Blockade of Orexin-1 Receptors Attenuates Orexin-2 Receptor Antagonism-Induced Sleep Promotion in the Rat

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ABSTRACT

Orexins are peptides produced by lateral hypothalamic neurons that exert a prominent role in the maintenance of wakefulness by activating orexin-1 (OX1R) and orexin-2 (OX2R) receptor located in wake-active structures. Pharmacological blockade of both receptors by the dual OX1/2R antagonist (2R)-2-[(1S)-6,7-dimethoxy-1-[2-[4-(trifluoromethyl)phenyl]ethyl]-3,4-dihydroisoquinolin-2(1H)-yl]-N-methyl-2-phenylethanolamide (almorexant) has been shown to promote sleep in animals and humans during their active period. However, the selective distribution of OX1R and OX2R in distinct neuronal circuits may result in a differential impact of these receptors in sleep-wake modulation. The respective role of OX1R and OX2R on sleep in correlation with monoamine release was evaluated in rats treated with selective antagonists alone or in combination. When administered in either phase of the light/dark cycle, the OX2R antagonist 1-(2,4-dibromophenyl)-3-[(4S,5S)-2,2-dimethyl-4-phenyl-1,3-dioxan-5-yl]urea (JNJ-10397049) decreased the latency for persistent sleep and increased nonrapid eye movement and rapid eye movement sleep time. Almorexant produced less hypnotic activity, whereas the OX1R antagonist 1-(6,8-difluoro-2-methylquinolinol-4-yl)-3-[4-(dimethylamino)phenyl]urea (SB-408124) had no effect. Microdialysis studies showed that either OX2R or OX1/2R antagonism decreased extracellular histamine concentration in the lateral hypothalamus, whereas both OX1R and OX1/2R antagonists increased dopamine release in the prefrontal cortex. Finally, coadministration of the OX1R with the OX2R antagonist greatly attenuated the sleep-promoting effects of the OX2R antagonist. These results indicate that blockade of OX2R is sufficient to initiate and prolong sleep, consistent with the hypothesis of a deactivation of the histaminergic system. In addition, it is suggested that simultaneous inhibition of OX1R attenuates the sleep-promoting effects mediated by selective OX2R blockade, possibly correlated with dopaminergic neurotransmission.

The neuropeptides orexins (orexin-A and orexin-B, also known as hypocretin-1 and hypocretin-2), which are produced by a cluster of neurons within the lateral posterior hypothalamus (de Lecea et al., 1998; Sakurai et al., 1998), project widely throughout the brain and play a major role in the regulation of sleep-wake states (Hagan et al., 1999). Activation of orexin neurons contributes to the maintenance of wakefulness, and endogenous loss of orexin neurons in humans results in narcolepsy, a condition characterized by excessive sleepiness, hypnagogic hallucinations, and cataplexy (Zeitzer et al., 2006). Orexins mediate their effect by stimulating two closely related G protein-coupled receptors, orexin-1 (OX1R) and orexin-2 (OX2R) receptors located in wake-active monoaminergic and cholinergic systems (Peyron et al., 1998; Sakurai et al., 1998; Marcus et al., 2001). It has thus been hypothesized that blockade of orexin receptors would be a novel pharmacological approach for the treatment of insomnia. Recently, it was reported that pharmacological blockade of both receptors by the dual OX1/2R antagonist (2R)-2-[(1S)-6,7-dimethoxy-1-[2-[4-(trifluoromethyl)phenyl]ethyl]-3,4-dihydroisoquinolin-2(1H)-yl]-N-methyl-2-phenylethanolamide (almorexant) promoted sleep in rats, dogs, and humans during their respective active period (Brisbare-Roch et al., 2007). It is noteworthy that OX1R and OX2R are differentially
distributed in brain areas that regulate sleep and wake, including the tuberomammillary nucleus, locus coeruleus, dorsal raphe, and laterodorsal tegmental/pedunculopontine tegmental nuclei (Sakurai et al., 1998; Marcus et al., 2001). Within the monoaminergic system, locus coeruleus neurons exclusively express OX1R. In contrast, OX2R are densely located in the tuberomammillary nucleus, whereas dorsal raphe neurons express both OX1R and OX2R. It has been demonstrated that the arousal effect of orexin-A depended on histaminergic activation via OX2R in the tuberomammillary nucleus (Eriksson et al., 2001; Huang et al., 2001). The wake-promoting effect of orexin-A was reduced by pretreatment with the histamine H1 receptor antagonist pyrilamine in rats (Yamanaka et al., 2002), and it was virtually absent in mice lacking H1 receptors (Huang et al., 2001). OX1R in the locus coeruleus have been shown to regulate rapid eye movement (REM) sleep. In rats, local administration of orexin-A into the locus coeruleus suppressed REM sleep (Bourgin et al., 2000), and the selective OX1R antagonist SB-334867 could reverse the REM sleep suppression induced by intracerebroventricular injection of orexin-A (Smith et al., 2003).

Significantly, investigators have also examined how the targeted disruption in the genes encoding the two orexin receptors influence the states of vigilance. Double OX1R and OX2R knockout mice display wake disturbances resembling human narcolepsy (Willie et al., 2001), characterized by excessive daytime sleepiness that can be accompanied by the sudden loss of muscular tone (cataplexy). Although a narcoleptic phenotype was not documented in the OX1 knockout mouse (Willie et al., 2001), OX2R knockout mice developed a mild disruption in wake patterns (Willie et al., 2003).

Taken together, these studies provide evidence that OX1R and OX2R may have a differential impact in the modulation of the sleep-wake states. The recent availability of selective antagonists of OX1R (SB-408124) (Langmead et al., 2004) or OX2R (JNJ-10397049) (McAtee et al., 2004), as well as the dual OX1/2R antagonist almorexant (Brisbare-Roch et al., 2007), provided the first opportunity to determine their respective functional roles and to analyze their possible interaction on sleep-wake modulation in correlation with orexin receptor occupancy and monoamine release during the light/dark cycle of the rat. Our results show that the sleep-promoting effects produced by OX2R antagonists correlated with a decrease in hypothalamic histamine (HA) release. The most striking finding is that additional blockade of OX1R greatly attenuated this hypnotic activity, possibly mediated by a transient increase in dopaminergic neurotransmission.

Materials and Methods

Animals. All the studies were carried out in male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) weighing 300 to 350 g. Animals were housed individually under controlled conditions, with a 12/12-h light/dark schedule (lights on at 6:00 AM) and temperature of 22 ± 2°C and allowed unrestricted access to food and water. All in vivo procedures detailed in this investigation were implemented in accordance with policies established by the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health (Bethesda, MD).

Ex Vivo OX1R and OX2R Binding Autoradiography. Animals were treated by subcutaneous administration of vehicle, almorexant (3 or 30 mg/kg), JNJ-10397049 (0.3, 3, or 30 mg/kg), or SB-408124 (30 mg/kg). The animals were euthanized at different time points (0.25, 0.5, 1, 2, 4, and 6 h; n = 3–6 animals/time point) using carbon dioxide and decapitated after drug administration. Tissue sections for ex vivo receptor autoradiography were prepared as described previously (Langlois et al., 2001). Ex vivo occupancy of OX1R and OX2R binding site was measured in the tenia tecta (OX1R) and frontal cortex (OX2R) of each individual rat. After thawing, the sections were dried under a stream of cold air and then incubated at room temperature for 10 min in Tris, 10 mM MgCl₂, and 5 mM EDTA buffer with the tracer. For OX1R, 5 nM [³²P]-H3442 was used (Langmead et al., 2004). For OX2R, 3 nM [³²P]-H101701-2-[6-methoxy-pyridin-3-yl]-(toluene-2-sulfonil)-aminol-N-pyridin-3-ylmethyl acetamide was used (Malherbe et al., 2009). The sections were not washed before incubation to avoid dissociation of the drug-receptor complex. Incubation was restricted to 10 min at room temperature to minimize dissociation of the drug from the receptor. Nonspecific binding was determined in the presence of 10 μM SB-408124 for OX1R or JNJ-10397049 for OX2R. After the incubation, the slides were washed in ice-cold buffer (Tris, 10 mM MgCl₂, and 5 mM EDTA buffer) followed by a quick rinse in ice-cold water. The sections were then dried under a stream of cold air. Quantitative analysis was performed as described previously (Langlois et al., 2001) using a beta imager (BioSpace, Paris, France). Ex vivo receptor labeling was expressed as the percentage of receptor labeling in corresponding brain areas of saline-treated animals. The percentage of receptor occupancy was plotted against time.

Sleep Recording and Analysis. For the determination of polysonmographic parameters, two stainless steel screw electrodes in the frontal and parietal cortex for the electroencephalogram (EEG) and wire electrodes in dorsal nuchal muscles for the electromyogram (EMG) were implanted in each rat under isoflurane anesthesia. Electrodes were connected to a sterile two-channel telemetric device (TL11 CTA-F20-EEF, Data Sciences International, St. Paul, MN) that had been implanted in the abdominal cavity and that allowed additional measurement of body temperature and locomotor activity. EEG and EMG signals were recorded for up to 12 h after drug administration and were digitized at a sampling rate of 100 Hz on an IBM PC-compatible computer using Dataquest A.R.T. software (Data Sciences International). High- and low-pass filters were set at 1 and 30 Hz, respectively, for the EEG signal and at 30 and 100 Hz, respectively, for the EMG signal.

Using the computer program SleepSign (Kissei Comtec, Nagano, Japan), consecutive EEG/EMG recordings were divided into individual 10-s epochs that were then visually assigned vigilance states based upon conventional criteria for wake, nonrapid eye movement (NREM) sleep and REM sleep as described previously (Bonaventure et al., 2007). Analysis of sleep-wake parameters included latency to persistent sleep or NREM sleep latency (time interval to the first six consecutive NREM sleep epochs after injection); REM sleep latency (time interval to the first two consecutive REM sleep episodes after injection); time spent in wake, NREM sleep, and REM sleep; and bout analysis (number and duration) for each vigilance state. Body temperature and locomotor activity counts were collected into 10- and 60-s bins, respectively, and averaged into 2- or 12-h periods for each animal. All the results were then averaged for each experimental group of animals and expressed as mean ± S.E.M. in defined time intervals. Statistical analyses were performed by a one-way analysis of variance (ANOVA) followed by either Dunnett’s multiple comparison test, Newman-Keuls test, or Tukey’s post hoc test.

Microdialysis in the Lateral Hypothalamus or Cortex of Freely Moving Rats. Animals were anesthetized with isoflurane and stereotaxically implanted with a guide cannula (Eicom, Kyoto, Japan) either in the lateral hypothalamus (LH) for HA detection (incisor bar, −3.5 mm, −1.6 mm anterior, 1.5 mm lateral, and 6 mm ventral to bregma) (Paxinos and Watson, 1997) or in the prefrontal cortex (PFC) for dopamine (DA), norepinephrine (NE), and 5-hydroxytryptamine (serotonin) (5-HT) detection (Barbier et al., 2007). Animals were allowed at least 4 days to recover before experimentation. Dialysis probes (Eicom; 2 mm 9 (LH) or 4 mm (PFC) active
membrane length) were perfused with artificial cerebral spinal fluid (147 mM NaCl, 4 mM KCl, 0.85 mM MgCl₂, and 2.3 mM CaCl₂, pH 7.4) at a flow rate of 1 μl/min and implanted the morning before sample collection. After stable baseline collection, animals were dosed 30 min before the onset of the dark phase, and 30-min samples were collected over 12 h.

Dialysis was quantified for HA using LC-tandem mass spectrometry (MS/MS). A Discovery HS F5 (2.1 × 100 mm; 3 μm) column (Supelco, Bellefonte, PA) was used with a mobile phase containing a 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The mobile phase was held on the B/A ratio of 100:0 for 1.5 min. The linear gradient was changed over the next 0.5 min to B/A ratio of 5:95, held for 2.5 min, and then returned to the starting conditions. The total run time was 6.5 min, and flow rate was 0.6 ml/min (LC-10AD VP with SCL-10A VP system controller; Shimadzu, Kyoto, Japan). MS/MS detection was carried out on an API4000 mass spectrometer (PerkinElmerSciex Instruments, Boston, MA) in the positive ion mode (electrospray ionization) by multiple reaction monitoring (MH+ daughter was mz 112.09 – 95.1). Dialysis experiments for DA, NE, and 5-HT were performed using high-performance liquid chromatography/electrochemical detection as described previously (Barbier et al., 2007). The concentration for each sample was calculated from the peak area of the chromatographic signal and the slope from the corresponding analytes standard curve.

The percentage of change from baseline values were calculated from the mean basal value of each neurotransmitter for each animal and are presented in the figures as mean ± S.E.M. The area under the curve value were calculated by the summation of the difference between each neurotransmitter after drug administration and the mean percentage of basal release value. Statistical analyses were performed on the area under the curve values by ANOVA, followed by Newman-Keuls multiple comparison post hoc test.

**Pharmacokinetics and Bioanalysis.** Dosing JNJ-10397049 (10 mg/kg) alone, SB-408124 (30 mg/kg) alone, or JNJ-10397049 (10 mg/kg) + SB-408124 (30 mg/kg) was followed by blood sampling via cardiac puncture over a time course. Brains were removed from the animals and homogenized for LC-MS/MS analysis. All blood samples were deproteinized by 1:4 dilution of the sample with acetonitrile with vigorous mixing. These samples were incubated for 5 min and then centrifuged at 14,000 rpm in a microcentrifuge for 4 min. The supernatant was recovered into autosampler vials and diluted 1:1 with sterile water. Samples were analyzed by LC-MS/MS. An SP C18 (2.1 × 50-mm analytical column (Grace Vydac, Hesperia, CA) was used for separation.

Statistical (paired t test) were calculated using Prism software (GraphPad Software Inc., San Diego, CA). A one-compartment pharmacokinetic model was also applied to these data using the software package WinNonlin, version 4.0.1 (Pharsight, Mountain View, CA). The model that was used was was a one-compartment first-order, no lag time, first-order elimination model (model 3). The parameters of the model were optimized using least-squares nonlinear regression. Pharmacokinetic parameters (C<sub>max</sub>) are given as the means ± CV%. The CV% is a measure of dispersion of a probability distribution. It is defined as the ratio of the S.D. to the mean. The CV% was calculated as the ratio of the S.E. for each parameter to its estimated value.

**Drugs.** JNJ-10397049 is a selective OX2R antagonist (pKi, OX2R = 8.2; pKi, OX1R = 5.7). SB-408124 is a selective OX1R antagonist (pKi, OX1R = 7.1; pKi, OX2R = 5.2). JNJ-10397049 and SB-408124 were screened in a panel of more than 50 other neurotransmitters and neuropeptide receptors and had no significant affinity for any receptor (<50% inhibition at 1 μM). Almorexant is a dual OX1/2R antagonist (pKi, OX1R = 7.8; pKi, OX2R = 8.0).

JNJ-10397049 and almorexant were synthesized at Johnson & Johnson Pharmaceutical Research & Development, L.L.C. SB-408124 was purchased from Tocris Bioscience (Ellisville, MO). The unlabeled precursor of [3H]N-ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulfon)-amino]-N-pyridin-3-ylmethyl acetamide and unlabeled precursor of [3H]SB-674042 were synthesized at Johnson & Johnson Pharmaceutical Research & Development, L.L.C. (San Diego, CA). The compounds were tritiated at Moravek Biochemicals (Brea, CA) (specific activity, 27 and 35.3 Ci/mmol, respectively). All compounds were administered via the subcutaneous route. JNJ-10397049 was formulated in 5% Pharmasolve (Sigma, St. Louis, MO), 20% Solutol (BASF, Ludwigshafen, Germany), and 75% hydroxypropyl-β-cyclodextrin (20%, w/v) and injected in a volume of 1 ml/kg. SB-408124 was formulated in (30%, w/v) sulfobutylether cyclodextrin, and almorexant was dissolved in (20%, w/v) hydroxypropyl-β-cyclodextrin. Both compounds were injected in a volume of 2 ml/kg.

**Results**

JNJ-10397049 and Almorexant Achieve High Levels of OX2R Occupancy in Rat Brain. Ex vivo receptor autoradiography as a function of time was performed to investigate OX2R occupancy within the rat frontal cortex (OX2R) or tenia tecta (OX1R) after the subcutaneous administration of 30 mg/kg JNJ-10397049, almorexant, and SB-408124.

Fifteen minutes after injection signaled the plateau for the maximal level of receptor occupancy for JNJ-10397049 (76 ± 4%). This degree of OX2R occupancy elicited by JNJ-10397049 was maintained until the 6-h time point (Fig. 1A). After 24 h, receptor occupancy for this compound was still measurable (20 ± 11%; data not shown). As expected, JNJ-10397049 did not show any significant level of OX1R occupancy at any time point (Fig. 1B). The pinnacle for maximal occupancy of the OX2R in the frontal cortex after the 30 mg/kg dose of almorexant was 30 min after dosing (89 ± 4%; Fig. 1A). The next sequential four time points revealed that this level of OX2 receptor occupancy after the administration of almorexant was sustained and slightly higher than the
level of OX2R occupancy achieved by JNJ-10397049 (Fig. 1A). At the 24-h time point, almost half of the OX2R still exhibited occupancy after the administration of almorexant at 30 mg/kg (data not shown). The OX1R occupancy profile of almorexant was similar to the OX2R profile, the only difference being the levels of OX1R occupancy were approximately 10% lower compared with the OX2R occupancy level (Fig. 1, A and B). SB-408124 did not show any level of OX2R occupancy at any time point (Fig. 1A). In contrast, SB-408124 achieved 90% OX1R occupancy 30 min after administration, which subsequently declined to approximately 30% by 6 h (Fig. 1B).

To parallel the sleep-wake EEG studies, a subsequent dose-dependent OX2R occupancy study was performed. Either JNJ-10397049 (0.3 or 3 mg/kg) or almorexant (3 mg/kg) was injected to determine the extent of OX2R occupancy at 60 min after injection. Similar to the findings with the 30 mg/kg dose of the two compounds, injection of 3 mg/kg almorexant resulted in a greater level of OX2R occupancy compared with JNJ-10397049 at the same dose (78 ± 5 versus 66 ± 2%, respectively). The lowest dose of JNJ-10397049 tested (0.3 mg/kg) occupied approximately 45% of the OX2R located within the frontal cortex at the 1-h time point.

**Promotion of Sleep Induced by Selective OX2R Antagonism versus Dual OX1/2R Blockade in Rats.** To investigate the specific role of OX1R and OX2R in sleep modulation, we compared the effects of selective or dual antagonists at each receptor subtype. The selective OX2R antagonist JNJ-10397049 (0.3, 3, and 30 mg/kg), the dual OX1/2R antagonist almorexant (3 and 30 mg/kg), and the selective OX1R antagonist SB-408124 (30 mg/kg) were subcutaneously administered at 2 h into the light phase in rats. At this point in the light/dark cycle, the diurnal concentrations of the orexin-A peptide are still relatively elevated but are starting to rapidly wane (Yoshida et al., 2001), whereas the sleep pressure is relatively high. For this facet of the study, sleep-wake parameters were presented for the first 2-h period after dosing based on the short-lasting sleep-promoting effect observed in these conditions.

The OX2R and OX1/2R antagonists significantly decreased the latency to persistent sleep (NREM sleep latency). This sleep-inducing effect occurred at a 10 times lower dosage with the OX2R antagonist (3 mg/kg) than with the OX1/2R antagonist (30 mg/kg) compared with the corresponding vehicle treatment (Fig. 2A). In contrast, the OX2R and OX1/2R antagonists induced an equipotent decrease in REM sleep latency at the highest dose tested (30 mg/kg) (Fig. 2B). Both compounds produced a significant increase in NREM sleep time, the OX2R antagonist from the dose of 3 mg/kg onward and the OX1/2R antagonist at the highest dose tested only (Fig. 2C). In addition, at the 30 mg/kg dose, REM sleep time was significantly increased after the OX1/2R antagonist administration, whereas a nonsignificant increase was also measured in rats treated with the OX2R antagonist (Fig. 2D). The increase in NREM sleep time produced by the OX2R antagonist was due to a prolongation of NREM bouts with no alteration in the bout numbers. In contrast, the NREM and REM sleep time-increasing effect after the OX1/2R antagonist treatment was apparently due to an enhancement in the number of NREM and REM sleep bouts, with no change in their duration. However, this discrepancy was rather due to the significantly lower number of NREM bouts with vehicle almorexant (OX1/2R) compared with vehicle JNJ-10397049 (OX2R) and vehicle SB-408124 (OX1R) (Table 1). Overall, a significant increase in total sleep time was observed after 3 mg/kg OX2R antagonist (+42%; P < 0.05) and after 30 mg/kg OX1/2R antagonist (+34%; P < 0.05) relative to vehicle treatment. The respective components for NREM and REM sleep as expressed as percentage of total sleep time were not different from those obtained after vehicle administration, indicating that NREM and REM sleep contributed approximately equally to this enhancement (data not shown). The selective OX1R antagonist was without effect on latency to either NREM or REM sleep and did not affect the duration of either NREM or REM sleep (Fig. 2).

In a subsequent investigation, the OX2R antagonist (3 and 30 mg/kg), the OX1/2R antagonist (3 and 30 mg/kg), and the OX1R antagonist (30 mg/kg) were tested at the onset of the dark (active) phase of the rat. At this time, the diurnal rhythm of the orexin-A peptide starts to increase (Yoshida et al., 2001), whereas the sleep pressure is at its nadir. For this aspect of the study, the data were garnered from the 12 h of the dark phase. Similar to our findings during the light phase, the OX2R antagonist elicited a significant dose-depen-
dient reduction in the latency to NREM sleep, although a nonsignificant trend existed for the OX1/2R antagonist (Fig. 3A). In contrast, these compounds did not alter the latency to the first episode of REM sleep (Fig. 3B). In comparison with vehicle-treated animals, the administration of the OX2R antagonist produced a significant prolongation of NREM sleep time during the 12-h dark phase at both 3 and 30 mg/kg, whereas the OX1/2R antagonist was able to produce a significant increase in NREM sleep time only at the highest dose tested (30 mg/kg) (Fig. 3C). For both compounds, the increased NREM sleep time was the result of an enhancement in the number of NREM bouts with no change in their mean duration (Table 1). In addition, rats treated with 30 mg/kg OX2R or the OX1/2R antagonist exhibited a significant increase in REM sleep time (Fig. 3D) due to a prolonged mean duration of REM bouts, with no change in their numbers (Table 1). As found after the light phase treatment, the OX1R antagonist did not affect the initiation and the duration of NREM and REM sleep during the dark phase (Fig. 3).

EEG power spectral analysis performed by Fast Fourier transform in specific sleep-wake states did not show any changes in power density values, specifically in regard to NREM delta power (0.5–4 Hz) or REM theta power (5–10 Hz) after the treatment with the OX2R, OX1/2R, or OX1R antagonists in either phase of the light/dark cycle (data not shown). In addition, a careful visual analysis of the EEG and EMG signals did not reveal any direct transitions from wake to REM sleep or any abrupt loss of muscular tone muscular atonia during wake (cataplexy-like behavior) with any of the three compounds.

Decrease in body temperature and locomotor activity was observed after administration of the OX2R and OX1/2R antagonists during the light phase that paralleled the effects on sleep. In the first 2 h after the treatment, activity counts were significantly reduced at all of the doses tested (0.3–30 mg/kg) of the selective OX2R antagonist and at the 30 mg/kg dose of the dual OX1/2R antagonist, whereas only the OX2R antagonist produced a moderate decrease in body temperature at 3 and 30 mg/kg (Fig. 4A). More limited effects were found during the total 12-h dark phase after injection. Rats treated with 30 mg/kg of either the OX2R antagonist or the

<table>
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<th>TABLE 1</th>
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<tr>
<td>NREM and REM sleep bout analysis after the subcutaneous administration of JNJ-10397049 (0.3, 3, and 30 mg/kg), almorexant (3 and 30 mg/kg), and SB-408124 (30 mg/kg) in rats</td>
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<td>Values (means ± S.E.M.; n = 6 animals/compound) are calculated for a 2-h period after the light phase treatment or for a 12-h period after the dark phase treatment.</td>
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<tr>
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<th>JNJ-10397049</th>
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<th>SB-408124</th>
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<tr>
<td></td>
<td>Vehicle</td>
<td>0.3</td>
<td>3</td>
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<tr>
<td>Light phase</td>
<td></td>
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<tr>
<td>No. of NREM bouts</td>
<td>30.2 (1.9)</td>
<td>30.8 (3.2)</td>
<td>30.8 (1.6)</td>
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<td>No. of REM bouts</td>
<td>5.8 (1.2)</td>
<td>4.5 (0.8)</td>
<td>6.8 (0.6)</td>
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<td>NREM bout duration (min)</td>
<td>1.82 (0.17)</td>
<td>2.62 (0.47)*</td>
<td>2.72 (0.34)*</td>
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<td>REM bout duration (min)</td>
<td>1.49 (0.21)</td>
<td>1.74 (0.21)</td>
<td>1.92 (0.28)</td>
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<td>Dark phase</td>
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<tr>
<td>No. of NREM bouts</td>
<td>182.0 (3.6)</td>
<td>194.0 (7.3)**</td>
<td>219.2 (5.1)**</td>
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<tr>
<td>No. of REM bouts</td>
<td>37.5 (3.0)</td>
<td>34.8 (3.0)</td>
<td>40.2 (2.5)</td>
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<td>NREM bout duration (min)</td>
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<td>1.21 (0.05)</td>
<td>1.13 (0.04)</td>
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<td>REM bout duration (min)</td>
<td>0.98 (0.08)</td>
<td>1.14 (0.11)</td>
<td>1.30 (0.13)*</td>
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* P < 0.05 and ** P < 0.01 for each dose vs. corresponding vehicle as determined by one-way ANOVA with Dunnett’s multiple comparison post hoc analysis.

* P < 0.05 for vehicle almorexant vs. vehicle JNJ-10397049 and vehicle SB-408124 as determined by one-way ANOVA with Tukey post hoc analysis.

* P < 0.05 for vehicle almorexant vs. vehicle JNJ-10397049 as determined by one-way ANOVA with Tukey post hoc analysis.

Fig. 3. Sleep-promoting effects of a selective OX2R antagonist (JNJ-10397049; 3 and 30 mg/kg) versus a dual OX1/2R antagonist (almorexant; 3 and 30 mg/kg) and a selective OX1R antagonist (SB-408124; 30 mg/kg) during the dark phase in rats. Latency to NREM (A) and REM sleep (B), and duration of NREM (C) and REM (D) sleep were calculated for 12 h after compound administration. Results are expressed in minutes and are represented as means ± S.E.M. of six animals per compound. * P < 0.05, treatment versus corresponding vehicle based on one-way ANOVA followed by Dunnett’s multiple comparison post hoc test.
OX1R antagonist displayed a slight but significant decrease in activity counts, and rats administered with the 30 mg/kg dose of the OX1/2R antagonist showed a moderate decrease in body temperature (Fig. 4B).

Coadministration of a Selective OX1R Antagonist Attenuates the Sleep-Promoting Effects Evoked by a Selective OX2R Antagonist in Rats. To try and mirror the actions of the dual OX1/2 receptor antagonist on the sleep-wake profile in rats, a study was undertaken in which rats received a selective OX1R antagonist and a selective OX2R antagonist in combination. For this investigation, rats were coadministered SB-408124 (30 mg/kg) and JNJ-10397049 (10 mg/kg) at 2 h into the light phase. Administration of the OX2R antagonist alone induced a significant reduction in NREM sleep latency and an increase in NREM sleep duration during the first 2 h after administration relative to vehicle treatment. Similar to our previous findings, the OX1R antagonist alone caused no change in any sleep parameters. Interestingly, when the OX1R antagonist was coadministered with the OX2R antagonist, the sleep-inducing effect produced by the selective OX2R antagonist alone was partially attenuated (Fig. 5A), whereas the increase in NREM sleep duration was abolished (Fig. 5C). In contrast, rats receiving the combined therapy entered in REM sleep significantly faster (Fig. 5B), whereas the overall time spent in REM sleep exhibited a nonsignificant increase (Fig. 5D).

To demonstrate that the diminished effect of the OX2R antagonist JNJ-10397049 in the presence of the OX1R antagonist SB-408124 is the result of a pharmacodynamic effect rather than a pharmacokinetic interaction, a drug-drug interaction study between these two compounds was carried out. The results from the drug-drug interaction study indicated that the OX2R antagonist plasma and brain exposure were not decreased by the coadministration of the OX1R antagonist (Fig. 6, A and B; plasma JNJ-10397049 alone $C_{\text{max}} = 1.01 \ \mu\text{M}, \ CV = 5.86\%$ and JNJ-10397049 + SB-408124 $C_{\text{max}} = 1.29 \ \mu\text{M}, \ CV = 5.78\%$; brain JNJ-10397049 alone $C_{\text{max}} = 1.03 \ \mu\text{M}, \ CV = 6.69\%$ and JNJ-10397049 + SB-408124 $C_{\text{max}} = 1.14 \ \mu\text{M}, \ CV = 6.84\%$) and that vice versa, the OX1R antagonist plasma and brain exposure was not affected ($P > 0.05$) by the coadministration of the OX2R antagonist (Fig. 6, C and D; plasma SB-408124 alone $C_{\text{max}} =$
84.29 μM, CV = 7.39% and SB-408124 + JNJ-10397049 C\text{max} = 64.08 μM, CV = 12.51%; brain SB-408124 alone C\text{max} = 1.09 μM, CV = 12.42% and SB-408124 + JNJ-10397049 C\text{max} = 1.30 μM, CV = 9.56%).

**Effect of Orexin Receptor Blockade on the Release of Monoamines in Various Rat Brain Regions.** As part of our comparative neurochemical profile of the three differential OXR antagonists, the effects of the selective OX2R antagonist JNJ-10397049, the dual OX1/2R antagonist almorexant, and the selective OX1R antagonist SB-408124 on monoamine release in defined brain regions were investigated. At 30 min before the onset of the dark phase, all three compounds were administered subcutaneously at a dose of 30 mg/kg. Through a microdialysis cannula in freely moving rats, samples for measurement of extracellular release of HA in the LH and DA, NE, and 5-HT in the PFC were collected at 30-min intervals during 2 h before and 4 h after the injection.

Overall, the two compounds with high affinity for OX2R significantly decreased extracellular HA levels in the LH with a similar magnitude during the first 4 h after the treatment. In contrast, the OX1R antagonist had no significant effect on extracellular HA release (Fig. 7A). The two compounds showing high affinity for OX1R elevated DA levels in rat PFC during the dark phase (Fig. 7B). Although the onset was relatively rapid, the elevation in PFC concentrations of DA was short-acting with DA levels returning to baseline value at approximately 1 h after the dual OX1/2R antagonist and 90 min after the selective OX1R antagonist treatment. In contrast, the OX2R antagonist did not significantly affect the extracellular DA release in the PFC (Fig. 7B). As a control experiment, extracellular DA levels were measured from samples acquired from the nucleus accumbens of rats that had been injected with the three OXR antagonists at 30 min before dark onset. The two compounds with high affinity for the OX1R, but not the selective OX2R antagonist, significantly decreased extracellular DA release in the nucleus accumbens (data not shown). Interestingly, none of the OXR antagonists tested modified the extracellular release of NE (Fig. 7C) and 5-HT (Fig. 7D) in the rat PFC when these compounds were administered 30 min before the dark phase.

**Discussion**

The present investigation demonstrated that selective blockade of OX2R promoted sleep, a likely consequence of the inhibition of hypothalamic HA release in rats. In addition, we show evidence that OX1R and OX2R do not contribute equally to the modulation of both sleep-wake states and monoaminergic release and in fact may serve counter-regulatory roles.

A recent study in animals and humans with the dual OX1/2R antagonist almorexant has provided evidence of its hypnotic activity during the active period (dark phase), whereas no significant sleep effect was detected during the light phase (sleep phase) in the rat (Brisbare-Roch et al., 2007). The present study partially confirms this finding, showing that almorexant given at dark onset was effective in promoting both NREM and REM sleep time without affecting sleep initiation. However, both sleep induction (decreased NREM and REM sleep latencies) and sleep lengthening (increase in NREM and REM sleep time) were observed in rats treated with almorexant at the beginning of the light phase. Therefore, these data indicate that the hypnotic activity of OXR antagonists can be revealed during the light phase (rest period) as well.

To unravel the pharmacological basis for these effects on sleep in both the dark and light phases, we examined the effects of either OX2R- or OX1R-selective compounds compared with the dual OX1/2R antagonist almorexant on various sleep parameters in rats. The selective OX2R antagonist JNJ-10397049 was fully capable of inducing sleep (decreas-
ing latency to NREM) and increasing NREM and REM sleep time in rats during both light and dark phases. In contrast, the selective OX1R antagonist SB-408124 had no effect on any sleep parameter tested. The differential sleep-promoting effects among a selective OX2R antagonist, a dual OX1/2R antagonist, and a selective OX1R antagonist that were found

Fig. 7. Differential effects of a selective OX2R antagonist (JNJ-10397049), a dual OX1/2R antagonist (almorexant), and a selective OX1R antagonist (SB-408124) on extracellular neurotransmitter release in the rat brain. Microdialysate samples from the lateral hypothalamus for HA (A) and from the prefrontal cortex for DA (B), NE (C), and 5-HT (D) were collected for 2 h before and 4 h after compound administration (30 mg/kg) in freely moving rats. Results are represented as means ± S.E.M. of three to 11 animals. HA, DA, NE, and 5-HT levels are expressed as a percentage of the predose baseline of each animal. Inset bar graphs illustrate area under the curves (calculated from time of injection to 240 min after injection) for all four neurotransmitters. *, P < 0.05 and ***, P < 0.001, treatment versus vehicle based on one-way ANOVA followed by Newman-Keuls post hoc test.
either during the light or the dark phase in rats indicate a key role for OX2R. The selective OX2R antagonist showed a 10-fold higher potency than the dual OX1/2R antagonist in inducing and prolonging sleep, whereas the selective OX1R antagonist had no effect. It was a surprise that the dual OX1/2R antagonist produced less hypnotic activity despite showing slightly higher levels of cortical OX2R occupancy than the selective OX2R antagonist, suggesting that the lower sleep response might be related to its OX1R affinity rather than OX2R occupancy. We tested this hypothesis by coadministering the two selective OX1R and OX2R antagonists, and we found that, indeed, the blockade of OX1R attenuated the sleep-promoting effects of the OX2R antagonist. These data, together with the drug-drug interaction results ruling out a possible pharmacokinetic contribution, clearly indicate a functional relationship between OX1R and OX2R on sleep regulation. It is interesting to note that the additional inhibition of OX1R counteracted the OX2R-induced NREM sleep initiation and prolongation without affecting REM sleep, implying a sleep state-specific action. Separate roles for the two OXR subtypes have been postulated that fit well with their distinctive anatomical distribution (Sakurai et al., 1998; Marcus et al., 2001), namely, a specific role in the regulation of REM sleep for OX1R in the locus coeruleus (Bourgin et al., 2000; Smith et al., 2003) and an implication of hypothalamic OX2R in the modulation of NREM sleep (Willie et al., 2003).

Activation of both OXR subtypes contributes to the physiological control of arousal state (Hagan et al., 1999; Akamnu and Honda, 2005). In our study, the virtual absence of sleep effect of the OX1R antagonist is in agreement with previous results reporting no significant change in the amount of sleep-wake states after administration of another OX1R antagonist, SB-334867 (Smith et al., 2003). These data are also in line with the minor sleep alterations (only mild sleep fragmentation) reported in OX1R knockout mice (Sakurai, 2007). Our observation that the transient concomitant inhibition of both OX1R and OX2R elicited a significantly lesser sleep-promoting effect than the selective blockade of OX2R was unexpected because the double OXR knockout mice display a more pronounced sleep phenotype compared with the OX2R knockout genotype (Willie et al., 2003). Furthermore, in human and animal narcolepsy models, orexin deficiency is associated with high prevalence of sleep fragmentation (Shelton et al., 1995; Chemelli et al., 1999; Overeem et al., 2001). In the present study, selective OX2R or dual OX1/2R antagonism during the dark phase did not cause sleep fragmentation because NREM and REM bout duration was not decreased. In contrast, during the light phase, NREM sleep time was increased after OX2R blockade due to a prolongation of NREM bout duration, which is an index of enhanced sleep consolidation.

Because the measurement of sleep parameters by EEG analysis is the output of a complex, integrated process, we attempted to determine whether we could measure more single parameter changes via neurotransmitter microdialysis. We demonstrated for the first time that the sleep-promoting action induced by pharmacological blockade of OX2R by both a selective OX2R antagonist and a dual OX1/2R antagonist paralleled a decrease in extracellular HA release within the LH. Conversely, intracerebroventricular administration of orexin-A has been reported to enhance hypothalamic HA release in rats (Ishizuka et al., 2002) and mice (Huang et al., 2001). Collectively, these findings strongly support a major role of the histaminergic system in the arousal effect of orexins (Bayer et al., 2001). In line with the reduced hypnotic activity of a dual OX1/2R antagonist relative to a selective OX2R antagonist, the decrease in extracellular HA levels within the LH was associated with a short-lasting increase in DA release within the PFC only in rats treated with the dual OX1/2R antagonist. Likewise, this enhancement of extracellular DA levels in the PFC occurred also after administration of the selective OX1R antagonist and, therefore, seems to be mediated through OX1R blockade. Because dopaminergic stimulation is associated with arousal, it is possible that the short-lasting elevation of extracellular cortical DA levels is responsible for the attenuated hypnotic activity elicited by the dual OX1/2R antagonist, despite its robust OX2R occupancy. In apparent contradiction with these data, orexin-induced hyperlocomotion has been shown to be mediated by the dopaminergic system (Nakamura et al., 2000) and to correlate with increased DA levels in the PFC (Vittoz and Berridge, 2006). However, DA concentrations in the rat medial PFC are increased not only during wake but also during REM sleep relative to NREM sleep (Léna et al., 2005), which may partly explain why the blockade of OX1R suppressed the NREM but not the REM sleep-promoting effects induced by OX2R inhibition. Although orexin-containing neurons in the ventral tegmental area have been associated with addiction and arousal (de Lecea et al., 2006; Harris and Aston-Jones, 2006), it is speculated that DA also plays an important role in OXR-mediated sleep, particularly REM sleep modulation that needs to be further clarified. In our hands, neither NE nor 5-HT release within the PFC was affected by the OX1R, OX2R, or OX1/2R antagonists. However, in addition to the monoaminergic system, orexins also activate cholinergic neurons through both OX1R and OX2R (Fadel and Frederick-Duas, 2008), and future investigations are required to examine the potential participation of the cholinergic system. From the visual examination of EEG/EMG traces, we did not detect any signs of abrupt loss of muscular tone during wake (cataplexy-like symptoms) after administration of any OXR antagonists. During cataplexy, the activity of histaminergic cells is maintained, whereas noradrenergic and serotonergic neurons are inactive (John et al., 2004). Therefore, the transient reduced HA release in the LH by selective OX2R inhibition, without any alteration of extracellular cortical NE and 5-HT levels, is not expected to particularly favor cataplexy-like behavior.

In conclusion, these findings highlight the complex interplay between OX1R and OX2R systems and demonstrate that unilateral blockade of all orexigenic activity may result in attenuation of specific behavioral responses such as sleep induction. Thus, selective OX2R antagonists may offer advantage for the treatment of insomnia and OX1R antagonists may be better suited to modulate DA-related behaviors such as addiction or reward.

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