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ORIGINAL INVESTIGATION

A role for the circadian transcription factor NPAS2 in the progressive loss of non-rapid eye movement sleep and increased arousal during fentanyl withdrawal in male mice

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Abstract

Rationale Synthetic opioids like fentanyl are contributing to the rise in rates of opioid use disorder and drug overdose deaths. Sleep dysfunction and circadian rhythm disruption may worsen during opioid withdrawal and persist during abstinence. Severe and persistent sleep and circadian alterations are putative factors in opioid craving and relapse. However, very little is known about the impact of fentanyl on sleep architecture and sleep–wake cycles, particularly opioid withdrawal. Further, circadian rhythms regulate sleep–wake cycles, and the circadian transcription factor, neuronal PAS domain 2 (NPAS2) is involved in the modulation of sleep architecture and drug reward. Here, we investigate the role of NPAS2 in fentanyl-induced sleep alterations.

Objectives To determine the effect of fentanyl administration and withdrawal on sleep architecture, and the role of NPAS2 as a factor in fentanyl-induced sleep changes.

Methods Electroencephalography (EEG) and electromyography (EMG) was used to measure non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS) at baseline and following acute and chronic fentanyl administration in wild-type and NPAS2-deficient male mice.

Results Acute and chronic administration of fentanyl led to increased wake and arousal in both wild-type and NPAS2-deficient mice, an effect that was more pronounced in NPAS2-deficient mice. Chronic fentanyl administration led to decreased NREMS, which persisted during withdrawal, progressively decreasing from day 1 to 4 of withdrawal. The impact of fentanyl on NREMS and arousal was more pronounced in NPAS2-deficient mice.

Conclusions Chronic fentanyl disrupts NREMS, leading to a progressive loss of NREMS during subsequent days of withdrawal. Loss of NPAS2 exacerbates the impact of fentanyl on sleep and wake, revealing a potential role for the circadian transcription factor in opioid-induced sleep changes.

Keywords Sleep · Opioids · Fentanyl · Withdrawal · Circadian rhythms · NPAS2 · Non-rapid eye movement sleep · Circadian genes

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Introduction

Fentanyl is a highly potent synthetic opioid used to alleviate pain in certain clinical contexts including during and after surgery and in the treatment of cancer and chronic pain (Comer and Cahill 2019). Fentanyl along with other synthetic opioids have surpassed heroin and oxycodone as the main drivers of overdose deaths and increased rates of opioid use disorder (OUD) in regions with already high base rates of opioid use (Gladden et al. 2016; O'Donnell et al. 2017). Thus, understanding the unique consequences of fentanyl use on health is imperative to developing strategies for treatments to curb the impact. An understudied health-related impact of chronic opioid use has been at the intersection of sleep and circadian rhythms.

Accumulating evidence suggests opioids lead to sleep and circadian disruptions, possibly contributing to increased craving and propensity to relapse (for review see Eacret et al. 2020; Logan et al. 2014). Sleep abnormalities are evident in OUD (Wang and Teichtahl 2007; Fathi et al. 2020) and likely contribute to relapse (Lydon-Staley et al. 2017). The standard treatment, methadone, for OUD and opioid dependence also leads to notable sleep disturbances (Tripathi et al. 2020). For example, morphine, heroin, and methadone acutely suppress rapid eye movement sleep (REMS) and promote wakefulness in humans (Kay et al. 1969; Lewis et al. 1970; Kay 1975). However, as tolerance to repeated use of opioids develops, the impact on REMS lessens with levels approaching individual baselines (Lewis et al. 1970; Kay 1975). Non-rapid eye movement sleep (NREMS) is also disrupted by opioids (Kay et al. 1969; Orr and Stahl 1978; Shaw et al. 2005). Notably, withdrawal from opioids leads to persistent disruptions in both NREMS and REMS, possibly lasting for months in patients (Oswald 1969; Lewis et al. 1970; Kay 1975). Similarly, in animal models, both NREMS and REMS are altered during and following opioid administration and withdrawal (Khazan and Colasanti 1972; De Andrés and Caballero 1989). In addition, disrupted sleep impacts opioid tolerance and withdrawal. For example, sleep deprivation delays the development of morphine tolerance in rats (Ahmadi-Soleimani et al. 2021) and reduces opioid-induced analgesia in humans (Smith et al. 2020). Further understanding of the complex relationships between sleep, circadian rhythms, and opioids is essential for advancing effective treatments for OUD and opioid dependence.

To begin to fill this gap, we investigated the impact of chronic fentanyl administration on sleep architecture and the diurnal pattern of sleep and wake in mice. We investigated the effects of acute and chronic fentanyl, along with withdrawal from fentanyl, on NREMS, REMS, and wake. In addition, little is known regarding the molecular factors which may underlie the relationships between sleep, diurnal rhythms, and substance use. Previous work highlights the circadian transcription factor, neuronal PAS domain 2 (NPAS2) (Garcia et al. 2000), as a key regulator of drug reward, craving and relapse (Ozburn et al. 2015; Becker-Krail et al. 2022; Depoy et al. 2021). Notably, NPAS2 also regulates sleep architecture, as NPAS2-deficient mice display reduced NREMS and REMS in a diurnal-dependent manner (Dudley et al. 2003). Together, these findings suggest NPAS2 may be involved in the impact of opioids on sleep and circadian rhythms. Using both wild-type (Wt) and NPAS2-deficient male mice, we investigated fentanyl-induced alterations in sleep, predicting NPAS2-deficiency exacerbates the consequences of fentanyl on sleep architecture.

Methods

Animals and housing

Experiments were conducted in Wt and NPAS2-deficient male mice (C57BL/6J background). NPAS2-deficient mice have the basic helix loop helix (bHLH) domain of the transcription factor replaced with a LacZ reporter, impairing NPAS2-mediated gene transcription (Garcia et al. 2000). Littermates were used as controls. Mice were initially group-housed then transferred to individual cages at least 5 days prior to surgical procedures ($n = 7$ per group per genotype). Mice were housed under standard 12:12-h light–dark schedule (lights on at 08:00 h and off at 20:00 h) with ad libitum food (Prolab RMH 3000 5P76, LabDiet) and water. Mice were habituated to pyramidal cages used for sleep recordings 2 h daily for at least 7 days prior to first sleep measurements. During sleep recordings, mice were placed into a pyramidal cage to allow for the recording cable to be tethered to the mouse and connected to the recording equipment via a commutator. Mice were then placed into a secondary housing container to insulate noise and other stimuli during sleep recordings. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh School of Medicine.

Sleep polysomnography surgery

Mice were implanted with electroencephalogram (EEG) and electromyogram (EMG) electrodes, as previously described (Tagaito et al. 2001). Briefly, an incision along the midline of the head was used to expose the skull and muscles immediately posterior to the skull. Underlying fascia was

then gently cleared from the skull and holes (1-mm diameter) were drilled through the skull (frontal and parietal). EEG electrodes (E363/1, Plastics One Inc.) were secured via screws into the holes (first electrode at 2–3 mm caudal to Bregma and 1–2 mm right of the midline; second electrode at 2–3 mm rostral to bregma and 2–3 right of the midline; and third electrode 0–1 mm rostral to bregma and 2–3 mm left of midline. EMG electrodes (E363/76, Plastics One Inc.) were then secured onto the surface of the muscle immediately posterior to the dorsal portion of the skull. EEG and EMG electrodes were inserted into a pedestal (MS363, Plastics One Inc.) and cemented to the skull with dental acrylic (1403 and 1420, Lang Dental). Skin caudal to the pedestal was sutured. Mice recovered for 5 days following surgery.

Sleep polysomnography recording

Mice were tethered to a preamplifier system via a custom cable (363-SL/6 80CM 6TCS, Plastics One Inc.) for sleep recordings. Our sleep–wake detection system has previously been described and validated in mice (Benington et al. 1994; O'Donnell et al. 2019). Sleep–wake states were determined using the frequency distribution of EEG and the amplitude of EMG. Temporal data was considered in terms of "epochs" of 10-s duration. Software thresholds using the polysomnographic parameters EEG frequency and EMG amplitude were set to assess sleep–wake state (Tagaito et al. 2001). For EEG, thresholds for β2 (20–30 Hz) and δ1 (2–4 Hz) from the EEG frequency distribution were used to parse NREMS (lower β2/δ1 ratio) from wakefulness or REMS (above β2/δ1 threshold). For EMG, above the highest threshold represented wakefulness (irrespective of the β2/δ1 ratio), while below the lowest threshold represented either NREMS or REMS depending on whether the β2/δ1 ratio was below (NREMS) or above (REMS) their threshold. The amplitude from EEG was also used to determine REMS, such that the amplitude needed to be below a threshold. Amplitude was included because in mice this measure invariably undergoes a uniform reduction during the transition from NREMS to REMS. Thresholds were optimized for each mouse using baseline EEG and EMG recordings (1–2 h).

Sleep–wake state scoring

Continuous EEG and EMG data was recorded as waveforms using WinDaq Data Acquisition Software (v.2.94, DATAQ Instruments, Inc.) and converted into an analyzable format (Stanford Sleep Structure Scoring System) via a custom program (Benington et al. 1994), subsequently validated in mice (Veasey et al. 2004). Manual sleep–wake state validation was based on previously described parameters (O'Donnell et al. 2019). Time spent in NREMS, REMS, and awake states were calculated as the percent of total spent in that state during a complete 24 h period. Bouts of NREMS and REMS were based on the following criteria: (1) NREMS bout began with more than three consecutive epochs of NREM and ended with either more than three consecutive epochs of wake or more than two consecutive epochs of REMS; (2) REMS bout began with more than two consecutive epochs of REMS and ended with more than three consecutive epochs of NREMS or wake; and (3) wakefulness bout began with more than three consecutive epochs of wake and ended with more than three consecutive epochs of NREMS and/or REMS. Propensity to remain awake was defined as the average duration from the beginning of more than three consecutive epochs of wake to the following of more than three consecutive epochs of NREMS and/or REMS over 24 h. Arousal (or awakening) was measured as one or more wake epochs followed by sleep (greater than three epochs of NREMS or REMS). Each arousal epoch had to last at least 10 s in duration. The frequency of arousals per hour of sleep over the 24 h of sleep recordings was calculated as the "arousal index." Independent investigators involved in the study separately scored sleep state for each mouse. Analyses presented were completed using the mean values between the scorers.

Fentanyl administration and sleep recordings

Male Wt and NPAS2-deficient mice underwent either acute or repeated administration of fentanyl (320 μg of fentanyl in sterile saline per kg body weight administered intraperitoneal, i.p.). For mice receiving acute fentanyl, a single injection of fentanyl was administered on day 12 after receiving 11 previous days of saline (Fig. 1). For mice receiving repeated fentanyl, fentanyl was injected every 12 h (08:00 h, lights on, and 20:00 h, lights off) from days 3 to 10 (Fig. 1). Sleep was recorded for all mice on day 0, prior to any saline or fentanyl administration. Sleep was also recorded on days 13 and 14 to determine the acute effects of fentanyl on sleep and during acute withdrawal (first 24 h following last fentanyl administration). Sleep was recorded on days 10 to 14 to determine the effects of repeated fentanyl on sleep and during prolonged withdrawal (initial 4 days following last fentanyl administration).

Statistical analyses

Two-way mixed ANOVA (between within design) or mixed-effects model when unbalanced was used to compare changes in sleep states between and NPAS2-deficient mice among acute and chronic fentanyl administration groups, and during fentanyl withdrawal across time. Dunnett's (experimental groups compared to control group), Sidak (repeated measure with two levels), and Tukey's post hoc (repeated measure with three levels) analyses were used to

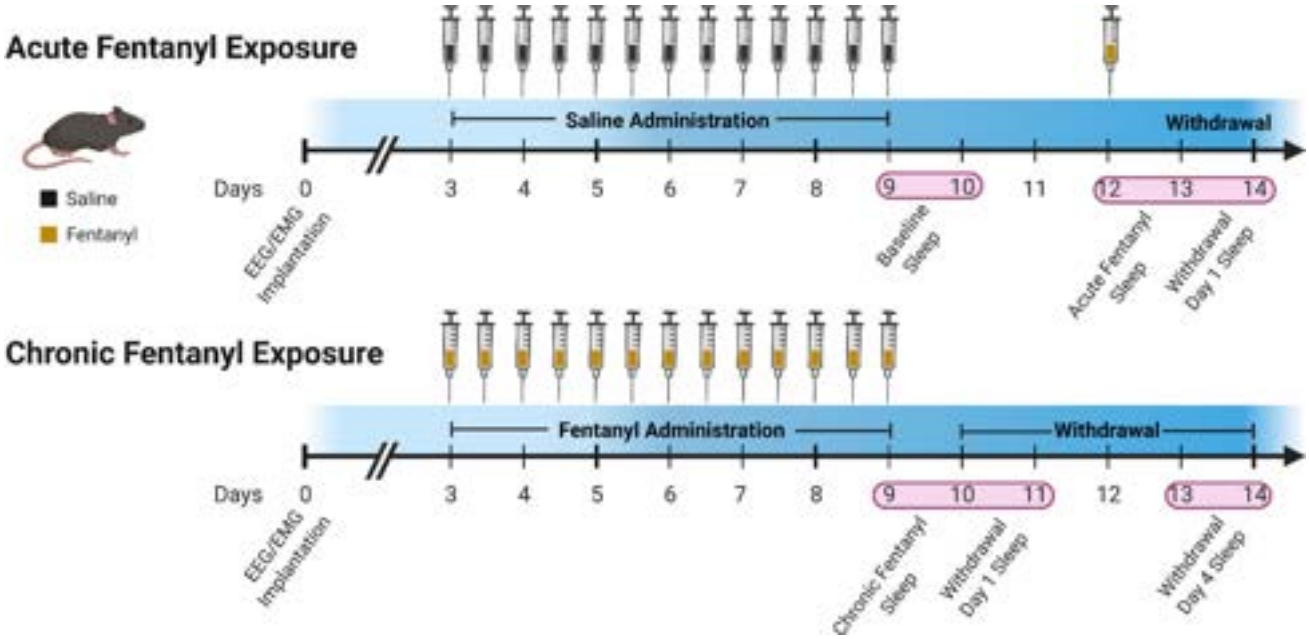

Fig. 1 Schematic of fentanyl administration in mice. Wild-type and NPAS2-deficient mice underwent electroencephalogram (EEG) and electromyography (EMG) implantation to record sleep–wake states, followed by chronic administration with saline (black) or fentanyl (orange) via i.p. injection twice a day for 7 days. Sleep–wake state recordings assessed sleep architecture at baseline, and during acute and chronic fentanyl administration, followed by withdrawal. Created using BioRender

test multiple comparisons. Geisser-Greenhouse correction was used for all applicable analyses. Outliers were determined using the three-sigma rule. This included 1 outlier in sleep bouts and sleep bout length measures for baseline, acute fentanyl administration, chronic fentanyl administration, and acute withdrawal in NPAS2-deficient mice. Results are presented as means ± standard error of the mean (SEM) and significance was set at $\alpha = 0.05$.

Results

The effects of saline administration on sleep and arousal in wild-type and NPAS2-deficient mice

Sleep was measured using EEG/EMG in mice following saline and fentanyl administration (Fig. 1). Baseline NREMS and REMS were measured immediately following repeated administration of saline. Overall, the amount of time in each sleep stage were similar between Wt (mean ± standard error of mean over 24 h for NREMS: 51.8 ± 1.36% and REMS: 3.3 ± 0.23%, and NPAS2-deficient (NREMS 49.83 ± 1.80% 24 h and REMS 2.917 ± 0.35%, Fig. 2A, B) mice. In addition, the time to transition from sleep to wake (arousal index) was similar between Wt (19.4 ± 1.18 min) and NPAS2-deficient male mice (26.75 ± 3.40 min Fig. 2C). Time to fall asleep (sleep propensity) was also similar (Wt: 2.77 ± 0.30 min to fall asleep after ≥ 3 wake epochs over 24 h compared to NPAS2 deficient: 2.38 ± 0.32 min, Fig. 2D). The number of sleep bouts and length of bouts were largely comparable between Wt (98.71 ± 8.68 bouts over 24 h with an average length of 8.54 ± 0.66 min, Fig. 2E) and NPAS2 deficient (92.17 ± 6.97 bouts over 24 h with an average length of 8.77 ± 0.52 min (Fig. 2F). Overall, baseline sleep measures of NREMS, REMS, bouts, and bout durations were similar between male Wt and NPAS2-deficient mice.

The effects of acute fentanyl on sleep and arousal in wild-type and NPAS2-deficient mice

To assess the impact of acute fentanyl on sleep, mice were administered an injection of fentanyl (320 ug/kg) following baseline recordings of sleep then sleep was measured for another 24 h (Fig. 1). Acute administration of fentanyl led to significant disruptions in sleep irrespective of genotype. Two-way ANOVA showed a main effect of acute fentanyl on NREMS ($F_{(1,12)} = 11.8$; $p < 0.01$) and no significant effect of genotype ($p = 0.06$) or an interaction, along with no effect on REMS. NREMS was significantly decreased following acute fentanyl (from 50.84 ± 1.12% at baseline to 46.61 ± 1.42% after acute fentanyl collapsed across genotypes, Fig. 3A, B). Opioid-induced alterations of NREMS were attributed to a pronounced reduction displayed in NPAS2-deficient mice (from 49.83 ± 1.80% at baseline to 43.56 ± 1.76% after acute fentanyl, Fig. 3A). Arousal index was reduced by fentanyl (main effect of

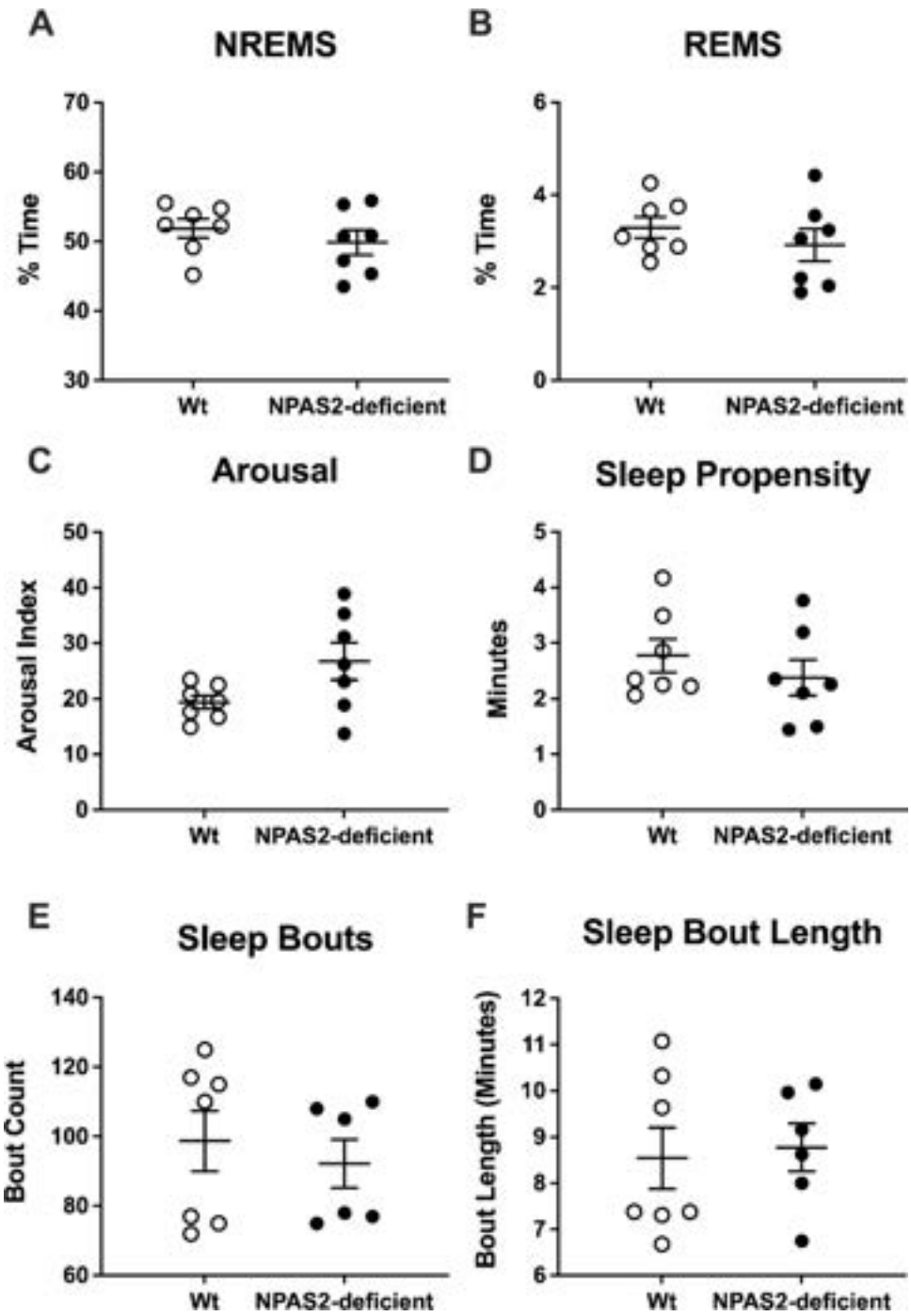

Fig. 2 Similar sleep–wake states between wild-type and NPAS2-deficient male mice. Sleep–wake analysis of wild-type (Wt) and NPAS2-deficient mice at baseline after 7 days of saline treatment. Sleep–wake state was recorded for a 24 h duration following the last saline injection on day 9. **A** Time spent in non-rapid eye movement sleep (NREMS) over 24 h. **B** Time spent in rapid eye movement sleep (REMS) over 24 h. **C** Average number of minutes to transitions from sleep to wake state per hour of sleep over 24 h. **D** Time to fall asleep after ≥ 3 awake epochs over 24 h. **E** Sleep bouts over 24 h. **F** Sleep bout length over 24 h

treatment, $F_{(1,12)} = 9.2$; $p < 0.05$, Fig. 3C), whereas sleep propensity significantly increased from 2.57 ± 0.22 min at baseline to 3.45 ± 0.26 min following acute fentanyl (main effect of treatment, $F_{(1,12)} = 7.99$; $p < 0.05$, Fig. 3D). The main effect of treatment for sleep propensity was mainly driven by an increase in NPAS2-deficient mice (from 2.38 ± 0.32 at baseline to 3.58 ± 0.35 min over 24 h following acute fentanyl, Fig. 3D). Total number of sleep bouts was significantly decreased from 95.69 ± 5.53 bouts across 24 h to 75.92 ± 4.82 bouts across genotypes (main effect of treatment, $F_{(1,12)} = 6.92$; $p < 0.05$, Fig. 3E), although no effects were observed for duration of sleep bouts (Fig. 3F). Thus, in the first 24 h following administration, acute fentanyl led to altered sleep, primarily in measures of arousal, sleep–wake transitions, and NREMS in both Wt and NPAS2-deficient male mice.

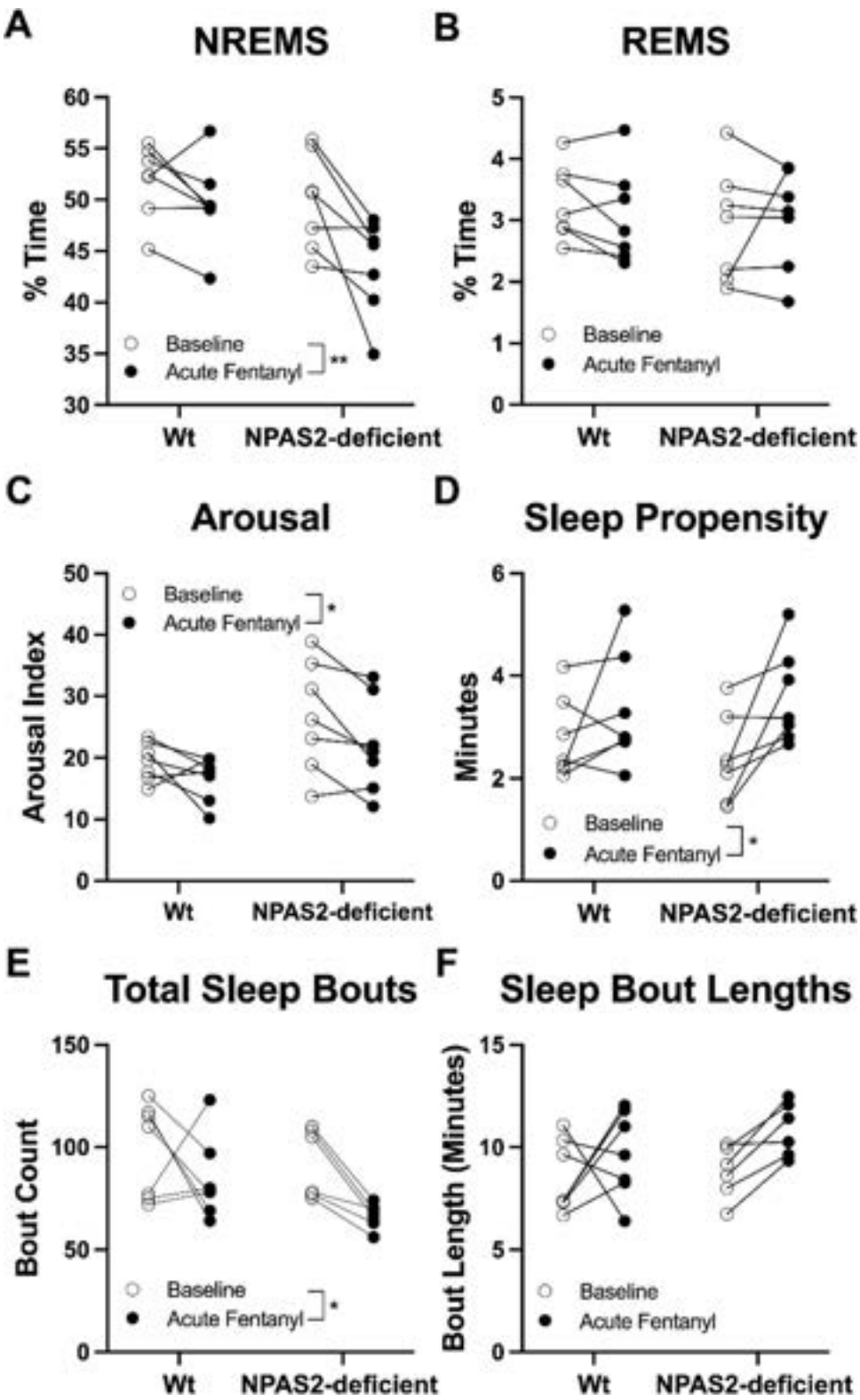

Fig. 3 Acute fentanyl administration leads to altered sleep and arousal in male mice. Sleep–wake analysis of wild-type (Wt) and NPAS2-deficient mice at baseline compared to acute fentanyl administration. **A** Time spent in non-rapid eye movement sleep (NREMS) over 24 h (main effect of treatment, $^{**}p < 0.01$). **B** Time spent in rapid eye movement sleep (REMS) over 24 h. **C** Average number of transitions from sleep to wake state per hour of sleep over 24 h (main effect of treatment, $^{*}p < 0.05$). **D** Time to fall asleep after ≥ 3 awake epochs over 24 h (main effect of treatment, $^{*}p < 0.05$). **E** Total number of sleep bouts over 24 h (main effect of treatment, $^{*}p < 0.05$). **F** Average length of sleep bouts over 24 h

The effects of acute fentanyl withdrawal on sleep and arousal in wild-type and NPAS2-deficient mice

Sleep was also measured during the acute withdrawal period 24–48 h following acute administration of fentanyl. Acute fentanyl administration led to an overall reduction in NREMS that remained during the acute withdrawal period mainly in NPAS2-deficient mice (saline: 52.60 ± 1.55% across 24 h; acute fentanyl: 43.56 ± 1.76%; and acute withdrawal: 46.13 ± 1.94%, Fig. 4A). Two-way ANOVA showed

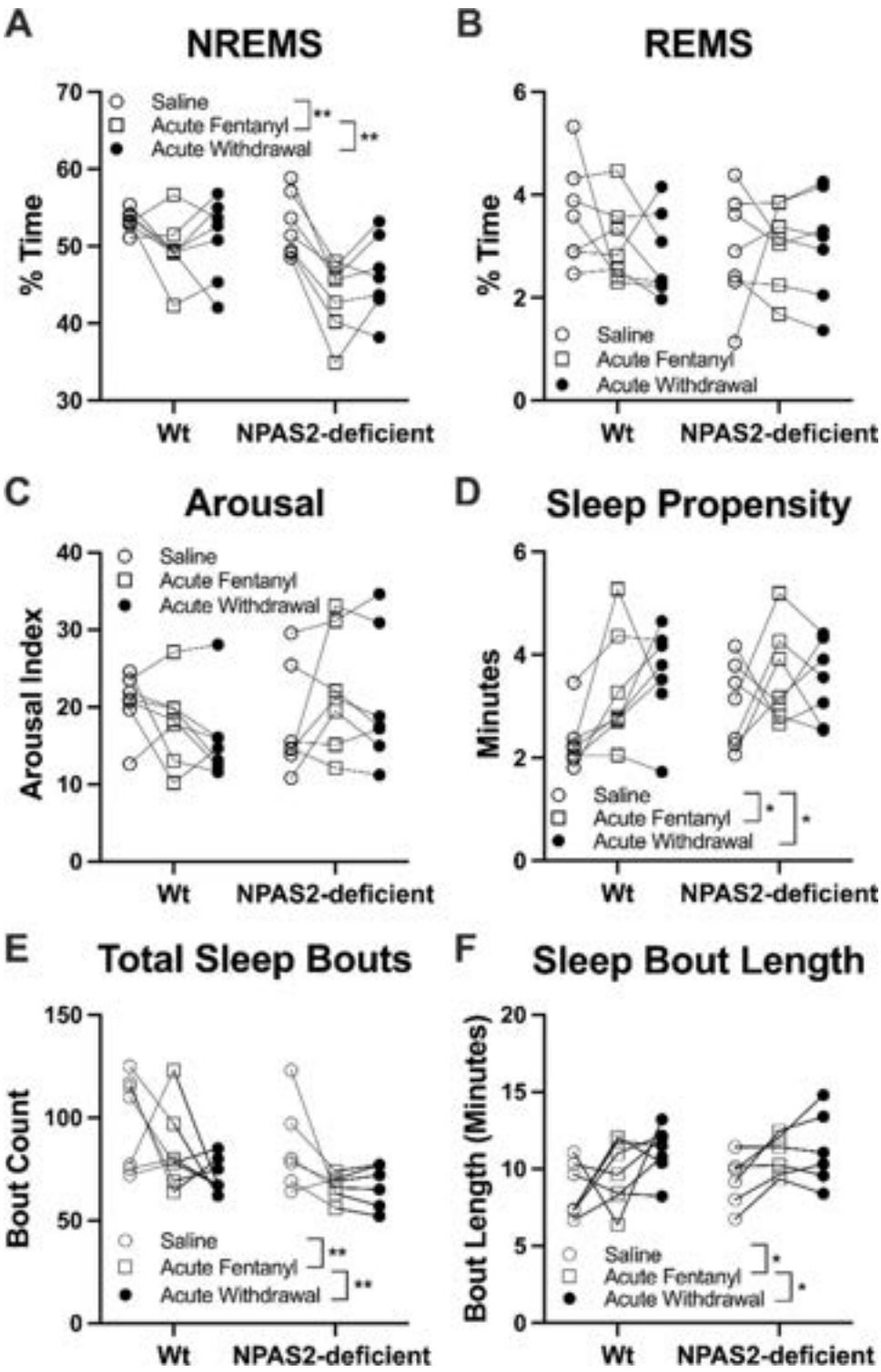

Fig. 4 Acute fentanyl withdrawal leads to altered sleep and wake states that persist during acute withdrawal. Wild-type (Wt) and NPAS2-deficient mice after saline injection, 24 h following acute fentanyl administration, and 24–48 h after fentanyl (acute withdrawal). **A** Time spent in non-rapid eye movement sleep (NREMS; saline vs. acute fentanyl, $^{**}p < 0.01$; acute fentanyl vs. acute withdrawal, $^{**}p < 0.01$). **B** Time spent in rapid eye movement sleep (REMS). **C** Average number of arousals (transition from sleep to wake state) per hour of sleep (Tukey's post hoc, saline vs. acute withdrawal in Wt mice, $^{*}p < 0.01$). **D** Time to fall asleep after ≥ 3 awake epochs (saline vs. acute fentanyl, $^{*}p < 0.05$; saline vs. acute withdrawal, $^{*}p < 0.05$). **E** Total number of sleep bouts over the 24-h recordings (saline vs. acute fentanyl, $^{**}p < 0.01$; acute fentanyl vs. acute withdrawal, $^{**}p < 0.01$). **F** Average length of sleep bouts (saline vs. acute fentanyl, $^{*}p < 0.05$; saline vs. acute withdrawal, $^{*}p < 0.05$)

a main effect of fentanyl condition ($F_{(1.78,\ 21.42)} = 13.78$; $p < 0.001$) and no significant effect of genotype ($p = 0.053$) or an interaction on NREMS, along with no effects on REMS (Fig. 4B) and arousal index (Fig. 4C). Sleep propensity was altered by condition (main effect of treatment, $F_{(1.89,\ 22.79)} = 5$; $p < 0.05$, Fig. 4D). Overall, duration to fall asleep increased from baseline (2.67 ± 0.21 min) to 3.45 ± 0.26 min following acute fentanyl, and finally to 3.56 ± 0.23 min during withdrawal. For sleep bouts, a mixed-effects model (outlier removal, repeated measures) revealed main effects for genotype ($F_{(1,34)} = 5.8$; $p < 0.05$) and treatment ($F_{(1.65,\ 28.06)} = 6.64$; $p < 0.01$), reflected by an overall

reduction in total sleep bouts by acute fentanyl administration that remained during the acute withdrawal period (Fig. 4E). An overall main effect of treatment was also found for sleep bout length ($F_{(1.84, 20.26)} = 6.13$; $p < 0.01$), whereby duration of sleep bouts were increased from baseline through acute withdrawal (8.86 ± 0.47 min per bout of sleep across 24 h during baseline, which increased to 10.21 ± 0.50 min after acute fentanyl administration, and further increased to 11.21 ± 0.53 min during acute withdrawal, Fig. 4F). Collectively, these findings indicate acute fentanyl led to alterations in sleep and arousal that were maintained during the acute withdrawal period similar across genotypes of mice, potentially reflecting the emergence of persistent changes in sleep.

The effects of chronic fentanyl administration and withdrawal on sleep and arousal in wild-type and NPAS2-deficient mice

In a separate cohort of mice, sleep was measured following chronic, repeated administration of fentanyl (7 days). In the initial 24 h following chronic administration of fentanyl, both NREMS and REMS were not significantly altered within and across genotypes of mice (Fig. 5A, B). A significant interaction was found for arousal index (genotype × treatment, $F_{(1,10)} = 5.27$; $p < 0.05$), with Sidak's post hoc analyses indicating a significant reduction in NPAS2-deficient mice (baseline: 26.75 ± 3.40 min to transition from sleep to wake per hour of sleep over 24 h on average to 17.75 ± 2.61 min, Fig. 5C). No significant effects were observed for sleep propensity (Fig. 5D). However, both the number ($F_{(1, 21)} = 13.09$; $p < 0.01$) and duration of sleep bouts ($F_{(1, 21)} = 8.09$; $p < 0.01$) were significantly altered by chronic fentanyl administration (Fig. 5E, F). The effects on sleep bouts were similar between acute (Fig. 4E, F) and chronic (Fig. 5E, F) among Wt and NPAS2-deficient mice, suggesting fentanyl led to an overall reduction in sleep bouts, while increasing bout duration in first 24–48 h following administration.

To determine whether opioid-induced alterations persisted during withdrawal, sleep was measured on days one and four of withdrawal. Both Wt and NPAS2-deficient mice exhibited a significant decrease in time spent in NREMS (main effect of treatment, $F_{(1.614,16.14)} = 38.95$; $p < 0.0001$) (Fig. 6A). In Wt mice, NREMS was 53.36 ± 0.69% after chronic fentanyl administration, which decreased to 50.96 ± 1.05% during the first day of withdrawal, then further decreased to 44.89 ± 0.75% after day four of withdrawal (~ 9% reduction in NREMS). Similarly, NREMS progressively decreased in NPAS2-deficient mice during withdrawal from fentanyl (chronic fentanyl: 52.60 ± 1.55%; day 1: 48.45 ± 0.77%; and day 4: 44.29 ± 1.30%), evident by an ~ 8% reduction in NREMS (Fig. 6A). However, inconsistent changes were observed in REMS between fentanyl administration and withdrawal from fentanyl regardless of NPAS2-deficiency (Fig. 6B). Further, while arousal was also unchanged, sleep propensity was significantly altered by fentanyl withdrawal (main effect of treatment, $F_{(1.127,11.27)} = 18.90$; $p < 0.001$), accompanied by an overall increase in both Wt and NPAS2-deficient mice (Fig. 6D). There were no significant changes in number of sleep bouts and duration of sleep bouts in Wt and NPAS2-deficient mice after either day 1 or day 4 of withdrawal from chronic fentanyl administration (Fig. 6E, F).

Diurnal effects of fentanyl administration and withdrawal on sleep and wake in wild-type and NPAS2-deficient mice

To investigate whether sleep–wake cycles were altered by fentanyl, sleep and wake states were analyzed across different times of day. Sleep and wake were largely disrupted during the dark phase of the light–dark cycle in Wt mice, both for acute and chronic fentanyl administration and during withdrawal. Two-way ANOVA indicated a significant effect of time of day on wake ($F_{(5,84)} = 22.69$; $p < 0.0001$, Fig. 7A), NREMS ($F_{(5,84)} = 21.95$; $p < 0.0001$, Fig. 7B), and REMS ($F_{(5,84)} = 10.75$; $p < 0.0001$, Fig. 7C), but no significant effect of fentanyl administration on each of these measures (Fig. 7A–C). Similarly, each of these measures varied by time of day (wake: $F_{(5,84)} = 57.29$; $p < 0.0001$; NREMS: $F_{(5,84)} = 50.89$; $p < 0.0001$; REMS: $F_{(5,84)} = 14.23$; $p < 0.0001$) during acute and chronic withdrawal from fentanyl (Fig. 7A–C). In addition, significant effects for fentanyl withdrawal were identified for wake ($F_{(2,84)} = 3.88$; $p = 0.02$; Fig. 7A) and NREMS ($F_{(2,84)} = 4.48$; $p = 0.01$; Fig. 7B). Post hoc analyses for fentanyl withdrawal condition revealed a significant increase ($p < 0.05$) in wake on day four of chronic withdrawal compared to acute withdrawal at ZT4 (increased ~ 12%; Fig. 7B). At ZT20, wake was significantly decreased ($p < 0.05$) on day one of chronic withdrawal (50.38 ± 3.82%) compared to acute withdrawal (62.55 ± 4.41%), suggesting chronic withdrawal alters the diurnal pattern of wakefulness in the early light phase and in the late dark phase (Fig. 7A). Fentanyl-induced alterations in REMS were inconsistent by time of day (Fig. 7C).

Fentanyl withdrawal led to significant alterations in wake and NREMS that were dependent on time of day. Repeated measures ANOVA identified significant interactions between time of day and fentanyl withdrawal condition for both wake ($F_{(10,72)} = 4.279$; $p = 0.0001$; Fig. 7A) and NREMS ($F_{(10,72)} = 4.222$; $p = 0.0001$; Fig. 7B). Post hoc analyses identified significantly reduced wake on days one and four of withdrawal at ZT4 compared to chronic fentanyl administration ($p < 0.05$). A significant increase in wake was found at ZT16 on day four of withdrawal compared to chronic fentanyl

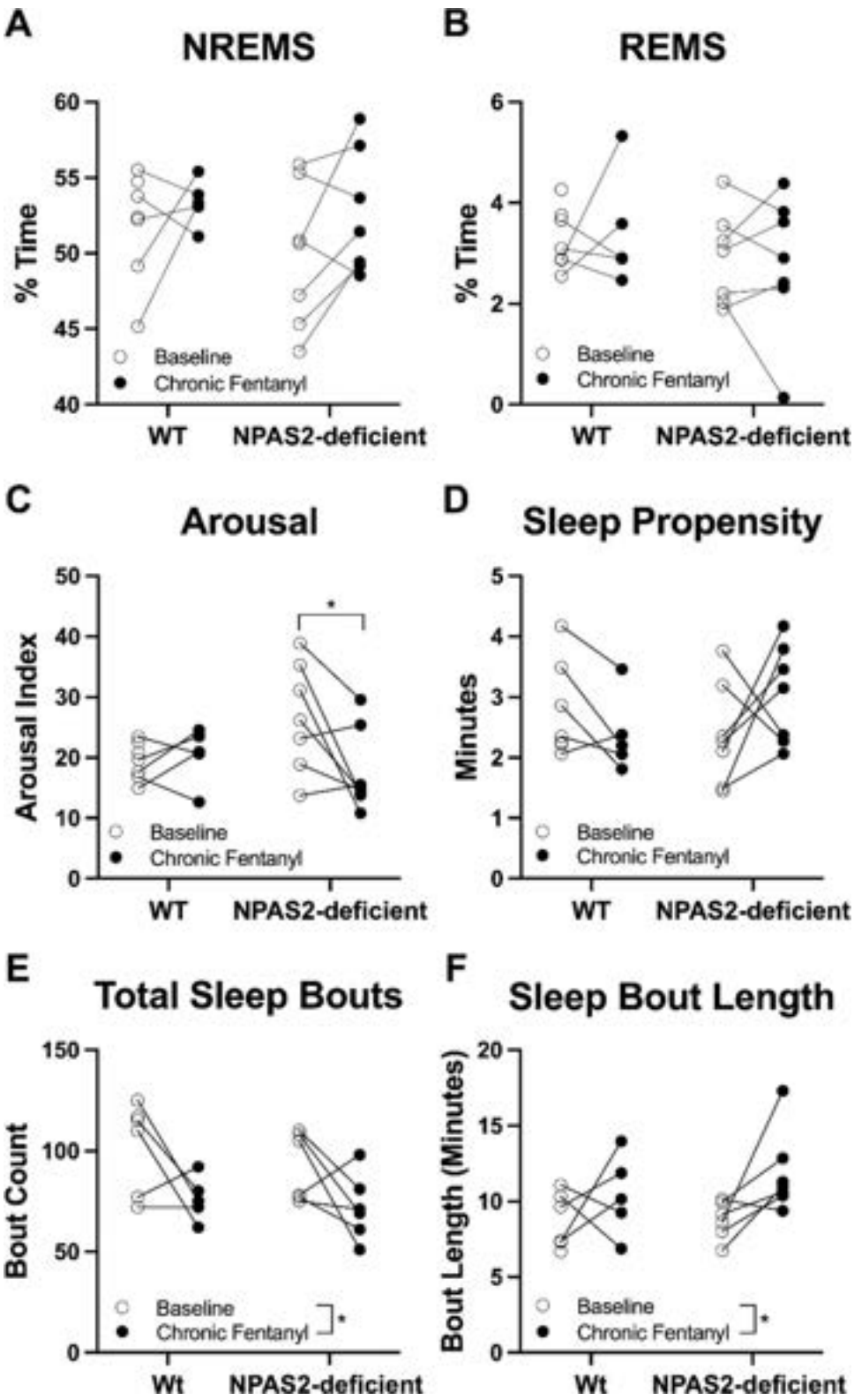

Fig. 5 Chronic fentanyl administration leads to altered sleep and wake states. Wild-type (Wt) and NPAS2-deficient mice at baseline and after seven days of chronic fentanyl administration. **A** Time spent in non-rapid eye movement sleep (NREMS) during 24 h of baseline compared to 24 h immediately following the final fentanyl administration (day 9). **B** Time spent in rapid eye movement sleep (REMS). **C** Average number of arousals (transition from sleep to wake state) per hour of sleep (baseline vs. chronic fentanyl in NPAS2-deficient mice, Sidak post hoc test, $*p < 0.05$). **D** Time to fall asleep after ≥ 3 awake epochs. **E** Total number of sleep bouts over 24 h (main effect of treatment, $*p < 0.05$). **F** Average length of sleep bouts (main effect of treatment, $*p < 0.05$). Comparisons are between unpaired mice as the baseline and chronic fentanyl mice are from separate groups

administration ($p < 0.05$), which remained significantly elevated at ZT20 (Fig. 7A). Conversely, NREMS was significantly reduced at ZT16 and ZT20 during fentanyl withdrawal on both days one and four ($p < 0.05$; Fig. 7B), suggesting the increased wakefulness contributed to reduced NREMS at specific times of day during withdrawal. A main effect for time of day was significant for wake ($F_{(5,72)} = 33.37$; $p < 0.0001$), NREMS

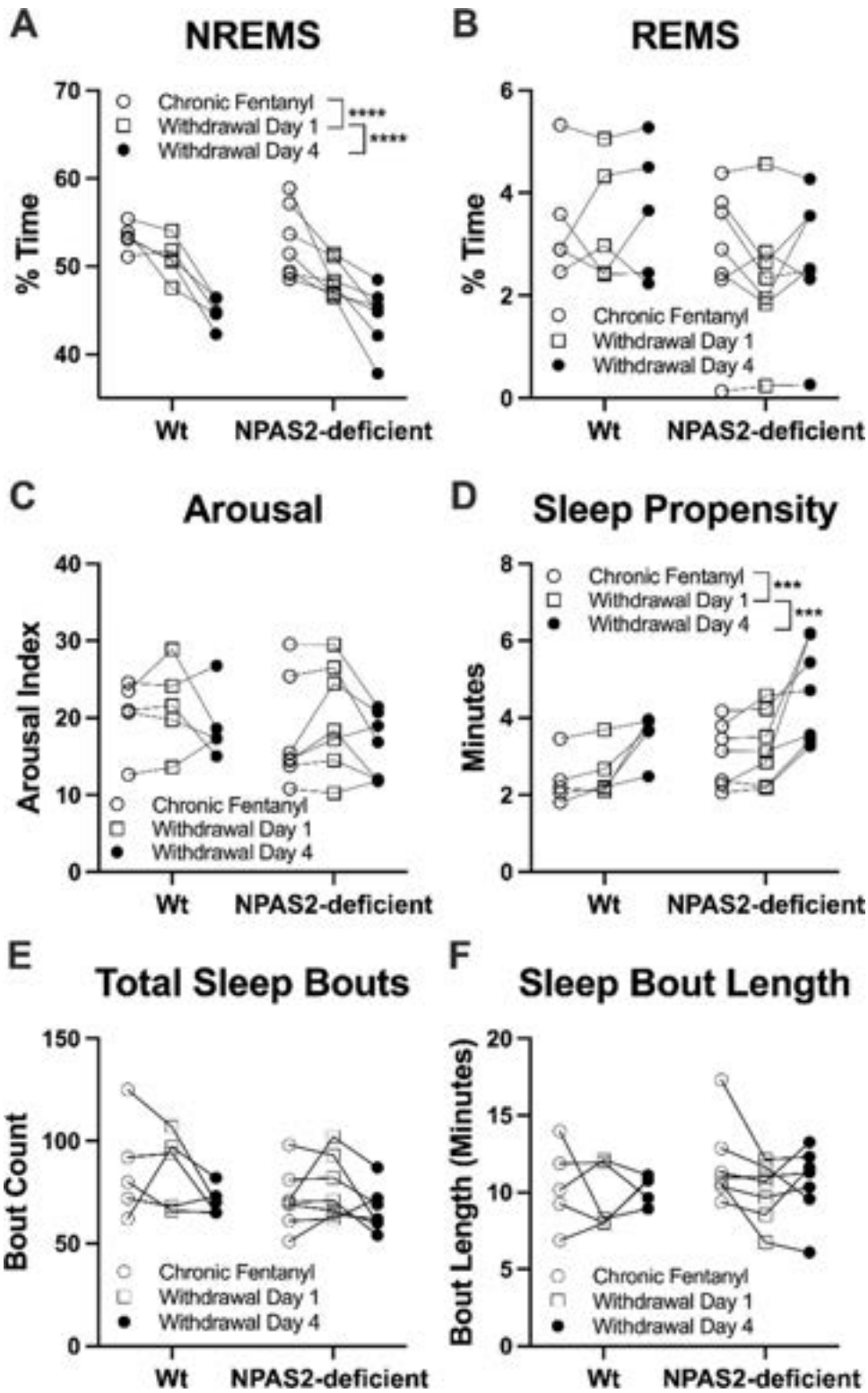

Fig. 6 Chronic fentanyl leads to altered sleep and wake states that progressively worsen during fentanyl withdrawal. Sleep–wake states were recorded from wild-type (Wt) and NPAS2-deficient mice after 7 days of fentanyl administration and during withdrawal. **A** Time spent in non-rapid eye movement sleep (NREMS; chronic fentanyl vs. withdrawal day 1, $^{****}p < 0.0001$; withdrawal day 1 vs. withdrawal day 4, $^{****}p < 0.0001$). **B** Time spent in rapid eye movement sleep (REMS). **C** Average number of arousals (transition from sleep to wake state) per hour of sleep. **D** Time to fall asleep after ≥ 3 awake epochs (chronic fentanyl vs. withdrawal day 1, $^{***}p < 0.001$; withdrawal day 1 vs. withdrawal day 4, $^{***}p < 0.001$). **E** Total number of sleep bouts over the 24 h. **F** Average length of sleep bouts

($F_{(5,72)} = 30.16$; $p < 0.0001$), and REMS ($F_{(5,72)} = 5.37$; $p = 0.0003$) during fentanyl withdrawal.

In NPAS2-deficient mice, sleep and wake alterations were also evident following fentanyl administration and withdrawal in a time-of-day dependent manner. Following acute and chronic administration of fentanyl, wake (treatment: $F_{(2,108)} = 6.68$; $p = 0.002$; and time: $F_{(5,108)} = 22.4$; $p < 0.0001$), and NREMS (treatment: $F_{(2,108)} = 8.28$; $p = 0.0004$; and time: $F_{(5,108)} = 23.2$; $p < 0.0001$) was significantly altered during the light phase of the light–dark cycle.

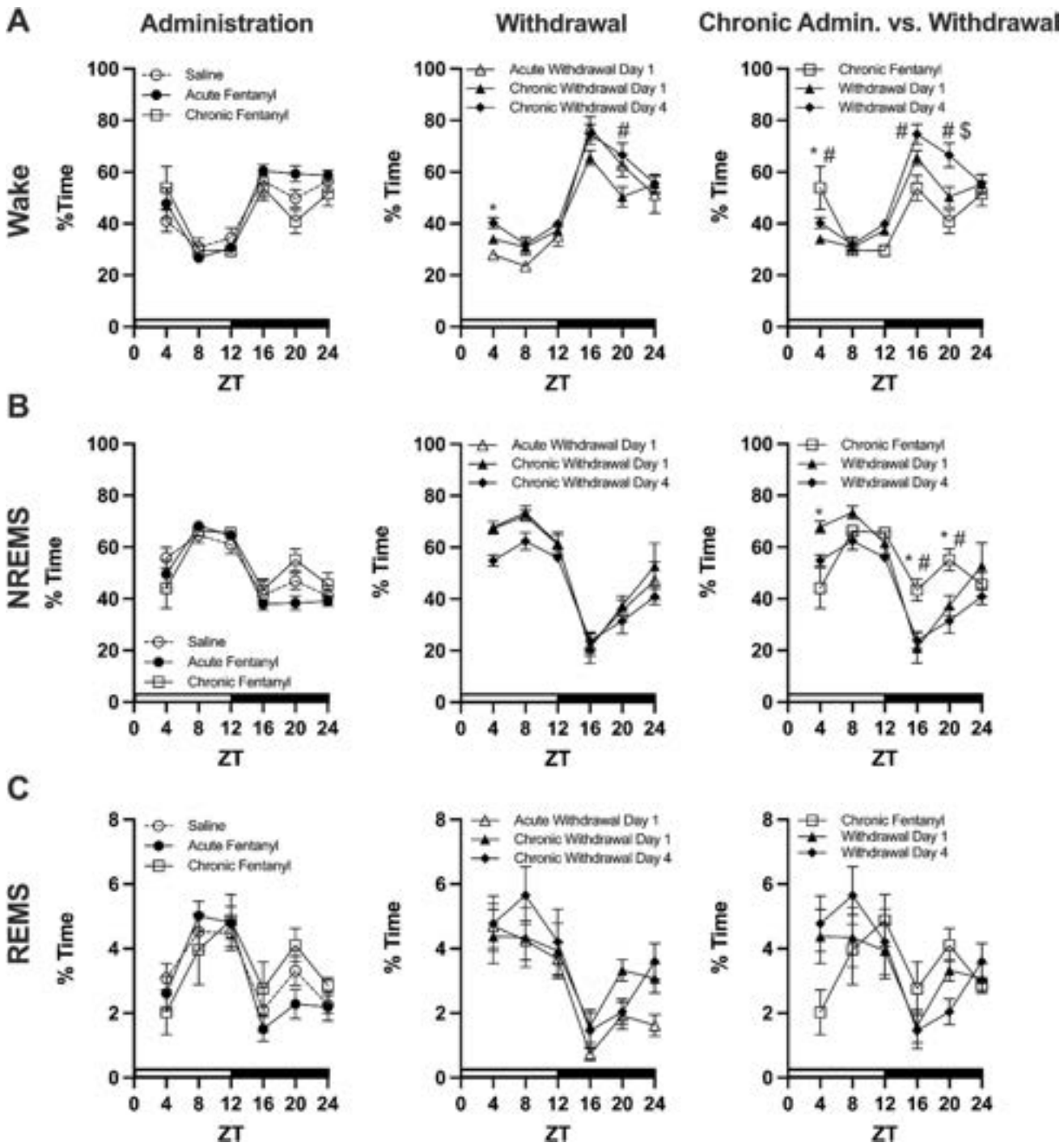

Fig. 7 Diurnal effects of fentanyl administration and withdrawal on sleep and wake in wild-type mice. Sleep–wake states across the light–dark cycle in wild-type (Wt) mice. Time spent in wake (**A**), non-rapid eye movement sleep (NREMS, **B**), and rapid eye movement sleep (REMS, **C**) following acute and chronic fentanyl relative to saline (Administration), during fentanyl withdrawal (withdrawal; #$p < 0.05$, fentanyl withdrawal day one vs. four), and chronic fentanyl administration relative to withdrawal (chronic admin. vs. withdrawal; * $p < 0.05$, chronic fentanyl vs. fentanyl withdrawal day one; #$p < 0.05$, chronic fentanyl vs. withdrawal day four; \$$p < 0.05$, fentanyl withdrawal day one vs. four)

This was evident by an increase in wake following acute fentanyl (saline: 40.16 ± 7.29%; acute fentanyl: 59.80 ± 3.27%; chronic fentanyl: 42.84 ± 2.84%), particularly at ZT4 ($p < 0.05$; Fig. 8A), accompanied by a significant decrease (saline: 56.79 ± 6.61%; acute fentanyl: 38.18 ± 3.48%; chronic fentanyl: 54.63 ± 2.41%) in NREMS at ZT4

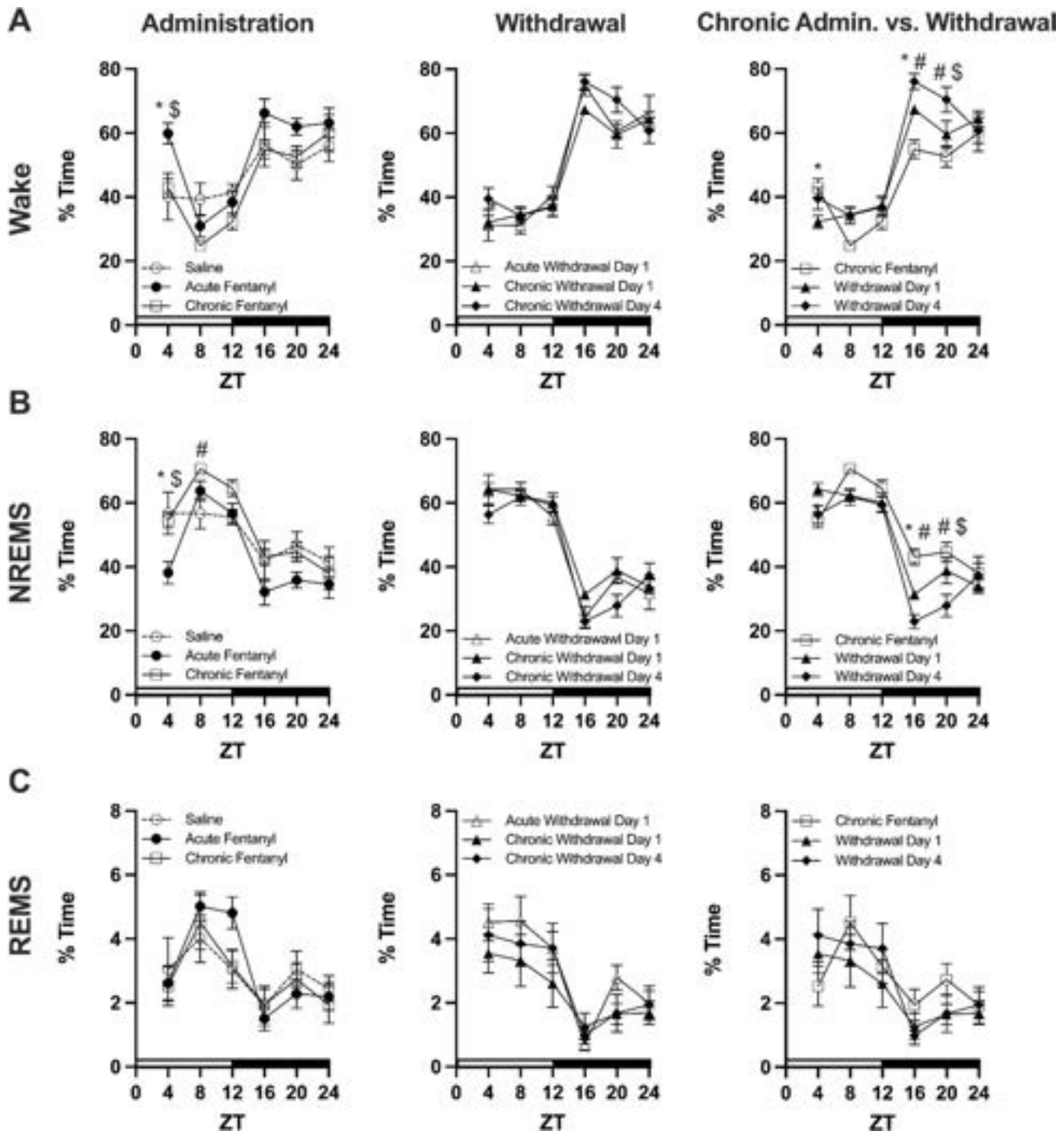

Fig. 8 Diurnal effects of fentanyl administration and withdrawal on sleep and wake in NPAS2-deficient mice. Sleep–wake states across the light–dark cycle in NPAS2-deficient mice. Time spent in wake (**A**), non-rapid eye movement sleep (NREMS, **B**), and rapid eye movement sleep (REMS, **C**) following acute and chronic fentanyl relative to saline (administration; *$p < 0.05$, saline vs. acute fentanyl; \$$p < 0.05$, acute vs. chronic fentanyl; #$p < 0.05$, saline vs. chronic fentanyl), during fentanyl withdrawal (withdrawal), and chronic fentanyl administration relative to withdrawal (chronic admin. vs. withdrawal; *$p < 0.05$, chronic fentanyl vs. fentanyl withdrawal day one; #$p < 0.05$, chronic fentanyl vs. withdrawal day four; \$$p < 0.05$, fentanyl withdrawal day one vs. four)

($p < 0.05$; Fig. 8B). NREMS was also increased by 14% by chronic fentanyl administration (70.63 ± 1.35%) compared to saline (56.70 ± 4.74%) at ZT8 ($p < 0.05$; Fig. 8B). No significant effects were observed in REMS between acute and chronic fentanyl administration, only varying by time of day ($F_{(5,108)} = 8.68$; $p < 0.0001$), with increased REMS

during the light, or inactive phase (Fig. 8C). Similarly, only main effects for time of day were identified for wake ($F_{(5,108)} = 88.37$; $p < 0.0001$), NREMS ($F_{(5,108)} = 90.43$; $p < 0.0001$), and REMS ($F_{(5,108)} = 14.05$; $p < 0.0001$), during fentanyl withdrawal (Fig. 8A–C).

Notably, withdrawal from chronic administration of fentanyl led to persistent alterations in sleep and wake in NPAS2-deficient mice. Repeated measures ANOVA revealed significant interactions between fentanyl withdrawal and time of day for wake ($F_{(10,108)} = 3.32$; $p = 0.0009$; Fig. 8A) and NREMS ($F_{(10,108)} = 3.33$; $p = 0.0008$; Fig. 8B). At ZT4, wake was significantly increased following chronic fentanyl (41.10 ± 2.49) relative to withdrawal day one (33.11 ± 2.79) ($p < 0.05$; Fig. 8A). Wakefulness was significantly elevated at ZT12 (chronic fentanyl: 29.56 ± 2.20 and withdrawal day four: 39.85 ± 1.93) and ZT16 (chronic fentanyl: 53.36 ± 3.76 and withdrawal day four: 76.18 ± 3.18) on day four of withdrawal in NPAS2-deficient mice, accompanied by significant reductions in NREMS at the same times of day ($p < 0.05$; Fig. 8B).

At ZT4, NREMS was lower for chronic fentanyl administration (ZT4: 56.35 ± 1.99) compared to withdrawal day 1 (ZT4: 63.72 ± 2.90), while at ZT8 and ZT16, NREMS was greater for chronic fentanyl administration (ZT8: 71.80 ± 1.33; ZT16: 44.63 ± 3.22) compared to withdrawal day 1 (ZT8: 62.60 ± 2.23; ZT16: 32.29 ± 1.36; Fig. 8B). Similarly, at ZT8, ZT16, and ZT20, NREMS was even lower by withdrawal day 4 (ZT8: 61.31 ± 3.25, ZT16: 22.93 ± 3.01, ZT20: 24.97 ± 3.31) compared to chronic fentanyl administration (ZT8: 71.81 ± 1.33, ZT16: 44.63 ± 3.22, ZT20: 44.68 ± 4.27; Fig. 8B). Together, these results demonstrate chronic fentanyl administration led to persistent changes in wake and NREMS through 4 days of withdrawal in NPAS2-deficient mice.

Discussion

Intense and severe withdrawal symptoms during abstinence can contribute to the risk of relapse to opioids. Among these symptoms is sleep dysfunction and disrupted circadian rhythms, recognized as a possible hallmark of OUD and opioid dependence (Beswick et al. 2003; Maulik et al. 2002; Lydon-Staley et al. 2017; Eacret et al. 2020). Most patients using prescription opioids or being treated for OUD or dependence with methadone breach the clinical threshold for sleep disorders (Hartwell et al. 2014; Stein et al., 2004). Few studies have characterized the impact of opioids on sleep architecture following acute and chronic opioid administration, as well as the several days into withdrawal. Synthetic opioids have surpassed prescription opioids in rates of use and the cause of drug-related overdose deaths. In the present study, we investigated the impact of the synthetic opioid fentanyl on sleep architecture and the diurnal pattern of sleep–wake states in male mice. In addition, given the recent findings that the circadian transcription factor NPAS2 modulates sleep and drug reward, we assessed whether fentanyl-induced sleep changes were further altered in NPAS2-deficient mice. Overall, our findings demonstrate that both acute and chronic fentanyl lead to changes in sleep architecture, particularly in NREMS, and following chronic administration of fentanyl, alterations in NREMS, accompanied by increased wakefulness and arousal, that persist during withdrawal. Further, augmented arousal and reduced NREMS was more pronounced in NPAS2-deficient mice, that was specific to transitions between the light and dark phase of the light–dark cycle (i.e., during the early light phase and again during the early dark phase).

Chronic fentanyl administration led to a robust reduction in NREMS that progressively became more severe during days one and four of withdrawal. The progressive loss of NREMS by chronic fentanyl and withdrawal occurred independent of genotype of the mice. Contrary to previous work, fentanyl (citrate) led to increased NREMS and decreased REMS, although these studies only monitored sleep for 1 h following an acute fentanyl dose in rats (Montandon and Horner 2019). Here, acute fentanyl administration led to minimal changes in sleep state and wakefulness in wild-type male mice. In contrast, acute fentanyl administration disrupted NREMS and arousal depending on time of day in NPAS2-deficient mice. Acute fentanyl reduced the duration of NREMS in NPAS2-deficient mice and increased the propensity to awaken compared to saline. This reduction in NREMS following fentanyl was more pronounced following either acute or chronic fentanyl administration in NPAS2-deficient mice relative to wild-type controls. Persistent reductions in NREMS were evident on days one and four of withdrawal. Together, our evidence suggests that the circadian transcription factor NPAS2 is involved in mitigating the impact of fentanyl administration on sleep and diurnal regulation of sleep and arousal. Interestingly, chronic fentanyl administration had minimal impacts on sleep and wake immediately following the last injection, suggesting the development of tolerance to opioids and sleep-related changes (Kay 1975).

In addition, NPAS2 may be critical for the regulation of sleep homeostasis in response to perturbations such as stress and sleep deprivation. For example, following sleep deprivation, mice typically experience a rebound of NREMS and REMS (Franken et al. 2006). However, in mice deficient of functional NPAS2, NREMS, and REMS rebound following sleep deprivation is significantly blunted (Franken et al. 2006). Moreover, previous work has shown that NPAS2-deficient mice exhibited lower NREMS and REMS in the dark (active) phase of the light–dark cycle (Dudley et al. 2003; Franken et al. 2006). Our findings revealed similar

amounts of NREMS and REMS between wild-type and NPAS2-deficient male mice. Opioids may entrain molecular and behavioral rhythms when administered repeatedly at similar times of day or circadian phases (Vansteensel et al. 2005; Hood et al. 2011). Our findings demonstrate that sleep architecture (i.e., REMS and NREMS) was mainly altered during the "active" phase (dark portion of the light–dark cycle) in both wild-type and NPAS2-deficient mice. Changes in the diurnal pattern of sleep architecture were mainly due to increases in wake and decreases in NREMS at specific times of day. Importantly, these sleep changes and their diurnal pattern alterations continued to "worsen" during withdrawal, days 1 and 4, despite the absence of fentanyl. Possibly, the repeated fentanyl administration led to time-dependent alterations in sleep and wake, possibly reflecting entrainment by fentanyl. Nevertheless, our work suggests NPAS2 may be important for mediating the impact of fentanyl on sleep and wake, and possibly, the homeostatic regulation of sleep during withdrawal from opioids. Although the mechanism by which NPAS2 mediates the effects of fentanyl withdrawal on sleep is unknown, the involvement of NPAS2 in the molecular clock is likely (Franken and Dijk 2009) and may be preferentially involved in modulating arousal and wakefulness through actions in the striatum (Becker-Krail et al. 2022; DePoy et al. 2021; Garcia et al. 2000; Luo et al. 2018; Ozburn et al. 2015, 2017; Parekh et al. 2019; Zhang et al. 2021).

NREMS is particularly critical for memory functions, especially declarative memory in humans. Evidence indicates that acute withdrawal impairs working memory in humans (Rapeli et al. 2006), however, studies in rodent models on the effects of acute opioid withdrawal on memory have been inconsistent (Morisot and Contarino 2016; Baidoo et al. 2020). Therefore, altered NREMS during opioid withdrawal may be involved in withdrawal-related impairments in working memory and cognitive function (Rapeli et al. 2006), postulated to contribute to relapse. Disrupted sleep may also contribute to cognitive impairments experienced by people with OUD (Eacret et al. 2020).

While other studies have reported differences in REMS following opioid administration (Eacret et al. 2020; Wang and Teichtahl 2007), our findings suggest that acute and chronic fentanyl administration have minimal effects on REMS in male mice. Additionally, fentanyl has unique pharmacological actions compared to other μ-opioid receptor agonists including morphine, likely contributing to differences between our findings and previous (Kelly et al. 2021). More studies are required to comprehensively understand the relationship between opioids and sleep. Here, we included only male mice, which is a major limitation of our work. Ongoing and future studies will include female mice using different models of opioid administration and sleep polysomnography. As OUD continues to rise in females (Barbosa-Leiker et al. 2021), further understanding the sex-specific mechanisms underlying the relationships between sleep, circadian rhythms, and opioids is imperative for developing interventions and therapeutics, as opioids differently impact males and females which impact clinical treatment (Huhn et al. 2019). While this study focused on the effects of opioids on sleep, the relationship between sleep and substance use disorders is bidirectional. For instance, chronic sleep deprivation reduced morphine drinking in male and female mice yet developed and maintained morphine conditioned reward (Eacret et al. 2022). Future studies will explicitly investigate sex-specific effects of opioids on sleep and the impact of sleep deprivation on opioid reward. Moreover, considering that fentanyl is often used in combination with other substances (Ciccarone 2019; Peppin et al. 2020), future work should expand into models of polysubstance use and sleep.

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Declarations

Conflict of interest The authors declare no competing interests.

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