Distinct effects of IPSU and suvorexant on mouse sleep architecture

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Dual orexin receptor (OXR) antagonists (DORAs) such as almorexant, SB-649868, suvorexant (MK-4305), and filorexant (MK-6096), have shown promise for the treatment of insomnias and sleep disorders. Whether antagonism of both OX1R and OX2R is necessary for sleep induction has been a matter of some debate. Experiments using knockout mice suggest that it may be sufficient to antagonize only OX2R. The recent identification of an orally bioavailable, brain penetrant OX2R preferring antagonist 2-((1H-indol-3-yl)methyl)-9-(4-methoxypyrimidin-2-yl)-2,9-diazaspiro[5.5]undecan-1-one (IPSU) has allowed us to test whether selective antagonism of OX2R may also be a viable strategy for induction of sleep. We previously demonstrated that IPSU and suvorexant increase sleep when dosed during the mouse active phase (lights off); IPSU inducing sleep primarily by increasing NREM sleep, suvorexant primarily by increasing REM sleep. Here, our goal was to determine whether suvorexant and IPSU affect sleep architecture independently of overall sleep induction. We therefore tested suvorexant (25 mg/kg) and IPSU (50 mg/kg) in mice during the inactive phase (lights on) when sleep is naturally more prevalent and when orexin levels are normally low. Whereas IPSU was devoid of effects on the time spent in NREM or REM, suvorexant substantially disturbed the sleep architecture by selectively increasing REM during the first 4 h after dosing. At the doses tested, suvorexant significantly decreased wake only during the first hour and IPSU did not affect wake time. These data suggest that OX2R preferring antagonists may have a reduced tendency for perturbing NREM/REM architecture in comparison with DORAs. Whether this effect will prove to be a general feature of OX2R antagonists vs. DORAs remains to be seen.

Keywords: orexin receptor antagonist, insomnia, pharmacology, REM and NREM sleep

INTRODUCTION

Since the link between the hypocretin/orexin system and sleep disorders was discovered (Chemelli et al., 1999; Lin et al., 1999; Nishino et al., 2000), there has been much interest in developing orexin receptor antagonists (ORAs) for the treatment of insomnia. Several dual orexin receptor antagonists (DORAs) have now been tested in the clinic and have demonstrated sleep inducing properties in healthy volunteers and/or in patients suffering from insomnia (Brisbare-Roch et al., 2007; Winrow et al., 2011; Bettica et al., 2012a). Whereas ORAs are expected to be without the side effects characteristic of currently available treatments, questions about mechanism related safety have accompanied their development. As the lack of orexin signaling causes narcolepsy with cataplexy, there is concern that DORAs may induce sudden loss of motor control or sleep attacks. Whereas the therapeutic potential for treating insomnia with ORAs is undisputedly high, there is less consensus regarding the necessity of targeting both receptors and whether selective antagonists might reduce potential safety concerns without loss of efficacy (Mieda et al., 2013).

Several lines of evidence suggest that selective OX2R antagonists may be sufficient for sleep induction and may have a reduced tendency for induction of cataplexy and/or narcolepsy in comparison with DORAs. In knockout mice, the sleep inducing properties of the DORA almorexant require intact OX2Rs but not OX1Rs (Mang et al., 2012). Also, selective OX2R antagonists induce sleep in rats and mice, whereas OX1R selective antagonists do not ([Dugovic et al., 2009; Steiner et al., 2013]; but see Morairity et al. (2012)]. Together these findings strongly suggest that antagonizing OX2R may be sufficient for sleep induction.
In humans, orexin deficiency leads to narcolepsy with cataplexy (Nishino et al., 2000). The narcolepsy/cataplexy phenotype is mimicked in mice lacking orexin peptides, lacking orexinergic neurons or mice lacking both orexin receptors (OXRs) (Chemelli et al., 1999; Hara et al., 2001; Kalogiannis et al., 2011). Although mice lacking OX1R also have sleep attacks, the incidence of cataplexy in these mice is close to null (Willie et al., 2003). Thus, there may be a reduced risk of inducing cataplectic events when only OX2R are antagonized. In dogs, mutations in OX2R alone are sufficient to cause narcolepsy with cataplexy. Interestingly, sporadic narcolepsy with cataplexy in dogs is associated with orexin deficiency resulting in more severe symptoms than the OX2R radic narcolepsy with cataplexy in dogs is associated with orexin signaling has a reduced propensity for inducing narcolepsy and/or cataplexy as compared to loss of the entire orexin signaling pathway.

We therefore have been interested in determining whether antagonists of OX2R are comparable to DORAs in their ability to induce sleep. Recently, we identified the orally bioavailable, brain penetrating OX2R preferring antagonist IPSU that induces sleep when administered at the start of the active (dark) phase in mice (Betschart et al., 2013). The aim of the present study was to investigate whether IPSU and the DORA suvorexant affect the natural sleep architecture, by testing low doses of the two compounds during the light phase when mice are primarily inactive and spend a high proportion of the time sleeping.

METHODS

All experiments were performed according to Swiss guidelines and law and were approved by the Veterinary Authority of Basel-Stadt, Switzerland. Every effort was made to minimize the number of animals used and to minimize any pain or discomfort. Male C57Bl/6 mice weighing 25–35 g were single or group-housed on wood shavings in type II (14 × 16 × 22 cm) and type III (15 × 22 × 37 cm) cages, respectively. Each cage contained a nest box, a piece of wood and tissue paper nesting materials, and animals had access to food and water ad libitum. The housing cages were placed in a temperature and humidity controlled room (20–24°C, 45% humidity) with a light/dark cycle of 12:12 (lights on at 03:00, max 80 Lux).

Suvorexant and IPSU were both synthesized and purified in house according to published procedures (Cox et al., 2008; Betschart et al., 2013). We selected doses that were effective at promoting sleep in mice for the first 4 h when administered at the start of the dark phase [Betschart et al. (2013) and unpublished observations]. At the mouse OXRs, IPSU has about 6.2× higher affinity at OX2R than OX1R (pKd OX1R 6.34, OX2R 7.23) whereas suvorexant is about 6.5× more potent at OX1R than OX2R [pKd OX1R 8.77, OX2R 8.06; FLIPR assay Callander et al. (2013)]. Both compounds are highly brain penetrant. One hour after oral dosing of 50 mg/kg, brain levels reached 8778 pmol/g for IPSU and 10329 pmol/g for suvorexant giving free levels of 53.6 and 67.0 pmol/g, respectively (Betschart et al., 2013). In the present study we decided to dose IPSU at the previously effective dose of 50 mg/kg and to reduce suvorexant to 25 mg/kg to better match the estimated OX2R occupancy. At 1 h following 25 mg/kg suvorexant brain levels reached 3605 pmol/g and free levels were therefore 23.4 pmol/g. Estimating the available antagonist concentrations to be 53.6 and 23.4 nM for IPSU and suvorexant, we estimated receptor occupancies according to:

\[ \text{Bound} = \frac{\text{Bmax}}{(1 + \text{Kd}/L)} \]

where Bmax is 100%, Kd the affinity from the FLIPR assay, and L the free brain concentrations at the doses tested.

The expected occupancies of suvorexant are therefore 93% at OX1R, 73% at OX2R and for IPSU 11% at OX1R and 48% at OX2R. These values are similar to the effective values for sleep induction at OX2R reported by Gotter et al. (2013).

IMPLANTATION OF ELECTROCORTICOGRAM/ ELECTROENCEPHALOGRAM (EEG) AND ELECTROMYOGRAM (EMG) ELECTRODES

Mice were administered buprenorphine (0.05 mg/kg s.c.) 1 h before surgery, anesthetized with ketamine/xylazine (110 mg/kg, 10:1, i.p.) and placed in a stereotaxic frame. The skull was exposed and four miniature stainless steel screws (SS-5/TA Science Products GmbH, Hofheim, Germany) attached to 36-gauge, Teflon-coated solid silver wires were placed in contact with the frontal and parietal cortex (3 mm posterior to bregma, ±2 mm from the sagittal suture) through bore holes. The frontal electrodes served as reference. The wires were crimped to a small 6-channel connector (CRISTEK Micro Strip Connector) that was affixed to the skull with dental acrylic. Electromyograph (EMG) signals were acquired by a pair of multistranded stainless steel wires (7SS-1T, Science Products GmbH, Hofheim, Germany) inserted into the neck muscles and also crimped to the headmount. After surgery, mice were kept singly in cages and allowed to recover on a heating pad. Buprenorphine, 0.05 mg/kg, s.c. was given 8 and 16 h after surgery to control pain. After 24 h, the mice were housed with their former cage mates and allowed to recover for 2 weeks.

SLEEP STUDIES

Mice were habituated to individual cages in a sound-attenuated recording chamber for 6–10 days (lights on 03:00, lights off 15:00, max 80 lux) at a temperature of about 23°C. During the studies, mice had access to food and water ad libitum, to one sheet of nesting paper and a piece of wood but no nesting box. Mice were weighed and attached to recording cables that connected their headmounts to a commutator (G-4-E, Gaueschi) allowing free movement in the experiment boxes, 1 day before beginning
the rodent scoring module of Somnologica into wake, NREM recordings were imported into and scored in 10 s epochs using vals by the software Labmaster V2.4.4. EEG/EMG and activity signals were acquired in 10 s intervals by the roof of the boxes. Activity signals were acquired in 10 s intervals during data collection, using an infrared video camera and locomotor activity was detected using infrared sensors (InfraMot Infrared Activity Sensor 30-2015 SENS, TSE Systems) placed in the roof of the boxes. Activity signals were acquired in 10 s intervals by the software Labmaster V2.4.4. EEG/EMG and activity recordings were imported into and scored in 10 s epochs using the rodent scoring module of Somnologica into wake, NREM sleep and REM sleep. Epochs during which there were state transitions were scored as the state present for at least 50% of the epoch. The time in each state per hour was calculated and mean ± s.e.m. is shown. Restricted maximum likelihood analysis (REML) was applied to the data from the first 6 h to determine if there was a statistically significant effect of treatment or a significant interaction between treatment and hour. When either treatment or the interaction was significant (p < 0.05), post-hoc Fisher’s Least Significant Difference (LSD) tests were applied to determine hour by hour where there were significant differences between the vehicle and compound days.

The first 2 h following drug treatment were, in addition, manually scored to assess sleep-wake transitions including very short awakenings i.e., those with durations of 1–5 s that are often seen in mice and which would not be detected using 10 s epochs.

RESULTS

The ORA IPSU (50 mg/kg) had no effect on the sleep architecture of mice when administered during the middle of the light phase (Figures 1A,C). The amount of time spent in wake, NREM and REM were unchanged following administration of IPSU relative to the previous day when vehicle was administered [treatment: wake F(1, 120) = 0.002, p = 0.96, NREM F(1, 120) = 0.002, p = 0.97, REM F(1, 120) = 0.001, p = 0.97, treatment × hour: wake F(5, 120) = 0.59, p = 0.71, NREM F(5, 120) = 0.56, p = 0.73, REM F(5, 120) = 0.44, p = 0.82, REML].

![Figure 1](image_url)

**FIGURE 1** | Sleep architecture during the inactive period is perturbed by a DORA but not by an OX2R antagonist in C57Bl/6 mice. (A) EEG/EMG/motility signals were used to score vigilance states into wake, NREM and REM beginning from time 0, 6 h into the light period. Vehicle (0.5% methylcellulose in water) or 50 mg/kg IPSU were applied per os 5–15 min prior to start of the recordings on successive days (n = 11). The mean ± s.e.m. minutes per hour spent in each stage are shown. Shading indicates the dark period. (B) Vehicle (0.5% methylcellulose in water) or 25 mg/kg suvorexant were applied per os 5–15 min prior to start of the recordings on successive days (n = 11). *p < 0.05, **p < 0.01, ***p < 0.001 Fisher’s LSD. (C) Quantification of the effect of IPSU and suvorexant on wake, NREM and REM during the first 4 h post-treatment. *p < 0.05, ***p < 0.001 paired t-test drug vs. vehicle.
In contrast, the DORA suvorexant (25 mg/kg) slightly decreased time spent in wake, had weak effects on time in NREMs but strongly increased the amount of time spent in REM sleep during the first 4 h immediately following application. Figures 1B,C, treatment: wake $F(1, 120) = 3.4, p = 0.066$, NREM $F(1, 120) = 0.44, p = 0.51$, REM $F(1, 120) = 28.9, p < 0.001$, treatment $\times$ hour: wake $F(5, 120) = 2.31, p = 0.048$, NREM $F(5, 120) = 1.76, p = 0.13$, REM $F(5, 120) = 3.37, p = 0.007$, REML).

Manual resoring of the first 2 h after vehicle or drug application confirmed that the automatic scoring was in excellent agreement when amount of time in each stage was compared, better than 90% as previously reported by us and others (Pick et al., 2011; Mang et al., 2012). The number of awakenings was, however, different. Very short awakenings were found within epochs scored as NREM (Léna et al., 2004). We therefore used the results from the manual scoring to quantify the latency to NREM or REM and the sleep-wake transitions (Table 1).

Both IPSU and suvorexant showed a tendency to shorten the latency to NREM and REM sleep but only the shortening of latency to REM by suvorexant was significantly different from the vehicle. Awakenings from NREM were unaffected by either compound. Whereas awakenings from REM were significantly increased by suvorexant, the OX2R antagonist IPSU had no effect.

**DISCUSSION**

Whereas IPSU did not perturb the normal sleep pattern of mice during the inactive period, suvorexant profoundly altered the sleep pattern, doubling the time spent in REM. The latency to REM and number of awakenings from REM was also selectively increased by suvorexant but not by IPSU. This pattern is similar to that found when these compounds were dosed at the start of the dark (active) period in mice (Betschart et al., 2013). Suvorexant promoted sleep primarily by increasing REM with small effects on NREM and IPSU promoted sleep primarily by promoting NREM and to a lesser degree REM (Betschart et al., 2013). The stronger enhancement of REM vs. NREM by suvorexant has also been seen in rats and in both healthy humans and humans suffering from insomnia (Winrow et al., 2011; Herring et al., 2012; Sun et al., 2013). Interestingly, our findings indicate that this DORA influences REM independently of the circadian dosing time, increasing the time in REM during both the light (active) phase and dark (active) phase whereas, at the dose tested, the OX2R preferring antagonist IPSU influences sleep only during the active phase in mice.

Influencing the balance between REM and NREM is an area of potential differentiation between OX2R preferring antagonists and DORAs. Classic benzodiazepines, “Z drugs” such as zolpidem, and antidepressants are well known for suppressing REM sleep whereas ORAs certainly lack this property. Although IPSU is not highly selective for OX2R vs. OX1R at mouse receptors ($\sim 6.2 \times$), the opposite is true for suvorexant, which prefers OX1R vs. OX2R ($\sim 6.5 \times$, Betschart et al., 2013; Callander et al., 2013). Thus, the balance between antagonism of OX1R and OX2R may contribute to the differential effects of ORAs on sleep architecture. Our findings suggest that reducing the level of OX1R antagonism shifts the sleep balance toward NREM. Supporting this hypothesis, almorexant induces a greater REM increase in OX1R−/− than in wildtype mice (Mang et al., 2012). Likewise, whereas both almorexant and the OX2R antagonist JNJ-10397049 increased REM during the light phase in rats, only almorexant also increased REM, and co-application of an OX1R antagonist significantly reduced the NREM induced by the OX2R antagonist (Dugovic et al., 2009). For the most part, DORAs increase REM more than NREM in rodent studies when % increase is considered (Brisbare-Roch et al., 2007; Winrow et al., 2011; Betschart et al., 2013; Black et al., 2013). However, the contribution of REM as a proportion of total sleep time varies for different compounds. For example, in mice almorexant-induced increases in REM remain within the proportion seen during normal sleep, even at high doses (Mang et al., 2012), whereas suvorexant increases REM proportion much above that seen during normal sleep (Betschart et al., 2013). Almorexant is unusual among the DORAs in that it appears to become a somewhat OX2R preferring antagonist in vivo. The ex vivo occupancy of almorexant was found to be about 2x higher and much longer lasting at OX2Rs (>12 h) vs. OX1Rs (Moriairy et al., 2012). This preference is most likely driven by the unusual kinetics (Malherbe et al., 2009; Mang et al., 2012; Callander et al., 2013) so that with short exposures the compound may act as a DORA, and when equilibrium is allowed to be reached almorexant has functional selectivity for OX1Rs. Evidence against our hypothesis includes the description of a newer DORA that increases NREM preferentially in rats (Sifferlen et al., 2013). The structure of this compound is quite similar to that of almorexant but whether the kinetics also bias it toward OX2R selectivity when equilibrated.

**Table 1 | Effect of the OX2R preferring antagonist IPSU and the DORA, suvorexant on latency to sleep and sleep to wake transitions during the first 2 h after administration during the inactive period.**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>IPSU</th>
<th>Diff</th>
<th>Vehicle</th>
<th>Suvorexant</th>
<th>Diff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency to NREM (minutes)</td>
<td>9.5 ± 3.0</td>
<td>2.6 ± 1.0</td>
<td>-7.0 ± 3.3</td>
<td>13.1 ± 5.9</td>
<td>4.8 ± 1.9</td>
<td>-8.3 ± 6.3</td>
</tr>
<tr>
<td>Latency to REM (minutes)</td>
<td>22.1 ± 3.4</td>
<td>14.5 ± 2.3</td>
<td>-7.6 ± 4.5</td>
<td>37.0 ± 10.1</td>
<td>8.4 ± 2.3*</td>
<td>-28.6 ± 8.8</td>
</tr>
<tr>
<td>Awakenings from NREM</td>
<td>25.1 ± 2.6</td>
<td>25.3 ± 2.0</td>
<td>0.2 ± 2.4</td>
<td>19.4 ± 1.6</td>
<td>18.6 ± 1.9</td>
<td>-0.8 ± 2.4</td>
</tr>
<tr>
<td>Awakenings from REM</td>
<td>10.2 ± 0.9</td>
<td>10.8 ± 1.1</td>
<td>0.6 ± 1.1</td>
<td>8.2 ± 1.4</td>
<td>15.6 ± 1.2**</td>
<td>7.4 ± 1.6</td>
</tr>
<tr>
<td>Short awakenings from NREM (&lt;5 s)</td>
<td>23.55 ± 2.8</td>
<td>26.5 ± 4.3</td>
<td>2.9 ± 1.9</td>
<td>25.7 ± 4.0</td>
<td>26.6 ± 3.1</td>
<td>0.9 ± 2.1</td>
</tr>
<tr>
<td>Total awakenings</td>
<td>58.8 ± 4.7</td>
<td>62.6 ± 5.1</td>
<td>3.7 ± 2.7</td>
<td>53.3 ± 4.9</td>
<td>60.8 ± 3.8***</td>
<td>7.5 ± 1.9</td>
</tr>
</tbody>
</table>

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, paired t-test.

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is unknown. Interestingly, species differences also exist in the effects of DORAs on sleep architecture. In dogs, suvorexant predominately increased NREM (Winrow et al., 2011). The effects of DORAs on REM in humans appear to more closely mimic the effects in rodents rather than dogs as in the clinical settings. REM was preferentially enhanced with SB-649868 (Bettica et al., 2012a,b,c) and suvorexant (Herring et al.; 2012; Sun et al., 2013).

With the limited data available, whether the differential effects of the DORAs and OX2R antagonists on sleep architecture are in fact due to differences in receptor affinity/occupancy or are due to other factors such as compound class and species, remains to be seen as more compounds from different chemical classes are developed.

Why might a DORA be expected to influence REM more strongly than an OX2R preferring antagonist? Intracerebroventricular or local application of orexin-A in the highly OX1R expressing locus coeruleus reduces REM sleep, an effect that is blocked by the OX1R antagonist SB-334867 (Smith et al., 2003; Mieda et al., 2011). Additionally, knock-down of OX1R receptors in the locus coeruleus selectively increases REM, without affecting NREM during the active phase (Chen et al., 2010). Interestingly, we did not see a similar dependence of circadian time on the REM enhancement by suvorexant. REM sleep is not however, exclusively modulated by OX1Rs. OX2R knockdown in the lateral pontomesencephalic tegmentum increased REM both during the active and inactive phases (Chen et al., 2013) while OX1R antagonists alone generally do not induce REM (Steiner et al., 2013), they may increase REM on top of the effects of OX2R antagonists (Dugovic et al., 2009). Overall, both OX1R and OX2R when activated or down regulated in the appropriate regions appear to be able to modulate REM sleep. However, the modulatory role of OX1R on REM may be greater than that of OX2R.

In conclusion, we hypothesize that selective OX2R antagonists have potential for the treatment of insomnia and may prove to perturb sleep architecture to a lesser degree than some of the DORAs. More highly selective antagonists from different chemical classes will be required to test this hypothesis further.

AUTHOR CONTRIBUTIONS
All authors read and commented on the manuscript. Daniel Hoyer and Claudia Betschart led the team and participated in writing the manuscript. Thomas Dürst planned and performed experiments and analyzed data. Claudia Betschart, Samuel Hintermann, Dirk Behnk, Silvio Ofner, and Simona Cotesta synthesized and/or designed compounds. Grit Laue performed in vivo analyses related to pharmacokinetics. Markus Fendt, Laura H. Jacobson, and Eric Legangneux contributed to experimental design and writing the manuscript. Christine E. Ge designed the study, performed analysis, and wrote the manuscript.

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Conflict of Interest Statement: All authors were or are employees of Novartis AG and may own stock in the company.

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