Rapid, noninvasive, and unsupervised detection of sleep/wake using piezoelectric monitoring for pharmacological studies in narcoleptic mice

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Abstract

Background: Assessment of sleep/wake by electroencephalography (EEG) and electromyography (EMG) is invasive, resource intensive, and not amenable to rapid screening at scale for drug discovery. In the preclinical development of therapeutics for narcolepsy, efficacy tests are hindered by the lack of a non-EEG/EMG based translational test of symptom severity.

New method: Noninvasive, unsupervised piezoelectric monitoring of gross body movement, together with respiration patterns during behavioral quiescence, was used to determine sleep/wake. In the PiezoSleep narcolepsy screen, these measurements were sampled during the dark period, and the ratio of time spent in long wake bouts to short sleep bouts was used to determine a wake-maintenance score by which to rank narcoleptic and wild type mice by sleepiness severity. PiezoSleep was validated for high-throughput screening of wake-promoting compounds using modafinil in narcoleptic and wild type mice.

Results: The PiezoSleep narcolepsy screen revealed a 15-fold difference in wake-maintenance scores between wild type mice and the most severely affected narcoleptic mice. The ED_{50} for wake-promotion by modafinil was approximately 50 mg/kg in both genotypes.

Comparison with existing method(s): The PiezoSleep narcolepsy screen increased by 16% the number of mice that could be identified that met the cataplexy inclusion criteria for more intensive studies. Using unsupervised piezoelectric monitoring, the efficacy of wake-promoting compounds can be determined in a 5-arm study with 60 mice in less than one week—a fraction of the time compared to EEG/EMG studies.

Conclusions: PiezoSleep offers rapid, scalable assessment of sleep/wake for high-throughput screening in drug discovery.

Highlights

- PiezoSleep narcolepsy screen provides a wake-maintenance score for narcoleptic mice
- The wake-maintenance score is the ratio of time spent in long wake bouts to short sleep bouts during ZT12-18
- The assay translates the Maintenance of Wakefulness and Multiple Sleep Latency Tests to mice
- PiezoSleep has been validated for the detection of sleep/wake at scale for drug discovery

Keywords

modafinil, mouse, narcolepsy, non-invasive sleep scoring, orexin, piezoelectric

Abbreviations

ANOVA, analysis of variance

Atax, *orexin/ataxin-3*

AUC, area under the curve

Cmax, maximum concentration

EDS, excessive daytime sleepiness

EEG, electroencephalography

EMG, electromyography

f, fornix

ic, internal capsule

LD12:12, 12 h light period, 12 h dark period

MOD, modafinil

MSLT, multiple sleep latency test

MWT, maintenance of wakefulness test

NREM, non-rapid-eye-movement

opt, optic tract

PBS, phosphate-buffered saline

PO, *per os*

REM, rapid-eye-movement

RM ANOVA, repeated-measures analysis of variance

SBD, sleep bout duration

SC, subcutaneous

S.E.M., standard error of the mean

mpk, mg per kg

MRT, mean residence time

$T_{1/2}$, half life

WBD, wake bout duration

WT, wild type

ZT, zeitgeber time

3V, third ventricle

1. Introduction

Pharmacological studies with arousal state endpoints are typically invasive, resource intensive, and time consuming. Rodent polysomnography requires surgical implantation of electrodes, recovery time, adaptation to sleep implant hardware, and manual scoring of arousal states for the highly accurate detection of sleep microstructure. Using standard electroencephalography (EEG) and electromyography (EMG), wakefulness is defined as low amplitude, desynchronous activity in the EEG across a broad frequency range with variable EMG activity. Sleep in rodents is classified as either rapid-eye-movement (REM) sleep, with pronounced activity in the EEG theta band (4.5-9.5 Hz) and atonia in the EMG, or non-REM (NREM) sleep, with slow, synchronous activity in the EEG delta band (0.5 to 4.5 Hz) and low, steady EMG tone. Some of the burden of rodent polysomnography has been relieved by the development of automated methods of scoring EEG/EMG records. However, the expediency and objectivity value of these methods is diminished for those that require a subset of manually scored samples from individual animals to train the algorithm (Gross et al., 2015; Gross et al., 2009; Rytkönen et al., 2011; Van Gelder et al., 1991). Unsupervised, automated scoring algorithms have addressed these limitations of machine learning with varying degrees of accuracy (72-96% agreement) compared to standard manual scoring (Libourel et al., 2015; Stephenson et al., 2009; Sunagawa et al., 2013). Nevertheless, all EEG/EMG based systems of arousal state monitoring are hindered by the time required for surgery, recovery, and in many cases, the repeated-measures experimental designs used for studies in which a small number of animals are used. There is a need in drug discovery for a fully automated method of sleep/wake staging that can scale to a sample size that is amenable to high-powered, between-subject designs for the rapid screening of compounds.

The use of non-invasive proxy-measures of sleep/wake, such as actigraphy or cardiovascular correlates of arousal states instead of traditional polysomnography, can expedite

sleep/wake determination in rodents (Fisher et al., 2012; Flores et al., 2007; Pack et al., 2007; Zeng et al., 2012). In particular, the use of a highly sensitive motion detector (piezoelectric sensor) on the cage floor to monitor gross motor activity, together with breathing patterns during periods of behavioral quiescence, yields unsupervised sleep/wake detection with 90% accuracy vs. manual EEG scoring (Mang et al., 2014). This piezoelectric sleep monitoring system (PiezoSleep) has been used to characterize daily sleep/wake amounts and patterns in a transgenic mouse model of Alzheimer's disease (Sethi et al., 2015b) and in 180 knockout lines as part of a large-scale phenotyping program (Sethi et al., 2015a).

Transgenic *orexin/ataxin-3* (Atax) mice model the human sleep disorder narcolepsy with high fidelity. Like their human counterparts, Atax mice experience degeneration of wake-promoting orexin (also known as hypocretin) neurons which leads to cataplexy (a sudden, reversible loss of muscle tone and REM-sleep like EEG) and/or increased sleep propensity at times of day when wakefulness would otherwise predominate (Hara et al., 2001). This excessive daytime sleepiness (EDS) leads to an inability of patients with narcolepsy to sustain long periods of wakefulness during the Maintenance of Wakefulness Test (MWT) and to fall asleep with short latency on the Multiple Sleep Latency Test (MSLT). Both tests are indicators of symptom severity; the MSLT is used in the diagnosis of narcolepsy and the MWT is used to assess treatment efficacy (Cao and Guilleminault, 2017). Modafinil (MOD) is a first-line choice of treatment for EDS in patients, and promotes EEG/EMG-defined wakefulness in narcoleptic mice, but is ineffective in cataplexy reduction (Black et al., 2017a; Cao and Guilleminault, 2017). Although Atax mice exhibit cataplexy that is readily identifiable according to EEG/EMG-dependent consensus criteria (Scammell et al., 2009), 34% of Atax mice have fewer than 3 cataplexy episodes per day, thus presenting a challenge to sort mice for inclusion in studies with cataplexy reduction as an endpoint (Black et al., 2017b).

Drug discovery efforts aimed at finding improved therapeutics for narcolepsy would benefit from a translational assay in mice similar to the MWT that is rapid, scalable, and not dependent upon polysomnography nor sleep deprivation protocols (Veasey et al., 2004). This assay may help prescreen Atax mice that are highly symptomatic and prone to cataplexy, as patients with severe cataplexy are the least able to stay awake on the MWT test (Mitler et al., 1998). While Atax mice have been monitored with piezoelectric detection of movement and heartrate for a few hours during the light period, the algorithm for autoscoring was only 73% accurate against manual EEG scoring (Sato et al., 2014) and missed the time of day in which cataplexy and sleep/wake fragmentation occurs most frequently (Black et al., 2017b). Here, we demonstrate how piezoelectric monitoring of sleep/wake states can be used to determine the severity of narcoleptic symptoms in Atax mice and to quantify sleep time and consolidation in pharmacological studies at a scale amenable to screening wake-promoting therapeutics in drug discovery.

2. Materials and Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Reset Therapeutics and were conducted in accordance with the principles set forth in the *Guide for Care and Use of Laboratory Animals of the National Institute of Health*.

2.1 Animals

Male and female hemizygous transgenic B6.Cg-Tg(HCRT-MJD)1Stak/J mice (Atax) and wild type (WT) littermates (JAX stock #023418, Jackson Labs, Bar Harbor, ME) were used (2001 Hara Neuron). Separate cohorts were used for immunohistochemistry, development of the narcolepsy phenotyping assay, EEG/EMG and video monitoring, and pharmacology. Male C57BL/6J mice (JAX stock #000664, Jackson Labs, Bar Harbor, ME) were also used in

pharmacological studies. Male CD-1 mice (Charles River Laboratories, Hollister, CA), 7-8 weeks old, were used in pharmacokinetic studies. Animals were individually housed, except for CD-1 mice which were housed 3 per cage, at 22-25 ^{o}C, 30-70% relative humidity, and LD12:12 with *ad libitum* access to food, water and nesting material.

2.2 Immunohistochemistry

All histological procedures and cell counts were performed at Jackson Laboratories (Bar Harbor, ME). Male and female Atax (*N* = 8 and 5, respectively) and WT littermates (*N* = 8 males and 6 females), aged 16.9 ± 0.1 and 28.5 ± 0.8 g were euthanized by pentobarbitol overdose and transcardially perfused with 0.4 % paraformaldehyde. Brains were harvested, postfixed overnight, and cryo sectioned at 40 µm from approximately -1.23 to -1.91 relative to Bregma in coronal sections. After blocking in normal donkey serum (5% in 0.3% triton in PBS), free-floating brain sections were incubated overnight at room temperature with goat anti-donkey orexin A antibody (SC-8070, Santa Cruz Biotechnology, CA) at 1:2000 dilution. After washing with phosphate-buffered saline, sections were incubated for 2 hours at room temperature with biotinylated donkey anti-goat (Jackson Immunoresearch, West Grove, PA) at 1:500 dilution in blocking solution. After further washes, sections were developed with the avidin-biotin-peroxidase system using diaminobenzidine as the chromagen (Vector Laboratories, Burlingame, CA). Sections were mounted onto slides, and anatomically matched sections between brains that represented the anterior, mid, and posterior regions of the orexin field were identified for manual cell counting. Counts were performed bilaterally on these sections at 20x magnification under light microscopy. Only somata with complete and intact cell membranes were included in the analysis.

2.3 Sleep/wake recordings

2.3.1. Piezoelectric monitoring

The narcoleptic phenotype of Atax mice and their sleep/wake responses in pharmacological studies were determined by piezoelectric recording of gross body movement and breath rate (PiezoSleep version 2.11, Signal Solutions, Lexington, KY). The PiezoSleep apparatus consisted of 60 individual polycarbonate cages, each of which contained a polyvinylidene difluoride piezo sensor beneath a thin (0.025 cm) plastic shield. The sensor covered the entire area of the cage floor (232.3 cm^2) and was connected to a single channel tube amplifier (RoMo version 1.2, Signal Solutions, Lexington, KY). Each amplifier fed into a digital acquisition device connected to a computer for data collection and sleep/wake determination as previously described (Donohue et al., 2008; Flores et al., 2007; Mang et al., 2014). Briefly, signals were amplified and filtered between 0.5 and 10 Hz and analog-to-digital converted at 120 Hz sampling rate. The frequency, amplitude, and peak spectral energy of the signal were evaluated in 2 second increments over a tapered, 8 second window to automatically calculate a sleep/wake decision statistic by simple linear discriminant analysis. Signals were classified as sleep if they exhibited a periodicity with a consistent, low relative amplitude at a frequency in the typical breathing range (1-4 Hz). Wakefulness was associated with high amplitude signals with variable frequency and broad spectral energy. Signal features related to these properties were extracted and combined to obtain a decision statistic for each interval. Over time, the decision statistics per increment accumulated in a bimodal distribution. The saddle point of the distribution was determined using an adaptive threshold for the classification of sleep and wake. Bouts of contiguous intervals of sleep or wake were defined to terminate when an interval, equal to the minimum bout length of 30 s, included less than 50% of the targeted state.

Piezoelectric experiments began by loading mice into individual PiezoSleep cages that contained standard rodent bedding (diamond dry cellulose approximately 1 cm thick) with food and nesting material from each mouse's home cage. Mice were permitted at least 24 h of

acclimation before experimental data were sampled. For development of the narcolepsy phenotype assay, after the acclimation period, Atax mice (*N* = 31, aged 15.7 ± 0.3 weeks, 29.4 ± 0.4 g) and their WT littermates (*N* = 17, aged 14.8 ± 0.5, 29.5 ± 0.4 g) were recorded undisturbed for 36 h beginning at zeitgeber time (ZT)12 (21:00). Hourly sleep percentages, mean sleep bout durations (SBD) and wake bout durations (WBD) per h, and histograms of the percent time spent asleep or awake in bouts from 0.5 to ≥ 64 min were calculated using SleepStats version 2.11 (Signal Solutions, Lexington, KY). Based on the circadian distribution of mean SBD and WBD per h, and on the increased propensity for cataplexy during the early dark period (Black et al., 2017b), sleep and wake data from ZT12-18 were used to differentiate mice based on their ability to maintain long bouts of wakefulness at that time of day. Scatterplots of time spent in short SBD (< 8 min) vs. long WBD (≥ 32 min) were used to screen Atax mice that were highly symptomatic for murine narcolepsy (i.e., compared with other Atax mice, they spent more than the mean amount of time in short SBD and less than the mean amount of time in long WBD.) All mice were assigned a wake-maintenance score based on their ratio of time in long WBD:short SBD during ZT12-18 for the PiezoSleep narcolepsy screen.

2.3.2. EEG/EMG & video monitoring

A second cohort of male Atax mice (*N* = 37, aged 11.6 weeks, 26.6 ± 0.4 g) was screened in the narcolepsy phenotype assay to identify the individuals that were the most highly symptomatic for narcolepsy and thus the best candidates for EEG and EMG studies. The 16 Atax mice with the lowest wake-maintenance scores were surgically implanted at 16.9 ± 0.2 weeks of age (30.2 ± 0.7 g) with transmitters (F20-EET, Data Sciences International, St. Paul, MN) for EEG and EMG recording as previously described (Black et al., 2013). Mice were anesthetized with isoflurane and transmitters were placed IP 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 subchranially over the dura and attached to the skull with cyanoacrylate and dental acrylic. Analgesia was managed with prophylactic ketoprofen (5 mg/kg, SC) followed by buprenorphine (0.1 mg/kg, SC) upon emergence from anesthesia. Ketoprofen (5 mg/kg, SC, QD) was continued for 3 days postsurgery. Mice received postoperative thermal support for 24 h and hydration (0.7 mL lactated Ringer's solution, SC and dietary recovery gel) for 3 days. Mice were permitted 3 weeks postsurgical recovery, which included 2 weeks adaptation to running wheels and the light-controlled (LD12:12 with ZT0 = 03:00), sound-attenuated and ventilated recording chamber.

Physiological and video-recorded behavioral data were simultaneously acquired with Ponemah version 5.2 (Data Sciences International, St. Paul, MN). Electrophysiological data were sampled at 500 Hz and digital videos were recorded at 15 frames per second (640 x 480 pixels) using Noldus Media Recorder (Leesburg, VA). Recordings from mice in their home cages (with nesting material and running wheels) began at ZT6 and continued for the following 30 h. Data were manually scored using NeuroScore version 3.2.1 (Data Sciences International, St. Paul, MN) by scorers trained with ≥ 96% interrater reliability by an expert scorer. Data were classified in 10-second epochs as wakefulness, wheel-running during wakefulness, NREM sleep, REM sleep, or cataplexy. Criteria for cataplexy were ≥ 10 seconds of EMG atonia, theta-dominated EEG, and video-confirmed behavioral immobility preceded by ≥ 40 seconds of wakefulness (Scammell et al., 2009). Bouts of each arousal state were defined to terminate with the occurrence of a single epoch of another state. Of the 16 implanted mice, 11 were included in the analysis (aged 19.9 ± 0.3 weeks at recording), 2 did not survive surgical recovery, and 3 had corrupted data files or unscorable EEG signals.

2.4. Compound formulation

Modafinil (MOD, 2-[(diphenylmethyl)sulfinyl]acetamide) was purchased from Focus Synthesis (San Diego, CA) for dosing in CD-1 and WT and from Advanced ChemBlocks, Inc. (Burlingame, CA) for dosing in Atax mice because MOD was no longer available from Focus Synthesis. For pharmacokinetic studies, MOD was formulated in 1.25% Hydroxypropyl Methylcellulose / 0.25% Methylcellulose in Water for Injection, which formed a homogenous suspension. For efficacy studies, after MOD was weighed, premade vehicle (20% propylene glycol, 25% polyethylene glycol 400 in water for injection) was added at a volume appropriate for the highest concentration, followed by overnight sonication, which produced a clear solution. Serial dilutions were created and re-sonicated approximately 6 h prior to dosing. All dosing solutions were formulated within 24 h prior to dosing.

2.5. Pharmacokinetic studies

Male CD-1 mice were acclimated for a minimum of 3 days prior to drug administration. Modafinil was administered as 10, 30, 50, or 100 mg/kg via oral gavage (PO) to CD-1 mice at ZT0 to ZT1. Blood and tissue samples were collected from three mice per time point at pre-dose, 15, 30, 60, 90, 180, 360, 720, and 1440 min following oral gavage from the CD-1 mice. Blood (500 µL) was obtained via cardiac puncture into Microtainer Blood Collection Tubes with K_2EDTA (BD Biosciences, San Jose, CA), and gently mixed. Blood was immediately centrifuged, and the resulting plasma was frozen on dry ice then stored at -80 °C until it was analyzed. Entire brain from each animal was harvested, weighed, frozen on dry ice, and stored at -80 °C until it was analyzed. The concentrations of MOD were determined by a liquid chromatography – mass tandem spectrometry (LC-MS/MS) method (Quintara Discovery, Hayward, CA) following the standard protocol. Briefly, Brain samples were homogenized in two volumes of ice cold water. Then 20 µL of each plasma or brain homogenate sample were processed by protein precipitation with methanol: acetonitrile (5:95 v:v) containing internal standard, verapamil. Extracts were analyzed by LC-MS/MS using positive electrospray

ionization under the multiple-reaction-monitoring mode for transitions m/z 274->167 (MOD) and m/z 455-> 165 (verapamil). The lower limit of quantitation was 1.8 nM for plasma and 5.5 nM for tissues. Pharmacokinetic parameters of MOD were generated by non-compartmental analysis with Phoenix™ WinNonlin® software (Pharsight Corporation, St. Louis, MO).

Table 1. Pharmacokinetic data after oral modafinil at ZT0 in CD-1 mice

Dose (mg/kg)	$T_{1/2}$ (hrs)		MRT (hrs)		Cmax (μM)		AUC (μM.h)	
	Plasma	Brain	Plasma	Brain	Plasma	Brain	Plasma	Brain
10	1.1	1.4	1.8	1.5	14.2	4.1	22.5	7.3
30	0.9	1.2	1.7	1.5	76.1	15.4	131.3	34.5
50	0.8	1.2	2.1	1.9	80.1	33.7	185.5	72.4
100	1.0	1.1	2.4	2.7	306.2	76.5	958.5	294.9

$T_{1/2}$: half-life
MRT: mean residence time
AUC: area under the curve

2.6. Efficacy studies

In a between-subjects design, male C57BL/6 mice (WT, 15.7 ± 0.1 weeks, 28.5 ± 0.2 g, *N* = 12/group) and Atax mice (16.4 ± 0.7 weeks, 30.0 ± 0.7 g, *N* = 9-10/group) were dosed separately in two pharmacological studies. Mice were loaded into PiezoSleep cages for 42 h acclimation, followed by 29 h baseline recording prior to dosing at ZT5 (14:00), the time of day in which wake-promoting/sleep-inhibiting effects would be expected to be most pronounced. Syringes were filled immediately prior to dosing, which was completed within a 15-minute window. After pharmacokinetic studies (Table 1) and based on efficacy in previous reports (Black et al., 2017a; Fujiki et al., 2009), mice received either MOD (10, 30, 50, 100 mg/kg, 10 mL/kg) or vehicle orally and were recorded for 44 h post dosing. Mice were undisturbed during the recording period except for dosing. Groups were balanced across dosing order, dosing personnel, and cage location per shelf and rack. Atax mice had previously been screened in the narcolepsy phenotype assay, and highly symptomatic mice were balanced across dosing groups.

2.7 Data analysis and statistics

Statistics were performed using GraphPad Prism version 7.03 (La Jolla, CA). Unless otherwise noted, all data are presented as the mean ± S.E.M. Orexin cell counts were compared between male and female Atax and WT mice using two-way analysis of variance (ANOVA) and between genotypes for combined sexes using two-tailed, unpaired *t* test. In phenotyping studies, sleep time and mean SBD and WBD were compared between Atax and WT mice using two-way mixed-model ANOVA on factors “genotype” (between subjects) and “hour” (within subjects). Histograms representing the distribution of time spent in various SBD and WBD were evaluated using two-way mixed-model ANOVA on factors “genotype” (between subjects) and “bout duration bin” (within subjects). Ratios of time spent in long wake bouts and short sleep bouts were compared between genotypes using two-tailed, unpaired *t* test. Comparisons of wake time measurements via piezoelectric monitoring vs. EEG/EMG with video recording over 24 h were made within subjects using two-way repeated-measures (RM) ANOVA, and for 24 h totals using two-tailed, paired *t* test. Pharmacological data were assessed within genotypes by two-way mixed-model ANOVA on between-subjects factor “drug condition” and within-subjects factor “hour” or “bout duration bin.” The maximum sleep deficit was calculated as the minimal value in the cumulative change from baseline in sleep % time during the 12 h post dosing. Consolidation of wakefulness after dosing was measured as the change from baseline in the % time spent in long wake bouts ≥ 8 min. Sleep deficits and consolidated wakefulness were compared between genotypes after dosing using two-way ANOVA. For each analysis, when ANOVA indicated significance ($\alpha = 0.05$), contrasts between relevant factor levels (vs. WT or vehicle, as appropriate) were detected *post hoc* with Bonferroni’s multiple comparisons test. The total sample size ($N = 97$) for the pharmacological study permitted sufficient power (0.8) for the detection of an effect size of 36% at $P < 0.05$ (computed using WebPower https://webpower.psychstat.org/wiki/).

3. Results

3.1. Histology

To confirm orexin cell loss from Atax mice taken out of cryopreservation at JAX, orexin cell counts were compared between the transgenics and their WT littermates at an adult age in which they would be used for sleep studies. Representative images (Figure 1) of the posterior hypothalamus and cell counts (Table 2) indicated that, as expected, Atax mice had fewer cells that stained positive for orexin A. Total numbers of orexin-positive cells counted from WT males vs. Atax males were 511 ± 21 vs. 12.9 ± 3.0, while from WT females vs. Atax females were 399 ± 54 vs. 19.0 ± 12. There were no sex differences in the number of orexin neurons that were sampled, $F(1, 23) = 3.58$, *n.s.* The extent of orexin neurodegeneration was consistent with that observed in a different colony of Atax mice (Black et al., 2013).

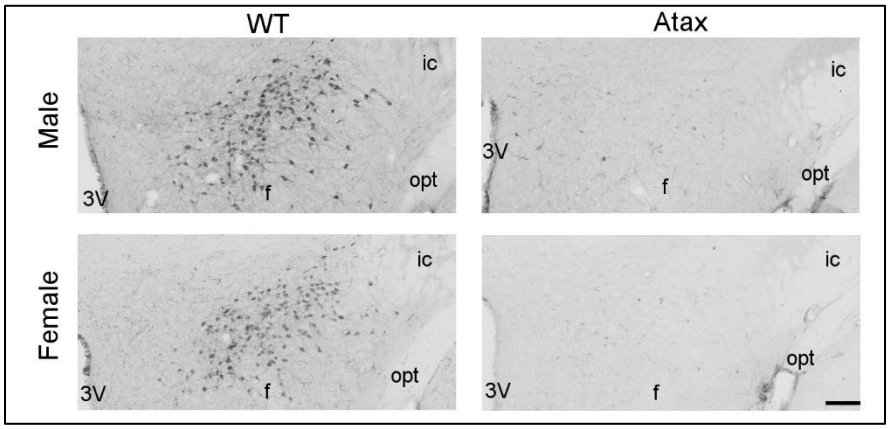

Figure 1. Loss of orexin neurons in orexin/ataxin-3 (Atax) mice. Representative images of Orexin A+ cells in the posterior lateral hypothalamus of male **(top)** and female **(bottom)** wild type littermates **(left)** and Atax mice **(right)**. Images taken at 10x magnification (bar = 100 μM), approximately -1.5 mm from Bregma; fornix (f), internal capsule (ic), optic tract (opt), third ventricle (3V).

Table 2. Normalized orexin cell counts in wild type (WT) and *orexin/ataxin-3* (Atax) mice

	N/group	WT	Atax	statistic	*p*
Males	8	100 ± 4.1	**2.5 ± 0.6**	***F*(1, 23) = 207.5**	**< 0.0001**
Females	5-6	100 ± 13.6	**5.1 ± 3.8**		
Both sexes	13-14	100 ± 6.0	**3.4 ± 1.2**	***t*(25) = 15.3**	**< 0.0001**

Data are the mean ± S.E.M. expressed as the % of WT mean per sex

Bold = main effect for genotype; no interaction or main effects for sex

3.2. Phenotyping of narcolepsy model

Piezoelectric monitoring of sleep/wake was used to evaluate the phenotype of Atax mice and to sort mice by the severity of murine narcolepsy symptoms. Compared to WT littermates, Atax mice slept more at a few time points during the dark period, particularly during the last hour prior to lights-on (Figure 2A), $F(36, 1656) = 4.81$, $P < 0.0001$. However, the total amount of sleep per 24 h was the same between genotypes (WT: 45.5 ± 0.8 % vs. Atax: 45.8 ± 1.0 %), $t(46) = 0.03$, *n.s.*, as expected. Genotype differences were observed in sleep and wake bout duration measures. Atax mice exhibited shorter mean SBD per h across the day (Figure 2B, $F(36, 1649) = 4.65$, $P < 0.0001$) and shorter mean WBD during the dark period (Figure 2C, $F(36, 1656) = 8.62$, $P < 0.0001$).

Because differences in both mean SBD and WBD were particularly robust during the first half of the dark period, and because that time of day is associated with an increased probability of cataplexy occurrence (Black et al., 2017b), ZT12-18 was chosen as the focus for determination of narcolepsy symptom severity. The percentage of time spent in sleep bouts that were 0.5 to 4 min long was greater in Atax than for WT mice, while WT mice spent more time than Atax mice in sleep bouts that were 8 to 32 min long (Figure 2D), $F(7, 368) = 24.31$, $P < 0.0001$. Compared to WT littermates, Atax mice spent more time in wake bouts that were 8-32

min long and less time in the longest wake bouts of ≥ 64 min duration (Figure 2E), $F(7, 368) = 25.74$, $P < 0.0001$. The percentage of time spent in short SBD (< 8 min) vs. time spent in long WBD (≥ 32 min) were plotted in Figure 2E. Atax mice clustered on the scatterplot in a manner indicative of more time spent in short sleep bouts and less time in long wake bouts than their WT littermates. The ratio of time spent in long WBD to short SBD was smaller in Atax mice (1.5 ± 0.2) than in WT (9.9 ± 2.7), $t(46) = 4.19$, $P < 0.0001$. This ratio was used in the PiezoSleep narcolepsy screen to sort individual Atax mice by murine narcolepsy symptom severity, in order to prioritize EEG/EMG instrumentation on the mice most likely to demonstrate cataplexy.

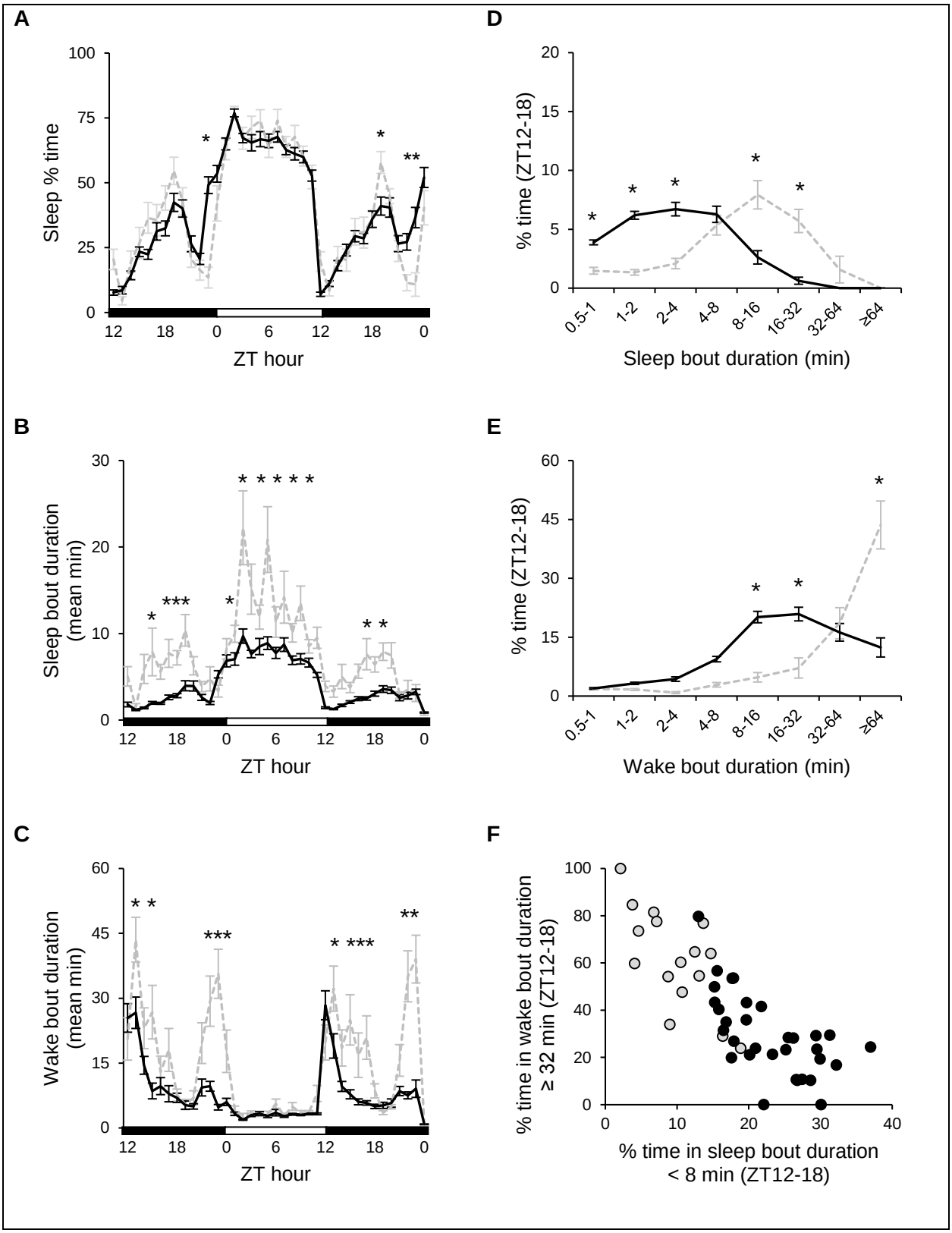
A
Sleep % time
ZT hour
B
Sleep bout duration (mean min)
ZT hour
C
Wake bout duration (mean min)
ZT hour
D
% time (ZT12-18)
Sleep bout duration (min)
E
% time (ZT12-18)
Wake bout duration (min)
F
% time in wake bout duration ≥ 32 min (ZT12-18)
% time in sleep bout duration < 8 min (ZT12-18)

Figure 2. The PiezoSleep narcolepsy phenotyping assay. Determination of narcoleptic phenotype in male orexin/ataxin-3 (Atax, *N* = 31) mice **(black)** vs. wild-type (WT, *N* = 17) littermates **(gray dashed lines)** using PiezoSleep. Sleep % per h **(A)** and mean bout durations per h of sleep **(B)** and wake **(C)** over the 36 h recording period. Percentage of time during the first half of the dark period (zeitgeber time (ZT)12-18) spent asleep **(D)** and awake **(E)** in bout durations lasting from 0.5 to ≥ 64 min. Percentage of time spent in short sleep bouts (< 8 min) vs. long wake bouts (≥ 32 min) during the first half of the dark period per individual mouse **(F)**. Two-way mixed-model analysis of variance with Bonferroni's multiple comparisons: * P < 0.05 vs. WT.

Using the PiezoSleep narcolepsy screen on a separate cohort of Atax mice, 16 mice were identified as "highly symptomatic" for murine narcolepsy, with lower wake-maintenance scores (0.68 ± 0.1) than the remaining Atax littermates (3.15 ± 0.4), and were implanted for EEG/EMG monitoring. The wake-maintenance scores of the 11 mice that were ultimately included in the EEG/EMG analysis (0.67 ± 0.1) were representative of the original 16 animals. The percentage of time from ZT12-18 spent in short SBD vs. time spent in long WBD for this cohort were plotted in Figure 3 for comparison of the subgroups based on narcolepsy symptom severity, EEG study inclusion or exclusion, and for the same individuals, comparison of values based on PiezoSleep vs. EEG/EMG/video data collection. The wake-maintenance scores determined by polysomnography (0.28 ± 0.05) were lower than those determined by PiezoSleep ($t(10) = 2.84$, $P < 0.02$). This difference occurred because the percentage of time spent in short SBD was greater after EEG-based methods, in which brief arousals fragment sleep (39.7 ± 1.7%), than after piezoelectric monitoring, which uses different criteria for sleep bout termination (26.3 ± 1.2%, $t(10) = 7.13$, $P < 0.0001$).

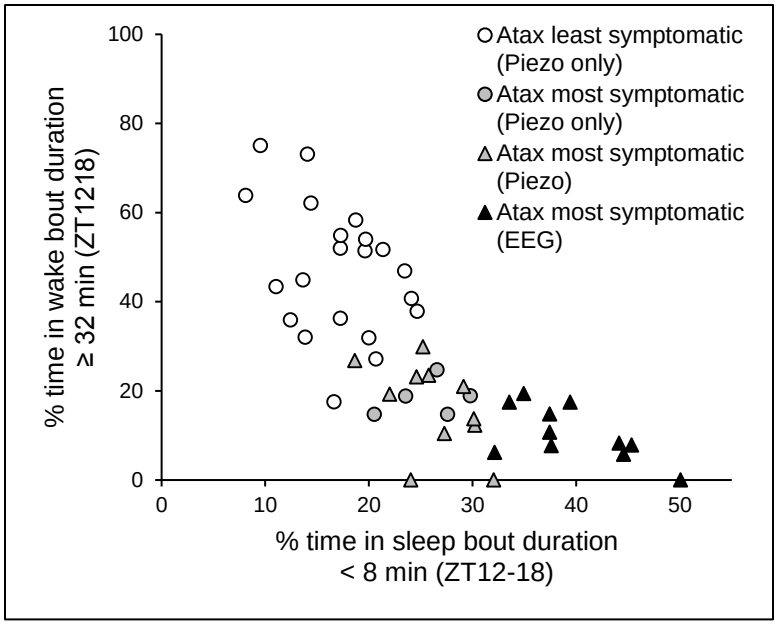

Figure 3. Short sleep bouts vs. long wake bouts in male orexin/ataxin-3 (Atax) mice. Percentage of time during the first half of the dark period (zeitgeber time (ZT)12-18) spent in sleep bout durations < 8 min vs. wake bout durations ≥ 32 min per individual mouse. Measurements based on PiezoSleep recordings were used to categorize Atax mice as either the least symptomatic for narcolepsy (*N* = 21, white circles) or the most symptomatic for narcolepsy (*N* = 16, gray symbols). Highly symptomatic Atax mice were either excluded (*N* = 5, gray circles) or included (*N* = 11, gray triangles) from EEG/EMG/video analysis. Data based on EEG/EMG/video measurements for the included mice (black triangles) were collected 8 weeks after the PiezoSleep recordings (gray triangles).

The percentage of time spent awake per hour across 24 h was compared between piezoelectric recordings from the phenotype screen and EEG/EMG with video monitoring that was performed 8 weeks later (Figure 4A). This comparison of non-simultaneous recordings from different environments was not intended to validate the accuracy of the piezoelectric sleep/wake detection against polysomnography, as has previously been done (Flores et al., 2007; Mang et al., 2014), but rather was intended to assess the predictive consistency between the two methods under different use cases. The pattern of the daily distribution of wakefulness appeared to be similar between sleep/wake recording methods, but piezoelectric determination yielded slightly more wakefulness per 24 h (53.3 ± 0.9%) than EEG/EMG with video monitoring (49.7 ± 1.3%), $t(20) = 2.31$, $P = 0.03$). However, the only time point in which the PiezoSleep

recordings differed from the EEG/EMG recordings was in the hour immediately prior to lights on, $F(23, 230) = 29.3$, $P < 0.0001$.

Time spent in NREM and REM sleep (Figure 4B) and during cataplexy and wheel-running (Figure 4C) were quantified in these mice from the EEG/EMG with video recordings. During the dark period, 9 of the 11 mice (82%) exhibited ≥ 3 bouts of cataplexy, the typical criteria for inclusion in pharmacological studies on cataplexy (Black et al., 2017b). These mice exhibited 5.4 ± 0.9 bouts of cataplexy that each lasted 1.2 ± 0.2 min long for 4.7 ± 1.2 cumulative minutes during the dark period.

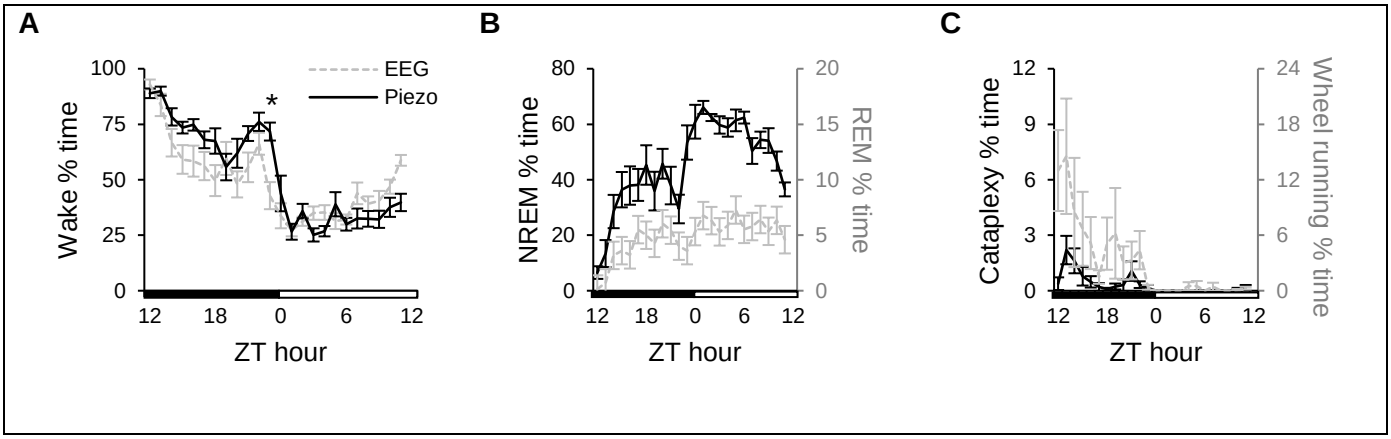

Figure 4. Quantification of arousal states in narcoleptic mice by EEG/EMG recording. Comparison of wake % per h in male orexin/ataxin-3 (Atax, *N* = 11) mice as measured by piezoelectric monitoring **(A, black line)** vs. manual scoring **(A, gray dashed line)** of EEG/EMG/video recordings from the same mice 8 weeks later. The % per h of NREM **(B, black line)**, REM **(B, gray dashed line)**, cataplexy **(C, black line)**, and wheel running **(C, gray dashed line)** as measured by manual scoring of EEG/EMG/video recordings. Two-way repeated-measures analysis of variance with Bonferroni's multiple comparisons: * P < 0.05 vs. EEG/EMG/video.

3.3. Sleep/wake pharmacology using PiezoSleep

To validate piezoelectric monitoring of sleep/wake for the screening of wake-promoting compounds for narcolepsy, male Atax mice (*N* = 46) and WT mice (*N* = 60) were dosed with MOD (10, 30, 50, 100 mg/kg, 10 mL/kg) or vehicle orally at ZT5 when sleep propensity is high.

As expected, MOD induced a dose-related inhibition of sleep for 3 h (30 and 50 mg/kg) and 6 h (100 mg/kg) post dosing vs. vehicle in WT (Figure 5A), $F(24, 330) = 11.4$, $P < 0.0001$. In Atax mice, MOD inhibited sleep for 2, 4, and at least 7 h post dosing at 30, 50, and 100 mg/kg, respectively, compared to vehicle (Figure 5B), $F(24, 246) = 8.25$, $P < 0.0001$. Although a dose-related response to MOD (10-100 mg/kg) was evident for the maximum sleep deficit in both Atax and WT mice (Figure 5C), $F(4, 96) = 96.0$, $P < 0.0001$ (main effect for dose), there was no interaction with genotype $F(4, 96) = 1.63$, *n.s.*

Modafinil fragmented sleep bouts in both mouse models. Under baseline and vehicle conditions during the last 7 h of the light period, WT mice spent most of their sleep time (26-31% of total time) in bouts that were 32-64 min long (Figure 5D). After MOD (10-100 mg/kg), the percentage of time spent in these long sleep bouts was reduced in dose-related manner, $F(28, 385) = 5.38$, $P < 0.0001$, and the distribution of time spent in the different SBD bins shifted such that more time was spent in shorter bouts than longer bouts (i.e., leftward shift of the peak), especially at the highest dose. Unlike WT mice, Atax mice in baseline and vehicle conditions spent 5-6% of the time in long sleep bouts ≥ 32 min, and most of their sleep time (22-25% of total time) was in bouts that were 8-32 min long (Figure 5E). Modafinil (10-100 mg/kg) further fragmented the sleep of Atax mice, as the percentage of time spent in sleep bouts 8-32 min long was reduced in a dose-related manner, $F(24, 246) = 8.25$, $P < 0.0001$. However, only the highest dose of MOD shifted the peak of the sleep bout distribution curve to the left toward shorter bouts. A dose-related increase in the percentage of time spent in wake bouts ≥ 8 min was evident after MOD (10-100 mg/kg) in WT and Atax mice (Figure 5F), $F(4, 96) = 98.6$, $P < 0.0001$ (main effect for dose), but the effect did not depend on genotype $F(4, 96) = 2.87$, $P = 0.03$, no significant contrasts per dose.

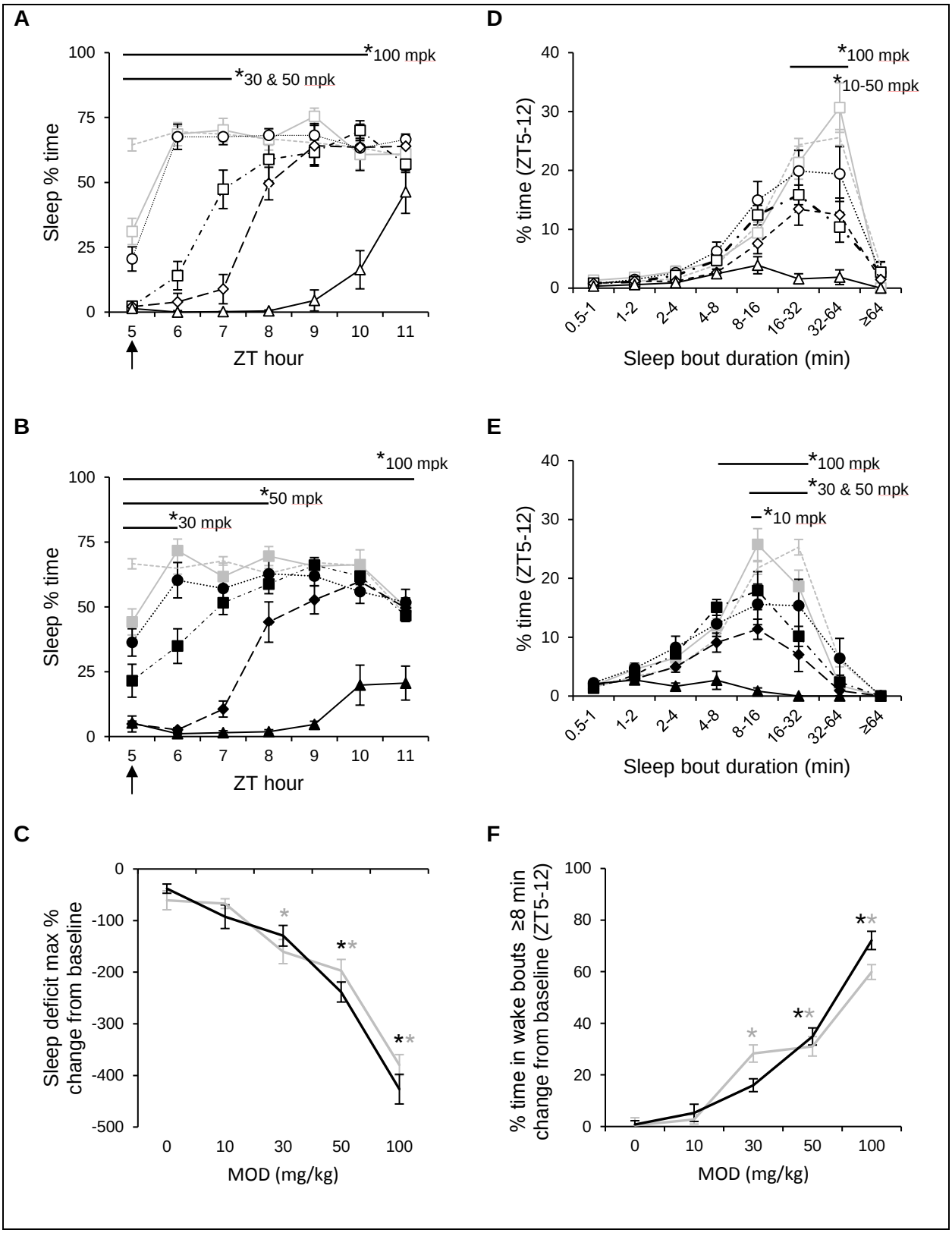
A
*100 mpk
*30 & 50 mpk
Sleep % time
ZT hour
B
*100 mpk
*50 mpk
*30 mpk
Sleep % time
ZT hour
C
Sleep deficit max % change from baseline
MOD (mg/kg)
D
*100 mpk
*10-50 mpk
% time (ZT5-12)
Sleep bout duration (min)
E
*100 mpk
*30 & 50 mpk
*10 mpk
% time (ZT5-12)
Sleep bout duration (min)
F
% time in wake bouts ≥8 min change from baseline (ZT5-12)
MOD (mg/kg)

Figure 5. Piezoelectric monitoring of sleep/wake in pharmacological studies. Inhibition of sleep and promotion of wakefulness by modafinil (MOD) in orexin/ataxin-3 (Atax, **filled symbols, B, E** and **black lines C, F,** *N* = 9-10/group) vs. wild type (**open symbols, A, D** and **gray lines C, F,** *N* = 12/group) male mice as detected by PiezoSleep. Mice were administered MOD at 0 (vehicle, **gray squares**), 10 (**circles**), 30 (**black squares**), 50 (**diamonds**), or 100 (**triangles**) mg/kg (mpk) at 5 h after lights on (zeitgeber time (ZT)5, **arrow**) after 30 h undisturbed baseline. Sleep % per h (**A-B**) and the percentage of time spent in sleep bout durations lasting from 0.5 to ≥ 64 min (**D-E**) for 7 h post dosing were plotted against values taken from undisturbed baseline (**gray dashed line**) for reference. The maximum sleep deficit (**C**) and the % of time spent in consolidated wake bouts ≥ 8 min for 7 h post dosing, expressed as the change from baseline (**F**), revealed dose-related wake-promotion by MOD (differences between genotypes, *n.s.*). Two-way analysis of variance with Bonferroni's multiple comparisons: * $P < 0.05$ vs. vehicle for doses and at time or bout duration bin ranges as indicated by bars at top of graphs.

4. Discussion

Piezoelectric monitoring of sleep/wake was used to identify key features of the narcoleptic phenotype that distinguish Atax mice by symptom severity, thus enabling rapid, non-invasive sorting of mice for EEG/EMG studies for cataplexy detection. The PiezoSleep narcolepsy screen was used to assign a symptom severity score—the ratio of time spent in long WBD (≥ 32 min) to time in short SBD (< 8 min) during the first half of the dark period—to individual mice. These wake-maintenance scores spanned a 15-fold difference between the most severely narcoleptic mice and their WT littermates. The PiezoSleep system was also demonstrated to detect changes in sleep/wake time and consolidation after administration of the wake-promoting therapeutic modafinil in normal and transgenic mice. Using this method, the efficacy of wake-promoting compounds can be determined in a 5-arm study with up to 60 mice in less than one week for high-throughput screening *in vivo*.

The PiezoSleep narcolepsy screen was designed to be a translational test of the ability to sustain long periods of wakefulness as is assessed in humans by the MWT and the MSLT. For the human tests, patients are provided 4-5 opportunities throughout the daytime to either

nap within 20 min (MSLT) or to try to stay awake for 20-40 min (MWT) in the absence of alerting factors. The latency to polysomnographically defined sleep is measured for each test. A mean sleep onset latency ≤ 8 min with ≥ 2 sleep-onset REM periods (abnormal transition into REM sleep within 15 min of sleep onset) is a positive result on the MSLT and aids in the diagnosis of narcolepsy (Cao and Guilleminault, 2017). A murine MSLT has been devised in which the latency to EEG/EMG-defined sleep is measured during four 20-min nap opportunities presented after 20 min of enforced wakefulness (Veasey et al., 2004). The PiezoSleep narcolepsy screen provides a rapid, automated alternative to the murine MSLT and does not require the manipulation of sleep homeostatic processes to obtain the measurements. The translational utility of the PiezoSleep phenotyping assay is limited by the inability of the current version of the algorithm to distinguish between NREM and REM sleep. Perhaps a new algorithm under development (Yaghouby et al., 2016) will permit quantification of short REM sleep latency into the assay as a murine equivalent of sleep-onset REM periods.

The PiezoSleep narcolepsy screen can be used to prescreen narcoleptic mice to identify those with the most fragmented sleep/wake for inclusion in resource-intensive EEG/EMG studies. The ratio of time spent in long WBD to short SBD clearly differentiated narcoleptic mice with high sleep propensity from WT littermates that were able to spontaneously sustain long bouts of wakefulness from ZT12-18 (Figure 2). It is unknown if the wake bouts in Atax mice terminated because of sleep onset or from the intrusion of cataplexy, which may register on the PiezoSleep assay as sleep due to the similar motor and autonomic physiology between REM sleep and cataplexy. Nevertheless, this distinction does not detract from the utility of the PiezoSleep narcolepsy screen to differentiate Atax mice by severity of narcolepsy symptoms. Out of the 37 Atax mice screened for inclusion in the EEG/EMG study, 16 mice with severe symptoms were identified through rank ordering of the wake-maintenance scores. These mice scored 4.7x lower on the PiezoSleep narcolepsy screen than the remainder of the Atax mice,

and only 2 out of the final 11 included in analysis (18%) had < 3 cataplexy bouts. Compared to a previous report that 34% of Atax mice fall below this study inclusion criteria (Black et al., 2017b), the current study represents a 16% improvement in yield. However, it is difficult to determine if the yield differences are due to sorting methodology or to differences between colonies and recording environments. The reason for high interindividual differences in cataplexy expression between Atax mice remains unclear, but may recapitulate the etiology of human narcolepsy type 2 (narcolepsy without cataplexy) which is also poorly understood (Black et al., 2017b). Determination of whether the wake-maintenance scores on the PiezoSleep narcolepsy screen correlate with the severity of cataplexy would require EEG/EMG measurement from mice across a broader range of symptom severity scores, and was beyond the scope of the current study. Comparison of EEG/EMG-defined sleep/wake between mice with low and high wake-maintenance scores identified by piezoelectric monitoring would be an important next step in the validation of the PiezoSleep narcolepsy screen.

Some differences between studies in the comparison of piezoelectric monitoring of sleep/wake and polysomnography are worth noting. Previous studies have validated the PiezoSleep sleep/wake classifier against manually scored EEG/EMG signals that had been simultaneously recorded from the same mice (across 4 strains) for direct epoch-to-epoch comparison (Flores et al., 2007; Mang et al., 2014). The current analysis in Atax mice does not permit this level of scrutiny, as the PiezoSleep and EEG recordings were staged in 2 and 10 second epochs, respectively, and occurred 8 weeks apart in different environments (e.g., the EEG recordings occurred in standard cages equipped with running wheels in a sound-attenuated recording chamber). Wakefulness over 24 h was 3.6% higher after piezoelectric monitoring than after EEG/EMG recording in the current study, vs. 2.3% lower as previously reported (Mang et al., 2014). In addition to the caveats between methods as noted above, the discrepancy could be due to the different mouse strains studied (Atax vs. CFW Swiss Webster).

Orexin knockout mice tend to breathe shallow and fast compared to WT when both asleep and awake as determined by EEG/EMG (Kuwaki, 2008). This respiration pattern during sleep could conceivably be misconstrued as quiet wakefulness in orexin-deficient Atax mice in the PiezoSleep assay, thus slightly overinflating the measurement of time awake compared to EEG/EMG in the current study. However, if this respiration-induced, false detection of wake in narcoleptic mice were true, then piezoelectric monitoring would be expected to detect more wakefulness in Atax mice than in WT littermates, which was not the case (Figure 2A). The differences in sleep/wake as determined by EEG/EMG and piezoelectric monitoring in the current study are likely due to the different environments used for recording. Future studies are needed in which simultaneous piezoelectric EEG/EMG recordings are collected during the dark period from narcoleptic mice to assess cataplexy detection using an unsupervised algorithm that has been tuned to extract features of REM sleep (Yaghouby et al., 2016) to determine if cataplexy can be identified by piezoelectric monitoring.

Perhaps more noteworthy than the small discrepancies between polysomnography and PiezoSleep monitoring in the current study was the consistency in the pattern of sleep/wake between the two recording methods (Figure 4A). The hour-by-hour increase and decrease in wake time after EEG/EMG recording paralleled the pattern observed after piezoelectric monitoring, despite the 8 week difference in recording dates. The hour immediately prior to lights-on was the only time point in which PiezoSleep registered more wakefulness in Atax mice than polysomnography (i.e., At ZT23, Atax mice slept more as determined by polysomnography than they did 8 weeks prior as measured with PiezoSleep). This time point was the same ZT in which Atax mice slept more than WT mice after piezoelectric monitoring of both genotypes. The increased sleep observed in Atax mice at the end of the dark period may reflect progressive worsening of EDS over time in this model. The differences in sleep/wake measurement between genotypes and between methods at ZT23 are less likely due to reduced accuracy of PiezoSleep

at that time point, as could be speculated, because the variance was similar at that hour between the different runs and between other ZT hours. It is also not clear what physiological or behavioral factors would differentially impact the detection of respiration rates and gross motor activity at ZT23 vs. other times of day.

In the current study, piezoelectric measurement of sleep/wake was validated as a method to screen wake-promoting compounds rapidly and at scale for the development of therapeutics for narcolepsy. Dosing WT mice at ZT5 was chosen as the first pass of screening using MOD because of the similarities between Atax and WT mice in the high amount of sleep per hour (Figure 2A) and in the very short WBD (Figure 2C) during the light period. Modafinil (50 mg/kg) with total brain exposure of 33.7 μM approximated the ED_{50} in both WT and Atax mice in terms of the time course of sleep inhibition, the maximum sleep deficit induced, and the consolidation of wakefulness (Figure 5) and is recommended as the positive control for screening the wake-promoting efficacy of compounds. Using the PiezoSleep assay, a total of 106 mice in two cohorts were screened across 4 concentrations of MOD vs. vehicle in two weeks, representing substantial improvement in efficiency over conventional sleep/wake bioassays (Black et al., 2017a; Fujiki et al., 2009). Future studies are needed to validate the PiezoSleep assay for pharmacological studies at different times of day and with hypnotics. Of particular interest would be to test the hypothesis that wake-promoting therapeutics administered at ZT12 would rescue the narcoleptic phenotype of Atax mice as measured by the wake-maintenance score on the PiezoSleep narcolepsy screen.

Piezoelectric monitoring of sleep/wake offers advantages over video-based methods, even those that claim to discriminate NREM and REM sleep (McShane et al., 2012) for several reasons. Because PiezoSleep does not depend on a visual signal, it permits mice to sleep while burrowed in nesting material for assessment of more ecologically relevant behavior. The highly sensitive piezoelectric sensor captures a detailed respiratory measure that enables it to

distinguish quiet, resting wakefulness from sleep using relevant physiology (Donohue et al., 2008; Flores et al., 2007; Mang et al., 2014; Yaghouby et al., 2016). To the best of our knowledge, sleep state discrimination based on postural changes in the aspect ratio of the body on video recordings (McShane et al., 2012) has not been replicated nor validated in mouse models characterized by obesity, such as Atax mice (Hara et al., 2001).

In conclusion, the non-invasive, unsupervised piezoelectric monitoring of sleep/wake is a new tool for efficacy studies with arousal state endpoints. The PiezoSleep narcolepsy screen translates as a murine test of the ability to sustain long bouts of wakefulness, as measured in humans with the MWT and MSLT, and can be used to improve phenotypic sorting for resource intensive EEG/EMG studies. PiezoSleep has been validated as a means to rapidly quantify sleep/wake time and consolidation for high-throughput screening *in vivo* in drug discovery.

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