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Orexin deficiency modulates cognitive flexibility in a sex-dependent manner

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Abstract

Cognitive flexibility is an important executive function and refers to the ability to adapt behaviors in response to changes in the environment. Of note, many brain disorders are associated with impairments in cognitive flexibility. Several classical neurotransmitter systems including dopamine, acetylcholine and noradrenaline are shown to be important for cognitive flexibility, however, there is not much known about the role of neuropeptides. The neuropeptide orexin, which is brain-widely released by neurons in the lateral hypothalamus, is a major player in maintaining sleep/wake cycle, feeding behavior, arousal, and motivational behavior. Recent studies showed a role of orexin in attention, cognition and stress-induced attenuation of cognitive flexibility by disrupting orexin signaling locally or systemically. However, it is not known so far whether brain-wide reduction or loss of orexin affects cognitive flexibility. We investigated this question by testing male and female orexin-deficient mice in the attentional set shifting task (ASST), an established paradigm of cognitive flexibility. We found that orexin deficiency impaired the intra-dimensional shift phase of the ASST selectively in female homozygous orexin-deficient mice and improved the first reversal learning phase selectively in male homozygous orexin-deficient mice. We also found that these orexin-mediated sex-based modulations of cognitive flexibility were not correlated with trait anxiety, narcoleptic episodes, and reward consumption. Our findings highlight a sexually dimorphic role of orexin in regulating cognitive flexibility and the need for further investigations of sex-specific functions of the orexin circuitry.

KEYWORDS

animal models of schizophrenia, attentional set shifting, anxiety, cognitive flexibility, food consumption, learning and memory, narcolepsy, neuropeptides, orexin, transgenic mice

1 | INTRODUCTION

Neuropsychiatric disorders are often associated with learning impairments especially in learning tasks that demand cognitive flexibility.[1,2] Cognitive flexibility is the ability to perform reversals or to transfer attention within the same perceptual dimension (IDS: intra-dimensional set shift) or between different perceptual dimensions (EDS: extra-dimensional set shift). Impairments in cognitive flexibility are characterized by a delay in inhibiting previously learned intrinsic rules, which is usually followed by difficulties in learning new rules. In those neuropsychiatric disorders which are associated with cognitive flexibility deficits, structural and functional changes of frontal cortex (FC) are often observed.[3] The important role of the FC in cognitive flexibility is confirmed by the observation that human patients with

damaged dorsolateral prefrontal cortex and orbitofrontal cortex (OFC) are usually impaired in EDS and reversal learning, respectively.[4-6] These findings comply with studies in laboratory rodents where lesions of the medial prefrontal cortex and OFC impaired EDS and reversal learning, respectively,[7] whereas lesions of the cingulate cortex (CC) impaired IDS.[8] Furthermore, blocking particular neurotransmitter systems including dopamine and noradrenaline impaired cognitive flexibility.[9-14] Although there is some research on the role of classical neurotransmitters in cognitive flexibility, there is not much known about the role of neuropeptides in cognitive flexibility. In our study, we investigated the role of orexin neuropeptides in cognitive flexibility.

Orexinergic neurons synthesize a pair of neuropeptides, orexin A and B, in the lateral hypothalamus, dorsomedial hypothalamus and perifornical area.[15,16] Their projections are distributed brain wide including locus coeruleus, amygdala, raphe nucleus, ventral tegmental area, brain stem and PFC.[17-21] Orexin A and B bind with different affinities to the two orexin receptors, OX1R and OX2R, and regulate multiple physiological functions through distinct neural pathways.[22] Given their strong localization in the tuberomammillary nucleus, a region known to be involved in regulating feeding, orexin was initially found to regulate feeding behavior.[15] A year after the discovery of orexin, orexin-deficient mice lacking prepro-orexin gene were found to exhibit narcoleptic episodes similar to human narcoleptic patients and canarc-1 mutant dogs, which were used as a canine model of narcolepsy, leading to the discovery of orexin's major role in regulating sleep/wake cycle.[23] Later, it was also found to be involved in physiological functions, arousal, anxiety- and fear-related behavior, and motivational behavior.[24-27] Of note, some recent studies indicate a role of orexin in cognition and attention in rodents[28-31] with supporting evidences pointing to impairments in executive control and working memory in human narcoleptic patients lacking orexin neurons.[32] Orexin is also believed to be involved in neuropsychiatric disorders such as schizophrenia or attention deficit hyperactivity disorder and neurodegenerative disorders such as Alzheimer's, which are also characterized by deficits in cognition.[33-36]

Given the widespread orexinergic projections to the FC which are known to regulate cognitive flexibility and the recent mounting evidences pointing to orexin's role in cognitive processes, we hypothesized that the loss of orexin in orexin-deficient mice modulates cognitive flexibility. We investigated our hypothesis by testing male and female heterozygous and homozygous orexin-deficient mice as well as their wildtype littermates in a well-established and widely used behavioral paradigm called attentional set shifting task (ASST). ASST is a rodent equivalent of the Wisconsin card sorting task (WCST) used to assess cognitive flexibility in humans.[37] We found that orexin deficiency impaired the IDS phase of ASST in homozygous orexin-deficient female mice and improved the first reversal phase of ASST in homozygous orexin-deficient male mice. Since, orexin deficiency can also affect anxiety, food consumption and induces narcoleptic episodes,[23,27,38,39] we additionally tested our mice in behavioral tests for these characteristics. Then, we first analyzed whether individual characteristics in these tests are in general correlated with ASST performance, and second, whether the observed changes in ASST performance were correlated with changes in these tests.

2 | METHODS

2.1 | Animal

Orexin-deficient mice (B6.129S6-Hcrt^{tm1Ywa}) were originally purchased from the University of Texas, Dallas, USA, and backcrossed for more than 20 generations with C57BL/6J mice. In orexin-deficient mice, the exon 1 of the prepro-orexin gene was replaced by a nuclear lacZ/neomycin resistance cassette which leads to the absence of orexin in these mice.[23] We used heterozygous x heterozygous breeding scheme. We genotyped the mice using a standard end-point PCR (DreamTaq™ Green PCR, Thermo Fischer Scientific). We tested wildtype (orexin +/+: males, n = 13; females, n = 11), heterozygous orexin-deficient mice (orexin +/−: males, *n* = 10; females, *n* = 10) and homozygous orexin-deficient mice (orexin −/−: males, *n* = 15; females, *n* = 10) in the experiments. Mice were housed in groups of up to 4/cage of mixed genotype under controlled conditions of humidity (50–55%), temperature (22 ± 2°C) and 12:12 light/dark cycle (lights on: 6:00 to 18:00). Mice were aged between 8 and 25 weeks during the experiment. Food and water were provided ad libitum except 1 week before and during the ASST where mice were restricted to the amount of food consumed (2 gm/mice/day) to maintain 80%–90% of their basal weight. All the experiments were conducted during the lights-on period and were performed according to the international guidelines of animal care and use for experimental procedures (2010/63/EU) with confirmed ethical approval (Landesverwaltungsamt Sachsen-Anhalt, 42,502–2-1351 Uni MD).

2.2 | Behavioral experiments

2.2.1 | Timeline of the experiments

Mice were subjected to various tests for a period of 3 weeks (Figure 1). On the first week, mice were subjected to light–dark box (LDB) and elevated plus maze (EPM) tests between days 1–3. From day 4 onwards, mice were food restricted to 2 gm/mice/day until the end of week 2. On the second week, habituation phases and testing phases of ASST were performed between days 8–14. At the end of the testing phases, mice were provided with ad libitum food. On the third week, food preference test was performed between days 18–21.

2.2.2 | Light–dark box

The light–dark box test was performed in four identical transparent chambers (49.5 cm × 49.5 cm × 41.5 cm) placed inside an animal detection infrared sensor frames (TSE systems, Bad Homburg, Germany). Each chamber consisted of two equally sized compartments

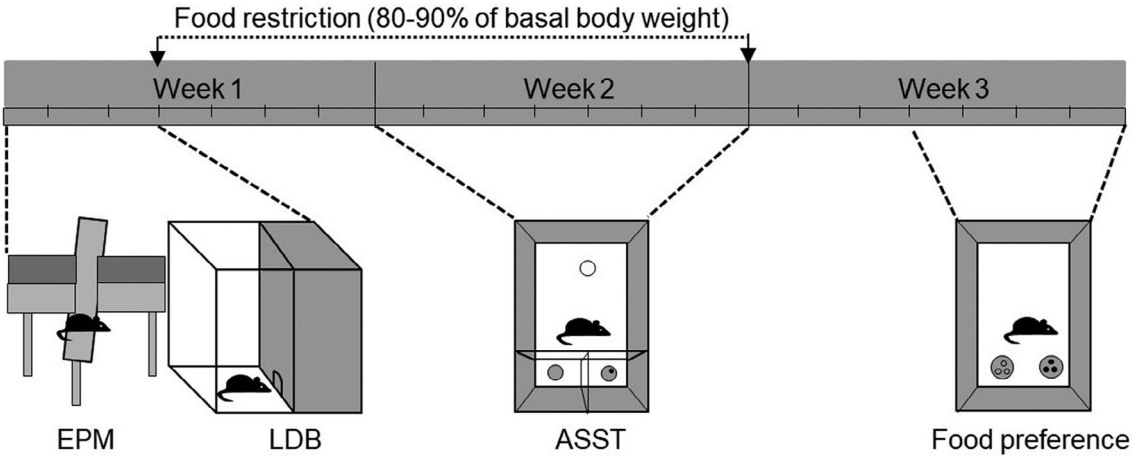

FIGURE 1 Timeline of the experiments. Trait anxiety (EPM and LDB), ASST and food preference test were performed in 3 weeks in the order illustrated above. ASST, attentional set shifting task; EPM, elevated plus maze; LDB, light–dark box

separated by a divider with an opening (8 cm × 6 cm) at the mid-bottom allowing the mice access to both the compartments. One of the compartments was bright (410–570 lux) and the other was dark (0.2–1.5 lux). The movement of the mice inside and between the two compartments was detected by the infrared sensors and was analyzed using TSE Phenomaster software. At the beginning of the experiment, mice were placed in the middle of the dark compartment and their movements were traced for 10 min.

2.2.3 | Elevated plus maze

The elevated plus maze test was performed in a custom-made maze consisting of four cross arms (94 cm × 10 cm) equidistant from the centre. Two of the four arms were enclosed by high walls (21 cm) and two were open. The maze was attached to a metal frame with four legs supporting each of the arms and raised from the floor at a height of 69 cm. The experiment started with placing the mice on the centre of the maze and the movement of the mice in the arms were recorded with a camera (Live! cam sync HD, Creative labs, Germany) for 5 min. The videos were later analyzed using videotracking software (EthoVision XT 11, Noldus, Wageningen, Netherlands).

2.2.4 | Attentional set shifting task

The ASST was slightly modified from the earlier protocols described for mice[14,40,41] and pictorially represented in Supporting Figure S1. Mice were handled and food restricted (80%–90% basal body weight) 1 week before and during ASST. Daily ration of food (2 gm/mice) was provided at the end of the lights-on period after all the habituation/testing phases for the day was over. Habituation and testing phases were performed in a low illuminated (50–70 lux) experimental room. The custom-built apparatus consisted of a rectangular chamber (41 cm × 22 cm × 24 cm), subdivided into a waiting compartment and two identical testing compartments. A bowl with water and testing bowls with medium (bedding material/digging medium) were placed in the waiting and testing compartments, respectively. In the test phase, we used odor (olfactory) and digging medium (visual/tactile) as the two-dimensional cues. Different odorants (e.g., citral, eucalyptol, s-(+)-carvone, R-(+)-carvone, valeric acid, 2-phenylethanol: Sigma-Aldrich Chemie GmbH, Germany) diluted in paraffin oil (1:20 dilution) were used as olfactory cues. Wooden pearls (Aduis GmbH, Kiefersfelden, Germany) of varying size (6 mm and 10 mm diameter) and color (green, yellow, and brown) served as digging media. Choco Rice (ca. 20 mg, Nordgetreide GmbH & Co. KG, Lübeck, Germany) was used as a reward. Before the actual ASST, there was an accommodation and pre-training phase: First, the mice were habituated to the set-up and the reward (ca. 45 min). Then, they were systematically trained to dig and retrieve the reward from the testing bowls in the absence of a dimensional cue prior to the test for about 30–60 min until the mice satisfactorily performed the training. After this habituation phase, the actual ASST was performed. During the following three test days, the bowls were presented with the dimensional cues and only one of the bowls was baited with the reward. The baited and unbaited bowls were placed randomly in the two testing compartments and the mice had to learn which dimensional cue was associated with the baited bowl in order to retrieve the reward. Importantly, the unbaited bowl was sprinkled with Choco Rice powder to prevent the mice from using the smell to retrieve the reward. Half of the mice were tested starting with odor as the relevant dimension and the other half with digging medium as the relevant dimension in the first testing phase (SD: simple discrimination). The first two trials were free trials where the mice were permitted to dig both the bowls to retrieve the reward (the reward was placed on top of the medium). These free trials were not included in the total number of trials to reach the criterion. From the third trial onwards, the reward was placed at the bottom of the bowl, hidden underneath the medium. Furthermore, when the mice began to dig in one of the bowls, the door to the other bowl was closed. Digging and retrieving the reward in the baited bowl was

recorded as a successful trial, digging in the unbaited bowl as an error. If successful, the mice could eat the reward completely before the beginning of the next trial. The trial at which the mice completed six consecutive correct responses (excluding the first two free trials) marked the end of the testing phase and was noted as the trial to criterion. On the first testing day, the mice had to learn simple discrimination (SD), where two different exemplars of only one of the dimensional cues (odor or digging medium) were presented. The second testing day started with two free trials in compound discrimination (CD), where an additional but irrelevant dimensional cue (digging medium, if odor was the starting dimensional cue, and odor, if digging medium was the starting dimensional cue) was introduced. CD was followed by the first reversal phase (Rev1), in which contingency of the relevant cues changed. The third testing day started again with two free trials in the IDS where new exemplars of both dimensional cues were presented and one of the new exemplars of the previously relevant dimension predicted the reward. The IDS phase was followed by the second reversal phase (Rev2). Then, the mice had to perform an extra-dimensional set shift (EDS), where new exemplars for both dimensions were presented and one of the new exemplars of the previously irrelevant dimension predicted the reward. EDS was followed by a third reversal phase (Rev3). If a mouse did not dig any of the bowls for more than 10 min, the experiment was temporarily stopped and resumed only after the mice expressed exploratory behavior again. Free trials were given only during the beginning of SD, CD, and IDS phase. Mice that failed to complete all the ASST phases were excluded from the final analysis (male: orexin +/+ = 1; orexin −/− = 1).

2.2.5 | Food preference test

At the end of the ASST, mice were provided ad libitum food for at least 3 days before the food preference test. The test was performed in a transparent rectangular Plexiglas chamber (41 cm × 26 cm). Mice were habituated to the empty chamber for 10 min at least an hour before the test. During the test, two bowls were placed in two corners of the chamber, one of them was filled with home cage chow food and the other was filled with the Choco Rice reward. Mice were placed inside the chamber for 20 min. Thereafter, the amount of chow food and reward consumed by the mice was measured.

2.3 | Statistical analysis

Data analysis was performed using SYSTAT 13 (SPSS Inc.) and Prism 7.0 (GraphPad Software Inc., La Jolla, USA). Normal distribution of the data was verified using D'Agostino-Pearson normality test. If the behavioral data were normally distributed, we performed analysis of variance (ANOVA), followed by post-hoc Sidak's multiple comparisons. If the data were not normally distributed, we performed Kruskal-Wallis test followed by Dunn's multiple comparisons. Outliers were omitted in the correlation analysis (males: orexin −/− = 1, females: orexin +/− = 3).

3 | RESULTS

3.1 | Trait anxiety

We measured several parameters in the EPM and LDB to assess trait anxiety in orexin-deficient mice. We observed a trend for a decrease in the percentage of open arm entries in orexin-deficient mice in the EPM (Figure 2(A); Kruskal-Wallis test: $H = 5.17$, $p = 0.08$) indicating increased level of anxiety in orexin-deficient mice. This was supported by data from the LDB, showing an effect of genotype in the percent distance traveled in the bright compartment (Figure 2(E); Kruskal-Wallis test: $H = 6.50$, $p = 0.04$). Post-hoc analysis showed that orexin-deficient mice had decreased percent distance traveled in the bright compartment compared to the wildtype ($p = 0.04$). Furthermore, we observed an increase in the latency to enter the bright compartment in males, irrespective of the genotype (Figure 2(D); main effect of sex: $F_{1,61} = 11.90$; $p = 0.001$) indicating a generally increased level of this measure of anxiety in males. There was neither an effect of genotype ($F_{[2,61]} = 0.07$; $p = 0.93$) nor interaction between sex and genotype ($F_{[2,61]} = 0.54$; $p = 0.58$). For all the other parameters in the EPM and LDB, we observed no effects of sex, genotype or interactions between sex and genotype (Figure 2(B), (C), (F); $Fs < 2.05$, $ps > 0.13$). Taken together, our results indicated increased levels of anxiety in homozygous orexin-deficient mice as illustrated in some of the parameters of EPM and LDB.

3.2 | Orexin deficiency attenuated cognitive flexibility in a sex-dependent manner

We measured the learning performance (trials to criterion) and the number of errors of wildtype, heterozygous and homozygous orexin-deficient mice in the six phases of ASST. The higher the trials to criterion or the numbers of errors, the poorer the performance in the respective phases of the ASST. We analyzed the learning performance using a three-way ANOVA with sex and genotype as between-subject factors and phase (CD, Rev1, IDS, Rev2, EDS, Rev3) as within-subject factor. We observed that the females generally required fewer number of trials to complete the phases compared to the males (main effect of sex: $F_{[1,61]} = 8.82$, $p = 0.004$) and that the performance of the mice is different in the different ASST phases (main effect of phase: $F_{[5305]} = 12.14$, $p < 0.0001$). In addition, there was a trend for an interaction between phase and sex ($F_{[5305]} = 2.17$, $p = 0.08$) while other interactions did not reach the level of significance (Fs < 1.45, ps > 0.17). Since there was a main effect of sex in the learning performance, we analyzed the data of females and males separately for each phase (Figure 3(A),(B)). In females, there was a main effect of genotype in the IDS phase (Figure 3(A); $F_{[2,28]} = 7.24$, $p = 0.003$). Post-hoc comparisons showed that the homozygous orexin-deficient mice required higher number of trials to complete the IDS phase compared to the wildtype ($t_{[28]} = 3.33$, $p = 0.007$) and heterozygous orexin-deficient mice ($t_{[28]} = 3.28$, $p = 0.007$). There was no effect of genotype in all the other phases (Fs < 0.40, ps > 0.67). In males, we observed a main effect of genotype in Rev1 phase (Figure 3(B); Kruskal-Wallis test: $H = 7.60$, $p = 0.04$). Post-hoc

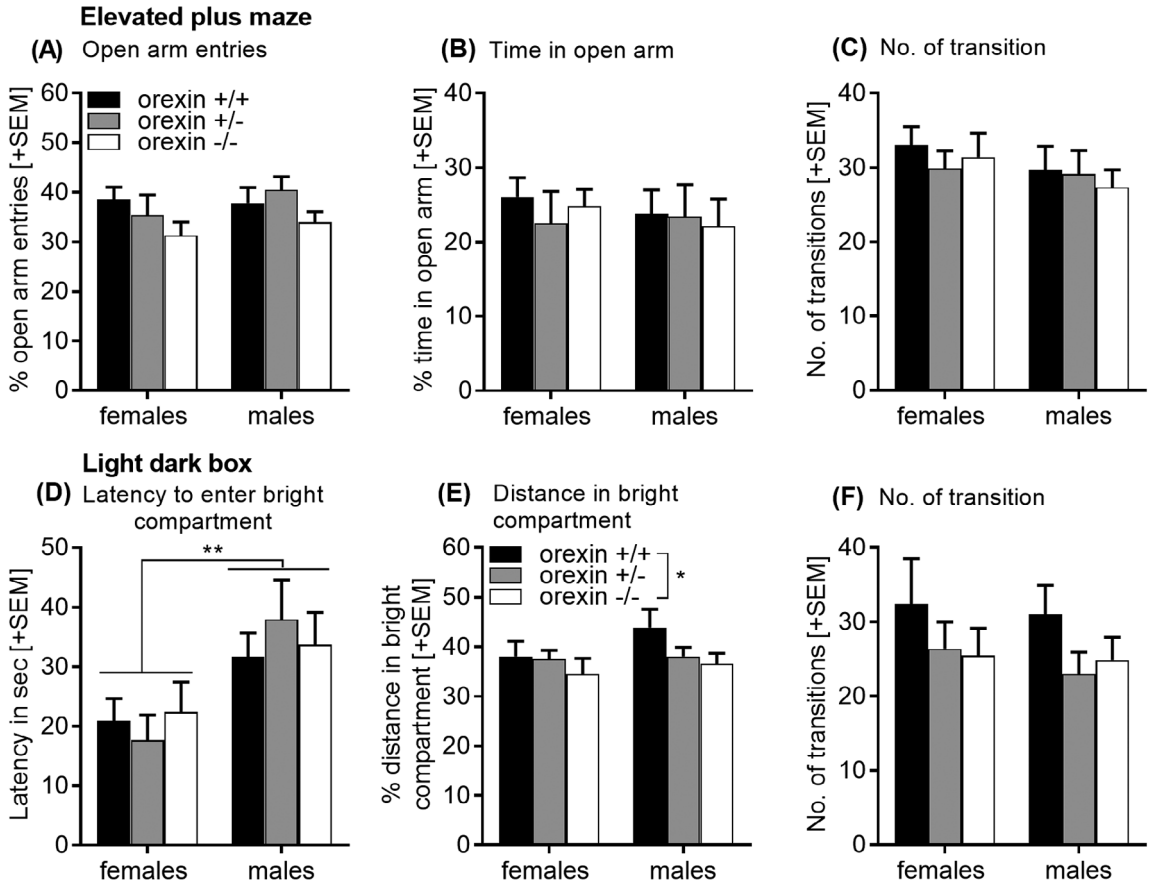

FIGURE 2 Trait anxiety. (A-C) represents percentage of open arm entries, time in open arm and the number of transition between open and closed arm in EPM. There was no main effect of sex or genotype. (D-F) represents the latency to enter the bright compartment, percentage of distance traveled in the bright compartment and the number of transitions between bright and dark compartment in LDB. Males, irrespective of the genotype, entered the bright compartment in LDB with increased latency, exhibiting anxiogenic-like behavior. Homozygous orexin-deficient mice traveled less percent distance in the bright compartment in LDB compared to the wildtypes indicating an anxiogenic-like phenotype. $^{*}p < 0.05$, $^{**}p < 0.01$. EPM, elevated plus maze; LDB, light–dark box

comparison showed that the homozygous orexin-deficient mice required fewer number of trials to complete the Rev1 phase compared to the wildtype ($p = 0.02$). There was no effect of genotype in all the other phases in males (Fs < 1.47, ps > 0.24). We also analyzed the number of errors in the six phases of ASST in females and males (Figure 3(C), (D)). The number of errors in the six phases mirrored the outcome of the learning performances in females and males (Figure 3(A),(B)). In females, there was a main effect of genotype in the IDS phase (Figure 3 (C): $F_{[2,28]} = 11.03$, $p = 0.0003$). Post-hoc comparison showed that homozygous orexin-deficient mice made significantly higher number of errors compared to the wildtype ($t_{[28]} = 4.01$, $p = 0.0008$) and the heterozygous orexin-deficient mice ($t_{[28]} = 4.15$, $p = 0.0008$). There were no genotype effects in all the other phases in females (Fs < 0.76, ps > 0.47). In males, there was a main effect of genotype in the Rev1 phase (Figure 3(D); Kruskal-Wallis test: $H = 6.90$, $p = 0.03$). Post-hoc comparison showed that homozygous orexin-deficient mice significantly made fewer number of errors compared to the wildtypes ($p = 0.03$). There was no main effect of genotype in all the other phases in the males (Fs < 1.50, ps > 0.24). Taken together, these results showed that orexin deficiency selectively impaired IDS phase in females and improved Rev1 phase in males. We further observed that the wildtype and heterozygous orexin-deficient female mice exhibited an ID-ED shift (i.e., the number of trials required to complete the EDS phase is higher than the IDS phase), whereas homozygous orexin-deficient female mice did not show this ID-ED shift (Figure 3(A)). On the contrary, they required higher number of trials to complete the IDS phase compared to the EDS phase indicating the absence of the formation of an attentional set. Males, irrespective of the genotype, did not exhibit an ID-ED shift (Figure 3(B)). We observed that the absence of the ID-ED shift in some of the mice was associated with the order of the dimensional cues presented during ASST. All the mice, irrespective of the genotype and sex showed more pronounced ID-ED shifts when presented with medium as the first relevant dimension at SD (Supporting Figure S2).

3.3 | Narcoleptic episodes

During the ASST, we observed narcoleptic episodes in some of the homozygous orexin-deficient mice but never in wild type and heterozygous orexin–deficient mice (Figure 4(A)). These narcoleptic episodes

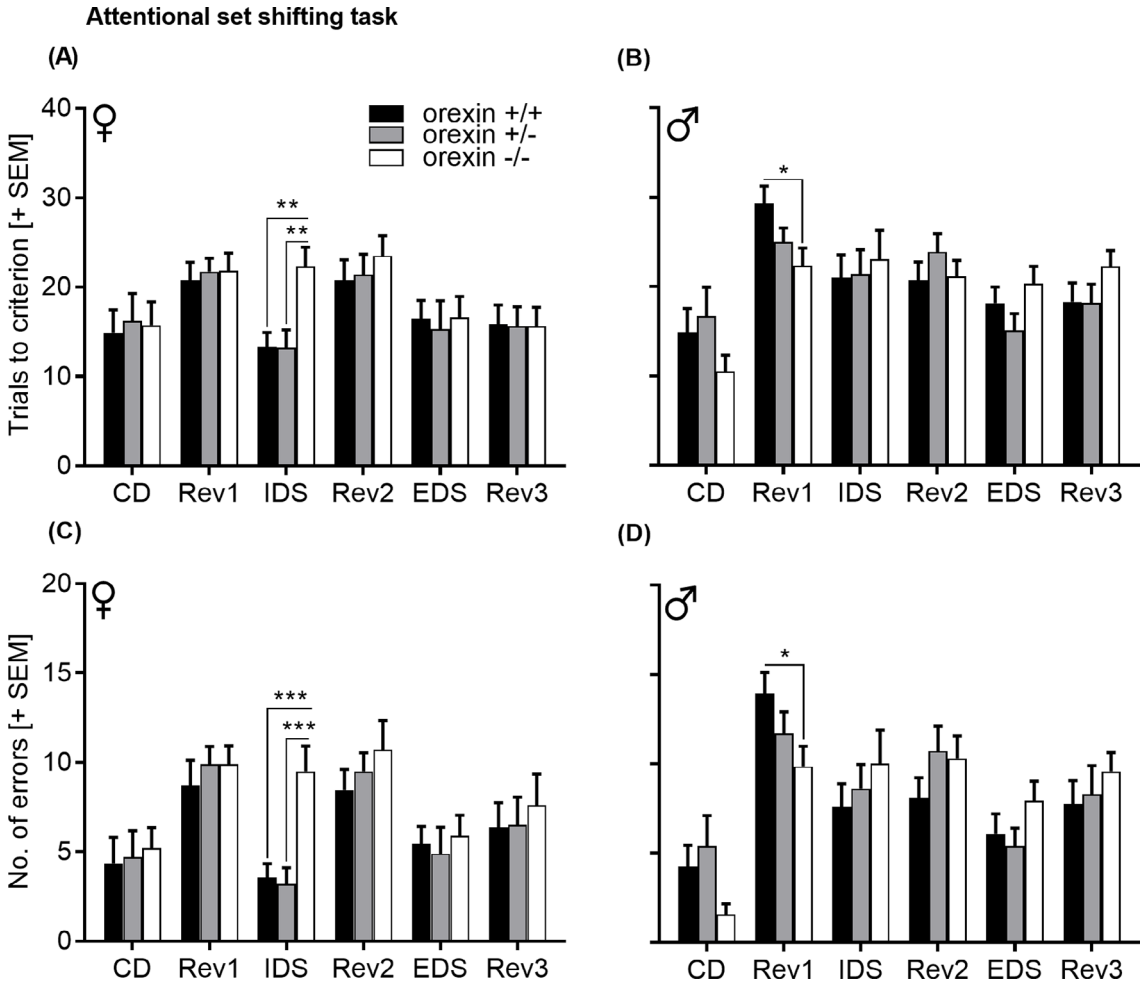

FIGURE 3 Orexin deficiency attenuated cognitive flexibility in a sex-dependent manner. (A and B) Learning performance expressed as trials to criterion in the six phases (CD, Rev1, IDS, Rev2, EDS, Rev3) of ASST in females and males, respectively. (A) In females, homozygous orexin-deficient mice showed impaired learning performance indicated by the higher number of trials to criterion in the IDS phase compared to the wildtype and heterozygous orexin-deficient mice. (B) In males, homozygous orexin-deficient mice showed improved learning performance indicated by the fewer number of trials to criterion in the Rev1 phase compared to the wildtype. (C and D) Number of errors in the six phases of ASST in females and males, respectively. (C) In females, homozygous orexin-deficient mice showed significantly higher number of errors compared to the wildtype and heterozygous orexin-deficient mice in the IDS phase. (D) In males, homozygous orexin-deficient mice significantly showed fewer number of errors compared to wildtype in the Rev1 phase. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ post-hoc analysis after significant ANOVA ($p < 0.05$) or Kruskal-Wallis test ($p < 0.05$). ASST, attentional set shifting task; EDS, extra-dimensional set shift

occurred randomly in the different phases of ASST (between 0 and 3 episodes/phase and mouse; median: 0.4). We did not observe any obvious deficits or improvements in the ASST performance in any phase after the occurrence of a narcoleptic episode. Females exhibited more narcoleptic episodes during the test compared to the males (*Mann–Whitney test*, $p = 0.03$). We did not observe narcoleptic episodes during the tests on trait anxiety or food preference.

3.4 | Reward consumption

After the ASST, we performed a food preference test. All the mice, irrespective of gender and genotype consumed exclusively the reward during this test and avoided the chow food. Therefore, we show only the data of the reward consumption (Figure 4(B)). There was neither an effect of genotype ($F_{[2,61]} = 0.56$, $p = 0.58$) nor an interaction between sex and genotype ($F_{[2,61]} = 0.08$, $p = 0.92$). However, we observed sex differences in the amount of reward consumption (Figure 4(B); $F_{[1,61]} = 4.49$, $p = 0.04$), that is, females consumed higher amount of reward compared to the males.

3.5 | Correlation analyses

We investigated by correlation analyses if trait anxiety, narcoleptic episodes or reward consumption affected ASST performance of the mice. In the first analytical approach, we checked whether the overall ASST performance (mean trials to criterion of all ASST phases) in male

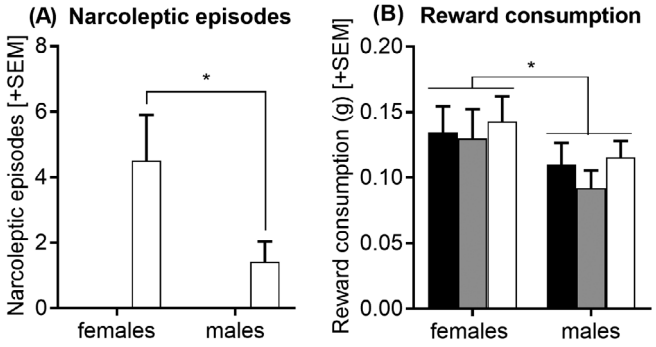

FIGURE 4 Narcoleptic episodes and reward consumption. (A) represents number of narcoleptic episodes in orexin-deficient mice during ASST. Females exhibited higher number of narcoleptic episodes compared to the males. (B) represents the amount of reward consumed during the food preference test. Females consumed higher amount of reward compared to the males, irrespective of the genotype. $^{*}p < 0.05$, $^{**}p < 0.01$. ASST, attentional set shifting task

and female mice, irrespective of genotype, is correlated with the different parameters measured in the trait anxiety test (EPM and LDB), number of narcoleptic episodes during ASST and amount of reward consumed during food preference test. With this approach, we only found some weak but significant correlations (Figure 5; Supporting Table 1 and 2): In males, percent time spent in bright compartment in LDB was positively correlated with the trials to criterion in ASST (Figure 5(A); $r^2 = 0.13$, $p = 0.02$). This means that lower anxiety levels were associated with poorer ASST performance. Furthermore, in males, the amount of reward consumption in the food preference test was negatively correlated with the trials to criterion in ASST (Figure 5 (B); $r^2 = 0.15$, $p = 0.02$). Whereas in females, the amount of reward consumption in the food preference test was positively correlated with the trials to criterion in ASST (Figure 5(C); $r^2 = 0.24$, $p = 0.005$). This means that in male mice, higher reward consumption in the food preference test was associated with stronger ASST performance, while in female mice it was associated with poorer ASST performance.

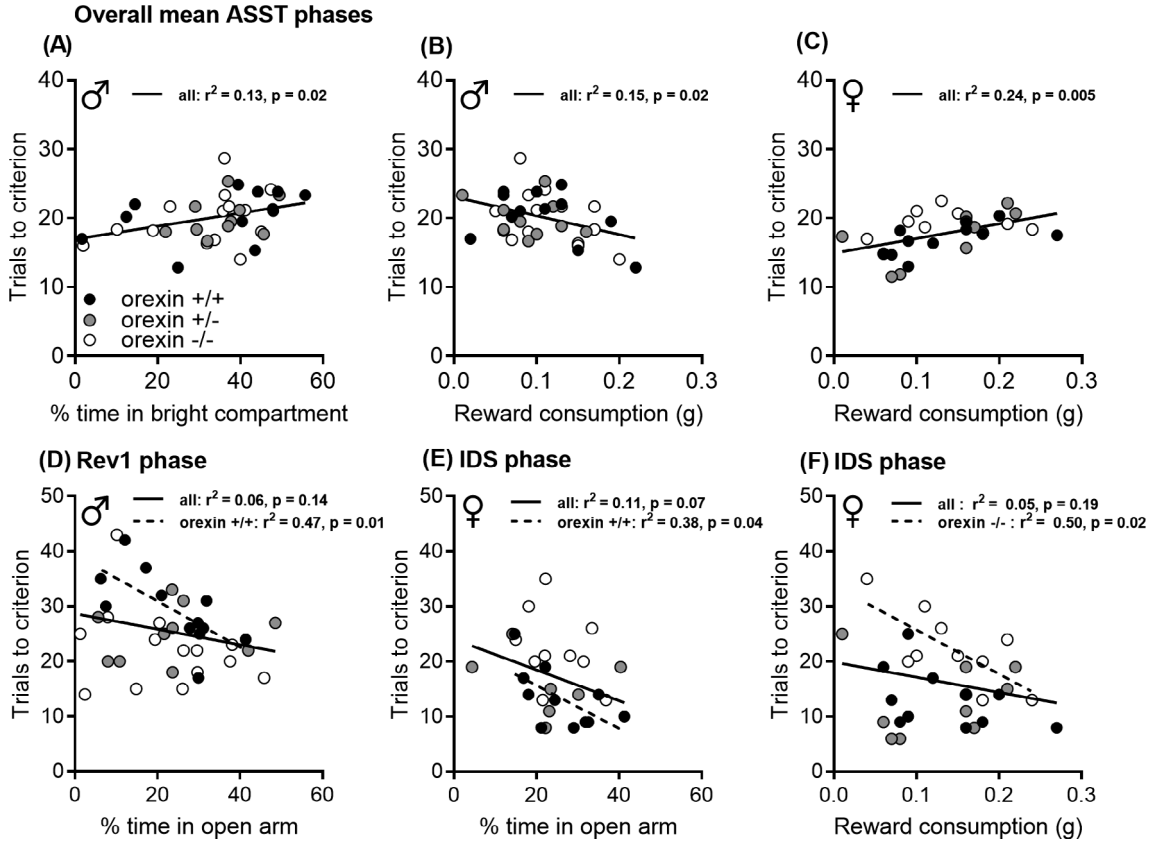

FIGURE 5 Correlation analyses. (A-C) represent correlations between overall ASST performance (mean of all phases) shown as trials to criterion and percent time in bright compartment in LDB and reward consumption. (A) In males, there was a positive correlation between the percent time traveled in the bright compartment in LDB and trials to criterion, irrespective of the genotype. (B) In males, there was a negative correlation between the amount of reward consumption and trials to criterion. (C) In females, there was a positive correlation between the amount of reward consumption and trials to criterion. (D) In male wildtypes, there was a negative correlation between the percent time in open arm in EPM and trials to criterion in Rev1phase. (E) In female wildtypes, there was a negative correlation between the percent time in open arm in EPM and trials to criterion in IDS phase. (F) In female homozygous orexin-deficient mice, there was a negative correlation between the reward consumption and the trials to criterion. Black lines represent correlation line of all genotypes together, dashed line represent the respective genotype with significant correlation and dots represent individual mice of the different genotypes. ASST, attentional set shifting task; EPM, elevated plus maze; LDB, light–dark box

Of note, we observed that during the ASST all mice, irrespective of genotype and sex, consumed the reward immediately after retrieving it. No other measures in these tests were correlated with overall ASST performance (Supporting Table 1 and 2).

In a second analytical approach, we also calculated correlation analyses between the different measures and the performance in the individual phases of ASST. In Figure 5(D),(F), we represent the genotype based significant correlation analyses in IDS phase in females and Rev1 phase in males. In males, the percent time in open arm in EPM was negatively correlated with trials to criterion in the Rev1 phase in wildtypes (Figure 5(D); $r^2 = 0.47$, $p = 0.01$). In females, the percent time in open arm in EPM was negatively correlated with trials to criterion in the IDS phase in wildtypes (Figure 5(E); $r^2 = 0.38$, $p = 0.04$). In females, the reward consumption in the food preference test was negatively correlated with the trials to criterion in the IDS phase in homozygous orexin-deficient mice (Figure 5(F); $r^2 = 0.50$, $p = 0.02$). However, we did not observe any genotype-based variation in reward consumption during the IDS phase of ASST. Most importantly, we could not find any other significant correlations between the genotype- and sex-related changes in the IDS and Rev1 phases of the ASST with behavioral measures in the EPM, LDB, and reward consumption (r^2s < 0.11, ps > 0.07). This indicates that the observed changes in ASST performance in orexin-deficient mice are not caused by other genotype-related behavioral changes. The results are shown in Supporting Table 1 and 2.

4 | DISCUSSION

The aim of our study was to investigate how brain-wide orexin deficiency affects cognitive flexibility. Although, there are existing research on orexin's role in attention and cognition,[28,30,42-44] this is the first study investigating how complete or partial loss of orexin neuropeptide affects cognitive flexibility in mice. Complete loss of orexin neuropeptide in the homozygous orexin-deficient mice attenuated or improved cognitive flexibility in a sex-dependent manner. Whereas, partial loss of orexin in heterozygous orexin-deficient mice did not significantly affect cognitive flexibility (Figure 3(A),(B)). We further tested anxiety and reward preference in our mice. There was slightly increased anxiety in orexin-deficient mice. Food preference was not affected by genotype but females consumed more reward in this test than males. Interestingly, food consumption was also correlated in a sex-dependent way with overall ASST performance. In addition, anxiety weakly correlated with ASST performance in males.

ASST is a well-established rodent test for cognitive flexibility[45] which is often used to evaluate cognitive flexibility in transgenic mice models of neurodegenerative and neuropsychiatric disorders.[14,41] In the present study, we found that orexin deficiency affected ASST performance in a sex- and phase-specific manner (Figure 3(A),(B)). Sex differences in cognitive impairment are regularly observed in neurodegenerative disorders[46] and often, cognitive impairment is more severe in females compared to males.[47] In orexin research, sex differences were repeatedly reported so far.[43,48-51] For example, a recent study reported that stress-induced activation of orexin signaling impairs cognitive flexibility in female rats but improved it in male rats. The stress-induced impairment in cognitive flexibility in female rats could be rescued by inhibiting orexin signaling prior to stress.[43] Our results are in line with these findings of orexin's role in attenuating/improving cognitive flexibility in a sex-specific manner. We demonstrated that – in females – orexin deficiency impairs learning to shift attention within the same perceptual dimension (IDS) suggesting that the presence of orexin supports this learning, whereas orexin deficiency improved the first reversal phase (Rev1) in males. It was previously shown that acute administration of orexin A in basal forebrain improved olfactory reversal learning in male rats.[30] These results are apparently contradictory to our findings where complete loss of orexin in male homozygous orexin-deficient mice improved the Rev1 phase. So far, it is not completely understood why and how orexin deficiency is advantageous to males. It might be possible that other neurotransmitters are compensating for the chronic loss of orexin neuropeptide to regulate cognitive flexibility in males but not in females. However, this would not explain why orexin deficiency is only advantageous in Rev1 phase. Of note, we also observed a general sex difference in mean ASST performance. In females, generally fewer trials were required to complete all the different ASST phases compared to males.

Formation of an attentional set is crucial for the validation of ID-ED shift in ASST.[7] It seems that mice have more difficulties in forming an attentional set compared to rats or marmosets. Absence of set formation and thereby absence of ID-ED shift has been previously reported in mice.[52,53] Some other studies had introduced multiple IDS phases or multiple reversal phases before the EDS phase to induce a stronger set-formation and thereby an ID-ED shift.[40,54] In our study, we introduced an IDS phase and a reversal phase (Rev2) prior to the EDS phase. However, we observed difficulties in the formation of attentional set in some mice which was sex-dependent as previously reported.[55] In females, wildtype and heterozygous orexin-deficient mice required slightly higher number of trials to complete the EDS phase compared to the IDS phase indicating an attentional set formation and an ID-ED shift. However, homozygous orexin-deficient mice did not exhibit this ID-ED shift. They were impaired in the IDS phase which could have disrupted the formation of an attentional set (Figure 3(A)). In males, irrespective of the genotype, there seem to be an absence of ID-ED shift with the present protocol (Figure 3(B)). However, we see ID-ED shift in males of other mouse lines in other experiments (unpublished data) which indicates a deficit to form an attentional set in the mouse line used in this current study. In addition, absence of ID-ED shift could also be due to testing the different phases of ASST on two consecutive days instead of testing on a single day as described in ASST in rats which might have enhanced formation of an attentional set.[7] We further observed that all mice, irrespective of genotype and sex, that began testing with digging medium as the starting relevant dimension, shifting to odor during EDS phase, showed an ID-ED shift (Supporting Figure S2). The other half of the mice that began testing with odor as the starting relevant dimension did not show an ID-ED shift. Therefore, we randomized

the order of stimulus presentation in our study to prevent the effect of stimulus cues on ASST performance.[56]

Different cortical areas, PFC, CC and OFC mediate different types of attentional shifts such as EDS, IDS and reversal learning, respectively.[7,8,40,57] Orexin neurons innervate many brain areas including PFC, CC and OFC.[17,20] Orexin innervated cholinergic neurons in basal forebrain that project to PFC and are involved in attention and cognition.[28,30] Intranasal orexin A administration attenuated cognitive performance in young rats,[44] while intraventricular infusion of orexin A attenuated distracter induced attention deficit.[42] Interestingly, orexin expression in female rats were found to be much higher than males[43,48] .This suggests a more important role of orexin in females, which could be the reason for the sex-specific IDS deficit in the present study. In addition, fluctuation in sex hormone levels might influence cognitive performance and/or cognitive flexibility suggesting that monitoring the oestrous cycle during the ASST could shed lights on the sex hormone-based modulation of cognitive flexibility. To understand the sex-specific role of orexin in cognitive flexibility, future studies should also investigate the sex differences in orexinergic projections to various cortical areas and their impact on cognition.

Since we previously showed that homozygous orexin-deficient mice exhibiting an anxiogenic-like phenotype in LDB,[27] we were also interested in the question of whether trait anxiety affected cognitive flexibility in the present experiment. This question has also a translational perspective since, anxiety disorders are often associated with cognitive impairment.[58] Decreased levels of orexin A were found in the cerebrospinal fluid of post-traumatic stress disorder patients.[59] However, increased levels of orexin A were also reported in panic disorders indicating bidirectional fluctuation of orexin levels in neuropsychiatric disorders.[60] In our study, we observed both, sex and genotype effects in the two used paradigms for trait anxiety. In the LDB, there was a main effect of sex in the latency to enter the bright compartment, that is, males were more anxious than females, irrespective of the genotype (Figure 2(D)), whereas the percent distance traveled in the bright compartment was generally reduced in homozygous orexin-deficient mice (Figure 2(E)). In EPM, homozygous orexin-deficient mice had slightly reduced open arm entries indicating tendency to be more anxious (Figure 2(A)). These results indicate mild anxiety in homozygous orexin-deficient mice. To dissect if this effect on trait anxiety contributes to the ASST performance, we analyzed whether overall ASST performance was correlated with the anxiety parameters. In males, we found a weaker positive correlation between mean trials to criterion and percent time in bright compartment in LDB. This means less anxiety was correlated with poor overall ASST performance which was contradictory to previous studies that reported increased anxiety levels associated with poorer cognitive performances.[58,61] We did not find any correlation between mean learning performance of all mice and any other anxiety measurements (Supporting Table 1 and 2). We also performed correlation between learning performance in individual phases and anxiety parameters. We observed stronger correlation ($r^2 > 0.50$) between trials to criterion and percent time in open arm in EPM in female homozygous orexin-deficient mice in EDS phase and in male wildtype mice in CD phase (Supporting Table 1 and 2). However, we observed only week correlation ($r^2 \leq 0.50$) in IDS and Rev1 phase, where ASST performance was attenuated or improved in orexin-deficient mice (Figure 5(D),(E), Supporting Table 1 and 2). This indicates that the changes in ASST performance observed in our study are independent of orexin's role in anxiety.

Orexin plays a major role in feeding behavior.[25,38] It could be possible that brain-wide orexin deficiency altered reward consumption and thereby the motivation to perform ASST. To understand if orexin deficiency affected motivation for reward consumption, we performed a food consumption and food preference test after the ASST. All mice, irrespective of genotype and sex, consumed only the reward and not the home cage chow food in the food preference test (Figure 4(B)). There was no main effect of genotype indicating that orexin deficiency does not affect reward consumption. However, we observed a main effect of sex in reward consumption. Females, irrespective of the genotype, consumed higher amount of reward compared to males. Correlation analyses of the amount of reward consumption and overall learning performance revealed that better performers consumed higher amount of reward in males. Whereas, better performers consumed lower amount of reward in females during the food preference test (Figure 5(B),(C)). Correlation analyses between individual phases and ASST performance revealed that better performing female orexin-deficient mice in IDS phase consumed more amount of reward during the food preference test (Figure 5(F)). However, it is difficult to interpret these outcomes as we did not observe any sex-specific, genotype-specific or phase-specific lack of motivation to consume reward during ASST. In addition, the duration of the food preference test was much shorter (20 min) compared to the approximate time taken to complete each ASST phase with six consecutive correct trials (30–90 min). A longer duration food preference test might shed lights on sustained motivation to consume reward.

In human narcolepsy, extremely low levels or even an absence of orexin A in the cerebrospinal fluid and a dysfunction of the orexinergic system is observed.[62-64] Of note, narcoleptic patients exhibit cognitive deficits especially in attention and executive function.[65] Orexin levels are not only altered in narcolepsy but also in inattentive subtype of attention-deficit/hyperactivity disorder (ADHD).[66] Homozygous orexin-deficient mice exhibited sleep disturbances similar to human narcolepsy and are used as narcoleptic mice models.[23,67] Narcoleptic episodes are triggered by emotional stimuli in human narcoleptic patients as well as in homozygous orexin-deficient mice.[68,69] We observed narcoleptic episodes in some of the homozygous orexin-deficient mice during ASST. In open field, fear and safety conditioning, EPM and LDB experiments, we did not observe such narcoleptic episodes before.[27] We speculate that the expectation of reward or the reward consumption itself, which are both positive events, triggered these narcoleptic episodes.[70] We observed a sex-difference in the total number of narcoleptic episodes during ASST. Females exhibited more narcoleptic episodes compared to males (Figure 4(A)). We investigated if the narcoleptic episodes affected ASST

performance by correlating total number of narcoleptic episodes and overall learning performance (Supporting Table 1 and 2). We did not find any correlation indicating that the attenuation/improvement of cognitive flexibility that we observed in the present study was not due to the narcoleptic episodes rather they are yet another behavioral phenotype of the orexin-deficient mice. Therefore, orexin might be involved in regulating sleep/wake cycle and cognitive flexibility through dissociated neural mechanisms.

Last, it should be mentioned that all our experiments were performed during the light-on phase, that is, the inactive phase of the mice. Whereas behavioral measures of anxiety are often more robust during the inactive phase,[71,72] measures of cognitive performance and/or cognitive flexibility may on a first glance less robust during the inactive phase. Latter assumption may not be correct since published data show that cognitive flexibility is not affected by the time of testing and even suggest that initial training takes longer in the active phase.[73] However, time of testing is especially critical for the present study due to the diurnal changes in orexin signaling. Brain orexin levels are generally higher during the active phase than during the inactive phase. Thereby, differences between wildtype and orexin-deficient mice might be masked or underestimated.

In summary, our study shows that orexin deficiency attenuates specific phases of cognitive flexibility in a sex-dependent manner. This supports the hypothesis that orexin plays an important role in cognition and sex-based regulation of behavioral endophenotypes underlying brain disorders. Further investigations should focus on the role of the different orexinergic projections to the different frontal cortex areas known to be involved in the ASST, as well on the effects of acute pharmacological orexinergic interventions on cognitive flexibility. Finally, it is important to better understand orexin's role in the sex-specific modulation of cognitive flexibility. This reiterates the necessity to perform comparative study in both sexes to design sex-based treatments to treat cognitive impairments.

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DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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SUPPORTING INFORMATION

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