Sex-specific role of the circadian transcription factor NPAS2 in opioid tolerance, withdrawal, and analgesia

Running Title: NPAS2, opioid analgesia and side-effects

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MAIN TEXT
Sex-specific role of the circadian transcription factor NPAS2 in opioid tolerance, withdrawal, and analgesia

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ABSTRACT

Opioids like fentanyl are the mainstay treatment for chronic pain. Unfortunately, opioids high addiction liability has led to the current opioid crisis. This is in part related to long-term use side-effects, including analgesic tolerance (gradual decrease in analgesia), and physical dependence (withdrawal symptoms upon opioid interruption), causing dose-escalation and preventing usage interruption. Altered circadian rhythmicity and sleep patterns are common in patients on opioid therapy. Prior research showed intricate bidirectional interactions between circadian rhythmicity, opioid analgesic efficacy, and side-effects. However, underlying mechanisms are largely unknown. Neuronal PAS domain protein 2 (NPAS2) is a circadian transcription factor that is highly expressed in structures of the central nervous system that modulate pain and opioids. In this study, we used male and female mice expressing non-functional NPAS2 (NPAS2 deficient, NPAS2-/-) to investigate the role of NPAS2 in fentanyl analgesia, tolerance, hyperalgesia, and dependence. In NPAS2-/- mice, we found that thermal pain thresholds, acute analgesia, and tolerance to a fixed dose of fentanyl were largely like wild-type mice. However, female NPAS2-/- mice augmented behavioral state of analgesic tolerance and exhibited significantly more behavioral symptoms of physical dependence. Conversely, only male NPAS2-/- mice had increased fentanyl-induced hypersensitivity, when compared to matched-sex littermate controls. Together, our findings suggest sex-specific effects of NPAS2 signaling in the regulation of fentanyl-induced tolerance, hyperalgesia, and dependence.

KEYWORDS
Circadian genes, NPAS2, fentanyl, opioid side-effects, analgesic tolerance, physical dependence, sex differences
INTRODUCTION

Prescription opioids are potent analgesics widely used to treat pain. Extended use, often necessary to treat chronic pain, is a risk factor for developing tolerance (e.g., reduced effect of analgesia), physical dependence (i.e., withdrawal symptoms during periods of abstinence), and pain hypersensitivity (i.e., opioid-induced increases in pain sensitivity)\(^1,2\), and serves as a risk factor for opioid use disorder\(^3\). Hallmarks of opioid dependence and opioid use disorder are profound disruptions to sleep and circadian rhythms that persist during abstinence and withdrawal\(^4-7\). In line with this, previous evidence suggests opioid tolerance, dependence, and analgesia\(^8-12\) are modulated by sleep and circadian rhythms. Altered circadian rhythms and sleep disruptions are commonly observed in patients being treated with opioids\(^13\). For example, sleep disturbances are a known indicator of opioid withdrawal symptoms in patients attempting to discontinue their opioid treatment\(^14\). Sleep and circadian rhythm disruptions may also contribute to relapse risk in opioid abstinent patients\(^15,16\). However, our understanding of the molecular pathways that underlie the relationship between circadian rhythms and opioid tolerance and withdrawal is limited.

Nearly every cell in the body expresses the necessary machinery that controls circadian rhythms at the molecular level\(^17\). The molecular clock is composed of a series of interacting transcriptional and translational feedback loops. Transcription is driven by heterodimers of CLOCK (Circadian Locomotor Output Cycles Kaput Protein), or NPAS2 (Neuronal PAS domain protein 2), with BMAL1 (brain muscle aryl nuclear translocase like-1), that bind to enhancer elements in gene promoters. These heterodimers of circadian transcription factors (CLOCK or NPAS2 and BMAL1) drive the transcription of circadian genes, \(Cry1,2\) (Cryptochrome) and \(Per1,2\) (Period). Accumulation of CRYs and PERs in the cytoplasm over 24-hours initiate the formation of their own dimers that eventually translocate to the nucleus to inhibit their own transcription via interactions with BMAL1 heterodimers\(^18\). Molecular clocks are critical to the function of most peripheral organs and many regions in the brain\(^19\).

In peripheral tissues and the central nervous system, the molecular clock modulates the circadian expression of endogenous opioid peptides and opioid receptors\(^20-23\). Opioids also impact the expression of circadian genes in the brain. For example, acute and chronic administration of opioids alters circadian genes in the hypothalamus\(^24-26\), while withdrawal from opioids alters the rhythmic expression of circadian genes in the midbrain and striatum\(^26-28\). Recent findings demonstrate circadian genes, including \(Per1\) and \(Per2\), regulate opioid reward\(^29\), tolerance, and withdrawal\(^30\). In a tissue-dependent manner, \(Per1\) and \(Per2\) transcription are driven by BMAL1 dimerization with CLOCK or NPAS2\(^31-33\). Notably, the circadian transcription
factor NPAS2 is enriched in major neural substrates of opioid-induced tolerance, dependence, and hyperalgesia, including the spinal cord, primary sensory neurons, and within the subregions of the striatum, including the nucleus accumbens. However, to our knowledge, the involvement of NPAS2 signaling in the emergence of opioid side-effects has not yet been studied.

In the present study, we investigated the role of NPAS2 in opioid tolerance, dependence, and analgesia, using the synthetic opioid, fentanyl. Fentanyl is a widely prescribed opioid with high addiction potential, found in most drug overdose deaths in the United States. Thus, further investigation into the potential pathways mediating the development of fentanyl-induced tolerance and dependence is imperative, as new therapeutic approaches are designed to improve opioid treatments and mitigate secondary effects of opioids. To explore the potential relationship between NPAS2 and opioids, we assayed behavioral phenotypes of fentanyl-induced tolerance, dependence, and hyperalgesia in NPAS2 deficient male and female mice. These transgenic mice possess a LacZ reporter in replacement of the basic Helix-loop-Helix (bHLH) domain required for NPAS2 to directly bind DNA and regulate transcription. Therefore, these NPAS2-/- mice retain the expression of NPAS2 protein, while lacking the ability to bind DNA and drive transcription. Overall, our findings reveal sex-specific effects of NPAS2 deficiency on thermal nociceptive thresholds, acute anti-nociception, and development of tolerance to fentanyl, as well as behavioral markers of physical dependence and pain hypersensitivity.
MATERIALS & METHODS

Animals

Adult male and female NPAS2 deficient (NPAS2-/-) mice and their respective wild-type (WT) littermates were used (aged 7-14 weeks) \( ^{32} \). Initial breeders were generously provided by Dr. David Weaver (University of Massachusetts Medical School). NPAS2-/- mice have a LacZ-Neo fusion gene in inserted into exon 2, replacing the locus that encodes the bHLH domain. Replacing the bHLH domain renders the NPAS2 protein deficient of capability to directly bind DNA and mediate NPAS2-dependent gene transcription. Mice were maintained on C57BL6/J background (The Jackson Laboratory, 000664; backcrossed to at least N10). Mice were group housed (2-4 mice per cage) and maintained under 12h:12h standard light-dark cycle with ad libitum access to food and water. Experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh School of Medicine.

Drugs

Fentanyl hydrochloride (NIH NIDA, Bethesda, MD) and Naloxone (Sigma, St Louis, MO) were dissolved in sterile saline vehicle (0.9%) under a biosafety cabinet. Fentanyl was administered intraperitoneally (i.p., 10ml/kg, v/w).

General Procedures and Experimental Timeline

Throughout the experiments, experimenters were blind to both the treatment and genotype groups. Prior to the each of the behavioral assays, mice were habituated to experimental rooms for at least one hour per day for three consecutive days. During habituation, mice were gently handled by the experimenters to prepare for various procedures required to conduct the behavioral assays. For the behavioral assays 9 to 11 mice per genotype per used. The experimental timeline is described in Figure 1. On day 0, baseline thermal thresholds were measured from tail flick latencies (TFL) averaged over three trials per mouse. On day 1, mice underwent a first dose-response procedure with fentanyl (i.p.). TFLs were measured 15 mins after fentanyl administration. The fentanyl dose was then doubled every 20 mins, and tested again until TFLs reached the threshold of 10 sec. The following fentanyl doses were administered on day 1: 10, 20, 40, 80, 160 and 320 \( \mu \)g/kg. Between days 4 through 8, mice were tested for the development of tolerance and hyperalgesia. Mice were administrated fentanyl (320 \( \mu \)g/kg, i.p.) twice daily at ~ZT2 (900h, lights on at 700h) and ~ZT9 (1600h). Testing of TFL was performed before hyperalgesia and 15 minutes after tolerance testing at the ZT2 administration. On day 11, mice a second dose-response was conducted using the
following fentanyl doses: 160, 320, 640 and 1280 μg/kg. Finally, on day 12, mice underwent naloxone-precipitated withdrawal. Mice were first administered fentanyl (320 μg/kg, i.p.), then ~15 mins later, naloxone (10mg/kg, i.p.). Mice were immediately placed in a novel cage and withdrawal behaviors were recorded.

**Tail-immersion test for fentanyl-induced analgesia and tolerance**

The tail-immersion behavioral assay was used to assess thresholds of thermal nociception, analgesia, and tolerance to fentanyl. A container filled with water maintained at 50±0.5°C using a thermoregulated water bath. Part of the mouse's tail was immersed in the water and the TFL was recorded with a maximum immersion limit of 10 seconds to avoid tissue damage. After tasting, mice were immediately returned to their home cage. TFL measurements were repeated 2-3 times per mouse at 2 mins intervals.

**Naloxone-precipitated fentanyl withdrawal**

Following the development of physical dependence to fentanyl, behavioral signs of withdrawal from chronic fentanyl were evaluated using the opioid receptor antagonist, naloxone. Mice were administered fentanyl (320 μg/kg, i.p.), then naloxone (10mg/kg, i.p.) ~15 mins later. Withdrawal behaviors were recorded for 10 mins in a novel home cage, which included escape jumps, wet-dog shakes, paw-shakes/grooming, teeth chattering. Presence of diarrhea was also recorded. Behaviors were compiled to calculate cumulative withdrawal scores as follows: Jumps: 1-10 = 2, 11-20 = 4, 21-30 = 6, 31-40 = 8, 41-50 = 10, etc.; wet dog shakes: 1-2 = 2, 3-10 = 4, 10 and more = 6; and presence of teeth chattering, paw shakes and diarrhea were scored a 2.

**Statistical analyses**

A combination of statistical analyses was used for the behavioral assays. Tolerance measures were analyzed using two-way mixed effect ANOVA (Time and Treatment) followed by Tukey’s multiple comparison post-hoc analyses. Thermal nociceptive thresholds, EC50s and withdrawal behaviors were analyzed using one-way ANOVA, followed by Tukey’s post-hoc multiple comparison tests, when appropriate. Dose-response curves were generated using a non-linear regression of log-transformed values compared to normalized values from 0 to 100. Comparisons of rightward shifts between mouse genotypes were done by calculating the ratio between EC50 obtained on day 11 / EC50 obtained on day 1. Unpaired t-test comparisons were performed to determine difference of rightward shift between NPAS2-/- and WT mice of the
same sex. Data were analyzed using GraphPad 9.0 and considered statistically significant if \( p \leq 0.05 \).
RESULTS

Impact of NPAS2 deficiency on thermal thresholds, fentanyl acute analgesia, fentanyl-mediated tolerance, and hypersensitivity

The impact of NPAS2 deficiency on thermal thresholds and development of tolerance and hypersensitivity with chronic fentanyl, was evaluated by measuring Tail Flick Latencies (TFLs) in NPAS2-/- and WT mice before and after a 5-day fentanyl administration regimen (Figure 1). The acute analgesic effect of fentanyl measured on day 1 was similar between NPAS2-/- and WT male (Figure 2A) and female (Figure 2B) mice. Additionally, all groups had a similar progressive reduction in TFLs over time without a significant difference between genotypes in males (Figure 2A, 2-way ANOVA, Interaction: $F_{5, 90} = 0.1601$, $P = 0.9764$, Days: $F_{3.058, 55.04} = 48.38$, $P < 0.0001$, Treatment: $F_{1, 18} = 0.7066$, $P < 0.0001$) or females (Figure 2B, 2-way ANOVA, Interaction: $F_{5, 80} = 2.233$, $P = 0.0590$, Days: $F_{3.211, 51.37} = 50.40$, $P < 0.0001$, Treatment: $F_{1, 16} = 0.001806$, $P < 0.9666$). Similarly, baseline TFLs measured in opioid naïve animals prior to fentanyl administration, were largely similar between NPAS2-/- and WT male and female mice (Figure 2C), suggesting that NPAS2 has minimal to no measurable impact on thermal nociceptive thresholds in opioid naïve mice (1-way ANOVA, $F_{3, 34} = 0.8418$, $P = 0.4805$). However, thermal thresholds measured prior to fentanyl administration on day 1 compared to day 5 revealed NPAS2-/- male mice developed thermal hypersensitivity, an effect not seen in WT male mice. Conversely, both NPAS2-/- and WT females developed thermal hypersensitivity (Figure 2D, day 5 – day 1 TFLs, $F_{3, 34} = 2.765$, $P = 0.0568$). Together, these findings support the possible involvement of NPAS2 in hyperalgesia development in a sex-specific manner.

Impact of NPAS2 deficiency on changes in fentanyl potency due to tolerance

To further test the impact of NPAS2 deficiency on fentanyl analgesic potency, we performed two dose-response procedures on male and female NPAS2-/- and WT mice. The first dose-response was conducted on day 1 when mice were naïve to opioids (Figure 3A), and the second dose-response was conducted on day 11, after mice had received the tolerance-inducing 5-day fentanyl administration regimen (Figure 3B). Dose-responses performed on day 11, revealed a rightward shift of the fentanyl dose-response curves in all groups, as compared to dose-responses measured on day 1 (Males, Figure 3A, Females, Figure 3B). Therefore, our regimen induced a robust state of analgesic tolerance in all mice. Based on best-fit values of these curves, we calculated effective concentration 50 (EC50) values, which represent the concentration of fentanyl that gives half-maximal analgesia, (EC50 day 1 values: WT males: 51.75 μg/kg; NPAS2-/- males: 61.25 μg/kg; WT females: 71.24 μg/kg; and NPAS2-/- females:
63.01 µg/kg. EC50 day 11 values: WT males: 273.4 µg/kg, NPAS2-/- males: 269.6 µg/kg, WT females: 291.0 µg/kg and NPAS2 females: 346.0 µg/kg). For EC50 values, no significant differences between NPAS2-/- and WT mice were found in pre-tolerance fentanyl potency on day 1 (Figure 3C, one-way ANOVA, F_{3,33} = 0.4501, P = 0.7189). On day 10, EC50 values were also similar between genotypes and among both sexes (Figure 3D, 1-way ANOVA, F_{3,33} = 2.056, P = 0.1251). Finally, calculation of the rightward shift factor between naïve (day 1) and tolerant (day 11) mice (EC50 day1 – EC50 day11), revealed that degree of rightward shift of curves in WT and NPAS2-/- males were similar (Figure 3E, two-tailed t-test, t=0.6714, df=18, p=0.5105). Conversely, comparison of shift factors in female mice revealed a greater shift in NPAS2-/- females (6.069 rightward shift average), than in WT females (4.382 rightward shift average) that was close to statistical significance (Figure 3F, two-tailed t-test, t=2.096, df=16, p=0.0523). Overall, our findings point toward a possible impact of NPAS2 deficiency on fentanyl potency in female, but not male, mice.

**Impact of NPAS2 deficiency on naloxone-precipitated withdrawal responses in fentanyl-dependent mice**

Following the post-tolerance dose-response regimen of fentanyl, mice received a fentanyl challenge, followed by naloxone, to precipitate withdrawal and induce dependence behaviors. Overall, female NPAS2-/- mice displayed significantly more withdrawal behaviors than NPAS2-/- and WT male mice. Fentanyl naloxone precipitated withdrawal led to more jumps in NPAS2-/- females compared to WT females (Figure 4A, 1-way ANOVA, F_{3,34} = 4.646, P = 0.0079), while these behaviors were similar between genotypes in males. The number of wet-dog shakes were overall unchanged in NPAS2-/- mice (Figure 4B), although female mice had significantly more wet-dog shakes than males, regardless of genotype (Figure 4B, 1-way ANOVA, F_{3,34} = 3.425, P = 0.0279). In addition, teeth-chattering episodes were more frequent in NPAS-/- mice as compared to WT mice, but this effect was not significant (Figure 4C, 1-way ANOVA, F_{3,34} = 2.624, P = 0.0663). Finally, no difference was observed in the number of grooming/paw shaking episodes across groups (Figure 4D, 1-way ANOVA, F_{3,34} = 0.8478, P = 0.4774). Global withdrawal scores, which involved the number of jumps, along with the number of teeth-chattering, wet dog shakes, grooming/paw shakes episodes, showed that NPAS2-/- females displayed significantly more withdrawal behaviors as compared WT female mice and male mice from both genotypes (Figure 4E, 1-way ANOVA, F_{3,34} = 6.543, P = 0.0013). Together, these results support a role of NPAS2 in the development and exhibition of physical dependence primarily in female than in male mice.
DISCUSSION

Long-term opioid use for the treatment of chronic pain, is hampered by analgesic tolerance\textsuperscript{42}, physical dependence\textsuperscript{43}, and profound disruptions of sleep and circadian rhythms \textsuperscript{4-7}. Previous studies implicated circadian regulation of pain, tolerance, and physical dependence \textsuperscript{11,12}, suggesting bidirectional interactions between circadian rhythms and opioids \textsuperscript{9}. NPAS2 is a circadian gene enriched in brain regions and spinal cord involved in pain and opioids, and previously shown to be involved in psychostimulant reward \textsuperscript{44,45}. However, the role of NPAS2 in opioid tolerance and dependence had yet to be established. To address this, we used NPAS2-deficient mice to investigate the involvement of NPAS2 on fentanyl-mediated tolerance, hypersensitivity, and physical dependence. Overall, we found that thermal pain thresholds, acute fentanyl analgesia, and fentanyl tolerance development were unchanged in male and female NPAS2-/- mice. Interestingly, NPAS2 deficiency led to a decrease in fentanyl potency after tolerance developed and led to markedly more symptoms of physical dependence only in female mice. Conversely, NPAS2 deficiency was associated with increased fentanyl-induced hypersensitivity in male compared to female mice.

Our results are consistent with prior studies which also evaluated the impact of genetic deletion of circadian genes on pain and opioids \textsuperscript{29,30}. In these studies, mice with global deletion of \textit{mPer1} (\textit{mPer1-KO})\textsuperscript{29} or \textit{mPer2} (\textit{mPer2-KO})\textsuperscript{30} genes showed no change in thermal pain thresholds and acute analgesia. This is similar to our observations with NPAS2-/- mice and suggests that these circadian genes may not be directly involved in these behaviors. Conversely, \textit{mPer1} and \textit{mPer2} clock genes were differentially involved in tolerance and dependence, as \textit{mPer2-KO} promoted morphine tolerance and mitigated physical dependence\textsuperscript{30}, while \textit{mPer1-KO} did not affect tolerance or physical dependence\textsuperscript{29}. In our study, which included both males and females, NPAS2-/- mice did show altered tolerance development to a fixed dose of fentanyl across both sexes. In addition, we also evaluated fentanyl potency, by conducting dose-response tests before and after tolerance development. Strikingly, NPAS2-/- mice developed higher analgesic tolerance compared to controls, an effect only observed in females. Together, these data could suggest that circadian genes are differentially involved in tolerance, with \textit{mPer2} being involved in its development, \textit{Npas2} in its expression, and \textit{mPer1} likely not involved. However, caveats of studies investigating the roles of \textit{Per} genes include the absence of testing opioid potency in tolerant animals with a dose-response assay, along with these studies including only male mice. Because \textit{Npas2} and \textit{Per} genes are both expressed in structures involved in tolerance such as the spinal cord \textsuperscript{39,46} or the NAc\textsuperscript{47}, and NPAS2 can...
directly regulate the transcription of *Per* genes, involvement of each of these circadian genes in tolerance remains a possibility.

Sex specific effects of NPAS2 deficiency were also observed with physical dependence symptoms. NPAS2-/- females showed more withdrawal behaviors than controls, also more than NPAS2-/- males. Conversely, when testing fentanyl-induced hypersensitivity, a symptom also emerging during opioid withdrawal, NPAS2-/- females were similar to WT females, while NPAS2-/- males had more pronounced hypersensitivity than WT males. Together, these results suggest that tolerance, physical dependence, and opioid-induced hypersensitivity behaviors could be modulated by NPAS2 signaling in a sex specific manner. Involvement of NPAS2 in these behaviors could be supported by the fact that NPAS2 expression is enriched in the NAc. The NAc is involved in tolerance, physical dependence, and opioid-induced hyperalgesia. NPAS2 modulates dopaminergic and glutamatergic neurotransmission in the striatum, both of which are altered during opioid tolerance and withdrawal. Interestingly, changes in the expression of circadian genes in the NAc were shown to occur in rodents with opioid-induced hyperalgesia during a state of withdrawal. Thus, together with our current findings, NPAS2 may modulate opioid-related behaviors and involve dopaminergic and glutamatergic signaling in the NAc. Ongoing and future studies are exploring possible involvement of NPAS2 in the NAc and the dopaminergic and glutamatergic neurotransmission in opioid tolerance and dependence.

Importantly, our results illustrate the importance of performing these further including both males and females to examine intersectional consequences between sex and opioid effects, and between sex and circadian genes. This is also supported by several previous studies, which examined whether sex could have a differential impact on opioid behaviors. Analgesic effect of opioids was shown to be variable depending on sex, with a higher and longer-lasting effect in male than female rodents, although, other studies did not find a sex difference in opioid analgesia. In addition, sexual dimorphism in opioid tolerance has not been extensively studied in rodents, yet the studies that have, report higher tolerance in males than females, with tolerance developing faster in females than males. However, these findings have not been supported by other studies. Finally, sexual dimorphism in opioid dependence-mediated withdrawal behaviors were also reported in rodent studies, with overall more dependence in WT males than WT female rats. Inconsistent findings between men and women have also been reported in humans. In our current study, we did not observe sex-related differences in opioid analgesia, tolerance development and expression, dependence, and hyperalgesia, between WT male and female mice. Together with these
aforementioned studies, our data illustrates the lack of consensus on the impact of sex on opioid behaviors, and thus require further investigation.

Nevertheless, in our study, we specifically examined intersectional consequences of NPAS2 deficiency and sex on opioid analgesia, tolerance, and dependence behaviors. Interestingly, NPAS2-/- females developed markedly more physical dependence behaviors and marginally more profound tolerance than female WT littermates, which was not observed in males. However, NPAS2 deficiency had no consequences on hyperalgesia development in females while it exacerbated that symptom in males. Thus, our data indicates that sex differences in our study are related to interacting effects between NPAS2 deficiency and sex. This could be explained by the fact that sex differences are also known to exist in circadian rhythms\textsuperscript{71,72} and in circadian genes rhythmicity between males and females in brains of humans\textsuperscript{73} and of rodents\textsuperscript{74}.

Our results are also consistent with prior studies which examined intersectional consequences between sex and circadian genes such as \textit{Clock} or \textit{Npas2}\textsuperscript{44,75}. Interestingly, \textit{Npas2} deletion had higher impact on cocaine reward and self-administration behaviors in female mice\textsuperscript{44}. More profound consequences on females than males could be explained by levels of circulating hormones, as sex differences in cocaine self-administration were abolished in ovariectomized females\textsuperscript{44}. This was consistent with the fact that circulating estrogens were shown to be essential in orchestrating rhythms of circadian genes in the SCN\textsuperscript{76}. In addition, estrogen signaling has been shown to influence opioid tolerance and dependence behaviors\textsuperscript{77}. Thus, female circulating hormones could also be involved in the sexually dimorphic consequences of NPAS2 deficiency on opioid tolerance and dependence. Further studies examining the mechanisms of interaction between NPAS2, opioids sexual hormones are now warranted.

Overall, our study and prior studies examining the involvement of circadian genes deletion on opioid analgesia, tolerance, and dependence, show a differential involvement of \textit{Npas2} and \textit{Per} genes\textsuperscript{29,30}. However, \textit{Npas2} and \textit{Per} genes bi-directionally influence each other’s expression levels, leading to variations in expression that follow circadian rhythmicity\textsuperscript{33}. Importantly, rhythmic expressions of circadian genes follow different circadian phases\textsuperscript{78,79}. Therefore, it is possible that rhythmic expression could have an impact on the involvement of each gene. In our study, we examined the role of NPAS2 deficiency at a similar time of day (ZT2) as the mPer1-KO and mPer2-KO studies (ZT3-5)\textsuperscript{29,30}. Ongoing studies are evaluating the impact of time of day and circadian genes on opioid-related behaviors.
It is important to note as well that while prior studies used morphine to evaluate the impact of \textit{mPer1} and \textit{mPer2} deletion, we used fentanyl to evaluate impact of NPAS2 deficiency. Although both opioids mediate analgesia via the mu-opioid receptor \textsuperscript{80,81}, they can activate different MOR downstream signaling pathways \textsuperscript{82-84}. We could therefore speculate that fentanyl and morphine may recruit different circadian genes and downstream signaling pathways involving the mu-opioid receptor.

In conclusion, our study provides evidence for a differential role of NPAS2 signaling in fentanyl mediated behaviors, with high impact on physical dependence and marginal effect on tolerance. Importantly, NPAS2 deficiency modulated these behaviors in a sexually dimorphic manner, with female mice more profoundly affected than males. Identification of NPAS2-controlled genes and signaling pathways that modulate opioid behaviors and that may interact with substrates of opioid tolerance and dependence, could provide better insight on understanding the impact of clock genes and circadian rhythmicity on the chronic use of prescription opioids in patients.
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**FIGURE LEGENDS**

**Figure 1. Experimental timeline.** All animals underwent the same procedures. On day 0, animals were tested for thermal nociceptive thresholds (baselines) with the tail flick (TFL) assay. On day 1, a first dose-response of the fentanyl analgesic effect on TFL was performed. The 5-day fentanyl administration regimen involving two fentanyl i.p. injections per day was conducted from day 4 to day 8. Tolerance and OIH development were evaluated simultaneously. On day 11, a second fentanyl dose-response was conducted. This series of experiments concluded with the naloxone-precipitated withdrawal assay on day 12.

**Figure 2. NPAS deficiency does not alter thermal nociceptive thresholds or fentanyl analgesic tolerance development but promotes fentanyl-induced hypersensitivity in males only.** Development of tolerance to a fixed dose of fentanyl (320μg/kg) administered twice
a day for 5 days was assessed my measuring tail flick latencies (TFLs), daily in males (A), and females (B). Two-way ANOVAs, males, N = 9-11, Interaction: $F_{5,90} = 0.1601$, $p = 0.9764$, Days: $F_{3.058, 55.04} = 48.38$, $p < 0.0001$, Treatment: $F_{1, 18} = 0.7066$, $p < 0.0001$; females, N = 11, Interaction: $F_{5,80} = 2.233$, $p = 0.0590$, Days: $F_{3.211, 51.37} = 50.40$, $p < 0.0001$, Treatment: $F_{1, 16} = 0.001806$, $p < 0.9666$. (C) Baseline thermal nociceptive thresholds measured prior to the beginning of fentanyl injections. One-way ANOVA, N = 9-11, $F_{3, 34} = 0.8418$, $p = 0.4805$. (D) Comparison of baseline thermal threshold measured on day 0, to threshold measured on day 5 before the fentanyl injection. Data represented as delta of TFL values measured on Day 5 – Day 0, 1-way ANOVA, N = 9-11, $F_{3, 34} = 2.765$, $p = 0.0568$. Data represented as mean +/-SEM. TFL: tail flick latency, BSL: Baseline.

**Figure 3. NPAS2-deficiency alters fentanyl potency in female tolerant mice.** Fentanyl dose-response curves in males (A), and females (B) measured before (day1) and after (day11) induction of tolerance with fentanyl. Doses administered ranged from 10μg/kg to 320μg/kg for during the pre-tolerance test, and from 160μg/kg to 1280μg/kg during the post-tolerance test. Data represented as normalized log[fentanyl] and normalized from 0 to 100. N = 9-11, Non-linear fit, Best-fit values for EC50 values calculations: Males: WT pre-tolerance: 51.75 μg/kg, WT post-tolerance: 273.4 μg/kg, 5.1-fold rightward shift; NPAS-/- pre-tolerance: 61.25 μg/kg, NPAS-/- post-tolerance: 269.6 μg/kg, 4.4-fold rightward shift. Females: WT pre-tolerance: 71.24 μg/kg, WT post-tolerance: 291.0 μg/kg, 4.08-fold rightward shift; NPAS-/- pre-tolerance: 63.01 μg/kg, NPAS-/- post-tolerance: 346.0 μg/kg, 5.5-fold rightward shift. (C) Day1 EC50s comparisons, N = 9-10, 1-way ANOVA, $F_{3.33} = 0.4501$, $p = 0.7189$. (D) Day11 EC50s comparisons, N = 9-10, 1-way ANOVA, $F_{3.33} = 2.056$, $p = 0.1251$. (E) Comparison of degree of rightward shift of EC50s between day1 and day11 (EC50 day1- EC50 day11) degree between WT and NPAS2/-/ males, N = 9-11, two-tailed t-test, $t = 0.6714$, df = 18, $p = 0.5105$. (F) Comparison of degree of rightward shift of EC50s between day1 and day11 (EC50 day1- EC50 day11) degree between WT and NPAS2/-/ females, N = 9, two-tailed t-test, $t = 2.096$, df = 16, $p = 0.0523$. (C-D) Data represented as mean +/- SEM. TFL = tail flick latency, EC50 = effective dose 50.

**Figure 4. NPAS2-deficiency potentiates physical dependence behaviors in female mice only.** Naloxone-precipitated withdrawal behaviors in NPAS2/- and WT littermate mice administered with a challenge dose of fentanyl (320μg/kg, i.p.). (A) total number of jumps, N = 9-11, 1-way ANOVA, $F_{3, 34} = 4.646$, $p = 0.0079$. (B) total number of wet dog shake episodes, N =
9-11, 1-way ANOVA, F_{3, 34} = 3.425, p = 0.0279. (C) total number of teeth chattering episodes, N = 9-11, 1-way ANOVA, F_{3, 34} = 2.624, p = 0.0663. (D) total number of paw shakes/grooming episodes, N = 9-11, 1-way ANOVA, F_{3, 34} = 0.8478, P = 0.4774. (E) withdrawal score, N = 9-11, 1-way ANOVA, F_{3, 34} = 6.543, p = 0.0013. Data represented as mean +/- SEM. Tukey’s multiple comparisons tests, * p < 0.05, ** p < 0.01, *** p < 0.001.