modifications which promote their dimerization and nuclear entry, where the complex can then inhibit CLOCK-BMAL1 mediated transcription. This feedback loop cycles over a twenty-four hour period (6). In forebrain regions or in conditions where CLOCK is nonfunctional, Neuronal PAS domain protein 2 (NPAS2), a protein homologous in structure and function to CLOCK, binds BMAL1 and induces expression of the *Per* and *Cry* genes (7, 8).

Circadian genes are expressed throughout the brain, and they form independent pacemakers that entrain to drugs of abuse and other non-photic stimuli such as food (9, 10). Most drugs of abuse can entrain locomotor activity rhythms, leading to an anticipatory activity just prior to drug administration which may enhance drug craving (11). In addition, behavioral responses and sensitivity to drugs of abuse exhibit diurnal variations in severity (12–14). Circadian gene expression is altered in response to drug treatment, and more recently, multiple studies have found that mutations in specific circadian genes in mice leads to an altered locomotor response and reward value for a variety of drugs of abuse (12, 13, 15–20).

Additional studies have revealed a critical role for circadian genes in mood and drug-related behaviors (21). Previous work from our laboratory has shown that mice with a dominant negative mutation (Δ19) in the *Clock* gene exhibit hyperactivity, decreased anxiety- and depression-like behaviors, as well as increased sensitivity in the rewarding value for cocaine as demonstrated in conditioned place preference, cocaine and alcohol self-administration, and intracranial self-stimulation paradigms (14, 16, 19, 22). Moreover, it has been shown that mice lacking a functional NPAS2 protein have defects in cued and contextual fear conditioning, altered patterns of sleep, and are unable to properly entrain to daytime food restriction (23, 24). NPAS2 is highly enriched in the striatum, especially in the NAc, which receives input from midbrain dopaminergic regions, thus NPAS2 may also be involved in regulating responses to drugs of abuse. Dopamine receptor expression as well as response to

quinpirole, a D2/D3 receptor agonist, has also been shown to be rhythmic (25). This suggests that diurnal variation in dopamine receptor expression accounts for time-dependent changes in quinpirole-induced locomotor behaviors or dopamine-mediated behaviors. Since CLOCK and NPAS2 are both expressed in the striatum, it is unclear whether this is a direct target effect of CLOCK or NPAS2, or if it is being mediated indirectly via other clock-controlled genes. Furthermore, the role of NPAS2 in drug reward has not yet been explored. Thus we wanted to determine whether NPAS2 and CLOCK are differentially involved in regulating cocaine reward-related behaviors, and identify factors that might underlie any differences with a particular focus on dopamine receptor expression.

METHODS AND MATERIALS

Animals

Npas2 (B6/129S6) mutant mice (23) were tested as homozygotes. Wild type littermate controls were utilized as a control for this mutation. C57BL/6J mice (The Jackson Laboratory) were utilized for *Npas2* knock-down, gene expression, and ChIP studies. GENSAT Drd1-GFP mice (Tg(Drd1a-EGFP)X60Gsat) (26, 27) used for FACS sorting were obtained from Jackson labs. All mice were group housed in a 12/12 light/dark (LD) cycle (lights on at 7am, lights off at 7pm) with food and water *ad libitum*. For the 24-hr time series studies, mice were group housed under the same LD schedule in temperature-controlled and sound-proof cabinets. Male mice were 8–10 weeks old at the start of all studies. Animal experiments were approved by the University of Texas Southwestern Medical Center and the University of Pittsburgh Institutional Animal Care and Use Committees.

Drug

Cocaine Hydrochloride (HCl) was generously provided by the National Institute on Drug Abuse. Animals were injected with a 15mg/kg cocaine or saline (i.p.) in gene expression studies and 2.5, 5.0, or 10.0 mg/kg in CPP studies.

Conditioned Place Preference (CPP)

An unbiased conditioning protocol, based on published methods (28), was used. Other details are in the supplemental material.

Locomotor response to novelty

Details are in the supplemental material.

Sucrose preference

Details are in the supplemental material.

Real time RT-PCR

RNA was isolated from mechanically homogenized tissue using the Trizol reagent (Invitrogen) according to the manufacturer's instructions and qPCR details are in the supplemental material. The Ct values used for these calculations are the mean of at least four biological replicates of the same reaction; each PCR reaction was done in duplicate.

Chromatin Immunoprecipitation (ChIP)

Brain tissue was processed as previously reported (29). For details see the supplemental material. Brain punches were taken in the NAc from 8 mice and pooled.

Deep Sequencing of ChIP Samples and Validation using quantitative RT-PCR

To identify genes that are direct transcriptional targets of NPAS2, CLOCK or both in the mouse striatum over the circadian cycle (at ZT 2, 6, 10, 14, 18, and 22), we performed deep sequencing of immunoprecipitated DNA. ChIP was performed as described in the supplemental methods and the immunoprecipitated DNA (obtained from three mouse striatums for each time point) was sequenced using a SOLiD next generation sequencer (SQX format). Reads were trimmed to 35bp as to avoid low quality bases at the 3' end of the reads. Trimmed reads were then mapped to the *Mus musculus* MM9-masked genome (http://hgdownload.cse.ucsc.edu/goldenpath/hg19/chromosomes/) using SHRiMP version 2.2.3 (http://compbio.cs.toronto.edu/shrimp/) (30). Putative peaks were identified using Model-based Analysis for CHiP version 1.4 (http://liulab.dfci.harvard.edu/MACS/) providing a negative control using IgG to emphasize the different binding profiles. All peak chromosomal locations were merged based on overlapping and book-ending peaks using Bedtools merge version 2.17.0 (http://code.google.com/p/bedtools/) (31) to obtain a clearer binding profile and to allow a one to one comparison of the putative peaks. Peaks were using annotation provided by UCSC Genome Browser using a custom perl wrapper that invoked Tabix (http://bioinformatics.oxfordjournals.org/content/27/5/718) to find nearby annotated features. Peak regions were extracted from the MM9 genome and provided to MEME-ChIP which was used to identify consensus sequences bound by CLOCK and/or NPAS2 (32). Predicted motifs were then compared to each other as well as JASPAR (http://jaspar.genereg.net/) and UniProbe databases of known transcription factor binding motifs (33, 34). Peaks were observed near known target genes of CLOCK and NPAS2 (i.e. Per1, Per2, Per3, Cry1, and Cry1). Novel target sequences of interest identified from ChIP-Seq results were validated using ChIP followed by quantitative RT-PCR and verified for size using agarose gel electrophoresis. Primer sequences are in the supplemental material.

Construction of shRNA constructs and viral packaging

A small hairpin RNA (shRNA) was constructed against the *Npas2* gene by selecting a 24 base sequence (5'- GAACACTGGATTCTTCCTGTTAAC - 3') in the 3'-UTR. The *Clock* shRNA was previously published (35). For the scrambled (Scr) shRNA, a random sequence of 24 bases (5'- CGGAATTTAGTTACGGGGATCCAC - 3') that had no sequence similarities with any known genes/mRNA was used. An antisense sequence of the selected mRNA region followed by a miR23 loop of 10 nucleotides (CTTCCTGTCA) was added at the 5'end of the above sequences. The miR23 loop facilitates the transfer of the hairpin RNA out of the nucleus. Additional details are in the supplemental material.

Stereotaxic surgery

Stereotaxic surgery was performed similarly to Mukherjee et al 2010 (35). Details are in the supplemental material.

Collection of AAV2- NPAS2 shRNA infected tissue

Details are in the supplemental material.

Immunohistochemical localization of AAV expression

Validation of AAV expression was performed as described by Mukherjee et al 2010 and in the supplemental material.

Enzymatic dissociation and FACS purification of striatal neurons—Details are in the supplemental material.

RNA extraction amplification, cDNA synthesis, and qPCR—Immediately after sorting, mRNA from isolated D1 containing MSNs using RNeasy Mini RNA purification kit with DNase treatment according to manufacturer's protocol. Other details are in the supplemental material.

Data analysis

For the time series experiments, one-way ANOVAs followed by Tukey's Multiple Comparison Test were used to establish diurnal rhythmicity for each gene and each condition, as previously published (36). Behavioral results from the CPP experiments were analyzed by ANOVA followed by posthoc tests. ChIP assays followed by qPCR were analyzed by one-sample t-tests, whereas changes in dopamine receptor expression were analyzed by Student's t-test. In all analyses, $P<0.05$ is considered significant.

RESULTS

Npas2 mutant mice show a decrease in the conditioned preference for cocaine

To determine if NPAS2 is important in regulating behavioral measures associated with cocaine reward, we employed homozygous mice that have a disruption in the PAS domain of NPAS2 (23). The mutation is considered a null, and the protein is nonfunctional. We first measured general locomotor activity in the *Npas2* mutant mice in response to novelty. This assay measures both general activity and habituation to a novel environment over two hours. In contrast to the *Clock* mutant mice which are hyperactive in this assay (16), the *Npas2* mutant mice have normal baseline locomotor activity and a normal habituation to novelty (Supplemental Figure S1). Since the *Clock* mutant mice exhibit increased sensitivity to the reinforcing properties of cocaine (16), we next wanted to determine if a mutation in NPAS2 leads to altered cocaine conditioned place preference (CPP). Surprisingly, we found that *Npas2* mutant mice have a marked decrease in the conditioned rewarding properties of cocaine ($F_{(5,57)}=2.41$, $P < 0.05$) (Figure 1). This decrease was observed at the low doses of 2.5 and 5.0 mg/kg, but not at 10 mg/kg, suggesting a ceiling effect at this dose. To determine if this response was indicative of general anhedonia we measured sucrose preference in these mice over multiple concentrations. We found no difference (Supplemental Figure S2), suggesting that this decrease in reward sensitivity is specific to drugs of abuse.

Effects of *Npas2* knockdown in the NAc

The NAc is well known to regulate reward and previous studies have found that NPAS2 is highly expressed in this region (23, 37). We wanted to know if NPAS2 expression in the NAc was important in cocaine reward. To reduce *Npas2* expression we utilized RNA interference via the injection of adeno-associated virus (AAV2) that expresses a short hairpin RNA (shRNA) homologous to a segment of *Npas2* 3'UTR. C57BL6/J mice were injected in the NAc with AAV-*Npas2* shRNA or -Scramble shRNA, allowed to recover, and tissue was processed for real-time RT-PCR to test the efficacy of viral-mediated knock-down of *Npas2*. Figure 2A shows the localization of a viral infection in the NAc at 10x magnification. Infection with the AAV-*Npas2* shRNA virus in the NAc led to a ~5-fold decrease of *Npas2* mRNA compared with mice injected with AAV-Scramble shRNA (Figure 2B). Localized knockdown of *Npas2* in the NAc had a modest, but significant effect on locomotor activity response to a novel environment ($F_{(1,408)}$=55.57, p<0.0001 treatment effect) (Figure 2C). AAV-*Npas2*-shRNA injected mice displayed a significant reduction in locomotor activity that was only present during the second hour of the test, suggesting an enhanced habituation to the environment over time. When we measured cocaine-induced CPP at doses that showed a differential response in the *Npas2* mutant mice (Figure 1), a reduction in NAc *Npas2* expression was sufficient to cause a significant decrease in cocaine preference at two separate cocaine doses, mimicking the effect observed in the *Npas2* mutant mice ($F_{(3,37)}$=2.85, p<0.01)(Figure 2D). The decreased locomotor response to novelty over time should not confound the interpretation of CPP measures since sessions last only 30 minutes and after 30 minutes in a novel environment, we saw no significant difference in locomotor activity between NAc *Npas2* knock-down and Scramble groups.

Effects of *Clock* knockdown in the NAc

To determine if the reduction in cocaine preference with *Npas2* knock-down was specific to *Npas2* or would also be seen with a knock-down of *Clock* expression in the NAc, we injected AAV-*Clock* shRNA or –Scramble shRNA into the NAc of C57BL/6J mice (Figure 3A). We have employed this AAV-*Clock* shRNA previously in the VTA and find that it is selective for reducing *Clock* expression and highly efficient at producing a knock-down in expression (35). When we measured the locomotor response to novelty we found no significant effect of NAc *Clock* knock-down as compared with Scramble controls (Figure 3B). Moreover, when we measured cocaine-induced CPP, we found that NAc infected AAV-*Clock* shRNA injected mice were indistinguishable from Scramble injected mice at doses in which there is an increase in preference in the *Clock* mutant mice (16) (Figure 3C). This is in contrast to the hyperactivity and increased alcohol preference which is observed when *Clock* expression is reduced specifically in the VTA (19, 35). Thus, a knock-down of NPAS2, but not CLOCK in the NAc is sufficient for decreased cocaine preference.

NPAS2 is preferentially expressed in D1 containing neurons in the striatum

Previous studies have identified distinct roles for the NAc neurons that express the D1 dopamine rector versus the D2 dopamine receptor in the regulation of conditioned place preference for cocaine (38). Specific stimulation of D1 containing neurons leads to an increase in cocaine preference (38). Thus, we wanted to determine if NPAS2 was

preferentially expressed in D1 containing neurons. We dissected and isolated striatal tissue from GENSET Drd1-Td-Tomato mice and performed FACS sorting to isolate D1 containing neurons and non-D1 containing neurons (as described by Lobo et al., 2010 (38)). We measured levels of *Drd1* and *Drd2* (to ensure that we were enriching for D1 expressing neurons) and 18S (as a control). We find that D1 expressing neurons are highly enriched with *Npas2* (~76 fold increase) as compared with non-D1 containing neurons ($F_{(5,30)}$=2.53, p<0.01) (Figure 4). *Clock* expression was present in both D1 and non-D1 expressing neurons with a less prominent bias towards D1 (P<0.05), suggesting these homologous proteins may exhibit differential functions in specific cell types.

NPAS2 and CLOCK binding to target genes in the striatum is enriched at different times of day and *Drd3* is bound by NPAS2

To determine the direct target genes of both CLOCK and NPAS2 in the striatum, we performed a ChIP-Seq experiment using striatal tissue collected at six different time points over the light/dark cycle and antibodies specific for NPAS2 or CLOCK. We identified 6,458 unique DNA binding sites for CLOCK, 18,573 unique binding sites for NPAS2, and 17,854 shared binding sites for CLOCK and NPAS2 (Figure 5A). Moreover, the patterns of DNA binding are different in that NPAS2 binding occurs with peak during the dark phase while CLOCK binding is more uniform through the light/dark cycle (Figure 5B). Furthermore, there are a number of genes which are specifically bound by either CLOCK or NPAS2. One of these specific target genes of NPAS2 was *Drd*3 (Figure 5C). NPAS2 bound both the promoter region and areas within the gene itself. Binding was confirmed by qPCR.

Npas2 knockdown in the NAc prevents diurnal expression of the *Drd3* receptor

We next wanted to determine whether NPAS2 mediates the diurnal variation in dopamine receptor expression. Following viral-mediated knockdown of *Npas2* in the NAc, expression of dopaminergic receptors at two time points (ZT 16 (night) and ZT4 (day)) was measured via quantitative RT-PCR. Interestingly, *Npas2* knock-down specifically in the NAc abolished diurnal expression of *Drd1*, *Drd2*, and *Drd3* dopamine receptor genes with the largest effect noted for the *Drd3* receptor (Figure 6 A, B, C).

Chronic cocaine treatment alters molecular rhythms of *Npas2* and *Drd3* in the striatum

Previously, we found that *Npas2* displays significant diurnal rhythmicity in the NAc and this rhythm is abolished following chronic cocaine treatment (20). *Clock* is also rhythmically expressed in the NAc. However, cocaine had no significant effect on its diurnal rhythms (20). To determine how cocaine might alter the diurnal rhythmicity of the dopamine receptors in the NAc, mice were treated with cocaine (15 mg/kg) or saline (i.p.) for 7 days at ZT 6 (1pm). Animals were sacrificed starting 26 hrs after the last injection (ZT8, 3pm) and subsequently every 4 hrs until ZT4 (11am). This last time point is exactly 2 hrs prior to drug administration time and thus, might provide some insight into possible anticipatory effects of expected drug administration. We find a striking reduction in *Drd3* diurnal variation and overall expression in response to cocaine ($F_{(5,25)}$=6.747, p<0.001 saline; $F_{(5,26)}$=1.215, p=0.332 cocaine) (Figure 7A). Chronic cocaine also blunts *Drd1* diurnal variation in expression in the NAc ($F_{(5,38)}$=4.068, p<0.01 saline; $F_{(5,43)}$=1.262, p=0.2978 cocaine)

(Figure 7B). However, cocaine treatment did not affect diurnal variation of *Drd2* ($F_{(5,38)}$=9.038, p<0.0001 saline; $F_{(5,42)}$=3.263, p<0.05 cocaine) (Figure 7C).

DISCUSSION

We have identified a unique role for *Npas2* in the regulation of cocaine reward and dopamine *Drd3* receptor expression. Although NPAS2 and CLOCK are highly homologous transcription factors, mutations in each lead to opposing effects on conditioned preference for cocaine. A mutation in *Clock* leads to increased place preference for cocaine (16) while here we show that *Npas2* mutant mice exhibit a decreased place preference for cocaine. Previous studies found that the *Npas2* mutant mice also have a decrease in fear conditioning, but show no differences in spatial memory in the Morris water maze (23). We do not suspect that the decrease in conditioned preference for cocaine is associated with a deficit in learning or memory since they have no differences in CPP with increased cocaine concentrations (10 mg/kg). Moreover, we find that these mice show no differences in sucrose preference, thus their response is not indicative of general anhedonia.

Regulation of cocaine reward by NPAS2 seems to be via actions in the NAc since localized knockdown of *Npas2* in this region was sufficient to recapitulate the decreased cocaine CPP observed in *Npas2* mutant mice. Although *Clock* is also expressed in the NAc, it does not appear to have a prominent role in the regulation of cocaine reward in this region. Previous studies have established that CLOCKs actions in the ventral tegmental area (VTA) are important for the regulation of dopaminergic activity, as well as mood, anxiety and reward-related behavior (19, 22, 35). Thus, we propose that *Clock* regulates drug reward primarily through its actions in the VTA, while NPAS2 primarily acts in the NAc. This is further supported by the expression pattern of *Npas2*; as it has high expression in striatal regions, but essentially no expression in the VTA (23).

The NAc is primarily composed of two sub-types of GABAergic medium spiny neurons. These sub-types are categorized primarily based on their expression of D1 or D2 receptors, among other genes, with each sub-type participating in parallel circuits that form the direct (D1-expressing) and indirect (D2-expressing) pathways to the VTA. Studies using optogenetics have found that stimulations of the D1 containing neurons leads to an increase in cocaine CPP while stimulating the D2 containing neurons results in a decrease in cocaine CPP (38). We find that *Npas2* is highly enriched in D1 expressing neurons whereas *Clock* is more uniformly expressed in both cell types. In general, *Clock* expression is ubiquitous throughout the brain and body, thus it is not surprising that it is ubiquitously expressed throughout the NAc. The specificity of *Npas2* expression in D1 expressing neurons is bolstered by pharmacological studies in neuronal culture. Imbesi et al. (2009) found an increase in *Npas2* expression following administration of a D1, but not D2, agonist in striatal cultures, whereas *Clock* expression was both increased and decreased following administration of D1 and D2 agonists, respectively (39). Moreover, a study performed in retinal cells found that D2-mediated signaling potentiated the transcriptional capacity of CLOCK:BMAL1 complexes, suggesting that CLOCK is expressed in these neurons and is activated downstream of D2 signaling (40). These studies demonstrate that effects on the circadian machinery can be differentially mediated by the different dopaminergic receptors.

Moreover, the distinct patterns of expression of *Clock* and *Npas2* within the NAc suggest that they differentially regulate gene expression within this region. Indeed, using ChIP-seq we find that CLOCK and NPAS2 DNA binding patterns are different with NPAS2 having a strong rhythm in binding while CLOCK binding does not display a robust pattern. We also find that there are many sites that are selectively bound by either CLOCK or NPAS2. Previously we showed that both *Clock* and *Npas2* mRNA expression have a significant diurnal rhythm in the NAc with peaks in *Clock* expression at ZT24 and peaks in *Npas2* expression at ZT16 (20). It is likely that the protein lags behind the mRNA such that NPAS2 function is optimal at ZT 18–22 which is the time that we find the highest levels of binding. Similar to the SCN, CLOCK binding to its targets may be more dependent upon BMAL1 rhythms in the NAc (5). It will be interesting in future studies to determine what other factors contribute to the differential temporal expression patterns and binding of these two proteins, particularly when they are expressed within the same neurons.

We find that NPAS2 is bound to the *Drd3* gene making it under direct transcriptional control. This is particularly interesting since we find that *Npas2* is highly enriched in D1 expressing neurons. Previous studies have found that D1 containing neurons in striatum also express D3, while D2 expressing neurons mostly represent a separate neuronal population (41, 42). D1 and D3 receptors dimerize and form a receptor complex which is important in the development of addiction (43, 44). D3 receptors can also inhibit D1 signaling which may be responsible for the decrease in cocaine preference seen with increased D3 expression in the *Npas2* mutant mice during the light cycle. It is possible that CPP tests at the opposite time of day would not produce the same result since D3 levels at ZT16 were not different between the controls and NPAS2 knock-down mice. Somewhat counterintuitively, D3 receptor antagonists have been proposed as a treatment for addiction since they inhibit drug self-administration (45, 46). It is possible that D3 antagonists lead to a sufficient increase in D1 signaling on their own such that additional cocaine is no longer rewarding or even becomes aversive, thus these drugs could be similar to other replacement therapies. The mechanism by which NPAS2 may be involved in the regulation of diurnal rhythms in *Drd1* or *Drd2* expression is unclear since these were not identified as direct target genes in our ChIP-seq analysis. It is possible that binding was simply not detected in this assay, however, a number of other possibilities exist for indirect mechanisms that could explain these findings. Future studies will investigate more specifically if the altered expression of specific dopamine receptors underlies the decrease in the conditioned preference for cocaine when *Npas2* is disrupted.

Chronic cocaine disrupts rhythmic expression of *Npas2* (20), as well as *Drd1* and *Drd3* in the NAc. Thus, this suggests that NPAS2 is not only involved in the initial vulnerability for cocaine-induced responses, but that chronic cocaine can also alter expression of NPAS2, leading to disrupted rhythms in dopamine receptor expression. Interestingly, continuous cocaine given via an osmotic pump for 14 days leads to an increase in D1 receptor occupation by dopamine during the day and a decrease in occupancy during the night compared to saline controls (47). The diurnal change in receptor function is abolished with co-administration of D1 antagonists, suggesting a feedback loop via D1 signaling. These disruptions by cocaine highlight the presumed Zeitgeber ability of the drug, since they can

be interpreted as the effect of a clash between the drug and light's synchronizing influences. Further studies are required to investigate the exact mechanism by which cocaine leads to alterations in NPAS2 and dopamine receptor expression. Indeed cocaine CPP has a strong diurnal rhythm with increased responses to cocaine seen during the light cycle and decreased responses during the dark cycle (13). However, cocaine self-administration is typically higher during the dark cycle than the light cycle (14). It will be interesting in future studies to determine how the rhythms in dopamine receptors are involved in these diurnal differences in addiction-related behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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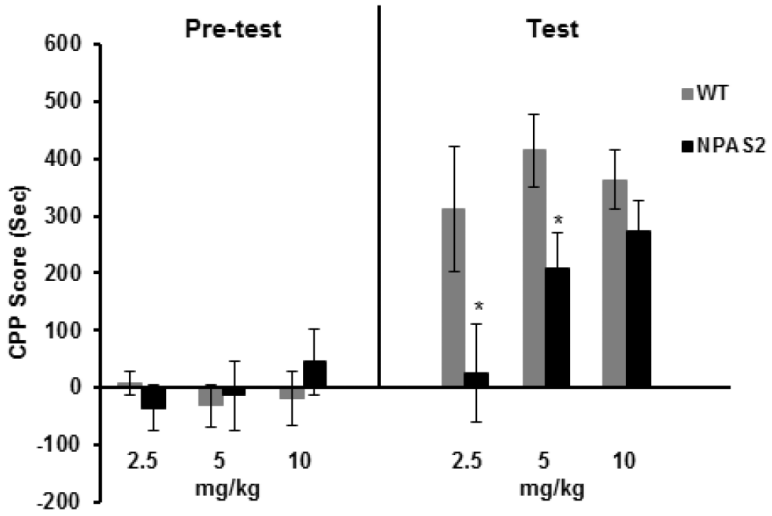

Figure 1.

Cocaine conditioned place preference in the *Npas2* mutant mice. *Npas2* mutant mice exhibited a decreased cocaine preference when compared to their wild-type counterparts at 2.5 and 5.0 mg/kg of cocaine. Tests were performed using an unbiased protocol at 2.5, 5, and 10 mg/kg cocaine. Data is mean±SEM. (*P < 0.05 by ANOVA, n=8-14/group).

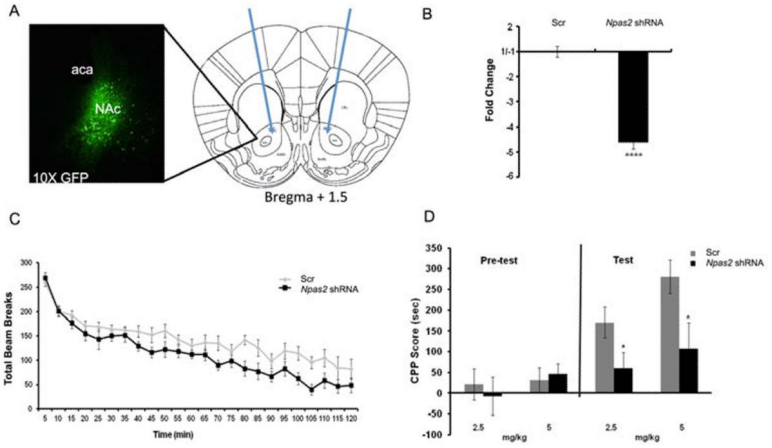

Figure 2.

Npas2 knock-down in the NAc. (A) Representative image showing viral expression in the NAc. (B) AAV-*Npas*2 shRNA infusion leads to a significant knockdown of *Npas2* expression in the NAc. (C) Localized knockdown in the NAc causes a significant decrease in locomotor activity that is noticeable during the second hour of the locomotor test ($p<0.0001$ treatment effect). (D) AAV-*Npas2* shRNA injected animals displayed a decrease in cocaine preference, similar to the *Npas2* mutant mice. (Data is mean±SEM. *$P < 0.05$ by ANOVA, n=9–10/treatment).

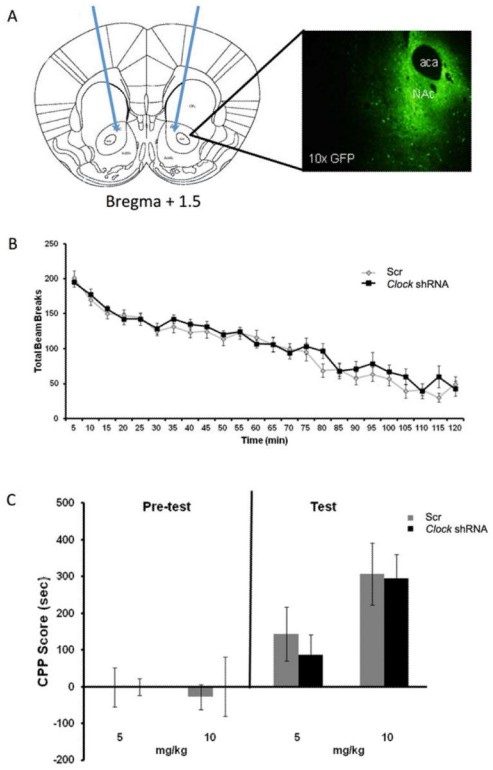

Figure 3.

Clock knock-down in the NAc. (A) Representative image showing viral expression in the NAc (B) AAV-*Clock* shRNA injected mice showed similar locomotor activity levels as control mice. (C) No difference in cocaine preference was observed between *Clock* shRNA injected mice and control mice under both doses of cocaine. Data is mean±SEM (n=8-14/ treatment for locomotor assay; n=6–9/treatment for CPP).

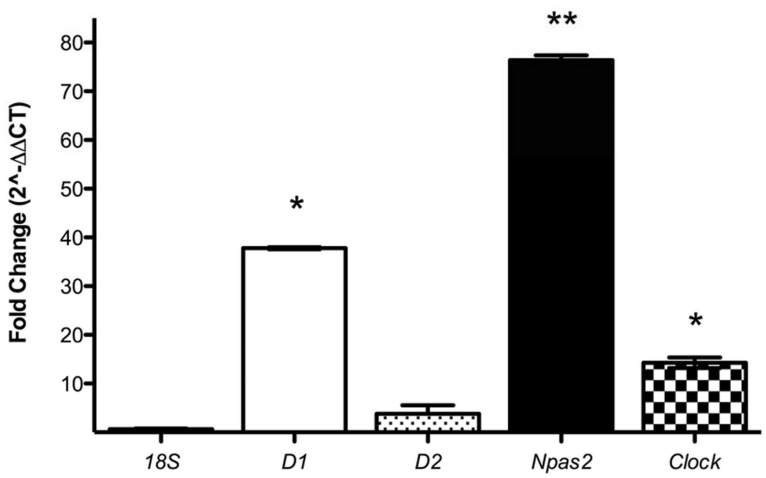

Figure 4.

Npas2 is enriched in D1 containing neurons (**p<0.01). *Clock* is also slightly enriched in D1 containing neurons (*p<0.05). Data is mean±SEM (n=4).

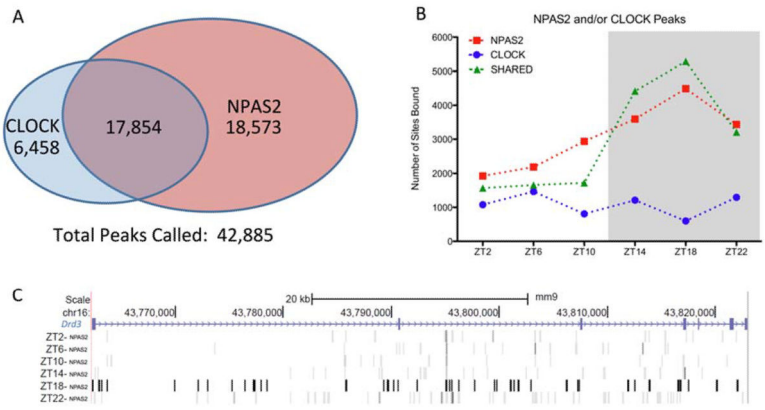

Figure 5.

NPAS2 and CLOCK binding to promoter sequences in the NAc. (A) Venn diagram showing the total number of sites bound by both CLOCK and NPAS2 over the 24 hr cycle. (B) graph showing the numbers of sites bound over the 24 hr cycle. (C) Figure showing the locations and relative intensity of binding for NPAS2 on the *Drd3* gene.

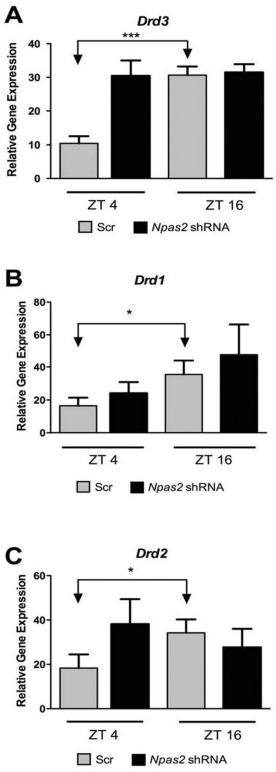

Figure 6.

Effect of *Npas2* knockdown in diurnal expression of dopaminergic receptors in the NAc. (A) *Npas2* knockdown abolishes diurnal expression of *Drd3* in the NAc. All AAV-Scr treated mice exhibited diurnal variation in expression, with higher expression at ZT 16. (B) *Npas2* knockdown disrupts diurnal expression of *Drd1*, although a trend towards an increase in expression at ZT 16 is observed (black bars). (C) *Drd2* diurnal expression is compromised following *Npas2* knockdown in the NAc. (*p<0.05, ***p<0.001 by t-test, data is mean ± SEM, n=5–7/treatment).

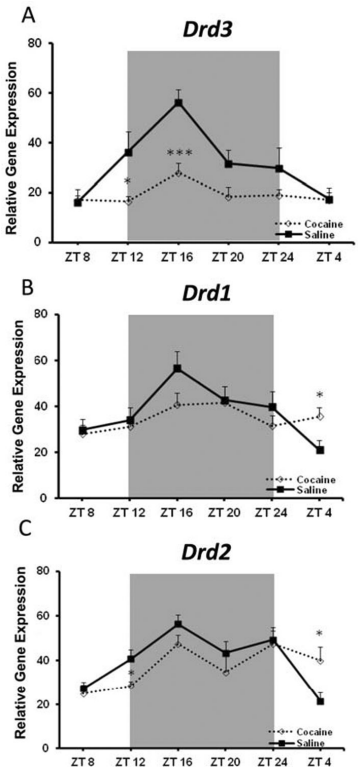

Figure 7.

Chronic cocaine alters diurnal expression of the *Drd3* gene in the NAc. (A) Cocaine significantly disrupts rhythms in *Drd3* ($F_{(5,25)}$=6.747, p<0.001 saline; $F_{(5,26)}$=1.215, p=0.332 cocaine). (B) Cocaine also alters *Drd1* expression in the NAc to a lesser extent ($F_{(5,38)}$=4.068, p<0.01 saline; $F_{(5,43)}$=1.262, p=0.2978 cocaine). A significant upregulation at ZT4 was observed (*p<0.05). (C) *Drd2* rhythmicity is unaltered in the NAc following cocaine treatment ($F_{(5,38)}$=9.038, p<0.0001 saline; $F_{(5,42)}$=3.263, p<0.05 cocaine). A significant upregulation at ZT 4 was observed (*p<0.05). Data is mean ± SEM, n=5–9.