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Activation of the nociceptin/orphanin-FQ receptor promotes NREM sleep and EEG slow wave activity

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Sleep/wake control involves several neurotransmitter and neuromodulatory systems yet the coordination of the behavioral and physiological processes underlying sleep is incompletely understood. Previous studies have suggested that activation of the Nociceptin/orphanin FQ (N/OFQ) receptor (NOPR) reduces locomotor activity and produces a sedation-like effect in rodents. In the present study, we systematically evaluated the efficacy of two NOPR agonists, Ro64-6198 and SR16835, on sleep/wake in rats, mice, and Cynomolgus macaques. We found a profound, dose-related increase in non-Rapid Eye Movement (NREM) sleep and electroencephalogram (EEG) slow wave activity (SWA) and suppression of Rapid Eye Movement sleep (REM) sleep in all three species. At the highest dose tested in rats, the increase in NREM sleep and EEG SWA was accompanied by a prolonged inhibition of REM sleep, hypothermia, and reduced locomotor activity. However, even at the highest dose tested, rats were immediately arousable upon sensory stimulation, suggesting sleep rather than an anesthetic state. NOPR agonism also resulted in increased expression of c-Fos in the anterodorsal preoptic and parastrial nuclei, two GABAergic nuclei that are highly interconnected with brain regions involved in physiological regulation. These results suggest that the N/OFQ–NOPR system may have a previously unrecognized role in sleep/wake control and potential promise as a therapeutic target for the treatment of insomnia.

NOP | REM sleep | EEG | body temperature | c-Fos

Significance

Although the control of sleep and wakefulness is known to involve coordination of multiple neurotransmitter and neuromodulatory systems, the mechanisms involved are only partially known. The N/OFQ–NOP receptor (NOPR) system has been implicated in nociception, stress-induced analgesia, cognition, reward, locomotion, anxiety, neuroendocrine control, cardiovascular and respiratory function, but some reports have indicated that activation of NOPRs also may also reduce activity and produce sedative effects. Consequently, we assessed the efficacy of two NOPR agonists in three mammalian species and report here robust, consistent effects on NREM sleep across these species, indicating that the N/OFQ–NOPR system has a pronounced, previously unrecognized role in sleep/wake control.

Insufficient sleep and insomnia, whether chronic—due to a pathological state—or acute—due to stress, jet lag, or shift work—has serious health and economic consequences. According to the Centers for Disease Control and Prevention, 30 to 40% of US adults and 65 to 80% of teens report sleep deficiency (i.e., insufficient sleep, irregular timing of sleep, poor quality of sleep) (1–3). Although cognitive behavioral training for insomnia (CBT-I) is now considered the first-line treatment worldwide for insomnia (4), sleep medications are often preferred by individuals because they offer near-immediate relief. Among prescription medications, the $GABA_A$ agonist zolpidem (ZOL) is widely used but has been associated with adverse complex behaviors such as somnambulism, nocturnal eating, sleep driving, and sexsomnia which, although rare, occur frequently enough to prompt the U.S. Food and Drug Administration to issue a warning (5). In contrast to the global inhibition of neural activity produced by ZOL, the newer hypocretin receptor antagonists create conditions that are permissive for sleep to occur by blocking the wake-promoting activity of the hypocretin neuropeptides. However, these compounds have had limited market success to date because of the delayed onset of efficacy relative to ZOL and the vivid dreaming that is disturbing to some individuals.

In searching for other receptor targets to influence sleep/wake control, we noted that the small molecule NOP receptor (NOPR) agonist Ro64-6198 had been reported to induce sedation and loss of the righting reflex in mice and hypoactivity in rats (6). NOPR is encoded by the *Oprl1* gene and has nucleotide and amino acid homology to the mu-, delta-, and kappa-opioid receptors comparable to that which they have with each other, but opiates do not bind to the NOPR with high affinity. Two groups independently discovered the endogenous 17-amino acid peptide ligand for the NOPR, which was initially called nociceptin (7) due to its putative pronociceptive activity, and orphanin FQ (8), because it bound to an orphan receptor whose first and last amino acids were Phe (F) and Gln (Q). This peptide ligand, now called N/OFQ, has low affinity for mu-, delta-, and kappa-opioid receptors (9). Similar to other opioid receptors, NOPRs are $G_{i/o}$-coupled and inhibit cAMP production, activate inwardly rectifying K^+ channels (10, 11) and inhibit L–, N–, and P/Q-type calcium channels (12) in widespread brain areas. The endogenous N/OFQ-NOPR system has been implicated in regulation of nociception, stress-induced analgesia, cognition, reward, motivation, locomotion, anxiety, neuroendocrine control, cardiovascular, and respiratory function (13–17). Agonists at each of the four receptors produce spinal analgesia and mu, delta, and

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kappa receptor agonists also elicit supraspinal analgesia whereas NOPR agonism results in supraspinal hyperalgesia. A mixed-function NOPR and mu-opioid receptor (MOPR) agonist is in clinical trials for pain (14) and a NOPR antagonist has been in clinical trials for depression (18). Encouraging results on pain have been recently reported for other mixed-acting NOPR/MOPR agonists (19, 20).

Both the N/OFQ peptide (21–23) and the NOPR (24–27) occur in hypothalamic and brainstem regions known to be involved in sleep/wake control. Among these brain regions, the N/OFQ peptide has been found in the suprachiasmatic nuclei (SCN) (28), although little to no N/OFQ mRNA is expressed there (21, 28). *Oprl1* mRNA is also sparsely expressed in the rodent SCN (24, 29) yet very high levels of radioligand binding occur there (24). Accordingly, N/OFQ suppressed firing in 88% of SCN neurons and inhibited both excitatory and inhibitory neurotransmission in the SCN (28, 30) and intra-SCN injections of N/OFQ attenuated the phase-shifting response to light pulses (28). NOPR activation downregulated the circadian clock protein PER2 in the SCN (31) and accelerated re-entrainment of rhythms following a shift in the light–dark cycle (31, 32). In the human prefrontal cortex, *OPRL1* mRNA expression varied across the day and was among the genes with the largest peak-to-trough amplitude (33). These results suggest a function for N/OFQ in the circadian system which, along with the prior reports of sedative activity of NOPR agonists (6, 34), encouraged us to evaluate a possible role for the N/OFQ-NOPR system in sleep/wake control.

Here, we determined the efficacy of two NOPR agonists, Ro64-6198 and SR16835, on sleep/wake in rats, mice, and non-human primates (NHPs). Ro64-6198 (Fig. 1*A*) was originally described as a full agonist at the NOPR (pK_i = 9.41 ± 0.06) with much lower affinity for the mu (pK_i = 7.33 ± 0.09), kappa (pK_i = 7.05 ± 0.07), and delta (pK_i = 5.86 ± 0.04) opiate receptors (35). SR16835 (Fig. 1*B*) is a full agonist at NOPR (K_i = 11.4 nM) and a partial agonist at the MOPR (K_i = 79.9 nM) (36). We found that NOPR agonism produced a profound, dose-related increase in NREM sleep and electroencephalogram (EEG) slow wave activity (SWA) and suppression of rapid eye movement sleep (REM) sleep in all three species. At the highest dose tested in rats, the increase in NREM and EEG SWA was accompanied by hypothermia and a prolonged inhibition of REM sleep. These responses to NOPR agonism also result in increased expression of c-Fos in the anterodorsal preoptic and parastrial nuclei, two GABAergic nuclei that are highly interconnected with brain regions involved in physiological regulation (37). Since NOPR activation induces such profound changes in NREM sleep and EEG SWA activity in both diurnal primates and nocturnal rodents, the N/OFQ-NOPR system may have a previously unrecognized role in sleep/wake control and the NOPR may be an interesting target for the development of insomnia therapeutics.

Results

NOPR Agonism Reduces Sleep Latency and Increases NREM Sleep and EEG Delta Power in Rats. In a repeated measures design, adult male Sprague–Dawley rats (n = 8) each received three concentrations of Ro64-6198 (1, 3, and 10 mg/kg), one concentration of the $GABA_A$ agonist zolpidem (ZOL; 10 mg/kg), and the hydroxypropyl methylcellulose (HPMC) vehicle control, all administered i.p. The two highest doses of Ro64-6198 reduced the latency to NREM sleep relative to vehicle (Fig. 1*C*). All three concentrations of Ro64-6198 decreased Wake for at least 4 h post-injection (6 h at the 10 mg/kg dose, Fig. 2 *A* and *B*) and potentiated NREM sleep for the same duration (Fig. 2 *C* and *D*). The magnitude of the NREM increase produced by the 3 and 10 mg/kg doses was greater than that produced by 10 mg/kg ZOL, the leading insomnia medication. After the 10 mg/kg dose of Ro64-6198, seven of eight rats exhibited uninterrupted NREM sleep for 1 to 2 h as indicated by the huge increases in NREM bout duration from ZT19-21 relative to both vehicle and ZOL (Fig. 2*G*). Consequently, the latency to REM sleep was delayed by the two highest concentrations of Ro64-6198 (Fig. 1*E*). Fig. 2*B* shows that the decrease in Wake produced by Ro64-6198 for the first 6-h postdosing was dose-related and Fig. 2*D* shows that the increase in NREM sleep was also dose-related for the same time bin. REM sleep was unaffected by the lowest dose but was almost completely eliminated for 8 h after injection at 10 mg/kg and only slowly recovered during the light phase (Fig. 2 *E* and *F*). These changes resulted in profound decreases in the REM:NREM ratios which, at the 10 mg/kg dose of Ro64-6198, exceeded even the well-known reduction in REM:NREM ratio produced by ZOL (*SI Appendix*, Fig. S1 *C* and *E*).

Locomotor activity (LMA) was decreased by Ro64-6198 (10 mg/kg) compared to vehicle for 4 of the 6 post-injection hours (ZT19-21 and ZT23) and during ZT21-ZT22 following Ro64-6198 at 1 and 3 mg/kg (Fig. 2*I*). A dose-related decrease in body temperature (T_b) was observed from ZT19-21 in association with increased NREM sleep (Fig. 2*J*). Following the 10 mg/kg dose, T_b was significantly lower than vehicle from ZT19-ZT22 and even lower than ZOL during ZT20-ZT21. However, T_b returned to normal levels by the end of the dark phase and increased during the subsequent light phase for ZOL and all three concentrations of Ro64-6198**.**

The increased NREM sleep subsequent to the 3 and 10 mg/kg concentrations of Ro64-6198 was accompanied by a sustained increase in NREM delta power for the first 6 h relative to vehicle (Fig. 3*B*; $P < 0.05$). NREM theta and alpha power also increased following Ro64-6198 (Fig. 3 *D* and *F* respectively). Dose-related increases in delta, theta, and alpha during wakefulness also occurred (Fig. 3 *A*, *C*, and *E* respectively). Despite these profound changes in the EEG, locomotor activity and T_b, experimenter observations indicated that rats remained responsive to auditory stimulation, even at the highest dose.

Mixed NOPR/MOPR Agonism also Dose-Dependently Reduces Sleep Latency and Increases NREM Sleep and EEG Delta Power in Rats. Since SR16835 is also a full agonist at NOPR, it was a useful tool compound to further establish the selectivity of the Ro64-6198 effects. Consequently, we replicated the experiment described above in a different cohort of Sprague–Dawley rats (n = 8) that were treated with three concentrations (1 to 10 mg/kg) of the NOPR agonist SR16385, ZOL (10 mg/kg), and the HPMC vehicle, all administered i.p. As with Ro64-6198, the two highest doses of SR16835 reduced the latency to NREM sleep relative to vehicle (Fig. 1*D*) and REM sleep latency was delayed (Fig. 1*F*). Furthermore, SR16835 produced nearly identical results with dose-dependent decreases in Wake (*SI Appendix,* Fig. S2 *A* and *B*), increases in NREM (*SI Appendix,* Fig. S2 *C* and *D*), and complete suppression of REM for 6h at the highest dose that only slowly recovered during the light phase (*SI Appendix,* Fig. S2 *E* and *F*). As with Ro64-6198, the reduced levels of REM sleep persisted for the first half of the light phase (*SI Appendix,* Fig. S2 *E* and *F*). The increased NREM sleep was due to longer NREM bouts for the first 2 h post-injection (*SI Appendix,* Fig. S2*G*) and was accompanied by reductions in both LMA and T_b (*SI Appendix,* Fig. S2 *I* and *J*). The REM/NREM ratio decreased for the 6-h post-administration of the two highest concentrations of SR16835 and for the first 6 h of the light period following the 10 mg/kg dose (*SI Appendix,* Fig. S1 *D* and *F*).

For the first 6 h following the 3 and 10 mg/kg concentrations of SR16835, NREM delta power underwent a sustained increase

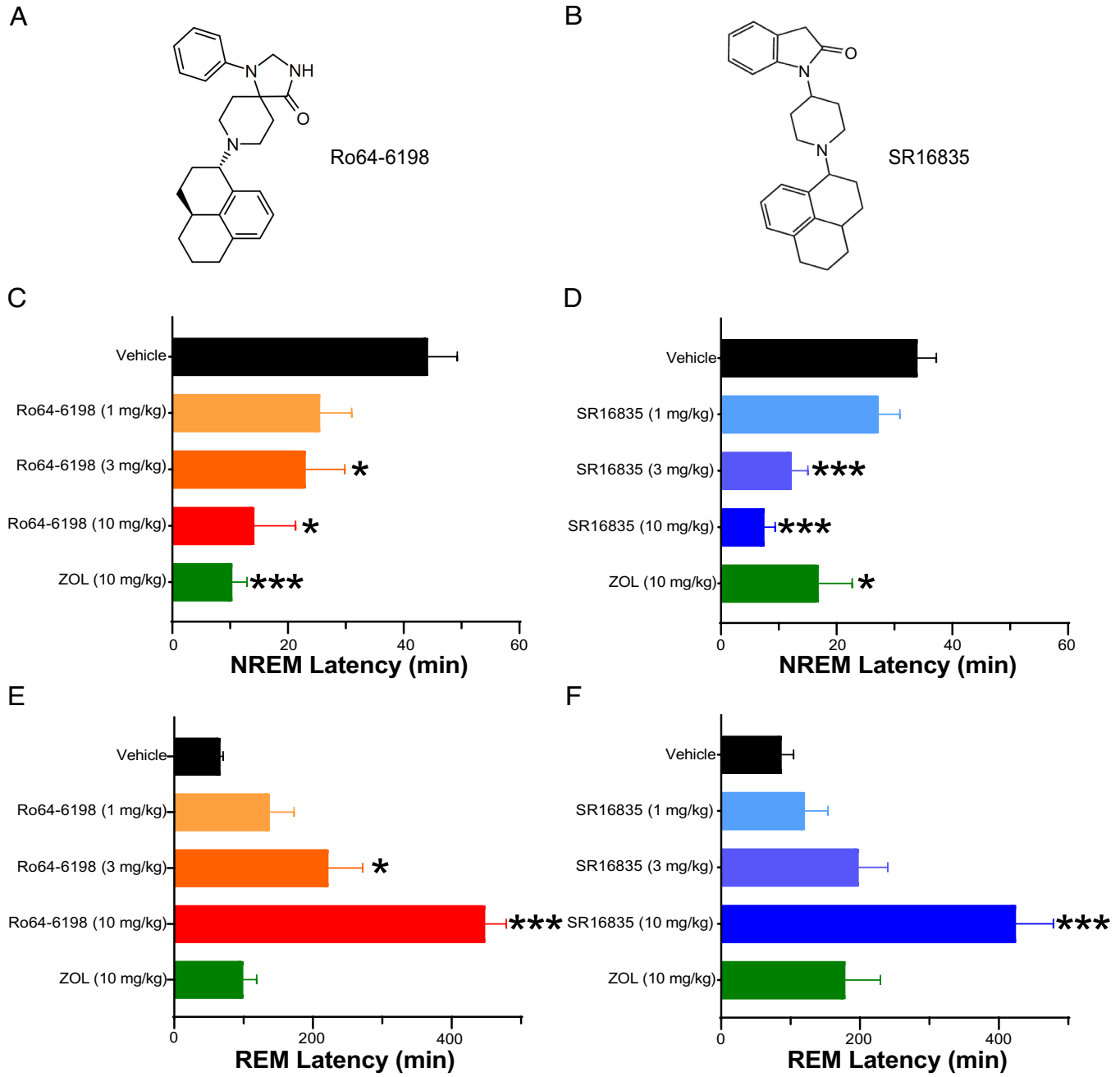

Fig. 1. Latencies to NREM and REM sleep in adult male Sprague–Dawley rats in response to two NOPR agonists. (*A*) Chemical structure of Ro64-6198 and (*B*) SR16835. (*C*) Latency to the onset of NREM sleep in response to treatment with Ro64-6198 and (*D*) SR16835. (*E*) Latency to the onset of REM sleep in response to treatment with Ro64-6198 and (*F*) SR16835. Values are mean ± SEM; $n = 8$. *$P < 0.05$; ***$P < 0.001$.

relative to vehicle treatment (*SI Appendix,* Fig. S3*B*; $P < 0.05$). NREM theta and alpha power also increased following SR16835 (*SI Appendix,* Fig. S3 *D* and *F* respectively). Similar to Ro64-6198, dose-related increases in Waking delta, theta, and alpha occurred (*SI Appendix,* Fig. S3 *A*, *C* and *E* respectively). These results demonstrate that two different NOPR agonists produce very similar effects on sleep/wake physiology.

Increased NREM Sleep Evoked by the NOPR Agonism is Absent in NOPR Null Mutant Mice. To determine whether the increased NREM sleep produced by Ro64-6198 was mediated through the NOPR, we evaluated the effects of Ro64-6198 on sleep/wake parameters in *Oprl1*$^{-/-}$, *Oprl1*$^{+/-}$ heterozygous and littermate wild type (WT) mice (30). Identical dose ranges and time of dosing were utilized for the three strains of mice as was used in the rat studies described above. Fig. 4 presents the cumulative time spent in NREM sleep for the three strains. As in rats, Ro64-6198 produced a dose-related decrease in Wake and increase in NREM sleep in WT mice ($P < 0.0001$ for both; $n = 6$; Fig. 4 *A* and *D*) and heterozygous *Oprl1*$^{+/-}$ ($P = 0.0003$ for Wake; $P = 0.0002$ for NREM; $n = 6$; Fig. 4 *B* and *E*) mice. In contrast, no changes In Wake or NREM sleep were found in *Oprl1*$^{-/-}$ null mutant mice ($n = 5$; Fig. 4 *C* and *F*). REM sleep decreased in WT mice following Ro64-6198 at 10 mg/kg (Fig. 4 *G*) but no significant effects on REM sleep were observed in heterozygous *Oprl1*$^{+/-}$ or *Oprl1*$^{-/-}$ null mutant mice (Fig. 4 *I* and *H*). These results demonstrate that the effects of Ro64-6198 on sleep are mediated through the NOPR.

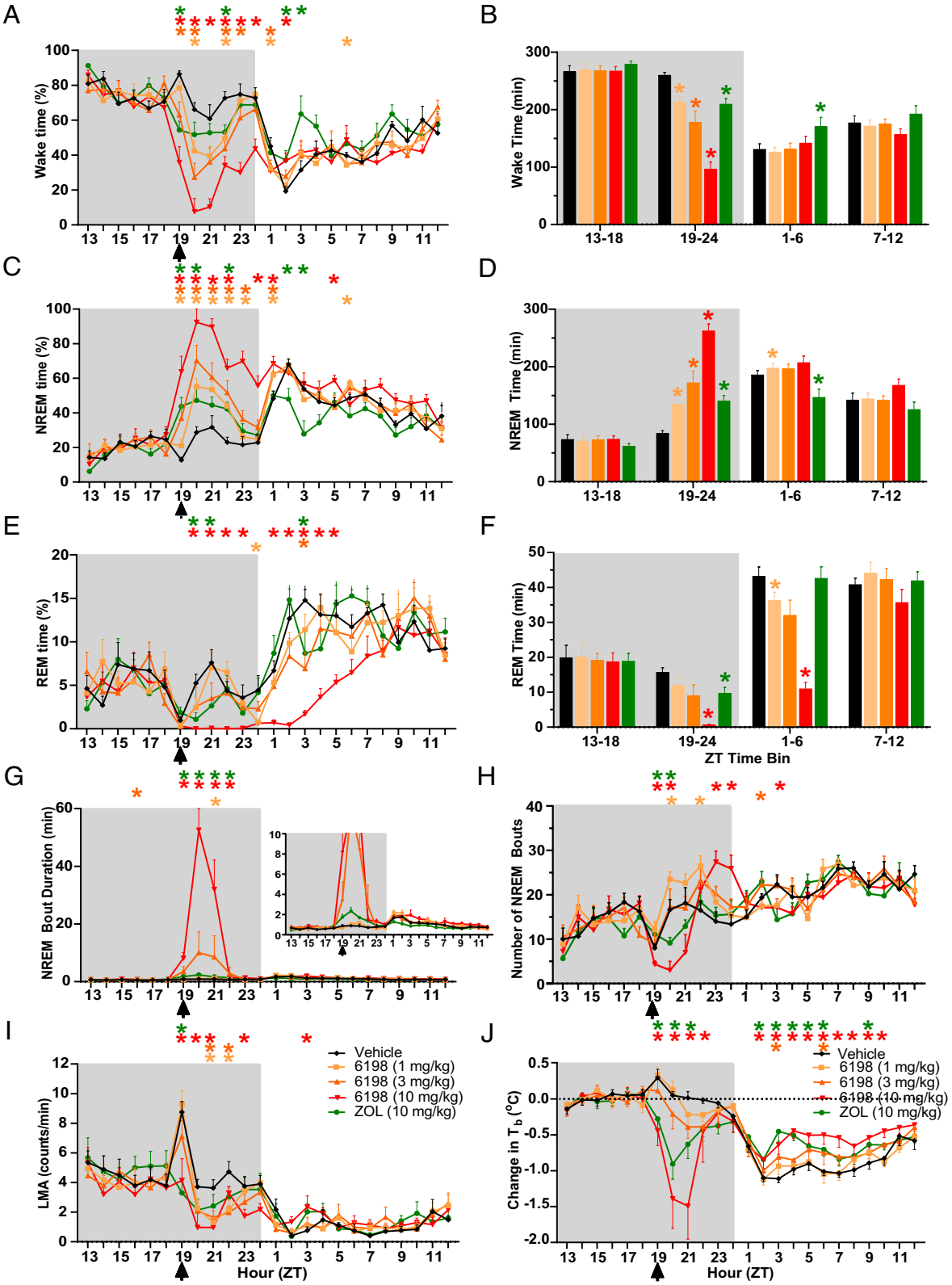

Fig. 2. Effects of the NOPR agonist Ro64-6198 on sleep/wake, body temperature (T_b) and locomotor activity (LMA) parameters in male Sprague–Dawley rats. Hourly percentage of time spent in Wake (*A*), NREM (*C*), and REM (*E*) sleep after i.p. injection of vehicle, three concentrations of the NOPR agonist Ro64-6198 or zolpidem (ZOL) in the middle of the dark period at ZT19, as indicated by the arrow on the abscissa of each panel. The amount of Wake (*B*), NREM sleep (*D*), and REM sleep (*F*) presented in 6-h bins for the 6 h prior to injection (ZT13-18) and 18 h after injection. The hourly duration (*G*) and number (*H*) of NREM bouts for the 6 h prior to and 18 h after injection. Inset in *G* shows the magnitude of the increase in NREM bout duration produced by Ro64-6198 relative to ZOL and Vehicle. At the 10 mg/kg dose, 7 of 8 rats had uninterrupted NR sleep for 1 to 2 h. LMA (*I*) and the change in core T_b (*J*) for the 6 h prior to and 18 h after injection. Colored asterisks above each graph indicate a significant difference ($P < 0.05$) for that treatment during that hour relative to vehicle treatment based on post hoc tests conducted when two-way ANOVA indicated a significant treatment × time effect. Values are mean ± SEM; $n = 8$. Shaded area indicates dark phase of LD12:12 cycle.

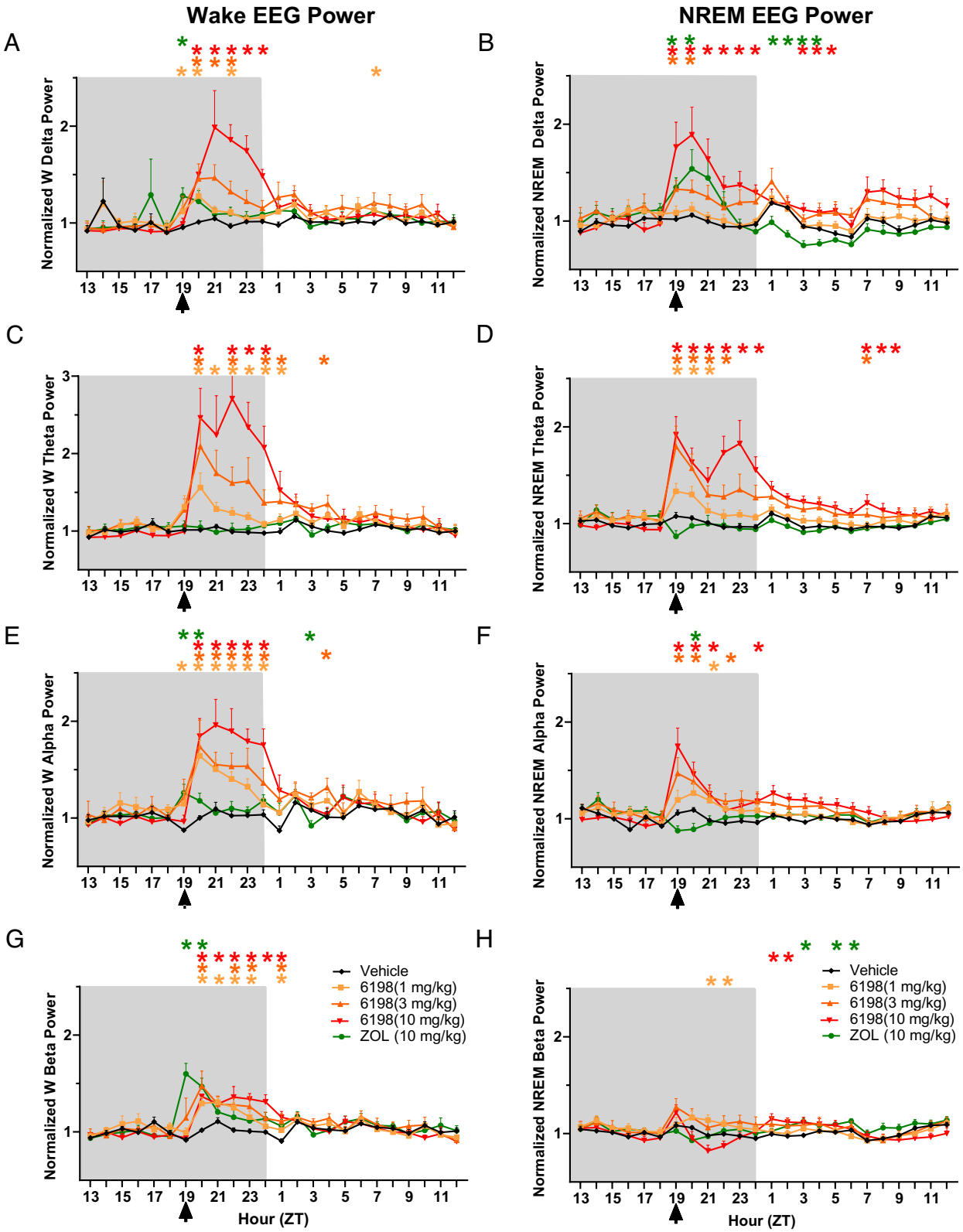

Fig. 3. Effects of the NOPR agonist Ro64-6198 on EEG spectra during Wakefulness (*Left*) and NREM sleep (*Right*) in male Sprague–Dawley rats after i.p. injection of vehicle, three concentrations of the NOPR agonist Ro64-6198 or zolpidem (ZOL) in the middle of the dark period at ZT19, as indicated by the arrow on the abscissa of each panel. (*A* and *B*) EEG delta power. (*C* and *D*) EEG theta power. (*E* and *F*) EEG alpha power. (*G* and *H*) EEG beta power. Colored asterisks above each graph indicate a significant change ($P < 0.05$) for that treatment during that hour relative to vehicle based on post hoc tests conducted when two-way ANOVA indicated a significant treatment × time effect. Values are mean ± SEM; $n = 8$. Shaded area indicates dark phase of LD12:12 cycle.

NOPR Agonism Reduces Sleep Latency and Increases NREM Sleep and EEG Delta Power in Non-human Primates. To determine whether the effects of NOPR agonism described for nocturnal rodent species could be observed in a diurnal primate, Cynomolgus macaques ($n = 5$) received either the HPMC vehicle or the NOPR agonist Ro64-6198 (0.1 mg/kg, s.c.) in a counterbalanced design, ~30 min before lights off at ZT12. A minimum of 3 d elapsed between treatments. Ro64-6198 at 0.1

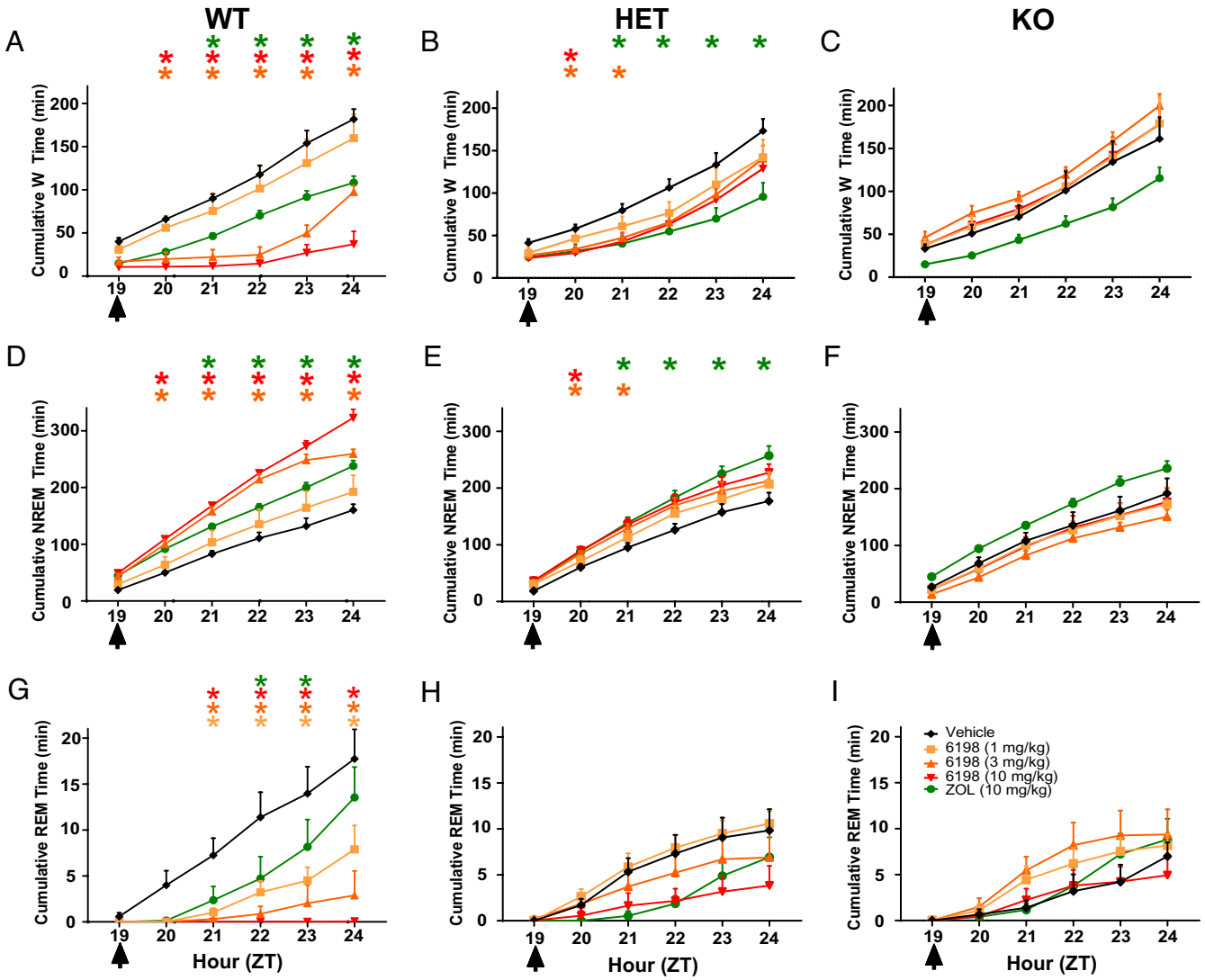

Fig. 4. Cumulative amounts of Wakefulness (*A–C*), NREM sleep (*D–F*) and REM sleep (*G–I*) in littermate wildtype (WT; $n = 6$, *Left* panels), heterozygous *Oprl1*$^{+/-}$ (HET; $n = 6$, *Middle* panels) and homozygous *Oprl1*$^{-/-}$ knockout (KO; $n = 5$, *Right* panels) mice after administration of the NOPR agonist Ro64-6198 in the middle of the dark period at ZT19, as indicated by the arrow on the abscissa of each panel. Colored asterisks above each graph indicate a significant change ($P < 0.05$) for that treatment during that hour relative to vehicle based on post hoc tests conducted when two-way ANOVA indicated a significant treatment × time effect. Values are mean ± SEM.

mg/kg reduced the latency to NREM sleep (N2; *SI Appendix*, Fig. S4*A*), decreased time in Wake, and increased NREM time (Fig. 5 *A–D*). Wake time decreased during both the first and second half of the 12-h dark period. N1 decreased and N3 increased during ZT13-ZT18 and N2 increased during ZT19-ZT24. Total NREM (N1+N2+N3) time increased overall (treatment effect) across the 12-h dark period and during ZT13-ZT14, ZT17-ZT19, and ZT21 (Fig. 5*D*). REM sleep decreased during both the first 6 h and across the 12-h dark period (Fig. 5 *A* and *E*); consequently, the REM/NREM ratio was reduced from ZT13-ZT18 (*SI Appendix*, Fig. S4*B*) but recovered during the second half of the night (*SI Appendix*, Fig. S4*D*). Delta power increased across the 12-h dark period for all three EEG derivations (Fig. 5 *F–K*). These results demonstrate that NOPR activation induces sleep in a NHP species that is diurnal and whose sleep structure more resembles that of humans.

NOPR Agonism Increases c-Fos Expression in the Preoptic Region. To identify neural structures influenced by NOPR agonism, we used c-Fos immunohistochemistry to identify region-specific induction of neural activity. A cohort of $n = 16$ Sprague–Dawley rats received an i.p. dose of either the NOPR agonist SR16385 (10 mg/kg, $n = 8$) or the HPMC vehicle ($n = 8$) at ZT18. After either 1 h or 2 h post-injection, $n = 4$ rats from each treatment group were deeply anesthetized and the brain removed for Fos immunohistochemistry. SR16835 (10 mg/kg) increased c-Fos expression in the anterodorsal preoptic (ADP) and parastrial (PS) nuclei of rats (Fig. 6). Significant increases in darkly stained c-Fos+ cells were evident at both 1 h (Fig. 6*A* vs. Fig. 6*B*) and 2 h (Fig. 6*C* vs. Fig. 6*D*) post-dose compared to vehicle (Fig. 6*F*). These results demonstrate that NOPR activation at a dose known to induce deep but reversible sleep also produced localized activation of neurons in the preoptic region, a brain area involved in sleep, thermoregulation and the regulation of other physiological functions.

Discussion

In the present study, we report that NOPR activation induces profound, dose-related increases in NREM sleep, EEG SWA and suppression of REM sleep in rats, mice and non-human primates. At the highest dose tested, the increased NREM and EEG SWA was accompanied by a prolonged inhibition of REM sleep in these three species and hypothermia in rats. These effects of Ro64-6198 were eliminated in *Oprl1* null mutant mice, further corroborating that

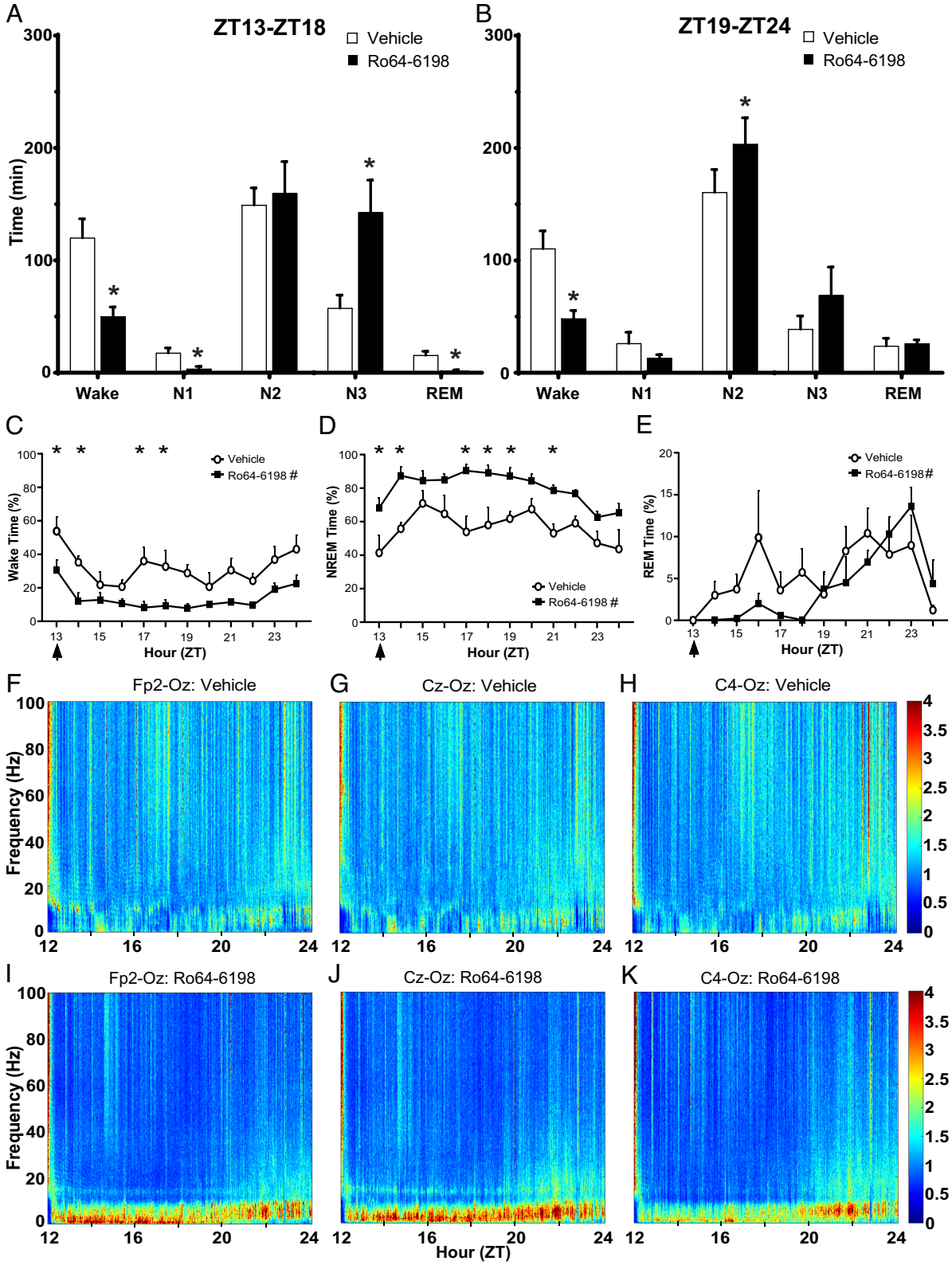

Fig. 5. Effects of the NOPR agonist Ro64-6198 (0.1 mg/kg, s.c.) on sleep/wake in male Cynomolgus macaques. (*A*) The amounts of Wake, N1, N2, N3 and REM sleep recorded during the first 6 h of the dark period (ZT13-18) after dosing just prior to light offset (just prior to light offset at ZT12). (*B*) The amounts of Wake, N1, N2, N3, and REM sleep recorded during the second 6 h of the dark period (ZT19-24) after dosing just prior to light offset. (*C*–*E*) Hourly percentage of Wake, NREM and REM sleep, respectively, during the 12-h dark phase for macaques treated with vehicle or Ro64-6198 (0.1 mg/kg, s.c.) just prior to light offset at ZT12. (*F*–*H*) EEG spectral density recorded across the dark from the Fp2-Oz, Cz-Oz, and C4-Oz derivations, respectively, after dosing with vehicle just prior to lights off. (*I*–*K*) EEG spectral density recorded across the dark from the Fp2-Oz, Cz-Oz, and C4-Oz derivations, respectively, after dosing with Ro64-6198 just prior to lights off. Values are mean ± SEM; *, # indicate $P < 0.05$ relative to Vehicle treatment; $n = 5$. # in legend denotes significant treatment effect by two-way ANOVA; * indicates significant treatment × time effect during that hour.

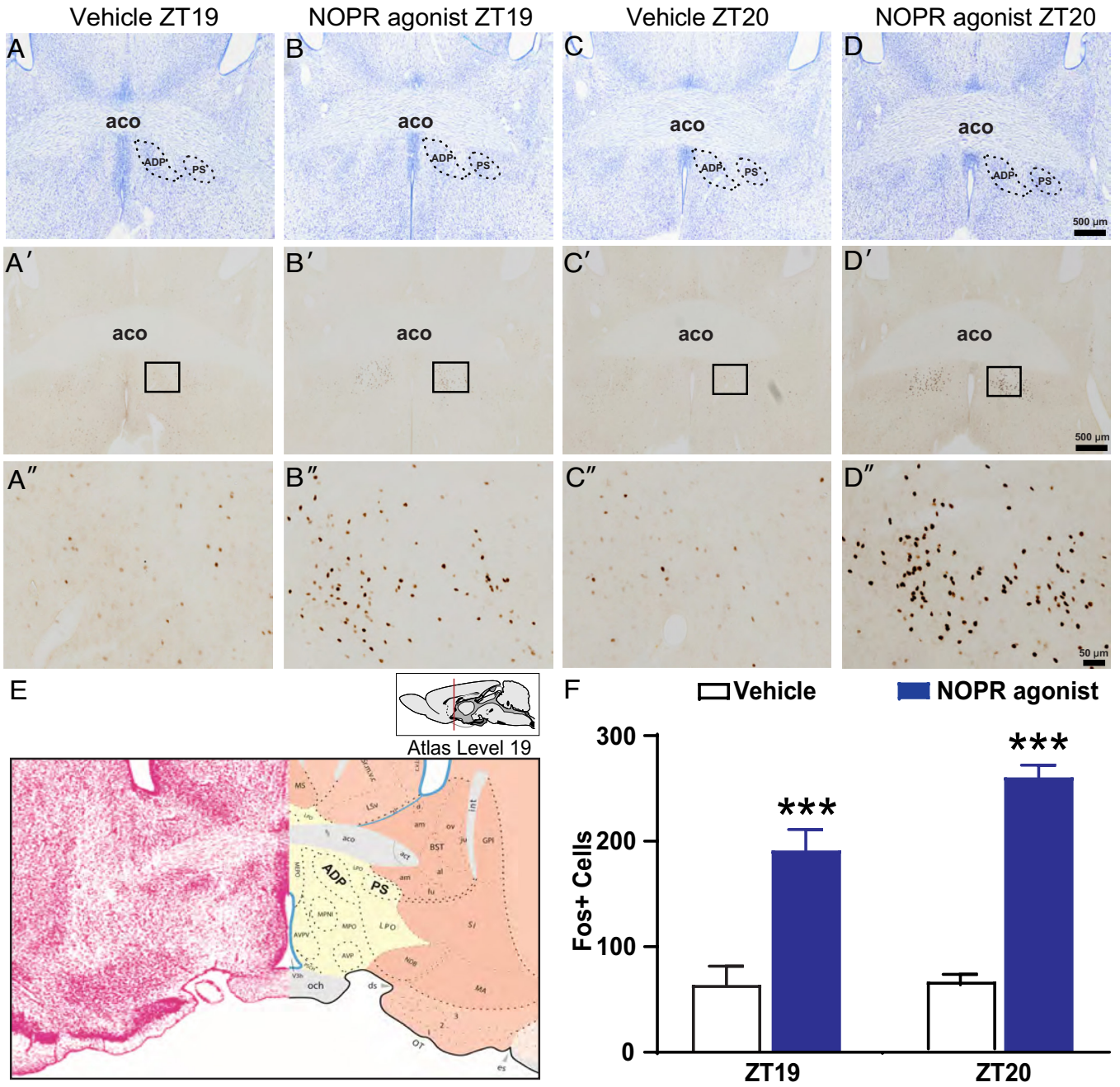

Fig. 6. Effects of the NOPR agonist SR16835 (10 mg/kg) on c-FOS expression in the anterodorsal preoptic (ADP) and parastrial (PS) nuclei of Sprague–Dawley rats. (*A*–*D*) Rat brain sections (40 µm) at the level of the anterior commissure stained with thionin. (*A′*–*D′*) Adjacent 40-µm brain sections immunostained for c-FOS. The areas enclosed by the rectangles in *A′*–*D′* are enlarged in *A″*–*D″* to show the density and intensity of c-Fos-immunoreactive nuclei. (*E*). Coronal section at Level 19 of the Swanson atlas (38) illustrating the location of the anterior dorsal preoptic (ADP) and parastrial (PS) nuclei. (*F*) Number (mean ± SEM) of c-FOS+ nuclei in the ADP and PS in the four experimental conditions. *$P < 0.05$ relative to Vehicle treatment; $n = 4$ per condition.

these systems physiology effects are mediated through NOPR action. The results obtained at lower doses of NOPR agonist administration thus encourage further exploration of NOPR as a unique target for sleep therapeutics and amplify the notion that the N/OFQ–NOPR system is an endogenous neuropeptidergic system that has heretofore been unrecognized with respect to sleep/wake control.

Previous Studies of NOPR Agonism Related to Behavioral States and Arousal. Systemic administration of the NOPR agonist Ro64-6198 was previously shown to elicit a loss of the righting reflex, hypoactivity and hypothermia of up to 5.5 °C in C57BL/6J mice given a 10 mg/kg dose, effects that were attenuated in hemizygous *Oprl1*$^{+/-}$ mice and absent in *Oprl1* null mutant mice (6). Hypoactivity also occurred at the 3 mg/kg dose in C57BL/6J mice. Hypoactivity without hypothermia has also been reported in mice in response to intracerebroventricular (ICV) injections of N/OFQ at doses of 10 nmol/animal (8, 39–41). In Lister hooded rats, the 10 mg/kg dose produced hypoactivity but, similar to low (0.01 to 0.05 nmol) ICV N/OFQ doses in the mouse (42), lower systemic doses of Ro64-6198 produced slight hyperactivity and there were no significant effects on T_b at any dose tested. Other investigators described similar behavioral effects in both rodent species when left undisturbed after treatment with 10 mg/kg Ro64-6198: "body posture became flattened, there was evidence for loss of muscular tone, and virtually complete absence of any locomotor activity" (6). In congruence with these findings, we also observed that the rats regained muscular tone,

resumed locomotor activity and showed reasonable neurological function when presented with sensory stimulation after NOPR agonist treatment. The investigators concluded that deficits recorded in those neurological tests that required experimenter handling were limited to the 10 mg/kg dose (6).

In rats, the NOPR agonists Ro 65-6570 and Org 26383 also produced loss of the righting reflex as well as EEG burst suppression that was reversed by a NOPR antagonist (34). Although these studies suggested promise for NOPR agonists as anesthetics (43), NOPR agonism at nonanesthetic doses has not been previously assessed for effects on sleep/wake. Furthermore, there have been no studies to date that assess the effects of manipulation of the endogenous N/OFQ–NOPR peptidergic system on sleep/wake.

Potential Sites of Action for Sleep-Inducing Effects of NOPR Agonists. N/OFQ (24) expression have been mapped in the rat brain by in situ hybridization. NOPR mRNA expression has been mapped in the rat brain by immunohistochemistry (44) and in situ hybridization (21) and in the mouse brain by histochemical staining for NOPR-LacZ (23, 29, 45). Both mRNAs are expressed throughout the brain, which presents a challenge to determine the neural circuitry that underlies the effects on sleep/wake and physiological systems described here. Among neural structures known to be involved in sleep/wake control, very high levels of NOPR mRNA have been found in the dorsal and median raphe nuclei and locus coeruleus (24, 29) but colocalization studies to define the chemical phenotype of the NOPR-expressing cells are yet to be conducted. We have previously shown that N/OFQ innervation of hypocretin/orexin (Hcrt) cells in the lateral hypothalamus and that application of N/OFQ in vitro hyperpolarizes Hcrt neurons (46). Although we did not assess sleep/wake in that study, since Hcrt neurons also receive corticotrophin-releasing factor (CRF) input (47), we assessed stress-induced analgesia (SIA), a behavioral response in which both CRF and N/OFQ had previously been implicated, and found that SIA was greatly attenuated in mice in which the Hcrt neurons had been ablated (46).

The c-Fos studies we report here indicate that NOPR agonism activates neurons in the ADP and PS nuclei. Although these nuclei have not been identified to have specific roles in sleep/wake control to date, neurons in these two nuclei are thought to be GABAergic and closely linked as part of Hypothalamic Module 1, Subsystem 1.1, which contains nuclei that are more involved in regulation of physiological systems rather than behavior control (37). It is unclear whether neurons in ADP and PS express the NOPR directly or whether the NOPR agonist-induced c-Fos activation in these cells is due to indirect inputs. Although c-Fos expression in these nuclei was robust, given the widespread nature of NOPR expression in the rat brain, additional efforts will be needed to identify the mechanisms and circuits activated by NOPR agonists.

Future Directions. Recently, novel transgenic mouse lines have been created that are enabling dissection of the role of N/OFQ neurons and the NOPR in complex behavior. A prepronociceptin-Cre (*Pnoc-Cre)* mouse line has been used to identify circuits in the central nucleus of the amygdala necessary for regulation of hedonic, but not homeostatic, feeding. Rodriguez-Romaguera (48) also used this line to identify *Pnoc* neurons in the bed nucleus of the stria terminalis that modulate rapid changes in physiological arousal that occur upon exposure to motivationally salient stimuli. Parker et al. (17) created a floxed NOPR line and used these mice along with *Pnoc-Cre* mice to identify a distinct population of N/OFQ neurons within the paranigral nucleus of the ventral tegmental area that regulates reward-related motivational states. Thus, critical tools are now available to manipulate the endogenous N/OFQ–NOPR system and efforts are underway to utilize systems neuroscience tools such as optogenetic and chemogenetic manipulation of specific N/OFQ populations and local or conditional deletion of the NOPR to determine the neural circuitry that underlies the role of this system in sleep/wake control.

Materials and Methods

Animals. Male Sprague–Dawley rats (n = 32; 300 ± 25 g at time of surgery) were purchased from Charles River Laboratories (Wilmington, MA). *Oprl1* null mutant, hemizygous *Oprl1*$^{+/-}$ and littermate wild-type mice were obtained from the laboratory of Chris Evans and Wendy Walwyn at the University of California, Los Angeles from a line originally described (30). Mice and rats were housed in separate temperature-controlled recording rooms under a reverse 12/12 light/dark cycle (lights on at 5:00 pm), with food and water available ad libitum. Room temperature (23 ± 2 °C), humidity (50 ± 20% relative humidity), and lighting conditions were monitored continuously via computer.

Adult male cynomolgus macaques (*Macaca fascicularis*; n = 5; 14 to 19 y, 7 to 10 kg) were maintained in constant environmental conditions (21 ± 3 °C; humidity 30 to 70%; 12:12-h light/dark cycle with lights on at 06:00 and off at 18:00). All animals received a full daily regimen of standard certified commercial chow (Purina Animal Nutrition, Gray Summit, MO) supplemented with fresh fruits and vegetables and had access to water *ad libitum* in their home cage. Animals were individually housed but had visual, auditory and olfactory contact with other monkeys throughout the study. Videos and music were played in the housing room to provide environmental enrichment during the day.

All animals were inspected daily in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and SRI International guidelines. All experimental procedures involving animals were approved by SRI International's Institutional Animal Care and Use Committee and were in accordance with National Institute of Health guidelines.

Experimental Design. In all rodent EEG studies, a repeated measures design was employed in which all animals received three concentrations of the test compound, a positive control (the $GABA_A$ agonist zolpidem; ZOL) and a vehicle control (HPMC: 1.25% hydroxypropylmethyl cellulose, 0.1% dioctyl sodium sulfosuccinate, and 0.25% methylcellulose in purified water). The dosing procedure began 6 h after lights off during the start of Zeitgeber hour 19 (ZT19) and was typically completed within 10 min. A minimum of 3 d elapsed between doses. The electroencephalogram (EEG), electromyogram (EMG), body temperature (T_b) and locomotor activity (LMA; EEG/EMG/T_b) were recorded for 24 h beginning at light offset (ZT12) including 6 h prior to and 18 h after each dose. NHPs received either the HPMC vehicle or the NOPR agonist Ro64-6198 (0.1 mg/kg, s.c.) in a counterbalanced design, ~30 min before lights off at ZT12. A minimum of 3 d elapsed between treatments. EEG/EMG/T_b and LMA were recorded for 12 h beginning at light offset (ZT12). All dosings were conducted by experimenters blind to treatment.

Scoring of EEG/EMG Recordings. Following completion of data collection, rodent EEG and EMG recordings were scored for waking (W), rapid eye movement (REM), and nonrapid eye movement (NREM) sleep by expert scorers who determined states of sleep and wakefulness by examining the records visually using Sleep Sign software (Kissei Comtec, Irvine CA) for rats and Neuroscore (DSI, Inc., St Paul, MN) for mice as described in our previous studies (49–55). Scorers were blind to experimental treatment.

Vigilance states for NHPs were determined by visual inspection of EEG and EMG signals using Neuroscore (DSI, St Paul, MN) according to the American Academy of Sleep Medicine guidelines for human sleep scoring (56, 57) and adapted for cynomolgus macaques as described previously (58, 59). Trained investigators classified each 10-s epoch as one of five states: Wake, NREM sleep stages N1, N2, or N3, or REM sleep.

c-Fos Immunohistochemistry. Rats were deeply anesthetized with a mixture of phenytoin and pentobarbital (Beuthanasia-D Special; 100 mg/kg) 1 or 2 h after injection with either the NOPR agonist SR16385 (10 mg/kg, n = 8) or the HPMC vehicle (n = 8) at ZT18 (i.e., at either ZT19 or ZT20), perfused with heparinized saline followed by 4% paraformaldehyde. Immunohistochemical details are described in *SI Appendix*. Brain sections were initially analyzed on a Leica

DM5000B microscope using Neurolucida (Microbrightfield Inc., Williston, VT) and subsequently on an Olympus VS200 slide scanner (Olympus America, Waltham, MA). After an initial visual analysis of c-Fos immunostaining throughout the brain, a region of interest (ROI) was identified in the preoptic area ventral and rostral to the anterior commissure. A comparison to a rat brain atlas (38, 60) revealed the structures to be the anterior dorsal preoptic (ADP) and parastrial (PS) nuclei. A counting box encompassing the entire ROI including both the ADP and the PS was established and all Fos+ nuclei within the box were counted for each of the 16 rats by individuals blind to the experimental condition. Data were analyzed via two-way ANOVA followed by post hoc comparisons.

Data Analysis. Statistics were calculated using the functions provided in the MATLAB statistics and machine learning toolbox and using GraphPad Prism (ver. 9.3.1). Results were considered significant at $P < 0.05$. See *SI Appendix*, Tables S1 and S2 for specific tests and results for each figure.

Data, Materials, and Software Availability. The data underlying the present work will be available at https://sleepdata.org/datasets/nop-nhp on the National Sleep Research Resource website (https://sleepdata.org) (61, 62).

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