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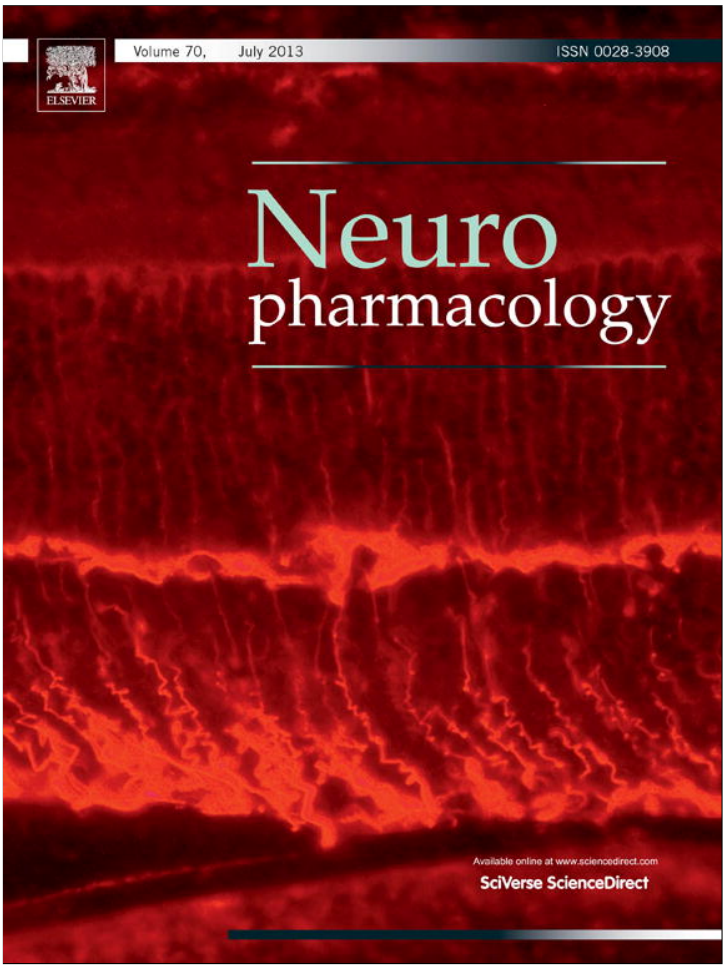

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Further evidence for the sleep-promoting effects of 5-HT_{2A} receptor antagonists and demonstration of synergistic effects with the hypnotic, zolpidem in rats

Guy Griebel [a,*], Sandra Beeské [a], Agnès Jacquet [a], Christian Laufrais [a], Richard Alonso [b], Michel Decobert [a], Patrick Avenet [a], Dominique Françon [a]

[a] Sanofi, Exploratory Unit, 1 Avenue Pierre Brossolette, 91385 Chilly-Mazarin, France
[b] Sanofi, Therapeutic Strategic Unit Aging, Montpellier, France

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ABSTRACT

5-Hydroxytryptamine (5-$HT)_{2A}$ antagonists are promising therapeutic agents for the treatment of sleep maintenance insomnias, but unlike hypnotics, they have limited effects on sleep initiation. This study evaluated the effects of several 5-HT_{2A} antagonists (eplivanserin, volinanserin and AVE8488) alone and/or in combination with the short-acting hypnotic, zolpidem, on the rat sleep profile. A repeated-measures design was used in which rats were treated with eplivanserin (3 and 10 mg/kg, i.p. or p.o.), volinanserin (0.3–3 mg/kg, i.p.), AVE8488 (0.1–3 mg/kg, i.p.) and zolpidem (3 and 10 mg/kg, p.o.). In addition, animals received a combination of eplivanserin (3 mg/kg, p.o.) and zolpidem (3 mg/kg, p.o.). Electroencephalogram was analyzed for 6 h after administration. Eplivanserin did not modify wakefulness and non-rapid eye movement sleep (NREMS), while zolpidem (10 mg/kg po) induced a marked increase in NREMS duration. Volinanserin (1 and 3 mg/kg) and AVE8488 (0.3 mg/kg) similarly increased NREMS, while reducing wakefulness. Moreover, the 5-HT_{2A} antagonists and, to a lesser extent, zolpidem, increased duration of NREMS episodes, while decreasing their frequency. When eplivanserin was co-administered with zolpidem, a synergistic effect was observed as the combination produced an increase in NREMS time and bouts duration. These findings confirm further that 5-HT_{2A} antagonists promote the maintenance of sleep, and suggest that combining a 5-HT_{2A} antagonist with a short-acting hypnotic may be a useful strategy for the treatment of insomnia.

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1. Introduction

Benzodiazepine (BZD) and non-BZD hypnotics are effective in treating sleep induction difficulties, but they do not induce a physiological sleep. During their administration, slow wave sleep (SWS) and rapid eye movement sleep (REMS) do not regain normal levels or can be even further suppressed (Monti, 2010). During the search for compounds that can increase SWS, attention was directed to a particular group of drugs, namely, the serotonin$_{2A}$ (5-HT_{2A}) receptor antagonists/inverse agonists. 5-HT has long been implicated in the regulation of sleep as demonstrated, for example, by electrophysiological studies showing that the firing of 5-HT neurons in the dorsal raphé nucleus (DRN) varies across the sleep-wake cycle, being highest during the waking state, slowing during non-rapid eye movement sleep (NREMS) and stopping during REMS (Trulson and Jacobs, 1979). This firing pattern is mimicked by changes in the extracellular 5-HT concentration across sleep-wake alternations (Portas and McCarley, 1994). Consistent with these findings, pharmacological stimulation of the 5-HT system consistently increases wakefulness and reduces NREMS and REMS (Monti et al., 2008).

The effects of 5-HT in the central nervous system are mediated by multiple receptor subtypes that have been classified into seven subfamilies (5-HT_1–5-HT_7) (Hoyer et al., 1994). Evidence from both clinical and preclinical studies suggests that, among all 5-HT receptor subtypes studied to date, primarily the 5-HT_{2A} and 5-HT_{1A} receptors are involved in quantitative and qualitative aspects of wakefulness, NREMS and REMS (Aloyo et al., 2009; Landolt and Wehrle, 2009; Monti, 2010; Teegarden et al., 2008). 5-HT_{2A} receptors are distributed in the brain regions of the rat involved in the

Abbreviations: BZD, benzodiazepine; DRN, dorsal raphé nucleus; EEG, electroencephalography; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; SWS, slow wave sleep.

* Corresponding author. Tel.: +33 6 73 68 97 96; fax: +33 1 53 77 41 33.
E-mail address: guy.griebel@sanofi.com (G. Griebel).

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physiological regulation of the sleep-wake cycle, including the basal forebrain, the raphé nuclei, the preoptic area, the anterior and lateral hypothalamic areas, the tegmental nuclei and the reticular formation (Cornea-Hebert et al., 1999). In human, high levels of 5-HT_{2A} receptors have been found in cortical regions such as the frontal, temporal and parietal cortices (Leysen, 2004). There is compelling evidence that nonselective 5-HT_{2A} receptor agonists reduce NREMS and REMS, and augment wakefulness in the rat, while 5-HT_{2A} receptor antagonists produce an increase of SWS (Monti, 2010). A study in transgenic mice, in which the 5-HT_{2A} receptor has been eliminated, provides additional support for a role of this subtype in sleep regulation and in the effects of 5-HT_{2A} receptor antagonists (Popa et al., 2005).

These findings in animals are now substantiated by clinical data indicating that 5-HT_{2A} receptor antagonists consistently increase SWS in healthy adult volunteers (Al-Shamma et al., 2010; Ancoli-Israel et al., 2011; Monti, 2010), poor sleepers (Adam and Oswald, 1989), patients suffering from primary insomnia (Rosenberg et al., 2008; Ruiz-Primo et al., 1989), generalized anxiety (da Roza Davis et al., 1992) and major depressive disorders (Staner et al., 1992), thereby showing promise as potential therapeutic agents for sleep maintenance insomnia. However, unlike BZD and non-BZD hypnotics, 5-HT_{2A} receptor antagonists have weak to no effects on sleep onset, thus potentially limiting their use in the treatment of transient and short-term insomnia. Therefore, combining a, preferentially, short-acting non-BZD hypnotic with a 5-HT_{2A} receptor antagonist may represent an interesting treatment option of insomnia as it would address both the architecture of sleep and sleep onset latency. To the best of our knowledge, until recently, no such study had been performed in either laboratory animals or in human.

In this context, the objectives of the current study are (1) to compare the sleep profile in rats of several earlier (i.e. eplivanserin and volinanserin) and novel (i.e. AVE8488) potent and 5-HT_{2A} receptor antagonists (Table 1) with that of the short-acting non-BZD $GABA_A$ hypnotic, zolpidem, and (2) to test the effects of a combination of low doses of eplivanserin and zolpidem to explore the potential synergistic effects on sleep-wakefulness patterns of such an association.

2. Material and methods

2.1. Animals and surgery

Male Sprague-Dawley rats (Iffa Credo, L'Arbresle; Charles River, Saint-Aubin-lès-Elbeuf, France), weighing between 300 and 350 g at the time of testing, were used. They were implanted under anesthesia with Zoletil®50, mounted in the stereotaxic apparatus and secured using blunt rodent ear bars. A scalp incision was made after local anesthesia with lidocaine 2% and the skin was retracted. The skull surface was cleaned to implant small stainless steel screw electrodes (0.9 mm in diameter). Three cortical electrodes were screwed into the bone over the sensorimotor, the visual cortice and over the cerebellum. They were attached to a connector (Winchester®, 5-led) and fixed with dental cement to the cranium. Animals were allowed to recover from surgery in their individual cage for three weeks prior recordings, housed individually in plexiglas cylinders with free access to food and water and kept for at least 2 weeks either under a 'normal' (lights on 7:00 AM–7:00 PM) or a 'reverse' (lights on 7:00 PM–7:00 AM) 12/12-h light/dark cycle, and a constant temperature of 21 °C. All experimental procedures described herein were approved by the Animal care and Use Committee of Sanofi or the Institutional Animal Care. Our animal facilities and animal care and use programs are in accordance with French legislation which implemented the European directive 86/609/EEC.

Table 1
Pharmacological properties of eplivanserin, volinanserin and AVE8488 at 5-HT_2 receptors.

	Eplivanserin[a]	Volinanserin[b]	AVE8488[c]
In vitro binding (Ki, nM)[d]			
5-HT_{2A}	0.38	1.5	1.28
5-HT_{2B}	>1000	–	>1000
5-HT_{2C}	4.8	30	62
In vitro functional assay of 5-HT_{2A} -antagonism			
Antagonism of 5-HT-induced calcium mobilization (IC_{50}, nM)[d]	29	0.7	1.9
In vivo functional assay of 5-HT_{2A} antagonism			
Antagonism of DMT- or 5-HTP-induced head-twitches in the mouse (ED_{50}, mg/kg p.o.)	0.85	2.8	1.21
Receptor occupancy			
Mouse brain membranes - (ED_{50}, mg/kg p.o.)	0.85	0.84	0.3

[a] From (Rinaldi-Carmona et al., 1992).
[b] From (Kehne et al., 1996).
[c] Personal communication
[d] Human cloned receptors.

2.2. Electroencephalogram recording procedure

The procedure used has been described previously (Depoortere et al., 1986; Griebel et al., 2001, 2012). Rats were habituated to the experimental room and recording apparatus for 4 days, and to the recording cable for one day prior to each EEG recording session. No saline or drug administration was performed during this period. Preliminary experiments have shown that this habituation period is sufficient to regain normal levels of sleep/wakefulness parameters. On the recording day, they were connected to the cable at ZT2 or at ZT14 (combination experiment). Recording sessions took place in the home cage between ZT3 and ZT9 or between ZT15 and ZT21 (combination experiment) and lasted 6 h. Sleep recording sessions were carried out after drug (D) or vehicle (V) administration over a 6 h period during 3 consecutive days: control day (D0), drug day (D1), control recovery day (D2) (data not presented). Drugs were administered orally via gavage (p.o.) (zolpidem and zolpidem + eplivanserin combination), intraperitoneally (i.p.) (AVE8488, volinanserin) or both p.o. and i.p. (eplivanserin) 15 min before recording, on D1 at 5 ml/kg. On D0 rats were administered vehicle (saline or distilled water with methylcellulose and Tween, for i.p. or p.o. respectively) order to attenuate potential stress effects of the drug administration procedure. The dose–response experiments with the 5-HT_{2A} receptor antagonists were performed under a normal light/dark cycle (lights on at 7:00 AM; NREMS quantity > wakefulness quantity), while the zolpidem/eplivanserin combination study was performed under a reverse light/dark cycle (lights on at 7:00 PM; wakefulness quantity > NREMS quantity). The use of the former cycle was more appropriate to test the effects of 5-HT_{2A} receptor antagonists as we expected the compounds to modify existing NREMS, rather then increasing its quantity.

2.3. Signal processing and sleep parameters

Implanted rats were connected to an EEG recording system (2 Grass, 12 tracks, 79D model, Deltamed, Paris) by a flexible cable with a rotating collector (APCL 12 channels, Air precision), which allowed rats to move freely. EEG signals were filtered at 1 and 100 Hz (6 dB/octave). They were then acquired and digitized at 256 Hz using the software Coherence 32 (Deltamed). Activities in the sensorimotor and visual cortices were recorded over the 6-hr recording period by comparison with the reference electrode placed over the cerebellar cortex. Three sleep/wakefulness states of vigilance were considered: (1) Wakefulness characterized by low voltage EEG signal and fast frequency (theta (θ) rhythm: within the 6–9 Hz range) on both cortical derivations; (2) NREMS characterized by high voltage with slow wave (delta (δ) rhythm: within the 1–4 Hz range) with bursts of sleep spindles (sigma (σ) rhythm: within the 10–15 Hz range) on the sensorimotor derivation; (3) REMS by hypersynchronisation of the θ rhythm (within the 4–9 Hz range) in the visual area. Analysis of the EEG signal was performed automatically by a computerized system able to discriminate between the various sleep phases as demonstrated previously (Depoortere et al., 2005; Griebel et al., 2012; Philbert et al., 2011; Stemmelin et al., 2008), before being accurately scored by expertly trained human scorers. The parameters examined were (1) total wakefulness time, (2) total NREMS time, (3) total REMS time, (4) mean duration of NREMS episodes, (5) mean number of NREMS episodes; (6) latency to REMS, (7) mean duration of REMS episodes, and (8) mean number of REMS episodes over the 6-hr recording sessions. Statistical analyses were performed for each parameter using paired (D0 vs. D1) Student's two-tailed *t*-test so that each rat was its own control.

2.4. Drugs

The drugs tested included AVE8488, volinanserin, eplivanserin and zolpidem (all synthesized by Sanofi medicinal chemistry). The drugs were prepared daily using saline (0.9% NaCl) for i.p. administrations, or distilled water containing methylcellulose (0.6%) with a drop of Tween 80 for p.o. administration. Volume of administration was 5 ml/kg. They were administered either at ZT3 or at ZT15 (combination experiment).

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3. Results

3.1. Dose-response effects of 5-HT_{2A} receptor antagonists

As will be detailed below, all three 5-HT_{2A} receptor antagonists used in this study produced relatively similar effects on sleep-wakefulness parameters in rats. The details of the statistical analyzes are summarized in Tables 2 and 4. Time-course analyzes of drug effects indicated no signification variation across hours.

3.1.1. AVE8488

AVE8488 induced sleep-wakefulness modifications shown in Fig. 1 and Table 3. While the drug did not modify latency to NREMS, it produced a significant decrease in wakefulness time, and increased NREMS time at 0.3 mg/kg. Furthermore, the compound produced a significant increase in the mean duration of NREMS episodes at all doses. This effect was accompanied by a significant decrease in the number of NREMS episodes. AVE8488 modified significantly REMS at 1 and 3 mg/kg. At 1 mg/kg, it increased the latency to REMS, decreased its duration and the mean number of episodes. The 3 mg/kg dose of AVE8488 similarly produced an increase in the latency to REMS and decreased the mean number of its episodes. In addition, the drug at this dose increased the mean duration of REMS episodes.

3.1.2. Volinanserin

The administration of volinanserin at 1 and 3 mg/kg was followed by a significant decrease in wakefulness time, while NREMS time was increased (Fig. 2). Like AVE8488, volinanserin, at all doses tested (i.e. 0.3, 1 and 3 mg/kg), did not modify latency to NREMS, but it increased significantly the mean duration of NREMS episodes, while decreasing their occurrence (Table 3). Finally, volinanserin at 1 and 3 mg/kg significantly decreased REMS duration, REMS latency to first occur and number of REMS episodes, and increased the mean duration of REMS bouts (Table 3).

3.1.3. Eplivanserin

No significant modification in any sleep-wakefulness stage duration was observed following 3 and 10 mg/kg i.p. eplivanserin (Fig. 3). Like AVE8488 and volinanserin, the drug did not affect latency to NREMS, but it significantly increased the mean duration of NREMS episodes, and concurrently decreased its frequency at 3 and 10 mg/kg, i.p. (Table 3). Moreover, eplivanserin increased the latency to REMS, increased its mean duration, while decreasing its occurrence at 10 mg/kg (Table 3).

3.2. Effects of eplivanserin combined with zolpidem

In a first group of rats treated with 3 and 10 mg/kg, p.o. eplivanserin, the duration of the different sleep-wakefulness stages was not modified (Fig. 4). Similar to the effects of eplivanserin in the light period, a significant increase in the mean duration of NREMS episodes and a decrease in their number was observed when the drug was administered in the dark period (Table 5). The administration of zolpidem at 10 mg/kg, p.o. resulted in a significant decrease in the latency to NREMS and wakefulness time, accompanied by an increase in NREMS time (Table 5 and Fig. 4). At this dose, zolpidem increased the mean duration of NREMS episodes, but did not modify their frequency (Table 5). When zolpidem was given at 3 mg/kg, it only affected the mean number of NREMS episodes, which was increased (Table 5).

Coadministration of sub- or weakly active doses of eplivanserin and zolpidem resulted in significant changes in sleep-wakefulness parameters (Fig. 4 and Table 5). When eplivanserin at 1 mg/kg was

Table 2
Summary of statistical analyses using paired (Day 0 vs. Day 1) Student's *t*-test. Statistically significant values are indicated by bold.

	mg/kg, i.p.	Latency NREMS	Wakefulness	NREMS	REMS	Mean duration NREMS episodes	Mean number NREMS episodes	Latency REMS	Mean duration REMS episodes	Mean number REMS episodes
AVE8488	0.1	$t = 1.39$, ns	$t = -0.72$, ns	$t = 0.87$, ns	$t = -0.06$, ns	$\mathbf{t = 2.95, P < 0.05}$	$\mathbf{t = -5.21, P < 0.01}$	$t = 0.78$, ns	$t = 1.99$, ns	$t = -0.91$, ns
	0.3	$t = -1.59$, ns	$\mathbf{t = -2.87, P < 0.05}$	$\mathbf{t = 3.23, P < 0.05}$	$t = 1.89$, ns	$\mathbf{t = 4.38, P < 0.01}$	$\mathbf{t = -5.32, P < 0.01}$	$t = 0.58$, ns	$t = 1.45$, ns	$t = 0.85$, ns
	1	$t = 3.00$, ns	$t = -0.67$, ns	$t = 1.69$, ns	$\mathbf{t = -3.45, P < 0.01}$	$\mathbf{t = 3.04, P < 0.05}$	$\mathbf{t = -3.36, P < 0.01}$	$\mathbf{t = 4.67, P < 0.01}$	$t = 2.18$, ns	$\mathbf{t = -6.17, P < 0.001}$
	3	$t = 1.29$, ns	$t = -1.60$, ns	$t = 2.51$, ns	$t = -2.06$, ns	$\mathbf{t = 3.63, P < 0.05}$	$\mathbf{t = -4.81, P < 0.01}$	$\mathbf{t = 3.06, P < 0.05}$	$\mathbf{t = 6.11, P < 0.01}$	$\mathbf{t = -3.36, P < 0.05}$
Volinanserin	0.3	$t = -0.32$, ns	$t = -0.32$, ns	$t = 0.47$, ns	$t = -0.25$, ns	$\mathbf{t = 4.51, P < 0.01}$	$\mathbf{t = -3.05, P < 0.05}$	$t = 1.42$, ns	$t = 0.74$, ns	$t = -0.42$, ns
	1	$t = 1.54$, ns	$\mathbf{t = -3.11, P < 0.05}$	$\mathbf{t = 6.64, P < 0.01}$	$\mathbf{t = -3.68, P < 0.05}$	$\mathbf{t = 3.28, P < 0.05}$	$\mathbf{t = -3.05, P < 0.05}$	$\mathbf{t = 3.53, P < 0.05}$	$\mathbf{t = 12.69, P < 0.001}$	$\mathbf{t = -6.23, P < 0.01}$
	3	$t = 1.96$, ns	$\mathbf{t = -4.80, P < 0.001}$	$\mathbf{t = 7.18, P < 0.001}$	$\mathbf{t = -2.34, P < 0.05}$	$\mathbf{t = 3.98, P < 0.01}$	$\mathbf{t = -4.80, P < 0.001}$	$\mathbf{t = 3.62, P < 0.01}$	$\mathbf{t = 2.74, P < 0.05}$	$\mathbf{t = -3.14, P < 0.05}$
Eplivanserin	3	$t = 1.86$, ns	$t = -0.88$, ns	$t = 1.51$, ns	$t = -1.02$, ns	$\mathbf{t = 3.53, P < 0.05}$	$\mathbf{t = -3.69, P < 0.01}$	$\mathbf{t = 10.10, P = 0.001}$	$t = 1.42$, ns	$t = -2.56$, ns
	10	$t = 1.86$, ns	$t = 0.38$, ns	$t = 0.40$, ns	$t = -1.38$, ns	$\mathbf{t = 5.33, P < 0.01}$	$\mathbf{t = -5.16, P < 0.01}$	$\mathbf{t = 5.79, P < 0.01}$	$\mathbf{t = 4.39, P < 0.01}$	$\mathbf{t = -3.49, P < 0.05}$

ns = non significant.

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Table 3
Summary of effect of 5-HT_{2A} receptor antagonists on sleep/wakefulness parameters during the light period (lights on at 7:00 AM).

	mg/kg, i.p.	Latency NREMS (min)		Mean duration NREMS episodes (min)		Mean number NREMS episodes		Latency REMS (min)		Mean duration REMS episodes (min)		Mean number REMS episodes	
		D0	D1	D0	D1	D0	D1	D0	D1	D0	D1	D0	D1
AVE8488	0.1	4.31 ± 2.0	7.51 ± 2.5	1.32 ± 0.1	1.56 ± 0.1*	135.1 ± 6.9	115.3 ± 7.9**	67.53 ± 30.0	88.51 ± 9.3	1.32 ± 0.1	1.58 ± 0.2	20.0 ± 0.0	17.8 ± 2.9
	0.3	6.27 ± 2.2	2.54 ± 1.1	1.13 ± 0.1	2.08 ± 0.2**	175.9 ± 14.2	118.7 ± 11.5**	86.11 ± 21.3	101.27 ± 9.1	1.47 ± 0.1	2.05 ± 0.1	10.3 ± 2.4	12.7 ± 1.5
	1	4.33 ± 1.2	15.32 ± 4.1	1.10 ± 0.1	1.57 ± 0.2*	182.0 ± 12.1	133.6 ± 16.7**	55.49 ± 13.3	146.52 ± 15.3**	1.58 ± 0.1	2.22 ± 0.1	17.2 ± 1.9	9.9 ± 1.8***
	3	9.55 ± 4.2	20.43 ± 5.5	1.49 ± 0.1	3.28 ± 0.3*	117.8 ± 6.4	74.7 ± 9.1**	96.22 ± 8.5	211.45 ± 35.4*	1.58 ± 0.1	3.00 ± 0.1**	12.8 ± 2.4	4.8 ± 1.4*
Volinanserin	0.3	11.37 ± 4.0	16.07 ± 2.6	1.39 ± 0.1	2.08 ± 0.1**	130.7 ± 10.2	117.7 ± 18.5*	63.22 ± 8.0	117.43 ± 35.2*	1.44 ± 0.1	1.58 ± 0.2	11.5 ± 1.6	10.2 ± 1.7
	1	19.18 ± 6.3	28.48 ± 5.5	1.29 ± 0.1	2.44 ± 0.3*	145.3 ± 17.0	100.2 ± 18.8*	56.22 ± 8.0	123.03 ± 15.3**	2.07 ± 0.1	2.52 ± 0.1***	18.0 ± 1.0	8.7 ± 1.1**
	3	15.27 ± 5.3	26.05 ± 3.6	1.33 ± 0.1	2.39 ± 0.2**	134.2 ± 10.4	98.4 ± 10.5***	114.08 ± 19.4	192.09 ± 19.4***	1.53 ± 0.1	2.25 ± 0.1*	11.5 ± 1.9	6.2 ± 0.9*
Eplivanserin	3	0.03 ± 0.1	2.20 ± 1.1	1.56 ± 0.2	2.50 ± 0.2*	116.2 ± 18.6	170.3 ± 24.9**	42.32 ± 12.0	113.37 ± 17.5**	1.59 ± 0.1	2.15 ± 0.1	17.5 ± 1.8	13.8 ± 1.9
	10	2.08 ± 0.4	11.18 ± 5.1	1.51 ± 0.2	3.44 ± 0.3**	150.3 ± 20.2	74.8 ± 10.4**	35.52 ± 3.6	147.12 ± 16.1***	1.45 ± 0.1	2.46 ± 0.2**	16.7 ± 2.1	8.3 ± 0.7*

D0 = Day 0, D1 = Day 1. Recording over a 6-h period. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (paired Student's *t*-test).

Table 4
Summary of statistical analyses using paired (Day 0 vs. Day 1) Student's *t*-test. Statistically significant values are indicated by bold.

	mg/kg, p.o.	Latency NREMS	Wakefulness	NREMS	Mean duration NREMS episodes	Mean number NREMS episodes	Latency REMS	Mean duration REMS episodes	Mean number REMS episodes
Eplivanserin	3	$t = 0.30$, ns	$t = -0.54$, ns	$t = -0.01$, ns	**$t = 2.97$, $P < 0.05$**	**$t = -3.24$, $P < 0.05$**	$t = -1.92$, ns	$t = 0.92$, ns	$t = 2.54$, ns
	10	$t = 1.90$, ns	$t = -1.45$, ns	$t = 1.53$, ns	**$t = 4.54$, $P < 0.01$**	**$t = -5.15$, $P < 0.01$**	$t = 0.58$, ns	$t = -0.87$, ns	$t = 0.80$, ns
Zolpidem	3	$t = -1.81$, ns	$t = -0.61$, ns	$t = 0.60$, ns	$t = -1.96$, ns	**$t = 3.32$, $P < 0.05$**	$t = -1.36$, ns	$t = 1.58$, ns	$t = 1.58$, ns
	10	**$t = -3.50$, $P < 0.05$**	**$t = -4.56$, $P < 0.01$**	**$t = 5.00$, $P < 0.01$**	**$t = 6.77$, $P < 0.01$**	$t = 0.45$, ns	$t = -0.07$, ns	$t = -0.96$, ns	$t = 2.08$, ns
Eplivanserin + zolpidem	1 + 3	$t = -0.39$, ns	**$t = -2.92$, $P < 0.05$**	**$t = 2.82$, $P < 0.05$**	**$t = 2.65$, $P < 0.05$**	$t = -2.26$, ns	$t = -1.19$, ns	$t = 0.46$, ns	$t = 1.95$, ns
	3 + 3	$t = -0.74$, ns	**$t = -4.52$, $P < 0.01$**	**$t = 4.31$, $P < 0.01$**	**$t = 6.43$, $P < 0.001$**	$t = -0.56$, ns	$t = -1.01$, ns	**$t = 2.56$, $P < 0.01$**	**$t = -2.87$, $P < 0.05$**

ns = non significant.

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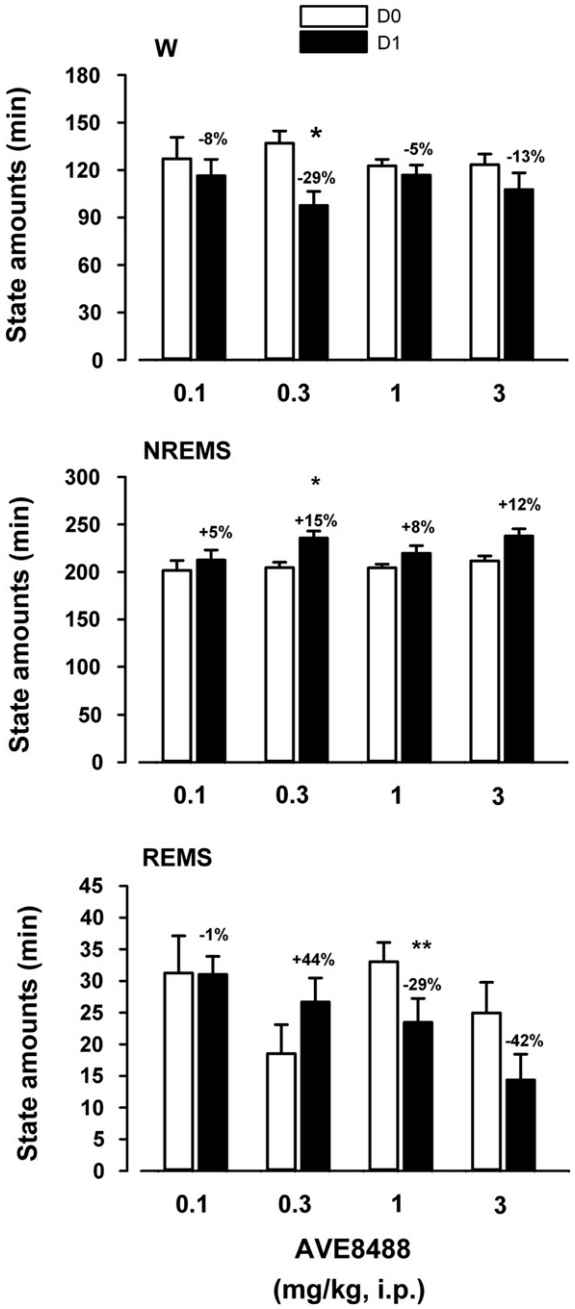

Fig. 1. Effects of acute treatment with the 5-HT_{2A} receptor antagonist AVE8488 on amounts of vigilance states in rats tested under a normal light/dark cycle (lights on at 7:00 AM). Data (mean ± SEM of 8 animals per) are expressed as minutes over a 6-h recording period. $^{*}P < 0.05$, $^{**}P < 0.01$ day 1 (D1) significantly different from paired-saline-treated injection on day 0 (D0).

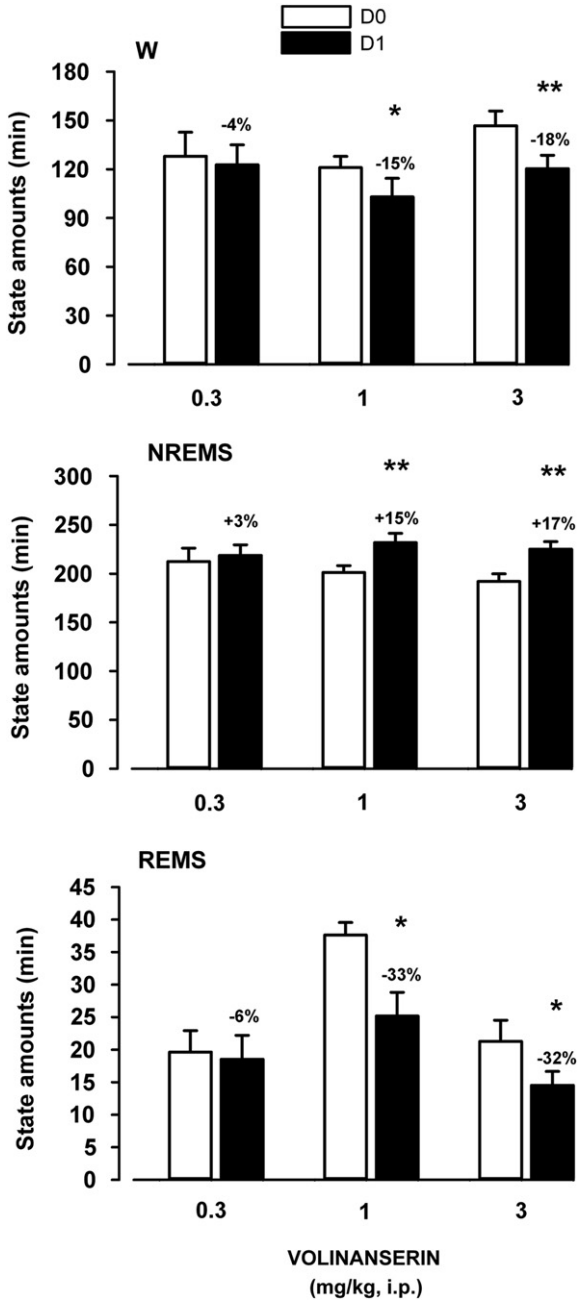

Fig. 2. Effects of acute treatment with the 5-HT_{2A} receptor antagonist volinanserin on amounts of vigilance states in rats tested under a normal light/dark cycle (lights on at 7:00 AM). Data (mean ± SEM of 11 animals per) are expressed as minutes over a 6-h recording period. $^{*}P < 0.05$, $^{**}P < 0.01$ day 1 (D1) significantly different from paired-saline-treated injection on day 0 (D0).

coadministered with zolpidem at 3 mg/kg, a significant decrease in wakefulness time and an increase in NREMS time were observed. These effects were accompanied by a significant increase in the mean duration of NREMS episodes, while their number remained unchanged. This combination also resulted in an increase in the duration and mean duration of REMS episodes, while their number was decreased. Finally, the combination of eplivanserin and zolpidem at 3 mg/kg each produced a decrease in wakefulness, while NREMS was increased as well as the mean duration of NREMS episodes. Neither combination modified the latency to NREMS or REMS.

4. Discussion

This study compared the sleep profile in rats of several potent 5-HT_{2A} receptor antagonists. The results confirm previous findings on the effects of 5-HT_{2A} receptor antagonists on sleep maintenance, while having modest effects on sleep amount compared to the non-BZD $GABA_A$ hypnotic, zolpidem. Furthermore, the data indicate a previously unreported synergistic effect between the 5-HT_{2A} receptor antagonist eplivanserin and zolpidem.

The three 5-HT_{2A} receptor antagonists used in the present study modified sleep-wakefulness parameters in a relatively similar manner. However, while volinanserin and, to a lesser extent,

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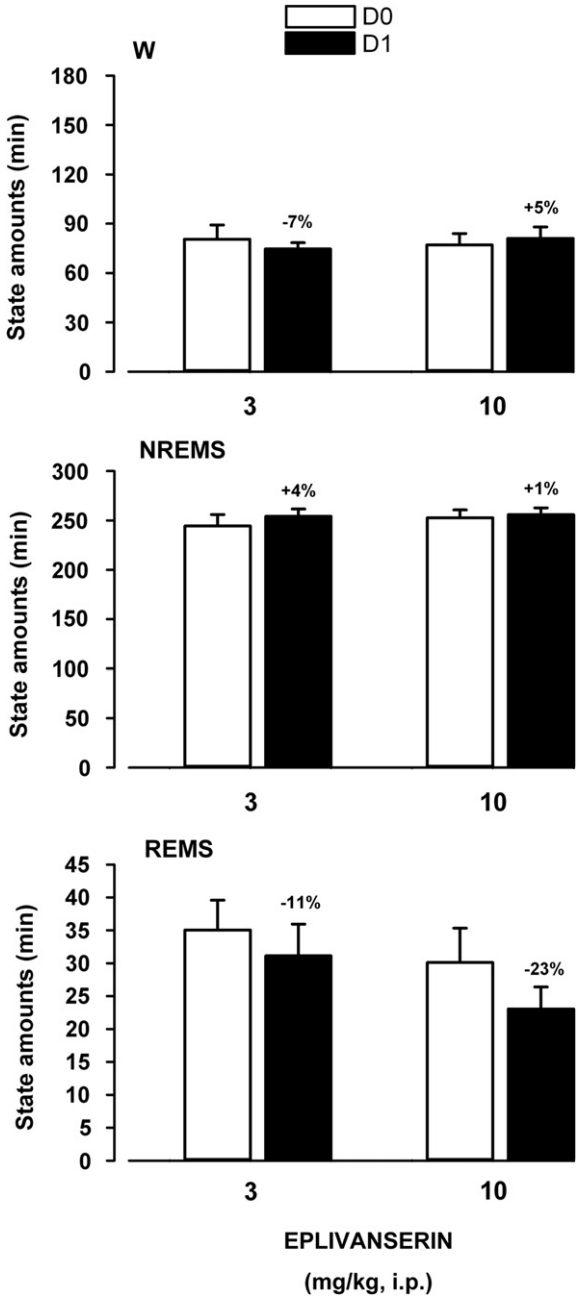

Fig. 3. Effects of acute treatment with the 5-HT_{2A} receptor antagonist eplivanserin on amounts of vigilance states in rats tested under a normal light/dark cycle (lights on at 7:00 AM). Data (mean ± SEM of 6 animals per) are expressed as minutes over a 6-h recording period.

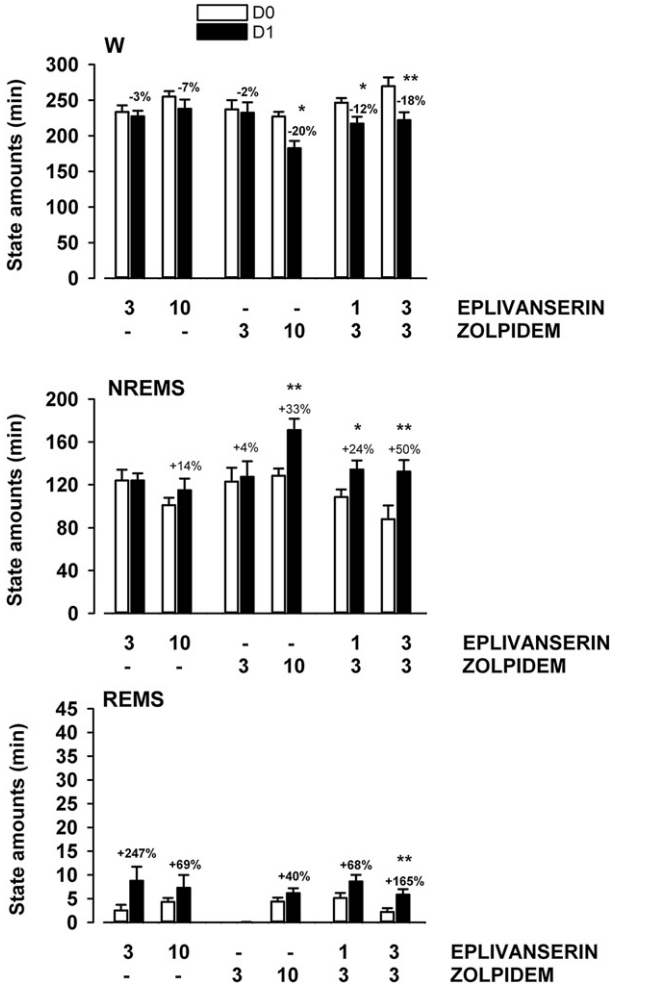

Fig. 4. Effects of acute treatment with the 5-HT_{2A} receptor antagonist eplivanserin, the non-BZD $GABA_A$ receptor agonist zolpidem, or combined treatment of both drugs on amounts of vigilance states in rats tested under a reverse light/dark cycle (lights on at 7:00 PM). Data (mean ± SEM of 6–9 animals per) are expressed as minutes over a 6-h recording period. $^{*}P < 0.05$, $^{**}P < 0.01$ day 1 (D1) significantly different from paired-saline-treated injection on day 0 (D0).

AVE8488 increased the amount of NREMS at the expense of wakefulness and REMS, eplivanserin did not affect these parameters, whether animals were tested under both light/dark cycle conditions. These findings with eplivanserin and volinanserin agree with previous studies, which showed that eplivanserin tested over a similar dose range did not modify NREMS in rats (Rinaldi-Carmona et al., 1992), while volinanserin induced a dose-dependent increase in NREMS in mice (Morairty et al., 2008; Popa et al., 2005). The reason for the differential profile between the current 5-HT_{2A} receptor antagonists on NREMS quantity is unclear. AVE8488, volinanserin and eplivanserin are all described as highly potent 5-HT_{2A} receptor antagonists, displaying comparable brain occupancy with relatively long duration of action (≈8 h) in the mouse (see Table 1) (Kehne et al., 1996; Rinaldi-Carmona et al., 1992). The doses employed in this study were selected to render near maximal receptor occupancy, and to exceed the reported minimum effective dose reported in *in vivo* studies in order to generate prolonged receptor occupancy over the 6 h of data capture. The only noticeable difference between eplivanserin and the two other 5-HT_{2A} receptor antagonists is the lower *in vitro* potency of the former compared to AVE8488 and volinanserin in antagonizing 5-HT-induced calcium mobilization using human recombinant 5-HT_{2A} receptors (IC_{50} = 29 vs. 0.7 and 1.9 nM, respectively). However, these experiments were not direct head-to-head comparisons, and the difference in *in vitro* potency findings did not translate in *in vivo* functional assays, where all three drugs displayed comparable potency (see Table 1). Perhaps, a more simpler explanation of the differences between eplivanserin and the other 5-HT_{2A} receptor antagonists on NREMS is that in the case of volinanserin and AVE8488 the reduction in sleep fragmentation, as will be discussed below, logically resulted in an increase in NREMS amounts, but such an effect was not observed with eplivanserin because of a ceiling effect. Indeed, baseline amounts of NREMS were higher in the eplivanserin experiment as compared to the volinanserin and AVE8488 experiments (≈250 vs. 200 min, respectively).

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Table 5
Summary of effect of eplivanserin or zolpidem alone, and both drugs in combination on sleep/wakefulness parameters during the dark period (lights on at 7:00 PM).

	mg/kg, p.o.	Latency NREMS (min)		Mean duration NREMS episodes (min)		Mean number NREMS episodes		Latency REMS (min)		Mean duration REMS episodes (min)		Mean number REMS episodes	
		D0	D1	D0	D1	D0	D1	D0	D1	D0	D1	D0	D1
Eplivanserin	3	10.27 ± 3.4	11.03 ± 4.3	1.04 ± 0.1	1.24 ± 0.1*	118.2 ± 11.6	91.5 ± 10.0*	232.30 ± 45.5	124.05 ± 23.6	0.45 ± 0.2	1.03 ± 0.1	2.2 ± 1.0	7.2 ± 2.2
	10	9.38 ± 4.3	17.13 ± 5.4	0.55 ± 0.1	1.38 ± 0.1*	111.3 ± 7.2	70.2 ± 5.0**	110.02 ± 16.2	144.10 ± 47.0	1.19 ± 0.1	1.07 ± 0.2	3.5 ± 0.7	5.0 ± 1.5
Zolpidem	3	12.05 ± 4.1	6.03 ± 1.3	0.60 ± 0.1	0.51 ± 0.1	126.5 ± 6.4	151.2 ± 8.9*	360.0 ± 0.0	349.10 ± 7.6	0.01 ± 0.0	0.03 ± 0.0	0.0 ± 0.0	0.3 ± 0.2
	10	1.38 ± 0.1	0.26 ± 0.1*	0.53 ± 0.1	1.10 ± 0.1*	152.4 ± 16.4	154.2 ± 16.4	132.14 ± 36.1	129.30 ± 10.3	1.02 ± 0.1	0.50 ± 0.1	4.6 ± 1.2	7.2 ± 0.5
Eplivanserin + zolpidem	1 + 3	7.21 ± 2.2	6.18 ± 1.2	0.44 ± 0.1	1.09 ± 0.1*	125.2 ± 19.1	118.7 ± 11.0	199.34 ± 50.5	151.52 ± 31.1	0.37 ± 0.1	1.00 ± 0.1*	2.4 ± 0.8	5.2 ± 0.9*
	3 + 3	11.32 ± 0.6	9.12 ± 2.3	1.07 ± 0.1	1.37 ± 0.1*	100.2 ± 8.9	84.5 ± 7.4	116.05 ± 20.4	96.45 ± 20.1	1.12 ± 0.1	1.18 ± 0.1	4.3 ± 1.0	6.8 ± 1.1

D0 = Day 0, D1 = Day 1. Recording over a 6-h period. *$P < 0.05$, **$P < 0.01$ (paired Student's *t*-test).

Eplivanserin, volinanserin and AVE8488 produced under normal light/dark cycle (lights off at 7:00 AM) comparable large effects across dose ranges on REMS and NREMS bouts, whose duration was increased, while their number was reduced. In addition, the latency of REMS was increased. When tested under reverse light/dark cycle, eplivanserin had similar effects on NREMS as compared to normal light/dark testing. Taken as a whole, the current findings parallel those of previous studies with volinanserin (Morairty et al., 2008) and other selective and non-selective 5-HT_{2A} receptor antagonists (Al-Shamma et al., 2010; Dugovic et al., 1989) that showed similar effects on NREMS episodes, strengthening further the idea that pharmacological blockade of 5-HT_{2A} receptors may improve sleep consolidation by decreasing sleep fragmentation.

The exact mechanisms underlying the 5-HT_{2A} receptor antagonist-induced sleep changes, beyond 5-HT_{2A} receptor blockade, are far from being established. It has been speculated that these compounds may promote NREMS via a GABAergic-mediated reduction of inhibitory input from the DRN to the sleep-active cells of the hypothalamic lateral preoptic (LPO) area (Landolt and Wehrle, 2009), which is thought to play an important role in the initiation and maintenance of sleep (Gallopin et al., 2000). In this context, it may be important to note that eplivanserin was found in a preliminary study to increase c-Fos expression in the LPO suggesting that its effects on sleep-wakefulness may be associated with an increase in LPO neuronal activity (Françon et al., 2007). However, other mechanisms of action of the effects of 5-HT_{2A} receptor antagonists on sleep cannot be excluded, notably at the level of the raphé neurons.

The profile displayed by the 5-HT_{2A} receptor antagonists somewhat differs from that obtained by the non-BZD hypnotic, zolpidem, in the current study, although it is important to note that AVE8488 and volinanserin have not been tested under a reverse light/dark cycle like eplivanserin and zolpidem. The strongest effects of zolpidem were observed on NREMS, particularly an increase in amount, a profile typically observed following the administration of BZD and non-BZD hypnotics, which represent the mainstay of drug treatment in sleep initiation difficulties. Unlike 5-HT_{2A} receptor antagonists, zolpidem had only partial effects on sleep episodes as it increased the duration of NREMS bouts, without modifying their number. This latter effect is not unexpected considering that zolpidem promotes NREMS. The increase in NREMS occurs via an increase in NREMS bout duration without changing the number of NREMS bouts. It is noteworthy that unlike in other studies (Depoortere et al., 1986; Steiner et al., 2011), zolpidem did not decrease REMS. However, baseline levels of REMS were low, suggesting that the lack of effect of zolpidem on REMS may be explained by a floor effect.

The current effects on sleep-wakefulness with the 5-HT_{2A} receptor antagonists and zolpidem given alone tend to indicate that the association of a 5-HT_{2A} receptor antagonist with a short-acting non-BZD hypnotic, such as zolpidem, could be a valid alternative to normalize sleep in patients with insomnia and, in case of a synergy between both drugs, potentially reduce the doses of each drug. This hypothesis was tested in this study. The results showed that the combined administration of subeffective or weakly active doses (depending the parameter) of eplivanserin (1 or 3 mg/kg) and zolpidem (3 mg/kg) resulted in a significant increase in the amount of NREMS and in the mean duration of NREMS bouts. This effect was accompanied (at 3 mg/kg of eplivanserin only) by an increase in the amount of REMS and duration of REMS episodes, while the number of REMS was decreased. The lack of effect of the combination on the number of NREMS bouts may not be surprising as eplivanserin and zolpidem, at the doses they were combined, produced opposite effects on this measure when given alone. Taken as a whole the findings from the drug association experiment

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demonstrate a synergistic effect between eplivanserin and zolpidem on both the amount and the fragmentation of NREMS.

Although drug levels for eplivanserin and zolpidem were not analyzed to support a pharmacokinetic mechanism underlying this behavioral drug–drug interaction, it is reasonable to argue for a pharmacodynamic action of this synergy. As mentioned above, 5-HT_{2A} receptor antagonists may exert their sleep-promoting action via a GABAergic-mediated mechanism involving the 5-HT-enriched DRN. It may be speculated that different pharmacological manipulations (here $GABA_A$ receptor activation and 5-HT_{2A} receptor blockade) promote a common final pathway involved in the modulation of sleep as proposed by Landolt and Wehrle (Landolt and Wehrle, 2009).

There are several methodological considerations with the current study. First, we did not perform an electromyogram (EMG), which is considered as a standard procedure for sleep recording, allowing accurate and reliable state scoring. Without EMG it may be difficult for example to extract REMS from the data. This may explain in part the low levels of REMS observed in the current experiments. Another important aspect to be discussed is that our design, which compared the first saline day to the second drug day, may not be ideal as the order is not counterbalanced and order effects are not accounted for. To overcome these two limitations, it will be necessary in future studies to record EMG and use a repeated-measures, Latin square design.

In conclusion, despite these limitations, the results of this study provide the first evidence of a synergistic effect between a 5-HT_{2A} receptor antagonist and a non-BZD $GABA_A$ receptor agonist on sleep-wakefulness, while confirming the effects of 5-HT_{2A} receptor antagonists on sleep promotion. These findings suggest that combining a 5-HT_{2A} antagonist with a short-acting hypnotic may be a useful strategy for the treatment of insomnia.

Disclosure statement

This study was supported by Sanofi. Dr. Griebel, Ms. Beeské, Ms Jacquet, Dr. Alonso, Dr Avenet and Dr. Françon are employees of Sanofi.

References

Adam, K., Oswald, I., 1989. Effects of repeated ritanserin on middle-aged poor sleepers. Psychopharmacology 99, 219–221.

Al-Shamma, H.A., Anderson, C., Chuang, E., Luthringer, R., Grottick, A.J., Hauser, E., Morgan, M., Shanahan, W., Teegarden, B.R., Thomsen, W.J., Behan, D., 2010. Nelotanserin, a novel selective human 5-hydroxytryptamine$_{2A}$ inverse agonist for the treatment of insomnia. J. Pharmacol. Exp. Ther. 332, 281–290.

Aloyo, V.J., Berg, K.A., Spampinato, U., Clarke, W.P., Harvey, J.A., 2009. Current status of inverse agonism at serotonin$_{2A}$ (5-HT_{2A}) and 5-HT_{2C} receptors. Pharmacol. Ther. 121, 160–173.

Ancoli-Israel, S., Vanover, K.E., Weiner, D.M., Davis, R.E., Van Kammen, D.P., 2011. Pimavanserin tartrate, a 5-HT_{2A} receptor inverse agonist, increases slow wave sleep as measured by polysomnography in healthy adult volunteers. Sleep Med. 12, 134–141.

Cornea-Hebert, V., Riad, M., Wu, C., Singh, S.K., Descarries, L., 1999. Cellular and subcellular distribution of the serotonin 5-HT_{2A} receptor in the central nervous system of adult rat. J. Comp. Neurol. 409, 187–209.

da Roza Davis, J.M., Sharpley, A.L., Cowen, P.J., 1992. Slow wave sleep and 5-HT_2 receptor sensitivity in generalised anxiety disorder: a pilot study with ritanserin. Psychopharmacology 108, 387–389.

Depoortere, H., Zivkovic, B., Lloyd, K.G., Sanger, D.J., Perrault, G., Langer, S.Z., Bartholini, G., 1986. Zolpidem, a novel non-benzodiazepine hypnotic: I. Neuropharmacological and behavioral effects. J. Pharmacol. Exp. Ther. 237, 649–658.

Depoortere, R., Dargazanli, G., Estenne-Bouhtou, G., Coste, A., Lanneau, C., Desvignes, C., Poncelet, M., Heaulme, M., Santucci, V., Decobert, M., Cudennec, A., Voltz, C., Boulay, D., Paul, T.J., Stemmelin, J., Roger, P., Marabout, B., Sevrin, M., Vige, X., Biton, B., Steinberg, R., Francon, D., Alonso, R., Avenet, P., Oury-Donat, F., Perrault, G., Griebel, G., George, P., Soubrie, P., Scatton, B., 2005. Neurochemical, electrophysiological and pharmacological profiles of the selective inhibitor of the glycine transporter-1 SSR504734, a potential new type of antipsychotic. Neuropsychopharmacology 30, 1963–1985.

Dugovic, C., Wauquier, A., Leysen, J.E., Marrannes, R., 1989. Functional role of 5-HT_2 receptors in the regulation of sleep and wakefulness in the rat. Psychopharmacology 97, 436–442.

Françon, D., Decobert, M., Bester, H., Alonso, R., Griebel, G., Avenet, P., Scatton, B., Le Fur, G., 2007. Eplivanserin promotes sleep maintenance in rats. Sleep Biol. Rhyth 5, A3.

Gallopin, T., Fort, P., Eggermann, E., Cauli, B., Luppi, P.H., Rossier, J., Audinat, E., Muhlethaler, M., Serafin, M., 2000. Identification of sleep-promoting neurons in vitro. Nature 404, 992–995.

Griebel, G., Decobert, M., Jacquet, A., Beeské, S., 2012. Awakening properties of newly discovered highly selective H_3 receptor antagonists in rats. Behav. Brain Res. 232, 416–420.

Griebel, G., Perrault, G., Simiand, J., Cohen, C., Granger, P., Decobert, M., Françon, D., Avenet, P., Depoortere, H., Tan, S., Oblin, A., Schoemaker, H., Evanno, Y., Sevrin, M., George, P., Scatton, B., 2001. SL651498: an anxioselective compound with functional selectivity for α_2- and α_3-containing γ-aminobutyric acid$_A$ ($GABA_A$) receptors. J. Pharmacol. Exp. Ther. 298, 753–768.

Hoyer, D., Clarke, D.E., Fozard, J.R., Hartig, P.R., Martin, G.R., Mylecharane, E.J., Saxena, P.R., Humphrey, P.P.A., 1994. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). Pharmacol. Rev. 46, 157–204.

Kehne, J.H., Baron, B.M., Carr, A.A., Chaney, S.F., Elands, J., Feldman, D.J., Frank, R.A., van Giersbergen, P.L., McCloskey, T.C., Johnson, M.P., McCarty, D.R., Poirot, M., Senyah, Y., Siegel, B.W., Widmaier, C., 1996. Preclinical characterization of the potential of the putative atypical antipsychotic MDL 100,907 as a potent 5-HT_{2A} antagonist with a favorable CNS safety profile. J. Pharmacol. Exp. Ther. 277, 968–981.

Landolt, H.P., Wehrle, R., 2009. Antagonism of serotonergic 5-$HT_{2A/2C}$ receptors: mutual improvement of sleep, cognition and mood? Eur. J. Neurosci. 29, 1795–1809.

Leysen, J.E., 2004. 5-HT_2 receptors. Curr. Drug Targets CNS. Neurol. Disord. 3, 11–26.

Monti, J.M., 2010. Serotonin 5-HT_{2A} receptor antagonists in the treatment of insomnia: present status and future prospects. Drugs Today 46, 183–193.

Monti, J.M., Jantos, H., Monti, D., 2008. Serotonin and sleep-wake regulation. In: Monti, J.M., Pandi-Perumal, S.R., Sinton, C.M. (Eds.), Neurochemistry of Sleep and Wakefulness. Cambridge University Press, Cambridge, pp. 244–279.

Morairty, S.R., Hedley, L., Flores, J., Martin, R., Kilduff, T.S., 2008. Selective 5HT_{2A} and 5HT_6 receptor antagonists promote sleep in rats. Sleep 31, 34–44.

Philbert, J., Pichat, P., Beeské, S., Decobert, M., Belzung, C., Griebel, G., 2011. Acute inescapable stress exposure induces long-term sleep disturbances and avoidance behavior: a mouse model of post-traumatic stress disorder (PTSD). Behav. Brain Res. 221, 149–154.

Popa, D., Lena, C., Fabre, V., Prenat, C., Gingrich, J., Escourrou, P., Hamon, M., Adrien, J., 2005. Contribution of 5-HT_2 receptor subtypes to sleep-wakefulness and respiratory control, and functional adaptations in knock-out mice lacking 5-HT_{2A} receptors. J. Neurosci. 25, 11231–11238.

Portas, C.M., McCarley, R.W., 1994. Behavioral state-related changes of extracellular serotonin concentration in the dorsal raphe nucleus: a microdialysis study in the freely moving cat. Brain Res. 648, 306–312.

Rinaldi-Carmona, M., Congy, C., Santucci, V., Simiand, J., Gautret, B., Neliat, G., Labeeuw, B., Le Fur, G., Soubrié, P., Brelière, J.C., 1992. Biochemical and pharmacological properties of SR 46349B, a new potent and selective 5-hydroxytryptamine$_2$ receptor antagonist. J. Pharmacol. Exp. Ther. 262, 759–768.

Rosenberg, R., Seiden, D.J., Hull, S.G., Erman, M., Schwartz, H., Anderson, C., Prosser, W., Shanahan, W., Sanchez, M., Chuang, E., Roth, T., 2008. APD125, a selective serotonin 5-HT_{2A} receptor inverse agonist, significantly improves sleep maintenance in primary insomnia. Sleep 31, 1663–1671.

Ruiz-Primo, E., Haro, R., Valencia, M., 1989. Polysomnographic effects of ritanserin in insomniacs. A crossed double-blind controlled study. Sleep Res. 18, 72.

Staner, L., Kempenaers, C., Simonnet, M.P., Fransolet, L., Mendlewicz, J., 1992. 5-HT_2 receptor antagonism and slow-wave sleep in major depression. Acta Psychiatr. Scand. 86, 133–137.

Steiner, M.A., Lecourt, H., Strasser, D.S., Brisbare-Roch, C., Jenck, F., 2011. Differential effects of the dual orexin receptor antagonist almorexant and the $GABA_A$-α1 receptor modulator zolpidem, alone or combined with ethanol, on motor performance in the rat. Neuropsychopharmacology 36, 848–856.

Stemmelin, J., Cohen, C., Terranova, J.P., Lopez-Grancha, M., Pichat, P., Bergis, O., Decobert, M., Santucci, V., Francon, D., Alonso, R., Stahl, S.M., Keane, P., Avenet, P., Scatton, B., Le, F.G., Griebel, G., 2008. Stimulation of the β3-Adrenoceptor as a novel treatment strategy for anxiety and depressive disorders. Neuropsychopharmacology 33, 574–587.

Teegarden, B.R., Al-Shamma, H.A., Xiong, Y., 2008. 5-HT_{2A} inverse-agonists for the treatment of insomnia. Curr. Top. Med. Chem. 8, 969–976.

Trulson, M.E., Jacobs, B.L., 1979. Raphe unit activity in freely moving cats: correlation with level of behavioral arousal. Brain Res. 163, 135–150.