Differential sleep-promoting effects of dual orexin receptor antagonists and GABA<sub>A</sub> receptor modulators

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Abstract

**Background:** The current standard of care for insomnia includes gamma-aminobutyric acid receptor A (GABA<sub>A</sub>) activators, which promote sleep as well as general central nervous system depression. Dual orexin receptor antagonists (DORAs) represent an alternative mechanism for insomnia treatment that induces somnolence by blocking the wake-promoting effects of orexin neuropeptides. The current study compares the role and interdependence of these two mechanisms on their ability to influence sleep architecture and quantitative electroencephalography (qEEG) spectral profiles across preclinical species.

**Results:** Active-phase dosing of DORA-22 induced consistent effects on sleep architecture in mice, rats, dogs, and rhesus monkeys; attenuation of active wake was accompanied by increases in both non-rapid eye movement (NREM) and rapid eye movement (REM) sleep. Eszopiclone, a representative GABA<sub>A</sub> receptor modulator, promoted sleep in rats and rhesus monkeys that was marked by REM sleep suppression, but had inconsistent effects in mice and paradoxically promoted wakefulness in dogs. Active-phase treatment of rats with DORA-12 similarly promoted NREM and REM sleep to magnitudes nearly identical to those seen during normal resting-phase sleep following vehicle treatment, whereas eszopiclone suppressed REM even to levels below those seen during the active phase. The qEEG changes induced by DORA-12 in rats also resembled normal resting-phase patterns, whereas eszopiclone induced changes distinct from normal active- or inactive-phase spectra. Co-dosing experiments, as well as studies in transgenic rats lacking orexin neurons, indicated partial overlap in the mechanism of sleep promotion by orexin and GABA modulation with the exception of the REM suppression exclusive to GABA<sub>A</sub> receptor modulation. Following REM deprivation in mice, eszopiclone further suppressed REM sleep while DORA-22 facilitated recovery including increased REM sleep.

**Conclusion:** DORAs promote NREM and importantly REM sleep that is similar in proportion and magnitude to that seen during the normal resting phase across mammalian animal models. While limited overlap exists between therapeutic mechanisms, orexin signaling does not appear involved in the REM suppression exhibited by GABA<sub>A</sub> receptor modulators. The ability of DORAs to promote proportional NREM and REM sleep following sleep deprivation suggests that this mechanism may be effective in alleviating recovery from sleep disturbance.

Keywords: Orexin, Hypocretin, Benzodiazepine, Insomnia, Sleep, Electroencephalography, Sleep deprivation, REM sleep, NREM sleep, Suvorexant, Belsomra
Background

Currently, most pharmacologic treatments for insomnia are central nervous system depressants that act by allosterically activating gamma-aminobutyric acid receptor A (GABA_A) [1–4]. More recently, the inhibition of orexin-sterically activating gamma-aminobutyric acid receptor are central nervous system depressants that act by allo-

The present study compared sleep induced by the standard of care, eszopiclone (a non-benzodiazepine GABA_A receptor modulator) and by two distinct DORAs, DORA-12 and DORA-22, using polysomnography (PSG) and quantitative electroencephalography (qEEG) spectral analysis following dose administration during the active phase across several species and following REM deprivation in rats. Direct comparisons with sleep architecture seen during the normal resting phase revealed that DORAs proportionately induced both NREM and REM sleep in a pattern no different from non-medicated sleep and induced qEEG changes consistent with those seen during the in- active phase. Eszopiclone had differential effects on sleep across species that were characterized by abnormal sleep architecture and qEEG profiles relative to vehicle-treated, inactive-phase control animals. Further, DORAs more immediately facilitated proportional recovery sleep following REM deprivation relative to vehicle or eszopiclone treatment. Co-dosing studies and experiments in orexin/ataxin-3 (Ox/Atx) transgenic rats lacking orexin neurons indicated only partial overlap of these two mechanisms.

Results

DORAs and standard of care differentially affect sleep
across mammalian species

To qualitatively compare the sleep architecture induced by orexin receptor antagonism versus GABA_A receptor modulation across species, PSG analyses were conducted in telemetry-implanted mice, rats, dogs, and rhesus monkeys receiving either DORA-22 or eszopiclone during their usual active period, and evaluated relative to vehicle treatment. Representative doses for each species were chosen based on prior dose response studies inducing sali- ent, active-wake reduction approximating that seen in rats, the highest dose tested (DORA-22 in rhesus monkeys), or a qEEG effect magnitude similar to that in other species (eszopiclone in mice, dogs). In all four species exam- ined, significant active-wake reduction at consecutive 30 min time points by DORA-22 ranged from 2 h (mice) to 7 h (dogs), and was associated with significant increases in both NREM and REM sleep (Figure 1A). In mice and rats, delta sleep showed immediate 2 and 2.5 hour in- creases (P ≤ 0.05 at four and five consecutive 30 min time points), respectively, while REM sleep was significantly in- creased for 3 and up to 5.5 h, respectively. Variable in- creases in light sleep, comprising the smallest proportion of vigilance state mean time, were also observed in mice and to a lesser extent in rats. In dogs and rhesus monkeys, NREM I sleep was similarly increased with a delay in dogs at time points up to 7 h after dosing and for 5.5 h in rhe- sus immediately after dosing coincident with significant
Figure 1 (See legend on next page.)
DORA-induced REM and NREM sleep mimics normal resting-phase sleep

To determine how the sleep architecture induced by both classes of drugs compares with that seen during the normal resting phase, we compared the effects of DORA-12 and eszopiclone administered during the active phase to the NREM and REM sleep seen normally during the inactive phase after vehicle dosing in rats. A DORA of distinct chemical structure, DORA-12 was used herein to further substantiate the observed effects of DORA-22 on general inactive-phase sleep effects and qEEG in a prior sleep-stage analysis [13]. Figure 2 directly compares the NREM and REM changes occurring during the normal inactive phase with those seen during the active phase by shifting the data collected during the normal inactive-phase onset of 6.5 h to coincide with the active-phase vehicle treatment time (Zeitgeber time [ZT] 17:30; see also Additional file 1: Figure S1A). As expected, the mean time spent in NREM and REM following vehicle treatment during the active phase was significantly lower than that seen during the 150 min following the onset of the vehicle treatment inactive phase (NREM: $F_{1,10} = 56.57$, $P < 0.0001$, 2-way ANOVA, $P < 0.0001$, Tukey HSD; REM: $F_{1,10} = 41.19$, $P < 0.0001$, 2-way ANOVA, $P < 0.0001$, Tukey HSD). Following active-phase treatment with eszopiclone (10 mg/kg), the time course of NREM was significantly increased relative to both active-phase vehicle ($F_{1,10} = 76.72$, $P < 0.0001$, 2-way ANOVA; $P < 0.0001$, Tukey HSD) and that occurring during the onset of the inactive phase ($F_{1,10} = 7.68$, $P = 0.0197$, 2-way ANOVA; $P < 0.0001$, Tukey HSD), whereas REM sleep was significantly decreased relative to both control conditions (active phase: $F_{1,10} = 8.86$, $P = 0.0139$, 2-way ANOVA; $P < 0.0001$, Tukey HSD; inactive phase: $F_{1,10} = 51.92$, $P < 0.0001$, 2-way ANOVA; $P < 0.0001$, Tukey HSD). On the other hand, treatment with DORA-12 (30 mg/kg) during the active phase increased both NREM and REM sleep similar to that observed following vehicle administration during the inactive phase onset. Remarkably, the time courses of these increases were statistically no different from those observed during the inactive-phase
onset following vehicle treatment (NREM: F$_{1,10}$ = 0.227, P = 0.644, 2-way ANOVA, P = 0.0661; Tukey HSD; REM: F$_{1,10}$ = 2.347, P = 0.157, 2-way ANOVA, P = 0.2933, Tukey HSD). A comprehensive comparison of the time courses of vigilance states with vehicle, DORA-12, and eszopiclone treatments during both the active phase and inactive phase is shown in Additional file 1: Figure S1; eszopiclone suppressed REM sleep while promoting NREM sleep in a manner distinct from that seen during the normal resting phase, whereas DORA-12 induced minimal changes relative to normal, inactive-phase sleep.

Quantitative EEG analysis in these same rats following treatment with DORA-12 (30 mg/kg) and eszopiclone (10 mg/kg) during both the active phase and at the onset of the resting phase further illustrates differences between these two mechanisms. DORA-12 administration during the active phase was associated with time-dependent decreases in high-frequency gamma power (30–100 Hz) (significant time points [P ≤ 0.05] up to 6.5 h following dosing), increases in middle-frequency spectral power (theta up to 3 h; alpha, 2.5 h; sigma, 2 h), and little change relative to vehicle in delta frequency (0.5–4 Hz) (Figure 3A). These changes largely dissipated 6.5 h later with the onset of the inactive phase, where DORA-12 responses approached those of vehicle-treated animals. In contrast, eszopiclone treatment during the active phase resulted in large increases in qEEG power in the beta band (19–30 Hz) (12 h) as well as sigma frequencies (12–16 Hz) (5.5 h) whereas minimal decreases were seen in theta and alpha power (4–7 Hz and 8–12 Hz, respectively). Relative to vehicle, eszopiclone induced little-to-no change in gamma and delta powers until the onset of the inactive phase 6.5 h later, when levels of gamma, delta, and theta power were maintained even though vehicle-treated animals exhibited substantial decreases in gamma power and increases in delta and theta power associated with sleep onset. These inactive-phase differences induced by eszopiclone were corroborated when the drug was administered 1 h prior to the inactive-phase onset, where substantial changes relative to the vehicle condition were seen in most frequency bands (Figure 3B). DORA-12, on the other hand, induced shorter-duration decreases in gamma frequencies and increases in low-to-mid frequency powers, changes that exaggerated the normal change displayed in the vehicle condition during the resting period. Overall, both DORA-12 and eszopiclone appeared to have similar effects on qEEG power during both the active and inactive phases, but their observed effects relative to vehicle were dependent on baseline changes occurring at different times of day. Relative to vehicle, DORAs appeared to have less effect on qEEG power during the inactive phase, since baseline levels already reflected these changes during the normal resting period.

**Figure 2** Sleep architecture induced by DORA-12 is no different from normal resting-phase sleep in rats. The time course and magnitude of mean time spent in NREM and REM sleep in 30-min intervals following active-phase treatment with DORA-12 (30 mg/kg, n = 14) and eszopicline (10 mg/kg, n = 16) is compared with that following vehicle treatment (vamin F TPGS, 20% solution, orally) at both the onset of the active phase (Zeitgeber time [ZT] 17:30) and the inactive phase (ZT 00:00). Values at 30-min intervals are expressed as percentage of the mean 30-min level calculated from times 90 to 150 min of the inactive-phase onset levels. Data represent a 3-h summary of the full time course and polysomnographic analysis presented in Additional file 1: Figure S1; light and delta sleep have been combined as NREM sleep. Comparison of treatment conditions analyzed by analysis of variance (ANOVA) followed by the Tukey multiple comparison (HSD) test revealed significant differences (P < 0.0001) between all conditions except the following: inactive-phase onset NREM vs DORA-12 NREM (P = 0.0661), eszopicline NREM vs DORA-12 NREM (P = 0.2582), and inactive-phase onset REM vs DORA-12 REM (P = 0.2933). Similar results were seen when each condition was evaluated pairwise by 2-way ANOVA: eszopicline vs DORA-12 (NREM, F$_{1,10}$ = 7.43, P = 0.0214; REM, F$_{1,10}$ = 12.09, P < 0.0001), active phase vehicle vs DORA-12 (NREM, F$_{1,10}$ = 26.67, P < 0.0001; REM, F$_{1,10}$ = 183.4, P < 0.0001), inactive phase vehicle vs DORA-12 (NREM, F$_{1,10}$ = 0.227, P = 0.644, REM, F$_{1,10}$ = 2.347, P = 0.157); active phase vehicle vs eszopicline (NREM, F$_{1,10}$ = 76.72, P < 0.0001; REM, F$_{1,10}$ = 8.86, P = 0.0339); inactive phase vehicle vs eszopicline (NREM, F$_{1,10}$ = 7.38, P = 0.0197; REM, F$_{1,10}$ = 51.92, P < 0.0001); inactive phase vehicle vs active phase vehicle (NREM, F$_{1,10}$ = 56.57, P < 0.0001; REM, F$_{1,10}$ = 41.19, P < 0.0001).

**Figure 3** Quantitative EEG analysis in rats following active- and inactive-phase treatment with DORA-12 (30 mg/kg) and eszopicline (10 mg/kg) during both the active phase and at the onset of the resting phase. A comprehensive comparison of the time courses of vigilance states with vehicle, DORA-12, and eszopicline treatments during both the active phase and inactive phase is shown in Additional file 1: Figure S1; eszopicline suppressed REM sleep while promoting NREM sleep in a manner distinct from that seen during the normal resting phase, whereas DORA-12 induced minimal changes relative to normal, inactive-phase sleep.

**DORA-22 promotes recovery from REM sleep deprivation**

Given the ability of DORA-22 to promote somnolence including REM sleep and, conversely, the potential for eszopicline to promote sleep associated with REM suppression, we evaluated the ability of both compounds to promote recovery from REM sleep deprivation. For these experiments, mice were first subjected to 28 h of REM deprivation using the platform or “flower pot” paradigm...
[29], followed immediately thereafter by administration of vehicle (vitamin E TPGS [d-alpha tocopheryl polyethylene glycol 1000 succinate], 20% solution, p.o.), DORA-22 (100 mg/kg) or eszopiclone (60 mg/kg), or no treatment. This manipulation involves placing mice in cages containing 3-cm-diameter platforms surrounded by water (REM deprivation condition), such that the atonia accompanying REM sleep is associated with the animals slipping into the water and arousing. In initial control experiments to demonstrate the feasibility of this approach, mice in cages containing pedestals with water were compared with mice housed similarly, but in the absence of water. As expected, mice in the REM deprivation condition exhibited significant reductions in the amount of time spent in REM sleep at time points throughout the duration of the manipulation (Figure 4A). Upon transfer to cages containing normal bedding at ZT 0:00, a condition representing a novel environment, both control and REM-deprived animals exhibited maximal active wake for approximately 1 h (Figure 4B). Shortly thereafter, mean time in active wake began to decrease in both groups with the REM-deprived group exhibiting significantly less active wake at 3 time points, and increased delta sleep at early time points. Similarly, REM sleep also increased in both groups with the REM deprivation group exhibiting more REM recovery relative to those in the control condition. The administration of DORA-22 occurred simultaneously with the transfer of animals to normal conditions and induced immediate active-wake attenuation coincident with increases in both delta and REM sleep, relative to vehicle-treated animals (significant changes at 1 hour and 50 min relative to vehicle) (Figure 4C). Unlike the changes seen during normal recovery, these changes occurred within 10 min after transfer to cages containing normal bedding. Eszopiclone also facilitated active-wake reductions for 1 hour immediately upon return to normal cages following REM deprivation (Figure 4D). However, in contrast to DORA-22 treatment, this manipulation was associated with increases in light and delta sleep at early time points with little change in REM sleep. Even in what was expected to be increased REM pressure, this GABA<sub>A</sub> receptor modulator significantly suppressed REM at later time points relative to vehicle-treated animals.

**GABA<sub>A</sub> modulators suppress REM independently of orexin signaling**

To determine the interaction between GABA<sub>A</sub> receptor modulator and DORA-mediated mechanisms, and the dependence of eszopiclone activity on orexin signaling, we first evaluated the effectiveness of both DORA-22 (30 mg/kg) and eszopiclone (10 mg/kg) in Ox/Atx transgenic rats deficient in orexin neurons (expressing the cytotoxic poly-Q-ataxin-3 gene product via the HCRT (hypocretin) gene promoter [30]). Relative to wild-type...
Sprague–Dawley rats evaluated simultaneously, DORA-22 resulted in diminished, but still detectable responses in Ox/Atx transgenic rats (Figure 5A), consistent with the diminished, yet incomplete, ablation of orexin signaling in this model (the selectivity of DORA-22 at 100 mg/kg has previously been demonstrated in mice lacking both OX1R and OX2R [12]). The sleep-promoting effects of eszopiclone were also diminished in Ox/Atx transgenic rats relative to the effects seen in wild-type Sprague–Dawley rats (Figure 5B), the exception being in the magnitude of REM reduction where significant reductions were seen up to 4 h post administration. Together these results suggest that eszopiclone mediates many of its sleep-promoting responses through orexin signaling with the exception of its effects on REM, which persist in the presence of reduced orexin signaling.
As these results suggest that orexin and GABA signaling may be largely redundant in their control of active wake and NREM sleep but diverge in their control of REM, the impact of combined administration of DORA-22 (30 mg/kg) and eszopiclone (10 mg/kg) on sleep parameters compared with administration of each agent alone was evaluated in wild-type rats. Relative to DORA-22 alone, the combination exhibited non-additive effects on active-wake reduction (biphasic 1.5 hour increase followed by 1 hour decrease) and only marginal increases in light and delta sleep (Figure 6A). REM sleep, however, was substantially reduced by combination treatment for 2.5 h following treatment. Compared with eszopiclone alone, the combination induced no clear decreases in active wake or increases in delta sleep with the possible exceptions of light and REM sleep (Figure 6B). Transient reductions in light sleep (1 hour) suggested a small influence of DORA-22 to counter the activity of eszopiclone. The combination of DORA-22 and eszopiclone, however, did significantly increased REM sleep relative to eszopiclone alone for up to 3.5 h following treatment. Together these results demonstrate that the pathways underlying the influence of orexin receptor antagonism on sleep parameters overlap with those of the GABA<sub>1</sub> receptor modulator with the exception of REM sleep, which appears to be mediated by distinct pathways.

**Discussion**

These analyses demonstrate that DORAs promote sleep architecture that is indistinguishable from normal resting-phase sleep in animal models, utilizing a mechanism that is distinct from the GABA<sub>1</sub> receptor modulator, eszopiclone. DORAs have been previously shown to promote NREM and REM sleep proportionately across mammalian species, including man [10-12,14-16]. Here we demonstrate that the sleep architecture promoted by DORA-12 during both the active and inactive phase closely mimics that seen during the normal resting phase following vehicle treatment, whereas eszopiclone suppresses REM sleep to levels below those seen even during the active phase (see Figure 2). Notably, both NREM and REM sleep induced by DORA-12 at 30 mg/kg is similar in both magnitude and time course to normal inactive-phase sleep. Further, the qEEG changes associated with DORA-12-induced somnolence
are also similar to those seen during inactive-phase sleep across frequency bands, the differences being an augmentation of increases in lower frequencies and decreases in higher frequencies typically associated with normal resting-phase sleep. While specific qEEG frequency band definitions (the frequency range assignment [in hertz] of delta, theta, alpha, sigma, beta, and gamma bands) can differ between species and laboratories, it is clear from these studies that the pattern of qEEG changes induced by DORA-12 is similar, if not indistinguishable, from that seen during the resting phase, regardless of band definitions. Eszopiclone responses, on the other hand, exhibit substantial differences from what is typically seen during the resting phase, including dramatically increased beta power, increased gamma power, and decreased lower-frequency alpha, delta, and theta power, the latter potentially being associated with REM suppression. Our qEEG findings corroborate prior evaluations in rats and humans, in which DORA-22 and SB-649868 each minimally disrupted sleep-stage–dependent qEEG spectral power in comparison with the GABA\textsubscript{A} receptor modulators, which substantially disrupted qEEG spectral power in both the active and inactive phases [13,14]. In fact, a recently published clinical study demonstrated that suvorexant minimally impacts qEEG spectral density during NREM and REM sleep relative to placebo in both healthy subjects and insomnia patients while trazadone and the GABA\textsubscript{A} receptor modulators, zolpidem and gaboxadol, induced distinct profiles in human subjects [31].

It has been suggested that the REM promotion by DORAs exceeds what might be expected for normal sleep [32,33], yet these assertions have been made in studies that lack a specific characterization of the time course and magnitude of REM sleep during the normal resting phase. By superimposing the sleep architecture observed during the inactive phase following vehicle treatment with that occurring during the active phase following vehicle treatment here, we have been able to demonstrate that both NREM and REM sleep increase substantially with the onset of sleep in rats, and that DORA-12 increases both vigilance states in a way that is similar to that seen normally at the onset of the inactive phase (see Figure 2; Additional file 1: Figure S1). Further, it is unclear what detrimental effects may result from normal or moderate increases in REM sleep during the inactive phase, and any causal relationships with behavior have yet to be demonstrated. On the other hand, it is clear that REM deprivation is associated with physiological and cognitive deficits. In rats, REM sleep deprivation by the disk-over-water method is associated with a severe phenotype characterized by weight loss with paradoxical hyperphagia, impaired thermoregulation, and eventual fatality [18,34,35]. Selective REM deprivation has also been shown to have hyperalgesic effects in sleep laboratory studies [24]. Perhaps the most well-studied effects are on impairments in learning and memory in rats; REM deprivation impairs hippocampus-dependent spatial learning, and is associated with molecular and cellular alterations in hippocampal function [19,20,36-38]. In fact, a recent study examining the effects of zolpidem in human subjects found that deficits in memory improve the day following administration, a time at which drug levels were expected to have diminished, interpreted to suggest that the quality of sleep induced by this GABA\textsubscript{A} receptor modulator affects cognitive performance [39].

On the other hand, our REM deprivation studies in mice demonstrate that DORA-22 effectively promotes both delta and REM sleep immediately upon transfer.
from deprivation cages to those containing normal bedding, a recovery that was more immediate than in untreated control mice. In this paradigm, transfer to a normal cage represents a novel environment such that untreated control mice continue to exhibit arousal for up to an hour. Both DORA-22 and eszopiclone significantly attenuated active-wake phase immediately upon transfer to the recovery condition, but DORA-22 facilitated REM recovery while eszopiclone actually suppressed REM sleep at later time points relative to vehicle despite a presumed homeostatic drive for REM recovery. In untreated control mice, delta sleep increased with a time course that preceded REM-sleep increases, while DORA-22 increased REM nearly coincident with delta sleep, suggesting that DORAs may facilitate recovery in response to homeostatic drive. Indeed, it has been suggested that the OX1R antagonism provided by DORAs may allow for disinhibition of REM sleep [33], which may underlie yet another favorable property of DORAs in their ability to respond to accumulated REM sleep debt. If this is indeed the case, variability in sleep architecture, including the magnitude and timing of REM sleep promotion observed in animal models at different laboratories, would be expected to be a function of the prior housing conditions; differences in total sleep debt or specific sleep stage debt in EEG-implanted mice or rats would be expected to give rise to differences in responses to DORA treatment. Further study is required to determine the ability of both ORAs and GABA_A receptor modulators to respond to homeostatic needs and the behavioral and psychiatric consequences resulting from those manipulations.

The hypnotics compared herein – GABA_A receptor modulators and DORAs – promote sleep via distinct, but overlapping mechanisms of action. Orexin neurons and their cognitive receptors are more discretely distributed in the brain relative to GABA_A receptor subtypes, and targeting orexin-mediated arousal with ORAs has more restricted effects relative to the GABA_A receptor modulators, which augment GABA_A receptor activity, resulting in widespread central nervous system depression. Within the sleep system, orexin neuron activity promotes arousal and vigilance-state control through their projections to tuberomammillary nuclei and brain stem structures including the dorsal raphe, locus coeruleus, and laterodorsal and pedunculopontine tegmental nuclei, while inhibitory GABAergic projections from ventrolateral preoptic nuclei project to many of the same structures as well as orexin neurons [40]. However, the widespread distribution and function of GABA_A receptor subtypes involved in other complex behaviors—including, but not limited to anxiety, locomotor coordination, addiction, learning, and memory [41,42]—may underlie the differential sleep-promoting effects of GABA_A receptor modulators across species (e.g., sleep promotion in rats, monkeys, and humans, but paradoxical hyperarousal in dogs). Species-dependent differences in signal strength mediated by the multitude of GABAergic pathways within and/or outside of the specific sleep pathways are likely responsible. Nevertheless, our findings indicate overlap between orexin and GABAergic pathways, as might be predicted from histological evidence. The sleep effects of DORA-22 were markedly attenuated in orexin neuron–deficient rats, indicating that these effects were mediated through the orexin pathway with some remaining effects likely due to incomplete ablation of orexin-containing neurons in these animals [30]. The selectivity of DORA-22 has previously been demonstrated at doses as high as 100 mg/kg in mice lacking both orexin receptors, where no detectable effects were seen [12]; as such, the residual REM promotion seen in Ox/Atx transgenic rats is likely due to residual orexin signaling in those animals. The effect of eszopiclone on active wake and NREM sleep was also attenuated in rats lacking orexin neurons, and the combined administration of both agents had similar effects on active wake compared with either administered alone. These results suggest that both classes of compounds reduce active wake through a common pathway. REM sleep, however, was similarly suppressed by eszopiclone in both Ox/Atx transgenic rats and in wild-type control rats. The combined administration of eszopiclone with DORA-22 markedly reduced REM relative to DORA-22 alone, and only slightly increased REM relative to eszopiclone alone, indicating that these effects on REM sleep are not entirely additive. Both orexinergic and GABAergic signaling influence the activity of brainstem nuclei, particularly the locus coeruleus, to affect vigilance-state control, including REM sleep. These results indicate that these influences are not entirely dependent on one another, but can influence this function through parallel means. From a therapeutic standpoint, these overlapping, yet distinct mechanisms have important implications regarding tolerance and dependence. GABA_A receptor modulators have been shown in both animal models and human subjects to exhibit tolerance to repetitive dosing, becoming less efficacious over time [43-45], while ORAs, including suvorexant (Belsomra®), have shown no tolerance or evidence of withdrawal even after a year of treatment in patients [17] and no diminution of efficacy in animal models (reviewed in [46]). While the role of orexin in reward and withdrawal suggest that ORAs may have the capacity to diminish the dependence on rewarding influences (reviewed in [47]), the effectiveness of ORAs in animal models and human subjects following treatment with the current standard of care, GABA_A receptor modulators, remains to be determined.
Conclusions
While no major differences in PSG and qEEG profiles were observed during DORA-induced sleep in this study, eszopiclone had marked effects on PSG and qEEG profiles, suggesting that GABA_A receptor modulators may have effects that do not resemble natural sleep or qEEG patterns. The observed effects of eszopiclone on REM sleep, but not active wake and NREM sleep in Ox/Atx mice or in the presence of co-administered DORA-22, support the idea that eszopiclone and DORA-22 impact REM sleep via divergent pathways but may share a common (orexin-dependent) mechanism for their influence on active wake and NREM sleep. The potential for ORAs to promote sleep in a way that is qualitatively similar to natural sleep suggests that orexin receptor antagonists may have promise as a novel therapeutic for insomnia.

Methods
Animals and compound administration
All animal experiments were performed in accordance with The National Research Council’s Guide for the Care and Use of Laboratory Animals (http://www.nap.edu/catalog.php?record_id=12910) and were approved by the Merck Institutional Animal Care and Use Committee. All efforts were made to minimize animal use and suffering. The animals were singly housed with food and water available ad libitum, and on a 12:12 light:dark cycle. All compounds were administered orally at the indicated dosages in vitamin E TPGS, 20% solution.

In adult male C57/BL6NTac wild-type mice (ages 9 to 14 weeks; Taconic Farms, Germantown, NY), DORA-22 (100 mg/kg), eszopiclone (60 mg/kg), or vehicle were administered during the active (dark) phase 4 h prior to lights-on (ZT 20:00, where ZT 00:00 is lights-on). Treatments were administered for 5 days in a balanced crossover design (5 days of compound or vehicle, followed by a 2-day washout and 5 days of conditional crossover), and the compound and vehicle conditions for each animal were combined and averaged over a 24-h time period before determining the effects relative to vehicle.

Adult male Sprague–Dawley wild-type rats (c.a. 600 g: 6–12 months of age; Taconic Farms, Germantown NY) were treated with DORA-22 (30 mg/kg), DORA-12 (30 mg/kg), eszopiclone (10 mg/kg), or vehicle during the active (dark) phase (7 h prior to lights-on, ZT 17:00) or inactive (light) phase (1 h prior to lights-off, ZT 23:00) in a balanced crossover design (1–2 days of vehicle run-in [all], 3 days on vehicle or compound treatment, 2–3 days of washout, 3 days on reverse arm). The orexin/ataxin-3 (Ox/Atx) rats, which exhibit postnatal loss of orexin neurons, have been described elsewhere [48] and were licensed from the University of Texas Southwestern Medical Center and maintained at Taconic Farms, Germantown NY.

Adult male beagles (9–17 kg; Marshall BioResources, North Rose, NY) were treated with DORA-22 (3 mg/kg) or eszopiclone (5 mg/kg) during the active phase 9 h prior to lights-off (ZT 03:00). Cognitive testing was performed 2.5 to 3 h following DORA-22 treatment, but found no differences from vehicle in PSG or qEEG recordings (not shown). For PSG analysis, a block repeated-measured design was employed, in which all dogs received vehicle for 5 days, followed by a 2-day washout and 5 consecutive days of DORA-22 treatment.

Adult male rhesus monkeys (Macaca mulatta, 6.9–13 kg; The Mannheimer Foundation, Homestead, FL, Covance Research Products, Denver, PA, and the University of Louisiana at Lafayette, Lafayette, LA) were treated with DORA-22 (30 mg/kg), eszopiclone (10 mg/kg), or vehicle during their active phase (6.5 h prior to lights-off, ZT 05:30). A 1-day block crossover design was used, in which all subjects received 1 day of vehicle and 1 day of compound treatment.

Sleep architecture and qEEG recordings
The durations of sleep stages were quantitated by PSG in mice and rats subcutaneously implanted with radio telemetry physiologic monitors (Data Sciences International, Arden Hills, MN) to simultaneously record continuous electrocorticogram (ECoG) and electromyogram (EMG) activities, as previously described [11,49]. Polysomnography was performed in telemetry-implanted dogs and rhesus monkeys via ECoG, EMG, and electrooculogram (EOG), as described previously [11,49]. Sleep scoring and methods for determining differences in the amount of time spent in active wake and various sleep stages (light, delta, and REM sleep) in mice and rats; NREM I/SWS I, NREM II/SWS II, and REM in dogs and rhesus monkeys) has been described in detail elsewhere [11,13,50].

Quantitative EEG scoring was performed on ECoG data collected during sleep experiments from telemetry-implanted C57/BL6 mice, Sprague–Dawley rats, beagles, and rhesus monkeys with modifications to that previously described [13]. Briefly, spectral analysis of continuous EEG was quantified for vehicle and compound conditions after scoring continuous frequencies into canonical frequency bands (delta, 0.5–4 Hz; theta, 4.0–7.0 Hz; alpha, 8.0–12 Hz; sigma, 12–16 Hz; beta, 19–30 Hz; and gamma, 35.0–100.0 Hz). Quantitative EEG values are spectral power (uV2) log transformed before analysis and averaged over 30-min intervals. Results are expressed as means ± standard error of the mean (SEM). Comparisons with vehicle were performed using a mixed-model analysis of variance (ANOVA) at each time point with random effects for subject and date within subject in the R statistical computing environment (cran.us.r-project.org; the R Foundation for Statistical Computing, Vienna, Austria). A linear mixed-effects model was used for significance
testing. Significant differences between conditions at 30-min intervals are indicated in the corresponding figures with gray vertical lines through significantly different data points and tic marks indicating significance level (short, medium, long, $P < 0.05$, $0.01, 0.001$).

**REM deprivation and recovery in mice**

REM deprivation in mice was performed using modifications to the “flower pot” method previously described [29]. Cages were prepared that contained four cylindrical pedestals (3 cm in diameter $\times 2.5$ cm tall) fixed to the bottom of the enclosure, roughly equidistant from one another, allowing mice to move from one location to another within the cage to access food and water. Under deprivation conditions, water was added to the bottom of the cage to a height of approximately 2 cm such that REM sleep – induced atonia was associated with water exposure and immediate arousal. Control conditions included cages containing pedestals without water. In all experiments, the deprivation (or no water control) condition proceeded for 28 h beginning at ZT 20:00 (late active phase) to ZT 24:00/00:00 (inactive-phase onset) on the subsequent day at which time the animals were returned to cages containing normal Bed o’ Cobb bedding to allow recovery sleep. All experiments were separated by at least 3 days of normal bedding conditions.

REM deprivation and recovery in the absence and presence of pharmacological treatment was performed in telemetry-implanted mice in which vigilance state was evaluated continuously (PSG described above). Experiments utilized a $2 \times 3$ day balanced crossover design in which each animal experienced both the control and experimental conditions on alternative arms of the experiment. In baseline studies measuring recovery after REM deprivation, the first arm of these experiments was initiated with 28 h of deprivation or control conditions (pedestal cages with or without water, respectively), followed by 44 h of recovery ($28 + 44 = 72$ h, or 3 days), and continued with a second 28-h deprivation and 44-h recovery period. After an additional day of washout, the second arm of the study proceeded identically to the first except that the experimental and control groups were reversed such that each animal received both treatments. In experiments evaluating the impact of DORA-22 and eszopiclone on REM recovery, all animals experienced deprivation for 28-h periods followed by REM recovery, at which time vehicle and drug (DORA-22 or eszopiclone) were administered in the $2 \times 3$ day balanced crossover design described above. REM recovery was evaluated in 10-min intervals following return to cages containing normal bedding.

**Statistical analyses**

Statistical comparison of control and experimental conditions at individual time points in PSG and qEEG experiments (Figures 1, 3, 4, 5 and 6) was determined using a linear mixed-effects statistical model with repeated measures analysis of variance (ANOVA) applied using the R statistical software application (cran.us.r-project.org; the R Foundation for Statistical Computing, Vienna, Austria, v3.0.1; nlme package v3.1-111) with fixed effects for treatment and random effects for subject at each time point to identify points of statistical significance. Biological/pharmacological significance was subsequently determined by consecutive significance points ($P < 0.05$) with similar sequential trend. Gray vertical lines indicate treatment differences of $p < 0.05$ with vertical black segment lines indicating level of significance (short: $P < 0.05$; medium: $P < 0.01$; long: $P < 0.001$). Significant differences in responses between multiple conditions over a defined time period (150 min for the experiments in Figure 2) were determined in the R statistical software application by two different methods and are reported separately. The first evaluated data combined from all four conditions by repeated measures ANOVA followed by Tukey multiple comparison (HSD) test to determine significant differences between each condition. A second method compared individual conditions in a pairwise manner by repeated measures ANOVA to determine the F statistic and P value for each of six comparisons between four conditions.

**Additional file**

**Additional file 1: Figure S1.** Showing detailed sleep architecture comparisons between active- and inactive-phase sleep following vehicle treatment, as well as active- and inactive-phase sleep architecture following treatment with DORA-12 (30 mg/kg) and eszopiclone (10 mg/kg), is supplied as supporting data to that summarized in Figure 2.

**Abbreviations**

DORA: Dual orexin receptor antagonist; EEG: Electroencephalography; qEEG: Quantitative electroencephalography; EMG: Electromyogram; EOG: Electrooculogram; GABA: Gamma-aminobutyric acid A receptor; NREM: Non-rapid eye movement; OX1R: Orexin 1 receptor; OX2R: Orexin 2 receptor; ORA: Orexin receptor antagonist; Ox/Alx: Transgene containing cytotoxic poly-Q-ataxin-3 gene product driven by the human hypocretin (HCRT) gene promoter; p.o.: Per os, or oral administration; PSG: Polysomnography; REM: Rapid eye movement; TPGS: d-alpha-tocopheryl polyethylene glycol 1000 succinate; VLPO: Ventrolateral preoptic nucleus; ZT: Zeitgeber time.

**Competing interests**

All authors are current or former employees of Merck Sharp & Dohme Corp, a subsidiary of Merck & Co., Inc., Whitehouse Station, NJ, and receive research and salary support and potentially own stock in the company. Continued support and employment is therefore not dependent on the validation of hypotheses put forward in specific aims developed prior to the initiation of studies.

**Authors’ contributions**

ALG, SLG, CJW, and JJR wrote and edited major portions of the manuscript with significant interpretive and editorial comments by all authors. SLG, JS, RLM, and SVF performed rodent polysomnography and quantitative electroencephalography studies with study design, additional data analysis, and interpretation contributed by ALG, PLT, TM, LY, and JJR. Dog and
monkey polysomnography studies were performed by JS, SLG, and SJT with study design, additional data analysis, and interpretation contributed by ALG, PLT, JMU, and JIR. Compound development, preparation, and analysis provided by SDR and PJC. REM deprivation studies were performed by RLM with analysis and interpretation by ALG, JS, and CIW. All authors read and approved the final manuscript.

Acknowledgements
This study was funded by Merck & Co., Inc., Whitehouse Station, NJ. The authors would like to thank Jason Skudlarek, Merck & Co., Inc., for supplying small molecule compounds used in these studies. Dosing and animal manipulation support was provided by Alan T. Savitz (Merck, In Vivo Pharmacology) and Merck’s Laboratory Animal Resources Department. Editorial assistance was provided by Erin Bekes, PhD, of Complete Medical Communications, Inc., Hackensack, NJ. This assistance was funded by Merck & Co., Inc., Whitehouse Station, NJ.

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Received: 11 July 2014 Accepted: 17 September 2014 Published: 22 September 2014

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doi:10.1186/1471-2202-15-109
Cite this article as: Gotter et al.: Differential sleep-promoting effects of dual orexin receptor antagonists and GABAA receptor modulators. BMC Neuroscience 2014 15:109.