

GABA_B Agonism Promotes Sleep and Reduces Cataplexy in Murine Narcolepsy

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γ-Hydroxybutyrate (GHB) is an approved therapeutic for the excessive sleepiness and sudden loss of muscle tone (cataplexy) characteristic of narcolepsy. The mechanism of action for these therapeutic effects is hypothesized to be GABA_B receptor dependent. We evaluated the effects of chronic administration of GHB and the GABA_B agonist R-baclofen (R-BAC) on arousal state and cataplexy in two models of narcolepsy: *orexin/ataxin-3* (Atax) and *orexin/tTA; TetO diphtheria toxin* mice (DTA). Mice were implanted for EEG/EMG monitoring and dosed with GHB (150 mg/kg), R-BAC (2.8 mg/kg), or vehicle (VEH) bid for 15 d—a treatment paradigm designed to model the twice nightly GHB dosing regimen used by human narcoleptics. In both models, R-BAC increased NREM sleep time, intensity, and consolidation during the light period; wake bout duration increased and cataplexy decreased during the subsequent dark period. GHB did not increase NREM sleep consolidation or duration, although NREM delta power increased in the first hour after dosing. Cataplexy decreased from baseline in 57 and 86% of mice after GHB and R-BAC, respectively, whereas cataplexy increased in 79% of the mice after VEH. At the doses tested, R-BAC suppressed cataplexy to a greater extent than GHB. These results suggest utility of R-BAC-based therapeutics for narcolepsy.

Key words: cataplexy; GABA; hypocretin; narcolepsy; orexin; therapeutics

Introduction

Narcolepsy is characterized by excessive sleepiness, fragmented sleep, short latency to rapid-eye-movement (REM) sleep, and cataplexy—a sudden, emotionally triggered loss of muscle tone—in both humans and animals (Sakurai and Mieda, 2011; Sinton, 2011). Disruption of the hypocretin (Hcrt; orexin) system results in a narcoleptic phenotype (Chemelli et al., 1999; Lin et al., 1999; Nishino et al., 2000; Peyron et al., 2000). Degeneration of ~90% of the Hcrt-producing neurons underlies human narcolepsy (Thannickal et al., 2000) and is recapitulated in *orexin/ataxin-3* (Atax) mice in which the Hcrt cells degenerate postnatally (Hara et al., 2001). A conditional model of Hcrt neuron ablation (*orexin/tTA; TetO diphtheria toxin A* or DTA mice)

has recently been described (Tabuchi et al., 2014). While Atax mice are a model of juvenile onset narcolepsy, DTA mice model the more typical postpubertal onset of narcolepsy. DTA mice exhibit high cataplexy levels and afford the opportunity to study physiology before and after Hcrt cell loss as well as symptom progression.

Current treatments for narcolepsy are symptomatic as no brain-penetrable Hcrt agonists are available. Only γ-hydroxybutyrate (GHB; sodium oxybate) simultaneously alleviates cataplexy, excessive daytime sleepiness, and nocturnal sleep disruption. Although its physiological relevance has been debated (Meerlo et al., 2004; Vienne et al., 2010, 2012), GHB increases slow-wave activity (SWA) during non-REM (NREM) sleep (Van Cauter et al., 1997; Black et al., 2010; Walsh et al., 2010; Boscolo-Berto et al., 2012), which has been hypothesized to mediate its therapeutic effectiveness. GHB is a controlled substance with abuse potential and possible neurotoxic and psychiatric side effects (Langford and Gross, 2011; van Amsterdam et al., 2012) and, due to its short half-life, must be administered in a split dose (Black et al., 2010), all of which underscore the need for improved narcolepsy pharmacotherapeutics. Despite its acute sedating effects, the therapeutic efficacy of GHB only develops after repeated administration and persists after an acute dose has been metabolized (Mamelak, 2009; Boscolo-Berto et al., 2012). The mechanism of action of GHB partially depends on the GABA_B receptor (Brown and Guilleminault, 2011), as GABA_B antagonists block GHB-induced inhibition of neurons (Jensen and Mody, 2001) and the GHB-prodrug γ-butyrolactone fails to produce behavioral or EEG effects in mice lacking GABA_{B1} and GABA_{B2} subunits (Kaupmann et al., 2003; Vienne et al., 2010).

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In the present study, we compared the efficacy of GHB and *R*-baclofen (*R*-BAC) in Atax and DTA mouse models in a chronic dosing paradigm. After a preliminary dose–response study established optimal doses, GHB and *R*-BAC were administered bidaily for 15 d during the rest phase to model the twice nightly dosing regimen used by narcolepsy patients. Arousal state variables were compared among GHB, *R*-BAC, and vehicle treatment groups in both mouse models. Chronic dosing with *R*-BAC promoted NREM sleep time, intensity, and consolidation during the light period, longer bouts of wakefulness during the dark period, and sustained suppression of REM sleep and cataplexy. GHB modestly reduced cataplexy and increased sleep intensity, but did not improve sleep/wake consolidation nor increase NREM sleep time.

Materials and Methods

Animals. Male, hemizygous transgenic C57BL/6-Tg(orexin/ataxin-3)/Sakurai mice (Atax), wild-type (WT) littermates, and transgenic C57BL/6-Tg(orexin/tTA; TetO diphtheria toxin A fragment)/Yamanaka (DTA) mice bred in our colony at SRI International were used. Mice from the Atax colony were backcrossed >10 generations on a C57BL/6 background. DTA mice were the double transgenic offspring of *orexin/tTA* mice (C57BL/6-Tg(orexin/tTA)G5/Yamanaka), which express the tetracycline transactivator exclusively in hypocretin neurons (Tabuchi et al., 2013) and mice that express diphtheria toxin A fragment in the absence of dietary doxycycline (B6.Cg-Tg(tetO-DTA)1Gfi/J). Both parental strains were obtained from the Yamanaka laboratory (Nagoya University, Japan), and were from a C57BL/6J genetic background. DTA mice underwent 29 d of dietary doxycycline withdrawal to induce ~97% Hcrt cell loss before experimental recording (Tabuchi et al., 2014). Ten Atax mice and 1 DTA mouse (21% of mice recorded) exhibited <3 bouts of cataplexy during 24 h baseline and were excluded from analysis. All experimental procedures were approved by the Institutional Animal Care and Use Committee at SRI and were conducted in accordance with the principles set forth in the Guide for Care and Use of Laboratory Animals, National Research Council.

Surgery. Mice (31 ± 0.8 g, aged 15 ± 0.4 weeks) were prepared for sterile surgical implantation of biotelemetry transmitters (F20-EET; Data Sciences) for chronic recording of EEG, EMG, core body temperature (T_b) and gross motor activity as previously described (Black et al., 2013). Briefly, mice were anesthetized with isoflurane and sterile transmitters were placed intraperitoneally along the midline. Biopotential leads were routed subcutaneously to the head, and EMG leads were positioned bilaterally through the nuchal muscles. Cranial holes were drilled 1 mm anterior to bregma and 1 mm lateral to midline and, contralaterally, 2 mm posterior to bregma and 2 mm lateral to midline. EEG leads were placed subcranially over the dura and were attached to the skull with cyanoacrylate and dental acrylic.

Arousal state recording. Mice were permitted at least 3 weeks postsurgical recovery and at least 1 week adaptation to running wheels, handling, and dosing procedures before data collection. Throughout the study, mice were housed individually in home cages with access to food, water, and running wheels *ad libitum*. Room temperature ($22 \pm 2^\circ\text{C}$), humidity ($50 \pm 20\%$ relative humidity), and lighting conditions (12 h light/dark) were monitored continuously via computer. Animals were inspected daily in accordance with Association for Assessment and Accreditation of Laboratory Animal Care and SRI guidelines, and body weights were taken during cage changes or immediately before dosing. Physiological data and video-recorded behavioral data were simultaneously acquired with DataQuest Art 4.2 software (Data Sciences). EEG and EMG were sampled at 250 Hz. Digital videos were recorded at 10 frames per second, 4CIF de-interlacing resolution.

Drugs. GHB (sodium oxybate oral solution 500 mg/ml; Orphan Medical) and powder (Lonza) were diluted in sterile 0.9% saline to concentrations of 8.75, 17.5, and 26.25 mg/ml. *R*-BAC ((*R*)-4-Amino-3-(4-chlorophenyl) butanoic acid; Tocris Bioscience) was diluted in sterile 0.9% saline to concentrations of 0.49, 0.98, and 1.96 mg/ml. All doses were delivered intraperitoneally at 5.7 ml/kg final volume.

Experimental protocol 1: dose–response study. Atax and WT mice ($n = 10$ per genotype) were dosed intraperitoneally in a counterbalanced crossover design with GHB (50, 100, and 150 mg/kg; Orphan Medical), *R*-BAC (2.8, 5.6, and 11.2 mg/kg), or saline vehicle (VEH). Treatments were administered every 3 d at Zeitgeber Time 2 (ZT2) and ZT6 to model the twice nightly GHB dosing regimen used by patients with narcolepsy (Black et al., 2010). Physiological data and video-recorded behavioral data were collected from ZT0–ZT12 on each dosing day.

Experimental protocol 2: chronic dosing study. Atax and DTA mice were dosed intraperitoneally in a two-way mixed (between-within subjects) design with GHB (150 mg/kg), *R*-BAC (2.8 mg/kg), or VEH; $n = 6–8$ per group per mouse model. GHB administered to Atax mice was diluted from sodium oxybate oral solution (Orphan Medical), and GHB received by DTA mice came from powdered stock (Lonza). After a baseline recording, mice were dosed at ZT2 and ZT6 daily for 15 d. The mean (\pm SE) time between baseline and day 1 of chronic dosing was 1.6 ± 0.3 weeks. Physiological data and video-recorded behavioral data were collected for 24 h beginning at ZT0 during undisturbed baseline and on chronic dosing day 15.

Data analysis. Data were manually scored in 10 s epochs by experts ($\geq 96\%$ inter-rater reliability) using NeuroScore 2.1 (Data Sciences). Epochs were classified as wakefulness (mixed-frequency, low-amplitude EEG and high-amplitude, variable EMG); wakefulness with wheel-running, REM sleep (theta-dominated EEG and EMG atonia); NREM sleep (low-frequency, high-amplitude EEG and low-amplitude, steady EMG); or cataplexy. Criteria for cataplexy were ≥ 10 s of EMG atonia, theta-dominated EEG, and video-confirmed behavioral immobility preceded by ≥ 40 s of W (Scammell et al., 2009). Some doses of GHB and *R*-BAC were associated with hypersynchronous slow waves and/or spike and wave discharge patterns in the EEG that could not be classified as any previously defined arousal state (Snead, 1984; Meerlo et al., 2004; Koek et al., 2007; Vienne et al., 2010). This drug-induced state occurred both before and following bouts of NREM sleep; began within the first hour after dosing; and was scored as a state distinct from wakefulness, NREM, or REM sleep or cataplexy. Only epochs with ≥ 5 s of the drug-induced state were scored as such; mixed-state epochs that contained <5 s of the drug-induced state were marked for exclusion from spectral analysis.

Data were analyzed as time spent in each scored classification per hour. Cumulative time spent in wakefulness, NREM, and REM sleep, or the drug-induced state was calculated over the entire recording period. Latency to NREM sleep onset was calculated from the time of injection at ZT6 to the first three continuous epochs of NREM. To assess sleep intensity, EEG spectra during NREM sleep were computed using the fast Fourier transform algorithm in NeuroScore (Data Sciences) on all 10 s epochs without visually detectable artifact. EEG delta power (0.5–4 Hz) in NREM sleep (NRD) was then calculated in hourly bins. In the dose–response study, NRD for each condition was normalized against the mean NRD value obtained during the 12 h VEH recording for each individual (unitless ratio). In the chronic dosing study, NRD for each group was normalized for each individual as the percentage of the mean NRD value obtained during the baseline recording. For full spectral analysis of the NREM EEG, data were normalized as the percentage of baseline values for each individual per each 0.12 Hz bin. To evaluate sleep/wake consolidation, the duration and number of bouts for each state were calculated. A bout of NREM sleep or wakefulness consisted of a minimum of two consecutive 10 s epochs of the specified state and was terminated by the occurrence of a single epoch of a different state. The distribution of NREM sleep bouts as a percentage of NREM sleep time was assessed during the last half of the light period, and the distribution of wake bouts as a percentage of time spent awake was examined during the first half of the dark period. Cataplexy density was defined as the number of cataplexy bouts per hour of wakefulness.

Statistical analysis. All statistical tests were performed using SigmaPlot 12.5 (Systat Software). For the dose–response study, hourly cumulative data between drug conditions were compared using two-way repeated measures (RM) ANOVA on factors “drug dose” and “hour.” Sleep latencies after different drug doses were evaluated by one-way RM-ANOVA. For the chronic dosing study, hourly cumulative data on dosing day 15 were expressed as the change from baseline and were compared between

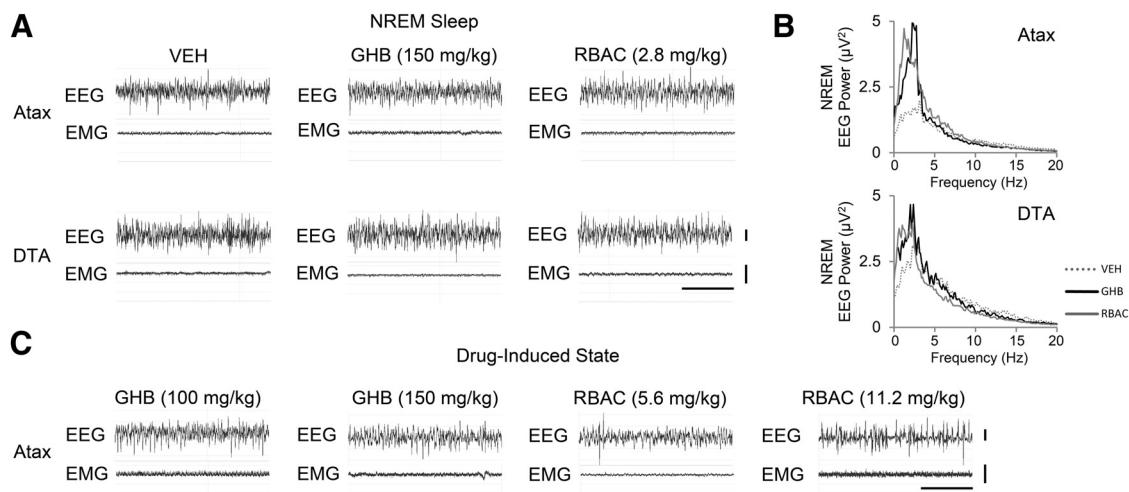


Figure 1. GHB and *R*-BAC occasionally induced hypersynchronous slow waves and/or a spike and wave discharge pattern that was distinct from NREM sleep. **A**, Representative NREM EEG and EMG traces in Atax and DTA transgenic mice ~1 h after dosing with VEH, GHB, or *R*-BAC (each trace from a different mouse). **B**, NREM EEG power density for VEH, GHB, or *R*-BAC corresponding to **A**. **C**, Representative EEG and EMG traces from a single Atax mouse during the drug-induced state within 1 h after dosing with GHB and *R*-BAC. Calibration: vertical bar, 200 μV; horizontal bar, 10 s.

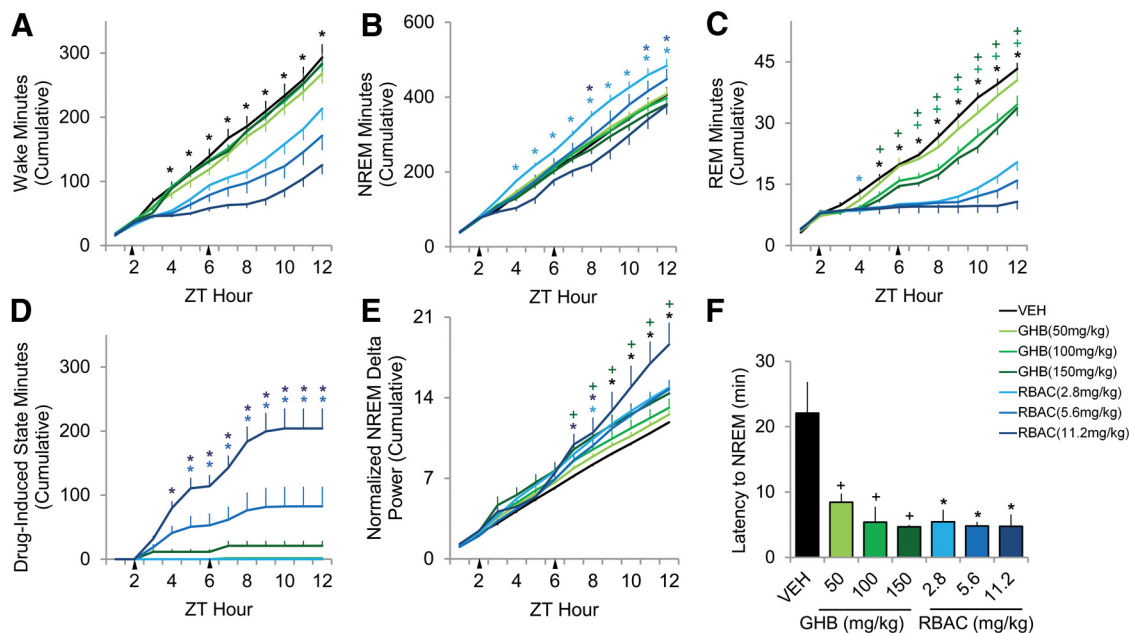


Figure 2. Cumulative minutes of wakefulness (**A**), NREM sleep (**B**), REM sleep (**C**), drug-induced state (**D**), and accumulated NREM delta power (**E**) after GHB, *R*-BAC, or VEH administered 2 h after light onset (ZT2) and ZT6 (triangles) in Atax mice (*n* = 10). Latency to NREM sleep (**F**) after dosing at ZT2. Data are mean ± SEM; **p* < 0.05 VEH versus *R*-BAC (light blue = 2.8 mg/kg; medium blue = 5.6 mg/kg; dark blue = 11.2 mg/kg; black = all doses); +*p* < 0.05 VEH versus GHB (medium green = 100 mg/kg; dark green = 150 mg/kg; black = all doses).

drug groups using two-way mixed-model ANOVA on factors “drug group” (between subjects) and “hour” (within subjects). NREM delta power, arousal state bout distributions, body weights, T_b , wheel running, and motor activity were compared between drug groups and recording days (within genotypes) with two-way mixed-model ANOVA. These variables were compared between genotypes and drug groups using two-way ANOVA. Time spent in cataplexy and cataplexy bout duration during the dark period on dosing day 15, expressed as the change from baseline, were compared between drug groups and narcoleptic mouse models using two-way ANOVA. When ANOVA indicated significance, contrasts between relevant factor levels were detected with *post hoc* Bonferroni *t* tests with α = 0.05. Because of the high variability in baseline cataplexy amounts between narcoleptic mouse models and individual mice, ANCOVA was used to compare changes in cataplexy density on dosing day 15 between drug groups on data pooled between mouse

models. Linear regressions were performed on the pooled data for each drug group to determine the relationship between baseline cataplexy density and the therapeutic response (cataplexy density change on dosing day 15). The proportions of mice that responded to each drug with an increase or decrease in cataplexy density were evaluated with a χ^2 test.

Results

Dose–response study

To determine the optimal sleep-promoting doses of GHB and *R*-BAC for use in the bi-daily chronic dosing study, an acute dose–response study was performed during the light period. GHB and *R*-BAC doses were chosen based on previous work as doses expected to enhance NREM SWA with minimal intrusion

of the hypersynchronous slow waves and/or spike and wave discharge EEG patterns that are indicative of an abnormal, drug-induced state (Snead, 1984; Meerlo et al., 2004; Koek et al., 2007; Vienne et al., 2010). Representative EEG and EMG traces during NREM sleep and the drug-induced state are depicted in Figure 1. NREM sleep observed ~1 h after GHB (150 mg/kg), R-BAC (2.8 mg/kg), and VEH appeared similar between Atax and DTA models (Fig. 1A). The NREM EEG power density during the hour from which the traces in Figure 1A were taken (and from the same individual mice per drug group; Fig. 1B) indicate that R-BAC (2.8 mg/kg) and GHB (150 mg/kg) increase NREM EEG power in the 0.5–4 Hz range (delta frequency band). EEG traces that contained highly synchronous waveforms in the slowest frequency range, and were often interspersed with spike and wave discharge patterns after high doses of R-BAC, were designated as a drug-induced state. Examples of the drug-induced state from a single Atax mouse within the first hour after GHB (100 and 150 mg/kg) and R-BAC (5.6 and 11.2 mg/kg) are shown in Figure 1C for comparison with the NREM EEG trace from this mouse after GHB (150 mg/kg) in Figure 1A. The drug-induced state occurred in a drug- and dose-related manner. The mean \pm SEM percentages of the 12 h recording that Atax mice spent in the drug-induced state after GHB (100 mg/kg), GHB (150 mg/kg), R-BAC (5.6 mg/kg), and R-BAC (11.2 mg/kg) were 0.2 ± 0.16 , 2.9 ± 1.1 , 11.5 ± 4.3 , and 28.3 ± 4.4 , respectively. The drug-induced state was not observed after GHB (50 mg/kg) or R-BAC (2.8 mg/kg).

The effects of GHB (50, 100, and 150 mg/kg) and R-BAC (2.8, 5.6, and 11.2 mg/kg) in Atax mice on the cumulative time spent awake, in NREM, and REM sleep, in the drug-induced state, on NREM delta power and NREM sleep latency are shown in Figure 2A–F. In Atax mice, R-BAC decreased the time spent awake in a dose-related manner, whereas GHB at all doses left the cumulative time awake unchanged from VEH (Fig. 2A; $F_{(66,839)} = 26.39$, $p < 0.001$). Only R-BAC (2.8 mg/kg) consistently increased the cumulative time spent in NREM sleep from ZT4 to ZT12; GHB did not increase NREM sleep time at any dose (Fig. 2B; $F_{(66,839)} = 6.62$, $p < 0.001$). However, GHB at the highest doses and R-BAC at all doses suppressed REM sleep for at least 6 h following the second injection (Fig. 2C; $F_{(66,839)} = 48.36$, $p < 0.001$). R-BAC at the highest doses produced a profound appearance of the abnormal, drug-induced state (Fig. 2D; $F_{(66,839)} = 19.93$, $p < 0.001$). This state was not significantly induced by R-BAC (2.8 mg/kg) nor GHB at any dose

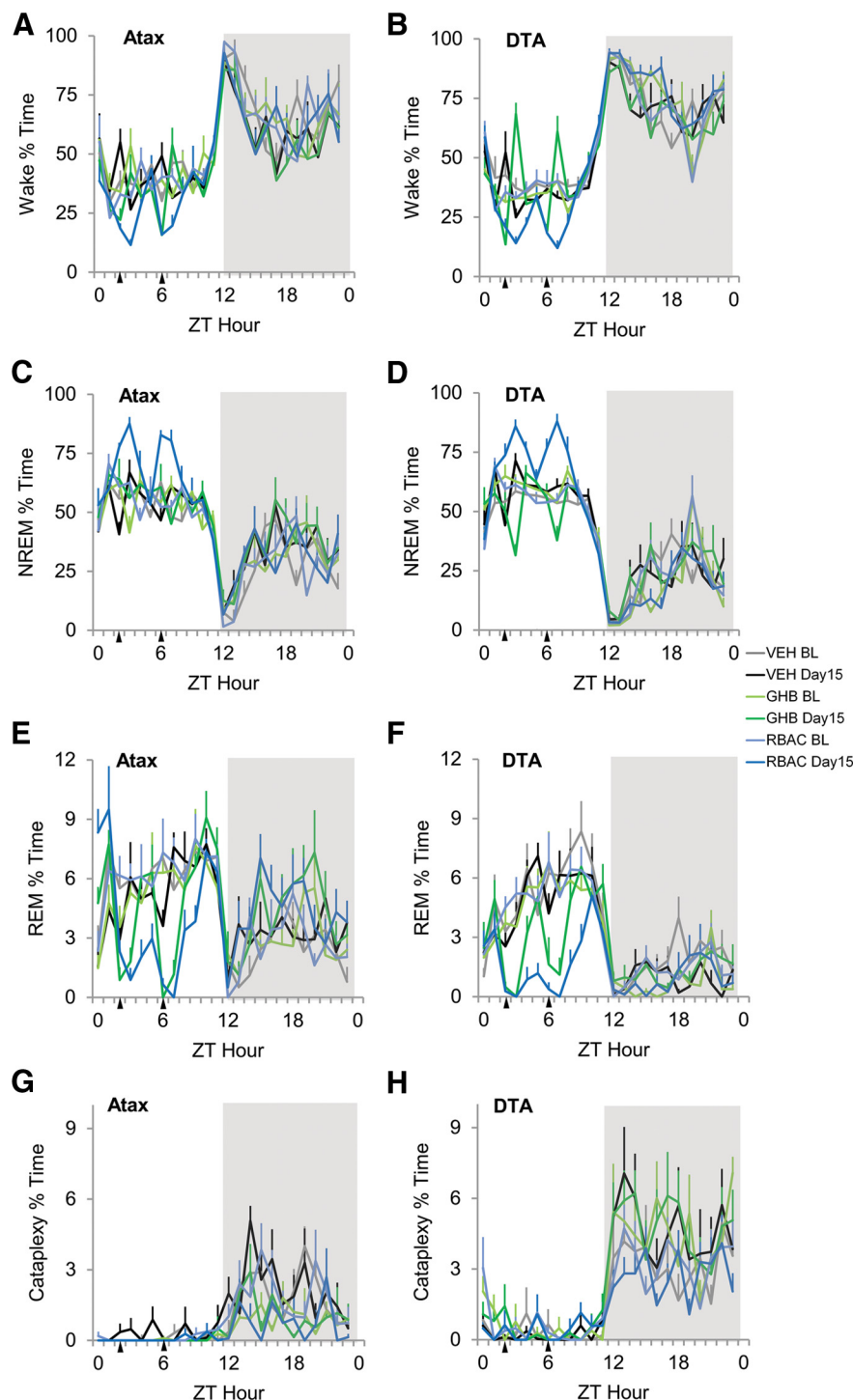


Figure 3. Percentage time spent awake (A, B), in NREM sleep (C, D), in REM sleep (E, F), and in cataplexy (G, H) on day 15 of chronic dosing with GHB (150 mg/kg), R-BAC (2.8 mg/kg), or VEH versus baseline in Atax and DTA mice ($n = 6–8$ /drug group per model). GHB, R-BAC, or VEH were administered 2 h after light onset (ZT2) and ZT6 (triangles). Data are the mean \pm SEM. Statistical comparisons of these data are shown as the cumulative change from baseline in Figures 4 and 7.

compared with VEH. NREM delta power increased after GHB (150 mg/kg), R-BAC (5.6 mg/kg), and the low dose of R-BAC (Fig. 2E; $F_{(66,839)} = 7.58$, $p < 0.001$). NREM sleep latency decreased after all GHB and R-BAC doses (Fig. 2F; $F_{(6,69)} = 6.49$, $p < 0.001$). Thus, based on the increased NREM sleep time after the low dose of R-BAC, the increased NREM delta power after the high dose of GHB, the reduction in NREM sleep latency, and the nonsignificant induction of the drug-induced state at these doses,

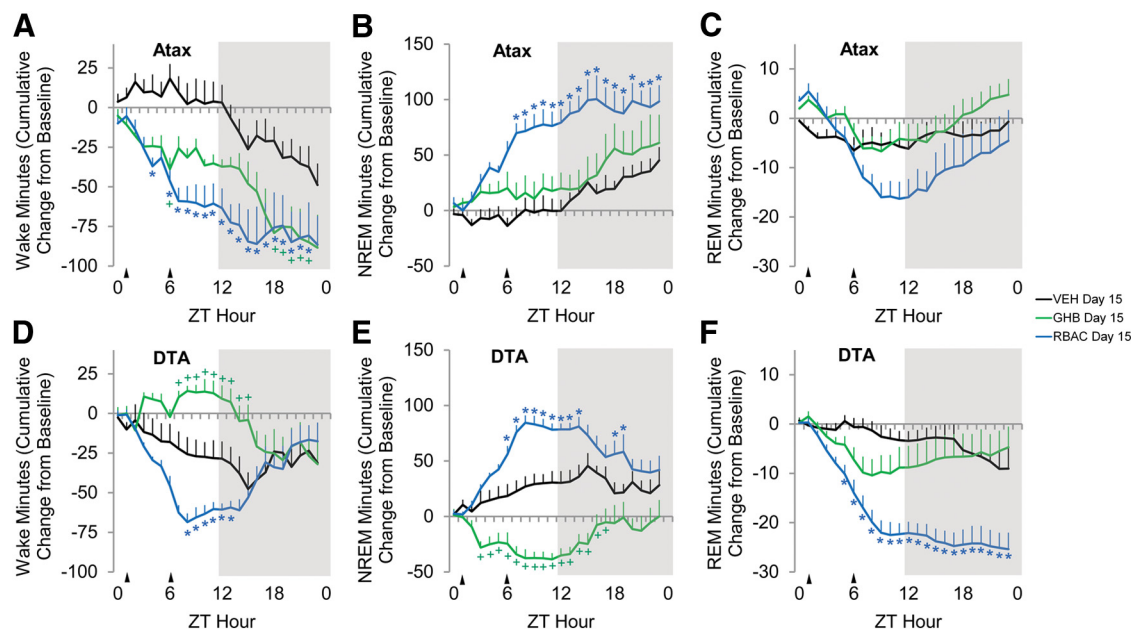


Figure 4. *R*-BAC promoted NREM sleep during the light period with sustained suppression of REM sleep. Minutes of wakefulness (**A**, **D**), NREM sleep (**B**, **E**), and REM sleep (**C**, **F**) on day 15 of chronic dosing with GHB (150 mg/kg), *R*-BAC (2.8 mg/kg), or VEH plotted as the cumulative change from baseline in Atax and DTA mice ($n = 6–8$ /drug group per model). Positive slopes indicate increased time in state relative to baseline. GHB, *R*-BAC, or VEH were administered at ZT2 and ZT6 (triangles). Data are mean \pm SEM; * $p < 0.05$ *R*-BAC versus VEH; + $p < 0.05$ GHB versus VEH. For data from which these plots were derived, see Figure 3.

GHB (150 mg/kg) and *R*-BAC (2.8 mg/kg) were chosen for the chronic dosing study.

Chronic dosing study

Effects on sleep/wake time

R-BAC promoted NREM sleep during the light period in both mouse strains with sustained suppression of REM sleep (Figs. 3, 4). Atax and DTA mice treated with GHB (150 mg/kg), *R*-BAC (2.8 mg/kg), or VEH were compared separately by genotype for effects on the time spent awake (Fig. 4*A*, *D*), in NREM sleep (Fig. 4*B*, *E*), or in REM sleep (Fig. 4*C*, *F*) over the 24 h of dosing day 15 (expressed as the cumulative change from baseline) using two-way mixed-model ANOVA. After *R*-BAC, wakefulness declined during the light period in Atax ($F_{(46,455)} = 1.77$, $p = 0.002$) and DTA ($F_{(46,551)} = 1.77$, $p < 0.001$) mice while NREM sleep time increased (Atax: $F_{(46,455)} = 3.18$, $p < 0.001$; DTA: $F_{(46,551)} = 9.98$, $p < 0.001$). During the dark period, NREM sleep time stopped accumulating (Fig. 4*B*, *E*), while wakefulness resumed in Atax mice and increased in DTA mice (Fig. 4*A*, *D*). GHB did not increase NREM sleep time during the light period at this dose in either mouse model. Surprisingly, GHB-induced wakefulness during the second hour after dosing (Figs. 3*A*, *B*, 4*A*, *D*) and compensatory NREM sleep did not occur until the dark period. After *R*-BAC, REM sleep decreased relative to baseline and remained suppressed well into the dark period, particularly in DTA mice (Atax: $F_{(46,455)} = 3.82$, $p < 0.001$; DTA: $F_{(46,551)} = 137.12$, $p < 0.001$). Of the 14 mice treated chronically with GHB (150 mg/kg), 11 exhibited the drug-induced state, which accounted for 3.9% of the light period. Chronic *R*-BAC (2.8 mg/kg) produced the drug-induced state in 2 of 14 mice studied, for 0.35% of the recording time.

The NREM EEG power spectra of Atax and DTA mice under the influence of GHB and *R*-BAC showed subtle differences across the 0–60 Hz range. Normalized NREM EEG power from the first 6 h (ZT7–Z12) following dosing with GHB (150 mg/kg), *R*-BAC (2.8 mg/kg), or VEH on day 15 in Atax and DTA mice

were assessed (Fig. 5). In both mouse models, GHB and *R*-BAC increased NREM delta power (0.5–4 Hz). Although we did not test for bin-by-bin statistical significance, *R*-BAC appeared to cause a greater increase in NREM EEG power than GHB (Fig. 5*A*, *B*).

Effects on sleep intensity and consolidation

R-BAC intensified and consolidated sleep during the light period, which resulted in longer bouts of wakefulness during the subsequent dark period (Fig. 6). Atax and DTA mice treated with GHB (150 mg/kg), *R*-BAC (2.8 mg/kg), or VEH were compared separately by genotype on NREM delta power (Fig. 6*A*, *D*), the distribution of NREM sleep bout durations (Fig. 6*B*, *E*) during the last half of the light period, and the distribution of wake bouts (Fig. 6*C*, *F*) during the first half of the dark period on dosing day 15 versus baseline using two-way mixed-model ANOVA. NREM delta power increased after *R*-BAC and GHB in Atax ($F_{(25,227)} = 5.80$, $p < 0.001$) and DTA ($F_{(25,238)} = 7.72$, $p < 0.001$) mice. The increase in NREM delta power was sustained longer after *R*-BAC than GHB in both models. *R*-BAC increased the proportion of NREM sleep time spent in long sleep bouts (i.e., ≥ 8 min) and decreased the proportion of short sleep bouts (< 4 min) in Atax ($F_{(35,303)} = 4.31$, $p < 0.001$) and DTA ($F_{(35,367)} = 4.44$, $p < 0.001$) mice. Consequently, during the next 6 h in the dark period, *R*-BAC increased the proportion of wake time spent in wake bouts longer than 16 min (Atax: $F_{(35,303)} = 2.19$, $p < 0.001$; DTA: $F_{(35,367)} = 1.53$, $p = 0.033$). In contrast, GHB did not appreciably affect sleep/wake consolidation in either mouse model.

Effects on cataplexy

R-BAC reduced the time spent in cataplexy and cataplexy density to a greater extent than GHB (Fig. 7). During baseline, DTA mice spent 2.5 \times as much time in cataplexy as Atax mice, but both models showed the most cataplexy during the dark period (Fig. 7*A*). Atax and DTA mice treated with GHB (150 mg/kg), *R*-BAC (2.8 mg/kg), or VEH were compared on time spent in cataplexy

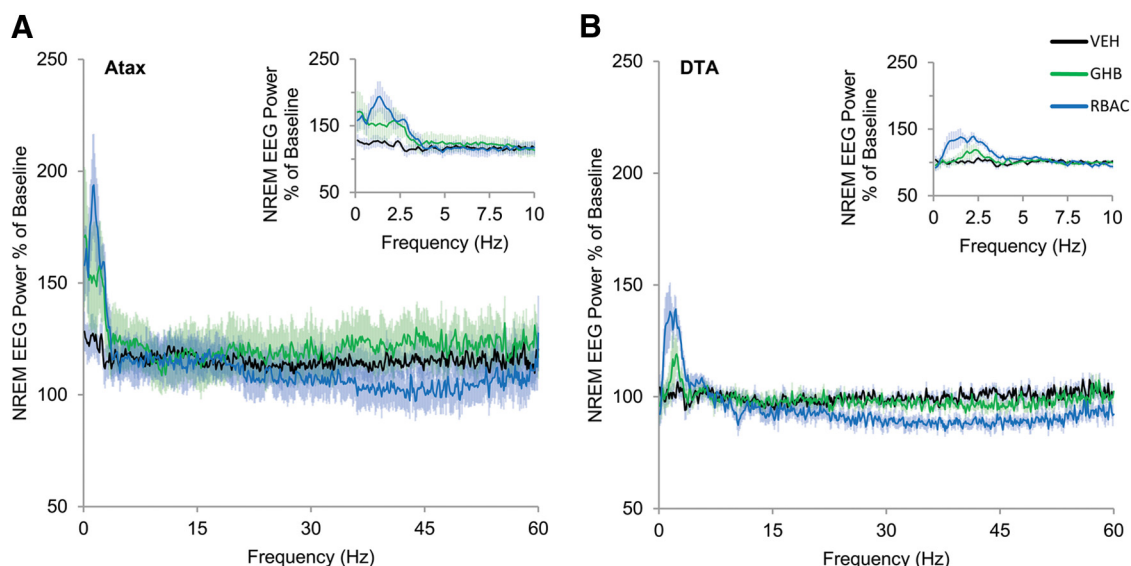


Figure 5. NREM EEG spectral power in Atax (**A**) and DTA (**B**) mice during the second half of the light period after GHB (150 mg/kg), *R*-BAC (2.8 mg/kg), or VEH. Insets, NREM EEG power spectra from 0.49–10 Hz. Data are the mean \pm SEM ($n = 6$ –7/drug group per model).

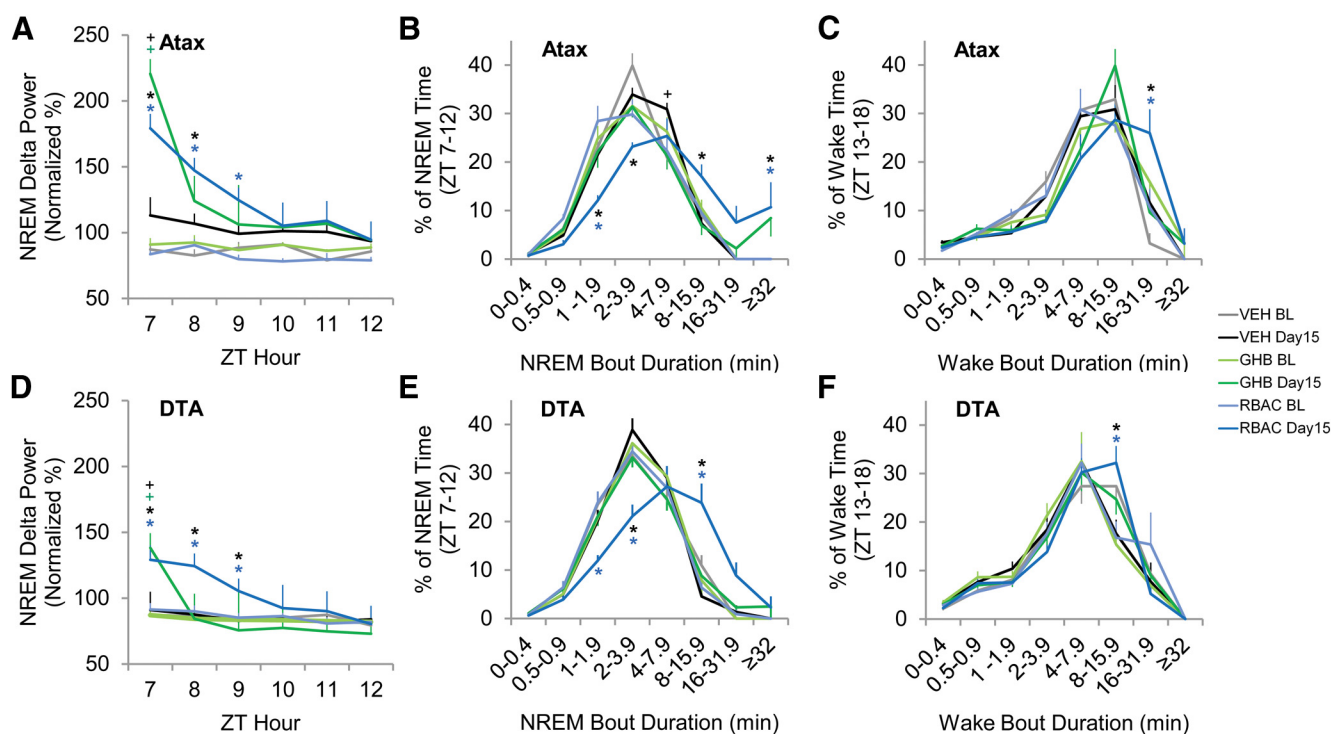


Figure 6. *R*-BAC increased NREM sleep intensity and consolidation during the light period and subsequently lengthened wake bouts during the dark period. NREM delta power (**A**, **D**) and distribution of NREM sleep bout durations by proportion of NREM sleep time (**B**, **E**) during the last 6 h of the light period on day 15 of chronic dosing with GHB (150 mg/kg), *R*-BAC (2.8 mg/kg), or VEH versus baseline (BL) in Atax and DTA mice ($n = 6$ –8/drug group per model). Distribution of wake bout durations by proportion of time spent awake (**C**, **F**) during the first 6 h of the dark period on dosing day 15. Data are mean \pm SEM. $p < 0.05$: black asterisk = *R*-BAC day 15 versus VEH day 15; blue asterisk = *R*-BAC day 15 versus *R*-BAC BL; black + = GHB day 15 versus VEH day 15; green + = GHB day 15 versus GHB BL.

during the dark period on dosing day 15 (expressed as the change from baseline) using two-way ANOVA (Fig. 7B). In both mouse models, time spent in cataplexy decreased after *R*-BAC and increased after VEH (main effect on factor drug group, $F_{(2,41)} = 5.84$, $p = 0.006$); however, there was no significant interaction between factors drug group and mouse model. Despite cataplexy bout duration differences between mouse models during baseline (Atax: 1.1 ± 0.08 min, DTA: 0.7 ± 0.07 min, two-tailed $t_{(40)} =$

3.42, $p < 0.05$), there was no significant interaction between factors drug group and mouse model in cataplexy bout duration on dosing day 15 (expressed as the change from baseline). Because both mouse models appeared to respond similarly to drug treatments, and because individual mice in the different mouse models show high variability in cataplexy amounts during baseline, the cataplexy data from both models were pooled for ANCOVA with baseline cataplexy density as the covariate with

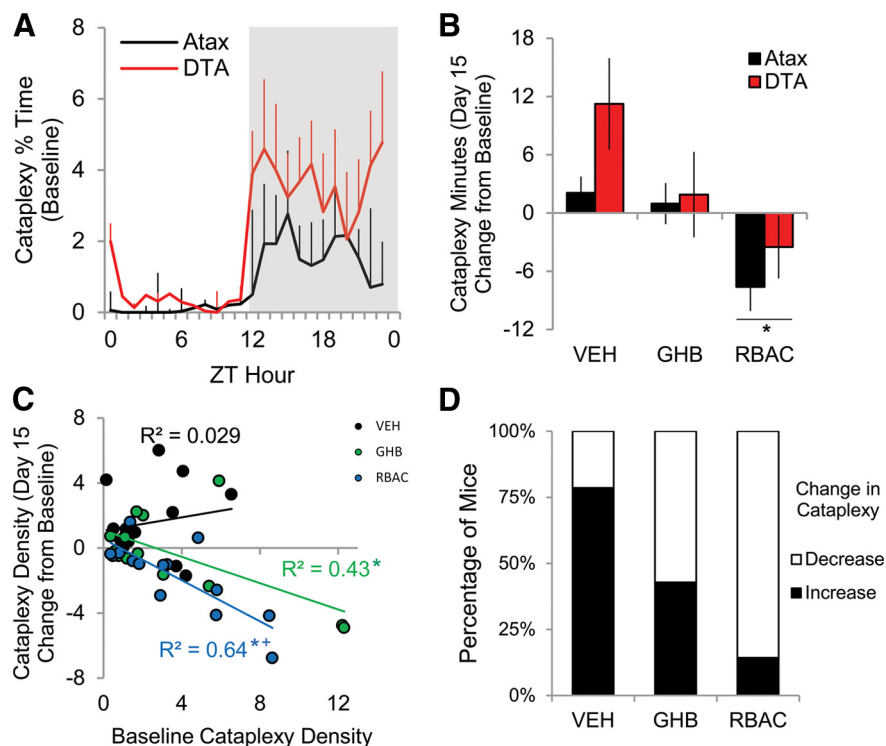


Figure 7. R-BAC reduced time in cataplexy and cataplexy density. **A**, Cataplexy time during 24 h baseline in Atax ($n = 19$) and DTA ($n = 23$) mice. **B**, Cataplexy time during the dark period on day 15 of chronic dosing with GHB (150 mg/kg), R-BAC (2.8 mg/kg), or VEH in Atax and DTA mice ($n = 6$ –8/drug group per model); $*p < 0.05$ versus VEH. For data from which these plots were derived, see Figure 3. **C**, Cataplexy density (number of cataplexy bouts per hour of wakefulness) during the dark period on baseline versus dosing day 15. Data pooled between mouse models ($n = 14$ per drug group). Linear regression: $*p < 0.05$; ANCOVA equality of intercepts: $^+p < 0.05$ versus GHB. **D**, Percentage of mice from both models that showed an increase or decrease in cataplexy density on dosing day 15 versus baseline; $\chi^2, p < 0.001$.

factor drug group (Fig. 7C). R-BAC was associated with a greater reduction in cataplexy density during the dark period on dosing day 15 than GHB, regardless of baseline cataplexy levels ($F_{(1,25)} = 4.3, p = 0.049$). In addition, linear regressions indicated a positive correlation between baseline cataplexy density and therapeutic responses for both GHB ($R^2 = 0.43$) and R-BAC ($R^2 = 0.64$). Figure 7D shows that the proportion of mice from both mouse models that decreased cataplexy density during the dark period after R-BAC on dosing day 15 (86%) was significantly different from the proportion of responders to GHB (57%) versus VEH (21%), ($\chi^2_{(2, n = 42)} = 85.6, p < 0.001$).

Effects on other parameters related to metabolism

The chronic dosing paradigm altered the weight of DTA mice (Fig. 8). The chronic dosing paradigm was initiated in mice with similar weights in the two strains (Atax: 35 ± 0.7 g, DTA: 35 ± 0.8 g). During Dox(–) to induce Hcrt cell loss, DTA mice steadily gained weight as described previously (Tabuchi et al., 2014) but weight gain was attenuated during the chronic dosing period (Fig. 8A; main effect on factor “recording day,” $F_{(3,91)} = 7.25, p < 0.001$). Atax mice lost a small amount of weight (<1 g) by the last day of dosing (main effect on factor recording day, $F_{(1,37)} = 5.33, p = 0.035$). However, the weight changes did not depend on the type of drug treatment for any genotype.

Atax mice had a higher mean T_b during the light period than DTA mice (Fig. 8B; baseline: $F_{(1,39)} = 6.48, p = 0.016$; dosing day 15: $F_{(1,39)} = 8.17, p = 0.007$) and T_b was higher on dosing day 15 versus baseline ($F_{(1,35)} = 5.42, p = 0.034$). During the dark period, Atax mice had lower mean T_b on dosing day 15 versus

baseline ($F_{(1,35)} = 80.05, p < 0.001$). DTA mice treated with GHB also had lower mean T_b during the dark period on dosing day 15 compared with baseline ($F_{(2,43)} = 5.84, p = 0.011$).

DTA mice were generally more active than Atax mice, as indicated by wheel running and gross motor activity during the first half of the dark period when most active wakefulness occurs (Fig. 8C,D). During baseline and on dosing day 15, DTA mice ran on their wheels more than Atax mice (baseline: $F_{(1,41)} = 19.05, p < 0.001$; dosing day 15: $F_{(1,41)} = 19.00, p = 0.001$); mice of both models ran less on dosing day 15 compared with baseline (Atax: $F_{(1,37)} = 10.09, p = 0.006$; DTA: $F_{(1,45)} = 11.89, p = 0.003$). However, only the Atax mice on dosing day 15 had reduced motor activity versus baseline ($F_{(1,37)} = 6.37, p = 0.023$) or compared with DTA mice ($F_{(1,41)} = 7.63, p = 0.009$). Neither wheel running nor gross motor activity depended on drug group in either mouse model.

Discussion

In the present study, the GABA_B agonists GHB and R-BAC were compared in two mouse models of narcolepsy as therapeutics to alleviate sleep disruption during the rest phase, consolidate wakefulness during the subsequent active phase, and suppress cataplexy. At the doses tested in a chronic dosing paradigm, R-BAC (2.8

mg/kg) increased the time spent in NREM sleep, NREM delta power, and the duration of NREM sleep bouts after dosing during the light phase. During the subsequent dark period, the duration of wake bouts in R-BAC-treated mice increased and cataplexy decreased to a greater extent than in GHB-treated mice (150 mg/kg).

Comparison of Atax and DTA models

The most notable difference between Atax and DTA mice is the basal amount of cataplexy they exhibit. This difference may be attributed to the number of remaining Hcrt cells and/or the timing of Hcrt cell loss. Atax mice slowly lose up to ~95% of their Hcrt neurons from 1 to 10 weeks of age (Hara et al., 2001) and adaptation and developmental compensation to Hcrt neurodegeneration may occur progressively during the postnatal period. In contrast, Hcrt neuron loss is rapid in DTA mice, which lose ~95% of their Hcrt neurons after only 2 weeks of Dox(–) and 97% by 4 weeks; DTA mice under Dox(–) continue to lose Hcrt neurons over at least the next 11 weeks (Tabuchi et al., 2014). In the present study, the continued weight gain after 4 weeks of Dox(–) (Fig. 8A; ~1 g/week vs <0.5 g/week in controls; Tabuchi et al., 2014) suggests that the DTA model continued to develop with perhaps another 1–2% of Hcrt neuronal loss into the dosing period. Additional Hcrt cell loss may underlie why DTA mice increase the time spent in cataplexy by 51% after 15 d of VEH (Fig. 7B). However, Atax mice also increase their time in cataplexy by 15%, suggesting that DTA mice may be more sensitive to the factors associated with the chronic dosing paradigm that may

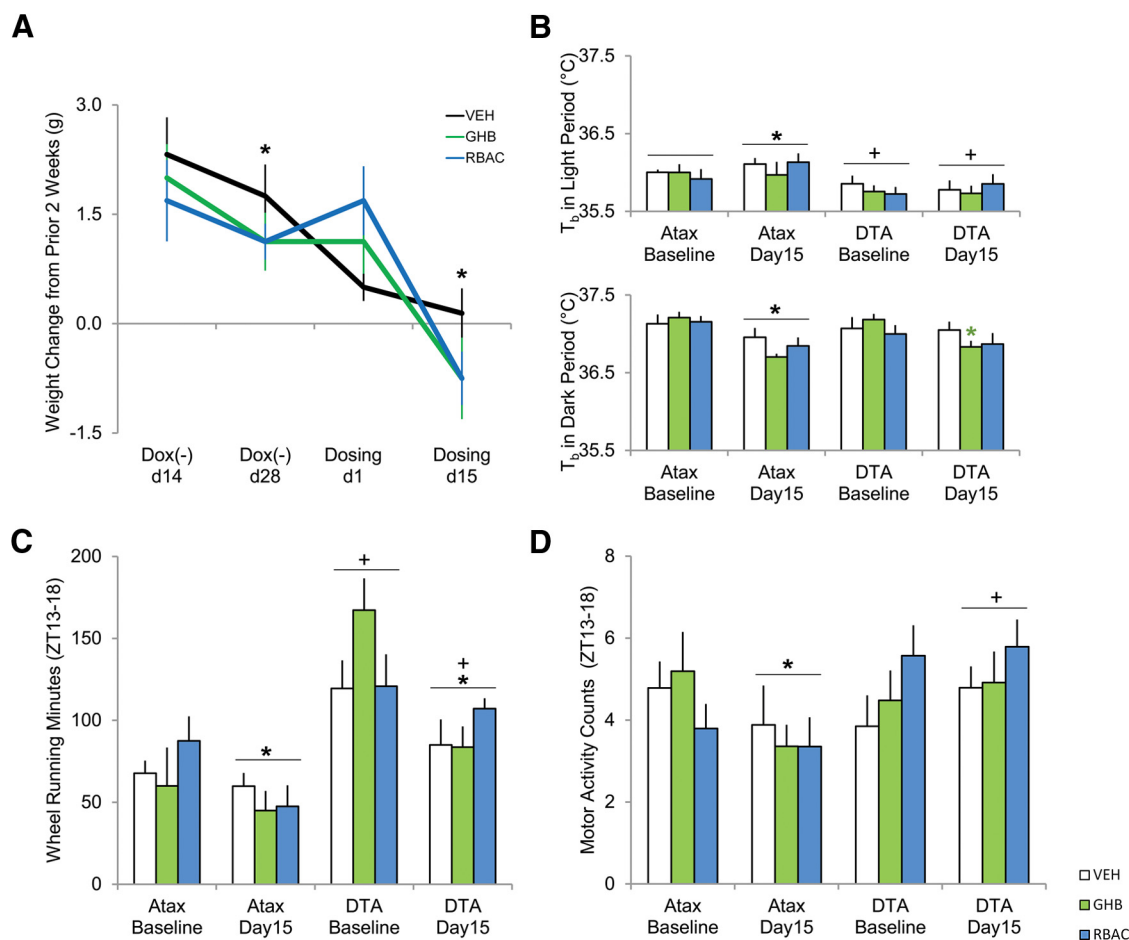


Figure 8. Chronic dosing protocol altered parameters related to metabolism in Atax and DTA mice administered GHB (150 mg/kg, green), *R*-BAC (2.8 mg/kg, blue), or VEH (white). **A**, Body weight change during Hcrt neurodegeneration (Dox(–)) and chronic dosing in DTA mice. 18 ± 3.3 d elapsed between Dox(–) day 28 and dosing day 1. **B**, Mean core body temperature (T_b) during the light (top) and dark (bottom) periods in Atax and DTA mice. **C**, Time spent wheel running and (**D**) gross motor activity during the first half of the dark period in Atax and DTA mice on dosing day 15 versus baseline. Data are mean \pm SEM ($n = 6–8$ /drug group per model); $p < 0.05$: black asterisk = main effect, day, + = main effect, model; green asterisk = interaction effect, GHB dosing day 15 versus baseline.

facilitate cataplexy. Positive emotional stimuli, such as the motivated behavior of wheel-running in mice, are triggers of cataplexy (España et al., 2007; Burgess and Scammell, 2012; Oishi et al., 2013) and DTA mice spent more time wheel running than Atax mice (Fig. 8C). Perhaps the high levels of cataplexy in DTA mice reflect underdeveloped compensatory mechanisms to dampen emotional triggering of cataplexy or reward anticipation, such as increased GABA in prefrontal cortex (Kim et al., 2008) or reduced activity in reward circuitry (Ponz et al., 2010).

Differences between the mouse models were evident in the arousal state dynamics after GHB and *R*-BAC administration. The strong inhibition of REM sleep by GHB and *R*-BAC did not appear to be recovered in DTA mice, yet compensatory REM sleep was observed in Atax mice (Fig. 4). During baseline, Atax mice exhibit less wakefulness and more REM sleep during the dark period than DTA mice (Fig. 3; Tabuchi et al., 2014). REM sleep in DTA mice steadily decreases from 2 to 13 weeks of Dox(–) (Tabuchi et al., 2014). The lack of compensatory REM sleep in DTA mice (evident in Fig. 4F as nonpositive slopes) may be due to the reduced levels of REM sleep that gradually emerge as Hcrt neurodegeneration (and, presumably, downstream network reorganization) proceeds in the DTA model. In contrast, the increased REM sleep during the dark period in Atax mice on dosing day 15 may be a consequence of more opportunities for

REM recovery because they sleep more than DTA mice at that time of day (Fig. 3E vs F).

Atax and DTA mice were similar in their therapeutic response to *R*-BAC, particularly in the consolidation of NREM sleep and wakefulness and reduction in cataplexy. For both mouse models, interaction effects between factors drug and recording day on physiological variables related to metabolism were generally not observed. These observations suggest that the therapeutic efficacy of *R*-BAC is not a consequence of altered activity levels, body weight, or temperature changes.

Translational considerations

The greater efficacy of *R*-BAC over GHB as a narcolepsy therapeutic in mouse models was surprising as it contrasts with previous human studies. The results presented here appear at variance with the finding by Huang and Guilleminault (2009) that sodium oxybate (~ 125 mg/kg) but not baclofen (~ 0.2 mg/kg) decreased the number of cataplexy bouts in patients with cataplexy and improved daytime sleepiness. Given that patients with narcolepsy are typically titrated with sodium oxybate from 4.5 to 9 g and that 3.75–6.26 g in humans is equivalent to a dose of 50 mg/kg (Black et al., 2010), it is not unreasonable to estimate that the 150 mg/kg dose of GHB used in the current study should be therapeutically relevant in the mouse. The 2.8 mg/kg dose of *R*-BAC used here is

within the range that *R*-baclofen (also known as STX209) has been shown to influence mouse behavior (Pacey et al., 2011; Henderson et al., 2012). The discrepancies between studies could be due to dose, route of administration, or species differences, but the enantiomer composition of the baclofen used in each study could also play a role. *R*-baclofen has a threefold higher affinity/efficacy for GABA_B receptors than the racemate (Filip and Frankowska, 2008), which is reflected in its higher potency on behavioral measures (Henderson et al., 2012). The *S*- enantiomer in racemic baclofen has been shown to interfere with the bioavailability of *R*-baclofen in the brain, which may explain, at least in part, the reduced efficacy of racemic baclofen versus *R*-baclofen in modulating behavior in mice (Shumway et al., 2012). Perhaps a similar mechanism in humans impedes sufficient concentrations of *R*-baclofen in a racemic mix to reach the necessary targets for cataplexy control.

The earliest systematic investigation of racemic baclofen reported dose-related increases in NREM and decreases in REM sleep (Guilleminault and Flagg, 1984). Racemic baclofen has also been shown to induce delayed hypersomnolence in humans (Huang and Guilleminault, 2009; Vienne et al., 2012) and BALB/c mice (Vienne et al., 2010) that appears to engage homeostatic sleep mechanisms, consistent with our results that *R*-BAC increased NREM sleep intensity and consolidation during the light period and, presumably as a consequence, lengthened wake bouts during the subsequent dark period. Unlike GHB, racemic baclofen was shown to increase time spent in NREM sleep and NREM delta power in mice deficient in GABA_{B1} and GABA_{B2} receptors, indicating the hypersomnolence may not be mediated by GABA_B receptors (Vienne et al., 2010). Accordingly, there is a possibility that the therapeutic effects of *R*-BAC observed in narcoleptic mice in the present study may be mediated by non-GABA_B mechanisms, or perhaps the *S*-isomer in racemic baclofen induces slow-wave sleep via an unknown binding site.

Mechanism of action

The different responses of narcoleptic mice to GHB and *R*-BAC presented here adds to a growing body of literature that shows these GABA_B agonists have substantially different modes of action. As a low-affinity GABA_B agonist, GHB preferentially inhibits GABAergic interneurons in the ventral tegmental area to disinhibit dopaminergic neurons, while the high-affinity agonist *R*-BAC directly inhibits both GABAergic interneurons and catecholamine cells (Cruz et al., 2004). GHB and baclofen both elicit outward currents in cholinergic neurons of the laterodorsal tegmentum (LDT) and serotonergic neurons of the dorsal raphe (DR; Kohlmeier et al., 2013). However, GHB and *R*-BAC evoke different patterns of Fos expression across the neuraxis (van Nieuwenhuijzen et al., 2009), some of which may be explained by the different kinetics of calcium responses to GHB or *R*-BAC in the LDT and DR (Kohlmeier et al., 2013). In addition, a high-affinity target for GHB has recently been identified as the $\alpha 4\beta\delta$ GABA_A receptor (Absalom et al., 2012), and sleep is differentially modulated depending on whether GABA_A or GABA_B receptors are activated (Manfridi et al., 2001; Gmeiner et al., 2013). Inhibition of REM sleep by baclofen can be rescued by activation of cAMP-PKA in the pedunculopontine tegmentum (Datta, 2007), but it is unknown if a similar effect can be achieved with GHB. All of these mechanisms could play a role in the arousal state differences observed in narcoleptic mice after GHB or *R*-BAC treatment.

Conclusion

The greater efficacy of *R*-BAC than GHB to reduce cataplexy, consolidate sleep and wakefulness, and increase time spent in NREM sleep and NREM delta power warrant further investigation of *R*-BAC as a therapeutic for human narcolepsy. The apparent effectiveness of *R*-BAC to engage sleep homeostatic mechanisms to evoke compensatory enhancement of wakefulness after an induction of NREM sleep suggests *R*-BAC may also be an effective treatment for insomnia. As a first step, preclinical comparisons of *R*-baclofen versus racemic baclofen on alleviating cataplexy and arousal state fragmentation need to be performed. Efficacy tests of *R*-baclofen and racemic baclofen in a mouse model of fragile X syndrome have led to clinical trials of STX209 (Pacey et al., 2011; Berry-Kravis et al., 2012) and encourages further development of enantiomer-specific baclofen for sleep disorders.

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