Nonpeptide Orexin-2 Receptor Agonist Attenuates Morphine-induced Sedative Effects in Rats

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ABSTRACT

Background: Sleepiness and decrease in attention are dose-limiting side effects of opioids. The orexin/hypocretin system plays an important role in maintaining wakefulness. This study aimed to explore the potential of a nonpeptide orexin receptor agonist to alleviate morphine-induced sedative effects.

Methods: Morphine sedative effects were evaluated as changes in electroencephalogram (EEG), locomotor activity, and acoustic startle response in rats (n = 5 to 9 per group). Effects of intracerebroventricular orexin-A and systemic orexin type-2 receptor agonist, YNT-185, on EEG changes induced by morphine were examined. Furthermore, the authors examined effects of morphine administered with or without YNT-185 on locomotor activity and on acoustic startle response.

Results: Morphine-induced, frequent, short epochs of increased power (total epoch duration: 0.5 [0.0 to 8.0] s/10 min during baseline *vs.* 74.0 [49.0 to 115.0] s/10 min during the post–morphine administration period; $P = 0.012$). EEG analyses revealed that morphine-induced, high-amplitude, slow activity (increase in spectral power of frequencies less than 15 Hz, baseline *vs.* postmorphine; $P < 0.001$). Orexin-A and YNT-185 attenuated these changes. Locomotor activity decreased after morphine (268 [103 to 889] ambulatory movement counts during baseline period [20 min] *vs.* 138 [7 to 434] counts during 40 to 59 min postadministration; $P = 0.012$), but did not change after morphine with YNT-185 (363 [121 to 636] *vs.* 864 [381 to 1092] counts, difference within morphine + YNT-185 group; $P = 0.071$). Startle response latency was longer after morphine (26 [20 to 28] ms) than after morphine with YNT-185 (17 [16 to 18] ms; $P = 0.012$).

Conclusions: Orexin-A and/or YNT-185 attenuated morphine-induced sedative effects assessed by EEG changes and behavioral measures in rats. The authors' results suggest that orexin-2 receptor activation alleviates morphine-induced sedative effects. **(Anesthesiology 2018; 128:992-1003)**

MORPHINE and other μ-opioids are potent analgesics used to treat acute and chronic pain. However, opioids have sedative effects that are characterized by sleepiness, decrease in attention, and reduction of motor control.[1] Such opioid-induced sedative effects can deteriorate patients' quality of life and limit pain control. Apart from decreasing the dose or administering an antagonist, there are no established agents recommended for the treatment of opioid-induced sedative effects. Small clinical studies suggested that psychostimulants, methylphenidate, and modafinil may be effective in patients.[2,3] However, usage of these drugs, especially methylphenidate, may be limited due to side effects. Methylphenidate acts by inhibiting catecholamine reuptake, and its common side effects are nervousness, dizziness, nausea, and anorexia. In addition, there may be cardiac risks, especially in patients with cardiac conditions.[4] Furthermore, methylphenidate has a potential to be abused and is a class II federally-controlled substance in the United States. In Japan, the use of methylphenidate is legally limited to attention-deficit disorder and narcolepsy due to its widespread abuse in the society. Thus, the development of innovative strategies to treat opioid-induced sedative effects is warranted.

What We Already Know about This Topic

- Sedation is a well-known and problematic side effect of μ-opioid agonists
- Orexins are peptides produced in the hypothalamus and orexin type-2 receptors regulate sleep and wakefulness in animals

What This Article Tells Us That Is New

- In a rat model, orexin-2 receptor agonists alleviated morphine-induced decreases in locomotor activity and in sedation, which was assessed by changes in electroencephalogram
- Orexin-2 receptor agonists did not attenuate the analgesic effect of morphine

Morphine produces a behavioral stupor in rats[5–8] and sleepiness and decrease in attention in humans. On the electroencephalogram (EEG), morphine induced dose-dependent slow-wave bursts and high-amplitude slow-wave activity in rats,[5–8] and analgesic doses reduced high-

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frequency EEG power.[9] Thus, morphine alters cortical activity and causes sedative effects.

Meanwhile, the orexin/hypocretin system plays important roles in maintaining wakefulness.[10] Orexins are peptides produced by neurons in the hypothalamus and the loss of orexin-producing neurons leads to narcolepsy.[11,12] Two subtypes of orexin receptors, orexin type-1 receptor (OX1R) and orexin type-2 receptor (OX2R), were identified.[13] Animal studies suggest that OX2R plays a major role in sleep/wakefulness regulation.[14,15] The lateral hypothalamus, in which orexin neurons occur, contains μ-opioid receptors,[16,17] and orexin cells express μ-opioid receptor immunoreactivity.[18] Morphine is shown to depress orexin neurons by direct actions on cell bodies and indirectly by presynaptic mechanisms.[19] Thus, it is likely that sedative effects of morphine involve inhibition of the orexinergic system and that drugs that activate orexin receptors—OX2R in particular—may attenuate morphine sedative effects, and may have the potential to be novel therapeutics of opioid-induced sedative effects. However, orexins cannot effectively cross the blood–brain barrier and it is impractical to develop orexins as therapeutic agents. Recently, a series of nonpeptide OX2R agonists were discovered through the lead optimization process.[20] YNT-185 is one of these agonists and has high potency (EC_{50} on OX2R: 0.028 μM) and selectivity (OX1R/OX2R EC_{50} ratio: 100, *i.e.*, binding affinity to OX2R is 100-fold higher than that to OX1R) to OX2R. YNT-185 ameliorated narcoleptic symptoms in murine models of narcolepsy-catalepsy.[21] In the current study, to explore the potential of OX2R agonists as a treatment of opioid-induced sedative effects, we examined whether YNT-185 can antagonize EEG signatures and behavioral manifestation of sedative effects induced by morphine in rats. In addition, since it is undesirable that treatment of opioid-induced sedative effects reduces opioid analgesia, we examined whether YNT-185 affects analgesic effects of morphine.

Materials and Methods

Experiments were approved by the Institutional Animal Use Committee of the Jikei University School of Medicine (Tokyo, Japan) and Teikyo University School of Medicine (Tokyo, Japan), and conducted in accordance with the National Institutes of Health guidelines and the International Association for the Study of Pain Committee for Research and Ethical Issues guidelines for animal research.[22] All experiments were performed during the light (rest) period. The experimenter who analyzed EEG was blinded to treatment allocations. The other tests were not performed in a blinded manner.

Animals and Drugs

Male Sprague-Dawley rats, 12 to 13 weeks old, were used in the experiments. All rats were housed on a 12:12 h dark–light cycle with food and water *ad libitum*. Orexin-A (Peptide Institute, Japan) was dissolved in normal saline for intracerebroventricular administration in a volume of 10 μl. YNT-185·2HCl (4'-methoxy-*N*,*N*-dimethyl-3'-[*N*-(3-{[2-(3-methylbenzamido)ethyl]amino}phenyl) sulfamoyl]-(1,1'-biphenyl)-3-carboxamide·2HCl) was synthesized by co-authors (H.N., T.S.). The complete synthesis and structure of YNT-185 are published elsewhere (Compound 31 in Nagahara *et al.*[20]). YNT-185·2HCl was dissolved in HCl-acidified saline (pH 2.3) for intraperitoneal administration in a volume of 0.1 ml/100 g rat weight. Morphine hydrochloride was obtained from Takeda Pharmaceuticals (Japan) and was dissolved in normal saline for subcutaneous administration in a volume of 0.1 ml/100 g rat weight. No randomization methods were used to assign animals to groups.

Pain Test and Dose-response Assay of Morphine Analgesia

The analgesic effect of morphine on acute thermal pain was assessed by the tail flick test. Radiant heat was applied to the tail at 3 cm from the tip using a tail flick apparatus (IITC, USA). The latency of the rat to flick the tail (tail flick latency) was measured. The intensity of the radiant heat was adjusted so that the latencies in the naive rat would fall between 2.5 and 3.5 s. To avoid tissue damage, the heat stimulus was discontinued after 10 s (cut-off latency).

Dose-response analysis of the morphine analgesic effect was performed using the tail flick test in naive rats. After the measurement of baseline latencies, morphine at 1.5, 2, 3, or 5 mg/kg was administered subcutaneously. Subsequent response latencies were determined 30 min after administration. The latency data were converted to a quantal form by determining the percentage of analgesic responders in each dosage group and a dose-response curve was constructed. An analgesic responder was defined as one whose response latency was more than two times the value of the baseline latency as previously reported.[23,24] Dose-response data were analyzed with nonlinear regression using a computerized program that estimates the 50% responding (ED_{50}) and 95% CI (Prism version 5.0; GraphPad Software, USA). The logarithms for morphine doses was entered as x values and the percentages of analgesic responders in each dosage group (% responding) were entered as y values. A variable slope E_{max} model sigmoidal (four-parametric logistic) dose-response curve was fitted to the data with the bottom parameter (minimum response) constrained to equal zero and the top parameter (maximum response) constrained to equal 100.

EEG Studies

Surgery and EEG/Electromyogram Recording. Rats were anesthetized with sevoflurane (2 to 3%) and stabilized in a rat stereotaxic apparatus (Narishige, Japan). The head was shaved and disinfected with povidone iodine before a midline incision of 1.5 cm was made in the scalp. Using bregma as needed for reference, four stainless steel screw electrodes

were implanted in the skull at locations in bilateral frontal and occipital regions, with the left frontal electrode serving as the reference electrode, and were connected to the head-mount of a EEG/electromyogram system (8239, Pinnacle Technology, USA). The four electrodes also served to anchor the head-mount. Two active electromyogram electrodes of the head-mount were implanted bilaterally in deep neck muscle. The complex was then affixed to the skull with dental acrylic. For rats receiving intracerebroventricular administration, a 26-gauge stainless guide cannula (PlasticsOne, USA) was also affixed to the skull and positioned toward the left lateral ventricle at the following coordinates: 1 mm posterior to the bregma, 1.5 mm lateral to the midline, and 3 mm from the surface of the skull. For intracerebroventricular administration, drug solution was injected into the lateral ventricle *via* an internal cannula (PlasticsOne) that was passed through the guide cannula and protruded 1 mm from the guide cannula.

Continuous EEG recordings from the right frontal and bilateral occipital regions were performed using the PAL 8200 three-channel monitoring system (Pinnacle Technology). Rats were tethered to the recording system and could move freely about the monitoring cage. The acquisition of the EEG/electromyogram was performed using the Sirenia Sleep Pro software (Pinnacle Technology). The signals were filtered using a bandpass filter (EEG, 0.5 to 45 Hz; electromyogram, 10 to 200 Hz), amplified, and stored at a sampling rate of 400 Hz. The EEG/electromyogram raw data were downloaded from the monitor to a laptop computer and were also converted to European Data Format for storage and further processing.

Experiment 1: Effect of Intracerebroventricular Orexin-A on Morphine-induced EEG Changes. Experiments were performed one week after the EEG electrode and intracerebroventricular cannula implantation. Each rat was tethered to the EEG recording system and EEG was recorded continuously. After an adaptation period of 1 h, baseline EEG/electromyogram recording was made for 10 min, and then morphine (5 mg/kg) was administered subcutaneously. One hour after the administration of morphine, orexin-A (3 nmol) was administered intracerebroventricularly *via* the implanted guide cannula.

Experiment 2: Effect of Systemic YNT-185 on Morphine-induced EEG Changes and Morphine Analgesia. Experiments were performed one week after the EEG electrode implantation. Each rat was tethered to the EEG recording system and left for 1 h for adaptation. After the adaptation period, baseline EEG recording was made for 10 min, and then baseline tail flick latency was measured before drug administration. After the tail flick test, morphine (5 mg/kg, subcutaneous) and YNT-185 (40 mg/kg, intraperitoneal) or vehicle (intraperitoneal) was administered concomitantly. Peak analgesic effect of morphine is observed at 30 min after subcutaneous administration of morphine at 5 mg/kg in rats.[25] A preliminary study showed that the onset of YNT-185 effect after intraperitoneal administration was 15 to 20 min and the duration was 1 to 2 h. Thus, the effect of YNT-185 was expected to be present during peak effect of morphine. Continuous EEG recording was made for 60 min after the administrations of the drugs, and tail flick latency was measured immediately after the cessation of the EEG recording.

EEG/Electromyogram Analyses. For EEG analysis, data from the right frontal region were used. The frontal region was chosen by considering previous reports.[8,26] The administration of morphine to rats induces frequent short-lasting clusters of high-amplitude activity. Such EEG activities have been termed *slow wave bursts* in previous reports.[5–8] The durations of slow wave bursts were shown to increase in a dose-dependent manner by morphine.[8] However, the frequencies of these high-amplitude activities extend beyond traditional slow waves. Thus, we termed these distinct activities *short epochs of increased power* and defined them as clusters of waves with amplitudes greater than 100 mV and durations of at least 1 s. The number and durations of these short epochs of increased power were assessed for 10 min at specific time points: in experiment 1, baseline (starting at 10 min before morphine administration), postmorphine/pre–orexin-A (starting at 50 min after morphine administration) and post–orexin-A (starting at 10 min after orexin-A administration); and in experiment 2, baseline (during a 10-min period immediately before the administrations of the study drugs) and during 10-min periods starting at 0, 10, 20, 30, 40, and 50 min after the administrations of study drugs.

Power spectral analysis was carried out off-line using Polyman for MS-Windows (PhysioNet, Massachusetts Institute of Technology, the Beth Israel Deaconess Medical Center, and Harvard Medical School, USA). The EEG/electromyogram raw data were segmented into 10-s epochs off-line. A standardized set of experimental states was identified in each rat at specific time points: in experiment 1, baseline (10 min before administration of morphine), postmorphine/pre–orexin-A (50 min after morphine administration) and post–orexin-A (10 min after orexin-A administration); and in experiment 2, baseline (10 min before drug administrations), 10, 20, 30, 40, 50, and 60 min after drug administrations. The artefact-free EEG/electromyogram from each epoch of the above specific points was converted from the time to the frequency domain using a fast Fourier transform algorithm. Artefact rejection was performed by visual inspection. Six consecutive epochs were analyzed at each time point, and power values of EEG/electromyogram were averaged. In electromyogram analysis, the mean power frequency was also calculated as follows:

$$Mean\ power\ frequency = \sum_{f=fl}^{fh} f*P(f) \Big/ \sum_{f=fl}^{fh} P(f)$$

where P is the electromyogram power spectrum and fl and fh are the sampling frequency.

Measurement of Locomotor Activity. Locomotor activity was examined using an infrared actimeter (IR Actimeter; Penlab, Spain).[27] This apparatus is composed of a two-dimensional square frame (45 × 45 cm), which counts with 16 × 16 infrared beams to detect locomotor activity and a clear plastic cage that fits the frame. Ambulatory movement is assessed by counting the number of additional beam interruptions that accompany displacement of the centroid of the animal and is expressed as ambulatory movement counts. Each rat was habituated inside the cage for 1 h. Measurement was made starting at 30 min before, and up to 1 h after, drug administrations. Morphine at 5 mg/kg (subcutaneous) and YNT-185 at 40 mg/kg or vehicle (intraperitoneal) were administered concomitantly. Locomotor activity was evaluated by the number of ambulatory movement counts during 20-min periods before (baseline period), and up to 1 h after, drug administration (0 to 19, 20 to 39, and 40 to 59 min-periods).

Acoustic Startle Test. Acoustic startle tests were performed using an SR-LAB Acoustic Startle System (SDI, USA).[28] Rats were placed in a clear plastic cylindrical case mounted on a piezoelectric accelerometer, which was placed inside a soundproof ventilated opaque chamber to avoid stimulatory effects of the environment. The piezoelectric accelerometer detected rat's movement in response to a brief pulse of white noise (102 dB) produced by a tweeter inside the chamber. The force exerted on the accelerometer was digitized and recorded. The latency of startle response was measured as the time between the acoustic stimulus and the first movement detected. Thirty min before testing, rats were administered test drugs: morphine (5 mg/kg, subcutaenous) and vehicle (acidic saline, intraperitoneal), morphine (5 mg/kg, subcutaneous) and YNT-185 (40 mg/kg, intraperitoneal), or saline (subcutaneous) and vehicle (acidic saline, intraperitoneal; control group). Each animal was habituated inside the chamber for 15 min before testing.

Determination of Respiratory Rates. Respiratory rates were determined immediately before the startle test by counting the number of respiratory movements of the rats. A web camera that allowed visualization of respiratory movement was placed inside the chamber.

Statistical Analysis

Sample sizes were determined based on a previously reported study[8] and our previous experience in rat behavioral studies. In the previously reported study, morphine-induced EEG changes could be assessed with a sample size of n = 5. Therefore, we used a sample size of at least n = 6 in the EEG studies, considering possible observational variations. The sample sizes in the study on locomotor activity were increased from n = 5 to n = 8 or 9 after peer review due to reviewer concerns over large data variability.

Values are presented as mean ± SD for parametric data and median (interquartile range) for nonparametric data. Statistical analyses were carried out with SigmaPlot statistical software package for Windows (version 11.2; Systat, USA). Spectral power data of EEG and electromyogram, and tail flick latency data were analyzed using two-way repeated analysis (ANOVA) for repeated measures followed by the Bonferroni multiple comparison tests. Mean power frequency of electromyogram was analyzed using paired *t* test. The number of EEG short epochs of increased power in experiment 1 were analyzed using the Friedman test with the Student-Newman-Keuls multiple comparison tests. The total duration of EEG short epochs of increased power in experiment 1 was analyzed using the Friedman test, and if differences were significant, Wilcoxon signed-rank tests with Bonferroni adjustments were used for pairwise comparisons. The total durations of EEG short epochs of increased power in experiment 2 and locomotor activity data were analyzed using Friedman test within each group, and if differences were significant, we used Wilcoxon signed-rank tests with Bonferroni adjustments to perform multiple comparisons between baseline data and data of specific postdrug time points within each group. Furthermore, for comparisons of data between groups at each time point, we used Mann–Whitney U test with Bonferroni adjustments. Latency data of the acoustic startle response were analyzed by the Kruskal-Wallis test, and if differences were significant, Mann–Whitney U tests with Bonferroni adjustments were used for pairwise comparisons. Respiratory rate was analyzed by one-way ANOVA. For each test, a *P* value less than 0.05 was considered significant. There were no missing data, lost data, and excluded data for all analyses.

Results

Dose-response Analgesic Effect of Morphine in the Tail Flick Test

Morphine produced dose-dependent analgesic effects in the tail flick test (fig. 1). The ED_{50} value of morphine was calculated as 2.5 mg/kg (2.2 to 2.9 mg/kg; 95% CI). A morphine dose twice the ED_{50} value (5 mg/kg) was used in the following experiments.

EEG Studies

Experiment 1: Morphine-induced EEG Changes and the Effects of Orexin-A Administration. *Short Epochs of Increased Power after Morphine Administration.* After morphine administration (postmorphine/pre–orexin-A), short epochs of increased power were frequently observed in the EEG, which were scarcely observed at baseline (fig. 2, A and B). EMG showed no difference between pre- and postmorphine administration in spectral power (frequency × group effect: $P = 1.000$; fig. 2C) and mean power frequency (56.0 ± 15.9 *vs.* 55.4 ± 14.9 Hz; $P = 0.450$; fig. 2D). The number and durations of these short epochs significantly decreased after the administration of orexin-A (post–orexin-A; fig. 3A). The total duration of the short epochs of increased power was markedly increased after morphine administration (postmorphine/pre–orexin-A: 74.0 [49.0 to 115.0]

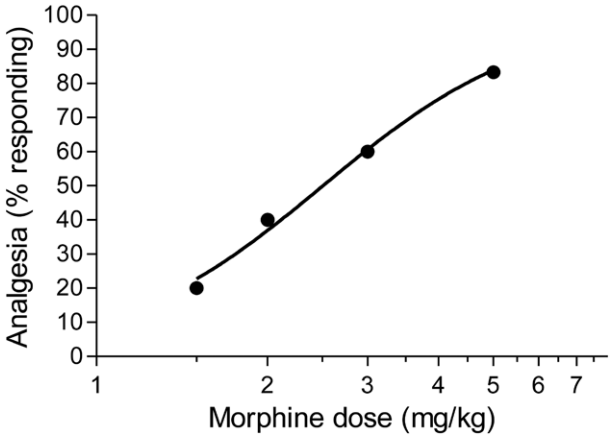

Fig. 1. Dose-response curve of morphine analgesic effect in the tail flick test. Tail flick latencies were determined before and 30 min after administration of morphine at 1.5 (n = 5), 2 (n = 5), 3 (n = 5), or 5 (n = 6) mg/kg. The latency data were converted to a quantal form by determining the percentage of analgesic responders in each dosage group. An analgesic responder was defined as one whose response latency was more than two times of the value of the baseline latency. Dose-response data were analyzed with nonlinear regression. Log-transformed morphine dose values were entered as *x* values and the percentages of analgesic responders in each dosage group (% responding) were entered as *y* values. A variable slope E_{max} model sigmoidal (four-parametric logistic) dose-response curve was fitted to the data, with the bottom parameter (minimum response) constrained to equal zero and the top parameter (maximum response) constrained to equal 100.

s/10 min) as compared to baseline (0.5 [0.0 to 8.0] s/10 min; P = 0.012; fig. 3B), and decreased to baseline level after orexin-A administration (post–orexin-A: 9.5 [4.0 to 26.0] s/10 min; post–orexin-A *vs.* postmorphine/pre–orexin-A, P = 0.021; post–orexin-A *vs.* baseline, P = 0.321; fig. 3B).

EEG Spectral Power Changes after Morphine Administration. EEG after morphine administration (postmorphine/pre–orexin-A) presented high-amplitude slow activity with short epochs of increased power, but EEG after orexin-A administration (post–orexin-A) presented low-amplitude fast activity similar to baseline EEG (fig. 4A). EEG spectral power analyses revealed that morphine administration induced increases in the spectral powers at 1 to 14 Hz, but orexin-A decreased the spectral powers at 1 to 14 Hz to baseline level (frequency effect, P < 0.001; group effect, P < 0.001; frequency × group effect, P < 0.001; fig. 4B).

Experiment 2: Effects of YNT-185 on Morphine-induced EEG Changes and Morphine Analgesia. *Short Epochs of Increased Power Induced by Morphine with and without Concomitant YNT-185 Administration.* YNT-185 significantly attenuated the increase in total duration of the short epochs of increased power induced by morphine administration as compared to vehicle control. The effect was present from 30 to 49 min after the administration of YNT-185 (P = 0.029; fig. 5).

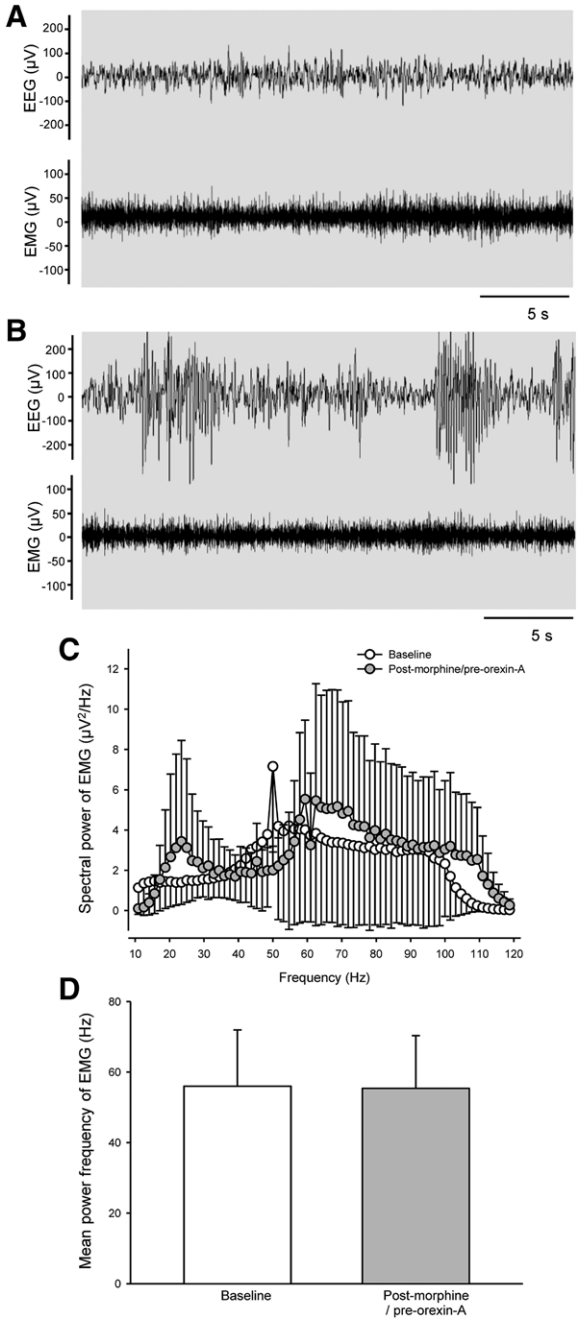

Fig. 2. Electroencephalogram (EEG) and electromyogram (EMG) after morphine administration. Morphine was administered subcutaneously at a dose of 5 mg/kg. (*A*) Raw EEG and electromyogram traces in a rat at 10 min before the administration of morphine (baseline). (*B*) Raw EEG and electromyogram traces of the same rat at 50 min after the subcutaneous administration of morphine before administration of orexin-A (postmorphine/pre–orexin-A). Short epochs of increased power were observed. (*C*) Spectral power of electromyogram did not differ between baseline and postmorphine/pre–orexin-A (n = 6 for each group; frequency × group effect: P = 1.000, two-way repeated measures ANOVA; *error bars* = SD). (*D*) Mean power frequency of electromyography did not differ between baseline and postmorphine/pre–orexin-A (n = 6 for each group; P = 0.450, paired *t* test; *error bars* = SD).

EEG Spectral Power Changes Induced by Morphine with and without Concomitant YNT-185 Administration. In rats given morphine (and vehicle control), EEG presented high-amplitude slow activity with two peaks in the spectral power, at 4 to 8 Hz and 12 to 14 Hz, which were observed from 10 min through 60 min after the administration of morphine (fig. 6). In contrast, these EEG changes were not present in rats given morphine with concomitant YNT-185 administration.

Effect of Morphine with or without Concomitant Administration of YNT-185 on Locomotor Activity. The administration of morphine (and vehicle) decreased locomotor activity from baseline values (difference within the group, $P = 0.003$; fig. 7). However, the rats showed occasional activity even at peak effects of morphine. No significant change from baseline values was observed in rats given YNT-185 concomitantly with morphine (difference within the group, $P = 0.071$; fig. 7). There was a significant difference between rats given YNT-185 and those given vehicle control at 40 to 59 min after the administrations of study drugs (864 [381 to 1092] *vs.* 138 [7 to 434] ambulatory movement counts; $P = 0.042$; fig. 7).

Effect on Latency of Acoustic Startle Response. The administration of morphine (and vehicle) significantly increased the latency of acoustic startle response (26 [20 to 28] ms) as compared to control (18 [16 to 18] ms; $P = 0.006$; fig. 8A). In rats given concomitant administration of YNT-185 with morphine, the latency (17 [16 to 18] ms) was significantly shorter than rats given morphine with vehicle ($P = 0.012$) and were not different from control ($P = 1.000$; fig. 8A).

Effect on Respiratory Rates. No difference in respiratory rates was observed among control rats, rats that were given morphine (with vehicle), and rats that were given morphine and YNT-185 (118 ± 10, 106 ± 9, 107 ± 12/min, respectively; $P = 0.110$; fig. 8B).

Effect of YNT-185 on Morphine Analgesia. Tail-flick latency increased significantly in both rats given morphine, with and without concomitant YNT-185 administration (3.2 ± 0.3 *vs.* 10.0 ± 0.0 s, $P < 0.001$; 3.3 ± 0.2 *vs.* 9.5 ± 1.2 s, $P < 0.001$; fig. 9). There was no difference between the two groups ($P = 0.161$).

Discussion

The major findings of the current study were that: (1) acute morphine administration induced short epochs of increased power and high-amplitude slow activity in the EEG with increases in spectral power at low (4 to 8 Hz) and middle (12 to 14 Hz) frequencies in particular; (2) orexin-A and the potent nonpeptide selective OX2R agonist, YNT-185, decreased the total duration of short epochs of increased power and attenuated the EEG changes induced by morphine; (3) YNT-185 inhibited the decrease in locomotor activity and the increase in latency of startle response induced by morphine; and (4) YNT-185 did not attenuate the analgesic effect of morphine.

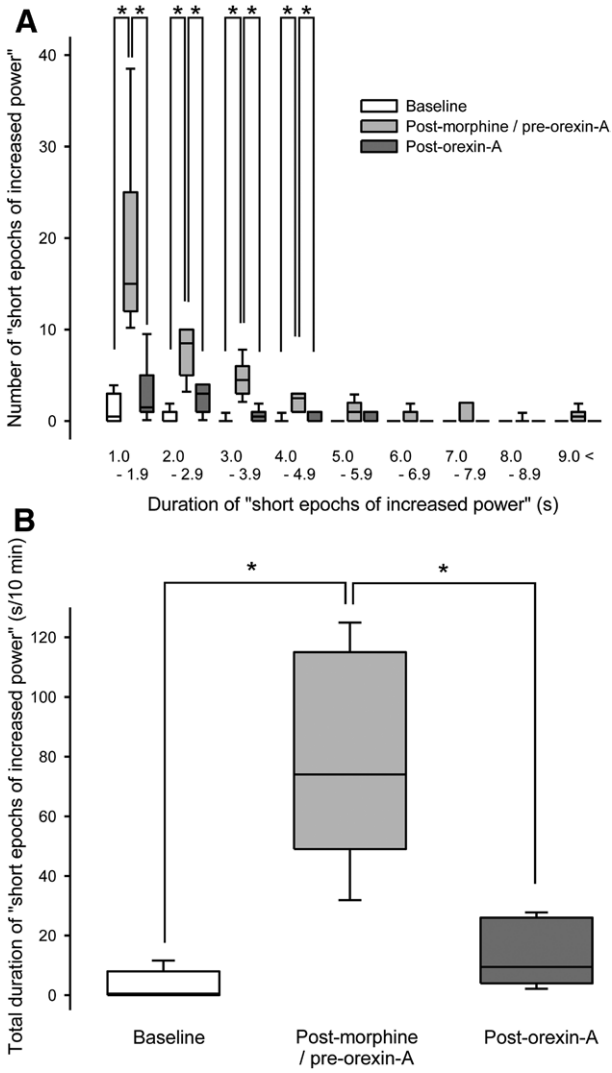

Fig. 3. Effect of orexin-A on electroencephalogram (EEG) short epochs of increased power induced by morphine. Orexin-A (3 nmol) was administered intracerebroventricularly 1 h after subcutaneous administration of morphine (5 mg/kg). Short epochs of increased power were analyzed for 10 min at specific time points: baseline (starting at 10 min before morphine administration), postmorphine/pre–orexin-A (starting at 50 min after morphine administration), and post–orexin-A (starting at 10 min after orexin-A administration). (*A*) The number of the short epochs categorized by duration at the above time points (n = 6 for each group; **P* < 0.05, Friedman test with Student-Newman-Keuls multiple comparison tests). The *box plots* show median with 25th and 75th percentile, and whiskers represent the 5% and 95% range. (*B*) Total duration of the short epochs at the above time points. Orexin-A decreased the total duration of the short epochs of increased power induced by morphine (n = 6 for each group; **P* < 0.05, Friedman test with Bonferroni adjusted Wilcoxon signed-rank test). The *box plots* show median with 25th and 75th percentile, and whiskers represent the 5% and 95% range.

In clinical practice, opioids are used as analgesic agents in a variety of pain states. Opioids are the mainstay in the treatment of cancer pain, and they are also used to treat acute and chronic pain. Although effective, opioids may present many

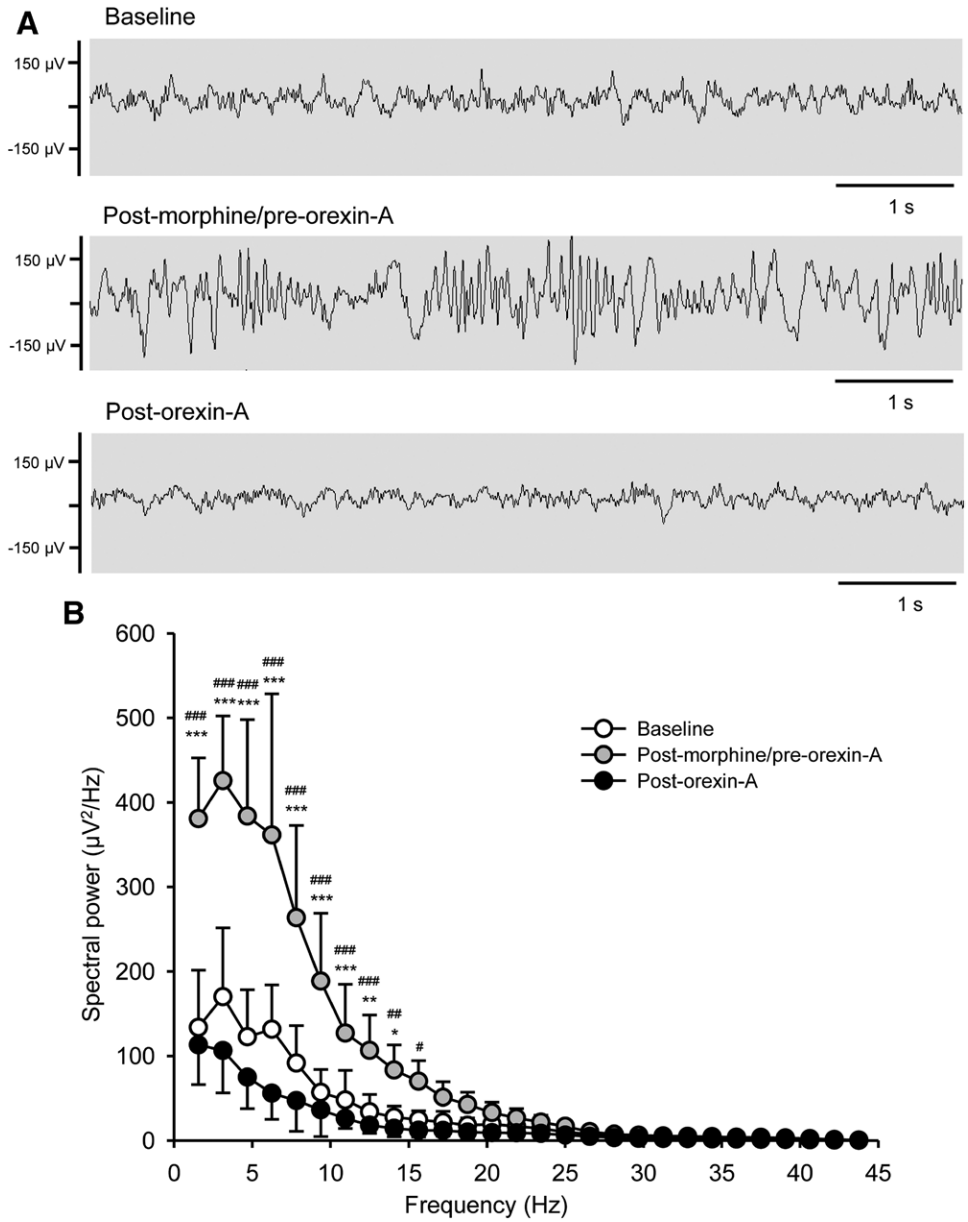

Fig. 4. Effect of orexin-A on morphine-induced spectral power changes in electroencephalogram (EEG). Orexin-A (0.3 nmol) was administered intracerebroventricularly 1 h after subcutaneous administration of morphine (5 mg/kg). (*A*) Raw EEG traces obtained from a rat at specific time points: baseline (starting at 10 min before morphine administration), postmorphine/pre–orexin-A (starting at 50 min after morphine administration), and post–orexin-A (starting at 10 min after orexin-A administration). (*B*) Spectral power analyses of EEG at the above time points. Morphine-induced high-amplitude slow activity, but orexin-A attenuated the EEG change (n = 6 for each group; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *vs*. baseline, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 *vs*. post–orexin-A; two-way repeated measures ANOVA with Bonferroni multiple comparison tests; *error bars* = SD).

side effects. Sedative effects such as sleepiness and decrease in attention[1] are undesirable side effects that negatively affect a patient's quality of life and may limit dosage resulting in inadequate pain control. Opioid sedative effects may be assessed by changes in EEG activity. The administration of opioid induces high-voltage slow wave EEG activity in humans.[9,29,30] In experimental animals, acute administration of morphine causes high voltage slow wave bursts on EEG, which are associated with behavioral stupor.[5–8] Consistent with previously reported studies,[5–8] we observed that morphine at 5 mg/kg induced high-amplitude slow activity with frequent short epochs of increased power in the EEG. EMG power did not change after morphine administration, whereas these changes in EEG were present. Thus, the high-amplitude activities after morphine were not due to artefacts produce by electromyogram waves. It should be noted that a transition from wake to non–rapid eye movement sleep in which the EEG exhibits low-amplitude pattern of

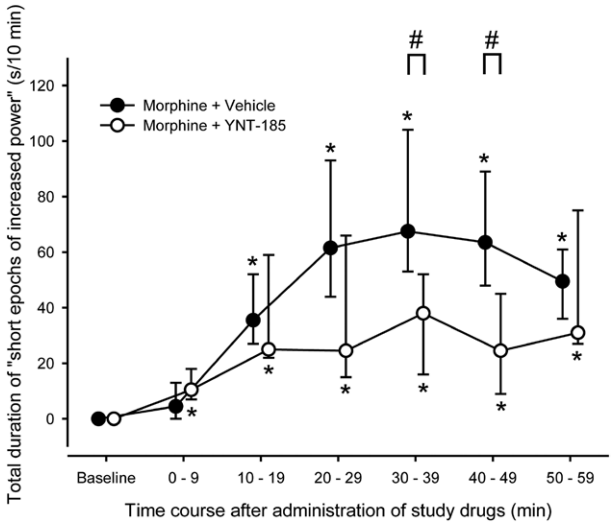

Fig. 5. Effect of YNT-185 on electroencephalogram (EEG) short epochs of increased power induced by morphine. Rats received intraperitoneal administration of YNT-185 (40 mg/kg; n = 7) or vehicle (saline; n = 6) concomitantly with subcutaneous administration of morphine (5 mg/kg). Total durations of short epochs of increased power were calculated during 10-min periods starting at 0, 10, 20, 30, 40, and 50 min after the administrations of study drugs. Baseline data were obtained during a 10-min period immediately before the administrations of the study drugs. In both groups, the total duration of short epochs of increased power increased after administration of study drugs (*P < 0.05 *vs*. baseline, Friedman test with Bonferroni adjusted Wilcoxon signed-rank test), but it was shorter in rats given morphine with concomitant YNT-185 administration than in rats given morphine and vehicle control (#P < 0.05, Mann–Whitney U test with Bonferroni correction). Data were given as median (interquartile range).

wakefulness flipping into a high-amplitude slow frequency pattern of non–REM sleep may have been counted as a short epoch of increased power. EEG spectral power analyses revealed that morphine-induced high-amplitude slow activity, specifically EEG power, increased at low (4 to 8 Hz) and middle (12 to 14 Hz) frequencies. The power in the 5 to 7 Hz frequency band was shown to be a good indicator of morphine effect in rats,[5] and the increases in absolute spectral power in 1.25 to 18.5 Hz frequency band has been demonstrated in rats after the administration of morphine.[8] Locomotor activity decreased in concurrence with the EEG changes induced by morphine. Furthermore, morphine increased the latency of acoustic startle response. Reaction time to auditory stimulus is considered to reflect attention and vigilance, and its increase by remifentanil has been reported in humans.[31] These results suggest that the EEG changes observed are signatures of the sedative effects induced by morphine. It should be noted, however, that the rats were not in a deeply sedated state. Their eyes were opened and there was occasional locomotor activity. The dose of morphine at 5 mg/kg used in this study is approximately twice the ED_{50} value (2.5 mg/kg) of morphine in the tail flick test. The dose did not reduce respiratory rates. It is of interest that a lower dose of morphine at 1 mg/kg has been shown to produce increased wakefulness.[32] In this study, morphine inhibited sleep-promoting neurons in the ventrolateral preoptic area, which exhibit inhibitory input to ascending arousal systems. Thus, it should be noted that the effect of morphine on wakefulness is dose dependent. Morphine at 1 mg/kg in rats, however, is not an analgesic dose according to our dose-response study. We do not know whether morphine at clinically effective doses can cause increased wakefulness in patients.

Maintenance of wakefulness is dependent on the hypothalamic orexinergic system.[33] Orexins are endogenous neuropeptides that are produced by neurons that exist in the lateral hypothalamus, and orexin-containing neurons have widespread projections throughout the central nervous system.[13,34] In particular, orexin neurons densely innervate regions that are thought to play an important role in sleep/wakefulness regulation, including the noradrenergic locus coeruleus, the serotonergic dorsal raphe nucleus, the histaminergic tuberomammillary nucleus, and the cholinergic pedunculopontine tegmental nucleus.[33] Noradrenergic cells of the locus coeruleus, histaminergic cells of the tuberomammillary nucleus, dopaminergic cells of the ventral tegmental area, and serotonergic cells of the dorsal raphe nucleus are shown to be activated by orexins.[35–37] These findings indicate that monoaminergic regions are important effector sites of orexin neurons, and orexin-mediated arousal/wakefulness involves activation of these monoaminergic neurons.[33] The sedative effects of morphine likely involves inhibitory effects on this arousal system *via* opioid receptor activation. μ-Opioid receptors are expressed in orexin neurons in the lateral hypothalamus,[18] and morphine depresses orexin neurons by direct actions on the cell body, and also indirectly by presynaptic mechanisms.[19] Furthermore, morphine has been shown to decrease activity of noradrenergic neurons in the locus coeruleus *via* μ-receptor activation.[38] In the current study, the administration of orexin-A decreased the total duration of morphine-induced EEG short epochs of increased power and reversed the changes in EEG spectral power induced by morphine. Our results suggest that activating orexin receptors, and thereby activating the orexinergic arousal system, attenuates morphine-induced sedative effects.

Two orexin receptors, OX1R and OX2R, have been identified with distinct expression patterns throughout the brain.[13,34] A study on orexin receptor–deficient mice suggested that OX2R-mediated pathway has a pivotal role in the promotion of wakefulness.[39] The potent non–peptide OX2R selective agonist, YNT-185, has been shown to increase wakefulness in mice and to ameliorate narcolepsy symptoms in murine models of narcolepsy.[21] In the current study, YNT-185 decreased the total duration of morphine-induced EEG short epochs of increased power and inhibited the increase in spectral power in the low and middle frequencies induced by morphine administration. Furthermore,

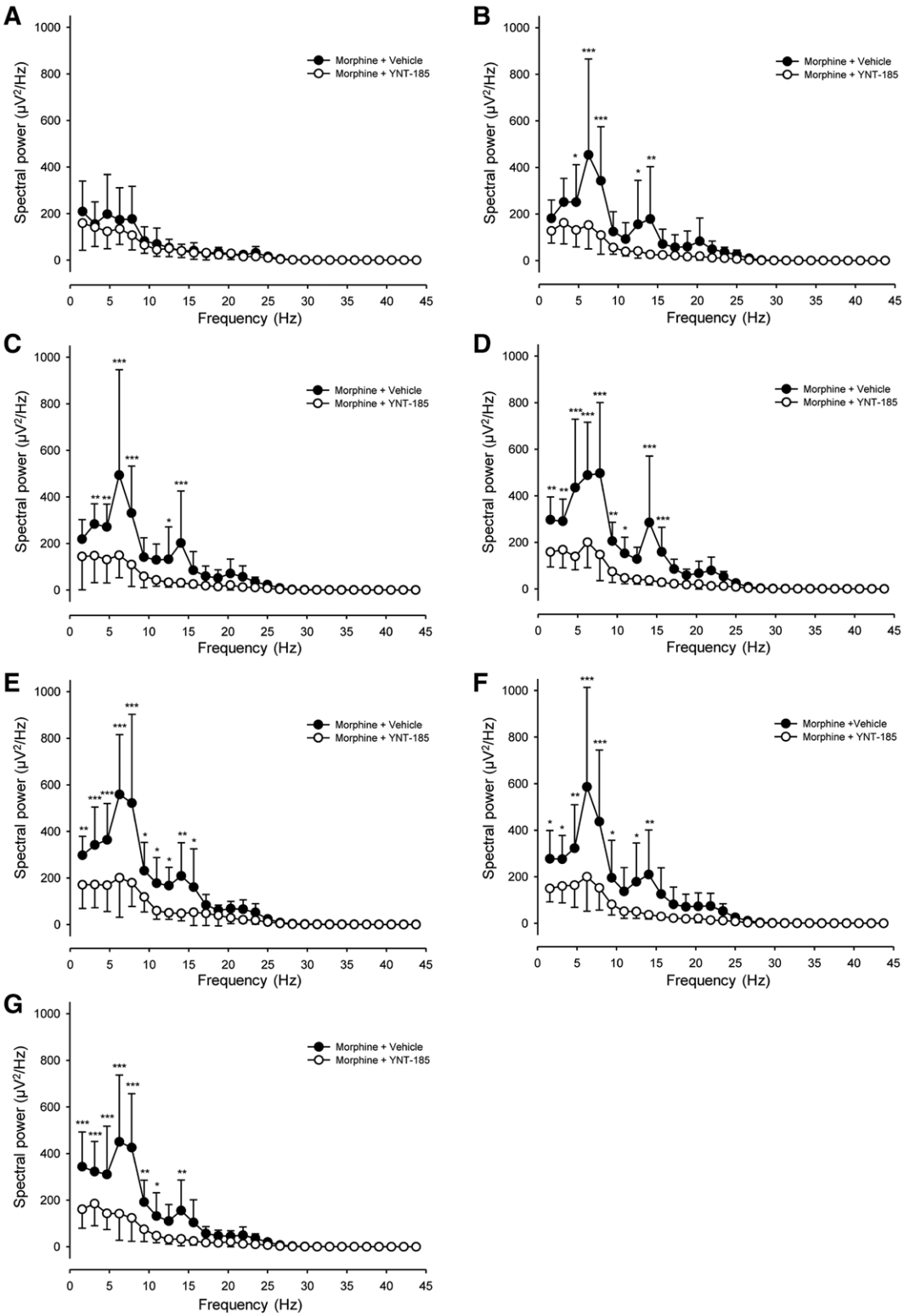

Fig. 6. Effect of YNT-185 on electroencephalogram (EEG) spectral power changes induced by morphine. Rats received intraperitoneal administration of YNT-185 (40 mg/kg; n = 7) or vehicle (saline; n = 6) concomitantly with subcutaneous administration of morphine (5 mg/kg). Spectral power analyses were performed at specific time points: 10 min before drug administrations (*A*), and 10 min (*B*), 20 min (*C*), 30 min (*D*), 40 min (*E*), 50 min (*F*), and 60 min (*G*) after drug administrations. EEG presented higher amplitude and slower activity with two peaks in the spectral power in rats given morphine and vehicle control compared with rats given morphine with concomitant YNT-185 administration (frequency × group effect: $P = 0.838$ for *A* and $P < 0.001$ for *B*, *C*, *D*, *E*, *F*, and *G*; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, two-way repeated measures ANOVA with Bonferroni multiple comparison tests; *error bars* = SD).

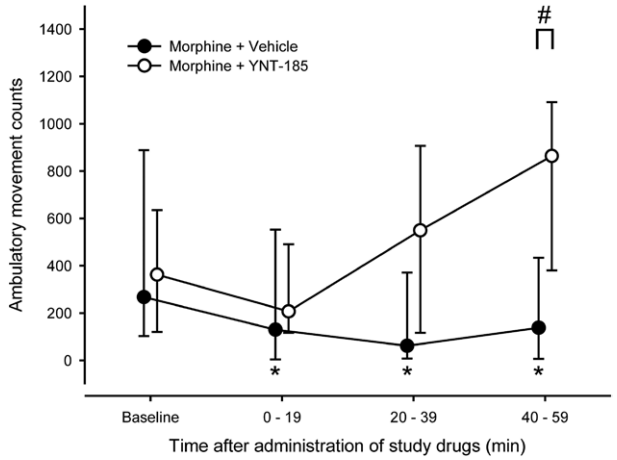

Fig. 7. Effect of morphine with or without concomitant administration of YNT-185 on locomotor activity. Rats received intraperitoneal administration of YNT-185 (40 mg/kg) (n = 8) or vehicle (n = 9) concomitantly with subcutaneous administration of morphine (5 mg/kg). Locomotor activity was measured before and up to 60 min after drug administration using an infrared actimeter. Locomotor activity was expressed as the number of ambulatory movement counts during a 20-min period before drug administrations (baseline) and during every 20-min period up to 1 h after drug administrations. Data are given as median and interquartile range (*error bars*). Locomotor activity decreased after morphine administration, but did not change after morphine administration with concomitant YNT-185 administration (*P < 0.05, *vs*. baseline, Friedman test with Bonferroni adjusted Wilcoxon signed-rank test). A significant difference between the two groups was observed at 40 to 59 min after administration of study drugs (#P < 0.05, Mann–Whitney U test with Bonferroni correction).

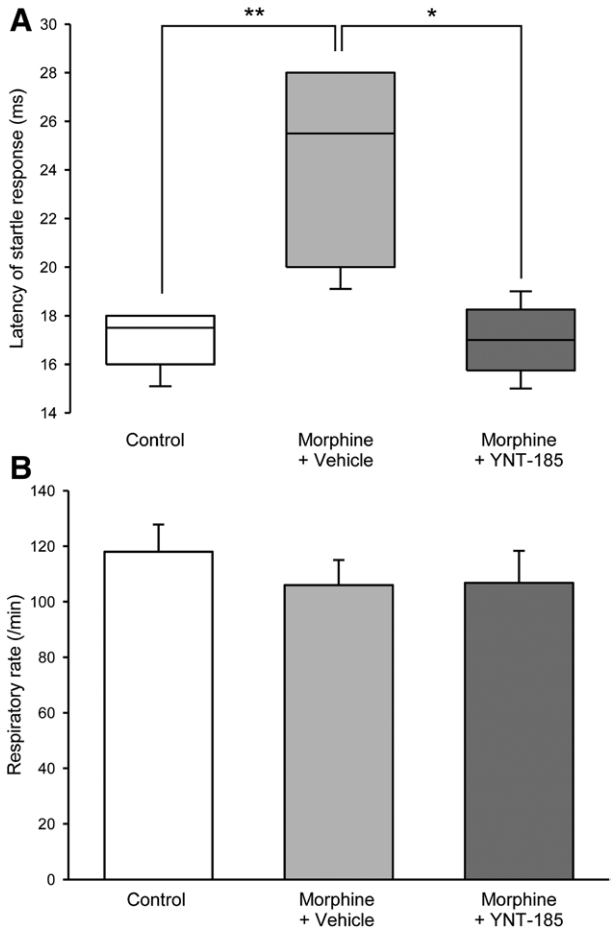

Fig. 8. Effects of morphine with or without concomitant administration of YNT-185 on the latency of acoustic startle response (*A*) and respiratory rate (*B*). Rats were administered morphine (5 mg/kg, subcutaneous) and vehicle (acidic saline, intraperitoneal; n = 6), morphine (5 mg/kg, subcutaneous) and YNT-185 (40 mg/kg, intraperitoneal; n = 5), or saline (subcutaenous) and vehicle (acidic saline, intraperitoneal; control group, n = 6). Thirty min after administration, acoustic startle test was performed with a 102-dB stimulus. The latency of startle response was measured as the time between the startle stimulus and the first movement detected. Latency of startle response was longer in rats given morphine and vehicle control compared with control rats and rats given morphine with concomitant YNT-185 administration (*P < 0.05, **P < 0.01, Kruskal-Wallis test with Bonferroni adjusted Mann–Whitney U test). The *box plots* show median with 25th and 75th percentile, and whiskers represent the 5% and 95% range. Respiratory rates were counted immediately before the acoustic startle test. Respiratory rate did not differ among the three groups (P = 0.110, one-way ANOVA). Data are presented as mean and SD (*error bars*).

YNT-185 inhibited the decrease in locomotor activity and the increase in acoustic startle response latency induced by morphine. These results demonstrate that morphine-induced sedative effects can be attenuated by OX2R activation. In addition, we showed that YNT-185 can alleviate morphine sedative effects without attenuating its analgesic effects. This is in clear contrast with opioid antagonists that act directly on opioid receptors and reverse all effects of morphine.

A limitation of this study is that we did not examine the effects of YNT-185 on different doses of morphine. Since morphine effect on wakefulness depends on dosage, the concomitant use of YNT-185 may cause different effects among different doses of morphine. Furthermore, this study only examined effects of acute administrations of morphine and YNT-185. Future studies will need to show whether the effect of YNT-185 is present with chronic morphine administration and whether the effects of YNT-185 develop tolerance. In addition, this study was performed with rats without pain. Since opioids are used in patients with pain, confirmation of the results by studies utilizing pain models may be necessary. Lastly, although YNT-185 may seem promising, we need caution to extrapolate the results of this study in rats to humans.

In summary, orexin-A and the non–peptide selective OX2R agonist, YNT-185, attenuated morphine-induced EEG changes in rats. YNT-185 also attenuated behavioral manifestation of morphine sedative effects. YNT-185 did not affect the analgesic effect of morphine. Our results suggest that the activation of OX2R alleviates morphine-induced sedative effects. YNT-185 may be a promising

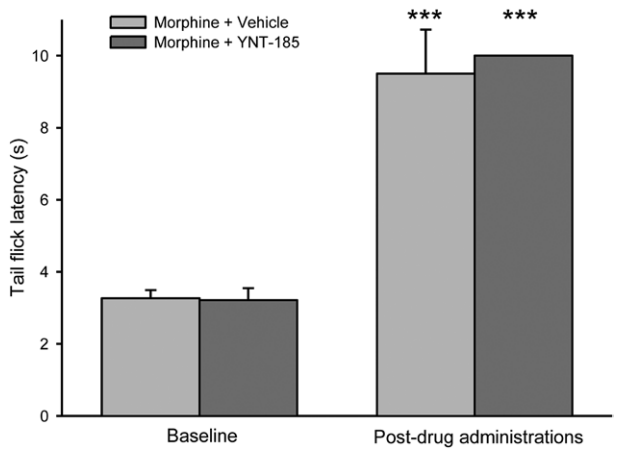

Fig. 9. Effect of YNT-185 on the analgesic effect of morphine in the tail flick test. Rats received intraperitoneal administration of YNT-185 (40 mg/kg) or vehicle concomitantly with subcutaneous administration of morphine (5 mg/kg; n = 7 for YNT-185 and n = 6 for vehicle). Baseline latencies were obtained 10 min before the administrations of the study drugs, and tail flick latencies were obtained again 60 min after the administrations of the study drugs. The analgesic effect of morphine did not attenuate by YNT-185 administration (time effect, $P < 0.001$; group effect, $P = 0.335$; time × group effect, $P = 0.315$; ***$P < 0.001$ *vs*. baseline within the same group, two-way repeated measures ANOVA with Bonferroni multiple comparison tests; *error bars* = SD).

candidate to treat the undesirable side effects due to opioid sedative effects.

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Competing Interests

The authors declare no competing interests.

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Address correspondence to Dr. M. Shimoyama: Department of Palliative Medicine, Jikei University Hospital, 3-19-18 Nishi-Shimbashi, Minato-Ku, Tokyo 105–8471, Japan. mshimoy@jikei.ac.jp. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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