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Longitudinal measures of cognition in the Ts65Dn mouse: refining windows and defining modalities for therapeutic intervention in Down syndrome

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Abstract

Mouse models have provided insights into adult changes in learning and memory in Down syndrome, but an in-depth assessment of how these abnormalities develop over time has never been conducted. To address this shortcoming, we conducted a longitudinal behavioral study from birth until late adulthood in the Ts65Dn mouse model to measure the emergence and continuity of learning and memory deficits in individuals with a broad array of tests. Our results demonstrate for the first time that the pace at which neonatal and perinatal milestones are acquired is correlated with later cognitive performance as an adult. In addition, we find that lifelong behavioral indexing stratifies mice within each genotype. Our expanded assessment reveals that diminished cognitive flexibility, as measured by reversal learning, is the most robust learning and memory impairment in both young and old Ts65Dn mice. Moreover, we find that reversal learning degrades with age and is therefore a useful biomarker for studying age-related decline in cognitive ability. Altogether, our results indicate that preclinical studies aiming to restore cognitive function in Ts65Dn should target both neonatal milestones and reversal learning in adulthood. Here we provide the quantitative framework for this type of approach.

Keywords

Down Syndrome; Ts65Dn; mouse model; cognition; aging; reversal learning; mouse behavior; developmental disorder; developmental milestones

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INTRODUCTION

Down syndrome (DS), the leading genetic cause of intellectual disability afflicting 1 in every 691 live births, is characterized by a constellation of phenotypes affecting many organ systems (Parker et al., 2010). Abnormalities in the nervous system include microcephaly, motor impairment and cognitive deficits (Cocchi et al., 2010; Parker et al., 2010).

Intellectual disability is characterized by deficits in problem solving, reasoning, and learning and memory, as well as deficiencies in conceptual, social and practical skills. It is a universal and life-long condition for individuals with DS and its severity varies widely, ranging from mild to moderate impairment on intelligence quotient tests (Patterson et al., 2013). Reversal learning, an adaptive behavior in which a learned response must be extinguished and replaced by an alternate response, is commonly impaired in DS (Campbell et al., 2013).

Proper modeling of these neuropsychological disabilities will enable an understanding of their etiology and their individual contribution to intellectual disability in DS. This will facilitate the development of tailored behavioral and medical therapies.

Due to their genetic similarities to humans, mouse models are used to replicate the behavioral landscape of DS. The trisomic Ts65Dn mouse is currently the most commonly employed model of DS, mainly because it displays several phenotypic abnormalities which parallel those found in DS, including delays in brain development, hyperactivity, and motor dysfunction (Davisson et al., 1993; Costa et al., 1999; Chakrabarti et al., 2007; Sanders et al., 2009). In addition, several studies have reported cognitive impairment in Ts65Dn mice using a variety of behavioral paradigms, primarily examining spatial learning and memory (L/M) (Das and Reeves, 2011; Ruparel et al., 2013). While these studies provide a framework of the L/M impairments in Ts65Dn, key questions about the onset and magnitude of these deficits as well as in-depth assessment over an extended period of time remain unanswered. This is particularly important when defining the optimal therapeutic window to treat intellectual disability in humans with DS since cognitive deficits appear early in life and can be varied within the population. Importantly, increasing lines of evidence suggest that failure to reach key developmental milestones, even during the first year of life, may at least partially predict cognitive challenges that manifest in adult life (Schillace, 1964; von Wendt et al., 1984; Taanila et al., 2005; Murray et al., 2006). Thus, identifying the severity and particular cognitive impairments on an individual basis at an early stage of life could facilitate targeted treatments. In addition, the high incidence of Alzheimer's disease (AD) pathology and dementia after 40 years of age is one of the most debilitating consequences of aging for people with DS (Zigman, 2013) and there is great interests in discovering whether biological changes in the DS brain precede the later dementia. A protracted study may therefore elucidate the biological basis for the association between trisomy 21 and AD, and could thereby improve outcomes for all individuals who develop AD pathology and dementia.

In this study, we performed a systematic behavioral analysis of Ts65Dn and control mice from birth to adulthood. Each animal was evaluated in a battery of developmental milestone tests, followed by several cognitive and innate behavioral tasks conducted during adolescence and then repeated after advanced aging. This allowed us to elucidate key correlations between development and adult cognitive function. Our results show that young

Ts65Dn mice acquire spatial learning at the same pace as euploid mice, but exhibit a pronounced reversal learning impairment at both young and old ages. Unsupervised principal component (PCA) and hierarchical clustering analyses (HCA) demonstrate that Ts65Dn mice can be clearly differentiated from euploid mice and identify variable phenotypes between individuals within each genotype. Our longitudinal study also shows that the developmental milestone performance of individual mice parallels their cognitive scores in adulthood. For the first time, these tests also uncover an age-dependent decline in learning in both Ts65Dn and euploid mice. Taken together, our results provide a detailed analysis of the timing and specificity of cognitive deficits expressed in Ts65Dn which serve as a novel and sensitive platform to assess treatment strategies aimed at improving cognitive function in DS.

MATERIAL & METHODS

Animals

Ts65Dn and euploid fetuses from 20 litters were generated by mating Ts65Dn females (Stock No. 005252 without the mutant *Pde6b* allele impacting retinal degeneration) with C57BL/6Jei × C3SnHeSnJ (B6EiC3) F1 hybrid males (Stock No. 003647), both obtained from The Jackson Laboratory, Bar Harbor, ME). Seventeen euploids and fifteen Ts65Dn males were used for developmental milestone tests. From these animals, fourteen euploids and fifteen Ts65Dn mice were chosen for their proximity in birthdates for adult behavior assessment. Weaning was performed at postnatal day (PND) 21 and sibling males were housed regardless of genotype. Animals were kept in the same cage until two weeks prior to the onset of adult behavioral assessments. Between the end of the behavioral assessment at 2 months of age and the completion of the behavioral assessment at 11 months of age, 5 Ts65Dn died. PCR genotyping was performed on genomic DNA extracted from tail tips. Genotyping was performed on the day of birth. For genotyping, we used primers previously described (Reinholdt et al., 2011). Mutant primers: Chr17fwd-5'-GTGGCAAGAGACTCAAATTCAAC-3' and Chr16rev-5'-TGGCTTATTATTATCAGGGCATT-3'. These primers amplify a 275 bp product. Positive control primers: IMR8545_5'-AAAGTCGCTCTGAGTTGTTAT-3' and IMR8546_5'-GGAGCGGGAGAAATGGATATG-3'. These primers amplify a 600 bp product from the *Rosa* locus. The PCR cycling conditions were: Step 1: 95 °C for 2 min; Step 2: 95 °C for 20 sec; Step 3: 55 °C for 30 sec; Step 4: 72 °C for 45 sec (Steps 2–4, 40 cycles); Step 5: 72 °C 5 min., followed by a final 5 min extension at 72 °C and hold at 4 °C. PCR products were separated on a 1% agarose gel.

Behavioral testing

All experiments involving animals were performed in accordance with institutional and federal guidelines. Standard rodent chow and water were available ad libitum. In addition to standard bedding, a Nestlet square was provided in each cage. The colony room was maintained on a 12:12 light/dark cycle, with lights on at 7:00 AM. All experiments were conducted in the light phase, between 8:00 AM and 1:00 PM. To minimize olfactory cues from previous trials, each apparatus was thoroughly cleaned with 10% ethanol after each animal. Each day of testing, mice were left in their home cages in the room used for the

experiment at least 1 h prior to the onset of the study for habituation. All behavioral tests were performed blindly without prior knowledge of genotype. Water T-maze and Morris water-maze tasks were the last experiments in the series.

Developmental Milestones

Ts65Dn pups and euploid littermate males were tested according to previous studies (Fox, 1965; J.M. Hill, 2008). A comprehensive set of neonatal behavioral tests to measure different sensory and motor development parameters were used including 1) body righting and coordination (surface righting, air righting and negative geotaxis), 2) motor strength (cliff aversion and forelimb grasp), 3) sensory system maturation (rooting, auditory startle, ear twitch and eye opening), and 4) extinction of rotatory behavior (open field). Briefly, the dam was temporarily transferred to a clean cage and then pups were placed with nesting material in a small bowl positioned on a heating pad at 37 °C. One pup was assessed at a time and was placed back into the home cage nest once finished its assessment. The time each pup was out of their home nest ranged from 3 min to 12 min. To mark the pups individually, we tattooed forelimbs and/or hindlimbs on PND0 using a sterile needle filled with non-toxic green ink. Pups were evaluated each day from PND0 to 21. Only weight data was collected and no other tests were performed on PND0. The amount of time (latency) or presence/absence of reflex was recorded and analyzed by a single experimenter. To calculate a developmental milestones (DM) score, we normalized the data to a 1 to 10 scale using the following formula; $y = 1 + (x - A) * (10 - 1) / B - A$ where x = data points, A = lowest score, B = highest score.

Nest-building

Mice were individually housed for at least 48 h in clean plastic cages with approximately 1 cm of bedding lining the floor and identification cards coded to render the experimenter blind to genotype of each subject. Around 6pm, one hour prior to the dark phase of the lighting cycle, animals were placed in new individual cage supplied with 3 g of Nestlet material (pressed cotton squares). The next morning (15 h later) cages were assessed for nest construction. Nest construction was scored using a slightly modified version of Deacon's scoring system (Deacon, 2006). Briefly, in this 6 point scale, 0= Nestlet intact, 1= >90% intact Nestlet, 2= Nestlet partially torn up, 3= Nestlet mostly shredded but no identifiable nest site, 4= an identifiable but flat nest, 5= Nestlet torn >90% and a clear nest crater.

Spontaneous alternation

The natural tendency to explore was assessed using the continuous variant of the spontaneous alternation procedure, as described previously (O'Tuathaigh et al., 2007; Desbonnet et al., 2012). Briefly, without prior habituation, animals were placed individually in the center of the maze and allowed to explore freely for 6 min. A video camera, mounted centrally above the maze, recorded each session. Alternation between arms, the number of arm entries, distance moved and velocity of movement were analyzed using Ethovision videotracking (Ethovision®, Noldus). The maze was made of opaque acrylic glass (Plastic-Craft, New York, USA). An arm entry was scored when four limbs were inside the arm. 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%).

Water T-maze

The Water T-maze (WTM) used was constructed from transparent Plexiglas (Plastic Craft Product Corp). Each arm is 20 cm long and 7 cm wide. The T-maze was filled with tap water and made opaque with white non-toxic paint (Crayola). The temperature of the water was maintained at 24 ± 0.5 °C. The platform is a 5 cm x 5 cm square made from Plexiglas and was submerged during the experiments. A recent study has been published using this behavioral paradigm (Frost et al., 2015). Mice were given 10 trials per day with an inter-trial interval (ITI) of approximately 5–8 min. Mice were released from two different locations (N or S) and were trained to find the location of the submerged platform in one of the arms (E). When mice were released from S or N, the other arm was blocked with a Plexiglas rectangle. Mice scored an error (0) when they swam two thirds of the wrong arm and scored a correct choice (1) when they found the platform. Once mice reached between 80–100% of correct choices, the platform was moved to the opposite arm for reversal learning (W). When mice reached 80–100% of correct choices, the platform was moved again to its first location (E) for assessing double reversal learning. Any errors before successfully finding the platform were scored as perseverative errors. Errors made after an errorless trial were scored as regressive errors. The time spent to find the platform in the first two trials for reversal and double reversal learning was also measured.

Morris water maze

Spatial and reversal learning were assessed in the Morris water maze (MWM) using procedures and equipment as previously described (Yang et al., 2012). The apparatus was a circular pool, 125 cm in diameter, filled with tap water rendered opaque with the addition of non-toxic white paint (Crayola). Water temperature was maintained at 24 ± 0.5 °C to reduce stress and potential hypothermia (Stasko and Costa, 2004). To facilitate spatial learning, room cues made of black and white cardboard were added to the walls surrounding the pool. Trials were videotaped and scored with Ethovision video tracking software (Noldus, Actimetrics, Inc., Wilmette, IL). The order of training was: cued trials, hidden acquisition trials, probe trial, hidden reversal trials and a final probe trial. Cued, acquisition and reversal training consisted of 4 trials a day. Each trial began by lowering the mouse into the water close to the pool edge in one of the 3 non-target quadrants. The start location for each trial was alternated in a semi-random order for each mouse. For cued training, the pool was surrounded by white curtains to occlude the extra-maze cues. The submerged platform was cued by means of a metal stick attached to a 5 cm high black ball and placed pseudo-randomly in different locations across trials. For the acquisition and reversal training, the hidden platform remained in the same quadrant for all trials for a given mouse. Mice were allowed a maximum of 60 sec to reach the platform. A mouse that failed to reach the platform in 60 sec was guided to the platform by the experimenter. Mice were left on the platform for 15 sec before being removed by the experimenter's hand. After each trial, subjects were put in a cage lined with absorbent paper towels. To confirm that the spatial learning task was acquired after acquisition and reversal training by using distal environmental room cues, subjects were tested in a 60 sec probe trial, 24 h after the

completion of the last training session. Reversal training began the day following the probe test in which the hidden platform was moved to the opposite quadrant. Parameters recorded during training days were latency to reach the platform, total distance swum, swimming speed and time spent in the periphery. Parameters recorded during the probe trial were (a) time spent in each quadrant, (b) number of virtual crossings over the trained platform location and (c) proximity as described previously (Maei et al., 2009).

Hierarchical clustering analysis

Two-way hierarchical clustering analysis was performed by Ward's method with Euclidian distance using the JMP software package (SAS, Cary, NC). All behavioral data was normalized to Z-scores prior to analysis by the clustering algorithm. Similarly, principal component analysis (PCA) was performed using JMP.

Statistical analysis

Statistical analyses were performed using Sigmaplot (Systat Software, Inc.). For developmental milestones and spontaneous alternation, mean was calculated and unpaired *t*-tests were performed. Two-side Fischer-exact test (FET) was used to determine statistical significance daily in developmental milestones. For adult behavioral tests, we employed two-way repeated measure ANOVA and the post hoc Tukey test for MWM and WTM and age comparisons. Time was considered as within subjects factor and Group as between subjects factor. For nesting behavior analyses, non-parametric Mann-Whitney *U* was used. Significance was set at a *p*-value of 0.05. For correlation analyses, we used the Pearson correlation coefficient. One Ts65Dn was identified as outlier in Nest-building and Spontaneous alternation tests at 11 months of age (yielding *N* = 13). One Ts65Dn was identified as outlier in reversal and double reversal for WTM at 2 months of age (yielding *N* = 14). One Ts65Dn was identified as outlier in hidden and hidden reversal for MWM at 2 months of age (yielding *N* = 14) but not for the probe trial (*N* = 15). Additionally, two Ts65Dn mice were identified as outliers and discarded for some correlation analyses. We identified outliers as individuals scoring greater than 2 times the standard deviation (SDTV) away the mean. We calculated the percentage of confidence interval for each correlation and we also estimated the slopes in a linear regression.

RESULTS

Developmental Milestones Acquisition is delayed in Ts65Dn

We performed a daily assessment of developmental milestones in euploid and Ts65Dn mice from birth until postnatal day (PND) 21, including motor strength and coordination tests as well as the appearance of neurological reflexes. Our results show that Ts65Dn mice exhibit a significant delay in the acquisition of a number of developmental milestones (Table 1). The greatest delays were observed in negative geotaxis, cliff aversion, air righting and surface righting. These delays happened at specific PNDs for each developmental milestone. For example, we found significant differences in negative geotaxis between PNDs 4–8, in cliff aversion between PNDs 4–9, in surface righting at PND 5, 9 and 10, in rooting reflex between PNDs 7–9, in ear twitch and open field at PND 10, in air righting between PNDs 11–15, in eye opening at PND 12, and in auditory startle at PND 15 (FET, *p* < 0.05; Fig.

1A–J; data not shown). These results show that Ts65Dn exhibit a profound delay in neurologic development and motor strength and coordination between birth and PND 21, starting within the first week of life. Therefore, this mouse model recapitulates features frequently observed in infants with DS and our data are consistent with previous reports on Ts65Dn indicating delays in achieving developmental milestones (Holtzman et al., 1996; Toso et al., 2008).

Innate behaviors are impaired in Ts65Dn

Nest building is a useful and easy procedure to assess innate behavior in mice (Deacon, 2006). Analyses of overnight nest-building were performed by assessing two separate measures, a detailed nesting scale and the weight of untorn nestlet material. Our results reveal that nesting behavior is dramatically impaired in 2 month-old Ts65Dn mice compared to euploids (nest score, Mann-Whitney $U = 27.500$, eup = 14, Ts65Dn = 15, $p < 0.001$ and untorn material, $t(27) = -6.078$, two-tailed $p < 0.00001$; Fig. 2A, B, D, E). The difference between genotypes did not appear to be related to slower nest building, as it persisted for up to 1 week (data not shown). When analyses of overnight nest-building were performed at 11 months of age, older Ts65Dn exhibited a similar impairment and no improvement was observed even following prolonged observation (nest score, $F_{1,50} = 18.482$, $p < 0.001$; two-tailed post hoc Tukey test, eup = 14, Ts65Dn = 13, $q = 6.080$, $p < 0.05$; untorn material, $F_{1,50} = 15.712$, $p < 0.001$; two-tailed post hoc Tukey test, eup = 14, Ts65Dn = 13, $q = 5.606$, $p < 0.05$; Fig. 2C, F).

To examine another innate behavior, we assessed spontaneous alternation in a Y-maze over a 6 min interval (Fig. 2G). Mice possess a strong tendency to alternate between arms on successive trials, and this behavior has been reported to be sensitive to the consequences of normal and pathological aging (Lalonde, 2002). Our analyses revealed a profound impairment in Ts65Dn mice at 2 and 11 months of age in the percentage of alternations between arms ($t(27) = 3.687$, $p = 0.001$, eup = 14, Ts65Dn = 15, and $t(25) = 5.328$, $p = 0.00001$, eup = 14, Ts65Dn = 13, respectively; Fig. 2H, I). We also performed analyses of number of entries at 2 and 11 months of age ($t(27) = -1.085$, $p = 0.288$, eup = 14, Ts65Dn = 15, and Mann-Whitney $U = 52.500$, eup = 14, Ts65Dn = 13, $p = 0.06$, respectively), distance traveled and velocity, but none of these parameters were significantly different between genotypes (data not shown). However, increased number of entries at 11 months of age in Ts65Dn mice is close to statistical significance. Significant differences in the number of entries have been reported recently in aged Ts65Dn (Lysenko et al., 2014). However, our longitudinal study demonstrates that the number of entries for Ts65Dn animals remain similar at 2 and 11 months of age. In fact, the decreased number of entries in euploids at 11 months of age compared to 2 months of age is the main driver for the apparent increase in Ts65Dn entries at 11 months of age.

Water T-Maze and Morris Water Maze reveal cognitive impairment in Ts65Dn

To test cognitive ability, euploid and Ts65Dn mice were examined using the water T-maze (WTM) and Morris water maze (MWM) spatial learning and memory paradigms. First, we assessed both groups using a 10 day protocol in the WTM, where a hidden platform is found using a two-choice system (Fig. 3A). Both groups improved their percentage of correct

choices over successive days during training, reversal and double reversal (Fig. 3B) demonstrating that learning can be measured using this paradigm. At 2 months, our analyses of training and reversal revealed no significant differences between groups in their percentage of correct choices ($F_{1,81} = 0.159$, $p = 0.693$, eup = 14, Ts65Dn = 15, and $F_{1,52} = 3.730$, $p = 0.064$, eup = 14, Ts65Dn = 14, respectively; Fig. 3B) although we found a strong trend towards a lower percentage of correct choices for Ts65Dn mice on day 1 of reversal (two-tailed Tukey post hoc analysis, eup = 14, Ts65Dn, 14, $q = 2.731$, $p = 0.051$). Furthermore, analysis of double reversal revealed significant differences between groups in their percentage of correct choices ($F_{1,52} = 5.556$, $p = 0.026$) on day 1 ($p < 0.05$, two-tailed Tukey post hoc test, eup = 14, Ts65Dn = 14, $q = 3.462$, $p = 0.017$; Fig. 3B). We then compared the percentage of correct choices within each genotype on day 1 of the training, reversal, and double reversal periods. Euploid mice showed no significant differences in the percentage of correct choices made between day 1 of training, reversal, or double reversal (at chance, ~50%; T1, R1 and DR1; $F_{2,26} = 0.177$, $p = 0.84$; Fig. 3B). In contrast, while Ts65Dn mice performed similarly to euploid on day 1 of training (57% correct choices), they showed a substantial reduction in their performance on day 1 of reversal and double reversal (34% and 38%, respectively; $F_{2,28} = 8.079$, $p = 0.002$). Altogether, these analyses support an impaired flexibility of learning in Ts65Dn mice.

We analyzed these same mice at 11 months of age following an identical protocol. It is noteworthy that both groups needed only 3 days of training to reach plateau compared to the 4 days needed at 2 months of age, even though the initial platform location was moved to the opposite arm of the last exposure at 2 months of age. Analysis of the training phase revealed no differences between groups in their percentage of correct choices ($F_{1,52} = 3.731$, $p = 0.064$, eup = 14, Ts65Dn = 14; Fig. 3C). In contrast to what we found in younger mice, significant differences between groups were found in the percentage of correct choices during reversal ($F_{1,52} = 10.920$, $p = 0.003$; Fig. 3C), particularly on the first and second day ($p < 0.05$ two-tailed Tukey post-hoc test, eup = 14, Ts65Dn = 14, first day $q = 5.279$, $p < 0.001$; second day, $q = 4.927$, $p = 0.001$). Analysis of the double reversal revealed a strong trend to significance between groups ($F_{1,50} = 3.627$, $p = 0.068$, eup = 14, Ts65Dn = 13; Fig. 3C). We characterized errors as perseverative or regressive as described previously (Guariglia and Chadman, 2013). Errors made before a mouse finds the platform are defined as perseverative while errors made after finding the platform are defined as regressive. Our analysis on day 1 of reversal and double reversal revealed no significant differences between euploid and Ts65Dn in either perseverative or regressive errors for both ages examined (Fig. 3D, E) although there was a strong trend towards increased perseverative errors during double reversal for Ts65Dn mice at 11 months of age (Fig. 3E; Mann-Whitney $U = 51.500$, eup = 14, Ts65Dn = 13, $p = 0.055$).

These initial trials at day 1 of the reversal periods, after the platform has just been moved to the opposite arm, are the most difficult for mice to learn and may be used as an indication of cognitive flexibility. To explore this in more detail we measured the latency to find the platform during only the first two trials on day 1 of reversal and double reversal periods. We found significant differences in latencies between groups in trial 1 of reversal ($F_{1,26} = 4.265$, $p = 0.049$, $p > 0.05$ two-tailed Tukey post hoc test, eup = 14, Ts65Dn = 14, $q = 2.858$, $p = 0.051$; Fig. 3F) and double reversal ($F_{1,25} = 9.994$, $p = 0.004$, $p < 0.05$ two-tailed Tukey post

hoc test, eup = 14, Ts65Dn = 13, $q = 4.712$, $p = 0.001$; Fig. 3F) and that Ts65Dn animals exhibit the most difficulty on trial 1 of the double reversal. We also measured the total combined latency in trial 1 and trial 2 for day 1 in reversal and double reversal and found that Ts65Dn mice showed longer latencies in each case (Fig. 3G; Mann-Whitney $U = 51.500$, $p = 0.034$ and Mann-Whitney $U = 32.500$, $p = 0.005$). Overall, these data suggest that WTM testing can uncover a significant impairment in cognitive flexibility in Ts65Dn which becomes more pronounced with age.

To provide another test of cognitive flexibility, we then tested these same mice using an extended MWM protocol with added reversal periods. We extended the numbers of testing days to distinguish whether Ts65Dn are permanently impaired in spatial learning and memory, as previously reported (Stasko and Costa, 2004; Seregaza et al., 2006; Herault et al., 2012), or if they learn at a slower pace compared to controls. We also expected that this increased testing duration might diminish confounding factors such as thigmotaxic behavior (the anxiety-based tendency of mice to swim close to the wall), jump-offs or swimovers previously reported in Ts65Dn. Proper consideration of these confounding factors is necessary for sound interpretations of MWM results (Stasko and Costa, 2004; Vorhees and Williams, 2006). We first employed a cued learning protocol to ensure that mice had the ability to learn to swim to a visual goal. At 2 months of age, both groups significantly decreased their latency to find the visible platform over 4 days ($F_{3,81} = 119.414$, $p < 0.001$, eup = 14, Ts65Dn = 15; Fig. 4A Visible). We did not find significant differences between groups, ($F_{1,81} = 1.182$, $p = 0.287$) indicating that both groups were able to learn the basic skill of swimming towards a visible goal and climbing onto the platform before being rescued. Euploid mice showed longer swim paths ($F_{1,81} = 6.655$, $p = 0.016$; Fig. 5A), and higher swimming speeds ($F_{1,81} = 7.628$, $p = 0.010$; Fig. 5B), yet Ts65Dn exhibited similar performance in the last two days compared to euploids. Analysis of time spent in the periphery during visible platform training revealed no significant difference between groups ($F_{1,81} = 1.067$, $p = 0.311$; Fig. 5C). We conclude that cued learning ability is similar between genotypes, ruling out procedural deficits. Interestingly, at 11 months of age, both groups exhibited very short latencies during the first day of cued testing (Fig. 4B Visible). Therefore, we only conducted our cued training for two days total in these older animals. Similar to their performance at 2 months-old, we did not find significant differences between euploid and Ts65Dn mice in their latency to find the visible platform, distance swum, swimming speed, or time spent in the periphery at 11 months-old (Fig. 4B; Fig. 5D–F). Clearly, both genotypes retained the cued learning ability and, interestingly, remembered the testing rules performed 9 months earlier.

The day after the visual test ended, both genotypes were tested for their ability to learn the location of a hidden platform. Overall, as expected, both genotypes improved their performance over successive trial days at 2 and 11 months of age, as measured by decreased latencies and swimming distance ($F_{11,286} = 27.998$, $p < 0.001$; $F_{11,286} = 30.887$, $p < 0.001$, eup = 14, Ts65Dn = 14, and $F_{11,242} = 13.244$, $p < 0.001$; $F_{11,242} = 14.671$, $p < 0.001$, eup = 14, Ts65Dn = 10, respectively; Fig. 4A, B; Fig. 5G–J). At 2 months, we found significant differences between genotypes in the latency and swimming distance during hidden platform period (Fig. 4A, $F_{1,286} = 14.162$, $p < 0.001$; Fig. 5G, $F_{1,286} = 9.693$, $p = 0.004$), but did not find significant differences between genotypes in swimming speed (Fig. 5H; $F_{1,286} = 0.771$,

$p = 0.388$). Specifically, Ts65Dn exhibited significantly longer latencies in the first four days (Fig. 4A; $p < 0.05$ two-tailed Tukey post hoc test, eup = 14, Ts65Dn = 14, day 1 $q = 5.322$, $p < 0.001$) and accordingly swam longer distances in the first, second and fourth day (Fig. 5G; $p < 0.05$ two-tailed Tukey post hoc test, eup = 14, Ts65Dn = 14, day 1 $q = 4.403$, $p = 0.005$). While these results imply differences in rates of learning between Ts65Dn and euploid mice, there was a large difference in time spent in the periphery of the tank between groups (Fig. 6A, $F_{1,286} = 8.319$, $p = 0.008$) between days one and four of hidden platform testing ($p < 0.05$ two-tailed Tukey post hoc test, eup = 14, Ts65Dn = 14, day 1 $q = 4.079$, $p = 0.008$). This time spent in the periphery of the tank may be due either to swimming path trajectory in search of the platform or to thigmotaxis.

Thigmotaxis was defined by continuous swimming along the periphery of the tank as previously described (Wagner et al., 2013). This behavior indicates a lack of focus on the task and is considered a confounding factor in the MWM test (Vorhees and Williams, 2006). To analyze this periphery swimming, we recorded the time spent in a virtual concentric ring within 10 cm of the wall of the tank. Our analysis showed that Ts65Dn mice exhibit long durations of periphery swimming mainly during the first 3 days of the hidden platform period at 2 months of age (Fig. 6A). As a general rule, this behavior diminished over the course of the first three days and disappeared almost entirely on day 4 in every trial and was hardly observed thereafter. To quantify whether this behavior was thigmotaxis, we reasoned that thigmotactic events prevent Ts65Dn animals to fully focus on the task. Therefore, we considered the mean latency to find the platform for Ts65Dn on day 4 (19.5 sec) as a threshold for a thigmotaxis event since this is the last day that time spent in the periphery is significantly different between genotypes. We calculated the percentage of each genotype that spent >19.5 sec in the periphery in at least one trial per day. We found that while most of the euploid animals exhibited thigmotaxis on day 1, this behavior rapidly diminished by day 2. In contrast, Ts65Dn animals showed increased frequency of thigmotactic events for the first three days of the testing compared to euploids (Fig. 6C). Heatmaps displaying the average range of location frequencies for all four trials per day indicate that thigmotaxis is indeed heightened in Ts65Dn mice during the initial days of the hidden platform period (Fig. 6D–F). Thus, Ts65Dn mice need 3 to 4 days to acclimate to the task before their underlying learning and memory capabilities can be fully measured. Importantly, we found that Ts65Dn mice exhibit normal spatial learning and memory following this thigmotaxis period. We therefore conclude that the higher initial latencies observed for Ts65Dn mice are primarily due to thigmotaxis rather than to a spatial learning deficit. Accordingly, once thigmotaxis dissipated, Ts65Dn mice were able to learn the location of the platform similarly to euploid mice (Fig. 4A, Hidden).

It has been established that adding a reversal phase to a MWM testing paradigm can uncover additional cognitive defects. Studies in a variety of mutant mouse models with drug treatments that show small or even no difference during hidden platform testing have found significant deficits during reversal training (Vorhees and Williams, 2014). We uncovered a similar pattern in Ts65Dn, as there were significant differences between groups in the latency and swimming distance during reversal learning (Fig. 4A, $F_{1,104} = 7.504$, $p = 0.011$; Fig. 5G, $F_{1,104} = 6.273$, $p = 0.019$; $p < 0.05$ two-tailed Tukey post hoc test, eup = 14, Ts65Dn = 14, latencies $q = 3.874$, $p = 0.011$; swimming distance $q = 3.542$, $p = 0.019$).

While we also found a main effect of time spent in the periphery between genotypes in the hidden reversal platform period (Fig. 6A, $F_{1,104} = 5.387$, $p = 0.028$; $p < 0.05$ two tailed Tukey post hoc test, eup = 14, Ts65Dn = 14, $q = 3.282$, $p = 0.029$), its cause in this period is different from that observed during the initial three days of the hidden platform period. The total time spent in the periphery was much lower compared to the hidden platform period and the percentage of animals that showed at least one thigmotaxic event per day was also very low (Fig. 6A, C). In addition, swimming trajectory heatmaps demonstrate that Ts65Dn animals spent significant time in the center of the tank searching for the platform (Fig. 6G, H). Therefore, we conclude that time spent in the periphery during the reversal period was due to transient visits into the virtual ring while actively searching for the platform and that longer latencies and swimming distances uncover a deficit in cognitive flexibility during the reversal period. This inflexibility of learning is illustrated by the swimming trajectories (Fig. 6G) which show that Ts65Dn spent a significant time in the former location of the platform on day 1 of the hidden reversal trials.

At 11 months, we found greater differences between genotypes in their ability to learn the platform location as measured by latency and swimming distance during the hidden platform period ($F_{1,242} = 18.551$, $p < 0.001$; $F_{1,242} = 15.700$, $p < 0.001$, respectively). Specifically, Ts65Dn showed longer latencies and swimming distance nearly every day (Fig. 4B; Fig. 5I; $p < 0.05$ two-tailed Tukey post hoc test, eup = 14, Ts65Dn = 10, latencies $q = 6.091$, $p < 0.001$; swimming distance $q = 5.604$, $p < 0.001$), while swimming speed was similar between genotypes ($F_{1,242} = 0.343$, $p = 0.564$; Fig. 5J). We also assessed cognitive flexibility by adding a reversal phase and found dramatic differences between groups in latency and swimming distance ($F_{1,242} = 16.812$, $p < 0.001$; $F_{1,242} = 20.090$, $p < 0.001$, respectively; Fig. 4B; Fig. 5I). Specifically, Ts65Dn showed greater latencies and swimming distances throughout the 12 days tested ($p < 0.05$ two-tailed Tukey post hoc test, eup = 14, Ts65Dn = 10, latencies $q = 5.799$, $p < 0.001$; swimming distance $q = 6.339$, $p < 0.001$). We also found significant differences in the time spent in the periphery during hidden and hidden reversal between genotypes ($F_{1,242} = 19.438$, $p < 0.001$; $F_{1,242} = 10.376$, $p < 0.004$, respectively). Similar to 2 months of age, we observed thigmotaxic behavior at the beginning of hidden platform and reversal periods, but this behavior decreased compared to the younger age and disappeared by the third day (Fig. 6B). Nevertheless, 11 month-old Ts65Dn never found the platform as quickly as controls even after the thigmotaxis had dissipated (Fig. 4B). These cognitive tests demonstrate that 11 month-old Ts65Dn mice exhibit impairments in spatial and reversal learning. Representative traces of paths clearly depict this (Fig. 4C).

We analyzed reference memory (using a probe trial) after each learning period 24 h after the last training trial. During this test, the platform was removed and mice were allowed to swim once freely for 60 sec. Both euploid and Ts65Dn mice displayed selective quadrant search in each probe trial performed demonstrating that both groups formed a cognitive map to find the platform (Fig. 4D–G). However, Ts65Dn spent significantly less time in the target quadrant compared to euploid mice after hidden and hidden reversal periods at 2 months of age but only after reversal period at 11 months of age (Fig. 4D–G), indicating a long-term memory deficit in Ts65Dn. To explore memory in more detail, we also measured the number of virtual platform crossings during each probe trial. We found that Ts65Dn mice made

significantly fewer platform crossings after reversal learning for both the initial 30 sec and the entire 60 sec at both ages (Fig. 4H, J and data not shown, respectively). In addition, we measured the mean distance to the virtual platform (Proximity) as it has been suggested to be the most sensitive measure of water maze probe test performance (Maei et al., 2009). We found that Ts65Dn mice were consistently farther from the platform location than euploids after hidden and hidden reversal periods at 2 months of age ($t(27) = -2.564$, $p = 0.016$ and $t(27) = -3.312$, $p = 0.002$, respectively). These differences were more pronounced in the initial 30 sec at 2 months of age (Fig. 4I, $t(27) = -4.014$, $p = 0.0004$; $t(27) = -3.592$, $p = 0.0002$, eup = 14, Ts65Dn = 15, respectively) and at 11 months of age (Fig. 4K, Mann-Whitney $U = 34.000$, eup = 14, Ts65Dn = 10, $p = 0.038$; $t(22) = -3.481$, $p = 0.002$, respectively). Overall, these data indicate that Ts65Dn have a spatial long-term memory impairment that is most accentuated during reversal periods.

Age-dependent cognitive decline

Since individuals with DS experience accelerated aging and a premature cognitive decline (Zigman, 2013), we asked whether aging has an effect on innate behavior and learning ability in euploid and Ts65Dn mice. This is important since mouse models of DS may advance our understanding of the neurobiological underpinnings of cognitive decline in both normal and pathological aging. Specifically, one important characteristic of the premature aging seen in DS is the early development of AD neuropathology prior to the appearance of clinical symptoms some years or decades later (Zigman, 2013). It may be that particular cognitive modalities are preferentially affected during this transition. For example, impairments in reversal learning but not spatial learning have previously been shown in a mouse model of AD (Zhuo et al., 2007).

To perform our analysis, we compared behavioral testing data from euploid and Ts65Dn mice acquired at 2 and 11 months of age. First, we found that while nesting behavior in euploid animals worsened with age, both young and old Ts65Dn mice performed equally poorly, although we identified a worse ability of tear nesting material in euploids as they aged (Fig. 7A, B; nesting score, $F_{1,25} = 0.0641$, $p < 0.802$; untorn material, $F_{1,25} = 4.516$, $p = 0.044$, $p < 0.05$ two-tailed Tukey post hoc test, eup_{2 month} = 14, eup_{11 month} = 14, $q = 3.105$, $p = 0.038$; Ts65Dn_{2 month} = 15, Ts65Dn_{11 month} = 13, $q = 1.181$, $p = 0.412$). For Y-maze spontaneous alternation, we found that both genotypes exhibited a slightly increased tendency to alternate between different arms of the Y-maze as they aged (Fig. 7C; $F_{1,25} = 10.727$, $p = 0.003$; $p < 0.05$ two-tailed Tukey post hoc test, eup_{2 month} = 14, eup_{11 month} = 14, $q = 3.358$, $p = 0.026$; Ts65Dn_{2 month} = 15, Ts65Dn_{11 month} = 13, $q = 3.197$, $p = 0.033$). This was somewhat surprising since this behavior has been suggested to decrease in an age-dependent manner (Lalonde, 2002). In the WTM, we found an improvement in both groups over time in the training phase (Fig. 7D, E; $F_{1,26} = 23.688$, $p < 0.001$, $p < 0.05$ two-tailed Tukey post hoc, eup_{2 month} = 14, eup_{11 month} = 14, $q = 6.555$, $p < 0.001$; Ts65Dn_{2 month} = 15, Ts65Dn_{11 month} = 14, $q = 3.179$, $p = 0.033$), supporting that aged mice remembered the rules of the test, but not aging effects in the reversal and the double reversal phase ($F_{1,27} = 0.0847$, $p = 0.773$; eup_{2 month} = 14, eup_{11 month} = 14, Ts65Dn_{2 month} = 14, Ts65Dn_{11 month} = 14, and $F_{1,24} = 4.275$, $p = 0.05$, eup_{2 month} = 14, eup_{11 month} = 14, Ts65Dn_{2 month} = 14, Ts65Dn_{11 month} = 13, respectively). To perform this analysis in the MWM test, we calculated

the total latency recorded (see methods) for each mouse during hidden platform and hidden reversal periods at both ages examined. Grouping animals by genotype, we found that young and aged euploid mice performed equally well during hidden platform acquisition, supporting the idea that basic components of spatial learning and memory are preserved with age. However, Ts65Dn animals were slightly worse at hidden platform learning at 11 months of age (Fig. 7F). In contrast, both genotypes performed significantly worse in the reversal learning at 11 months (Fig. 7G; $F_{1,22} = 22.206$, $p < 0.001$; $p < 0.05$ two tailed Tukey post hoc test, $eup_{2\text{ month}} = 14$, $eup_{11\text{ month}} = 14$, $q = 3.252$, $p = 0.031$; $Ts65Dn_{2\text{ month}} = 15$, $Ts65Dn_{11\text{ month}} = 10$, $q = 5.977$, $p < 0.001$), indicating that aging has a major impact on this more difficult task. We also looked at each individual mouse at 2 and 11 months of age to compare whether their cognitive performance improved or declined over time. We found that there was not a consistent worsening in the hidden platform performance over aging for either the euploid or Ts65Dn groups. For example, only 50% of euploid and 70% of Ts65Dn individuals performed worse at 11 months old in this task (Table 2). In contrast, when latencies for the hidden reversal period were analyzed, 100% Ts65Dn mice showed cognitive performance decline at 11 months while only 57% of the euploid animals performed worse (Table 3). These results indicate that the hidden reversal task dramatically reveals the consequence of aging on cognition in Ts65Dn animals.

Hierarchical clustering and principal component analyses stratify Ts65Dn and euploid mice into distinct groups

We then combined all of our developmental and adult datasets into a longitudinal record of each animal's performance. This allowed us to analyze the entire behavioral history for each animal using unsupervised two way hierarchical clustering analysis (HCA) (see methods). We performed three different HCAs: 1) comparing developmental milestone data alone, 2) adult behavioral data alone, and 3) combining all of the behavioral data together (Fig. 8). The results demonstrate that euploid and Ts65Dn mice can be clearly segregated into different groups in an unbiased fashion when using either developmental milestones, adult behavior, or all behavioral data combined (Fig. 8A–C). These analyses uncovered three different clusters in each dendrogram, two of which were populated by euploid individuals separated into high and middle performance groups. The third cluster contained the Ts65Dn mice which are characterized by relatively lower scores for each task. Interestingly, the membership of the two euploid clusters was largely independent of age, suggesting that an individual's performance during developmental milestones parallels its behavioral performance as an adult. We then used principal component analysis (PCA) to visually inspect the variability in performance on these behavioral tasks within and between genotypes (Fig. 9). Interestingly, in each case, PCA resolved euploid and Ts65Dn mice into clearly separable groups. It is noteworthy that both HCA and PCA analyses suggest that mice with equivalent developmental milestones performance also performed similarly on the adult behavior tasks. For example, animals found within the same developmental milestones dendrogram branch (Fig. 8A) are also found within the same adult behavior dendrogram branch (Fig. 8B, C).

Developmental Milestones partially influence adult behavior performance

To explore this apparent connection between juvenile and adult behavioral scores, we conducted a correlation analysis to test whether developmental milestone scores could potentially predict performance in adult behavior (Fig. 10). First, we consolidated the developmental milestones data into a single “DM” score (see methods). We then plotted the relationship between adult task performance and the DM score, using the latter as a predictor. Interestingly, we found a strong negative correlation ($r(27) = -0.636$, $p = 0.0002$; 95% CI $[-0.831, -0.351]$; slope = -0.14) between DM score and nesting behavior at 2 months of age (Fig. 10A), suggesting that delays in acquisition of developmental milestones during the first three weeks of life are highly correlated with poor nest building performance at 2 months of age. This correlation decreased substantially at 11 months of age ($r(25) = -0.311$, $p = 0.1140$; 95% CI $[-0.618, -0.077]$; slope = -0.04). We found a strong positive correlation between DM score and the acquisition period of the MWM at 2 ($r(26) = 0.467$, $p = 0.0122$; 95% CI $[0.113, 0.715]$; slope = 13.14) and 11 ($r(21) = 0.501$, $p = 0.0149$; 95% CI $[0.111, 0.756]$; slope = 15.67) months of age (Fig. 10B, C). In each correlation, one outlier was identified and removed from the analysis. Also, the correlation shown in Figure 10C has only $n = 10$ for Ts65Dn instead of 15 animals due to death of 5 Ts65Dn adults during the course of the longitudinal study (see Materials and Methods). We also found positive correlations between DM and the reversal period latencies in the MWM at 2 ($r(25) = 0.325$, $p = 0.098$; 95% CI $[-0.062, 0.627]$; slope = 4.37) and 11 ($r(22) = 0.37$, $p = 0.0752$; 95% CI $[-0.0393, 0.672]$; slope = 9.54) months of age. These comparisons indicate that individuals with poor developmental milestone performance also perform poorly in the MWM. Finally, we found a strong negative correlation between DM score and spontaneous alternation at 2 ($r(27) = -0.46$, $p = 0.0119$; 95% CI $[-0.707, -0.113]$; slope = -0.64) and 11 ($r(25) = -0.575$, $p = 0.0017$; 95% CI $[-0.783, -0.249]$; slope = -0.74) months of age (Fig. 10D, E), indicating that slower developmental milestone completion is found in animals with innate behavioral defects measured with the Y-maze. These correlations clearly suggest that acquisition of developmental milestones is a predictor of adult cognitive performance.

DISCUSSION

Intellectual disability is a universal yet complex feature of DS that has a tremendous impact on individuals and on society. Intellectual disability in DS affects many different aspects of intelligence such as cognitive flexibility, problem solving and reasoning and memory, all of which are impaired to different degrees. For example, individuals with DS are able to learn many tasks, although over a protracted time frame due in part to their difficulty in changing between tasks (Campbell et al., 2013; Phillips et al., 2014; de Sola et al., 2015). Our findings in Ts65Dn are in agreement with these learning deficits in DS. Rather than a “baseline” spatial L/M deficit, our findings uncover a prominent impairment of reversal learning in young and old Ts65Dn mice. This greater difficulty in L/M performance for complex tasks is also consistent with individuals with DS who commonly show deficits in executive function and/or cognitive flexibility (Campbell et al., 2013; Breslin et al., 2014; Heyman and Hauser-Cram, 2015). We believe that in-depth analyses of cognitive performance in mouse models of DS, such as those presented here, are necessary for a deeper understanding of the temporal components of learning disabilities and to clearly measure the effects of treatment

and genetic studies. This discrimination is especially crucial in treatment studies aimed at improving cognition in DS animal models.

It is important to note that although there are approximately a dozen different mouse models of DS, all of which differ genetically, the Ts65Dn mouse is the most widely studied and therefore has the most abundant data relating to cellular development, behavior and neurophysiology. However, whether the Ts65Dn mouse is the best model of DS is indeed an important and ongoing question in the field because this model carries a triplication of the centromeric part of the mouse chromosome 17 (Duchon et al., 2011; Reinholdt et al., 2011). This centromeric region contains approximately 50 genes not triplicated in DS; the specific effects of these genes have not yet been elucidated, but their triplication in Ts65Dn may alter development and function in ways not related to DS pathophysiology. Despite these genetic dissimilarities, Ts65Dn mice exhibit many morphological, physiological and neurological phenocopies to humans with DS.

Cognitive deficits in Ts65Dn mice have been extensively reported and one of the most consistent findings are deficits in spatial memory elucidated by the MWM and other learning and memory paradigms (Stasko and Costa, 2004; Seregaza et al., 2006; Herault et al., 2012). Our results confirm that Ts65Dn mice exhibit spatial learning deficits, but the high levels of thigmotaxis we have measured modify our current understanding of the character of these spatial memory deficits. While several studies employing the MWM paradigm never assessed thigmotaxic behavior, other studies considered thigmotaxis as a non-spatial strategy to find the platform (Shichiri et al., 2011; Altafaj et al., 2013; Garcia-Cerro et al., 2014; Souchet et al., 2014; Zhang et al., 2014). We believe that thigmotaxis behavior reflects sensorimotor and/or emotional issues that are separate from spatial L/M deficits, as discussed previously (Holmes et al., 2002; Stasko and Costa, 2004; Inostroza et al., 2011; Vorhees and Williams, 2014). Importantly, by employing an extended testing procedure with four trials per day, our study elucidates significantly different L/M disabilities in Ts65Dn. Contrary to the previously published work, we show for the first time that Ts65Dn mice are indeed able to learn the location of a hidden platform with similar latencies as euploids. In particular, our results on the initial hidden platform for 2 month-old Ts65Dn show increased latencies which we show are due to increased thigmotaxic behavior. In fact, once thigmotaxis dissipated after the first 3–4 days of testing, differences in latencies between Ts65Dn and euploid animals were minimal; these results indicate that Ts65Dn do not exhibit a spatial learning deficit. With these results, we have extended previous findings which analyzed thigmotaxis only during the probe trial (Stasko and Costa, 2004) We have demonstrated that thigmotaxis assessment during learning trials is crucial to infer proper L/M abilities in Ts65Dn.

Our results also uncover a temporal decline in cognitive and innate abilities in both Ts65Dn and euploid mice although it is clear that this decline is exacerbated in Ts65Dn. It is possible that neurodegenerative changes may be contributing to the worsening of Ts65Dn brain function. For example, the loss of cholinergic neurons in the basal forebrain of 4 month-old Ts65Dn animals has been previously correlated with cognitive decline (Granholm et al., 2000). Behavioral studies on young Ts65Dn are important because they provide a baseline of cognitive deficits prior to neurodegenerative changes. Our data clearly show that

significant defects in cognition are present in Ts65Dn prior to this neurodegeneration (i.e. our tests at 2 months) and that they intensify with aging. Further cellular and morphometric studies on young Ts65Dn are needed to understand the mechanisms underlying these early cognitive deficits. An intriguing possibility is that early proliferation impairments in peripheral tissues in Ts65Dn might be a critical determinant that contributes to the widespread phenotypes present in DS, including intellectual disability (Fuchs et al., 2012).

Studies in infants with DS have reported delays in the achievement of developmental milestones during the first year of life which has led to the implementation of early intervention programs consisting of a variety of exercises and activities (Hartley, 1986; Wisniewski, 1990; Wishart, 1991; Vicari et al., 2013; Cardoso et al., 2015). Previous studies and our present findings have shown delays in the acquisition of developmental milestones during the first 3 weeks of life in Ts65Dn mice (Holtzman et al., 1996; Toso et al., 2008). Indeed, as with infants with DS, Ts65Dn mice eventually reach developmental milestones but do so later than euploids. Interestingly, we found a strong correlation between the developmental milestone score for each animal and its ability to build a nest at 2 month of age, a species-typical behavior that has been related to attentional processes and frontal cortical function (Kolb and Whishaw, 1985; de Brabander et al., 1991). We have also uncovered a moderate correlation between developmental milestone score and cognitive performance, consistent with previous mouse studies which show the importance of early development on subsequent social and cognitive skills (Branchi et al., 2013; Cutuli et al., 2015). A developmental progression linking infant sensory and motor development to cognition and motor skills in adulthood has been reported in humans as well (Taanila et al., 2005; Murray et al., 2006; Murray et al., 2007; Piek et al., 2007; Iverson, 2010; Clearfield, 2011; Kobayashi et al., 2014). Individuals markedly late in achieving developmental milestones have been found to be at high risk for a subsequent diagnosis of intellectual disability (Schillace, 1964; von Wendt et al., 1984). Indeed, a recent study has uncovered a relationship between early motor skills and executive function in DS (Schott and Holfelder, 2015). As in humans, it is intriguing to suggest that deficits in innate, sensory and motor abilities during early life in Ts65Dn, as we have shown in this study, might have a direct consequence on cognitive abilities in the adult. Thus, this work highlights the need to more comprehensively measure the cellular and molecular aspects occurring during fetal and perinatal brain development in DS.

This study also supports Ts65Dn as a potential mouse model for the AD-related cognitive decline observed in DS. Ts65Dn mice are characterized by a number of phenotypes including progressive loss of working memory, cholinergic deficit in the basal forebrain, neuroinflammation, and endosomal and brain amyloid precursor protein abnormalities, all of which have been observed in AD and mouse models of AD (Hartley et al., 2015). The current findings of impairments in nesting behavior, spontaneous alternation and reversal learning are also consistent with findings in mouse models of AD (Filali and Lalonde, 2009; Morales-Corraliza et al., 2013; Torres-Lista and Gimenez-Llort, 2013; Webster et al., 2014; Devi et al., 2015). Moreover, inflexibility of learning is an early feature of AD dementia (Albert, 1996; Traykov et al., 2007), further linking Ts65Dn as a mouse model of AD. Because of these similarities, Ts65Dn may prove to be very useful in drug development to improve cognition in AD. It is crucially important to conduct similar longitudinal behavioral

studies in other DS mouse models to determine whether additional components of AD pathogenesis may be observed.

Our finding that reversal learning is greatly impaired in Ts65Dn suggests specific abnormalities are present in the prefrontal cortex (PFC) (Cutuli et al., 2015). The PFC is a key structure involved in modulating executive function (EF), which allows hierarchical and timely control of actions leading to specific behaviors (Alvarez and Emory, 2006; Robbins and Roberts, 2007; Chudasama, 2011). Behavioral studies in adolescents and adults with DS have shown a broad impairment in EF, and one of the key components of EF is cognitive flexibility (Jurado and Rosselli, 2007; Pritchard et al., 2015). Despite substantial clinical research into this core deficit in DS, there is only a single study, to our knowledge, that has specifically addressed the role of PFC in Ts65Dn and other animal models of DS (Heller et al., 2014). Interestingly, this study reported that both serotonin content and 5HT_{2a} receptors are increased in the frontal cortex of Ts65Dn mice and that pharmacological blockage of 5HT_{2a} receptors in Ts65Dn mice rescues their context-dependent nest building impairment. Thus, further research on PFC could potentially help to elucidate the development and molecular aspects of PFC dysfunction, and ultimately cognitive inflexibility in DS.

In this study, we provide a comprehensive and novel longitudinal behavioral analysis in Ts65Dn and euploid mice over the course of life, including assessment of developmental milestones, innate behavior, and cognitive function. Analysis of this behavioral performance highlights that impaired reversal learning, but not naive learning, is a major feature of Ts65Dn cognitive function. While this particular deficit is clearly detectable in young Ts65Dn, it significantly worsens with age. We also show that Ts65Dn pups exhibit delays in reaching several early developmental milestones and that the degree of their impairment at this stage of life is strongly correlated with impairments in innate and cognitive abilities observed later in adulthood. We have corroborated these results using unsupervised HCA and PCA which demonstrate that Ts65Dn mice can be clearly differentiated from euploid mice when all the behavioral data are considered. Interestingly, we were able to uncover heterogeneity within groups by tracking the performance of each mouse throughout life, reminiscent of the variations in intellectual disability found in the DS population. Altogether, these studies provide a new benchmark for behavioral study in Ts65Dn, defining the particular tasks useful for probing neurological deficits across the lifespan.

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HIGHLIGHTS

- Naïve learning impairment in Ts65Dn is affected by thigmotaxis
- Impairment in reversal learning is a major feature of Ts65Dn cognitive function
- Delays in developmental milestones predict adult behavioral function
- Reversal learning is impacted with age in Ts65Dn and controls
- Individual behavioral performance uncover heterogeneity within both genotypes

Figure 1. Developmental milestones of Ts65Dn versus euploid mice

A–J, Early and late-acquisition developmental milestones of surface righting (A), negative geotaxis (B), cliff aversion (C), forelimb grasp (D), open field (E), rooting (F), ear twitch (G), air righting (H), auditory startle (I) and eye opening (J). Each task was assessed at specific time-window during postnatal development and each dot represents the percentage of animals that acquired the developmental milestone on a specific postnatal day. Fischer Exact test was used to examine statistically significant differences between genotypes at each postnatal day (Euploid, $n = 17$, Ts65Dn, $n = 15$). PND, postnatal day. * $p < 0.05$.

Figure 2. Innate behaviors of Ts65Dn versus euploid mice

A, D, Representatives images of nesting abilities within 15 h (overnight) upon providing nesting material into the cage. A well-built nest in a euploid cage is illustrated in A, while a largely intact nestlet is illustrated in the Ts65Dn cage in D. **B, C,** Nesting abilities of 2 month and 11 month-old Ts65Dn and control mice. **E, F,** Represents the ability of tear nesting material in a novel cage by measuring the weight of the untorn material overnight at 2 months of age and up to 6 days at 11 months of age. **G,** Cartoon depicting the Y-maze employed to assess spontaneous alternation. **H, I,** The percentage of spontaneous alternation is reduced in ts65Dn animals at 2 months of age (H) and at 11 months of age (I). In each graph, the bars represent the mean $\pm$ SEM. Statistical analyses were performed using non-parametric Mann-Whitney Rank Sum test for nesting scale score and two-tailed Student's t test analysis for untorn nesting material and spontaneous alternation. Euploid_{2month}, n = 14, Ts65Dn_{2month}, n = 15. Euploid_{11month}, n = 14, Ts65Dn_{11month}, n = 13. * $p < 0.05$.

Figure 3. Water T-maze spatial learning and memory performance of Ts65Dn versus euploid mice

A, Cartoons depicting the cross-maze and the experimental design employed for the water T-maze paradigm. Each mouse performed 10 trials per day and on each trial the platform was in one of two possible locations. The correct choice per trial was scored as 1 and, wrong choice was scored as 0. Percentage of correct choices was calculated for each day for each mouse. **B**, **C**, The percentage of correct choices over time are shown for the training phase (days T1–T4), the reversal phase (days R1–R3) and the double reversal phase (DR1–DR3) at both 2 and 11 months of age. Note that one Ts65Dn mouse was discarded as an outlier for the reversal and double reversal phase. **D**, **E**, The number and type of perseverative errors (errors made before finding the platform) and regressive errors (errors made after finding the platform) were measured at 2 and 11 months of age. **F**, **G**, The latency to find the new location of the platform during trial 1 and 2 of the reversal and double reversal on day 1 of testing was significantly longer in Ts65Dn animals. Euploid_{2month}, *n* = 14,

Ts65Dn_{2monthtraining}, n = 15, Ts65Dn_{2monthreversal&doublereversal}, n = 14, Euploid_{11month}, n = 14, Ts65Dn_{11monthtraining&reversal}, n = 14, Ts65Dn_{11monthdoublereversal}, n = 13. * $p < 0.05$.

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Figure 4. MWM spatial learning and memory performance of Ts65Dn versus euploid mice
A, B, Escape latencies were measured over a number protracted of days at 2 (A) and 11 months of age (B). All animals progressed through visible, hidden platform and hidden platform reversal components. Platform locations are denoted. **C**, Representative traces of swimming-paths from euploid and Ts65Dn mice during hidden and hidden reversal learning. **D–G**, The graphs show percentages of time spent in each quadrant during the probe test after hidden and hidden reversal at 2 and 11 months of age. **H, K**, Proximity defined as mean average distance to the platform is plotted at 2 and 11 months of age. **I, J**, The number of virtual platform crossings is plotted at 2 and 11 months of age. These parameters were assessed in the initial thirty seconds and in the entire sixty seconds of the test. Each dot represents the mean $\pm$ SEM. In each graph, the bars represent the mean $\pm$ SEM.
 Euploid_{2month}, n = 14, Ts65Dn_{2monthcued&probetrial}, n = 15, Ts65Dn_{2monthhidden&hiddenreversal}, n = 14; Euploid_{11month}, n = 14, Ts65Dn_{11monthcued}, n = 12, Ts65Dn_{11monthhidden&hiddenreversal&probetrial}, n = 10. * $p < 0.05$; ** $p < 0.01$.

Figure 5. MWM swimming velocity and distance in Ts65Dn versus euploid mice

A–F, Swimming distance (A, D), swimming velocity (B, E) and time spent in the periphery (C, F) over a number of days at 2 months (A–C) and 11 months of age (D–F) during cued training. G–J, Swimming distance (G, I) and swimming velocity (H, J) over a number of days at 2 months (G, H) and 11 months of age (I, J) during the hidden platform period. Each dot represents the mean ± SEM. In each graph, the bars represent the mean ± SEM.

Euploid_{2month}, n = 14, Ts65Dn_{2monthcued&probetrial}, n = 15, Ts65Dn_{2monthhidden&hiddenreversal}, n = 14; Euploid_{11month}, n = 14, Ts65Dn_{11monthcued}, n = 12,

Ts65Dn_{11monthhidden&hiddenreversal&probetrial}, n = 10. *p < 0.05.

Figure 6. Analysis of thigmotaxis in MWM spatial learning and memory performance
A, B, Total time spent in the periphery of the tank on each day over a number of days at 2 months (A) and 11 months of age (B) during hidden and hidden reversal platform periods.
C, The percentage of animals that spent more than 19.5 sec to find the platform during hidden platform at 2 months of age. **D–H,** Heatmaps plotted as a range of location frequency averaging the four trials per day. Euploid and Ts65Dn swimming paths are depicted for day 1, 3 and 12 during hidden platform period (D–F) and on days 1 and 4 during hidden reversal period (G, H). * $p < 0.05$.

Figure 7. Age comparisons between euploid and Ts65Dn mice

A, B, Comparison of nesting score and the ability to tear nesting material at 2 and 11 months of age between euploids and Ts65Dn. **C,** Comparison of spontaneous alternation at 2 and 11 months of age between euploids and Ts65Dn. **D, E,** Comparison of the percentage of correct choices during acquisition (D), reversal and double reversal (E) at 2 and 11 months of age between euploids and Ts65Dn. **F, G,** Comparisons of total escape latencies during acquisition and reversal at 2 and 11 months of age (F and G, respectively). In each graph, the bars represent the mean $\pm$ SEM. * $p < 0.05$. ** $p < 0.01$. Euploid_{2&11monthnesting}, $n = 14$; Ts65Dn_{2monthnesting}, $n = 15$, Ts65Dn_{11monthnesting}, $n = 13$; Euploid_{2&11monthspontaneousalternation}, $n = 14$, Ts65Dn_{2monthspontaneousalternation}, $n = 15$, Ts65Dn_{11monthspontaneousalternation}, $n = 13$; for WTM, Euploid_{2&11month}, $n = 14$, Ts65Dn_{2monthtraining}, $n = 15$, Ts65Dn_{2monthreversal&doublereversal}, $n = 14$, Ts65Dn_{11monthtraining&reversal}, $n = 14$, Ts65Dn_{11monthdoublereversal}, $n = 13$; for MWM, Euploid_{2&11month}, $n = 14$, Ts65Dn_{2monthcued&probetrial}, $n = 15$, Ts65Dn_{2monthhidden&hiddenreversal}, $n = 14$, Ts65Dn_{11monthcued}, $n = 12$, Ts65Dn_{11monthhidden&hiddenreversal&probetrial}, $n = 10$.

Figure 8. Hierarchical cluster analysis of behavioral performance in euploids and Ts65Dn
 A–C, Dendrograms and heatmaps from the two-way hierarchical cluster analysis of performance in the battery of developmental milestones (A), adult behavior (B) and developmental milestones plus adult behavior combined (C) tasks. Tasks lists are listed along the x-axis of the heat map. The 3-clusters are emphasized by the boxes at the left of the figure. Euploid, n = 14, Ts65Dn, n = 10.

Figure 9. Principal Component Analysis of behavioral performance in euploids and Ts65Dn
A–C, Comparisons between genotypes with respect to the PCA Milestones (A), PCA Adult Behavior (B) and PCA All tests (C). Black = Euploid, Red = Ts65Dn. Triangles and circles represent subsets of clusters within euploids. Euploids = 14, Ts65Dn = 10.

Figure 10. Correlations between developmental milestones and adult behaviors

A, Graph representing a negative correlation between developmental milestones achievement and nesting behavior at 2 month of age. **B, E**, Graphs representing a positive correlation between developmental milestones achievement and total latency during acquisition period at 2 (B) and 11 (E) months of age. **C, D**, Graphs representing a negative correlation between developmental milestones achievement and percentage of spontaneous alternation at 2 (C) and 11 (D) months of age. Euploid = 14. Ts65Dn = 15 in A,D; Ts65Dn = 14 in B; Ts65Dn = 9 in C; Ts65Dn = 13 in E.

Table 1

Average postnatal day at which developmental milestone is achieved per genotype. Data is presented as mean first day of milestone acquisition $\pm$ SDTV. Student's t-test was performed for statistical analysis. Euploid N = 17, Ts65Dn N = 15.

	Euploid	Ts65Dn	Student's t-test
Surface righting	6.71 $\pm$ 1.62	8.8 $\pm$ 1.68	<u>0.0029</u>
Negative geotaxis	3.85 $\pm$ 1.72	7.28 $\pm$ 2.15	<u>0.00013</u>
Cliff aversion	5.64 $\pm$ 1.79	9.07 $\pm$ 1.93	<u>0.0003</u>
Rooting	7.92 $\pm$ 0.79	9.21 $\pm$ 0.77	<u>0.0003</u>
Forelimb grasp	8.78 $\pm$ 1.20	8.64 $\pm$ 1.44	0.7864
Ear twitch	10.92 $\pm$ 1.03	10.66 $\pm$ 1.13	0.5367
Open field	11.07 $\pm$ 1.70	12.13 $\pm$ 1.78	0.1264
Air Righting	11.64 $\pm$ 1.23	13.71 $\pm$ 1.82	<u>0.0037</u>
Auditory startle	14.35 $\pm$ 1.23	15.53 $\pm$ 1.39	<u>0.0334</u>
Eye opening	12.71 $\pm$ 0.88	13.14 $\pm$ 0.83	0.2136

Table 2

Mouse individual total latencies comparison between 2 month of age and 11 month of age during acquisition in the Morris Water Maze test. Positive value under the column “Change” reflects worse performance at 11 month of age while negative values under the column “Change” reflects better performance at 11 month of age.

	ID	ACQUISITION		CHANGE	DM Score
		Latency (s) 3 month	Latency (s) 11 month		
EUPLOID	3	340.140119	423.823805	83.683686	51.9
	5	538.338315	353.486803	-184.85151	37.55
	8	659.526172	682.415729	22.889557	35.34
	12	532.732712	700.834146	168.10143	43.74
	13	352.552533	452.118771	99.566238	42.17
	15	802.535847	1049.249232	246.71339	47.28
	17	655.388705	477.47746	-177.91125	38.35
	19	454.32097	483.750399	29.429429	61.52
	21	750.150132	593.259907	-156.89023	34.93
	22	436.10275	376.710024	-59.392726	32.78
	23	363.430073	311.111091	-52.318982	49.94
	25	418.885533	424.29094	5.405407	56.69
	28	883.283261	733.266579	-150.01668	48.93
	29	608.80879	372.305619	-236.50317	30.42
	1	865.79911	2008.608593	1142.8095	57.07
	4	960.160137	1003.937258	43.777121	66.11
	6	1497.163803	1363.897209	-133.26659	65.82
Ts65Dn	7	654.7881	840.306953	185.51885	66.29
	9	710.5104	790.657303	80.146813	36.72
	14	1077.143794	950.417065	-126.72673	61.05
	16	628.895541	662.462445	33.566904	62.74
	24	952.685999	817.684332	-135.00167	62.54
	26	691.958603	958.291603	266.333	69.55
	27	670.336986	1112.912894	442.57591	43.17

Table 3

Mouse individual total latencies comparison between 2 month of age and 11 month of age during reversal in the Morris Water Maze test. Positive value under the column “Change” reflects worse performance at 11 month of age while negative values under the column “Change” reflects better performance at 11 month of age.

	ID	REVERSAL		CHANGE	DM Score
		Latency (s) 3 month	Latency (s) 11 month		
EUPLOID	3	148.281609	323.323314	175.041705	51.9
	5	436.503159	286.686678	-149.81648	37.55
	8	380.447105	835.835828	455.388723	35.34
	12	284.484476	675.275265	390.790789	43.74
	13	233.700358	216.616609	-17.083749	42.17
	15	520.186844	823.957283	303.770439	47.28
	17	572.906229	261.327989	-311.57824	38.35
	19	366.232894	301.835158	-64.397736	61.52
	21	403.5134	682.88287	279.36947	34.93
	22	283.735435	396.262921	112.527486	32.78
	23	201.276333	174.174168	-27.102165	49.94
	25	245.4886	370.904231	125.415631	56.69
	28	431.461599	731.998657	300.537058	48.93
	29	425.086788	414.014004	-11.072784	30.42
	1	374.574568	634.034025	259.459457	57.07
	4	578.978968	835.835828	256.85686	66.11
	6	674.674668	737.470795	62.796127	65.82
	7	401.801794	752.686009	350.884215	66.29
Ts65Dn	9	512.912905	745.545537	232.632632	36.72
	14	482.01534	580.046701	98.031361	61.05
	16	552.218879	879.479471	327.260592	62.74
	24	454.198227	829.496154	375.297927	62.54
	26	513.986121	701.768429	187.782308	69.55
	27	415.460599	690.090082	274.629483	43.17