SUPPLEMENTARY INFORMATION

The Impact of *Mmu17* Non-Hsa21 Orthologous Genes in the Ts65Dn Mouse Model of Down Syndrome: The "Gold Standard" Refuted

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Supplementary Materials

Neonatal developmental milestones

Pups were separated from their mother for 25 min before testing and placed with nesting material in a bowl positioned on a heating pad at 37°C. Male and female Ts66Yah pups (19 males and 11 females for Cohort 1; 40 males and 21 females for Cohort 2) and Ts65Dn pups (16 males and 17 females) and their euploid littermates (19 males and 26 females for Ts66Yah Cohort 1; 37 males and 50 females for Ts66Yah Cohort 2; 22 males and 35 females for Ts65Dn) were tested as previously described (1-3). In addition to the Fox scale, ultrasonic vocalization (USVs), motor activity in an open field were used as translational measures of early language, motor and cognitive development in DS.

Ultrasonic Vocalization and Motor Development

Baseline communication and motor development were analyzed and compared between Ts66Yah, Ts65Dn and their euploid littermates between postnatal days (PND) 2 and 12. Individual pups were placed in SMART chambers and their vocalization recorded during a 5 min session (1 session/day) using the Sonotrack system (Metris BV, Hoofddorp, The Netherlands). The total number of USVs, the percent of the different USV classes, the average power and the average frequency were analyzed using the Automated Class Classification software (Metris BV, Hoofddorp, The Netherlands) that uses the different USV classes defined in Vogel et al (4). The SMART chambers were equipped with IP cameras that allowed the analysis of motor development in an open field (20 cm x 20 cm x 10 cm) using ANYmaze tracking software (Stoelting Co., Wood Dale, IL).

Homing Test

In this test, spatial olfactory memory in neonates (P12) was assessed as described previously (5). Pups were separated from the dam as described above and placed in the testing arena in the presence of home cage bedding (goal zone) on one side of the arena and clean bedding on the opposite side. Each pup performed one trial of 180s. The latency to first goal zone entry as well as the time spent in the goal zone versus clean zone were recorded and analyzed in trisomic and euploid littermates.

Adult Behavior

The open field (OF), rotarod, Y-maze, contextual fear conditioning (CFC), novel object recognition (NOR) and Morris water maze (MWM) tests were used to investigate adult behavior in Ts66Yah mice. The number of animals used in these tests is indicated in Supplementary Table 3. Behavioral findings in Ts66Yah mice were compared to previously published data in the Ts65Dn mice (3,6). The number of animals used for each mouse model and sex is as follows: Cohort 1 mice (17 males and 11 females for Ts66Yah; 17 males and 24 females for Eup); Cohort 2 mice (36 males and 13 females for Ts66Yah; 34 males and 13 females for Eup).

Exploratory behavior and spontaneous locomotor activity (Open Field)

Adult mice were individually placed in an open field arena consisting of a white opaque plastic box 40 cm x 40 cm x 40 cm divided into a center zone measuring 20 cm x 20 cm x 20 cm and periphery. Exploratory behavior was tracked during a 60 min unique trial using ANYmaze tracking

software (Stoelting Co., Wood Dale, IL). The total distance traveled (cm) in the center versus periphery as well as the average velocity (cm/s) were analyzed for each genotype. Data were collected as time bins of 20 min and as total time over the course of the experiment.

Motor coordination (Rotarod Test)

Motor coordination was investigated using the rotarod test (Med Associates, Fairfax, VT) using two different protocols (fixed speed protocol on day 1 and accelerating speed protocol on day 2). Prior to testing with the fixed speed protocol on day 1, each mouse was given two 120 s practice sessions at 16 RPM. Mice were then tested at five different fixed speeds (4, 8, 16, 24 then 32 RPM) for three trials (300 s/trial) at each speed with an inter-trial interval of 15 min. On day 2, mice were tested in three trials under conditions of increasing difficulty in which the speed of the rotation gradually increased from 4 to 40 RPM over 300 s. The latency to fall was recorded and analyzed between genotypes and sexes.

Working Memory (Y-Maze Alternation)

Mice were placed individually in the center of the Y-maze and allowed to explore freely for a 10 min session. A video camera, mounted centrally above the maze, recorded each session. A normal mouse usually explores the three branches of the Y-maze and in an ordered way (branch A then branch B and last branch C for example). The percent alternation, number of arm entries, distance travelled, and average speed were analyzed using ANYmaze tracking software (Stoelting Co., Wood Dale, IL). Spontaneous alternation was defined as successive entries into the three arms of the Y-maze, in overlapping triplet sets, with arm choices differing from the previous two choices expressed as a percentage of the total number of arm entries: Percent alternation = [number of alternations/(total number of arm entries − 2)] × 100 (chance level = 50%).

Hippocampal-dependent contextual memory (Contextual Fear Conditioning)

Hippocampal-dependent memory was analyzed using the fear conditioning test in a conditioning chamber as described previously (8). On day 1 (training session), each mouse was individually placed for 360 s into the conditioning chamber and allowed to explore freely (habituate) for 180 s. Following exploration/habituation, four mild foot shocks (0.5 mA for 2 s) were administered at 180 s, 240 s, 300 s and 360 s. On day 2 (testing session), the mice were placed into an identical conditioning chamber for 360s with no foot shocks. Each mouse was monitored for freezing (fear) behavior. The extent of (or percent of time spent) freezing, was analyzed in bins of 60 s and as a total over the course of the experiment using the Freeze View software (Med Associates, Fairfax, VT). These measurements were used as a proxy of the animal's memory of a noxious stimulus.

Long-Term Memory (Novel Object Recognition)

On day1, mice were habituated in an empty arena (40 cm x 40 cm x 40 cm) for 30 min prior to the acquisition session. During the acquisition session (2 trials of 10 min with an inter-trial interval of 60 min) mice were exposed to two identical objects, and exploration of these objects was tracked using ANYmaze tracking software (Stoelting Co., Wood Dale, IL). Twenty-four hours later, long-term memory was tested in a 10 min session by replacing one of the familiar objects with a novel object. The performance of each animal was measured using the Recognition Index [RI=TN/(TN+TF)] where TN is the time spent exploring the novel object and TF corresponds to the time spent exploring the familiar object. Memory of the object was considered to be present for a group if animals spend more time exploring the new object than the familiar one (i.e., RI was

higher than 50%). Several same size and different contrast objects were alternated as familiar and novel objects between animals and testing chambers (cube, sphere, butterfly and cupcake).

Hippocampal-dependent spatial memory (Morris Water Maze)

Hippocampal-dependent spatial memory was analyzed using the Morris water maze (MWM) test in a 125 cm diameter circular water maze as described previously (3). Mice were trained using the following sequence of trials: Cued (4 trials/day for 4 days), hidden platform (4 trials/day for 5 days) and probe trial (1 trial). Each trial lasted for a maximum period of 60 s after which the mouse was guided to the platform and allowed to recover for 15 s before being gently removed by the experimenter. Twenty-four hours after the hidden platform training sessions, each mouse was subjected to a probe trial to test reference memory. During this test, the platform was removed, and mice were allowed to swim once freely for 60 s. Video tracking was performed using ANYmaze tracking software (Stoelting Co., Wood Dale, IL). Latency to reach the platform, swimming speed, total distance, time spent in the center versus periphery, as well as the time spent in each quadrant were recorded and analyzed.

Supplemental References

1. Fox WM (1965): Reflex-ontogeny and behavioral development of the mouse. Anim Behav. 13:234-241.
2. Hill JM, Lim MA, Stone MM (2008). Developmental milestones in the newborn mouse. *Neuromethods.* 39:131–149.
3. Olmos-Serrano JL, Tyler WA, Cabral HJ, Haydar TF (2016): Longitudinal measures of cognition in the Ts65Dn mouse: Refining windows and defining modalities for therapeutic intervention in Down syndrome. Exp Neurol. 279:40-56.
4. Vogel AP, Tsanas A, Scattoni ML (2019): Quantifying ultrasonic mouse vocalizations using acoustic analysis in a supervised statistical machine learning framework. Sci Rep. 9:8100.
5. Guedj F, Pennings JL, Ferres MA, Graham LC, Wick HC, Miczek KA, et al. (2015): The fetal brain transcriptome and neonatal behavioral phenotype in the Ts1Cje mouse model of Down syndrome. Am J Med Genet A. 167A:1993-2008.
6. Aziz NM, Guedj F, Pennings JLA, Olmos-Serrano JL, Siegel A, Haydar TF, et al. (2018): Lifespan analysis of brain development, gene expression and behavioral phenotypes in the Ts1Cje, Ts65Dn and Dp(16)1/Yey mouse models of Down syndrome. Dis Model Mech. 11(6): dmm031013. Doi :10.1242/dmm.031013.
7. Guedj F, Siegel AE, Pennings JLA, Alsebaa F, Massingham LJ, Tantravahi U, et al. (2020): Apigenin as a Candidate Prenatal Treatment for Trisomy 21: Effects in Human Amniocytes and the Ts1Cje Mouse Model. Am J Hum Genet. 107:911-931.

Figure S1

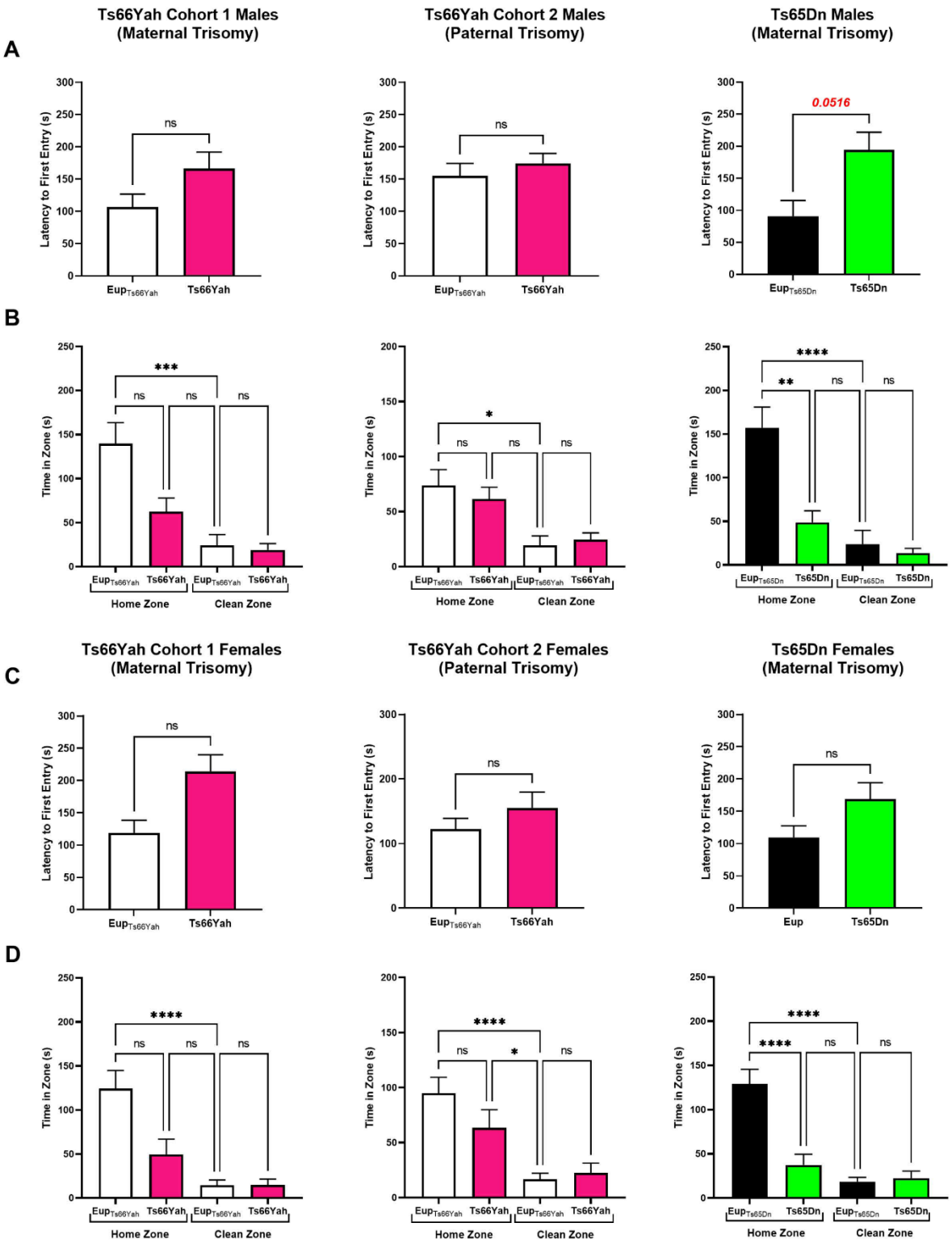
A
Ts66Yah Cohort 1 Males (Maternal Trisomy)
Ts66Yah Cohort 2 Males (Paternal Trisomy)
Ts65Dn Males (Maternal Trisomy)
Latency to First Entry (s)
ns
0.0516
Eup Ts66Yah
Ts66Yah
Eup Ts65Dn
Ts65Dn
B
Time in Zone (s)
***
****
**
*
Home Zone
Clean Zone
C
Ts66Yah Cohort 1 Females (Maternal Trisomy)
Ts66Yah Cohort 2 Females (Paternal Trisomy)
Ts65Dn Females (Maternal Trisomy)
Eup
D

Figure S1: Olfactory spatial memory measured with the homing test in Ts66Yah and Ts65Dn neonates. Latency to first zone entry and time spent in the "Home Zone" Versus "Clean Zone" in Cohort 1 Ts66Yah, Cohort 2 Ts66Yah and Ts65Dn male pups (A-B) and female pups (C-D) at postnatal days 12. Significant differences are indicated as # ($0.09>p>0.05$), * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p<0.0001$).

Figure S2

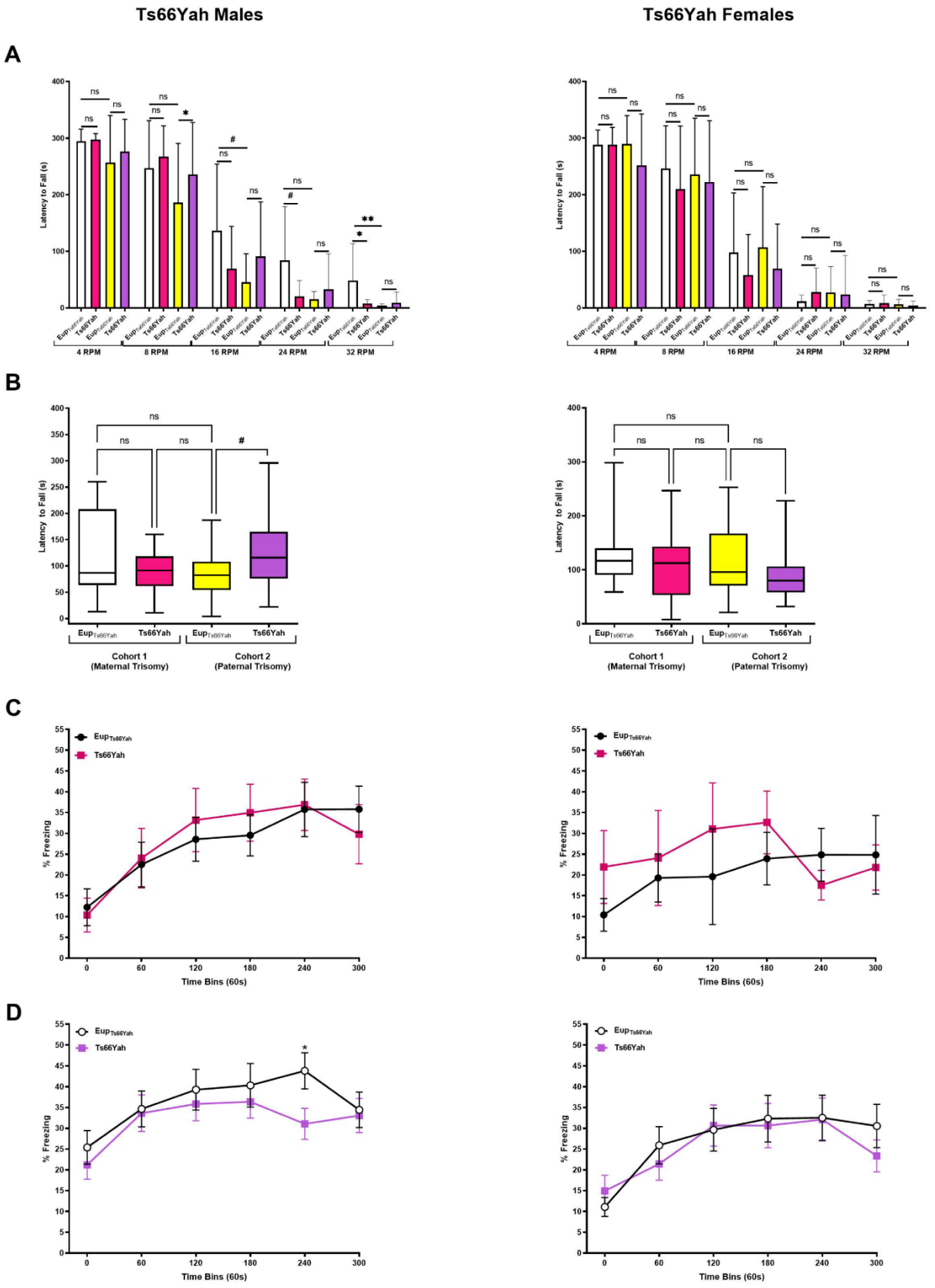
Ts66Yah Males
Ts66Yah Females
A
B
C
D
Latency to Fall (s)
4 RPM
8 RPM
16 RPM
24 RPM
32 RPM
Cohort 1
(Maternal Trisomy)
Cohort 2
(Paternal Trisomy)
% Freezing
Time Bins (60s)

Figure S2: Motor coordination and contextual hippocampal memory in Ts66Yah adult mice. (A-B) Motor coordination was investigated using the rotarod test. The latency to fall from the rotarod was measured in adult Cohort 1 and Cohort 2 Ts66Yah male and female mice in the static speed (A) and accelerating speed (B) versions of the rotarod. (C-D) The contextual fear conditioning test was used to investigate hippocampal contextual memory in the Ts66Yah mouse model. Percent freezing was compared in Cohort 1 (**C**) and Cohort 2 (**D**) male and female Ts66Yah and Eup littermates. Significant differences are indicated as # ($0.09>p>0.05$), * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$), **** ($p<0.0001$).

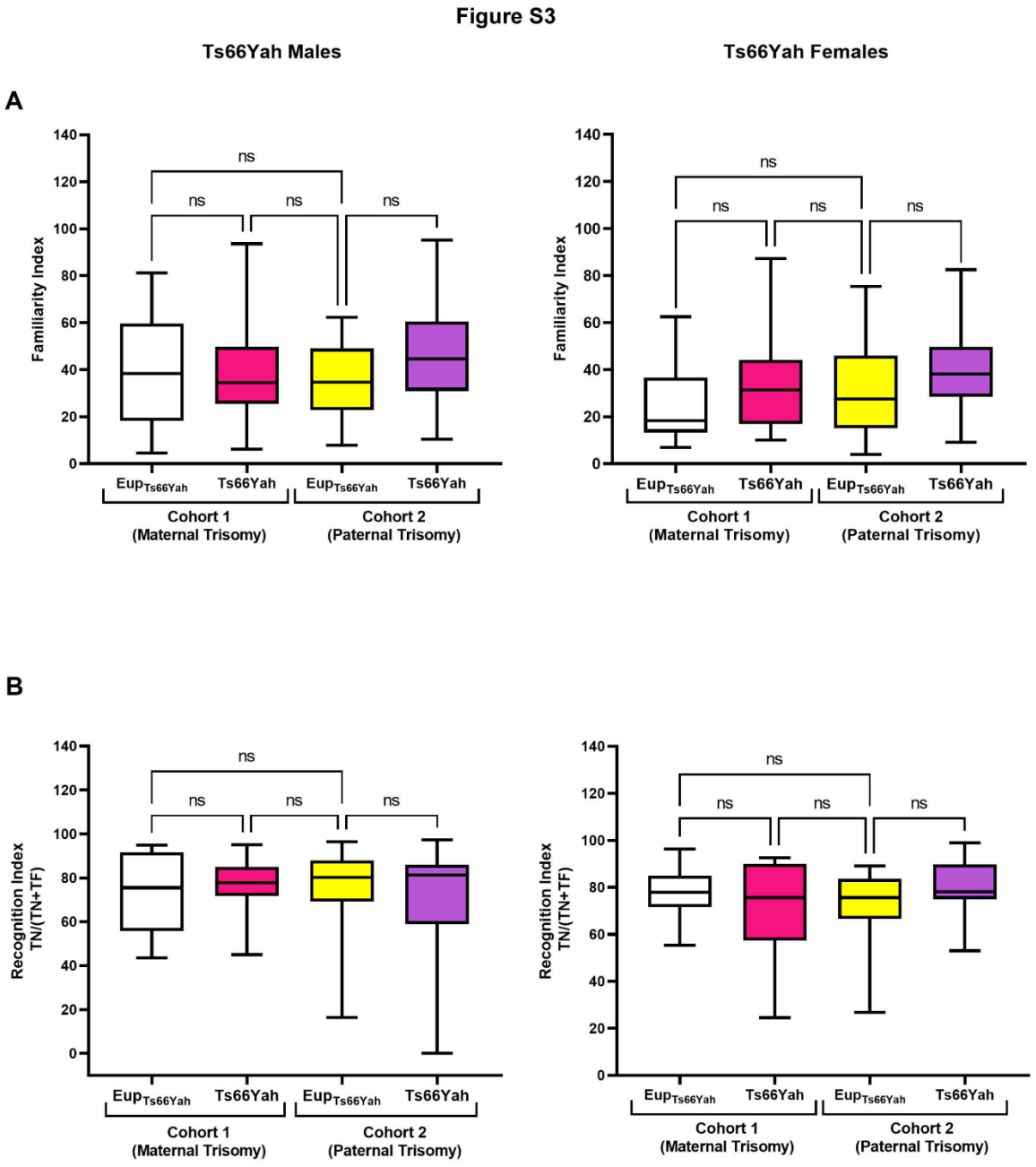

Figure S3: Novel object recognition in Ts66Yah adult mice. In (A) results of the familiarity index are shown in Ts66Yah adult males and females. No significant differences were seen between Ts66Yah and Eup littermates in either cohort. In (B) results of the familiarity index are shown in Ts66Yah adult males and females. No significant differences were seen between Ts66Yah and Eup littermates in either cohort.