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RESEARCH PAPER

Differential effects of acute and repeat dosing with the H_3 antagonist GSK189254 on the sleep–wake cycle and narcoleptic episodes in Ox–/– mice

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Background and purpose: Histamine H_3 receptor antagonists are currently being evaluated in clinical trials for a number of central nervous system disorders including narcolepsy. These agents can increase wakefulness (W) in cats and rodents following acute administration, but their effects after repeat dosing have not been reported previously.
Experimental approach: EEG and EMG recordings were used to investigate the effects of acute and repeat administration of the novel H_3 antagonist GSK189254 on the sleep–wake cycle in wild-type (Ox+/+) and orexin knockout (Ox–/–) mice, the latter being genetically susceptible to narcoleptic episodes. In addition, we investigated H_3 and H_1 receptor expression in this model using radioligand binding and autoradiography.
Key results: In Ox+/+ and Ox–/– mice, acute administration of GSK189254 (3 and 10 mg·kg^{-1} p.o.) increased W and decreased slow wave and paradoxical sleep to a similar degree to modafinil (64 mg·kg^{-1}), while it reduced narcoleptic episodes in Ox–/– mice. After twice daily dosing for 8 days, the effect of GSK189254 (10 mg·kg^{-1}) on W in both Ox+/+ and Ox–/– mice was significantly reduced, while the effect on narcoleptic episodes in Ox–/– mice was significantly increased. Binding studies revealed no significant differences in H_3 or H_1 receptor expression between Ox+/+ and Ox–/– mice.
Conclusions and implications: These studies provide further evidence to support the potential use of H_3 antagonists in the treatment of narcolepsy and excessive daytime sleepiness. Moreover, the differential effects observed on W and narcoleptic episodes following repeat dosing could have important implications in clinical studies.
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Keywords: Orexin knockout mice; GSK189254; H_3 receptor antagonist; narcolepsy; modafinil; sleep–wake cycle

Abbreviations: DREMs, direct onset of rapid eye movement sleep from wakefulness; Ox+/+, wild-type mouse; Ox–/–, orexin knockout mouse; PS, paradoxical sleep; REM, rapid eye movement; SWS, slow wave sleep; W, wakefulness

Introduction

Histamine plays a major role in the control of arousal and the sleep–wake cycle (Lin, 2000; Brown *et al.*, 2001; Haas and Panula, 2003). Histaminergic neurons fire in a wake-specific pattern (Vanni-Mercier *et al.*, 2003; Takahashi *et al.*, 2006), and histamine release is subject to circadian variation being higher in periods of wakefulness (W) (Brown *et al.*, 2001). Histamine can increase wake and decrease slow wave sleep (SWS) when administered into cerebral structures important for sleep–wake control in the cat (Lin *et al.*, 1996). In contrast, decreased W occurs following inhibition of histamine synthesis, either pharmacologically using histidine decarboxylase inhibitors or genetically as in histidine decarboxylase knockout mice (Monti *et al.*, 1996; Parmentier *et al.*, 2002).

The wake-promoting effects of histamine are believed to be mediated via histamine H_1 receptors in the central nervous system (CNS). It is well documented that brain penetrant H_1 antagonists (classical antihistamines) can cause sedation in humans (Roth *et al.*, 1987) and affect psychomotor performance (Richardson *et al.*, 2002). H_1 antagonists also show similar sedative activity in a number of preclinical species including rats (Saitou *et al.*, 1999).

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Histamine can also exert its effects via other histamine receptors including presynaptic H_3 receptors. H_3 receptor activation results in the inhibition of histamine synthesis and release from histaminergic neurons via an autoreceptor function (Arrang *et al.*, 1983), and the inhibition of release of other neurotransmitters, such as acetylcholine, dopamine, 5-HT and noradrenaline, from non-histaminergic neurons via a heteroreceptor function (Schlicker *et al.*, 1994; Blandina *et el.,* 1996). Conversely, *in vivo* blockade of H_3 receptors with selective antagonists/inverse agonists can increase the release of histamine (Arrang *et al.*, 1988) and other neurotransmitters (Fox *et al.*, 2005; Medhurst *et al.*, 2007) involved in cognition and arousal.

H_3 receptor agonists have been shown to produce sedation in preclinical species while H_3 antagonists have the opposite effect and can increase W (Monti *et al.*, 1996; Ligneau *et al.*, 1998; Lin, 2000; Barbier *et al.*, 2004). Transgenic mice devoid of the H_3 receptor display characteristics of behavioural inactivation and are unresponsive to the wake-promoting actions of H_3 receptor antagonists (Toyota *et al.*, 2002). The increase in wakefulness observed with H_3 antagonists can be blocked with H_1 antagonists such as mepyramine (Lin *et al.*, 1990), while a recent study in H_1 receptor knockout mice confirmed that the W effect of the H_3 antagonist ciproxifan is dependent on the presence of H_1 receptors (Parmentier *et al.*, 2007). These effects on W have led to the hypothesis that H_3 receptor antagonists may be beneficial in diseases where excessive daytime sleepiness occurs, such as in narcolepsy. Moreover, histamine levels have been shown to be decreased in human narcoleptics and in dogs genetically susceptible to narcolepsy (Nishino *et al.*, 2001; Kanbayashi *et al.*, 2004), while H_3 antagonists can reverse food-elicited cataplexy in narcoleptic dogs (Bonaventure *et al.*, 2007). In addition, H_3 antagonists have recently been shown to increase W in narcoleptic mice (Lin *et al.*, 2008).

A number of H_3 antagonists/inverse agonists are currently in clinical trials to evaluate their potential utility in various CNS disorders, such as narcolepsy, Alzheimer's disease, schizophrenia and attention-deficit hyperactivity disorder (Celanire *et al.*, 2005; Esbenshade *et al.*, 2008; Lin *et al.*, 2008). Recently, tiprolisant (BF2.642; Ligneau *et al.*, 2007) has been shown to improve excessive daytime sleepiness in narcoleptic patients in a small phase II clinical trial (Lin *et al.*, 2008), consistent with a potential elevation of histamine in humans following blockade of H_3 autoreceptors.

GSK189254 {6-[(3-cyclobutyl-2,3,4,5-tetrahydro-1*H*-3-benzazepin-7-yl)oxy]-*N*-methyl-3-pyridinecarboxamide hydrochloride} is a highly potent and selective H_3 receptor antagonist/inverse agonist that shows efficacy in a number of cognition paradigms and pain models in rats after acute and repeat administration for up to 8 days (Medhurst *et al.*, 2007; 2008). However, certain effects of other H_3 antagonists have been shown to tolerate out after repeat dosing in preclinical species (Pan *et al.*, 2006), although the effect of repeat dosing on the sleep–wake cycle has not been reported previously. In the present study, we therefore investigated the effects of acute and repeat administration of GSK189254 on the sleep–wake cycle in wild-type (Ox+/+) and orexin knockout (Ox–/–) mice that are genetically susceptible to narcoleptic episodes. Ox–/– mice show direct transitions from W to paradoxical sleep (PS)/rapid eye movement (REM) sleep (also known as direct REM sleep, DREMs), a characteristic symptom in human narcolepsy (Mignot, 2005; Billiard *et al.*, 2006). In addition, we investigated whether there were any changes in H_3 and H_1 receptor levels in Ox–/– mice compared with Ox+/+ mice. These studies reveal interesting differences in the effects of repeat dosing of an H_3 antagonist on W and narcoleptic episodes in the mouse model of narcolepsy.

Methods

Animals and surgery

All experiments followed EEC (86/609/EEC) and French National Committee (decree 87/848) directives and every effort was made to minimize any pain and discomfort and the number of animals used. Procedures were also reviewed and approved by the GlaxoSmithKline Procedures Review panel. Orexin knockout (Ox–/–) mice were generated as previously described (Lin *et al.*, 2008). Prepro-orexin KO (Ox–/–) mice were descendants of the mouse strain generated by Chemelli *et al.* (1999) and kept on C57BL/6J genomic background by five to nine more backcrosses during the present study. To obtain experimental animals, male Ox–/– mice were backcrossed with female wild-type (Ox+/+) mice, and the generated Ox+/– mice were crossed between them resulting in both heterozygotes and homozygotes. Only littermate homozygote Ox+/+ and Ox–/– mice ($n = 14$ pairs) were used in this study to ensure that any detected phenotype resulted from the deletion of prepro-orexin gene rather than the genetic heterogeneity between individual animals. Mouse genotypes were determined as previously described using tail biopsies performed at 4 weeks of age (Chemelli *et al.*, 1999; Lin *et al.*, 2008).

At the age of 11–13 weeks and with a body weight of 30 ± 2 g, mice used for EEG and sleep–wake studies were chronically implanted, under deep gas anaesthesia using isoflurane (2%, 200 mL·min^{-1}) and a TEM anaesthesia system, with six cortical electrodes (gold-plated tinned copper wire, Ø = 0.4 mm) and three muscle electrodes (fluorocarbon-coated gold-plated stainless steel wire, Ø = 0.03 mm) to record the EEG and EMG, and to monitor the sleep–wake cycle. All electrodes were previously soldered to a multi-channel electrical connector and each was separately insulated with a covering of heat-shrinkable polyolefin/polyester tubing. Cortical electrodes were inserted into the dura through three pairs of holes (Ø = 0.5 mm) made in the skull, located respectively in the frontal (1 mm lateral and anterior to the bregma), parietal (1 mm lateral to the midline at the midpoint between the bregma and lambda) and occipital (2 mm lateral to the midline and 1 mm anterior to the lambda) cortices. Muscle electrodes were inserted into the neck muscles. Finally, the electrode assembly was anchored and fixed to the skull with Super-Bond and dental cement. This implantation allows stable and long-lasting polygraphic recordings (Parmentier *et al.*, 2002; Lin *et al.*, 2008).

Polygraphic recording in the mouse and data acquisition and analysis

After surgery, the animals were housed individually in transparent barrels (Ø = 20 cm, height = 30 cm) placed in an

insulated sound-proof recording room maintained at an ambient temperature of 22 ± 1°C and on a 12 h light/dark cycle (lights on at 07 h 00 min), standard food and water being available *ad libitum*. A video camera with infrared and digital time recording capabilities was set up in the recording room to observe and score, when necessary, the animals' behaviour during the light or dark phase. After a 7 day recovery period, mice were habituated to the recording cable for 7 days before polygraphic recordings were started. Cortical EEG and EMG signals were amplified, digitized with a resolution of 256 and 128 Hz respectively, and computed on a CED 1401 Plus. Using a Spike2 script and with the assistance of spectral analysis using the fast Fourier transform, polygraphic records were visually scored by 30 s epochs for W, SWS and PS/REM sleep according to previously described criteria validated for mice (Valatx, 1971; Valatx and Bugat, 1974; Parmentier *et al.*, 2002). DREMs onset episodes, also called narcoleptic episodes or sleep onset REM periods (Chemelli *et al.*, 1999; Mignot, 2005), were defined as the occurrence of REM sleep directly from W, namely a REM episode that follows directly a W episode lasting more than 60 s without being preceded by any cortical slow activity of more than 5 s during the 60 s.

Drug administration and experimental procedures in the mouse

After recovery from the surgery and habituation to the recording cables, each mouse was subjected to a recording session of two continuous days, beginning at 07 h 00 min. During these periods, the animals were left undisturbed to obtain baseline parameters on the cortical EEG, sleep–wake cycle and circadian rhythm. After baseline recordings, animals were subjected to cortical EEG and sleep–wake recordings following administration of either a vehicle (placebo) or drug (GSK189254 or modafinil). The vehicle consisted of 0.05 mL NaCl at 0.9% containing methylcellulose at 1%. The drug doses were expressed as compound weight. They were dissolved in the vehicle, fresh before each administration, and were administered orally using a mouse gavage probe. The following dosing schedules were investigated:

(1) Effect of acute administration of GSK189254 (3 and 10 mg·kg^{-1}, p.o.) on the sleep–wake cycle in Ox+/+ and Ox–/– mice, dosed at 10 h 00 min (i.e. 3 h after lights on) – mice exhibit maximal sleep between 10 and 16 h (Parmentier *et al.*, 2002; Lin *et al.*, 2008), so this period was appropriate for detecting any awakening effect.

(2) Effects of acute administration of GSK189254 (10 mg·kg^{-1}, p.o.) on narcoleptic episodes, dosed at 18 h 45 min just before lights-off phase at 19 h 00 min – both Ox+/+ and Ox–/– mice were active and awake at this time, and Ox–/– mice display narcoleptic attacks/DREMs almost exclusively during the lights-off phase. This period was therefore appropriate for detecting any anti-narcoleptic effects. Modafinil (64 mg·kg^{-1}, p.o.) was used as a comparator as previously described (Lin *et al.*, 2008), because of its wide use in sleep medicine including narcolepsy.

(3) Effects of repeat oral administration (8 days b.i.d. at 10 h 00 min and 19 h 00 min) of GSK189254 (10 mg·kg^{-1}) either on W or narcoleptic episodes during the lights-off phase. We sought to investigate whether the effects of GSK189254 (10 mg·kg^{-1}) on W or narcolepsy seen during acute dosing were maintained.

Oral doses of GSK189254 (3 and 10 mg·kg^{-1}) were selected to achieve >80% receptor occupancy. The ED_{50} in CD1 mice had previously been determined to be ~0.6 mg·kg^{-1} in *ex vivo* binding assays 1–2 h post dose (B. Crook, unpubl. obs.). The pharmacokinetic properties of GSK189254 in CD1 mice are comparable to rats, with the plasma half life following oral dosing being around 1.7 h and pharmacokinetics being linear with dose (M. Briggs, unpubl. obs.). Concentrations of GSK189254 determined in CD1 mice were around 1–3 μmol·L^{-1} following oral dosing of 3 and 10 mg·kg^{-1} and there was no evidence for drug accumulation. These brain concentrations are well above the K_i required for efficacy as previously reported for a number of other H_3 antagonists (Barbier *et al.*, 2004; Medhurst *et al.*, 2007).

The order of drug administration was randomized and each animal received both placebo and drug treatment. Polygraphic recordings were made immediately after any administration and were maintained for 12 h or during the whole lights-off period (12 h). Two acute administrations were separated by a 7 day washout period. In the repeat dose studies, recordings commenced on day 9 after the dose at 10 h 00 min.

Autoradiography

Receptor autoradiography was carried out based on methods previously described (Roberts *et al.*, 2004; Medhurst *et al.*, 2007). Frozen half brains from drug naïve Ox+/+ and Ox–/– mice (n = 4 per group) were sectioned coronally (12 μm), thaw-mounted onto gelatin-coated slides and stored at –80°C until use. For H_3 receptor binding, sections were incubated with 1 nmol·L^{-1} [^{3}H]-GSK189254 at room temperature for 60 min (Medhurst *et al.*, 2007). Non-specific binding was determined in the presence of 10 μmol·L^{-1} imetit on adjacent slides. For H_1 receptor binding, sections were incubated with 5 nmol·L^{-1} [^{3}H]-mepyramine for 45 min at room temperature (Palacios *et al.*, 1981). Non-specific binding was determined in the presence of 10 μmol·L^{-1} chlorpheniramine maleate on adjacent slides. After incubation, all sections were rinsed, dried and analysed by digital autoradiography using a Beta-Imager 2000 instrument as previously described (Medhurst *et al.*, 2007). Ten measurements were made from each brain area (cortex, hippocampus and hypothalamus) for each animal (five sections per animal and two measurements per region).

Saturation binding in brain homogenates

H_3 and H_1 receptor saturation binding experiments were carried out in cortical membranes from Ox+/+ and Ox–/– mice based on methodology previously described (Tran *et al.*, 1978; Medhurst *et al.*, 2007). Half brains were homogenized using a polytron P10 (2 × 10 s bursts at full speed) in 30 volumes of 50 mmol·L^{-1} Tris-HCl, pH 7.7, at 25°C containing 5 mmol·L^{-1} ethylenediamineteraacetic acid buffer. The homogenate was centrifuged in a Sorval Evolution RC centrifuge at 4°C for 20 min (SS34 rotor – approximately 48 000× g). The final pellet was resuspended in 10 mL buffer.

Membranes (corresponding to approximately 20 μg of protein per well) and [^{3}H]-GSK189254 (0.02–20 nmol·L^{-1}) for

H_3 binding, or [^{3}H]-mepyramine (0.05–50 nmol·L^{-1}) for H_1 binding were incubated in polypropylene tubes in a final volume of 200 µL of 50 mmol·L^{-1} Tris-HCl, pH 7.7, at 25°C containing 5 mmol·L^{-1} ethylenediamineteraacetic acid. Non-specific binding was determined in the presence of 10 µmol·L^{-1} imetit for H_3 and 10 µmol·L^{-1} chlorpheniramine for H_1. Reactions were conducted at 30°C for 45 min. The experiments were terminated by rapid filtration through Whatman GF/B filters [presoaked in 0.3% (v/v) polyethyleneimine]. The filters were washed with 4 × 2 mL aliquots of ice-cold buffer containing 50 mmol·L^{-1} Tris-HCl, pH 7.7, at 25°C and 5 mmol·L^{-1} $mgCl_2$. Filters were dried and added to vials each containing 4 mL of Ultima Gold MV scintillation fluid. Radioactivity was determined by liquid scintillation spectrometry using a Packard Tri-Carb 2500TR liquid scintillation counter. Protein concentrations were determined using the Bradford assay method (Bio-Rad protein assay Kit) with bovine serum albumin as a standard.

Statistical analysis

The sleep–wake amounts obtained after GSK189254 or modafinil dosing were compared with those obtained with vehicle. Individual animals acted as their own controls. Using Excel and/or Statgrafy software, one-way and multiple factorial ANOVA and the *post hoc* Student's *t*-test (two-tailed) were applied to compare and evaluate any difference in the effects obtained between: (i) compound and vehicle; (ii) compounds; (iii) doses; (iv) the first and the last administrations of GSK189254; and (v) mouse genotypes.

For autoradiography studies [^{3}H]-GSK189254 and [^{3}H]-mepyramine binding signals in cortex, hippocampus and hypothalamus were measured from five sections per animal and two measurements per region. This was determined previously to be a statistically validated number of sections. The results are expressed as mean specific binding cpm mm^{-2} ($n = 4$ animals per group), and the data were analysed using a repeated-measures ANOVA approach. For homogenate binding studies, H_3 and H_1 specific binding was analysed to estimate binding parameters B_{max} (reflective of total number of binding sites) and K_D (reflective of the binding affinity) using GraphPad Prism 3.0 by GraphPad Software Inc (San Diego, CA, USA).

Materials

GSK189254 {6-[(3-cyclobutyl-2,3,4,5-tetrahydro-1*H*-3-benzazepin-7-yl)oxy]-*N*-methyl-3-pyridinecarboxamide hydrochloride} was synthesized at GlaxoSmithKline (Harlow, UK). [^{3}H]-GSK189254 (specific activity 81 Ci·mmol^{-1}) was prepared through a contract with GE Healthcare. Modafinil was obtained from Laboratoires L. Lafon (Paris), imetit from Tocris Cookson Inc (Bristol UK) and chlorpheniramine from Sigma. [^{3}H]-mepyramine (specific activity 20–30 Ci·mmol^{-1}) was obtained from Amersham Bioscience (UK).

TEM anaesthesia system (Bordeaux, France), cortical electrodes (gold-plated tinned copper wire, Ø = 0.4 mm, Filotex, Draveil, France) and the muscle electrodes (fluorocarbon-coated gold-plated stainless steel wire, Ø = 0.03 mm, Cooner Wire Chatworth, CA, USA); Super-Bond (Sun Medical Co., Shiga, Japan). The CED 1401 Plus was from Cambridge (UK). The mouse gavage probe (20G) was obtained from Phymep (Paris). The Beta-Imager 2000 instrument was obtained from Biospace (Paris, France); Whatman GF/B filters (Whatman, Maidstone, UK); Ultima Gold MV scintillation fluid (Hewlett Packard, Palo Alto, CA); Packard Tri-Carb 2500TR liquid scintillation counter (PerkinElmer Life and Analytical sciences, Boston, MA); Bio-Rad protein assay Kit, Bio-Rad (York, UK); GraphPad Software Inc (San Diego, CA, USA).

Results

Effects of acute administration of GSK189254 on the sleep–wake cycle in Ox+/+ and Ox–/– littermates during a 4 h recording in the lights-on period

In wild-type Ox+/+ mice during the lights-on phase (when the mice slept most of the time at baseline), acute oral administration of GSK189254 (3 and 10 mg·kg^{-1}) caused an increase in W and a corresponding decrease in SWS and PS compared with vehicle-treated mice. Representative examples of hypnograms from Ox+/+ mice are shown in Figure 1A. In EEG spectral analysis, these effects were manifest as a suppression of neocortical slow activity (including δ range 0.8–2.5 Hz) and spindles (8–15 Hz) and an increase in power spectral density of neocortical fast rhythms (β and γ bands, mainly 30–60 Hz), resulting in marked enhancement of cortical activation (i.e. low voltage and fast electrical activity). Very similar effects on the sleep–wake cycle and cortical EEG were observed in Ox–/– littermates following acute oral administration of GSK189254 (3 and 10 mg·kg^{-1} p.o.), and representative examples of hypnograms from Ox–/– mice are shown in Figure 1B.

As shown in the analysis of sleep–wake states over the 4 h post-dose period (Figure 2A), acute single administration of GSK189254 (3 and 10 mg·kg^{-1}) to wild-type Ox+/+ mice significantly increased ($P < 0.05$, *t*-test between groups after significant ANOVA) the duration of W compared with vehicle-treated (placebo control) mice. The increase in W occurred at the expense of SWS and PS, which was reduced significantly during the same period ($P < 0.05$, *t*-test between groups after significant ANOVA). Similar effects following acute administration of GSK189254 (3 and 10 mg·kg^{-1}) were observed in Ox–/– mice (Figure 2B), with duration of W significantly increased, and SWS and PS significantly reduced ($P < 0.05$, *t*-test between groups after significant ANOVA).

Effects of repeat dosing of GSK189254 on the sleep–wake cycle in Ox+/+ and Ox–/– littermates during a 4 h recording in the lights-on period

We were also interested in exploring the effects of repeat dosing with GSK189254 given that this would potentially be required for future therapeutic use in narcolepsy or other sleep disorders. Both Ox+/+ and Ox–/– mice were treated with GSK189254 (3 and 10 mg·kg^{-1} p.o.) twice a day for 8 days respectively at 10 h 00 min and 19 h 00 min and effects on the sleep–wake cycle were studied on the ninth day when GSK189254 was also administered at 10 h 00 min and 19 h 00 min.

During the lights-on phase when untreated mice were sleepy and inactive at baseline, GSK189254 (10 mg·kg^{-1}, p.o.)

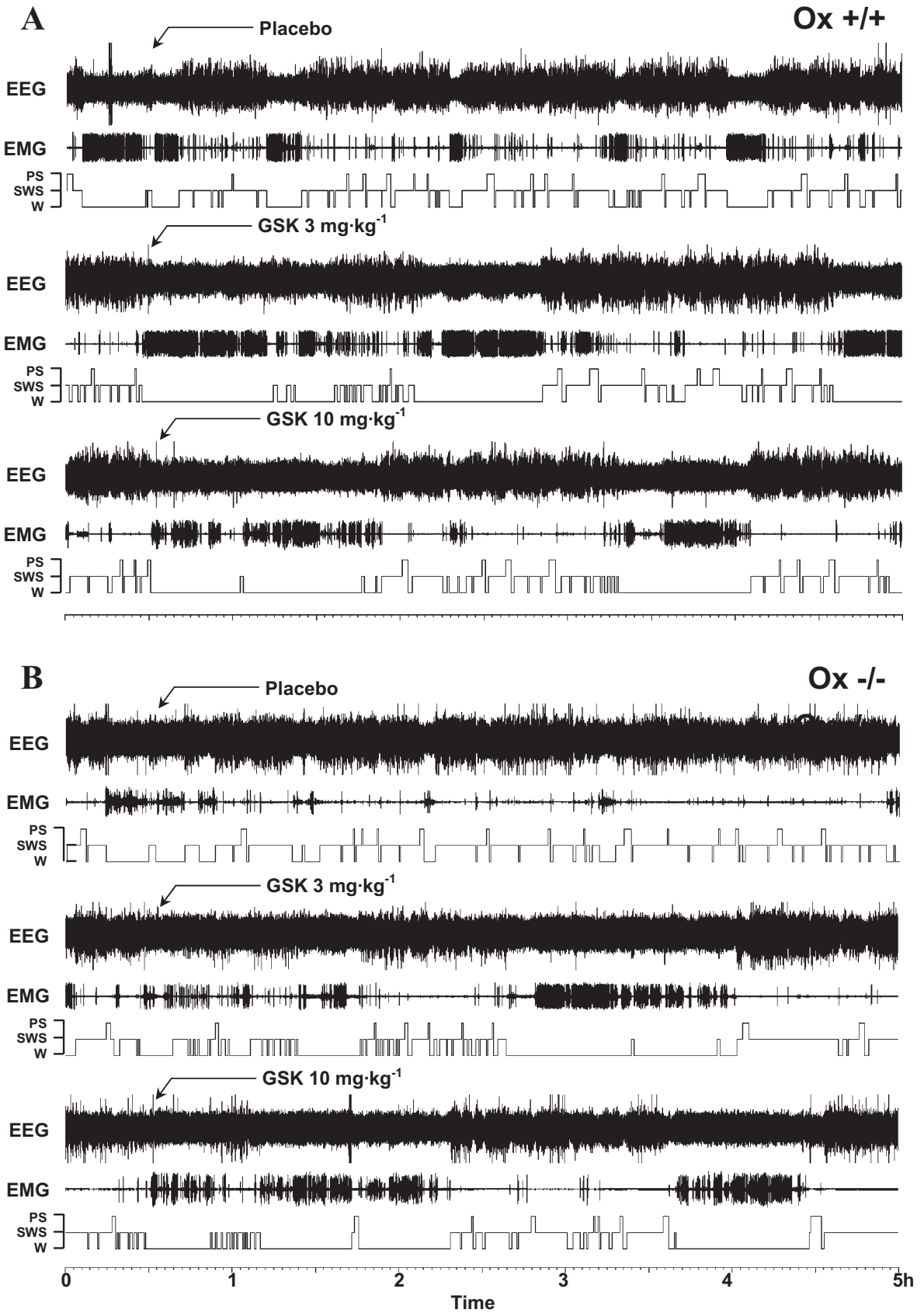

Figure 1 Representative examples of hypnograms showing the sleep–wake cycle in (A) wild-type (Ox+/+) and (B) Ox–/– mice over a 5 h period in the lights-on phase. Arrows show where vehicle (placebo) and GSK189254 (3 and 10 mg·kg^{-1}) were administered. GSK189254 increased wakefulness (W) and decreased slow wave sleep (SWS) and paradoxical sleep (PS) in both Ox+/+ and Ox–/– mice compared with vehicle-treated mice.

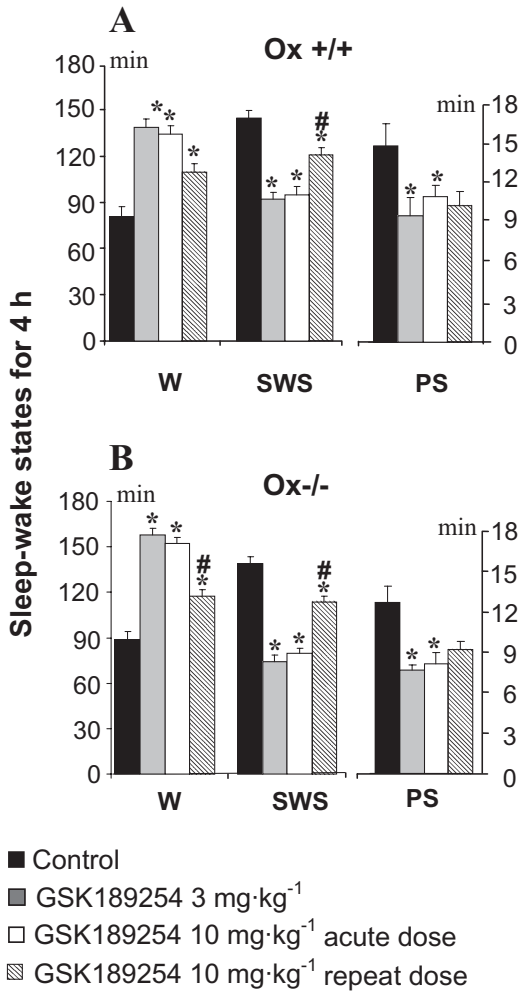

Figure 2 Effects of acute and repeat administration of GSK189254 on mean duration (±SEM) of sleep–wake stages in a 4 h recording within the lights-on period in (A) wild-type (Ox+/+) and (B) Ox–/– mice ($n = 14$ per group). GSK189254 (3 and 10 mg·kg^{-1}) significantly increased wakefulness (W) and decreased slow wave sleep (SWS) and paradoxical sleep (PS) following acute dosing. Repeat administration of GSK189254 (10 mg·kg^{-1}, twice daily for 8 days) resulted in a reduction in effects on W, SWS and PS compared with acute dosing with 10 mg·kg^{-1}. *$P < 0.05$, *t*-test between groups (compared with vehicle-treated mice) after significant ANOVA. #$P < 0.05$, *t*-test between groups (compared with acute 10 mg·kg^{-1} dose group) after significant ANOVA.

was still able to significantly increase W and decrease SWS and PS ($P < 0.05$, *t*-test between groups after significant ANOVA) following 8 days of repeat dosing in both Ox+/+ and Ox–/– mice (Figure 2A and B respectively). However, these effects were significantly but not completely reduced compared with those observed following a single acute dose of GSK189254 (10 mg·kg^{-1}, p.o.; $P < 0.05$, *t*-test between groups after significant ANOVA). Thus, after repeat dosing, GSK189254 maintained about half its waking potency compared with that seen with acute dosing. For example, during the 4 h recording session, there was an increase by 38% in W in both Ox+/+ and Ox–/– mice following repeat dosing, compared with a 66% (Ox+/+) and 69% (Ox–/–) increase seen with acute dosing. In both Ox+/+ and Ox–/– mice, the decrease in SWS following repeat dosing was also about a half of that seen with acute dosing, whereas the amount of PS was similar between acute and repeat dosing (Figure 2).

Cumulative effects of acute and repeat dosing of GSK189254 on the sleep–wake cycle in Ox+/+ and Ox–/– littermates over 8 h during the lights-on period

We also measured the cumulative effects of GSK189254 on the sleep–wake cycle over an 8 h period to get an indication of whether any sleep rebound occurred after the waking effect. During the recovery periods between the fifth and eighth hours after dosing, total amount of W and SWS in both Ox+/+ and Ox–/– mice remained significantly increased and decreased respectively, indicating that no significant sleep rebound occurred following the effect of GSK189254 on W (Figure 3A and B respectively). It should be noted that there is not a prolonged action of GSK189254 as the data in Figure 3 (and also Figure 5) represent hourly cumulative values of sleep–wake amounts. Hourly analysis showed that the effects of GSK189254 did not last more than 4 h, hence the 4 h sleep–wake amounts presented in Figures 2 and 4. The amount of PS also remained significantly lower than that of control for 8 h in both Ox+/+ and Ox–/– mice (Figure 3A and B respectively). During the lights-on period, the effects on sleep–wake amount elicited by single doses of 3 and 10 mg·kg^{-1} GSK189254 were similar (Figures 2 and 3), although the activating effect of GSK189254 on the EEG appeared to be greater with 10 mg·kg^{-1} than with 3 mg·kg^{-1} (Figure 1). A similar ratio between the effects of acute dosing and those after repeated dosing to that observed during the 4 h measurements (Figure 2) was found for all the sleep–wake states in the 8 h cumulative data set (Figure 3).

Acute effects of GSK189254 and modafinil on the sleep–wake cycle in Ox+/+ and Ox–/– littermates during a 4 h recording in the lights-off phase

Given the clear arousing effect observed in the lights-on phase, a 10 mg·kg^{-1} dose was chosen to assess the effects of acute administration of GSK189254 on the sleep–wake cycle of Ox+/+ and Ox–/– mice during the lights-off phase, when the animals were already highly awake and active. For comparison, modafinil, a wake-promoting compound (Lin *et al.*, 1996; Parmentier *et al.*, 2007) now used in sleep medicine for the treatment of narcolepsy and hypersomnia, was also applied to mice at a dose of 64 mg·kg^{-1}. During 4 h post-dose recording, GSK189254 still significantly ($P < 0.05$, *t*-test between groups after significant ANOVA) increased the time spent in W, decreased SWS and to a lesser extent decreased PS in both Ox+/+ (Figure 4A) and Ox–/– (Figure 4B) mice when applied just before lights-off, although these effects were less marked than those observed in the lights-on period, as expected given the higher baseline W and general activity. Modafinil also increased W and decreased SWS and PS in both Ox+/+ (Figure 4A) and Ox–/– mice (Figure 4B). During a recording session of 4 h, the effects of GSK189254 (10 mg·kg^{-1}) on sleep–wake states appeared to be similar to those of modafinil in Ox+/+ mice (Figure 4A) but marginally less in Ox–/– mice (Figure 4B).

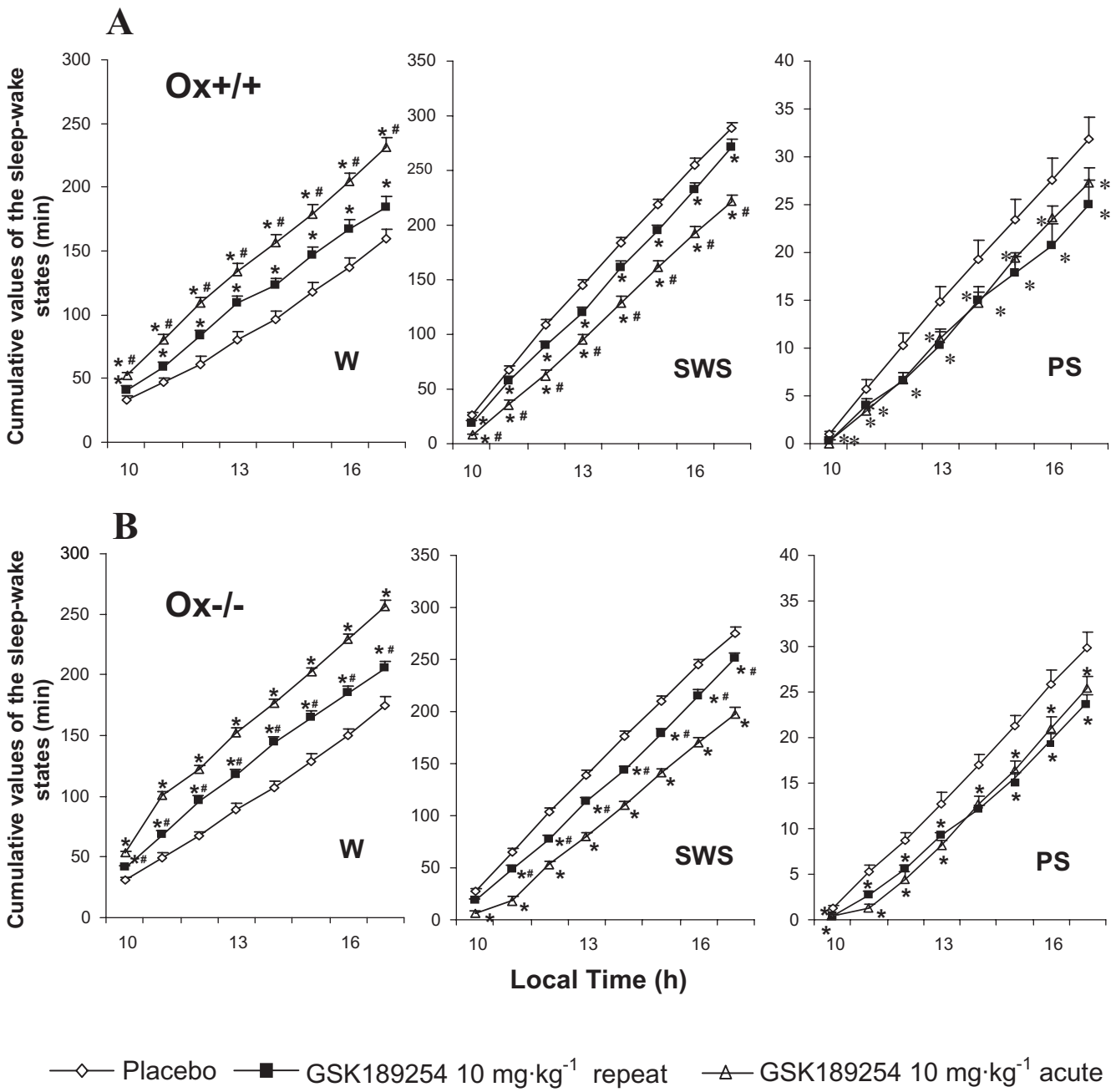

Figure 3 Cumulative effects of acute and repeat administration of GSK189254 on mean duration of sleep–wake stages over 8 h of the lights-on period in (A) wild-type (Ox+/+) and (B) Ox–/– mice (n = 14 per group). GSK189254 (3 and 10 mg·kg^{-1}) significantly increased wakefulness (W) and decreased slow wave sleep (SWS) and paradoxical sleep (PS) following acute dosing. Repeat administration of GSK189254 (10 mg·kg^{-1}, twice daily for 8 days) resulted in a reduction in effects on W, SWS and PS compared with acute dosing with 10 mg·kg^{-1}. *P < 0.05, t-test between groups (compared with vehicle-treated mice) after significant ANOVA. #P < 0.05, t-test between groups (compared with acute 10 mg·kg^{-1} dose group) after significant ANOVA. There was no evidence of sleep rebound and effects of GSK189254 lasted for the 8 h duration of measurements.

Effects of repeat dosing of GSK189254 on the sleep–wake cycle in Ox+/+ and Ox–/– littermates during a 4 h recording in the lights-off phase

In Ox+/+ mice during a 4 h recording period in the lights-off phase when untreated mice are highly awake and active, GSK189254 (10 mg·kg^{-1} p.o.) had no clear effect on W, SWS and PS following 8 days of b.i.d. dosing (Figure 4A) compared with vehicle-treated animals. This was in complete contrast to the effects of a single dose of GSK189254. Interestingly after repeat dosing in Ox–/– mice, GSK189254 (10 mg·kg^{-1}) significantly decreased W and increased SWS (P < 0.05, t-test between groups after significant ANOVA) and to a lesser extent, PS (Figure 4B). These effects in Ox–/– mice were opposite to those observed following a single dose.

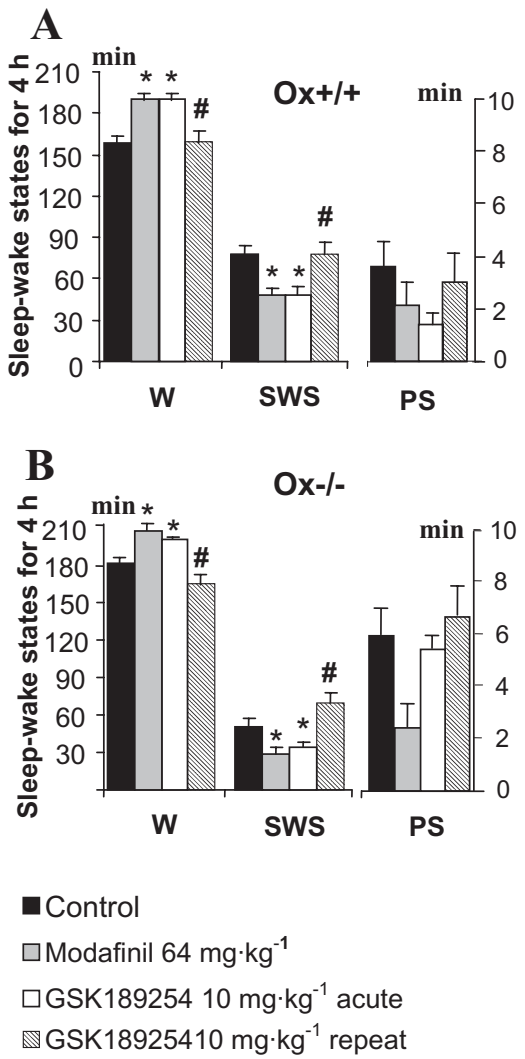

Figure 4 Effects of acute and repeat administration of GSK189254 on mean duration (±SEM) of sleep–wake stages in a 4 h recording within the lights-off period in (A) wild-type (Ox+/+) and (B) Ox–/– mice ($n = 14$ per group). GSK189254 (3 and 10 mg·kg^{-1}) significantly increased wakefulness (W) and decreased slow wave sleep (SWS) and paradoxical sleep (PS) following acute dosing. Repeat administration of GSK189254 (10 mg·kg^{-1}, twice daily for 8 days) had no effect on W, SWS and PS compared with acute dosing with 10 mg·kg^{-1} on Ox+/+ mice. In Ox–/– mice, GSK189254 actually reduced W and increased SWS and PS, effects that were the reverse of those seen with acute dosing. *$P < 0.05$, *t*-test between groups (compared with vehicle-treated mice) after significant ANOVA. #$P < 0.05$, *t*-test between groups (compared with acute 10 mg·kg^{-1} dose group) after significant ANOVA.

Cumulative effects of acute and repeat dosing of GSK189254 on the sleep–wake cycle in Ox+/+ and Ox–/– littermates over 12 h of the lights-off period

As in the lights-on phase, we also measured the cumulative effects of acute and repeat administration of GSK189254 on the sleep–wake cycle over an extended time period (in this case 12 h) to get an indication whether any sleep rebound occurred after the waking effect. In Ox+/+ mice, the cumulative effects of acute administration of GSK189254 (10 mg·kg^{-1}) on W and SWS were somewhat greater than those seen with modafinil, while the effects on PS were comparable (Figure 5A). In Ox–/– mice the effect of GSK189254 at 10 mg·kg^{-1} on W and SWS appeared slightly greater than that of modafinil during the 12 h recording session whereas the decrease in PS seen with GSK189254 appeared less than that seen with modafinil (Figure 5B). In terms of repeat dosing effects with GSK189254 in the 12 h recording period in Ox+/+ mice, there was no difference in W, SWS or PS compared with vehicle-treated animals, in contrast to the effects of acute dosing (Figure 5A). However, in Ox–/– mice, W duration was significantly reduced, while SWS, and to a lesser extent, PS, were significantly increased compared with vehicle-treated mice, an opposite effect to that observed with acute dosing (Figure 5B) and consistent with observations over the 4 h time frame (Figure 4B).

Acute effects of GSK189254 and modafinil on narcoleptic episodes in Ox–/– mice during lights-off

As shown in previous studies (Chemelli *et al.*, 1999; Lin *et al.*, 2008), Ox–/–mice display, almost exclusively during lights-off phase, a narcoleptic phenotype characterized by DREMs. In spite of their similar effect on W, GSK189254 and modafinil showed a different profile against narcoleptic episodes when applied to Ox–/– mice in the current study.

The waking effect of GSK189254 was accompanied by a significant decrease in the narcoleptic phenotype in Ox–/– mice. During the first 4 h after dosing (Figure 6A), the number of DREMs was significantly reduced ($P < 0.05$, *t*-test between groups after significant ANOVA). In addition, the mean cumulative number (Figure 6C) and duration (Figure 6B) of narcoleptic episodes were significantly reduced by GSK189254 ($P < 0.05$, *t*-test between groups after significant ANOVA).

In contrast, despite causing an increase in W and a reduction in SWS and PS, modafinil had no effect on the narcolepsy phenotype in Ox–/– mice. As shown in Figure 6, the total duration and cumulated number of narcoleptic attacks after modafinil dosing were not significantly different to those obtained in vehicle-treated mice throughout the 12 h recording period.

Repeat dose effects of GSK189254 on narcolepsy in Ox–/–mice

To our surprise, both the number and duration of narcoleptic episodes were dramatically reduced following 8 days of repeat dosing of GSK189254 (10 mg·kg^{-1}, b.i.d.). The anti-narcoleptic effect of GSK189254 was significantly greater after repeat dosing than after acute dosing, with narcoleptic attacks being quasi-absent for 6 h and their occurrence remaining very rare during the whole lights-off period; that is, the mean number per night of narcoleptic attacks was reduced from 6 to 1 and the total duration from 8 to 2 min (Figure 6).

Autoradiography

Autoradiographic analysis of coronal half brain sections from Ox+/+ and Ox–/– mice revealed extensive specific [^{3}H]-GSK189254 binding (>90%) to H_3 receptors within the cerebral cortex, hippocampus and hypothalamus, while binding levels were negligible following co-incubation with 10 μmol·L^{-1} imetit to define non-specific binding (Figure 7A,

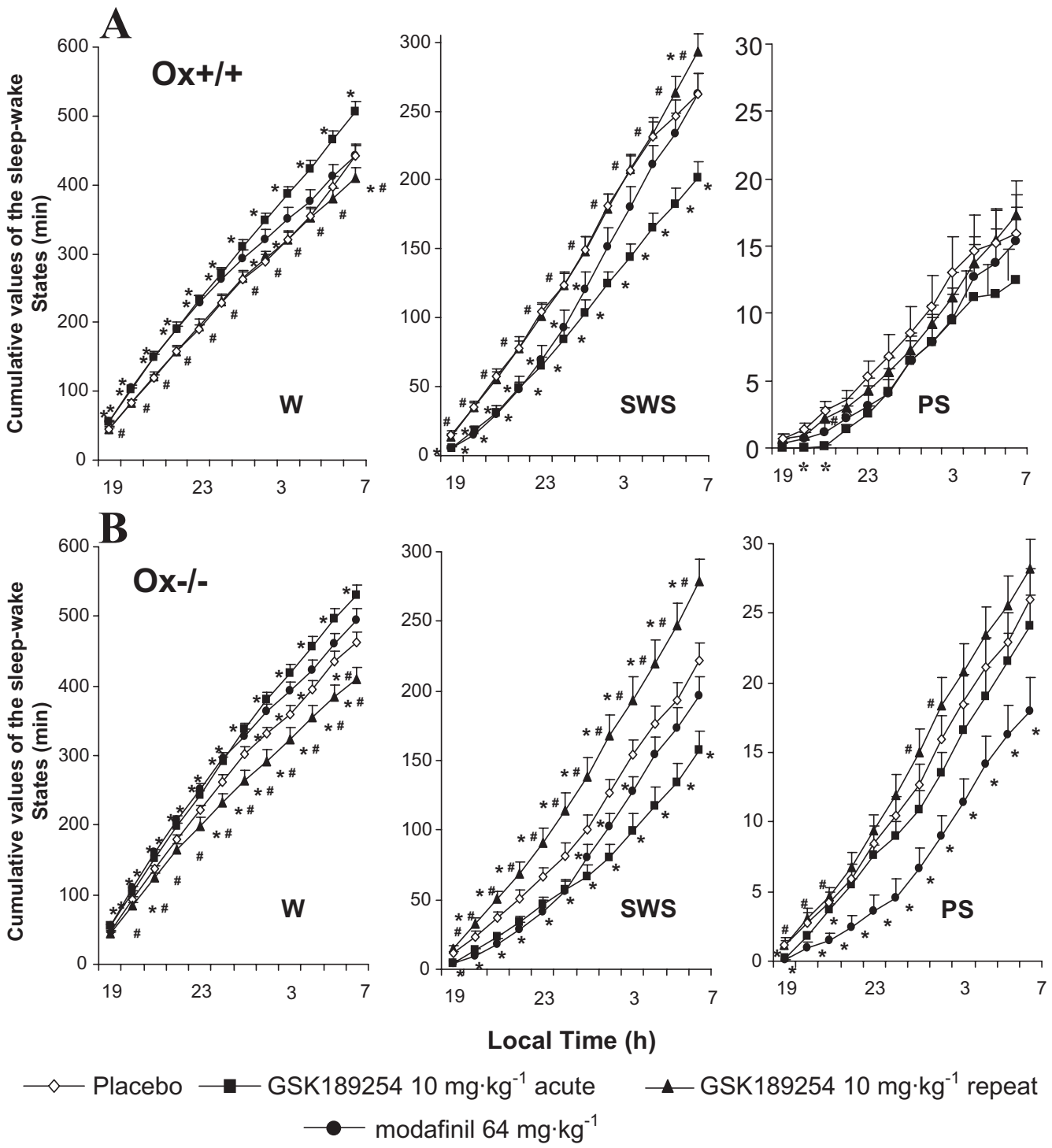

Figure 5 Cumulative effects of acute and repeat administration of GSK189254 and acute administration of modafinil on mean duration of sleep–wake stages over 12 h of the lights-off period in (A) wild-type (Ox+/+) and (B) Ox–/– mice (n = 14 per group). GSK189254 (3 and 10 mg·kg^{-1}) significantly increased wakefulness (W) and decreased slow wave sleep (SWS) and paradoxical sleep (PS) following acute dosing, as did modafinil (64 mg·kg^{-1}). Repeat administration of GSK189254 (10 mg·kg^{-1}, twice daily for 8 days) resulted in a reduction in effects on W, SWS and PS compared with acute dosing with 10 mg·kg^{-1}. *P < 0.05, t-test between groups (compared with vehicle-treated mice) after significant ANOVA. #P < 0.05, t-test between groups (compared with acute 10 mg·kg^{-1} dose group) after significant ANOVA. There was no evidence of sleep rebound and effects of GSK189254 lasted for the 12 h duration of measurements.

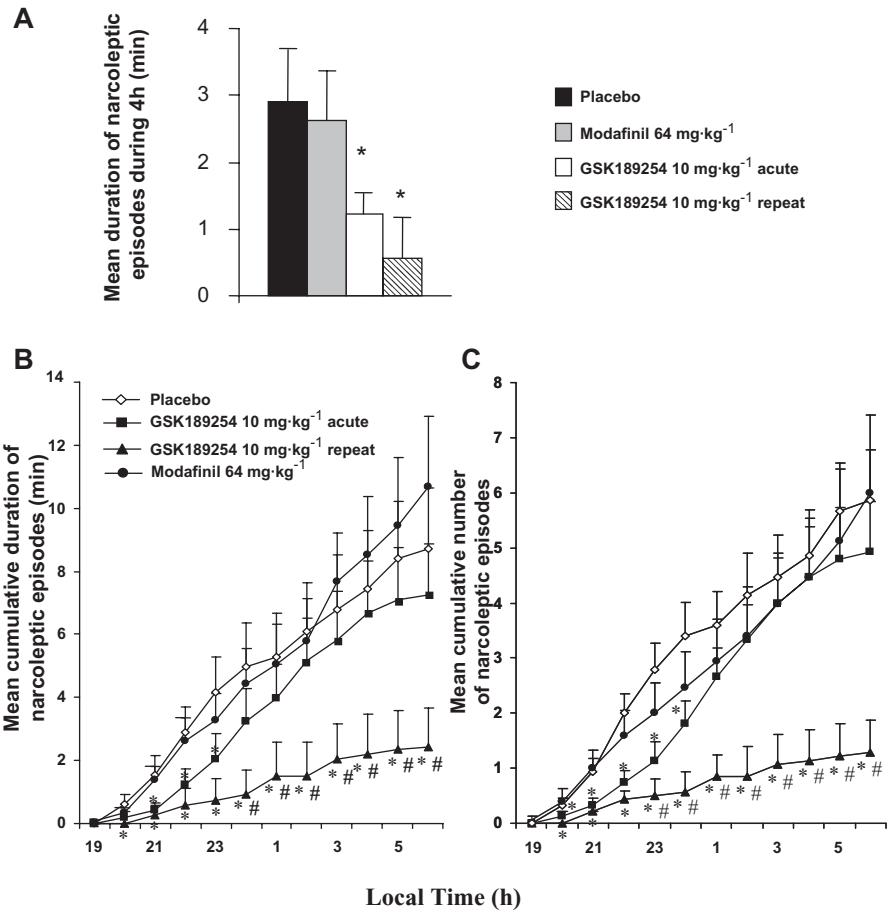

Figure 6 Effects of acute and repeat administration of GSK189254 and effect of acute administration of modafinil on narcoleptic attacks in Ox–/– mice in the lights-off period compared with vehicle-treated mice. (A) Mean (±SEM) number of narcoleptic episodes in the 4 h period after dosing. (B) Mean (±SEM) cumulative duration of narcoleptic attacks and (C) mean (±SEM) cumulative number of narcoleptic attacks over the 12 h lights-off period. *$P < 0.05$, *t*-test between groups (compared with vehicle-treated mice) after significant ANOVA. #$P < 0.05$, *t*-test between groups (compared with acute 10 mg·kg^{-1} dose group) after significant ANOVA.

left panel). Quantification of H_3 binding revealed no significant differences between Ox+/+ and Ox–/– mice in cortex, hippocampus and hypothalamus (Figure 7B, left panel). Autoradiographic analysis in adjacent coronal half brain sections using [^{3}H]-mepyramine showed H_1 receptor expression in similar areas to H_3 receptors but at much lower density (Figure 7A and B, right panels). Binding levels were negligible following co-incubation with 10 μmol·L^{-1} chlorpheniramine to define non-specific binding. No significant differences were observed in H_1 receptor density between Ox+/+ and Ox–/– mice in cortex, hippocampus and hypothalamus ($P > 0.05$; repeated-measures ANOVA).

Saturation binding

H_3 and H_1 receptor saturation binding analysis was carried out in half brain homogenates from Ox+/+ ($n = 3$) and Ox–/– ($n = 4$) brains using [^{3}H]-GSK189254 and [^{3}H]-mepyramine respectively (Figure 7C). Specific binding represented >90% of total binding for both ligands, similar to that observed in autoradiography. Saturation analysis for H_3 receptors with [^{3}H]-GSK189254 in Ox+/+ and Ox–/– mice yielded B_{max} values of 390 ± 21 and 350 ± 17 fmol mg^{-1} respectively, and K_D values of 0.46 ± 0.03 and 0.5 ± 0.04 nmol·L^{-1} respectively, with no significant differences ($P > 0.05$, Student's *t*-test) being observed between the groups. Saturation analysis for H_1 receptors with [^{3}H]-mepyramine in Ox+/+ and Ox–/– mice yielded B_{max} values of 750 ± 107 and 589 ± 85 fmol mg^{-1} respectively and K_D values of 4 ± 1.2 and 3 ± 0.66 nmol·L^{-1} respectively, with no significant differences ($P > 0.05$, Student's *t*-test) being observed between the groups. This lack of significant difference in B_{max} or K_D between Ox+/+ and Ox–/– mice for H_3 and H_1 receptor binding was consistent with the lack of differences in binding observed in autoradiography studies.

Discussion

In the present study, we have identified differential effects of the novel H_3 receptor antagonist GSK189254 following acute and repeat oral administration, in both narcoleptic Ox–/– mice and in wild-type littermates (Ox+/+). While the effects of GSK189254 on the sleep–wake cycle decreased following repeat dosing in Ox+/+ and Ox–/– mice, its effects on narcoleptic episodes in Ox–/– mice increased, compared with acute

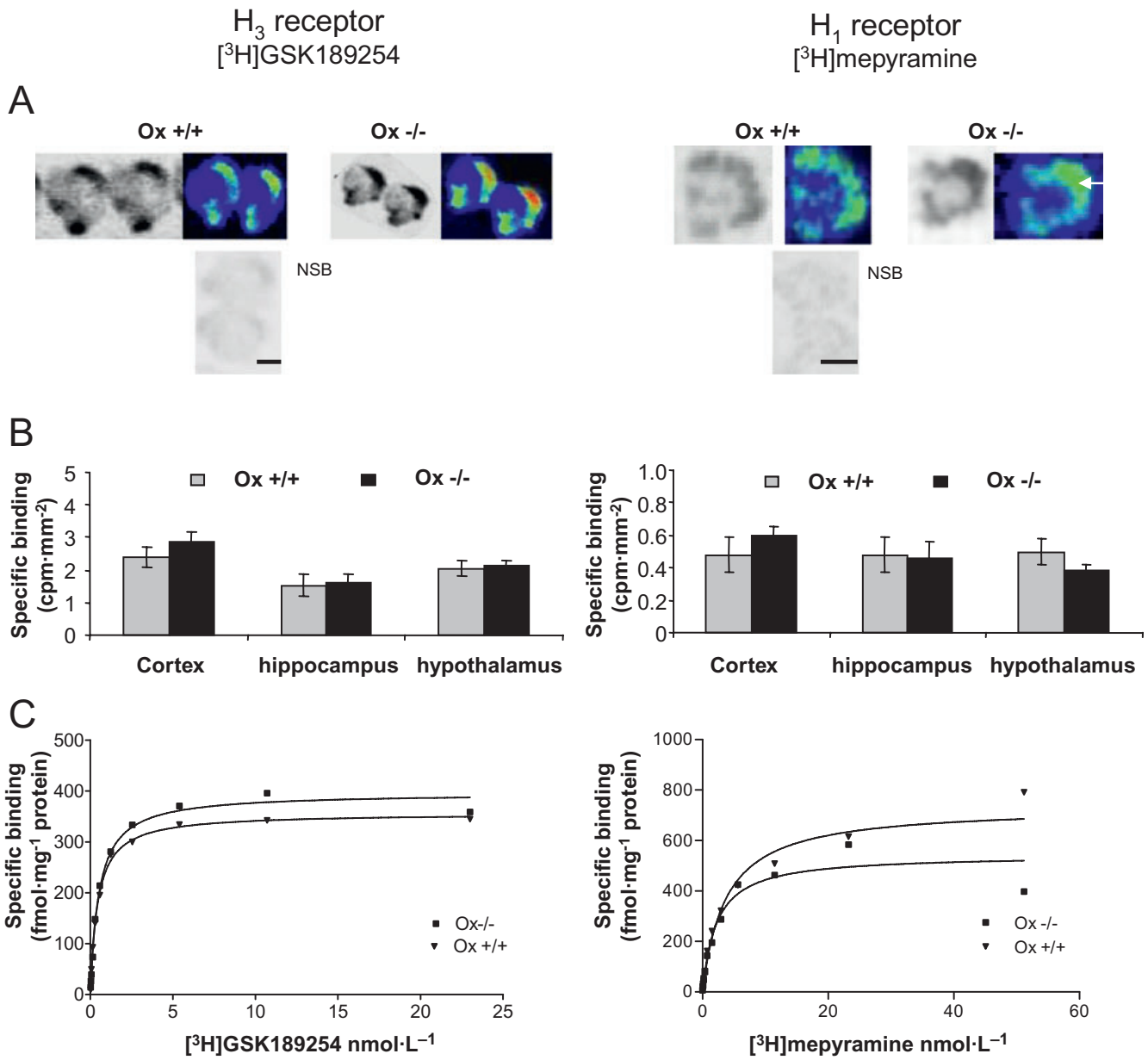

Figure 7 H_3 (left panels) and H_1 (right panels) receptor binding in Ox+/+ and Ox–/– mice measured using [^{3}H]-GSK189254 and [^{3}H]-mepyramine respectively, with real-time autoradiography. (A) Representative pseudo-coloured images of coronal half brain sections showing specific [^{3}H]-GSK189254 and [^{3}H]-mepyramine binding in cortex and hypothalamus of Ox+/+ and Ox–/– mice. Non-specific binding (NSB) for H_3 and H_1 was determined in the presence of 10 μmol·L^{-1} imetit and 10 μmol·L^{-1} chlorphenimramine respectively. Scale bars = 1 mm. (B) Quantitative histograms showing specific [^{3}H]-GSK189254 and [^{3}H]-mepyramine binding in cortex, hippocampus and hypothalamus of Ox+/+ and Ox–/– mice (mean ± SEM, n = 4 per group). No significant differences were observed between Ox+/+ and Ox–/– mice in any of these brain regions. (C) Saturation binding analysis for [^{3}H]-GSK189254 to H_3 receptors and [^{3}H]-mepyramine to H_1 receptors in whole brain of Ox+/+ and Ox–/– mice. Representative curves are shown. No significant differences were observed in mean B_{max} or K_D (n = 3–4 per group) as shown in *Results* section.

administration. These data are important given that several H_3 antagonists/inverse agonists are currently being evaluated in clinical trials for a number of CNS indications including narcolepsy (Celanire *et al.*, 2005; Esbenshade *et al.*, 2008; Lin *et al.*, 2008).

In the present study, acute administration of GSK189254 significantly increased W and decreased SWS and PS in both Ox+/+ and Ox–/– mice consistent with previous data with structurally unrelated H_3 antagonists/inverse agonists, such as BF2.649 (tiprolisant), JNJ-5207852, thioperamide and ciproxifan, in several species, including rats, cats and mice, and also Ox–/– mice (Lin *et al.*, 1990; 2008; Monti *et al.*, 1991; Ligneau *et al.*, 1998; 2007; Barbier *et al.*, 2004; Bonaventure *et al.*, 2007; Parmentier *et al.*, 2007). Evidence accumulated from studies with H_1 antagonists, as well as H_1 and H_3 receptor knockout mice suggest that the increase in W observed with H_3 antagonists is mediated by an increase in histamine release following blockade of H_3 autoreceptors, which then activates

postsynaptic H_1 receptors resulting in increased cortical arousal (Lin *et al.*, 1990; Toyota *et al.*, 2002; Parmentier *et al.*, 2007). The fact that we observed no difference in the effect of GSK189254 on the sleep–wake cycle between Ox+/+ and Ox–/– would suggest that H_3 receptor function is not affected in this model. This is in contrast to another study that suggested that Ox–/– mice were more susceptible to the H_3 antagonist JNJ10181457, although this compound is thought to be a neutral antagonist rather than exhibiting inverse agonist properties (Fujiki *et al.*, 2006; Bonaventure *et al.*, 2007).

It is interesting to note that the doses of GSK189254 required to induce W in the present study (i.e. 3 and 10 $mg \cdot kg^{-1}$) were somewhat higher than those used in cognition studies previously in rats (Medhurst *et al.*, 2007). These doses were known to give ~80% and 90% occupancy respectively in *ex vivo* binding studies in CD1 mice 1–2 h after oral administration where brain concentrations of ~1–3 $\mu mol \cdot L^{-1}$ were achieved (M. Briggs, unpubl. obs.). This suggests that higher H_3 receptor occupancy (>80%) is probably required to induce effects on W than to improve cognition, consistent with recent findings in rats with a number of H_3 antagonists (Le *et al.*, 2008).

The Ox–/– mouse model is not only useful for investigating the effects of drugs on the sleep–wake cycle, but for the assessment of potential anti-narcoleptic agents (Lin *et al.*, 2008). This model displays episodes known as DREMs, which are one of the characteristic symptoms of human narcolepsy (Chemelli *et al.*, 1999; Mignot, 2005; Lin *et al.*, 2008). Previously, it has been reported that modafinil does not alleviate DREMs but increases W in Ox–/– mice (Lin *et al.*, 2008), and we confirmed this in the present study. In contrast to modafinil, acute administration of GSK189254 to Ox–/– mice significantly reduced the number and duration of DREMs, in addition to increasing W. These data are consistent with a previous study with the H_3 inverse agonist BF2.649 (tiprolisant; Lin *et al.*, 2008), and suggests that H_3 antagonists/inverse agonists may potentially offer a dual approach to alleviating narcoleptic attacks and excessive daytime sleepiness in narcoleptics. Other symptoms observed in narcoleptics can include cataplexy and cognitive deficits (Mignot, 2005; Dauvilliers *et al.*, 2007) and recent preclinical data support the possibility that H_3 antagonists might help alleviate these. For example, H_3 antagonists can increase cognitive performance in rats (e.g. Fox *et al.*, 2005; Medhurst *et al.*, 2007; Esbenshade *et al.*, 2008) and reduce cataplexy in narcoleptic dogs (Bonaventure *et al.*, 2007). Thus H_3 antagonists may offer advantages over current therapies, which tend to help to alleviate some but not all symptoms (Billiard *et al.*, 2006; Siegel and Boehmer, 2006).

Recent studies have demonstrated that certain effects of particular H_3 antagonists may be susceptible to tolerance in preclinical species. For example, the effects of ciproxifan on food intake and locomotor activity were decreased following repeat dosing, but the effects of a non-imidazole H_3 antagonist A304121 were maintained (Pan *et al.*, 2006). However, in other studies tolerance has not been observed. For example, efficacious effects of ABT-239 in cognition models (Fox *et al.*, 2005) and of GSK189254 in cognition and pain models were observed after repeat dosing for several days (Medhurst *et al.*, 2007; 2008). While there is a wealth of evidence to support the acute effects of H_3 antagonists on the sleep–wake cycle, to our knowledge there are no previous reports describing the effects of repeat administration of H_3 antagonists. We were therefore keen to investigate the effects of repeat dosing with GSK189254 (twice daily for 8 days) on the sleep–wake cycle in Ox+/+ and Ox–/– mice and DREMs in Ox–/– mice.

To our surprise, the effects of GSK189254 on the sleep–wake cycle (i.e. increased W and decreased SWS and PS) seen after acute administration were significantly reduced following repeat dosing in both Ox+/+ and Ox–/– mice. In addition, in the lights-off phase there was a change in direction of effects of GSK189254 in Ox–/– mice compared with acute dosing. In marked contrast, the beneficial effects of GSK189254 on narcoleptic attacks (i.e. reduction in duration and number of DREMs) in Ox–/– mice were further improved significantly following repeat dosing compared with acute administration. Interestingly, this dramatic effect observed on narcoleptic episodes following repeat dosing with GSK189254 in Ox–/– mice was comparable to the effect observed in the same model with co-administration of BF2.649 and modafinil (Lin *et al.*, 2008).

The divergence of effects observed with GSK189254 on the sleep–wake cycle compared with narcoleptic episodes after repeat dosing is both intriguing and unexpected. It is likely that the W effects are mediated by histaminergic neurons, while narcoleptic episodes may be driven additionally by the noradrenergic system (Nishino *et al.*, 2000; Lin *et al.*, 2008). It could be hypothesized that the W effects of GSK189254 in Ox–/– mice are primarily driven by increased histamine release following autoreceptor blockade, while the anti-narcoleptic effects are driven primarily by the increased monoamine release (most likely noradrenaline) apparent following heteroreceptor blockade. Therefore, it might be possible that heteroreceptors are potentially less susceptible to tolerance than autoreceptors, although complex repeat dose microdialysis studies would be required to investigate this further in these mice.

Further support for this hypothesis is provided by the observations that GSK189254 shows similar efficacy in rat cognition and pain models following single or repeat (up to 8 days b.i.d.) dosing (Medhurst *et al.*, 2007; 2008), in contrast to the reduction in effect on W observed in the present study with repeat dosing compared with acute administration. Efficacy observed with H_3 antagonists in these cognition and pain models is more likely to involve heteroreceptor blockade (e.g. facilitation of acetylcholine release), compared with the sleep–wake cycle when autoreceptor blockade (i.e. facilitation of histamine release) is likely to be important (Passani *et al.*, 2004; Esbenshade *et al.*, 2008). Another possibility is that different H_3 receptor splice variants are involved (Hancock *et al.*, 2003) and that they have different susceptibility to tolerance. However, it is not known which of the described splice variants are autoreceptors and which are heteroreceptors, as well as how each variant contributes to different behavioural responses.

The mechanism of behavioural tolerance observed in the current sleep–wake studies in Ox+/+ and Ox–/– mice is not clear. Further studies are required with other H_3 antagonists to confirm this phenomenon, given that previous reports demonstrating tolerance in other behav-

ioural parameters were compound specific (Pan *et al.*, 2006). It is possible that H_3 receptors are up-regulated following repeat dosing of H_3 antagonists in Ox–/– mice, as previously described in rats (Pan *et al.*, 2006) and wild-type mice (Morisset *et al.*, 2000), although this up-regulation was dependent on brain region measured and the H_3 antagonist investigated. Alternatively, other targets affected by enhanced neurotransmitter release following H_3 receptor blockade, such as H_1 receptors, acetylcholine or dopamine receptors, could be modulated in some way. However, we were not able to investigate this in the current available cohort of mice.

The current results are potentially important for the future clinical investigation of H_3 antagonists not only in narcolepsy, but in other CNS disorders including Alzheimer's disease. While the potential tolerance to the W effects of H_3 antagonists could be a downside for the treatment of narcolepsy, it could be beneficial in Alzheimer's disease where long-term W at night would be highly undesirable. In addition, the increase in effect on narcoleptic episodes could be inferred following repeat dosing. Recently, a small clinical study provided the first evidence for a potential beneficial effect of an H_3 inverse agonist in human narcoleptics after 1 week of dosing (Lin *et al.*, 2008) and the results of further long-term dosing studies are eagerly awaited.

Finally, as part of the present study we investigated H_3 and H_1 receptor binding in Ox–/– mice because there was no information on the status of these receptors in narcolepsy, despite histamine levels being shown to be decreased in human narcoleptics and narcoleptic Dobermans, indicative of a deficit in the histaminergic system (Nishino *et al.*, 2000). Using autoradiography and saturation binding in brain homogenates, we did not observe any differences in [^{3}H]-GSK189254 binding to H_3 receptors or [^{3}H]-mepyramine binding to H_1 receptors between Ox+/+ and Ox–/– mice. This is consistent with a previous report, which eluded to the fact that H_3 receptor mRNA levels in hypothalamus, cortex and hippocampus were not significantly different between Ox+/+ and Ox–/– mice (Lin *et al.*, 2008). In addition, no changes in turnover of histamine and other monamines have been observed in Ox–/– mice previously, and tiprolisant had a similar effect on turnover in Ox+/+ and Ox–/– mice (Lin *et al.*, 2008).

In conclusion, the present study provides further supportive data for the potential use of H_3 antagonists in narcolepsy. However, following repeat dosing, the effects of the H_3 antagonist GSK189254 on W and narcoleptic episodes in Ox–/– mice were differentially affected. Further studies are required to understand the mechanistic basis of these differences and their relevance to human narcolepsy.

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Conflict of interest

A.D.M., J.C.R., S.H.B. and N.U. are employed by GlaxoSmith-Kline.

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