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ORIGINAL ARTICLE

Effects of sex and estrous cycle on sleep and cataplexy in narcoleptic mice

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Abstract

Narcolepsy type 1 (NT1) is a rare neurology disorder caused by the loss of orexin/hypocretin neurons. NT1 is characterized by excessive daytime sleepiness, sleep and wake fragmentation, and cataplexy. These symptoms have been equally described in both women and men, although influences of gender and hormonal cycles have been poorly studied. Unfortunately, most studies with NT1 preclinical mouse models, use only male mice to limit potential variations due to the hormonal cycle. Therefore, whether gender and/or hormonal cycles impact the expression of narcoleptic symptoms remains to be determined. To address this question, we analyzed vigilance states and cataplexy in 20 female and 17 male adult orexin knock-out narcoleptic mice, with half of the females being recorded over multiple days. Mice had access to chocolate to encourage the occurrence of cataplectic episodes. A vaginal smear was performed daily in female mice to establish the state of the estrous cycle (EC) of the previous recorded night. We found that vigilance states were more fragmented in males than females, and that females had less paradoxical sleep ($p = 0.0315$) but more cataplexy ($p = 0.0375$). Interestingly, sleep and wake features were unchanged across the female EC, but the total amount of cataplexy was doubled during estrus compared to other stages of the cycle ($p = 0.001$), due to a large increase in the number of cataplexy episodes ($p = 0.0002$). Altogether these data highlight sex differences in the expression of narcolepsy symptoms in orexin knock-out mice. Notably, cataplexy occurrence was greatly influenced by estrous cycle. Whether it is due to hormonal changes would need to be further explored.

Statement of Significance

This study provides evidence that sex and the estrous cycle influence cataplexy severity in a preclinical mouse model of narcolepsy type 1. Of particular interest, it shows for the first time that the amount of cataplexy is doubled during the estrus stage in female orexin-deficient mice. Gender differences in narcolepsy type 1 are largely overlooked and studies on the influence of the menstrual cycle on the severity of symptoms are largely absent. Future studies evaluating the influence of gender and hormonal fluctuations on symptom severity of narcolepsy would be of great interest to the benefit of patients and to get a better understanding of the disease.

Key words: narcolepsy; hormone; hypothalamus; REM sleep; female; neuroendocrine

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Introduction

Female-male differences are common at the cellular level and can arise from many factors such as sex-chromosome gene expression, specializations in receptors expression, or levels of circulating hormones. In turn, these cell-based sex differences can give rise to female-male differences in brain networks and behaviors. Such effects may have substantial implications in sleep regulation and sleep disorders, some showing a clear difference in men-women prevalence. Accordingly, sex differences in sleep amount, sleep architecture, slow gamma power or sleep spindle characteristics have been reported [1–4]. Hormonal fluctuations over the estrous or menstrual cycle also induce variations in sleep architecture [1, 4–8].

Narcolepsy type 1 (NT1) is an orphan neurological disorder that is characterized by excessive daytime sleepiness, paradoxical sleep (PS, as called REM [rapid eye movement] sleep) dysregulation, hypnagogic hallucinations, sleep paralysis, and episodes of cataplexy [9]. A few studies have evaluated the men–women prevalence of NT1 but some showed no major gender effect [10], a slight predominance for men [11], or a higher incidence in women [12, 13].

The etiology of NT1 is caused by the specific postnatal loss of orexin/hypocretin (OREX) neurons of the lateral hypothalamus [14, 15]. Cataplexy—a pathognomonic symptom of NT1 depicted by a bilateral loss of postural muscle tone during wakefulness and triggered by a strong, generally positive emotion without loss of consciousness [16, 17]—is strongly associated with the loss of OREX [18–22]. Interestingly, a few studies have shown that preprohypocretin mRNA and OREX-A protein levels in lateral hypothalamus and the cerebrospinal fluid are higher in female rats compared to males [23–25]. Additionally, it has been reported that sex hormone fluctuations across the estrous cycle (EC) affect OREX expression [26, 27].

In light of these data, it can be hypothesized that the loss of OREX may have a different impact on females vs males with a potential gender difference in symptom severity in NT1, such as sleepiness, PS dysregulation, and/or cataplexy.

Of particular interest, the severity of cataplexy—in its frequency and extent—varies greatly among patients [28]. Gender differences have been largely understudied with no consensus between studies [10, 29–31], and to the best of our knowledge, the impact of the menstrual cycle has not yet been evaluated. Similarly, except for the most recent studies [32–34], only male mice were classically included in preclinical studies on narcolepsy. To fill this gap in our knowledge, two recent studies [35, 36] investigated sex-related differences on sleep in an inducible mouse model of narcolepsy and reported no difference in sleep and wake amounts. Coffey et al. [35] additionally found that females experienced more cataplexy than males. However, none of those studies evaluated whether EC influences the expression of cataplexy.

Here, we first evaluated whether sex influences sleep-wake features and cataplexy in a well-established mouse model of narcolepsy, the OREX Knock-Out mice (Orex-KO). Secondly, we quantified sleep, wake, and cataplexy across the EC of these mice.

Methods

Animals

Experiments were conducted with a mouse model of NT1 exhibiting sleep fragmentation and cataplexy, the Orex-KO mice [18] of C57Bl/6J genetic background. Twenty nulliparous female and 17 male mice were implanted for polysomnography recordings at 12 weeks of age (23.2 ± 0.5 g and 28.6 ± 0.8 g, respectively; $p < 0.0001$). Mice were recorded between 13 and 17 weeks of age, some being recorded for several weeks, across several ECs. Animals were maintained under a constant light/dark cycle (12:12 h, lights on at 07:00 am) with *ad libitum* access to water and regular chow throughout the experiment. Room temperature was kept at 23 ± 1°C. The experimental procedures were approved by the CELYNE Ethics Research Committee (protocol #21759) in accordance with the European Community guidelines for care in animal research.

Experimental design

Seven to ten days after surgery, mice were individually hosted in transparent Plexiglas barrels (30 cm in diameter) and placed in an open-space recording chamber containing 4 barrels. Mice were able to see, hear and smell but not touch each other. Barrels were enriched with a running wheel (diameter: 15.5 cm) and paper cuttings for nest building to improve animal welfare [37–39]. After 3 days of habituation to this new environment, mice were recorded continuously.

In the first set of experiments, Orex-KO mice—10 females and 9 males—were recorded in groups of up to 4 mice of the same sex. They were first recorded in baseline conditions for 24 h. The following day, a piece of milk chocolate was given to each mouse at 6:30 pm in addition to regular food to encourage the occurrence of cataplexy [38]. Note that among the 10 female mice, 8 were recorded in both baseline and chocolate conditions, 1 was only recorded in baseline condition and 1 was recorded under chocolate treatment only. The latter two females were integrated into the analyses but only when unpaired comparisons were made. All males were recorded in baseline and under chocolate conditions.

In the second set of experiments, 4 series of 2–3 females and 1 male were recorded for several nights in a row to obtain multiple recordings per EC stage. The presence of a male in the chamber encouraged female estrus and reduced any anestrus. To determine the stage of the EC of the previous night, a vaginal smear was performed by a trained experimenter each morning between 9:00 and 10:00 am. For each 12 h of night of recording, one male was analyzed in parallel with the females to uncover potential effects due to their presence. Note that one night of baseline was recorded, then chocolate was added on each of the following recorded nights. Unfortunately, for two of these 10 females and one of the 4 males, recordings were not available without chocolate. Furthermore, to evaluate whether the presence of females would affect sleep and cataplexy in males, 4 additional males were recorded in the presence of 3 females (for a total of 8 males). Note that these females were not recorded but their EC was evaluated.

Surgical procedure

Anesthesia was induced by an intraperitoneal injection of a cocktail of ketamine:xylazine (100:10 mg/kg) then mice were placed on a stereotaxic frame (David Kopf Instruments, USA) where they were implanted with three electroencephalogram (EEG) electrodes: one above the parietal cortex (1.5 mm lateral to midline and –2.5 mm posterior to Bregma) and one above the

frontal cortex (1 mm lateral to midline and 1.5 mm anterior to Bregma), the last one above the cerebellum (1.5 mm lateral to midline and 7 mm posterior to Bregma) to be used as reference. Two electromyogram (EMG) electrodes were slipped between neck muscles. Then, all EEG and EMG electrodes were connected to a miniature plug (Plastics One, Bilaney, Germany), fixed to the skull using acrylic cement (Superbond, C&B Sun Medical), and further coated using dental cement (Paladur, Heraeus Kulzer). A subcutaneous injection of carprofen (5 mg/kg) was administered at the end of surgery for pain caring.

Polysomnographic recordings

Mice were connected to a cable plugged into a rotating connector (Plastics One, Bilaney, Germany) to allow free movements during recordings and were acclimated to the recording chamber and EEG/EMG cable for at least 3 days. Each frontal and parietal EEG signal was referenced to the cerebellar EEG electrode, and muscle tone was assessed by a differential EMG signal [40]. Briefly, EEG and EMG signals were amplified (EEG: 2000×; EMG: 5000×; A-M systems, Model 3500, Sequim, WA 98382, USA), filtered (bandwidth 1–100 Hz for the EEG, 10–100 Hz for the EMG), digitized (NI USB-6343 card, National Instruments, Austin, TX, USA, sampling rate 1024 Hz) and collected using Sleepscore software (Viewpoint, Lyon, France). Video recordings were continuously collected with an infrared camera fixed above each barrel (Point Grey firefly MV, Black & White, 640 × 480, 15 frames/sec).

Quantification of the vigilance states and cataplexy

Vigilance states were scored using a 5-sec window frame according to standard criteria using Sleepscore [40]. Wake (WK), slow-wave sleep (SWS), and PS were assigned using the standard mouse EEG/EMG scoring method, based on rules established in wild-type mice [41]. Cataplexy was identified according to the consensual definition [42] using both EEG/EMG and simultaneous video recordings [43] and the four following criteria (1) an abrupt episode of nuchal muscle atonia lasting for at least 10 sec, (2) with immobility of the animal during the entire episode, (3) an EEG signal rich in theta and low in delta activities, and (4) at least 40 sec of WK prior to the episode (Supplementary Video). In addition, attacks were considered as cataplexy when occurring outside the nest, during a period of high motor activity [39].

Estrous cycle staging

Mouse EC is classically divided into 4 stages: proestrus (P), estrus (E), metestrus (M), and diestrus (D) using vaginal cytology, a cycle that repeats itself every 4–5 days unless interrupted by pregnancy or anestrus [44]. A vaginal lavage was performed daily between 09:00 and 10:00 am to collect vaginal cells as previously described [45]. The smear was air-dried at room temperature, stained using crystal violet (Sigma Aldrich) for 1 min, gently washed twice in distilled water, and coverslipped with glycerol (Sigma Aldrich). The observation was performed under a light microscope immediately after staining. Each EC stage was identified according to its relative ratio of polymorphonuclear leukocytes, cornified squamous epithelial cells, and nucleated epithelial cells [1, 44, 45]. P was defined by the predominance of nucleated epithelial cells accompanied by a few cornified epithelial cells (Supplementary Figure S1A). E was determined when cornified epithelial cells were mostly present (Supplementary Figure S1B). In M, cornified epithelial cells and leukocytes were present while nucleated cells were absent (Supplementary Figure S1C). In D, leukocytes were predominant but epithelial cells were also found (Supplementary Figure S1D).

Photomicrographs

They were taken using an Axioskop microscope (Axioskop 2 plus, Zeiss) and a color video camera (ProgRes CF, Jenoptik) connected to an imaging analysis system (MorphoLight, Explora Nova, France).

Statistical analysis

Graphs and statistical analyses were performed using GraphPad Prism (v9), and the significance was set at 0.05.

To compare females to males, since mice were recorded for several nights in a row, we first calculated sleep/wake and cataplexy median values of the repeated recordings for each mouse regardless of the EC. Normality of distribution was assessed using the Shapiro-Wilk test. Unpaired *t*-test was performed on data with normal distributions while the non-parametric Mann-Whitney test was applied when the normality assumption was violated. When comparing females or males in baseline vs chocolate, we performed Wilcoxon or paired *t*-tests depending on normality of the data distribution. Weight difference between groups was evaluated using an unpaired *t*-test and relations between weight and the occurrence of cataplexy were verified by using a Pearson correlation test.

To analyze vigilance states and cataplexy across EC, a repeated-measures analysis of variance (ANOVA) with one dimension (EC stages) was performed, followed by a post-hoc Tukey's test for multiple comparisons when appropriate. To limit any masking effects due to the previously described high intra- and inter-individual variability in cataplexy occurrence (Supplementary Figure S2) [39, 46], data were normalized. For each parameter studied and each female mouse, we first determined a reference value, the median of each EC stage median value. Then each measure was normalized by calculating the ratio of the value to its reference. We were thus able to evaluate the expression of cataplexy according to the different stages of the EC and compare mice with each other. We used a similar strategy to normalize the amount of cataplexy in males recorded in the presence of 0, 1, 2, or 3 females with concomitant E.

The data underlying this article will be shared on reasonable request to the corresponding author.

Results

Influence of sex on vigilance states

To evaluate whether sex could influence sleep and wake features and cataplexy in Orex-KO mice, females and males recorded in the absence of the opposite sex were first compared in baseline condition then they were compared while being under chocolate treatment.

Nine female and nine male mice were first compared in terms of WK, SWS, and PS quantity, number of episodes, and

mean episode duration over 24 h. Female mice had the same amount of WK and SWS with a similar level of fragmentation than males (Table 1) but had less PS ($p = 0.0315$) due to a highly reduced number of PS bouts ($p = 0.0025$) (Table 1). During the light phase, only the number of PS episodes was mildly reduced in females compared to males ($p = 0.0448$) (Supplementary Table S1). However, during the dark phase, all vigilance states—WK, SWS, and PS—were more fragmented in males than females with a higher number of bouts of shorter mean duration (Figure 1; Supplementary Table S2). Accordingly, PS latency was significantly shorter in males than females (4.81 ± 1.5 vs 28.80 ± 7.2 min, respectively; unpaired *t*-test $p = 0.0050$).

With the aim of combining females and males in future studies and recording them in parallel in an open space, we evaluated whether the presence of mice of the opposite sex would influence vigilance states in Orex-KO mice during the dark phase. We found no significant differences between females recorded in the presence or absence of males for all parameters studied (total amount, number of episodes, and mean episode duration) of WK, SWS, and PS (Figure 1; Supplementary Table S2). Similarly, the presence of females did not alter vigilance states in males (Figure 1; Supplementary Table S2). When comparing animals recorded in the presence of a member of the opposite sex, we found no female-male differences for WK, SWS, and PS (Figure 1; Supplementary Table S2).

In most studies, chocolate is given to mice just before light-off to favor the occurrence of cataplexy. We thus evaluated whether chocolate would influence vigilance states during the dark phase and whether it would do it in a similar proportion in both females and males.

Chocolate treatment increased WK, decreased SWS, and had no significant effect on PS in female and male Orex-KO mice compared to their respective baseline (paired *t*-tests; Table 2). However, these chocolate effects tended to be of different amplitudes in females vs males (Table 2). In particular, females showed a nearly 50% reduction in SWS ($p = 0.0012$) while males had only a 28% reduction ($p = 0.0115$). In both males and females, the SWS mean episode duration was reduced but females also had less SWS bouts while having access to chocolate, than during baseline ($p = 0.0038$) (Table 2). As a result, females were slightly more awake ($p = 0.0464$) and had significantly less SWS ($p = 0.0135$) than males under chocolate treatment (Table 2). Furthermore, vigilance states were less fragmented in females than males with a lower number of episodes (unpaired *t*-tests; Table 2). However, when animals were recorded with mice of the opposite sex and with chocolate, no female-male difference was found in the amount of WK ($p = 0.46$), SWS ($p = 0.97$), or PS ($p = 0.09$), nor in their fragmentation index as evaluated by the number of bouts and their mean bout duration (Figure 2).

Altogether, these data showed that chocolate treatment strongly modulates vigilance states in Orex-KO mice while the presence of animals of the opposite sex has no influence.

Effects of sex on cataplexy

Cataplexy occurs essentially during the dark phase (Figure 1) and only rarely during the light phase at light-on/light-off transitions (Supplementary Table S1). We thus focused on the dark phase. We found that the total amount of cataplexy in baseline condition was higher in females than male Orex-KO mice ($p = 0.0375$) due to a longer mean episode duration ($p = 0.0188$) (Figure 1J–L; Supplementary Table S2). The number of cataplexy episodes tended to be higher in females than males, but it did not reach significance due to high inter-individual variability ($p = 0.08$) (Figure 1K; Supplementary Figure S2).

We used chocolate to encourage the occurrence of cataplexy in Orex-KO mice. Accordingly, we found a large increase in the total amount and the number of cataplexy episodes in females ($p = 0.0001$ and $p = 0.0003$, respectively) and males ($p = 0.0039$ for both), although the increase was larger in males than females (929% vs 378%, respectively) (Table 2). As a result, the female-male differences in the amount of cataplexy that we observed in baseline condition (Figure 1J–L), were less prominent under chocolate treatment, although still significant ($p = 0.0294$) (Table 2).

As female mice were leaner than males ($p < 0.0001$), we verified whether the higher amount of cataplexy in females could be due to weight differences. We found no correlation between weight and the total amount of cataplexy, the number of episodes, or mean episode duration of cataplexy within each group (females or males), whether we considered baseline (data not

Table 1. Vigilance states and cataplexy quantification of female and male mice recorded over 24 h of baseline

	Wakefulness			Slow-wave sleep			Paradoxical sleep			Cataplexy		
	Total amount (min)	Number of episodes	Mean episodes duration (sec)	Total amount (min)	Number of episodes	Mean episodes duration (sec)	Total amount (min)	Number of episodes	Mean episodes duration (sec)	Total amount (min)	Number of episodes	Mean episodes duration (sec)
Females ($n = 9$)	741.78 ± 28.0	440.33 ± 20.5	103.08 ± 6.9	586.70 ± 29.6	418.44 ± 24.6	85.69 ± 4.8	96.83 ± 4.1	135.89 ± 7.4	43.25 ± 1.9	14.85 ± 4.2	21.67 ± 6.5	36.61 ± 5.7
Males ($n = 9$)	742.44 ± 15.0	496.44 ± 24.9	91.13 ± 3.8	584.69 ± 16.4	491.33 ± 25.1	73.49 ± 5.4	110.08 ± 2.4	178.22 ± 6.5	37.42 ± 1.1	2.57 ± 1.6	5.56 ± 2.0	17.51 ± 3.9
Test	Unpaired *t*-test	Unpaired *t*-test	Unpaired *t*-test	Unpaired *t*-test	Unpaired *t*-test	Unpaired *t*-test	Mann-Whitney	Mann-Whitney	Unpaired *t*-test	Mann-Whitney	Mann-Whitney	Mann-Whitney
t, *df* values or *U* value	$t = 0.021$ $df = 16$	$t = 1.738$ $df = 16$	$t = 1.528$ $df = 16$	$t = 0.059$ $df = 16$	$t = 2.074$ $df = 16$	$t = 1.676$ $df = 16$	$U = 16$	$U = 8$	$t = 2.617$ $df = 16$	$U = 17$	$U = 20.5$	$U = 13$
p value	0.9836 ns	0.1015 ns	0.1461 ns	0.9532 ns	0.0546 ns	0.1131 ns	0.0315*	0.0025**	0.0487*	0.0375*	0.0801 ns	0.0142*

The total amount, number of episodes, and mean episode duration of vigilance states and cataplexy are represented by the mean ± SEM in females ($n = 9$) and males ($n = 9$). Data are extracted from experiment 1 when mice were recorded in the absence of chocolate and of animals of the opposite sex. Statistical tests: unpaired *t*-test or Mann-Whitney test were used depending on whether data followed a normal distribution or not, respectively; ns: non-significant.
*$p < 0.05$; **$p < 0.01$.

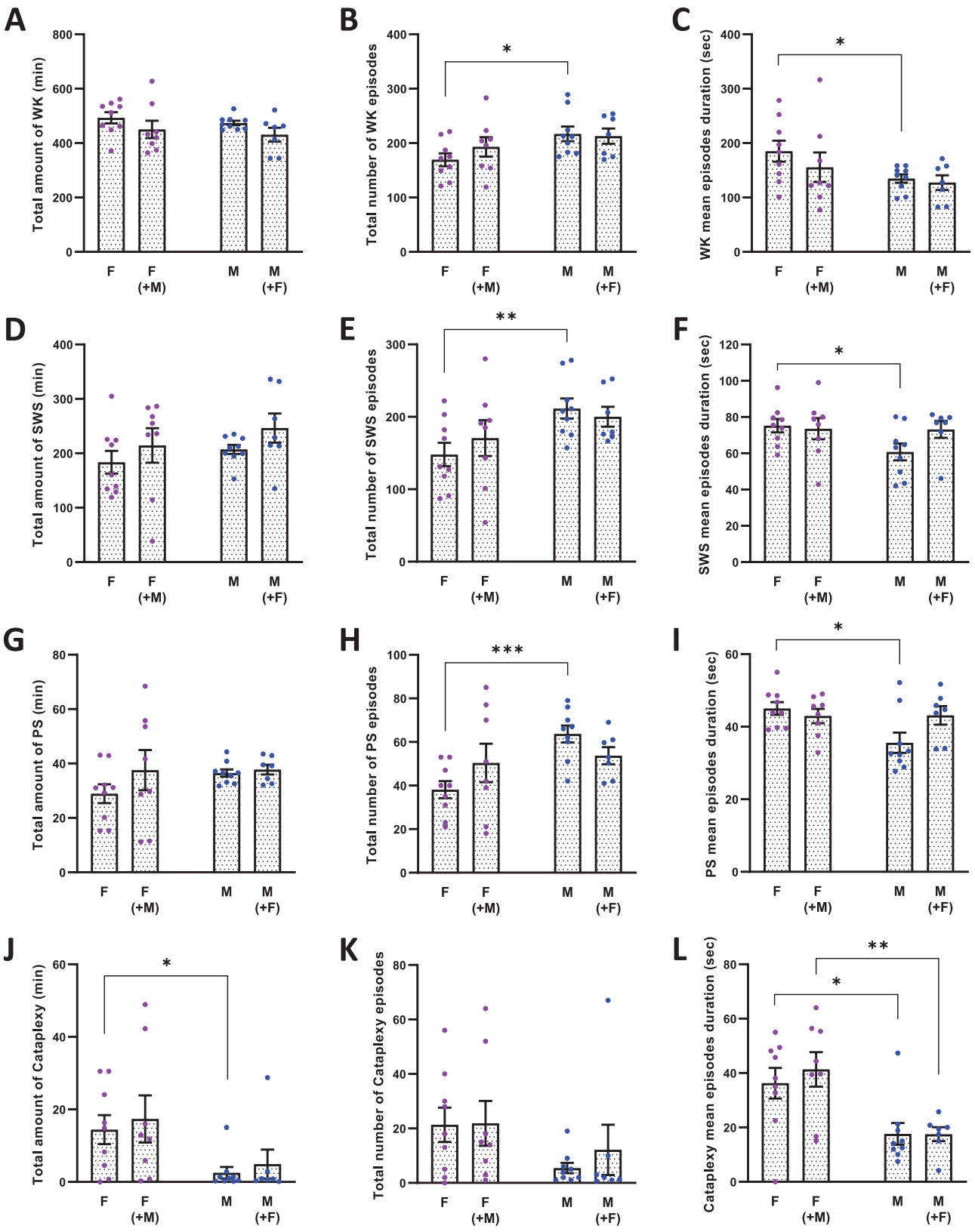

Figure 1. Effects on vigilance states and cataplexy of female and male Orex-KO mice of the presence of mice of the opposite sex. Histograms on the left illustrate the total amount of time (mean ± SEM) spent in WK (A), SWS (D), PS (G), and cataplexy (J) over the 12 h of the dark phase. Histograms in the middle illustrate the number of WK (B), SWS (E), PS (H), and cataplexy (K) episodes (mean ± SEM) while those on the right illustrate the mean episode duration (mean ± SEM) for WK (C), SWS (F), PS (I) and cataplexy (L) in the 4 experimental groups, female (F; $n = 9$), females in the presence of males (F(+M); $n = 8$), males (M; $n = 9$) and males in the presence of females (M(+F); $n = 7$). Note that mice of the F group are different mice than those of the F(+M) group, (same for the male population). Statistical tests for independent measures were thus used. Each dot represents one animal, female (purple) and male (blue). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 2. Quantification of vigilance states and cataplexy during the 12 h of dark phase in female and male Orex-KO mice having access, or not, to chocolate

	Wakefulness			Slow-wave sleep			Paradoxical sleep			Cataplexy		
	Total amount (min)	Number of episodes	Mean episodes duration (sec)	Total amount (min)	Number of episodes	Mean episodes duration (sec)	Total amount (min)	Number of episodes)	Mean episodes duration (sec)	Total amount (min)	Number of episodes	Mean episodes duration (sec)
Females (n = 8)	484.40 ± 21.0	175.40 ± 11.4	173.50 ± 19.4	191.60 ± 21.8	155.00 ± 16.4	74.85 ± 4.1	30.64 ± 3.4	40.00 ± 3.9	45.69 ± 1.9	13.22 ± 4.3	20.25 ± 7.0	34.81 ± 6.2
Females with CHOCO (n = 8)	556.80 ± 12.7	172.30 ± 10.6	200.2 ± 14.6	88.00 ± 11.8	90.75 ± 12.5	58.88 ± 5.3	23.91 ± 3.8	32.63 ± 6.1	45.33 ± 4.1	51.25 ± 7.9	80.88 ± 14.1	38.97 ± 1.7
Males (n = 9)	473.82 ± 8.3	216.89 ± 13.5	134.76 ± 7.7	207.03 ± 8.3	211.44 ± 13.9	60.77 ± 4.7	36.50 ± 1.3	63.67 ± 3.9	35.56 ± 2.8	2.53 ± 1.6	5.44 ± 1.9	17.63 ± 4.0
Males with CHOCO (n = 9)	509.91 ± 17.4	228.11 ± 14.9	140.50 ± 14.8	148.79 ± 18.8	190.67 ± 21.4	46.87 ± 3.4	37.69 ± 5.8	63.44 ± 9.5	37.69 ± 5.8	23.50 ± 8.8	37.33 ± 11.1	31.50 ± 4.1
Females vs Females with CHOCO	Paired t-test t = 3.030 df = 7 p = 0.0191*	Paired t-test t = 0.192 df = 7 p = 0.8531ns	Paired t-test t = 1.011 df = 7 p = 0.3458ns	Paired t-test t = 5.267 df = 7 p = 0.0012**	Paired t-test t = 4.245 df = 7 p = 0.0038**	Paired t-test t = 3.412 df = 7 p = 0.0113*	Paired t-test t = 1.751 df = 7 p = 0.1234ns	Paired t-test t = 1.234 df = 7 p = 0.2570ns	Paired t-test t = 0.078 df = 7 p = 0.9402ns	Paired t-test t = 7.430 df = 7 p = 0.0001***	Paired t-test t = 6.674 df = 7 p = 0.0003***	Paired t-test t = 0.666 df = 7 p = 0.5269ns
Males vs Males with CHOCO	Wilcoxon test W = 39 p = 0.0195*	Paired t-test t = 0.546 df = 8 p = 0.6003ns	Wilcoxon test W = −7 p = 0.7344ns	Paired t-test t = 3.262 df = 8 p = 0.0115*	Wilcoxon test W = 3 p = 0.9102ns	Paired t-test t = 4.731 df = 8 p = 0.0015**	Paired t-test t = 0.188 df = 8 p = 0.8558ns	Paired t-test t = 0.029 df = 8 p = 0.9779ns	Wilcoxon test W = 11 p = 0.5703ns	Wilcoxon test W = 45 p = 0.0039**	Wilcoxon test W = 45 p = 0.0039**	Wilcoxon test W = 45 p = 0.0039**
Females with CHOCO vs Males with CHOCO	Mann-Whitney test U = 15 p = 0.0464*	Unpaired t-test t = 3.102 df = 15 p = 0.0073**	Mann-Whitney test U = 9 p = 0.0079**	Unpaired t-test t = 2.798 df = 15 p = 0.0135*	Mann-Whitney test U = 7 p = 0.0037**	Unpaired t-test t = 1.988 df = 15 p = 0.0654ns	Unpaired t-test t = 2.029 df = 15 p = 0.0606ns	Unpaired t-test t = 2.764 df = 15 p = 0.0145*	Mann-Whitney test U = 19 p = 0.1139ns	Unpaired t-test t = 2.407 df = 15 p = 0.0294*	Unpaired t-test t = 2.513 df = 15 p = 0.0239*	Unpaired t-test t = 1.704 df = 15 p = 0.1089ns

The total amount, number of episodes, and mean episode duration of vigilance states and cataplexy are reported as mean ± SEM in 8 female and 9 male mice with or without chocolate. These animals were recorded in the absence of mice of the opposite sex. Statistical tests: paired t-test or Wilcoxon test to compare females vs females with chocolate and males vs males with chocolate; unpaired t-test or Mann-Whitney test to compare males with chocolate vs females with chocolate; ns: non-significant.

*p < 0.05; **p < 0.01; ***p < 0.001.

shown) or chocolate treatment (Supplementary Figure S3) conditions, indicating that the amount of cataplexy was influenced by sex but not weight.

The presence of an animal of the opposite sex in the environment had no effect on the total amount of cataplexy, the number of episodes or their mean duration in female or male mice compared to their baseline (Figure 1J–L; Supplementary Table S2). However, when mice were recorded in the presence of the opposite sex and under chocolate treatment, the amount of cataplexy and the mean episode duration were significantly higher in females compared to males (p = 0.0434 and p = 0.0404) (Figure 2J and L), as in baseline (Figure 1J and L) and under chocolate (Table 2) conditions.

Altogether these results indicated that females had less fragmented sleep and WK during the dark phase but had more cataplexy than males in baseline or after chocolate treatment whether an animal of the opposite sex was present in the environment or not. Therefore, in order to determine whether the increased cataplexy was a constant or limited to a specific stage of the EC, we next quantified SWS, PS, WK, and cataplexy across the female EC, attributing an EC stage to each recorded night by performing a vaginal smear on the following morning.

Effects of the estrous cycle on vigilance states and cataplexy of Orex-KO female mice

Vigilance states were not modulated in females across the EC. We found no significant differences in the amount of time spent in WK (p = 0.1440), SWS (p = 0.0976), or PS (p = 0.9054) between P, E, M, and D (Supplementary Figure S4A, D, and G). Equally, we found no significant differences in the number of WK (p = 0.2709), SWS (p = 0.1286), and PS (p = 0.7954) episodes (Supplementary Figure S4B, E, and H) between EC stages nor in their mean duration (WK, p = 0.1032; SWS, p = 0.0996; PS, p = 0.4778) (Supplementary Figure S4C, F, and I). Altogether, these data indicate that the EC did not modulate vigilance states in Orex-KO female mice.

The occurrence of cataplexy in females was increased during the E stage. As previously described, we found a large intra- and inter-individual variability in the total amount and number of cataplexy episodes (Supplementary Figure S2). Furthermore, due to differences in the duration of EC stages, the number of recorded nights obtained for each EC stage varied. Accordingly, cataplexy data were normalized (see methods for details). We found that the total amount of cataplexy was strongly modulated across the EC (p = 0.001) (Figure 3A and B). It was doubled during E compared to the other EC stages (E: 1.76 ± 0.2 vs P: 0.92 ± 0.1; p = 0.03; vs M: 0.81 ± 0.2; p = 0.0428; vs D: 0.81 ± 0.1; p = 0.0435) while it was not different between any other EC stage (P vs M: p = 0.9597; P vs D: p = 0.9432; M vs D: p > 0.9999).

Similarly, the number of cataplexy episodes was also modulated across the EC (p = 0.0002) and doubled during E compared to the other EC stages (E: 1.46 ± 0.1 vs P: 0.78 ± 0.1; p = 0.0285; vs M: 0.66 ± 0.1; p = 0.0335; vs D: 0.82 ± 0.1; p = 0.0261) and not different between other EC stages (P vs M: p = 0.8860; P vs D: p = 0.9937; M vs D: p = 0.7948) (Figure 3C and D). However, the mean cataplexy episode duration was not modulated by the EC (p = 0.8511) (Figure 3E and F).

Effect of EC on sleep and cataplexy in males

As described above, the presence of an animal of the opposite sex in the environment had no effect on vigilance states of female and male mice (Figure 1) and we found that vigilance

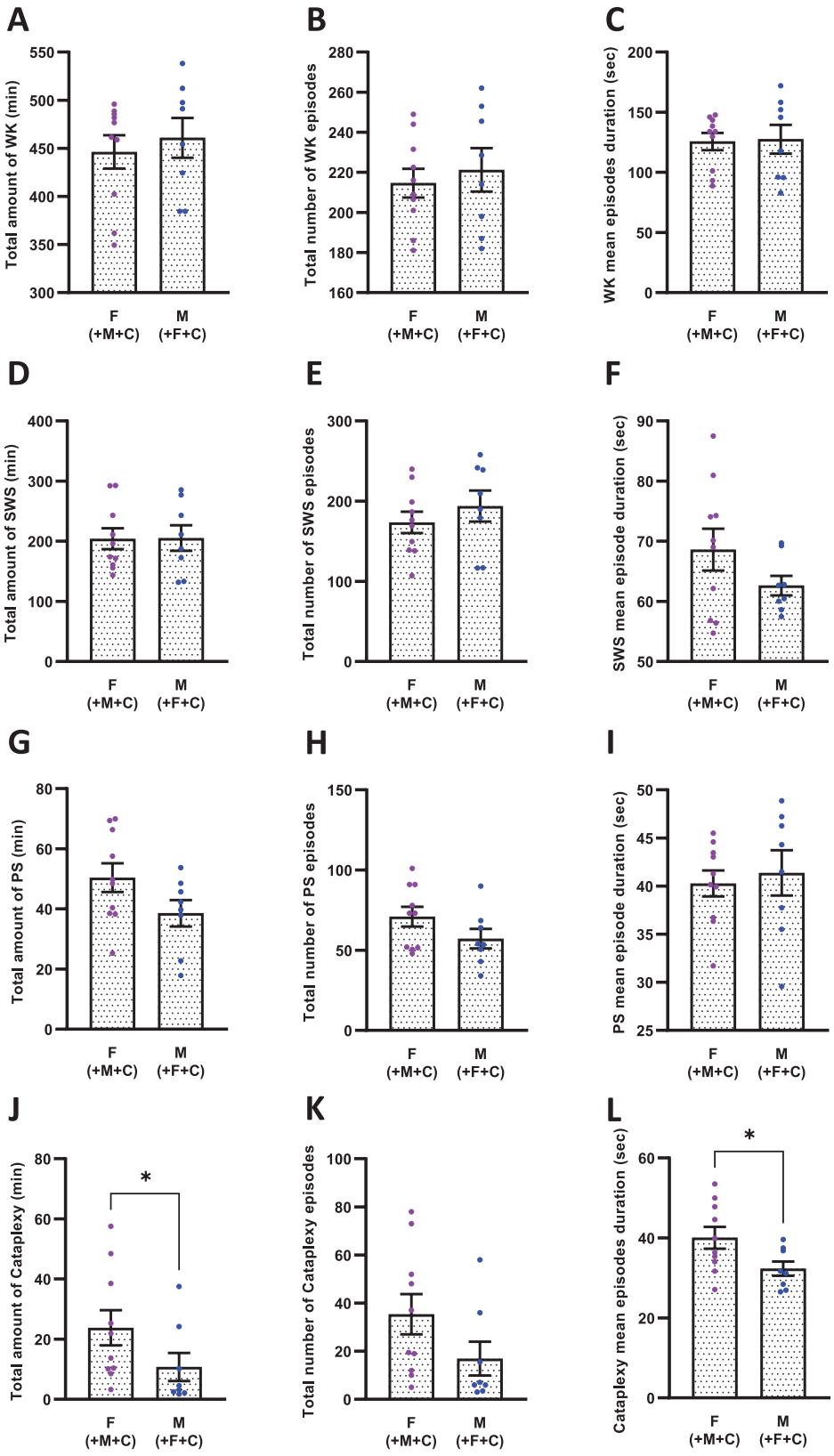

Figure 2. Quantification of vigilance states and cataplexy under chocolate treatment and in the presence of a mouse of the opposite sex in female vs male Orex-KO mice. Histograms on the left illustrate the total amount of time (mean ± SEM) spent in WK (A), SWS (D), PS (G), and cataplexy (J) of 10 females (+M+C) and 8 males (+M+C) during the dark phase. Histograms in the middle illustrate the number of WK (B), SWS (E), PS (H), and cataplexy (K) episodes (mean ± SEM) while those on the right illustrate the mean episode duration (mean ± SEM) for WK (C), SWS (F), PS (I), and cataplexy (L). Each dot represents one animal. *$p < 0.05$.

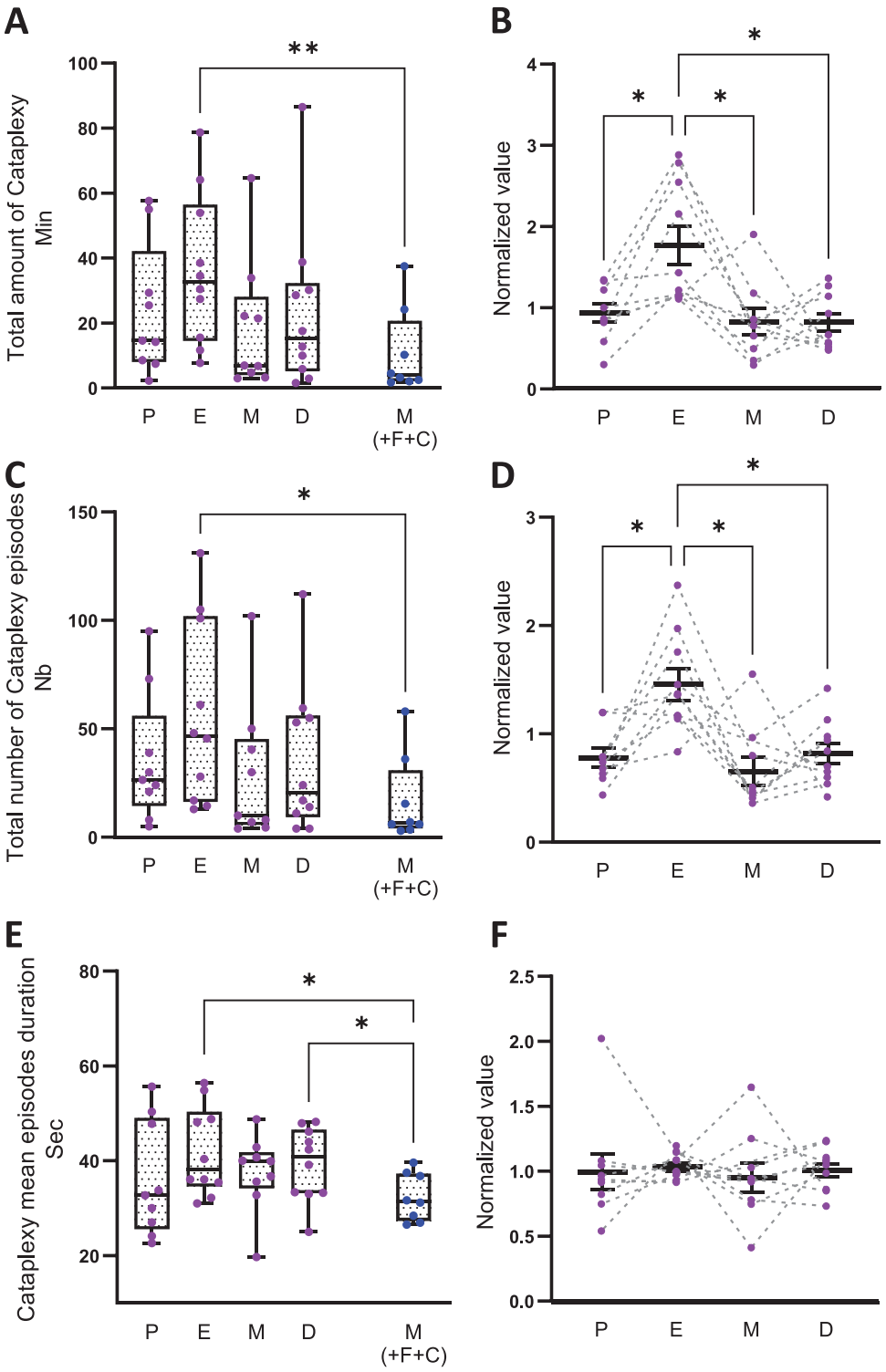

Figure 3. Illustration of cataplexy in Orex-KO female mice across the EC. Box plots report median (min; max) of the total amount (A), the number of episodes (C), and the mean episode duration (E) of cataplexy (A, C, and E, respectively) in P, E, M, and D of female mice (n = 10). Each purple dot represents the median value for one female animal. Females and males (n = 8) were recorded in the presence of mice of the opposite sex and under chocolate treatment. Note that values obtained from males are also illustrated in Figure 2 but are reported here again to illustrate which phases of the EC in females are different compared to males. B, D, and F panels illustrate the normalized values corresponding to A, C, and E, respectively, for cataplexy in females across the EC. Dotted gray lines in B, D, and F trace cataplexy across the EC for each given female. Thick black horizontal lines represent the mean values (±SEM). *p < 0.05; **p < 0.01. P, proestrus; E, estrus; EC, estrous cycle; M, metestrus; D, diestrus.

states were not influenced by the EC in females (Supplementary Figure S4). Accordingly, we found no difference between males and females at any of the 4 estrous states for WK, SWS, and PS, with the exception of a slight significant reduction in SWS mean episode duration in males compared to females in M (Supplementary Figure S4).

Interestingly, a significant increase in the total amount of cataplexy was observed between females in E and males (p = 0.0062) but not between males and females in other EC stages (Figure 3A). A similar observation was made for the number of cataplexy episodes (p = 0.0112) (Figure 3C) and the mean episode duration (p = 0.0193) (Figure 3E), indicating that the larger amount of cataplexy in females compared to males was attributable to the E stage. It is interesting to note that those males were recorded multiple times with up to 3 females in their environment. Whether one, two, three, or none of the females were in E stage (the ovulation period) had no influence on cataplexy, in terms of total duration or number of episodes (Supplementary Figure S5).

Discussion

With this study, we show that vigilance states are more fragmented in males and that cataplexy is prominent in females. Altogether, our data highlight sex differences in the expression of narcolepsy symptoms in Orex-KO mice. We also illustrate the high intra- and inter-individual variability of cataplexy in females and males and show for the first time that cataplexy is modulated across the EC, the occurrence of cataplexy being doubled during E compared to all other EC stages.

Effects of sex and EC on vigilance states in Orex-KO mice

Only a few sleep studies have explored sex differences in mice. It was reported that C57Bl6/J female mice had less SWS during the dark phase than males, but similar PS architecture [3]. Here, we found no female-male differences in the amount of WK, SWS, and PS during the dark period of C57Bl6/J Orex-KO mice but vigilance states were more fragmented in males. Vigilance state fragmentation as well as shorter PS latency are well-documented features of narcolepsy in mice [18, 46, 47]. It may thus reflect symptom severity.

A recent study looking at sex differences in another mouse model of narcolepsy, the C57Bl6/J DTA narcoleptic mice, reported no differences in WK and sleep quantities between males and females except for a tendency for males to spend more time in PS than females [35]. Similarly, in our study, we found higher amounts of PS with a larger number of PS episodes over 24 h in males, an observation that was only significant for the number of PS episodes during the dark phase. Of interest, these two murine models of narcolepsy differ by the fact that only OREX is genetically deleted in the Orex-KO mice while OREX neurons are killed in narcoleptic DTA mice. OREX neurons express other neurotransmitters in addition to OREX, such as dynorphin, glutamate, or galanin [48], suggesting that these latter neuropeptides might only mildly intervene in the sleep phenotype. In agreement with the similarity of these two mouse models, a recent study [49] has demonstrated that OREX neuropeptide is essential for the maintenance of neuronal electrophysiological properties. OREX neurons without OREX neuropeptides have lower spontaneous firing frequencies, lower input resistances, and a hyperpolarized membrane potential, suggesting that these neurons are less excitable and function poorly in the absence of OREX neuropeptides. The study also showed that OREX neuropeptides are responsible for the major effect on the maintenance of WK and the inhibition of PS.

Interestingly, several studies have reported that female patients display increased daytime sleepiness compared to men, as well as shorter sleep latencies on the multiple sleep latency test [10, 29, 31]. We found, however, that PS latency in males was greatly shortened compared to their female counterparts. We evaluated PS latency as the length of time between light-on and the first PS bout. The discrepancies might be due to species-related differences or to the difficulty in evaluating sleep latencies in mice where vigilance states are naturally more highly fragmented.

Healthy women tend to report a decreased quality of sleep during their menstruations and at the beginning of the follicular phase (corresponding to P + E in mice EC) [8, 50]. Objective measures using polysomnography or actigraphy, reported no menstrual cycle variability in sleep [8, 50, 51] or slight changes with increased WK and decreased PS during the luteal phase (M + D in mice) compared to the follicular phase [5, 6, 8, 52]. To the best of our knowledge, it has not been evaluated in patients with NT1.

Hormonal fluctuations across EC have more or less influences on sleep architecture depending on the species of rodents [3, 4, 7]. These changes have been attributed to the progesterone and estradiol reproductive hormones [53] but only partially since, although they disappear in ovariectomized females, they are only partially recovered by hormonal supplementation [3, 54]. According to Koehl et al. [7], genotypes seemed to have a stronger effect on sleep than sex hormones. In rats, females were more alert at the expense of sleep and in particular of PS during P, when ovulation occurs [4, 53, 55] followed by a SWS and PS rebound during E [4]. The EC seems to have limited effects on sleep and WK in wild-type female mice of C57Bl6/J genotype, with a slight PS reduction in P compared to D during the dark period and no difference with the other EC stages [7]. Similarly, we found no modulation for all sleep and WK features studied across the EC in C57Bl6/J Orex-KO narcoleptic female mice. It is important to note, however, that shorter ECs in mice might preclude the uncovering of mild hormonal influences on sleep.

Effects of sex and EC on cataplexy in mice

We found that females Orex-KO mice had more cataplexy than males. Similarly, female narcoleptic dogs showed more severe cataplexy attacks than males [56], DTA narcoleptic female mice exhibited earlier occurrence of cataplexy than males when NT1 was developing [36] and showed more cataplexy than males when the disease had settled [35]. Taken altogether, animal models of NT1 suggest that the severity of cataplexy would be elevated in females compared to males. Gender differences in the severity of cataplexy have rarely been evaluated and results are quite controversial, probably due to the limited number of patients included in these studies. Indeed, Luca et al. [10] reported no differences while Mattarozzi et al. [30] found a larger proportion of men with a higher frequency of cataplexy than women, and Won et al. [31] declared that women reported more occurrences of cataplexy than men. Further studies are thus needed to clarify whether gender might influence the severity of cataplexy in human patients with NT1.

We show here, for the first time, that cataplexy is influenced by the EC in Orex-KO narcoleptic mice with cataplexy occurring twice more frequently during the E stage compared to the other EC stages. This effect is due to a higher number

of bouts during E. Although we found that the amount of cataplexy generally correlates with time spent in WK (data not shown), we found that a similar amount of WK during E compared to the other EC stages. It thus does not explain the increased number of cataplexy episodes during E. In mice, a peak in follicle-stimulating hormone levels and a small surge in prolactin signal ovulation and entry into E. During E, 17-β-estradiol levels decline while progesterone levels are low and prolactin levels peak [45]. Whether and how sex hormones or prolactin levels would influence cataplexy expression is unknown and ought to be explored. Similarly, to the best of our knowledge, severity of cataplexy in patients has not been evaluated regarding the menstrual cycle, it needs to be explored.

Technical considerations

We focused most of our observations on the dark phase because the main symptoms of narcolepsy (sleepiness and cataplexy) are observed during that phase when mice are highly active. Accordingly, we found only a small female-male effect on the number of PS episodes during the light phase.

Interestingly, when animals were recorded with or without chocolate but in the absence of a mate of the opposite sex, vigilance states were more fragmented in males than females. However, under the best conditions, when recording males and females together and under chocolate treatment, we found no sex-related differences in vigilance states, indicating that males and females may be equally included in studies and preferentially be recorded in the same open space.

Furthermore, we confirmed here the well-known cataplexy eliciting effect of chocolate. We show, however, that chocolate had more effect on males than females, possibly because males had only a few cataplexies in baseline condition. However, it is remarkable that for both sex, chocolate was approximately 2.5 times less efficient when mice of the opposite sex were present. Such observation should be reevaluated for confirmation.

We had no difficulties in identifying the 4 EC stages based on cytology. The EC lasts only 4–5 days in mice and some of the stages are relatively short [57]. Therefore, we recorded mice over 3–4 cycles to detect all of them. Nevertheless, M could not be detected in one of the females. The shortness of M, often lasting for less than 12 h, conducted some authors to exclude it from the analysis [7]. In our analysis, we chose to keep it. Nevertheless, we cannot exclude that part of the variability observed in each EC stage is due to transition between stages.

It has been reported in C57Bl6/J mice that sleep duration increases with weight [58]. Here, females were leaner than males although they were of similar age and came from the same litters. Weight differences were highly significant but mild. Accordingly, we found no correlation between weight and cataplexy in baseline (sometimes called spontaneous cataplexy) and under chocolate treatment (food-elicited cataplexy).

Of interest, 8 males were recorded for several nights with females in their close environment. They showed no difference in the number of cataplexy whether 0–3 of the female mice present in the environment where in E, a stage where ovulation occurs. It suggests that the hormonal stage of the female does not induce cataplexy in males. Note that females were in the same open space but not in physical contact with males, which could make a difference.

Conclusion

To conclude, our data indicate that the narcoleptic profile is slightly different in female and male Orex-KO mice, males having more fragmented sleep while females have more cataplexy. In particular, we show that cataplexy is greatly influenced by EC, with a major increase in occurrence during the E stage. How sex hormones modulate the occurrence of cataplexy in mice still needs to be explored. Whether these effects are present in patients with NT1 and due to hormonal changes or the emotional state associated with these hormonal changes would also need to be addressed to get a fuller picture and a better understanding of the physiopathology of NT1.

Supplementary Material

Supplementary material is available at *SLEEP* online.

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Author Contributions

S.A., M.V., and C.P. conceived and designed the study. S.A., M.V., and C.B. performed experiments. A.L.M. performed genotyping and helped with the microscopy. S.A. performed most of the analyses. M.V. wrote the first draft of the manuscript. S.A., M.V., and C.P. wrote the paper. All authors reviewed and approved the submitted version.

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