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Cognitive, Behavioral, and Sleep Characterization of Sex Differences in the Ts65Dn Mouse Model of Down syndrome

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Plain English summary

Down syndrome (DS) is due to a full or partial trisomy of human chromosome 21, and it is the most common neurodevelopmental disability with a known genetic cause. The triplication of the expression of so many genes makes DS a complex disability. Of the many altered traits that characterize DS in humans, we focus on learning, memory, and sleep. These phenotypic characteristics are also found in the Ts65Dn mouse model of DS making it a useful experimental subject for investigating underlying causes as well as potential mitigations. Sex differences in cognition and sleep have been shown in humans with DS, however research on animal models of DS have rarely reported results as a function of sex. The aim of our research was to establish whether or not sex differences in the critical traits of learning, memory, and sleep exist in our mouse model of DS. Control and Ts65Dn mice were tested for memory, anxiety, risk taking, depression, and social behavior. Female Ts65Dn mice had better memory, more anxious and social and risk-taking behavior, than male Ts65Dn mice, as well as less depression-like behavior. The sleep of control and Ts65Dn mice were also recorded. The males have a more fragmented sleep, which could explain their memory deficits compared to female Ts65Dn mice.

Abstract (243/350 words)

Background: Down syndrome (DS), arising from full or partial trisomy of human chromosome 21, is the most common neurodevelopmental disability with a known genetic cause. People with DS experience altered learning, memory, and sleep. These phenotypic characteristics are exhibited in the Ts65Dn mouse, a genetically engineered murine model of DS. Sex differences in cognition, sleep, and behavior have been shown in humans with DS. However, in Ts65Dn mice, there is little research into sex-specific cognitive and behavioral distinctions despite the necessity of using both sexes in research being well-accepted.

Methods: We studied male and female Ts65Dn mice using Novel Object Recognition (NOR), Novel Object Location (NOL), T-Maze, Open-Field, Elevated Plus Maze, Social test, and Splash tests to examine long-term and short-term memory, anxiety-like behavior, risk-taking, sociability, and depression-like behavior. We also recorded and compared the sleep of male and female Ts65Dn mice

Results: Our results show that female Ts65Dn mice have better long-term recognition memory, more anxiety, more risk-taking behavior, more social behavior, and less depression-like behavior than male Ts65Dn. No significant impact of sex in Ts65Dn mouse performance was found on long term spatial memory and short-term memory. Sleep recording shows no differences in the global amount of wake, NREM and REM between the sexes but more fragmentated sleep in the male Ts65Dn mice.

Conclusions: There are clear sex differences in a broad spectrum of behaviors in the Ts65Dn mouse model of DS. These differences should be taken into consideration when planning and analyzing future work that uses this mouse model. Using single sex cohorts or mixed sex cohorts may obscure sex related differences in the etiology of DS characteristics in humans as well as potential therapeutic strategies for improving cognitive functions.

Highlights (51/150 words)

- Female Ts65Dn show better long-term memory, more anxiety, social and risk-taking behavior as well as less depressive behavior,
- Male Ts65Dn present more sleep fragmentation,
- No differences between sex were observed regarding the short-term memory, long term spatial memory and global amount of wake, NREM and REM.

Introduction

Down syndrome (DS), arising from full or partial trisomy of human chromosome 21, is one of the most common neurodevelopmental disabilities with a known genetic cause (Stallings et al., 2024). People with DS experience alternations in learning, memory (Roizen & Patterson, 2003) and sleep (Miano et al., 2008). These characteristics are exhibited in the Ts65Dn mouse, a widely used transgenic mouse model of DS that carries triplicated orthologous genes of human chromosome 21, corresponding to regions on mouse chromosomes 16 and 17 (Reeves et al., 1995).

Cognitive impairments observed in Ts65Dn mice are presumably due to abnormal neural signaling resulting from the effects of gene triplication on the development and functioning of neural networks and signaling. One neurotransmitter that has been studied extensively for its role in memory processing in the Ts65Dn mouse is gamma-aminobutyric acid (GABA). Multiple GABA antagonists have been shown to improve and even normalize performance in hippocampal dependent learning task learning in Ts65Dn mice (Fernandez et al. 2007, Colas et al. 2013, Colas et al. 2017). These tasks include novel object and novel location object recognition as well as working memory tasks. GABA is a major inhibitory neurotransmitter, so the prominent involvement of $GABA_A$ signaling in the learning and memory abilities of the Ts65Dn mice led to the hypothesis that the problem was over-inhibition in the hippocampal circuits (Fernandez et al 2007). Where could the GABA responsible for over-inhibition come from? The suprachiasmatic nucleus (SCN) was implicated by the fact that the $GABA_AR$ antagonist treatment was only effective if it were given at the right circadian phase – the sleep phase, and the SCN is a GABAergic nucleus. Also, SCN neurons project to the hippocampus through the lateral and medial septal pathways (Watts, 1991). The most convincing support for the SCN being involved in the over-inhibition was the discovery that spatial learning and memory in the Ts65Dn mouse could be restored by lesioning of their SCNs (Chuluun et al., 2020).

Stepping back from the molecular, cellular, circuit investigations of the possible causes of the cognitive dysfunction of DS, we can appreciate that multiple systems play roles in learning and memory – sleep, circadian rhythms, emotional and social controls. Humans have been shown to have sex differences in all these physiological/behavioral systems. Yet, sex differences in the animal models we use to study DS and other conditions have rarely been recognized. Awareness

of those differences could be relevant for the interpretation of the results we obtain from our model systems.

Deficits in sleep are exhibited both in humans with DS and in the Ts65Dn mouse. Smaller bone dimensions as well as morphological differences in the upper airway of patients with DS predispose them to obstructive sleep apnea (OSA) (Lal et al., 2015). Individuals with DS exhibit increased OSA events during REM. OSA leads to hypoxemia, producing decreased sleep quality, increased sleep fragmentation, and cognition deficits. Like humans wiexhibitsTs65Dn mice have increased OSA events during REM sleep compared to wild type (Bartolucci et al., 2021). Consequently, Ts65Dn mice exhibit increased wakefulness, decreased NREM sleep, and increased sleep fragmentation that compromises memory consolidation (Rolls et al. 2011).

In humans with DS, research has connected sex with differences in cognition and behavior and other physiological measures. Female children with DS have higher IQ scores than males across Wechsler Intelligence Scale for Children – Revised and Stanford-Binet testing metrics (Kittler et al., 2004, Clements et al., 1976). Female children with DS also have significantly faster and higher developmental trajectories of non-verbal cognitive and language ability development than males, whose cognition is more likely to be negatively affected by neonatal risk factors (Aoki et al., 2018). Females with DS show an association between verbal IQ and autism symptom presentation that is absent in male counterparts, showing sex has a differential effect on the role of verbal cognition in restrictive and repetitive behaviors (del Hoyo Soriano et al., 2024). Hormonal sex differences and the neuroprotective effect of estrogen in females with DS could account for their apparently better cognitive performance. However, females with DS show earlier onset of menopause (Coppus et al., 2010) than typically developing females, which is hypothesized to have sex-specific adverse hormonal effects on cognition and the development of Alzheimer's pathology. Numerous studies have investigated the association between sex and sleep in humans with DS, with some suggesting that males have a greater occurrence of OSA (Hanna et al., 2022). The array of sex differences in humans with DS have significant implications for the clinical profile of DS and provoke questions as to whether they are represented by the Ts65Dn mouse model. To our knowledge, no research has focused on sex differences specifically in mouse model of DS even if they are well documented in humans.

Existing Ts65Dn studies that used both sexes, but never specifically studied sex differences, have shown neuroanatomical sex differences in the Nucleus Basalis of Meynert /

Substantia Innominata of the cholinergic basal forebrain (Kelley et al., 2014) and sex-specific alterations in trisomic gene expression during brain development (Hawley et al., 2024). Ts65Dn sex differences in bone degeneration, with impaired healing in male Ts65Dn mice (Sherman et al., 2025), and differences in motor activity (Faizi et al., 2011, Martinez-Cue et al., 2002) have also been reported, but there has been little research into sex differences in behavioral analyses and sleep recordings. One study investigating the effect of environmental enrichment on behavior, spatial learning, and working memory showed a sex-dependent response to treatment, with a positive response in female Ts65Dn mice and a negative response in male Ts65Dn mice (Martinez-Cue et al. 2006), suggesting a modulatory relationship between sex and Ts65Dn cognition. Martinez-Cue et al. (2006) also showed enhanced biochemical and behavioral anxiety responses in female Ts65Dn mice compared to males and controls. However, sex differences across more extensive cognitive and sleep paradigms have yet to be elucidated. A major question is whether behavioral results in the study of the mouse model should be averaged across sexes. Discerning potential sex differences in greater detail will help establish baseline research for future use of the Ts65Dn mouse and its representation of sleep and cognition in DS.

Therefore, the aim of this paper is to characterize the sex-differences, in cognition and sleep, between male and female Ts65Dn. To do so we did numerous behavioral tests and recorded the sleep of the male and female Ts65Dn mice and their wild-type controls.

Methods

Animals

All experiments were performed in accordance with the guidelines described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the Stanford University Institutional Animal Care and Use Committee and are in accordance with the ARRIVE Guideline (https://arriveguidelines.org/arrive-guidelines). Efforts were made to minimize the number of animals used and the pain and distress they experienced. All experiments were randomized and blinded. Male and female mice, between 3 and 5 months old, were used in this study. Mice were housed in groups of 2–4 per cage except the mice that went through surgeries. Animal rooms were at 22°C and 40%–60% of humidity. The light cycle was 12 hours light/12 hours dark with lights on at 08:00 am. Food and water were available *ad libitum*.

Ts65Dn (Ts (1716)65Dn, genetic background: C57Bl/6JEi × C3H) mice are a popular mouse model of DS because they have three copies of most of the mouse genes that are orthologous to the genes triplicated in humans with DS on chromosome 21. Segmental trisomic 16 (Ts65Dn) mice were obtained by mating female carriers of the 1716 chromosome (B6EiC3H—a/ATs65Dn) with C57BL/6J Ei × C3H/HeSnJ (C3H) F1 hybrid males (Reeves et al., 1995) and produced at Jackson West Laboratories, (Davis, CA). As a control, we used 2N mice that are the non-trisomic littermates of the Ts65Dn mice and, therefore, do not have three copies of genes orthologous to genes on human chromosome 21.

Behavioral experiments

T-Maze

The T-Maze test measures short term spatial memory through spontaneous alternations. For 7 minutes, the mouse is able to explore a clear acrylic maze with three arms: one long stem arm (49.2x9.7x20.3cm) that opens at one end into two lateral arms (each 34.5x9.7x 20.3 cm) that are at right angles to the left and right of the stem arm to form a T-configuration. Where the left and right arms meet the stem arm, there is a clear vertical barrier in the stem arm that requires the animal to decide on which arm to enter and for this decision to be observed objectively. Test subjects are first placed in a start chamber at the base of the stem arm set off by a clear barrier 20 cm from the end of the arm. The animal spends 10 seconds behind the barrier before it is lifted giving the animal access to the T-maze for 7 minutes. The mouse explores the maze by entering the left or right arm, returning to the stem arm, and then exploring the other arm (an alteration) or returning to the arm it just left (non-alternation). Each entry into the left or right arm was manually recorded. Percentages of alternations were calculated using the formula

$$\%\ \text{altermations} = \frac{(\text{Number of alternations})}{(\text{Number of alternations} + \text{non} - \text{alternations})} * 100$$

Open Field (OF) Test

The open field test was used to evaluate the general activity, exploratory behavior, and anxiety-like behavior of mice. This assessment was also used as a baseline control for the novel object recognition and novel object location tests.

Testing took place in an enclosed square arena (50x50x50 cm) that was comprised of an acrylic floor set under a tracking camera. The mice were gently placed in the center of the open arena and allowed to move freely for 10 minutes while being tracked with Viewpoint Life Sciences, Inc. software (Montreal, Canada). To quantify anxiety-like behavior, the arena was divided into central (15x15x15 cm) and peripheral zones. The percentage of total time spent in the center of the arena was used as the primary measure for anxiety-like behavior. We also measured the general activity, and exploration of the animals.

$$\%\ \text{time center} = \frac{(\text{time in center})}{(\text{Total time})} * 100$$

Novel Object Recognition (NOR)

The Novel Object Recognition (NOR) test assessed the animal's ability to remember objects encountered at an earlier time. This assay was conducted in three phases:

1. Habituation (Open Field Test): The animals were first habituated to the testing arena (see above) before being placed in the same arena along with test objects.
2. Training Phase: Following habituation, each mouse was placed in the arena containing two identical objects (e.g. wooden blocks, plastic tubes, plastic barrels) placed near the center of the arena, with one positioned slightly anterior and lateral to the other, forming an offset central configuration. Both objects were placed at an equal distance from the wall and were securely adhered to the acrylic floor to prevent movement or toppling, which could startle the animals. Mice were allowed to explore freely for 10 minutes.
3. Testing Phase: The following day, the animal was reintroduced into the arena with one of the two familiar objects replaced with a novel object and allowed to explore for 7 minutes. Exploration time directed towards each object was recorded and calculated, with the primary measure being the percentage of time spent near the novel object.

$$\%\ \text{Time spent near the novel object} = \frac{(\text{Time spent near to the novel object})}{(\text{Time spent near both objects})} * 100$$

All trials were recorded and analyzed using Viewpoint Life Sciences, Inc. software (Montreal, Canada), which automatically tracked the position of the mouse's nose within a 2cm radius surrounding each object.

Novel Object Location (NOL)

The novel object location (NOL) test was used to assess spatial memory in mice. Like the novel object recognition test, this assay was conducted in three phases, with the first two nearly identical to the NOR test. Different sets of objects that differ by shape, texture and pattern, were used during this test.

After habituation in the open field test (as described above), there were two following phases:

1. Training Phase: Each mouse was placed in the arena with two identical objects placed at the same distance from the arena wall. The objects were fixed to the acrylic floor to prevent movement, and the mice were allowed to explore freely for 10 minutes.
2. Testing Phase: The following day, one of the objects was placed in a new position, resulting in a side-by-side configuration near wall of the arena. Each animal was then placed in the center of the arena and allowed to explore for 10 minutes. The exploration time directed towards the moved object (novel location) versus the unmoved object (familiar location) was recorded.

$$\%\ \text{Time spent near object at new location} = \frac{(\text{Time spent near object at new location})}{(\text{Time spent near both objects})} * 100$$

Exploratory behavior was automatically tracked using Viewpoint Life Sciences, Inc. software (Montreal, Canada), which detected when the animal's nose entered a 2cm radius of each object. The percentage of total exploration time spent investigating the object in the novel location was the primary measure of spatial memory.

EPM

The Elevated Plus Maze (EPM) is a measure of anxiety-like and risky behavior. Animals are placed in the center of an acrylic maze with four perpendicular arms, each 30x5 cm, that meet in the center to form the shape of a plus sign. Two collinear arms are walled with 25 cm tall acrylic, while the other two arms are unwalled "open arms" associated with greater risk. Mice are first placed in the center of the maze, with trials lasting 7 minutes and data points excluded if the mouse falls off the maze before the full duration of the trial. Manual observation with a stopwatch was conducted to record the amount of time test subjects spent in the open arms of the maze, the number of entries into the open arms, the time in and number of entries into the "risky" area of the open arms (the third of the open arm farthest from the center), and number of head dips, where test mice dip their heads over the edge of a maze arm and look below.

Social

The Social test is a measure of prosocial behavior. The test animal is placed in a 50x50x50 cm enclosure for 10 minutes with a novel stimulus mouse, of the same genotype, in the center of the enclosure. The stimulus mouse is positioned underneath a 15x15x15cm wire cage through which the mice can see, smell, and touch. Viewpoint Behavioral Technology Video Track was used to record videos of each trial and the amount of time test mice spent in the "Social Zone," defined as the region within 2cm around the perimeter of the wire cage. Manual scoring of the trial videos using Behavioral Observation Research Interactive Software measured (1) the duration and count of nose-nose interactions, a prosocial behavior in which the test and stimulus mice sniff each other while making nose-to-nose contact, and (2) the time test subjects spent climbing on or sitting on top of the wire cage.

Splash Test

The splash test was used as a measure of anhedonia, as seen in depression. This assay evaluated self-care motivation by quantifying the percentage of time grooming after the application of a sucrose solution to the animal's fur. A 10% sucrose solution was prepared by diluting sucrose in distilled water, and sprayed four times on the mouse's dorsal coat, bilateral flanks, and facial region while they were held in a metal wire cage. Immediately following application, the mouse was gently placed in the center of the open field arena, and their behavior was recorded for 15 minutes.

The video was manually scored to track grooming behavior, including nose/face grooming, head washing, body grooming, and full-body shakes. The following parameters were collected: time to initiate grooming after placement in the arena (Latency), total number of discrete grooming bouts (Grooming count), the cumulative duration of all grooming episodes (Time grooming), proportion of the 15-minute session spent grooming (% of Total Time Spent Grooming), and the grooming frequency normalized to the session duration (Groom per minute). These measures, together with the proportion of inactive time (periods without active grooming), were used to evaluate anhedonia-like behavior.

Sleep recording

To record the cortical encephalographic (EEG) and electromyographic (EMG) signals, we surgically implanted EEG and EMG electrodes as previously described (Pittaras et al., 2025). Animals were anesthetized using 5% of isoflurane (Dechra, Isospire™) and maintained under anesthesia with a flow of 2% of isoflurane. Buprenorphine (SR-LAB, 0.5 mg/mL) was also injected subcutaneously at 0.5 mg/kg before starting the surgery. EEG electrodes were placed over the frontal and parietal cortices. The EMG electrodes were placed in the neck musculature. After 2 weeks of recovery, animals were connected to lightweight recording cables leading to freely rotating commutators and adapted to single occupancy recording chambers for a few days before the beginning of the experiments. EEG and EMG signals from the surgically implanted electrodes were collected using commercial hardware (Embla; Broomfield, CO), digitized at 256 Hz, and visualized using sleep recording software Somnologica-3 (Medcare, Reykjavik, Iceland, 79).

Sleep analysis

All recordings were scored with an automated EEG scoring program using machine learning algorithms (Somnivore Pty. Ltd., Parkville, VIC, Australia). To do so, the software was first trained by manually scoring 100, 4-second epochs per mouse using visual inspection of EEG and EMG signals of NREM sleep (high voltage, low-frequency EEG), REM sleep (prominent theta activity, low voltage EMG), and wake (low voltage, high-frequency EEG). After this training, the scoring was done automatically by the software. The rules applied for the auto-score were a minimum of one epoch for wake, a minimum of two epochs for NREM sleep, and a minimum of

three epochs for REM sleep. We also used one force rule: if the previous epoch is scored as wake and the epoch in question is scored as REM, then change the REM scoring to wake. Visual inspection was done to identify and correct mistakes. We analyzed the sleep organization as follows: percentage of total recording time (TRT) spent in wake, NREM sleep, and REM sleep; and number of transitions from NREM sleep to REM sleep, average bout length of wake, NREM sleep, and REM sleep and longest bout length of wake, NREM sleep, and REM sleep; the number of bouts of wake, NREM sleep, and REM sleep; the total amount of wake, NREM sleep and REM sleep, and the average inter-bout distance for wake, NREM sleep, and REM sleep. Power in the 1–80 Hz range of the recording was averaged, and the mean values were plotted in 0.8 Hz bins. Power spectrum, relative power and peak frequency of delta (δ), theta (θ), alpha (α), sigma (σ), beta (β), gamma (γ) range, and in spindles were calculated by using the aggregate power between 1–3 Hz, 4–7 Hz, 8–12 Hz, 11–12 Hz, 13–17 Hz, 31–40 Hz, and 13–14 Hz, respectively. All results are shown as the average for the 24-hour recordings.

Timeline

The male and female groups that did not undergo surgery were treated with the following protocol of behavioral and cognitive experiments: one day of Novel Object Recognition (NOR) and open field, two days of NOR, one day of T-Maze, four days of Sucrose, one day Social/Splash, one day of Novel Object Location (NOL) and open field, one day of just NOL, one day NOL and Elevated Plus Maze (EPM), with an additional day of EPM if needed (**Figure 1**). In total, these experiments were performed over the course of twelve days.

Mice that received electroencephalogram (EEG) and electromyogram (EMG) implants had 10 to 19 days recovery before starting the same set of behavioral experiments. Three days after finishing behavioral tests, animals were plugged in and habituated to being plugged in for five days, with sleep recorded on the following two days.

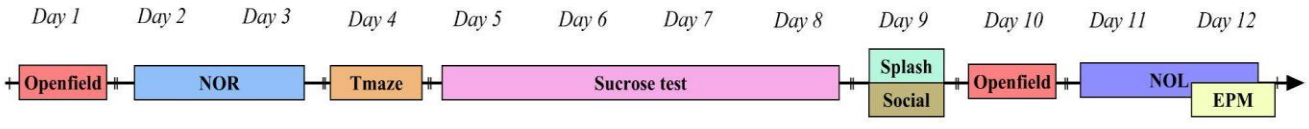


Figure 1: Order of the behavioral tests done during the two weeks of experiments. The sleep was recorded before the behavioral experiments.

Statistical analysis

Statistical analyses were performed using Statview software. If the data showed normal distribution (Shapiro–Wilk test) and passed equal variance tests (F test), statistical analyses were performed using t-tests or analyses of variances (ANOVAs). If not, statistical analyses were performed using Wilcoxon task sign-rank, Kruskal Wallis or Mann–Whitney non-parametric tests. For the behavioral tests, ANOVAs were used to test the effect of genotype/sex, time (training vs. testing), and the combined effect of genotype/sex × time as independent values.

For the sleep recording analysis, we used ANOVAs to test the effect of genotype, sex and the combined effect of genotype × sex. To compare the sleep of the 2N *vs*. the Ts65Dn mice, we used a t-test.

For multiple comparisons, a post hoc correction was applied.

Results were reported as means ± standard error of the mean. p values ≤ .05 are considered statistically significant. All statistical analyses are available in the supplement.

Results

1. **Results on behavioral tests**
    a. *Memory tests*

T-maze

The 2N mice groups show a percentage of alternation that is larger than the chance level (2N, female: t=2.531, **p=0.02** - male, t=3.817, **p=0.01**; Ts65Dn, p>0.5). The Ts65Dn mice male and female show impaired short-term memory (p>0.5; **Figure 2A**).

Novel Object Location

The ANOVA repeated measurement did not show any effect of groups (p>0.5), training *vs.* testing (p>0.5) and no combined effect of groups x training *vs.* testing (p>0.5; **Figure 2B**).

Novel Object Recognition

The ANOVA repeated measurement showed an effect of groups (F(3)=4.219, **p=0.008**), training *vs.* testing (F(1)=18.462, **p<0.0001**) but no combined effect of groups x training *vs.* testing (p>0.5). Only the female Ts65Dn and 2N mice spent more time close to the new object during the testing

compared to the chance level (2N: female, Z=-3.657,**p=0.0003**; Ts65Dn: female, t=3.901, **p=0.0007**; male, p>0.5) and compared to the training for the 2N female (2N: female, Z=-2.704, **p=0.007; Figure 2C**).

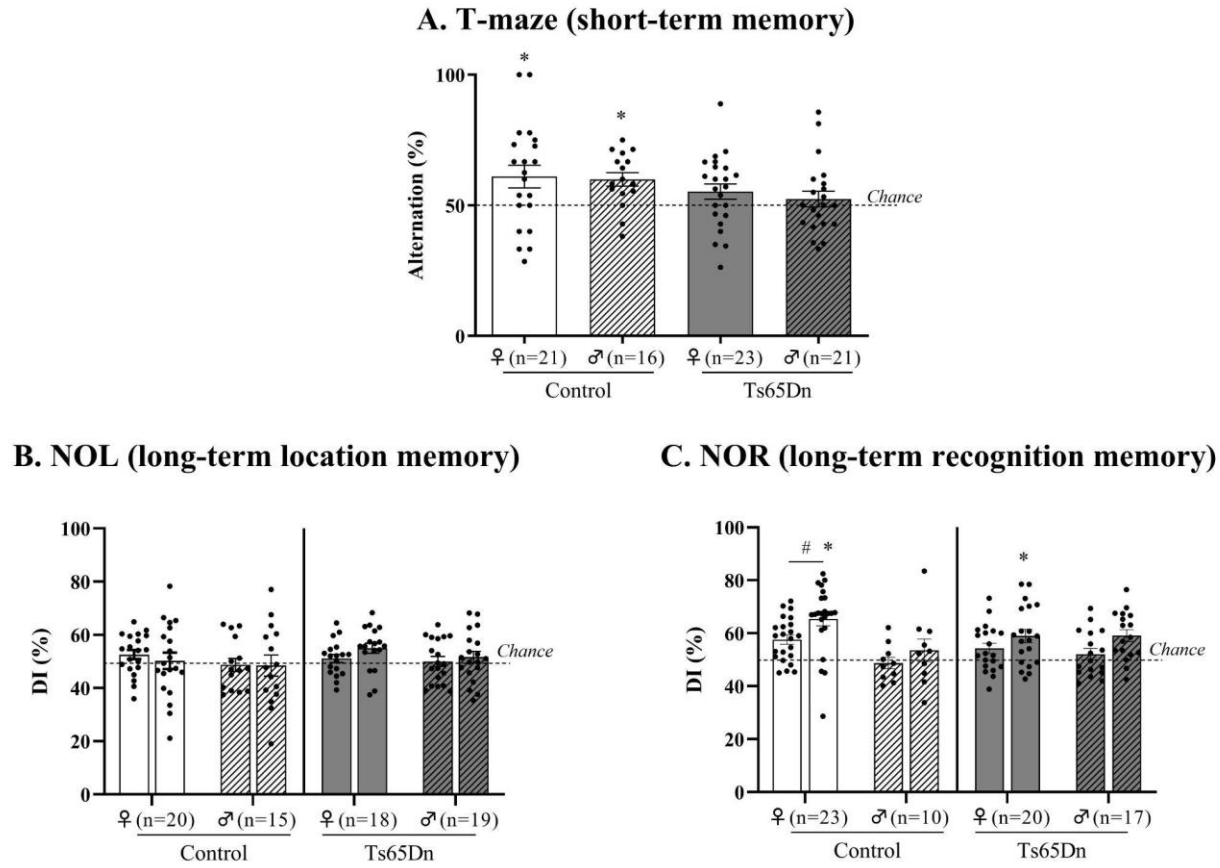


Figure 2: A. Percentage of alternation, during the T-maze test, reflecting short term memory. Only the 2N mice show a percentage of alternation superior to the chance level. **B.** Discrimination index (DI) during the Novel Object Location test (NOL) reflecting long-term location memory. Only the female Ts65Dn mice show good long-term location memory. The bar on left represents the training, and the bar on the right the testing, for each group. **C.** Discrimination index (DI) during the Novel Object Recognition test (NOR) reflecting long-term recognition memory. The bar on left represents the training, and the bar on the right the testing, for each group. Only the females 2N and Ts65Dn mice show good long-term recognition memory. *, $p<0.05$ represents a significant difference from the chance level. #, $p<0.05$ represents a significant difference between the training and the testing of the test.

b. *Anxiety/risk taken*

Open field

As shown in **Figure 3A**, there is a significant effect of groups regarding the time spent in the center of the open field (H(3)=10.225, **p=0.02**). The female Ts65Dn spent significantly less time in the center area than the Ts65Dn male (t=-2.864, **p=0.007, Figure 3A**).

Elevated Plus Maze

In the Elevated Plus Maze we observed a significant effect of sex and genotype for the head dips (H(3)=23.991, **p<0.0001**), the time spent (H(3)=21.002, **p=0.0001**) and number of entries (H(3)=56.826, **p<0.0001**) in the open arms as well as the number of entries in the extremities of the open arms (H(3)=10.049, **p=0.01**) but not for the time spent at the extremity of the open arms (p>0.5; **Figure 3B**).

. Genotype differences

The female and male Ts65Dn mice did more head dips than the 2N male mice (Female: t=4.323, **p<0.0001**; male: Z=-2.789, **p=0.009**), spent more time in the opened arms than the female 2N (female: t=-3.978, **p=0.004**; male: Z=-3.914, **p<0.0001**) and entered more open arms than the 2N male and female mice (Male 2N *vs.* male Ts65Dn: Z=-2.338, **p=0.01**; Male 2N *vs.* female Ts65Dn: Z=-5.838, **p<0.0001**; Female 2N *vs.* female Ts65Dn: t=-3.492, **p=0.01**; Female 2N *vs.* male Ts65Dn: Z=-4.366, **p<0.0001**). Female Ts65Dn also did more entries at the extremity of the open arms than the male 2N (Z=-2.474, **p=0.01; Figure 3B**).

. Sex differences

The Ts65Dn female did significantly more head dips (t=2.0680, **p=0.01**) and spent more time in the open arms than the Ts65Dn male mice (Z=-2.521, **p=0.01**). Females' mice, for both genotypes, also significantly entered more into the open arms than males' mice (2N: Z=-4.825, **p<0.0001**; Ts65Dn: Z=-5.672, **p<0.0001; Figure 3B**).

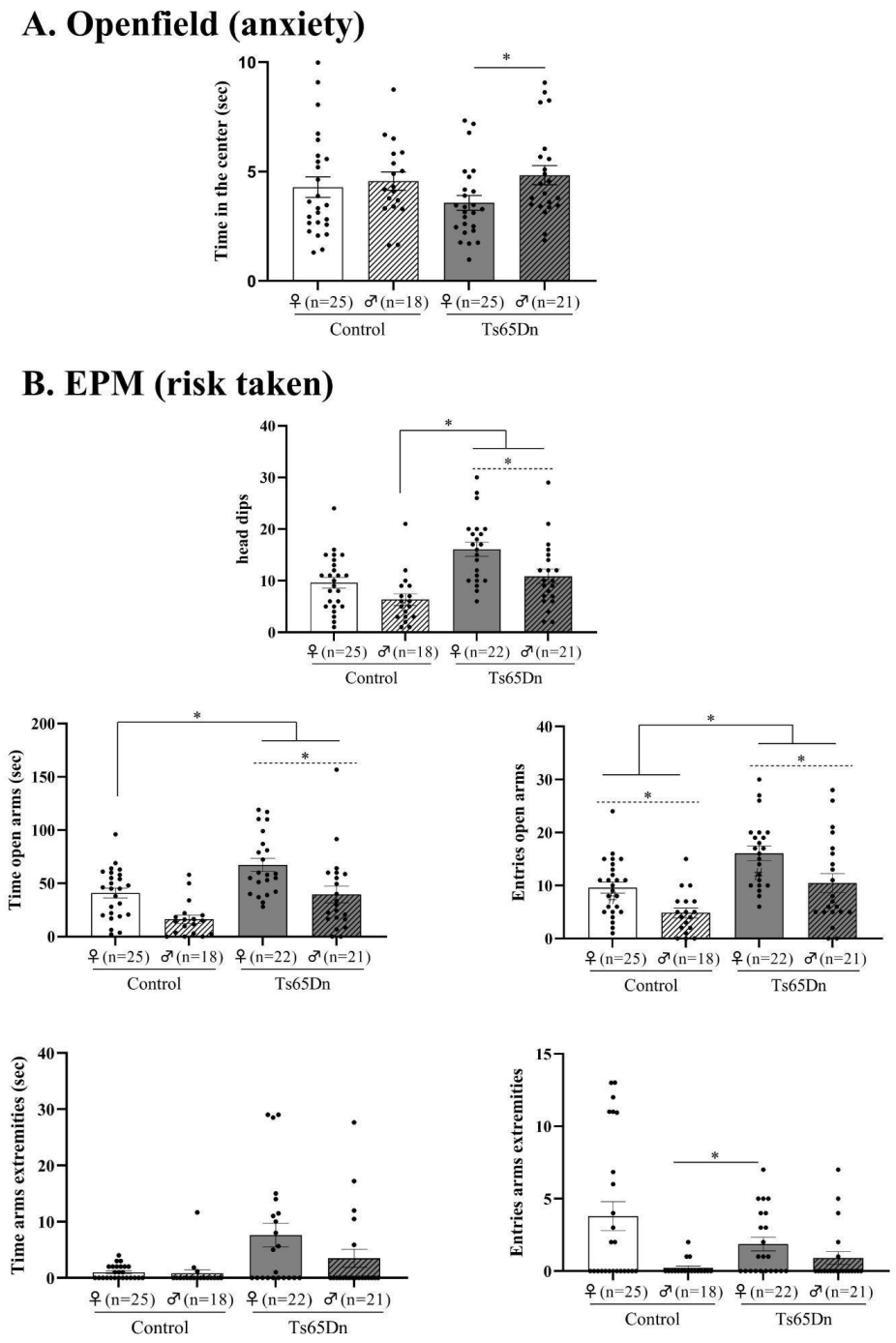


Figure 3: **A.** Time spent in the middle of the Openfield, more anxiogenic area, during the Openfield test. The female Ts65Dn significantly spent less time in this area. **B.** Elevated Plus Maze (EPM) test reflecting the risk taken of the animals. The Ts65Dn mice took more risk during this test than the 2N mice. The female Ts65Dn mice also took more risk than the male Ts65Dn during the EPM. *, $p<0.05$ represents a significant difference between groups.

c. Social behavior

As shown in **Figure 4**, a significant effect of sex and genotype was observed for the number of entries in the social zone (H(3)=7.985, **p=0.04**), the time spent on the top of the social cage (H(3)=9.071, **p=0.03**), in the social zone (H(3)=19.635, **p=0.0002**) and the duration of the nose touches (H(3)=15.842, **p=0.0012**). However, no significant effect of sex and/or genotype was observed for the time spent in the social zone (p>0.5) and the number of nose touches (p>0.5).

. Sex differences

The female 2N significantly entered less into the social zone than the male 2N (Bonferroni correction, p=0.01; t=-2.250, **p=0.01; Figure 4A**).

Both female 2N and Ts65Dn mice spent more time in this social zone (2N: Z=-3.494, **p=0.0003**; Ts65Dn: Z=-2.419, **p=0.01; Figure 4D**) and in nose touch (2N: Z=-2.391, **p=0.01**; Ts65Dn: Z=-2.771, **p=0.005; Figure 4F**) compared to males.

. Genotype differences

The male 2N mice spent significantly less time in the social zone (Z=-2.796, **p=0.005**) and in nose touches (Z=-3.383, **p=0.0001**) than the female Ts65Dn mice. The female 2N spent more time in the social zone than the Ts65Dn male mice (Z=-3.656, **p=0.003; Figure 4**).

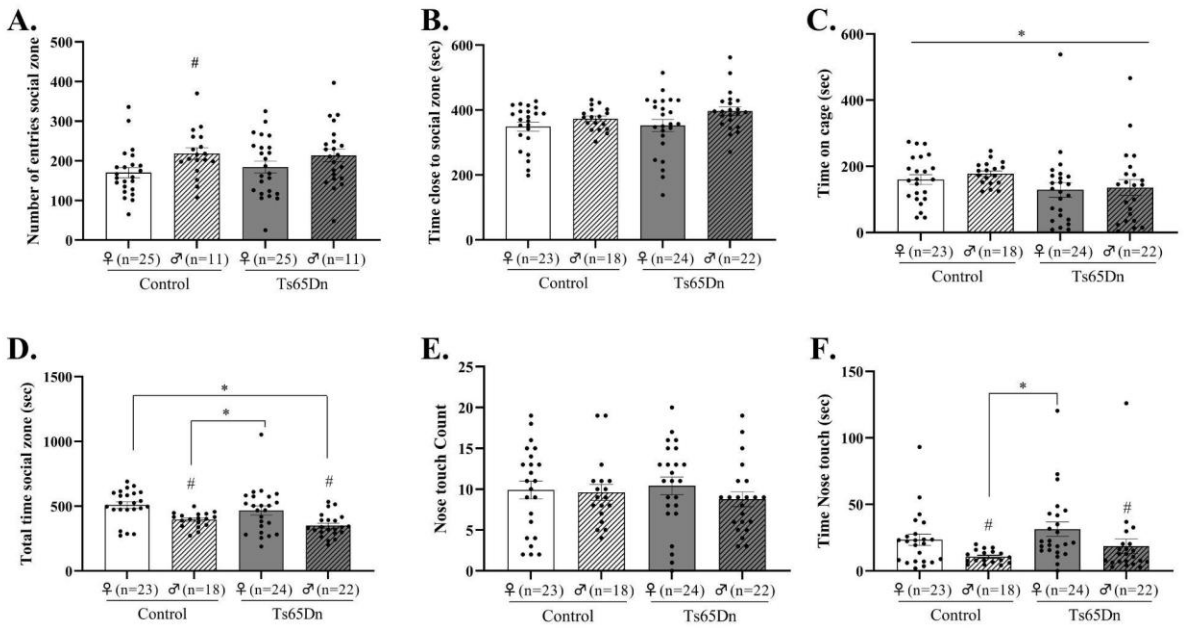


Figure 4: Number of entries in the social zone (**A.**), time close to the social zone (**B.**), time on the top of the social cage (**C.**), total time in social area (close to the social zone + on the top of the social cage, **D.**) and number (**E.**) and time (**F.**) of nose touches. The females are more social than the males. *, $p<0.05$ represents a difference between genotypes. #, $p<0.05$ represents a difference between sexes.

d. Anhedonia

As shown in **Figure 5**, there is an effect of sex and genotype regarding the percentage of time spent grooming ($H(3)=11.854$, **p=0.008**) and the percentage of time immobile ($H(3)=14.743$, **p=0.002**). Indeed, the male 2N did spend less time grooming than the female 2N ($t=3.124$, **p=0.0049**) and Ts65Dn ($t=3.740$, **p=0.001**). The female 2N spent also significantly more time immobile than the male 2N ($Z=-2.833$, **p=0.005**) and the female Ts65Dn ($Z=-3.599$, **p=0.0003**).

No significant effects of sex and genotype were observed for the latency to start grooming, the number of grooming and the number of grooming per minute ($p>0.5$).

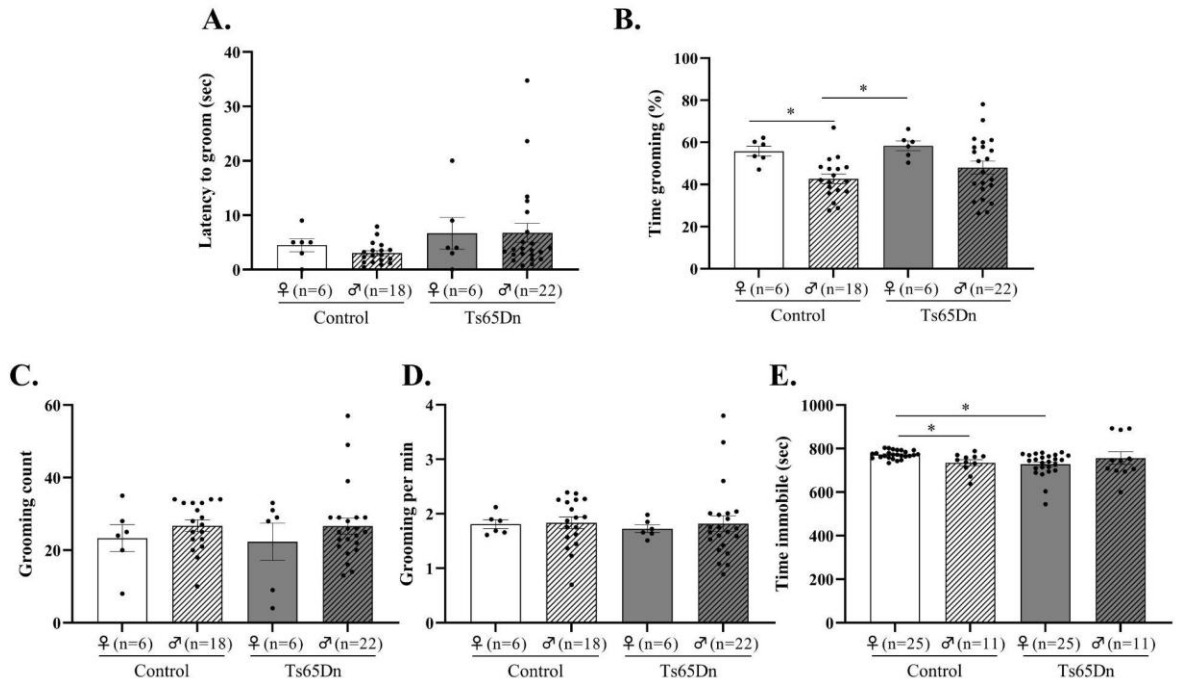


Figure 5: Latency to start grooming (**A.**), percentage of time spent grooming (**B.**), number of grooms (**C.**), number of grooming per minute (**D.**) and time immobile (**E.**) during the Splash test. The 2N male spent significantly less time grooming than the female mice. The female 2N spent significantly more time immobile than the male 2N and the female Ts65Dn. *, $p<0.05$ represents a significant difference between groups.

2. Results on sleep recording

A significant effect of genotype x sex was observed for the percentage of wake ($F(26,3)=6.419$, **p=0.0002**), NREM ($F(26,3)=8.794$, **p=0.0003**) and REM ($H=7.786$, **p=0.05**), the number of wake bouts ($F(26,3)=2.802$, **p=0.05**), the number of NREM bouts ($F(26,3)=2.820$, **p=0.05**) and the number of REM bouts ($F(26,3)=7.515$, **p=0.0009**) as well as for the total time spent in wake ($F(26,3)=6.419$, **p=0.002**), NREM ($F(26,3)=8.794$, **p=0.0003**) and REM ($H=7.786$, **p=0.05; Figure 6**).

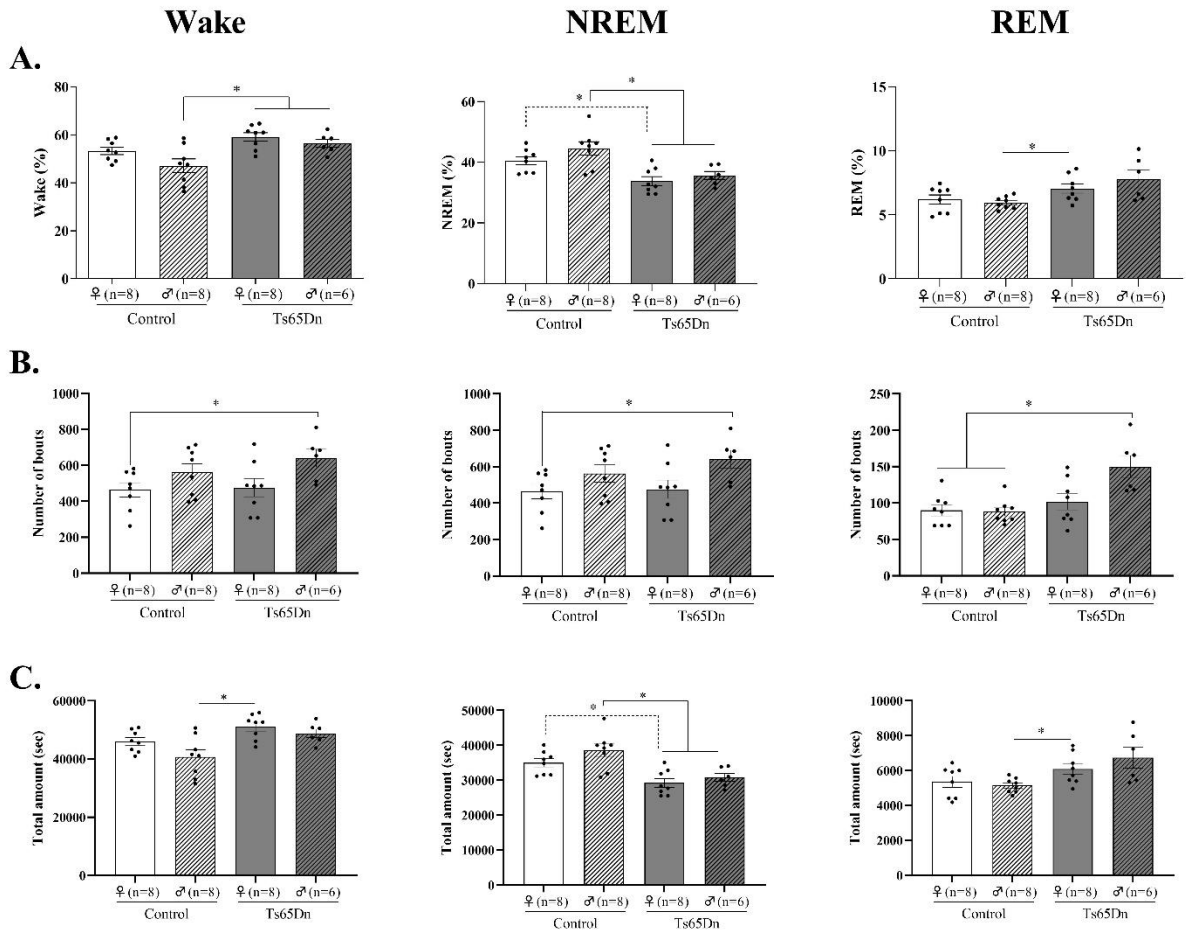


Figure 6: Percentage (**A.**), number of bouts (**B.**) and total amount (**C.**) of Wake, NREM and REM. The Ts65Dn mice show more wake and REM as well as less NREM. No differences were observed between the male and female Ts65Dn mice. *, $p<0.05$ represents a significant difference between groups.

. Genotype differences

Compared to the Ts65Dn male and female mice, the 2N male mice spent less time in wake (male Ts65Dn, t=-2.574, **p=0.002**; female Ts65Dn, t=3.568, **p=0.003**), more time in NREM (male Ts65Dn, t=3.222, **p=0.007**; female Ts65Dn, t=-4.106, **p=0.001**), and show amounts of NREM (male Ts65Dn, t=3.222, **p=0.007**; female Ts65Dn, t=-4.106, **p=0.001**). The male 2N also had a lower percentage of REM (t=2.737, **p=0.01**), total amount of wake (t=-4.016, **p=0.001**) and REM (t=3.568, **p=0.003**) than the female Ts65Dn mice. The female 2N spent more time in NREM and showed a longer total amount of NREM than the female Ts65Dn (t=3.327, **p=0.005**). The female 2N also had a lower number of wake bouts (t=-2.823**, p=0.01**) and NREM bouts (t=-2.828, **p=0.01**) than the male Ts65Dn mice. The number of REM bouts was significantly lower for the 2N male

(t=-4.235, **p=0.002**) and female (t=-3.831, **p=0.002**) compared to the male Ts65Dn mice (**Figure 6 and 7**).

A significant effect of genotype was observed for the average bout lengths of wake (H=9.076, **p=0.02**), NREM (H=9.352, **p=0.02**) and REM (H=6.547, **p=0.004**) as well as for the longest bouts of wake (F(26,3)=3.165, **p=0.04**) and NREM (H=9.817, **p=0.02**) but not for the longest REM bouts (p>0.5). We also observed the effect of genotype x sex for the average interbout length between REM sleep bouts (F(26,3)=7.663, **p=0.0008; Figure 7**).

The male and female 2N showed a significant higher average bout length (male: t=4.395, **p=0.0009**; female: t=4.839, **p=0.0004**) and inter-bout duration (Female: t=2.796, **p=0.01**; male: t=4.708, **p=0.0005**) of REM compared to the Ts65DN male mice. The female 2N had an average bout length (Z=-3.098, **p=0.001**) and longest bout (Z=-3.098, **p=0.001**) of NREM shorter than the male Ts65Dn mice (**Figure 7**).

. Sex differences

The male Ts65Dn mice showed a shorter longest NREM (t=2.746, **p=0.01**) and average bout length of REM (Z=-2.840, **p=0.04**) than the female Ts65Dn mice. No other differences were observed between sex (p>0.5; **Figure 7**).

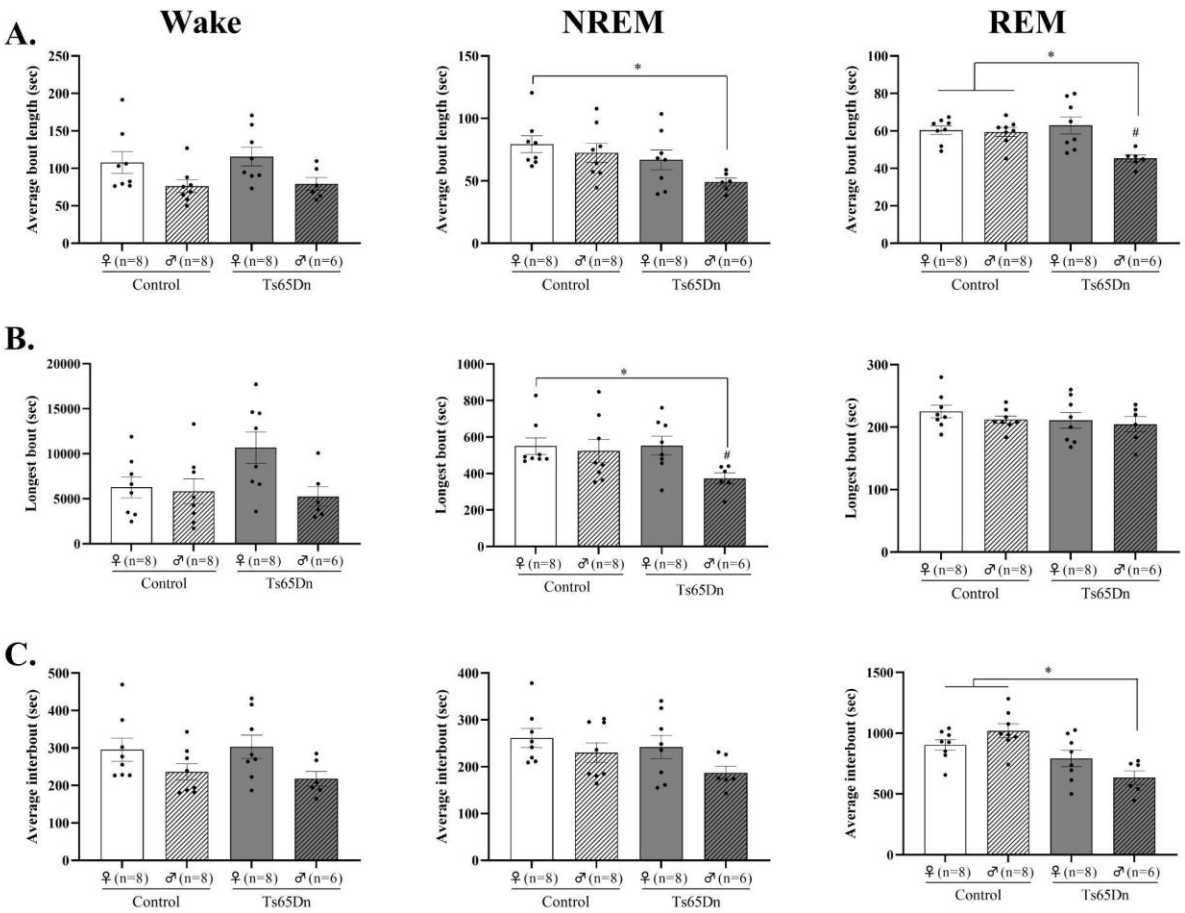


Figure 7: The average bout length (**A.**), the longest bout (**B.**) and the average interbout duration (**C.**) for Wake, NREM and REM. The Ts65Dn mice show more sleep fragmentation than the 2N mice. *, $p<0.05$ represents a significant difference between genotypes. #, $p<0.05$ represents a significant difference between sexes.

Discussion

This study investigated the role of sex differences in Ts65Dn mice and their 2N controls across cognitive performance, behavioral characteristics, and sleep to establish baseline research for using the mouse model to investigate causes of characteristics of DS in humans and being able to decode whether or not the results of such testing can be averaged across sexes. Using NOR, NOL, open field, EPM, Social, and Splash tests as well as sleep recordings, we evaluated the varying effects of sex and genotype on memory and cognition, anxiety, and risk-taking behavior, sociability, anhedonia, and sleep architecture.

In the T-Maze test, 2N mice groups show a percentage of alteration that is larger than the chance level, indicating intact short-term spatial working memory. In contrast, both male and female Ts65Dn mice alternate at chance level, implicating impaired short-term working memory in both sexes. The T-maze relies on hippocampal-prefrontal circuit function, so the deficits observed confirm previous studies that revealed working memory deficits in hippocampal-dependent tasks in Ts65Dn mice (Freeburn and Munn 2021, Munn et al. 2022). Previous studies attributed the observed impairments to deficits in synaptic plasticity and alterations in the inhibitory-excitatory balance (Kleschevnikov et al., 2004). There were no attempts to investigate sex differences in the results from these experiments on Ts65Dn mice.

The discrimination index (DI) in the Novel Object Location (NOL) task experiments reflect long-term spatial memory. No differences in NOL performance across genotypes or sex were observed. These results replicate the findings of González-Parra et al., (2025). The Novel Object Recognition (NOR) test assesses long-term recognition memory. In this task, only the female 2N and Ts65Dn mice showed pronounced differences between training and testing, as well as significant differences from chance level indicating intact recognition memory. In contrast, male mice of both genotypes failed to discriminate the novel objects under the same testing conditions. Object recognition memory relies on neural circuits in the perirhinal cortex and hippocampus. Our results indicate that these circuits are compromised in both the control and Ts65Dn male mice while such memory ability appears to be intact in the female Ts65Dn. Since most research on DS model mice have used only males, more investigations are necessary to identify the molecular differences between male and female Ts65Dn in those brain areas. Our conclusions on the Ts65Dn mice are that there are no sex differences in object location memory, but female Ts65Dn showed better long-term object recognition memory than male Ts65Dn.

In the open field test, female Ts65Dn mice spent significantly less time than male Ts65Dn in the central area, indicating a higher level of anxiety-like behavior. This is consistent with previous research (Martinez-Cue et al., 2006) that observed enhanced behavioral and biochemical anxiety and panic responses in female Ts65Dn mice. Those investigators hypothesized that the causative factor was the effect of higher estradiol levels on the hypothalamic-pituitary-adrenal (HPA) axis, which altered the release of the stress hormone corticosterone. Different levels of corticosterone could be responsible for different stress response levels between sexes and may cause female mice to have higher levels of anxiety-like behavior. These sex-associated effects on stress by estradiol are mirrored in the general human population, where females are more prone to anxiety disorders (Seeman, 1997, McLean & Anderson, 2009, Nouri et al., 2022).

Ts65Dn mice exhibited more risk-like behavior than 2N controls in the elevated plus maze test, as observed in previous studies (Coussons-Read & Crnic, 1996). Interestingly, female Ts65Dn mice showed more risk taking than male Ts65Dn mice, with significantly more head dips and entries into and time in the open arms. Past studies utilizing both the open field and elevated plus maze tests as measures of anxiety report a trend of Ts65Dn spending more time in the anxiogenic center of the open field as well as the risk-associated open arms of the elevated plus maze (Coussons-Read & Crnic, 1996; Escorihuela et al., 1995), and observe that female control mice that display increased anxiety-like behavior show decreased entries in both the center and open arms, so it is surprising that our female Ts65Dn mice show decreased time in the anxiogenic open field center but increased time in the elevated plus maze open arms. Some research posits that Ts65Dn performance on open field and elevated plus maze tests measures hyperactivity and lack of inhibition rather than exclusively anxiety-like behavior (Martinez-Cue et al. 2006), and that the relationship between anxiety and risk-taking behavior in humans may depend on the ambiguity of negative outcome likelihood, which may suggest the effect of factors other than sex and genotype in our results. The discrepancy we observed between open field and elevated plus maze sex differences warrants further study, potentially including additional testing paradigms of anxiety and risk to further define sex and genotype-associated differences. However, our study showed that female Ts65Dn mice display more anxiety and risky behavior.

Like the open field and elevated plus maze tests, sex differences were also found in the social test, with females displaying greater social engagement than males. The females spent more time in the social zone and longer durations of nose-to-nose interactions. This display of greater social engagement in Ts65Dn females is consistent with the literature (González-Parra et al., 2025). No significant differences were observed for total time spent near the social zone or for the number of nose touches, indicating that males and females approached the stimulus mouse with similar frequency, but females-maintained interactions for longer periods. Sex differences were the most consistent effect across groups. Both female control and female Ts65Dn mice spent more time in the social zone and engaged in longer nose-touch interactions than males indicating stronger sustained social engagement in females independent of genotype. Overall, females demonstrated greater sustained engagement with the stimulus mouse.

In the Splash test, male 2N control mice spent significantly less time grooming than 2N and Ts65Dn female mice, showing an effect of sex within the 2N population on anhedonia and depression-like behavior. It is interesting to note that unlike the mice in this study, in humans, the general population of women has higher rates of depression compared to males (Altemus et al., 2014), and that individuals with Down syndrome have a higher prevalence of depression (Rivelli et al., 2022), however these reports do not directly measure anhedonia specifically. Between male and female Ts65Dn mice, there was no effect of sex on displays of anhedonia. It is important to note that affective disturbances such as anhedonia do not occur in isolation.

Sleep plays a crucial role in mood but also in cognition. Therefore, we compared the sleep of male and female Ts65Dn. Our results showed that both female and male Ts65Dn mice spent more time spent in wake than the 2N controls. This finding is consistent with the previous literature (Colas et al., 2008); however, it is important to note no sex differences for percent time spent in wake were observed in our study. In agreement with our previous studies and others, Ts65Dn exhibited decreased percent time spent in NREM (Chuluun et al., 2020). Furthermore, the Ts65Dn male and female mice displayed a statistically significant decrease in total amount of NREM compared to control. We hypothesize that the deficits in cognition experienced by the Ts65Dn mice may be linked to decreased time in NREM, as NREM plays a key role in learning and memory (Wilckens, 2018).

REM is crucial to memory consolidation (Boyce et al., 2016), but sex specific characteristics of REM sleep were not explored prior to this study. Ts65Dn males showed an

increased number of REM bouts when compared to 2N males and females. The increased number of REM bouts indicated increased fragmentation of sleep for the Ts65Dn males, which could explain the deficits in long-term spatial memory in NOL for the Ts65Dn males. In conclusion, no sex differences were observed between the male and female Ts65Dn regarding the percentage of wake, NREM and REM but the male Ts65Dn showed a more fragmentated sleep. The increase of sleep fragmentation for Ts65Dn males has been previously observed (Colas et al., 2008); however, this represents the first report of Ts65Dn females not exhibiting as much sleep fragmentation as the male.

A few limitations should be noted: estrous cycle stage was not tracked in females, so cyclical estradiol fluctuations could not be directly tested, and no hormonal assays were performed to confirm this hypothesis. These findings also derive from a single DS mouse model, which should be considered when analyzing these results.

All together, we have found sex-specific differences in long-term recognition memory, anxiety, depression-like, risk-taking, social behavior and sleep fragmentation. No differences in long-term recognition memory, short-term memory and global sleep percentages were observed (**Figure 8**). This is the first study focusing only on sex differences in the Ts65Dn mouse model of Down syndrome. These results will be of interest for any researchers working on this model and using both sexes.

	Male	Female
Short term memory	=	=
Long term location memory	=	=
Long term recognition memory		+
Anxiety		+
Risk taken		+
Social behavior		+
Anhedonia		-
Sleep -Wake	=	=
Sleep - NREM	=	=
Sleep - REM	=	=
Sleep fragmentation	+	

Figure 8: Comparison table between male and female Ts65Dn mice. = means no difference between sex. + means that males or females have better memory or show more anxiety, risk taken or REM. - means that the females are less anhedonic.

List of abbreviations

DS Down Syndrome
REM Rapid Eye Movement
NREM Non-Rapid Eye Movement
NOL Novel Object Location
NOR Novel Object Recognition
EEG Electroencephalogram
EMG Electromyogram/Electromyographic
EPM Elevated Plus Maze
OF Open Field
OSA Obstructive Sleep Apnea
GABA Gamma-Aminobutyric Acid
GABAA GABA type A
GABAAR GABA-A Receptor
SCN Suprachiasmatic Nucleus
HPA Hypothalamic-Pituitary-Adrenal
DI Discrimination Index
IQ Intelligence Quotient
ANOVA Analysis of Variance
TRT Total Recording Time
DYRK1A Dual-specificity tyrosine-phosphorylation-regulated kinase 1A
CA1 Cornu Ammonis 1 (hippocampal subregion)
F1 First-generation hybrid
2N Diploid control mice

Declarations

- Ethics approval and consent to participate

All experiments were performed in accordance with the guidelines described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the Stanford University Institutional Animal Care and Use Committee and are in accordance with the ARRIVE Guideline (https://arriveguidelines.org/arrive-guidelines). The protocol number is 14926.

- Consent for publication

Not applicable.

- Availability of data and material

All data supporting the findings of this study are available within the paper and its Supplementary Information.

- Competing interests
The authors declare that they have no competing interests.

- Funding
This work was founded by the Matthew foundation (FZAHB) And the Fondation Jérôme Lejeune (UDCXE).

- Authors' contributions
T.K. performed experiments, analyzed the data, wrote and reviewed the paper. S.L., S.P. and L.C. performed experiments, wrote and reviewed the paper. C.H. conceptualized the study, acquired funding, and reviewed the paper. E.P. conceptualized the study, supervised the project, performed experiments, analyzed the data, and wrote and reviewed the paper.

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Not applicable.

- Authors' information (optional)
Not applicable.

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