Circadian- and sex-dependent increases in intravenous cocaine self-administration in *Npas2* mutant mice

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Abstract

Background: Addiction is associated with disruptions in circadian rhythms. The circadian transcription factor neuronal PAS domain protein 2 (NPAS2) is highly enriched in reward-related brain regions, but its role in addiction is largely unknown.

Methods: To examine the role of NPAS2 in reward, we measured intravenous cocaine self-administration in wild-type (WT) and *Npas2* mutant male and female mice at different times of day (light or dark phase). Mice underwent acquisition, dose-response, progressive ratio, extinction and cue-induced reinstatement.

Results: Cocaine self-administration was elevated in *Npas2* mutant mice, particularly in females. Cocaine reinforcement was increased in all mutant mice, whereas motivation was only increased in females. Sex differences were amplified during the dark (active) phase with *Npas2* mutation increasing self-administration, reinforcement, motivation, extinction responding and reinstatement in females, but only reinforcement in males. To determine whether circulating ovarian hormones are driving these sex differences we ovariectomized WT and *Npas2* mutant females before cocaine self-administration. Unlike sham controls, ovariectomized mutant mice showed no increase in self-administration. To identify whether striatal brain regions are differentially activated in *Npas2* mutant females we measured cocaine-induced ΔFosB expression. ΔFosB expression was increased in D1+ neurons in the nucleus accumbens core and dorsolateral striatum in *Npas2* mutant, relative to WT, females after dark phase self-administration.

Conclusions: These results suggest NPAS2 regulates reward and cocaine-induced ΔFosB expression in specific striatal regions in a sex and time of day specific manner. Striatal activation could be augmented by circulating sex hormones, leading to an increased effect of *Npas2* mutation in females.

Introduction

Circadian disruptions are a common symptom of many psychiatric disorders (1–3) and are thought to contribute to their etiology, including addiction (4–7). Diurnal rhythms in the behavioral responses to drugs of abuse are common, including drug sensitivity and motivation to seek and take drugs, which are typically more pronounced during active (dark) phase compared to the inactive (light) phase in nocturnal rodents (8, 9). Chronic exposure to drugs of abuse alters circadian rhythms and these alterations may contribute to subsequent substance use and abuse (4, 5). Emerging evidence from rodents and humans links disrupted circadian genes to substance abuse vulnerability (10–17), suggesting drug-induced alterations to these genes may augment drug-seeking.

Almost every cell in the brain and body expresses a molecular clock, comprised of several interlocking transcriptional-translational feedback loops (18–20). The molecular clock is regulated by Circadian Locomotor Output Cycles Kaput (CLOCK) or its homologue, neuronal PAS domain protein 2 (NPAS2), which dimerize with Brain and Muscle ARNT-like 1 (BMAL1) to control transcription of many genes, including *Period* and *Cryptochrome*. After translation, these proteins enter the nucleus and inhibit the transcriptional activity of CLOCK/BMAL1, closing the negative feedback loop (20). NPAS2 is similar to CLOCK in structure and function, yet these proteins are differentially enriched across reward-related brain regions. Relative to CLOCK, NPAS2 is highly expressed in the striatum (21), including the nucleus accumbens (NAc), a major neural substrate of reward (reviewed in 22), suggesting NPAS2 may play an integral role in drug reward. Our laboratory has previously demonstrated that mutations in *Clock* lead to increased cocaine and alcohol intake in mice (15, 16, 23) and a loss of diurnal rhythmicity in cocaine self-administration (9). We have also previously shown NPAS2 modulates cocaine conditioned reward. Interestingly, *Clock* mutation increases cocaine preference, while *Npas2* mutation attenuates preference (24). These results suggest that, despite their homology, CLOCK and NPAS2 might play unique roles in the regulation of reward.

This study aimed to determine whether NPAS2 regulates intravenous cocaine self-administration, a more translational model of drug taking, reinforcement, motivation, and relapse-like behavior. To investigate this, we used mice with a mutation in *Npas2* which renders the protein non-functional (25). We aimed to determine whether *Npas2* mutation modulates the diurnal rhythm in drug taking by measuring cocaine self-administration during both the light and dark phase. Furthermore, evidence suggests both circadian rhythms (26) and addiction

(27) are differentially regulated by sex, therefore we investigated whether *Npas2* mutation will differentially impact male and female mice. Here, we found that NPAS2 regulates cocaine self-administration differentially across sex and time of day (TOD). Furthermore, ovarian hormones and cocaine-induced expression of striatal ΔFosB were associated with increased self-administration in *Npas2* mutant females during the dark phase.

Methods and Materials

Subjects. Male and female *Npas2* mutant mice or wild-type (WT) littermates, maintained on the C57BL/6J background, were used. These mice were originally described by Garcia et al., 2000. This mutation results in a form of NPAS2 lacking the bHLH domain, leaving NPAS2 intact, but incapable of binding to its partner BMAL1 (25). Adult mice were maintained on a 12:12 light-dark cycle with lights on (Zeitgeber Time (ZT0)) at 0700 and 1900. Behavioral testing occurred from approximately ZT2-7 or ZT14-19 respectively for light and dark phase experiments. All mice were provided *ad libitum* food and water unless otherwise indicated. Procedures were approved by the University of Pittsburgh IACUC. Throughout, additional methodological details can be found in the supplement.

Drug. Cocaine hydrochloride was generously provided by the National Institute on Drug Abuse. Animals were injected with 2.5, 5 or 15 mg/kg (intraperitoneal, i.p.; volume 10 ml/kg) in conditioned place preference and locomotor sensitization studies and 0-1 mg/kg/infusion for cocaine self-administrations studies.

Surgery. Jugular catheterization (15, 28) and ovariectomy (29) procedures were performed similarly to those previously described.

Behavioral testing.

Conditioned place preference (CPP). A biased conditioning protocol, adapted from published methods (30), was used.

Locomotor sensitization. Locomotor activity was measured on day 1 for habituation, days 2-3 for saline treatment and days 4-8 and 16-17 for cocaine treatment.

Food and intravenous cocaine self-administration. Mice were trained to self-administer food (15) and then cocaine (adapted from 19) based on published methods.

Dual RNAscope and immunohistochemistry.

In order to measure the percent of D1+ and D1- neurons expressing ΔFosB, RNAscope in situ hybridization (ISH) for *Drd1* was performed followed by immunohistochemistry (IHC) for ΔFosB. Mice were trained to self-administer cocaine and brains were extracted and flash frozen 24 hr after the last self-administration session (timeline Fig.6a). Frozen coronal sections were processed using an RNAscope fluorescent multiplex kit. Briefly, tissue sections were fixed, permeabilized to unmask target RNA, and hybridized with *Drd1* probe. The signal was amplified and mounted sections were then blocked, incubated in rabbit anti-FosB primary antibody, rinsed and incubated in donkey anti-rabbit Alexa Fluor 555 secondary antibody. Sections were counterstained with DAPI and cover-slipped with prolong gold. Although a pan-FosB antibody was used, full-length FosB should degrade by the time of tissue harvesting (31), 24 hr after the last rewarding stimuli. Therefore, all immunoreactivity reflects ΔFosB.

Imaging and cell counting.

Immunofluorescence images were captured on an Olympus IX83 confocal microscope using a 20x objective at x2 magnification (DAPI, *Drd1* and ΔFosB). Approximately 300 x 300 μm images were taken from sections along the anterior-posterior extent of the NAc core and shell, and dorsomedial (DMS) and dorsolateral (DLS) striatum.

The total number of D1+ and D1- cells as well as the D1+ and D1- ΔFosB expressing cells (see Fig.6c) were counted using ImageJ. The percentage of D1+ and D1- ΔFosB expressing cells was calculated for all conditions and brain regions.

Statistical Analyses.

GraphPad Prism 7 and IBM SPSS statistics (version 24) were used. Throughout, two-way ANOVAs were performed with significant interactions followed by Bonferroni post-hoc tests corrected for multiple comparisons. Data are expressed as means+SEM with $\alpha=0.05$ considered statistically significant and $0.05<\alpha<0.1$ considered a statistical trend.

Results

Sex differences in cocaine sensitization and place preference in *Npas2* mutant mice

We first aimed to expand upon earlier evidence that NPAS2 regulates the behavioral effects of cocaine. Prior work showed that male *Npas2* mutant mice have reduced CPP for cocaine (30), but female mice were not tested. Here, we conditioned mice to 2.5 or 5 mg/kg cocaine and found that sex plays a pivotal role in cocaine reward [sex x genotype interaction: 2.5 mg/kg ($F_{(1,58)}$=4.4,p=0.04) and 5 mg/kg ($F_{(1,57)}$=7.01,p=0.01)]. Male *Npas2* mutant mice showed reduced CPP, replicating our prior findings, while female mutant mice showed no change (Fig.1a).

We next investigated whether another cocaine-related behavior, locomotor sensitization, is altered in a sex-dependent manner. Again, we found a significant effect of sex (sex x genotype interaction: $F_{(1,32)}$=4.01,p=0.05). Female mutant mice showed elevated locomotor-activating properties of cocaine (genotype main effect: $F_{(1,14)}$=8.16,p=0.013)(Fig.1bc). All mice sensitized equally to cocaine [day main effect: males ($F_{(4,72)}$=24.11,p<0.0001), females ($F_{(4,56)}$=7.17,p<0.0001)]. These results together indicate sex plays a critical role in how NPAS2 regulates the behavioral effects of cocaine.

Npas2 mutant mice self-administer more cocaine, particularly females in the dark phase

We next examined the role of NPAS2 in a more translational model, intravenous cocaine self-administration. In addition to sex and genotype, we also measured behavior at 2 times of day since *Npas2* can influence circadian rhythms. Animals were first trained to respond for food during the light or dark phase; all mice acquired this response (session main effect: $F_{(4,92)}$=524.72,p<0.0001) and discriminated between the levers (lever main effect: $F_{(1,173)}$=212.83,p<0.0001). Response rates varied based on genotype and TOD (session x genotype x TOD interaction: $F_{(4,173)}$=4.19,p=0.002), but not sex. Since prior behaviors showed explicit sex differences, the sexes were analyzed individually, but in this case only trending effects were found (Fig.S1). Male *Npas2* mutant mice responded less on the last day of light phase food training (genotype x session interaction: $F_{(4,204)}$=2.30,p=0.06), while female *Npas2* mutant mice responded moderately more on session 4 of dark phase training (genotype x session interaction: $F_{(4,128)}$=2.21,p=0.071).

After recovery from jugular catheterization, mice were trained to self-administer cocaine. Regardless of TOD, the *Npas2* mutation differentially affected males and females (sex x genotype interaction:

$F_{(1,95)}$=4.18,p=0.044). When pooled across TOD, female *Npas2* mutants self-administer significantly more cocaine than male mutants ($F_{(1,49)}$=5.89,p=0.019) and female WT mice ($F_{(1,41)}$=18.1,p=0.0001)(Fig.2a). We also confirmed that all mice self-administer more cocaine during the dark (active) phase (session x TOD interaction: $F_{(13,1300)}$=2.11,p=0.012), which is expected since mice are nocturnal (Fig.2b). In order to further parse the effects of mutating *Npas2* on cocaine self-administration, female and male data will be shown separately for the light and dark phases throughout. During the light phase, males (genotype main effect: $F_{(1,29)}$=7.27,p=0.012), but more so females (session x genotype interaction: $F_{(13,351)}$=1.82,p=0.039), take more infusions of cocaine relative to WT mice (Fig.2cd). During the dark phase, this sex difference was exacerbated with mutant females continuing to self-administer more cocaine (session x genotype interaction: $F_{(13,247)}$=6.43,p=0.05), while mutant males showed no differences (Fig2.ef).

To further illustrate the effects of loss of NPAS2 function on cocaine self-administration, we analyzed sessions required to reach criteria and total drug intake (infusions). We again found the *Npas2* mutation differentially affected males and females (sex x genotype interaction: infusions $F_{(1,95)}$=3.76,p=0.055). TOD was also critically important (sex x genotype x TOD interaction: criteria $F_{(1,95)}$=3.08,p=0.08), with sex differences emerging during dark phase self-administration. All *Npas2* mutant mice acquired self-administration faster and took more infusions than WT mice in the light phase [genotype main effects: criteria ($F_{(1,52)}$=4.32,p=0.043), infusion ($F_{(1,57)}$=16.41,p=0.0002)](Fig.S2ab), but in the dark phase these effects were only found in females [sex x genotype interaction: criteria ($F_{(1,39)}$=3.92,p=0.055), infusion ($F_{(1,39)}$=3.12,p=0.085)](Fig.S2cd).

Increased reinforcing properties of cocaine in *Npas2* mutant mice

Following acquisition, we investigated the reinforcing properties of cocaine using a dose-response analysis. No sex or TOD differences were found (Fig.3ab). Throughout, *Npas2* mutant mice earned more infusions, indicating an increase in the efficacy of cocaine regardless of other factors [dose x genotype interaction: light phase ($F_{(5,190)}$=4.21,p=0.0012), dark phase ($F_{(5,190)}$=9.99,p<0.0001)]. During the light phase, female *Npas2* mutants took more infusions across dose (genotype main effect: $F_{(1,17)}$=5.05,p=0.038), while male mutants took more infusions at lower doses (dose x genotype interaction: $F_{(5,95)}$=3.55,p=0.006)(Fig.3ef). During the dark phase, male mutants took more infusions at the lowest dose (dose x genotype interaction: $F_{(5,90)}$=2.84,p=0.0198), while females took more at the three lowest doses (dose x genotype interaction:

$F_{(5,90)}$=8.55,p<0.0001)(Fig.3ij). Saline infusions were also increased in all female mutant mice, which is likely due to the descending unit dose procedure used or greater responding under extinction conditions in these mice.

Increased motivation, extinction responding, and cue-induced reinstatement in female *Npas2* mutant mice in the dark phase

Next, a progressive ratio schedule was used to measure motivation for cocaine as shown by increases in a breakpoint ratio. Similar to the reinforcing properties of cocaine, we found no TOD or sex differences associated with motivation (Fig.3cd). However, when males and females were analyzed separately, genotype only affected motivation in females (Fig.3gk). This was particularly true in the dark phase (genotype main effect: $F_{(1,13)}$=4.50,p=0.05)(Fig.3k), compared to the light phase (main effect: $F_{(1,12)}$=3.52,p=0.085)(Fig.3g).

Mice then underwent extinction and cue-induced reinstatement to model relapse-like drug seeking in the absence of cocaine. We confirmed that all mice extinguished responding (session main effect: $F_{(9,432)}$=28.68,p<0.0001) and found a trending 4-factor interaction (session x sex x genotype x TOD interaction: $F_{(9,432)}$=1.85,p=0.058) indicating divergent effects of genotype on males and females in the light and dark phase. Female *Npas2* mutants respond significantly more during extinction compared to male mutants ($F_{(1,31)}$=5.3,p=0.028) and female WT mice ($F_{(1,26)}$=6.86,p=0.015) when pooled across TOD (Fig.4a). When male and female mice were pooled, *Npas2* mutants responded more during extinction compared to WT mice, but only during the dark phase ($F_{(1,27)}$=4.27,p=0.049) (Fig.4b). When analyzed separately by sex and TOD, only female *Npas2* mutant mice in the dark phase were affected (Fig.4i), as shown by increased responding during early extinction (session x genotype interaction: $F_{(9,108)}$=2.71,p=0.007). This indicates that in the absence of cocaine, female *Npas2* mutants will escalate their drug seeking, but only in the dark phase.

After extinction, mice were presented with a previously cocaine associated audio-visual cue, followed by contingent cues for each subsequent lever press. In the absence of cocaine, these cues invigorated or reinstated responding in all mice (lever main effect: $F_{(1,48)}$=53.38,p<0.0001). Although no TOD differences were found, a sex x genotype interaction was detected ($F_{(1,48)}$=4.49,p=0.039) again indicating that mutating *Npas2* differentially affects males and females (Fig.4cd). Female *Npas2* mutants self-administer significantly more cocaine than male mutants ($F_{(1,31)}$=9.26,p=0.005) and female WT mice ($F_{(1,26)}$=5.11,p=0.033)(Fig.4a). Similar to extinction, in subsequent analysis we only detected an increase in active lever pressing in female *Npas2* mutants in the dark

phase (lever x genotype interaction: $F_{(1,12)}$=4.58,p=0.054)(Fig.4k), further indicating an increase in drug seeking in the absence of cocaine.

Circulating sex hormones contribute to increased cocaine self-administration in female *Npas2* mutant mice

In order to determine whether ovarian hormones are contributing to sex differences in cocaine self-administration in *Npas2* mutant mice, a separate cohort of female mice underwent a sham surgery or ovariectomy (OVX). Mice were then trained to self-administer food and later drug following recovery from jugular catheterization. This experiment was conducted during the dark phase where we observed the largest behavioral effect. While sham surgery mice recapitulated our original finding, increased self-administration of cocaine in dark phase mutant females (genotype main effect: $F_{(1,18)}$=4.09,p=0.058)(Fig.5a), no genotype effect was found in OVX mice (Fs<1)(Fig.5b). Furthermore, daily infusions self-administered during maintenance (days 7-14 based on stable responding onset in Fig.2e and Fig.5ab) were increased in sham mutant females ($t_{(18)}$=1.83,p=0.042)(Fig.5c), but not OVX mutant females compared to WT controls ($t_{(15)}$=1.06,p=0.153)(Fig.5d). These findings suggest that circulating sex hormones could be contributing to the greater effects of *Npas2* mutation seen in female mice.

Increased ΔFosB expression in D1+ neurons in *Npas2* mutant females following dark phase cocaine self-administration

In order to determine which striatal regions might be mediating increased self-administration in *Npas2* mutant females, we measured cocaine-induced expression of ΔFosB, a stable, long-lasting variant of FosB, in the dorsal and ventral striatum. Female mice were trained to self-administer cocaine during either the light or dark phase. In order to control for total cocaine intake, infusions were limited to 25; cocaine self-administration was normalized between WT and *Npas2* mutant mice [no genotype main effect: light ($F_{(1,9)}$=2.73,p=0.133), dark (F<1); genotype x session interaction: light (F<1), dark ($F_{(13,117)}$=2.23,p=0.012, no significant post-hoc tests)], as was total drug intake [light ($t_{(9)}$=1.91,p=0.089), dark (t<1)](Fig.S3). Tissue was harvested 24 hr after the last self-administration session (Fig.6a).

The percentage of D1+ and D1- cells expressing ΔFosB in the NAc core, NAc shell, DLS and DMS were quantified (Fig.6c). No genotype differences were found in ΔFosB expression after light phase self-

administration, but dark phase *Npas2* mutant females had increased ΔFosB expression in the NAc shell (genotype main effect: $F_{(1,9)}$=6.85,p=0.031). In the NAc core and DLS this increase in ΔFosB was specific to D1+ cells [cell x genotype interaction: NAc core ($F_{(1,8)}$=3.97,p=0.082), DLS ($F_{(1,10)}$=5.64,p=0.039)]. No effects of genotype were seen in the DMS. Throughout all regions, cell-type differences were found where ΔFosB expression was higher in D1+ compared to D1- cells [cell-type main effect: NAc ($F_{(1,18)}$=30.47,p<0.0001), DS ($F_{(1,19)}$=27.66,p<0.0001].

Discussion

Overall, loss of NPAS2 function increases vulnerability to substance use, particularly in females during the active phase. Specifically, a non-functional variant of NPAS2 increases cocaine intake and the propensity to self-administer cocaine, as well as the reinforcing and motivational properties of cocaine. Interestingly, NPAS2 seems to affect reward in a circadian-dependent manner, where effects are not only greater and more frequent in females during the dark phase, but sex differences are exacerbated (Table1).

Interestingly, these changes occur without a baseline increase in cocaine reward in female *Npas2* mutants as measured by CPP. While males show contradicting effects of *Npas2* mutation on cocaine reward and self-administration during the light phase, females show a more synergistic phenotype with no change in cocaine reward, but an increase in self-administration. Although it isn't uncommon to find opposing results for these disparate drug-related behaviors (32, 33), it is more common to only detect a change in self-administration where active, volitional, chronic drug intake is being measured, as with females here.

Here we found a collective phenotype across behaviors where females are affected by *Npas2* mutation differently than males: females show no change in CPP, but an increase in the locomotor-activating effects of cocaine and self-administration, whereas males show a decrease, no change, and a slight increase respectively. The only behavior tested that is consistently increased across groups is drug intake during a dose-response analysis. Since cocaine reinforcement is similarly affected, while other addiction-related behaviors are only affected in females, it is possible that estradiol is interacting with increased reinforcement to drive drug taking and seeking in females.

Despite these and previous findings suggesting circadian genes regulate drug taking and reward, little is known about *how* alterations in circadian genes contribute to problematic drug use. In order to examine possible mechanisms, we first ovariectomized female mice before dark phase cocaine self-administration to determine whether circulating ovarian hormones contribute to the effects of *Npas2* mutation. We confirmed that increased self-administration in female mice with non-functional NPAS2 is absent in ovariectomized mice. This suggests that ovarian hormones contribute to the role of *Npas2* in drug taking and could be driving the enhanced effects seen in mutant females compared to males.

It is well known that sex differences exist in circadian rhythms and that circulating estradiol contributes to these differences in rodents. Locomotor activity rhythms vary by sex (reviewed in 35) where wheel-running in

constant darkness shows increased daily activity in females, but longer activity duration in males (35). It is also typical to observe sex differences in the rhythmicity of peripheral circadian genes (*Rev-erbα*, *Cry1* and *Bmal1*)(36). While few studies have been done in brain tissue, recent findings demonstrate that human females show earlier peaks in several circadian genes (*Per2*, *Per3*, and *Bmal1*) in the prefrontal cortex (37). In rodents, the robustness of diurnal variations in clock genes can vary by sex and brain region (38). Sex differences are also extremely robust in the master pacemaker, the suprachiasmatic nucleus (SCN) (39). In rodents, SCN volume is increased in males (40), as are firing rates in the SCN shell during the light phase (41). This parallels our current work and supports the idea that sex differences can be varied across the 24-hr period.

Despite the abundance of evidence indicating an intersection between rhythms and hormones, neuroscience and behavioral research investigating the link between molecular rhythms and sex is limited. Whereas estrogens affecting the output of behavioral rhythms is likely due to sexual dimorphisms in the SCN, evidence that estrogens regulate the expression of circadian clock genes in the extra-SCN brain indicates SCN-independent behaviors could also be affected (37, 38). These findings are relatively recent, therefore very few papers have examined sex differences in how altered circadian rhythms, for example from mutating circadian genes, affect behavior. As with our results, studies that *have* utilized both sexes found significant differences. For example, female *Clock* mutant mice show more robust increases in exploratory and escape-seeking behavior (42) and *Npas2* mutation affects sleep homeostasis differentially in male and female mice (43).

Given the critical role sex plays in circadian rhythms and the behaviors they regulate, future studies examining the role circadian genes play in behavior across sex are imperative. This is particularly true in neuroscience, since there are strong sex differences in the occurrence and features of many psychiatric disorders, including addiction. In humans, women tend to escalate drug use more quickly, have more severe cocaine use disorder when seeking treatment and report more cue-related craving (27, 44–46). It is also important to study extra-hormonal factors, such as chromosomal sex. For example, chromosomal female mice (XX) have a longer activity duration regardless of gonadal state (41). Future studies could take advantage of the available four core genotype mice (47) in order to dissect the organizational, activational and chromosomal effects of sex on circadian rhythms and the behaviors regulated by these rhythms.

We also sought to determine which brain regions might be involved in the increased self-administration in *Npas2* mutant females using ΔFosB expression as a marker of activation. While other Fos family proteins are

transiently induced by drug exposure, ΔFosB is a stable, long-lasting variant induced by chronic administration of drugs of abuse, including cocaine, in the NAc of mice and humans (48). This allows activation due to chronic drug exposure to be measured, in lieu of acute cocaine-induced activation. Here, female WT and *Npas2* mutant mice self-administered cocaine, but drug intake was limited in order to avoid greater cocaine intake, and therefore greater ΔFosB induction, in mutant mice.

We measured ΔFosB expression in D1+ and D1- cells in various subregions of the striatum. The striatum is primarily comprised of medium spiny neurons, subdivided into populations expressing D1 or D2 dopamine receptors (49), which contribute differentially to reward-related behaviors (50–52). For example, activation of D1-expressing neurons in the NAc promotes cocaine preference (52). In addition, cocaine activates D1 and D2 receptors differently (53) and induces ΔFosB specifically in D1 medium spiny neurons (54). NPAS2 is also enriched in D1-expressing neurons (30) and recent work from our lab has shown that *Npas2* knockdown in the NAc increases glutamatergic excitability (AMPA/NMDAR ratio) specifically in D1 cells (55). Therefore, we expected *Npas2* mutation to affect ΔFosB expression in D1+ cells. Indeed, we found that ΔFosB expression was increased in D1+ neurons in the NAc core and DLS of *Npas2* mutant females.

Although ΔFosB expression was increased in mutant mice in both the NAc core and shell (across cell-type), it was confined to the DLS. Interestingly, the DLS is thought to mediate habitual drug seeking, whereas the DMS mediates goal-directed decision making (56). This finding suggests the DLS is more highly recruited, and therefore, habitual decision-making strategies are more heavily relied upon during cocaine self-administration in *Npas2* mutant females than in WT controls. Since a bias towards habitual decision-making strategies is considered an etiological factor in addiction (57), future studies could utilize other behavioral paradigms, such as action-outcome contingency degradation or outcome devaluation, to measure the biases of *Npas2* mutant females towards habitual response strategies.

Increased induction of ΔFosB in *Npas2* mutants was also only found during the dark phase when the behavioral effects of *Npas2* mutation were greatest. *Npas2* expression is normally highest in the NAc during the dark phase around ZT16 (58) when behavior was occurring during these experiments. It stands to reason that mutating *Npas2* would have a greater impact on behavior when mRNA expression is normally elevated. Higher expression suggests a functional necessity for NPAS2, however, a parallel rhythm in protein expression has not

yet been confirmed. Furthermore, drug intake is typically higher during the dark phase, therefore, mice could be more vulnerable to other insults during this time of predisposed drug seeking.

Cocaine-induced expression of the transcription factor ΔFosB is also a hypothesized mechanism underlying the long-term effects of cocaine. Similar to the effects of *Npas2* mutation, overexpression of ΔFosB in D1 neurons in the NAc increases self-administration of cocaine (59, 60), indicating ΔFosB accumulation after drug administration is functionally relevant. Overexpression of ΔFosB also impacts neuronal physiology and morphology, increasing dendritic spine density (61) and decreasing excitatory synaptic strength onto D1-expressing neurons (62). ΔFosB-induced changes in cellular morphology and physiology could underlie increases in addiction-related behaviors in *Npas2* mutant mice, particularly long-term changes which would allow for greater accumulation, such as maintenance levels of self-administration and cue-induced reinstatement. Future studies could examine whether augmented ΔFosB expression in *Npas2* mutant females contributes to increased cocaine self-administration, as well as how *Npas2* mutation affects physiology and dendritic spine morphology in the NAc and DLS, specifically in the dark phase.

Ultimately, these results demonstrate that decreased function in NPAS2 drives vulnerability for substance use through increased cocaine intake, increased locomotor-activating, reinforcing and motivational effects of cocaine, impaired extinction, and enhanced drug seeking during cue-induced reinstatement. NPAS2 appears to affect reward in a circadian-dependent manner and is associated with cocaine-induced ΔFosB expression in the NAc and DLS. These effects could be augmented by circulating sex hormones, leading to an increased effect of *Npas2* mutation in females.

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Figure and Table Legends

Figure 1. Sex differences in cocaine sensitization and place preference in *Npas2* mutant mice. (A) Male *Npas2* mutant mice showed a decrease in conditioned place preference to cocaine (2.5 and 5 mg/kg), whereas females showed no change. (B) One cohort of mice were then tested for locomotor sensitization. All mice showed sensitization to cocaine, but female *Npas2* mutant mice also showed elevated locomotor-activating effects of cocaine. (C) No effect of genotype was seen in male mice. Means+SEMs, *p<0.05, ****p<0.0001, *n*=12-20.

Figure 2. *Npas2* mutant mice self-administer more cocaine, particularly females in the dark phase. *Npas2* mutant mice were trained to self-administer cocaine (0.5mg/kg/infusion). (A) Male and female mice are differentially affected by *Npas2* mutation with only female mutant mice showing increased self-administration when pooled across time of day. (B) Cocaine intake during self-administration was elevated during the dark, or active phase, in all mice. Due to sex and time of day differences, female and male mice were graphed and analyzed separately in order to better demonstrate sex differences. (C) Female and (D) male mutant mice self-administered significantly more infusions at ZT2, during the light phase. A greater effect is seen in females compared to males. At ZT14, during the dark phase (shaded gray), this increase was still observed in (E) females but absent in (F) males. Means+SEMs, $^{\#}$p<0.1; *p<0.05; **p<0.01; ***p<0.001, *n*=10-19.

Figure 3. The reinforcing and motivational properties of cocaine were increased in *Npas2* mutant mice. *Npas2* mutant mice self-administered significantly more infusions of cocaine during a dose-response analysis (0-1 mg/kg/infusion). Neither (A) sex, nor (B) time of day affected how *Npas2* mutant mice self-administered cocaine. Both (E) female (F) and male mutant mice similarly took more infusions during the light phase. During the dark phase, (I) female *Npas2* mutant mice had a slightly greater increase in intake compared to (J) male mutants. During progressive ratio testing, again no (C) sex or (D) time of day differences were found. However, when data were separated by sex and phase similarly to prior experiments, we found that (G) female *Npas2* mutant mice tended to work harder for each infusion of cocaine, as shown by a higher breakpoint ratio, (K) particularly in the dark phase. (H,L) On the other hand, male *Npas2* mutant mice during the light and dark

phase did not show changes in the motivational properties of cocaine. Means+SEMs, #p<0.1; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, *n*=4-11 E-L.

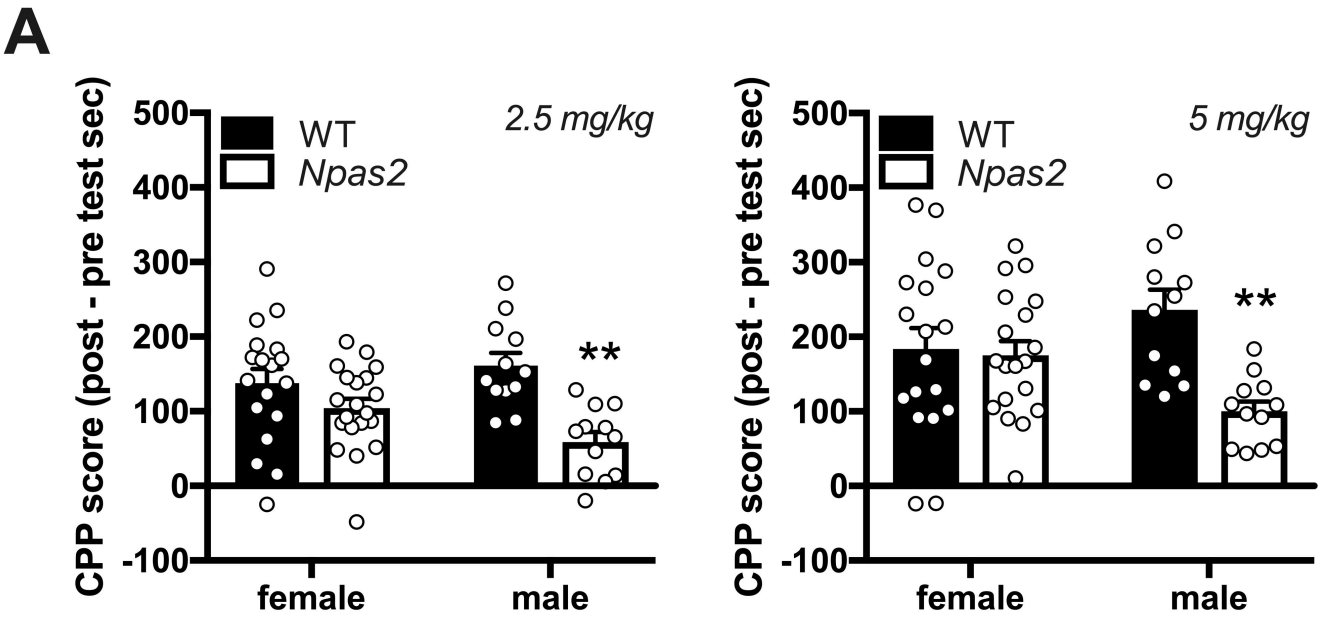
Figure 4. Increased extinction responding and cue-induced reinstatement in female *Npas2* mutant mice during the dark phase. Following progressive ratio, responding on the cocaine-associated lever was extinguished over the course of at least 10 days. Following extinction, responding on the active lever was reinstated with the presence of previously cocaine-associated cues. (A,C) Again, male and female mice are differentially affected by *Npas2* mutation with only female mutant mice showing increased responding during extinction and reinstatement when pooled across time of day. (B) Furthermore, *Npas2* mutants only responded more during extinction in the dark phase, not the light phase, when pooled by sex, with (D) no differences seen in reinstatement across time of day. (E-F) During the light phase, no differences were seen in extinction responding or (G-H) reinstatement between genotypes. However, during the dark phase (I,K) female *Npas2* mutant mice responded significantly more during extinction and cue-induced reinstatement, (J,L) with no differences observed in males. Means+SEMs, *p<0.05; **p<0.01; ***p<0.001, *n*=4-11.

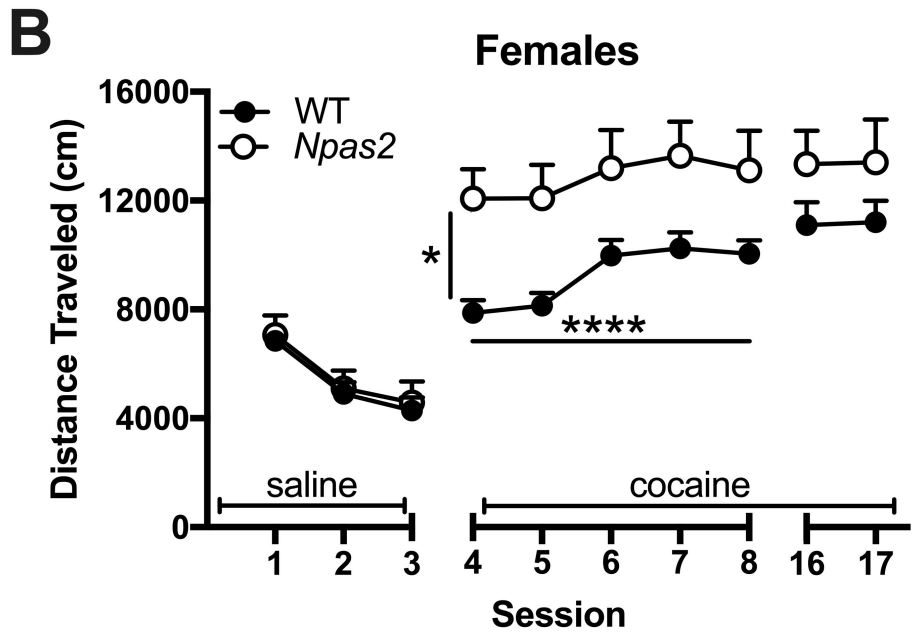
Figure 5. Ovariectomy reversed increased cocaine self-administration in *Npas2* mutant females in the dark phase. A separate cohort of female mice underwent sham surgery or ovariectomy (OVX) before being trained to self-administer intravenous cocaine. (A) Sham surgery treated mice recapitulated our original finding that *Npas2* mutant females take more cocaine than their WT counterparts during acquisition. (B) On the other hand, OVX seemed to reverse this effect with no differences seen between mutant and WT mice. These results were confirmed by average infusions during maintenance responding, which are (C) increased in sham treated *Npas2* mutant mice, but not (D) OVX mice. Means+SEMs, #p<0.1; *p<0.05, *n*=7-12.

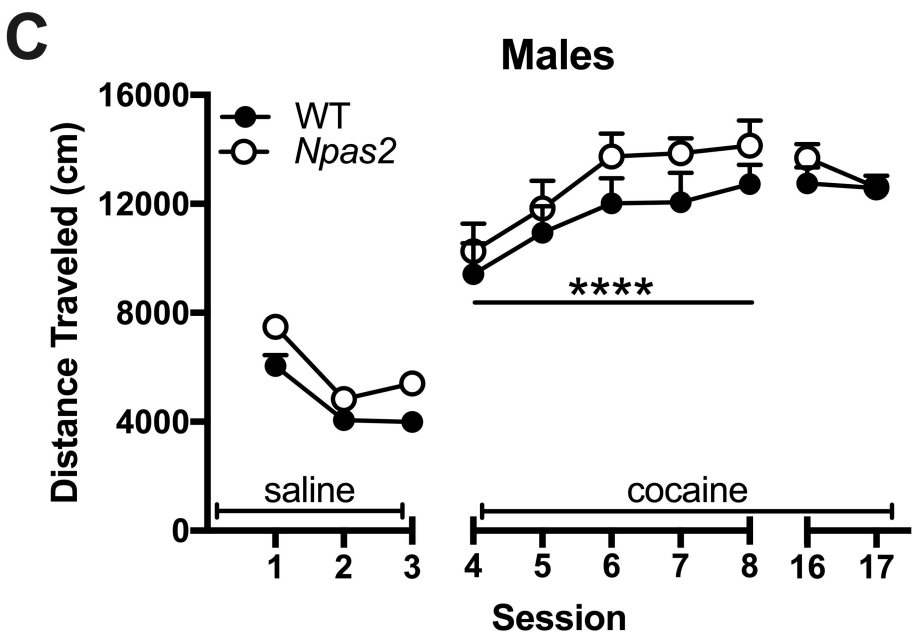
Figure 6. Cocaine induced more ΔFosB in *Npas2* mutant females in the dark phase. (A) Timeline for experimental design, wherein a separate cohort of female mice were trained to self-administer cocaine during either the light phase or dark phase. Infusions were limited to 25 in order to normalize cocaine intake between groups. (B) No differences were found between genotypes in ΔFosB expression following light phase cocaine self-administration. However, following dark phase self-administration ΔFosB expression was increased in

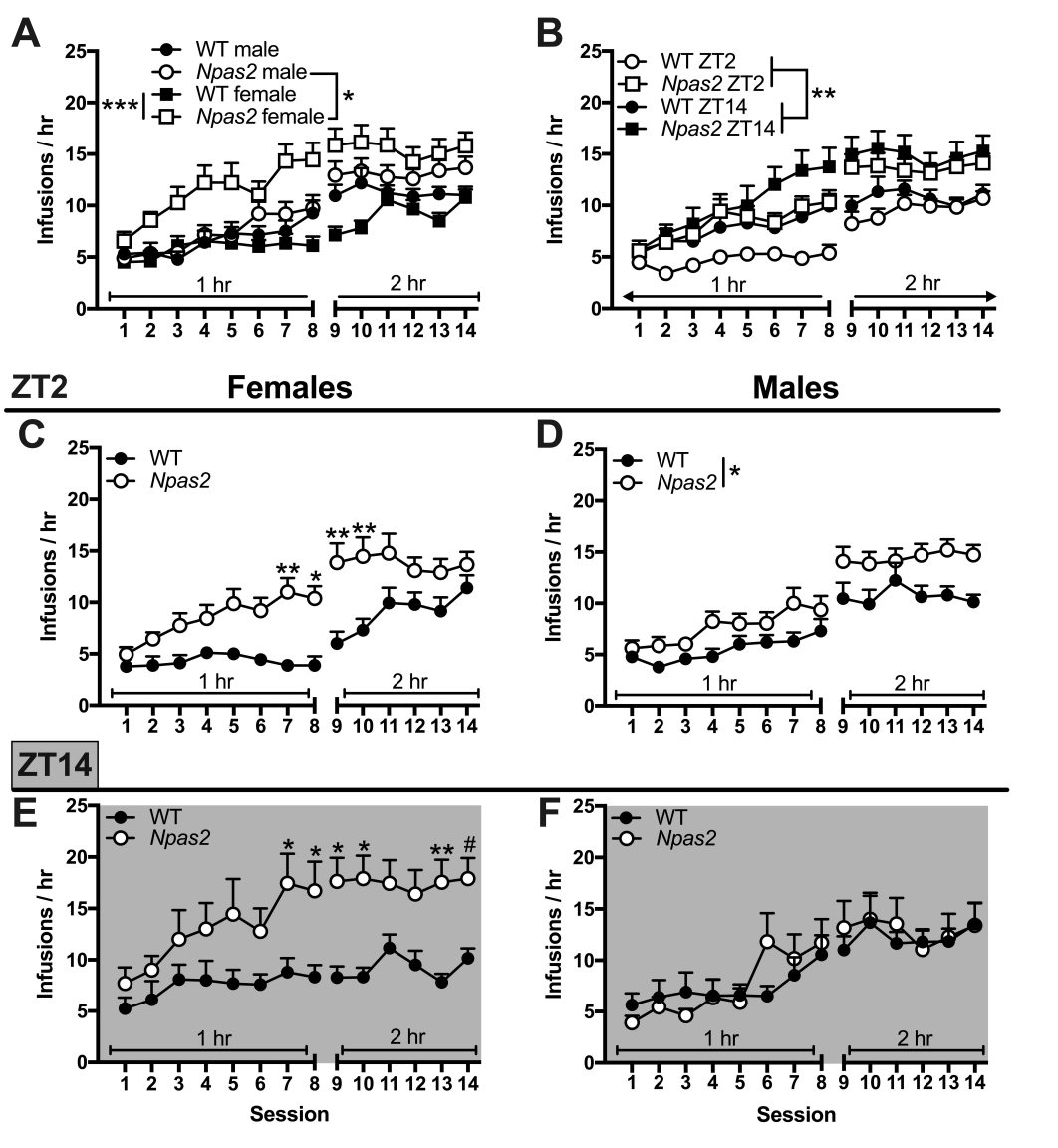
Npas2 mutant females in the NAc core, shell and DLS. This expression was specific to D1+ cells in the NAc core and DLS. Throughout, cocaine-induced **Δ**FosB expression was greater in D1+ compared to D1- cells. (C) Representative staining is shown with the nucleus (DAPI, blue), D1 (green) and **Δ**FosB (red) shown individually and as a composite image with 20 μm scale bar shown. Means+SEMs, $^{\#}p<0.1$; $^{*}p<0.05$, *n*=4-8.

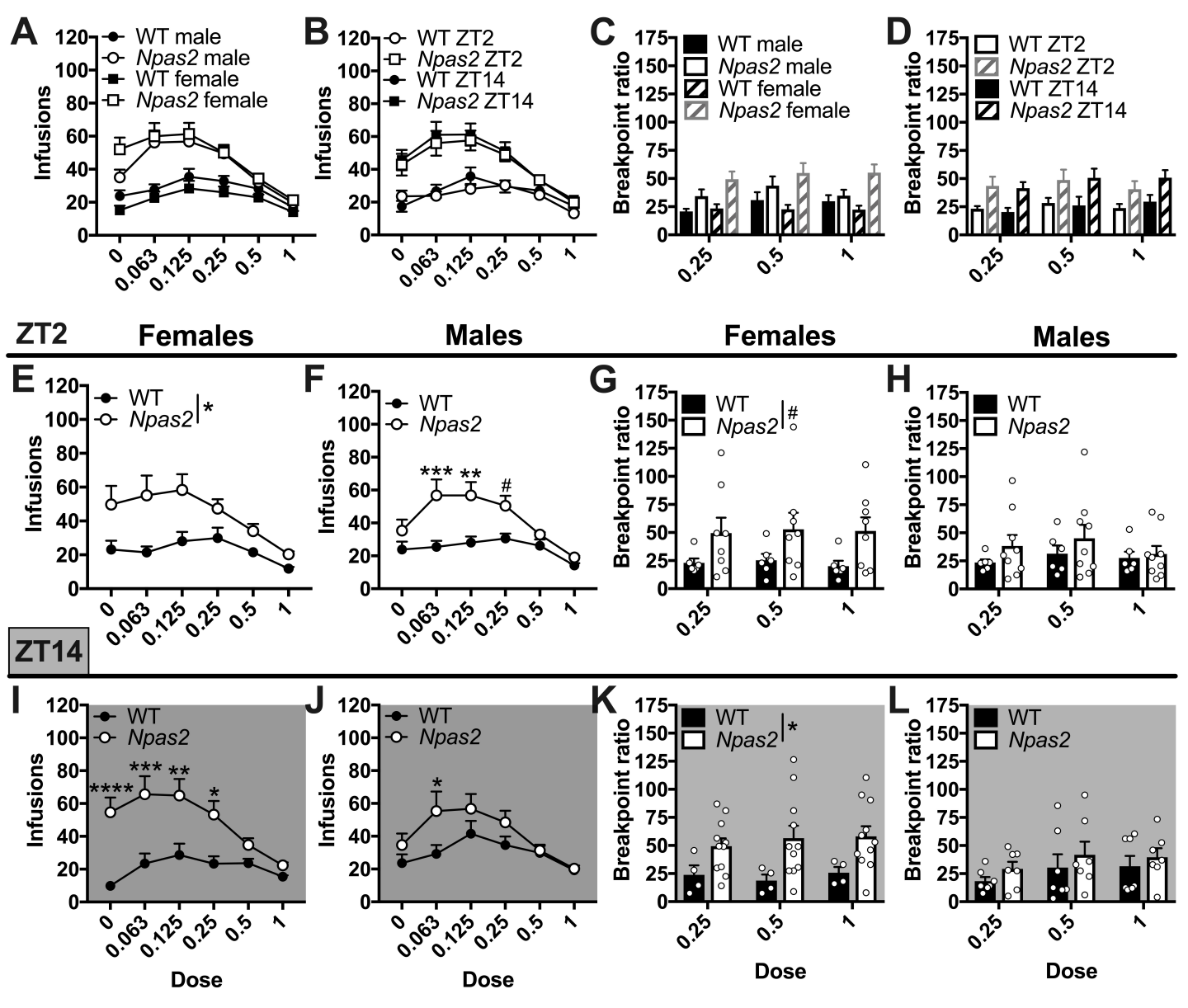
Table 1. Sex differences in *Npas2* mutant mice are exacerbated during the dark phase. Effects are shown for various cocaine self-administration behaviors: acquisition, dose response, progressive ratio, extinction and cue-induced reinstatement. NC indicates no change seen, arrows indicate significant increases by main effects (smaller arrows indicate trends), and circles indicate significant interactions of genotype x another factor, which is explicitly stated inside the circle in the table.

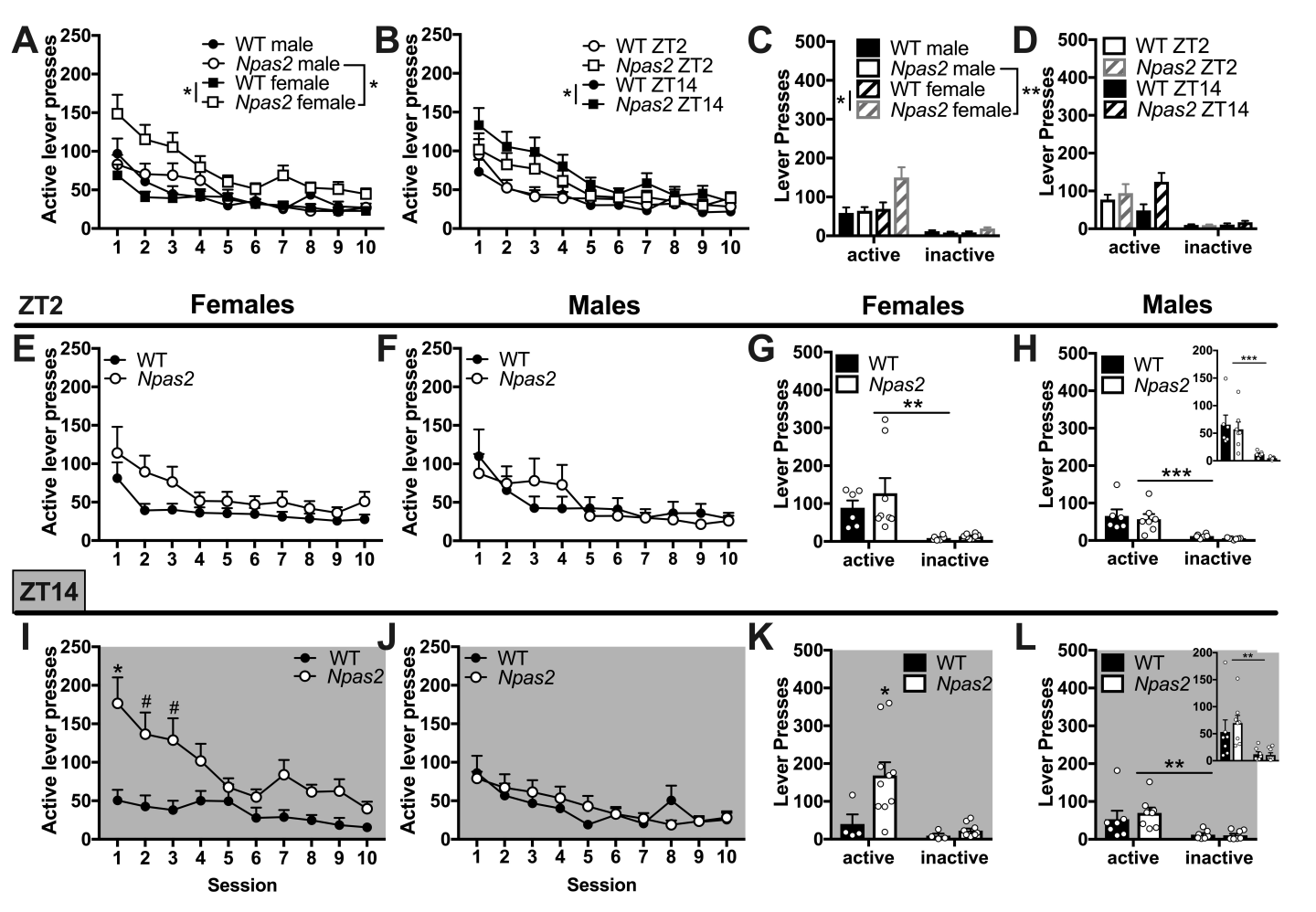

A
CPP score (post - pre test sec)
500
400
300
200
100
0
-100
WT
Npas2
2.5 mg/kg
**
female
male
5 mg/kg
**

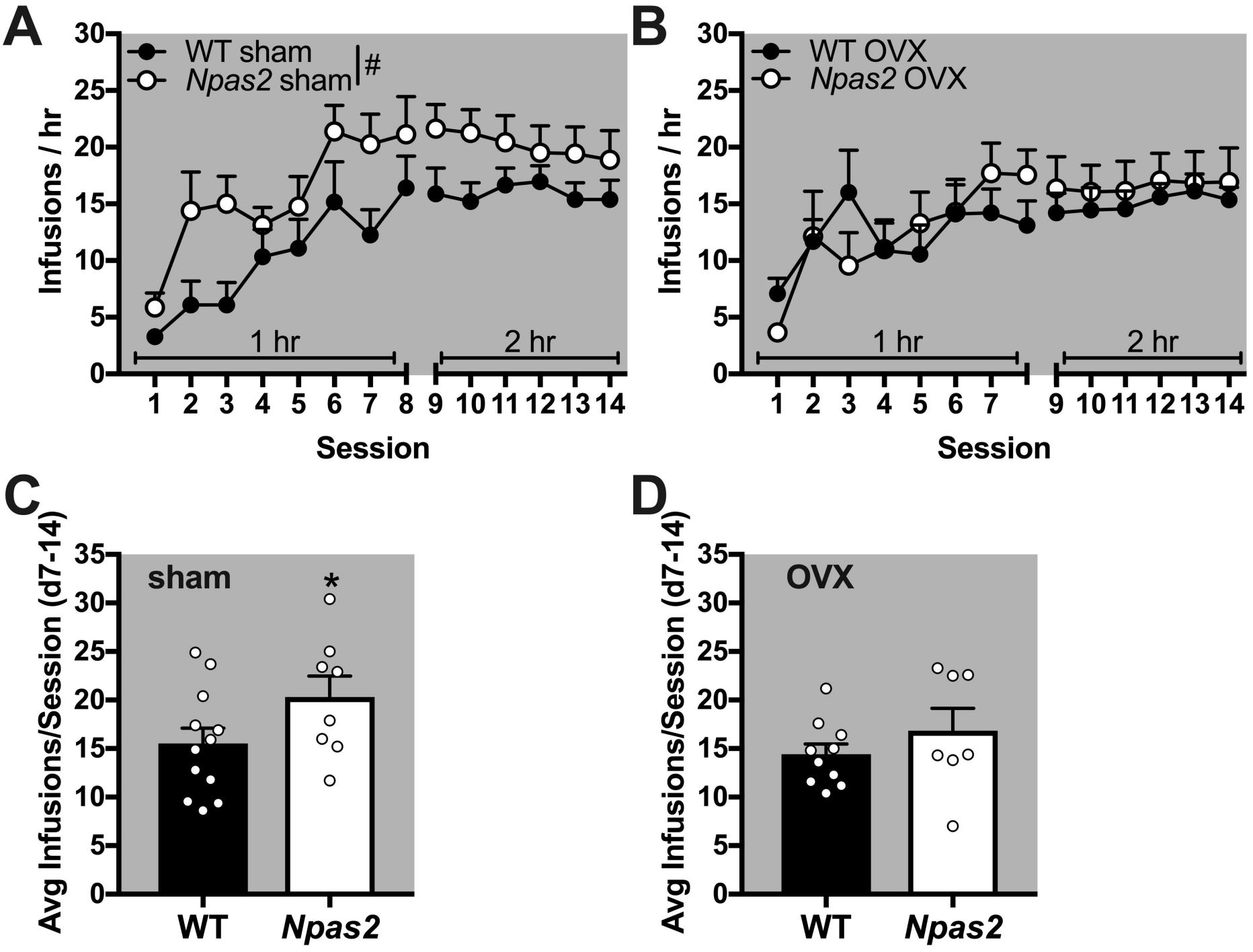

B
Females
Distance Traveled (cm)
16000
12000
8000
4000
0
WT
Npas2
*
****
saline
cocaine
1
2
3
4
5
6
7
8
16
17
Session

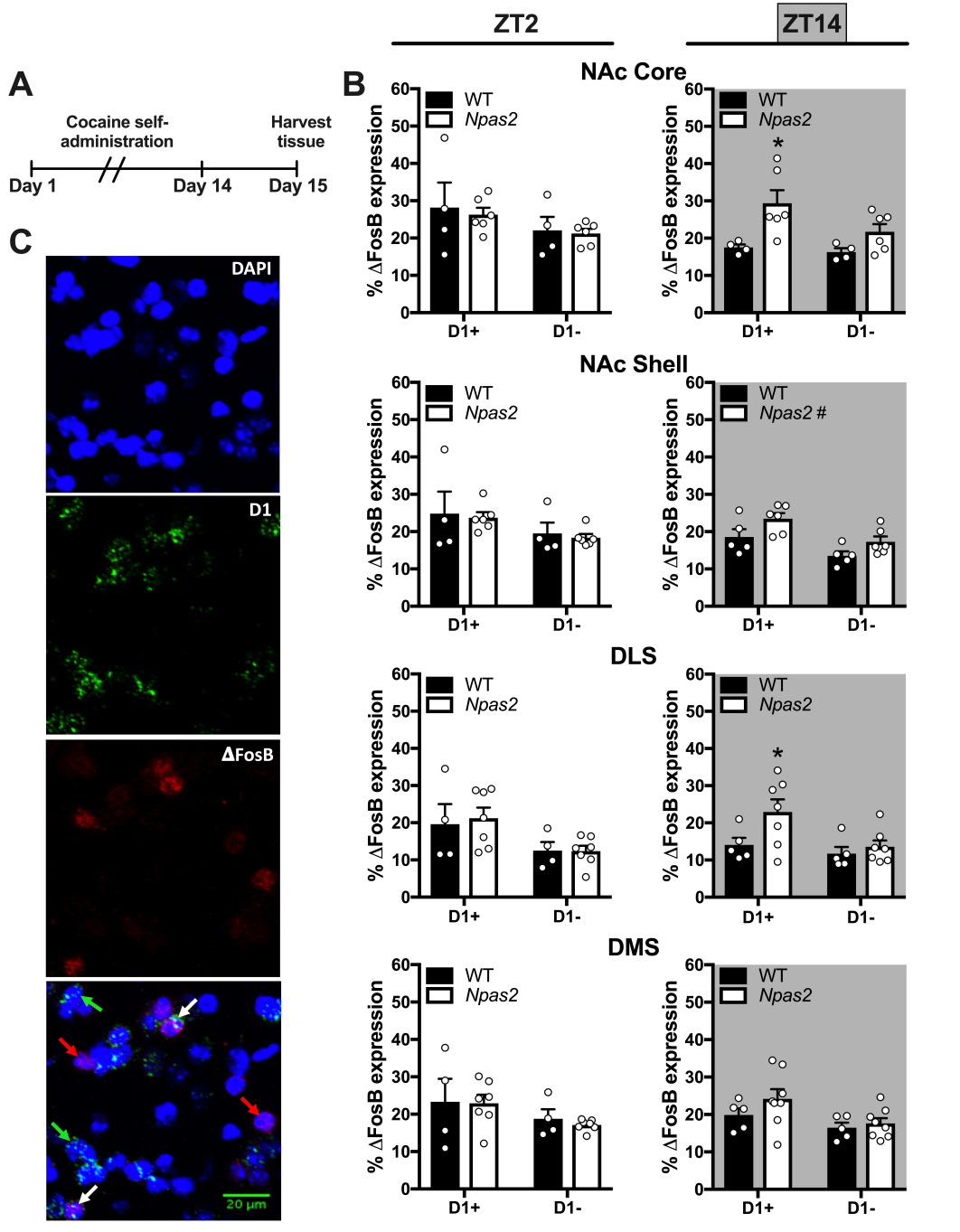

C
Males
Distance Traveled (cm)
16000
12000
8000
4000
0
WT
Npas2
****
saline
cocaine
1
2
3
4
5
6
7
8
16
17
Session


A
WT male
Npas2 male
WT female
Npas2 female
***
*
Infusions / hr
1 hr
2 hr
B
WT ZT2
Npas2 ZT2
WT ZT14
Npas2 ZT14
**
ZT2
Females
Males
C
WT
Npas2
D
*
ZT14
E
F
Session


A
WT male
Npas2 male
WT female
Npas2 female
Infusions
B
WT ZT2
Npas2 ZT2
WT ZT14
Npas2 ZT14
C
Breakpoint ratio
D
ZT2
Females
Males
Females
Males
E
WT
Npas2
*
F
***
**
#
G
#
H
ZT14
I
****
***
**
*
J
K
*
L
Dose


A
B
C
D
WT male
Npas2 male
WT female
Npas2 female
WT ZT2
Npas2 ZT2
WT ZT14
Npas2 ZT14
Active lever presses
Lever Presses
active
inactive
ZT2
Females
Males
Females
Males
E
F
G
H
WT
Npas2
ZT14
I
J
K
L
Session


A
WT sham
Npas2 sham
#
Infusions / hr
1 hr
2 hr
Session
B
WT OVX
Npas2 OVX
Infusions / hr
1 hr
2 hr
Session
C
sham
*
Avg Infusions/Session (d7-14)
WT
Npas2
D
OVX
Avg Infusions/Session (d7-14)
WT
Npas2


ZT2
ZT14
A
Cocaine self-administration
Harvest tissue
Day 1
Day 14
Day 15
B
NAc Core
NAc Shell
DLS
DMS
WT
Npas2
Npas2 #
% ΔFosB expression
D1+
D1-
C
DAPI
D1
ΔFosB
20 µm

NC No change

↑/⇑ Trend / significant increase

◯ Interaction (genotype x __)

	Light		Dark	
	Female	Male	Female	Male
acquisition	◯ Session	⇑	◯ Session	NC
dose response (reinforcing)	⇑	◯ Dose	◯ Dose	◯ Dose
progressive ratio (motivation)	↑	NC	⇑	NC
extinction	NC	NC	◯ Session	NC
reinstatement	NC	NC	◯ Lever	NC