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Behavioral Phenotyping for Down Syndrome in Mice

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Abstract

Down syndrome (DS) is the most frequent genetic cause of intellectual disability characterized by alterations in different behavioral symptom domains: neurodevelopment, motor behavior, and cognition. As mouse models have the potential to generate data regarding the neurological basis for the specific behavioral profile of DS, and may indicate pharmacological treatments with the potential to affect their behavioral phenotype, it is important to be able to assess disease-relevant behavioral traits in animal models to provide biological plausibility to the potential findings. The field is at a juncture that requires assessments that may effectively translate the findings acquired in mouse models to humans with DS. In this protocol, behavioral tests are described that are relevant to the domains affected in DS. A neurodevelopmental behavioral screen, the balance beam test, and the Multivariate Concentric Square Field test to assess multiple behavioral phenotypes and locomotion are described discussing the ways to merge these findings to more fully understand cognitive strengths and weaknesses in this population. New directions for approaches to cognitive assessment in mice and humans are discussed.

Introduction

Down syndrome (DS) is the most common genetic form of intellectual disability caused by trisomy of Homo sapiens autosome 21 (Hsa21) (Antonarakis et al., 2020). Individuals with DS present with a distinct collection of symptoms and manifestations that affect multiple organs and systems, although notable variation exists among individuals. Individuals with DS present unique neurocognitive and neurobehavioral profiles that emerge within specific developmental periods. These profiles reflect underlying neuroanatomic findings, that evolve differentially across the lifespan (Grieco et al., 2015). In early childhood, deviations from

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neurotypically developing trajectories emerge that get more pronounced by school-age. Aspects of language skills are the most compromised and deficits in attention/executive functions are present in childhood and become more pronounced with age. Changes in emotional and behavioral functioning in adulthood are typically associated with neurodegeneration and detected in individuals with DS that develop dementia of Alzheimer's type. Individuals with DS also show higher prevalence of certain comorbidities compared with the general population, including some that may affect behavior such as hearing and vision problems, recurrent infections, anxiety, depression or early-onset Alzheimer disease. To date, a myriad of mouse models relevant to DS has been developed (Herault et al., 2017). These mouse models have been important to get insight into the pathogenetic mechanisms of the altered phenotypes found in this condition (Rueda et al., 2020). They have also allowed to determine genotype-phenotype relationships, identify dosage-sensitive genes involved in DS pathophysiology, and explore the impact of the additional chromosome on the whole genome. Finally most of the new therapeutic targets, and the assessment of different pharmacotherapies, have been performed in mice, thus providing the basis for developing clinical trials in DS individuals (Martinez Cue & Dierssen, 2020). Although new interesting experimental models of DS, such as stem cells and organoids (Gough et al., 2020) have emerged in the last years, the mouse models are crucial to understand the behavioral phenotype.

Most models of DS have been based on the homology of Hsa21 and the murine chromosomes (Mmu)16, 17, and 10 or adding an additional copy of Hsa21 (Deitz & Roper, 2011). Although these mouse lines do not completely resemble DS aneuploidy (Muniz Moreno et al., 2020), they exhibit many DS-like phenotypes, including cognitive, behavioral, molecular, physiological and neuromorphological alterations (Das & Reeves, 2011; Dierssen, 2012; Rueda et al., 2012; Xing et al., 2016). However, they do not always reproduce DS pathophysiology, probably due to the incomplete synteny between Hsa21 and homologous mouse regions and to the fact that the neural circuitry and plasticity, underlying the neural functions of humans are much more complex than in mice (Benavides-Piccione et al., 2004; Dierssen & Ramakers, 2006). Even so, the mouse has proven to be a useful model organism for DS because many of the protein-coding genes currently identified on Hsa21 are conserved on Mmu 10, 16, and 17 (Yu et al., 2020). While most of the research to date has been carried out with the Ts65Dn mouse (Reeves et al., 1995), models that more closely approximate the genetic basis of DS in humans have recently been developed, involving triplication of different chromosomal regions (Yasuhiro Kazuki, 2020).

Over time, a variety of behavioral protocols have been established and validated for their capacity to reflect specific brain systems activity (Crawley, 2007). Specific behavioral tasks are then designed to test hypotheses about the function of the gene and to model the symptoms of the human disease. However, behavior is a complex domain and as such relevant behavioral phenotypes are often influenced by other factors, and may be discovered during sensory or motor analyses or modified by diet, housing conditions of experiment-related stress conditions. There are several aspects of behavioral phenotyping that specifically concern to DS mouse models. One aspect to consider is that the phenotype is not constant along lifespan and thus behavioral profiling should be performed at different stages to capture age-related disease-relevant phenotypes (Hunter et al., 2003; Olmos-Serrano et

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al., 2016; Aziz et al., 2018). DS is characterized by delays in the achievement of developmental milestones and cognitive deficits that appear early in life, but there is an age-dependent change in the observed phenotypes (Granholm et al., 2000). Additionally, male and female mice of different DS mouse models differ in several physiological and behavioral parameters (Martinez-Cue et al., 2002; Martinez-Cue et al., 2006; Stewart et al., 2007; Dierssen et al., 2011; Faizi et al., 2011; Kelley et al., 2014); for example, basal alterations in stress hormones, including increased corticosterone and decreased adrenocorticotropic hormone in females, and differential responses to predators between Ts65Dn males and females have been reported (Martinez-Cue et al., 2002). Some of the major challenges in maximizing the use of the mouse as a model system in the areas of phenotyping is to also appreciate the interdependent relationships with other factors such as diet, social and environmental factors (Martinez-Cue et al., 2005; Begenisic et al., 2011; De Toma et al., 2016) that may also interact with sex (Martinez-Cue et al., 2002) and age (Sansevero et al., 2016). In summary, the ability of reproducing the relevant behavioral traits in animals is of the utmost importance.

General considerations

Unique attributes of Down syndrome model mice

Working with DS mouse models requires specific knowledge pertaining to the DS models in general as well as particular nuances of specific models; this awareness is beyond parameters usually recommended for other mouse models (Fisher & Bannerman, 2019). Important features of mouse models are the ability to accurately represent the genetic components (construct validity) and phenotypes displayed (face validity) in the corresponding human disorder (Rueda et al., 2012; Garner, 2014). Construct and face validity facilitate the use of mouse models to generate new knowledge that may be applied to the human condition (predictive validity (Baxter et al., 2000)). In the study of DS, mouse models may be used to analyze the influence of many genes in three copies (Sago et al., 2000; Olson et al., 2004b; Aziz et al., 2018), the contribution of triplication of a region of genes (Olson et al., 2004a; Yu et al., 2010c), or a reductionist approach that investigates the influence of a single gene in three copies in a transgenic model. Other common approaches use offspring from a knockout mouse bred to a DS mouse model to investigate the normalization of the particular gene in an otherwise trisomic mouse (Salehi et al., 2006). Each of these approaches offers different perspective on the contribution of three copy genes to phenotypes associated with DS (Nguyen et al., 2018). Because corresponding genetic information Hsa21 is found on Mmu16, 17 and 10, it is difficult to create an accurate and complete genetic model of DS in a mouse. To achieve construct validity, DS mouse models have been generated with an extra mouse or human chromosome, duplications of homologous chromosome material, or by expression of a single or a few homologous transgenes. Through the use of cre-lox engineered duplications, DS model mice have been made with three copies of all Hsa21 homologous genes (Yu et al., 2010b; Belichenko et al., 2015). Yet, these mice require extensive breeding and are not readily produced in numbers sufficient to investigate most phenotypes associated with DS. Transchromosomal models with a human chromosome 21 in mouse cells have been generated (O'Doherty et al., 2005). The Tc1 mouse model has a number of gaps in the chromosome, is mosaic, and ~20% of the

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protein coding genes of the extra chromosome are disrupted (Gribble et al., 2013). A new transchromosomal model has been made, and contains ~93% of Hsa21q (Yasuhiro Kazuki, 2020). Additional testing with this mouse model is necessary to see if it effectively replicates DS phenotypes. One potential drawback of these transchromosomal mouse models is that the expression of the human genes may not be controlled in the same way as the mouse genes.

Ts65Dn, with ~104 of the Hsa21 homologous genes on the distal end of Mmu16 on an additional small marker chromosome, is the most widely used model and displays DS associated cognitive and behavioral phenotypes (Davisson et al., 1990; Davisson et al., 1993; Reeves et al., 1995). This model is unique because it possesses a freely segregating extra chromosome, like most individuals with DS. However, this model has the extra Hsa21 orthologous chromosomal material attached to the centromere of Mmu17 with ~35 non-homologous Mmu17 protein coding genes (Duchon et al., 2011; Reinholdt et al., 2011; Gupta et al., 2016). Ts1Cje mice have a Robertsonian translocation with ~94 homologous Mmu16 genes in three copies (Sago et al., 1998). It has also disrupted some genes on Mmu12 and fewer genes in three copies have been shown to attenuate some phenotypes (Olson et al., 2004b). Cre-lox engineered duplication models have been generated for homologous regions on Mmu16, 17 and 10 and tested for different phenotypes (Li et al., 2007; Yu et al., 2010c). These mouse models effectively contribute three copies of the Hsa21 homologous mouse regions, but do not have a freely segregating extra chromosome.

Other caveats exist in DS mouse models. DS mouse models are often difficult to generate and frequently sufficient mice cannot be purchased from a vendor to perform experiments; these mice must be generated in colonies maintained by each laboratory. Additionally, some models with an extra segregating chromosome cannot be placed on a uniform genetic background. There may be some genetic drift from the original mice as mice are maintained in separate colonies for long periods of time (Roper et al., 2020). Ts65Dn mice are maintained on a recombinant B6 and C3H background, and C3H mice carry the *Pde6b^{rd1}* gene, which causes blindness in the homozygous state. A model of Ts65Dn has been made that does not carry the mutation causing blindness, but these mice may not have exactly the same phenotypes as Ts65Dn mice (Costa et al., 2010). Two problems arise from this example that may be extended to all DS mouse models: background genes have to be accounted for, especially when breeding to Ts65Dn mice to other mouse strains, and there are two seemingly similar Ts65Dn mouse strains that may be very different (Costa et al., 2010; Roper et al., 2020).

This Unit describes three different behavioral tests in the main areas affected in DS (neurodevelopment, motor coordination, adaptive behavior). These tests have been modified and adjusted to DS specific characteristics from similar tests that have been used to characterize genetic animal models of intellectual disability. The tests presented would have application in studies aimed at defining the neurobiological underpinnings and potential therapeutic strategies of known or novel genetic animal models.

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Order of Testing

We recommend an order of testing that begins with the neurodevelopmental studies, home cage observations, continues with observations of general health and neurologic reflexes, then addresses sensory and motor abilities, and finally focuses on the behavioral domains relevant to the specific hypotheses. This allows the detection of underlying physiologic abnormalities, which are known in the DS mice that might limit their ability to perform the procedures necessary for complex behavioral tasks. This prevents false positives, caused by artifacts such as blindness (Costa et al., 2010) limiting performance in a visual learning task, or motor and emotion-related disturbances impeding, for example elevated plus maze arm entries (Coussons-Read & Crnic, 1996). To avoid false negatives, it is recommended to choose three or more tasks within the behavioral domain of interest. Different types of memory, different types of anxiety, different components of feeding, different types of parental care, different symptoms relevant to DS and so forth may be differentially regulated by the gene of interest.

All protocols must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to governmental regulations regarding the care and use of laboratory animals before experiments can proceed.

Basic Protocol 1: Preweaning Neurodevelopmental Battery

DS is a neurodevelopmental disorder in which behavioral differences arise from early stages (Horovitz & Matson, 2011). Delays in the achievement of developmental milestones, delays in behavioral pattern acquisition and cognitive deficits appear early in life. Thus, the evaluation of neurobehavioral development represents an essential study paradigm for DS and other neurodevelopmental disorders. In fact, the age at which neonatal and perinatal behavioral milestones are acquired in DS murine models can be correlated with later cognitive performance in adult (Olmos-Serrano et al., 2016). Numerous test batteries exist for analyzing the development of behavioral reflexes (Fox, 1965; Kodama, 1993; Rogers et al., 1997), sensory-motor development (Noel, 1989; Heuze et al., 1997), locomotor behavior (Feather-Schussler & Ferguson, 2016), grooming, exploration, ultrasonic vocalizations (Chang et al., 2017), and social behavior (Chang et al., 2017).

The protocol we present for DS models is a modified Fox scale (Fox, 1965) and its later adaptations (Wahlsten, 1974; Tremml et al., 1998). This test battery evaluates the acquisition of neurological, sensory and motor developmental milestones during the mice postnatal stage (Dierssen et al., 2002). The battery mimics some of the clinical explorations performed in babies and includes exploration of body righting mechanisms, coordination and strength (surface righting and negative geotaxis), strength and coordination (cliff aversion and forelimb grasp), sensory system maturation (startle response, pinna, and eye opening), labyrinthine reflex (blast), and the developmental transition from rotatory locomotion behavior to straight-line walking, reflecting the rostrocaudal development of limb coordination. The battery of tests is applied longitudinally from postnatal day (PD) PD1 to PD21 (Figure 1). The age of observation of each milestone should be adjusted to reduce handling. The day of achievement of a developmental milestone is defined as the day at

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which the pup performs the task successfully for 2 consecutive days. Once the mice reach this criterion, they are no longer tested in one specific test to reduce handling.

Material—• Experimental animals (6-8 litters); starting at age PD1

Notes: In order to better plan the number of litters needed, for the Ts65Dn strain, the most frequently used for DS research, it should be considered that only 20–40% of the offspring of Ts65Dn mothers are trisomic at weaning, due to loss of trisomic offspring in late gestation that continues through birth to weaning. The proportion of trisomic mice per litter decreases with age of the Ts65Dn mother (Roper et al., 2006).

- Heating pad
- Bowl of nesting material
- Paper towel
- India ink
- Needle
- Balance with automated compensation of movements during weighing
- Ruler
- Toothpick
- Swab
- Wiremesh grid
- Wooden bar (4-mm diameter)
- Cage for homing test
- Timer
- Infrared light

Preparation of the experimental setup

General considerations: On delivery, the litter size of each dam is recorded and each pup checked for gross abnormalities. The day of delivery is designated as PD1 of age of the neonates (erroneous estimates on time of birth ± 6 h). At PD1, the pups are individually marked with India ink on the paws with the aid of a needle. Pups nursed by their natural dams until weaning.

1. Conduct the neurodevelopment experiments in a quiet testing room with sufficient space on the bench for the breeding cage, balance, bowl of nesting material, heating pad and the rest of required materials (see Figure 1 and list of Materials below). It is desirable that the experimentation room is the same as the housing room, with low levels of noise throughout the entire protocol, to maintain conditions of reduced levels of stress for the mother to avoid further abnormal maternal behavior (e.g. pup rejection, cannibalism etc.). Always perform the behavioral assessment during the dark phase of the light cycle, when pups can be more

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reactive to the handling. To this aim, the use of infrared lamps can be very helpful for the manipulation of the pups.

Note: *Maternal behavior is impaired in Ts65Dn dams* (Heller et al., 2014) *and in other DS mouse models* (Fotaki et al., 2002)

2. Set up a daily recording containing: order and identification of pups within each day; pup ID should include information such as age, sex, genotype, and dam ID.

3. Pups are tested at the same time each day in order to eliminate time of day differences in behavior. During the testing period whole litters are separated from the dams and maintained in a small bowl nesting material placed on a heating pad at 37 °C. Separation from the dam should not be longer than 30 min to prevent rapid loss of body heat and hunger/separation issues. In addition, pups are allowed to rest in between tests so that maximal efforts will be elicited on each test. Impregnate hands with the litter bedding to avoid the rejection by the mother once the pups are returned to the cage.

Note: Temporary separation from the mother has a beneficial effect by increasing the time and amount of maternal behavior (Baamonde et al., 1999; Gariepy et al., 2002).

4. The assessment of body growth is performed daily starting on PD1 until PD21. Place gently the pups on a balance covered by paper towel, weighing to the nearest 0.01 g on a balance with automated compensation of pup's movements during weighing. Length of the body is measured from the tip of the nose to the base of the tail using a ruler.

Testing procedure

1. Developmental landmarks: Retrieve the mouse pup from its home cage gently and inspect it carefully. For this experiment, the unit of analysis is the day of attainment of the criterion for each landmark.

PD2: Pinna detachment. Inspect the pups for the complete separation of the distal portion of the pinnae from the cranium. Prior to detachment, the distal portion of the pinnae folds over the auditory meatus and detachment is defined as the pinna being raised to a position of less than 90° of the final position.

PD7: Incisor eruption. Gently inspect the gingiva of the pups. Record the day of the emergence of both lower and upper incisor from the gingiva.

PD9: Eye opening. Inspect the pups and record the day of the complete opening of both eyelids.

2. Neurobehavioral development: The test includes sensorial and motor responses. These reflexologic and behavioral tests reflect the maturation of the central nervous system and are ordered according to starting day of testing.

PD3: Surface righting response.

1. Gently place the pup on its back on a flat surface.

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2. Observe the ability of the pup to turn over to rest in the prone position with all four paws on the floor (Figure 2).

3. Record the latency in seconds for pups placed in a supine position to return to the prone position with all four paws on the ground. Cut off time of 30 seconds. Register the following scoring criteria:

   (3) four paws on the floor;

   (2) one or more paws remain beneath the body;

   (1) vigorous response, but no efficacious attempts to right;

   (0) No response.

4. Record the day of positive response when the animal reaches the maximum score.

   This test can be used to test pups from PD 3-9. Note: information derived from vestibular organs, eyes, neck and body surface reflexly evoke movements required to bring first head and eyes (see Fig 2b), and then the rest of the body, into the normal position with respect to gravity. At the same time, the position of all four limbs must be adjusted to promote the balanced posture.

PD3: Forepaw/hindpaw grasping/suspension.

The grasping reflex in humans is present at birth and disappears around 5 - 6 months. Note: The test can detect right/left side strength differences. Mice falling immediately when released or failure to grasp when placed on the wire are indicative of non-participation.

This test can be used to test pups from PD 3-7.

1. Hold the pups firmly by the body.

2. Enable the pup to grasp the rod with both forepaws. *The paw flexes to grasp a thin rod (toothpick) that it is gently stroking the paw.*

3. Release the pup.

4. Record the total time to fall using a timer or stopwatch. Repeat test for a total of three times. *Paw weakness is determined if a pup consistently falls from the wire with one paw before the other rather than releasing from the wire with both paws at the same time.*

5. The appearance of the reflex is considered the first day that pups remain suspended for at least 5 sec after grasping with their forepaws

   The testing area is over a padded drop zone. The test can detect right/left side strength differences. Paw weakness is determined if a pup consistently falls from the rod with one paw before the other rather than releasing from the rod with both paws at the same time.

PD3: Cliff drop aversion.

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Cliff aversion tests vestibular and proprioceptive responses, as well as strength and coordination and can be used to test pups from PD 3-7.

1. Place the pup gently on the edge of a box with an elevated edge ensuring that the forepaws and snout lie over the edge of a shelf. Note: Use a pre-scented box (e.g. a box where a minimum of 5 mice have been allowed to freely roam)
2. Release the pup and start the timer.
3. Record the time using the timer in which both the snout and paws have been removed from the edge. If the pup does not move away from cliff within 30 sec, no score is given. The response is positive if it turns and crawls away from the cliff. Record first day and time in seconds for pups to turn and crawl away.

PD3. Negative geotaxis test.

This test can be used to test pups from PD 3-10, the average day of appearance of this reflex is PD7. *This test assesses vestibular nociception and the paw strength of all four paws at the same time.*

1. Place the pup head downward on a 45° incline wire mesh grid.
2. 2. The latency to turn 180 is recorded (cut off of 120 seconds). Register the following score:

    (0) absence of a turning response

    (1) incomplete response, the pup turning about 90 but then freezes

    (2) complete turning response.

Note: Mice that fall down the incline or fail to turn can be either re-tested, eliminated, or given a zero score.

PD5: Disappearance of crossed extensor reflex.

This test can be used to test pups from PD 5-10. Poking the paw of one hind limb causes the flexion of the stimulated limb, while the opposite hind limb is extended (reflex mediated at the spinal level). This test measures the maturation of the reflex pathways that convey sensory information from one leg to the contralateral leg (crossed reflex pathways). It provides also insight into the maturation of muscle bundles and the formation of neuromuscular connections.

1. Hold the pups firmly by the body.
2. Poke a hindlimb with a toothpick.
3. A positive response occurs when the stimulated limb flexes while the opposite limb extends.

PD6: Disappearance of rooting response.

This test can be used to test pups from PD 6-11

1. Place the pup on a flat surface.

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2. Touch gentle both sides of the head with a swab.
3. Positive response in considered when the pup crawls forwards, pushing the head in a rooting fashion.

PD7: Forelimb placing.

This test can be used to test pups from PD 7-12

1. Hold the pups firmly by the body.
2. Gently touch the dorsum of the forepaw with the edge of a swab.
3. The response is positive when the hand lift is placed itself on the object.

PD7: Tactile orientation.

This test can be used to test pups from PD 7-14. This test evaluates the appearance of tactile sensitivity.

1. Place the pup on a flat surface.
2. Touch one side of the perioral area with a cotton Q-Tip.
3. Positive response is registered when pup turns the head (orienting response) against the cotton.

PD7: Vertical climbing.

This test can be used to test pups from PD 7-12.

1. Gently place the pup on a wire-mesh grid and allow the pup to adjust to this environment for approximately 5 seconds.
2. Rotate the grid slowly to a vertical position, to challenge the grasping of all four limbs.
3. Record the angle with the aid of a template of increasing 10 degrees at which the pup falls and the latency to climb in the vertical position of the grid. If the mouse holds on to the mesh screen when inverted to 180°, record the latency to fall.

PD8: Vibrissae placing.

This test can be used to test pups from PD 8-12.

1. Gently suspend the pup by the tail with the fingers, in such a position that it cannot escape or hold on to nearby surfaces.
2. During each placing trial the motion should be gently lowering the pup towards the tip of a swab. Ensure that the majority of unilateral whiskers are touched along the swab. Otherwise the sensory input may be insufficient, and the motor response can be incomplete.
3. Positive response is registered when at contact of the cotton with the vibrissae, the pup raises its head and performs a forepaw placing response. Only count the trials in which the animal completes one full flexion–extension movement:

forelimb along body side (flexion) and forelimb resting upon the swab (extension).

PD10: Preyer reflex/startle response.

This test can be used to test pups from PD 10-17.

1. Place the pup on a flat surface.
2. Finger snap at a distance of 30 cm directly above the mouse.
3. The response of the pup (Preyer reflex), consisting of a moderately brisk flick of the pinna is recorded.

PD10: Visual placing response.

This test can be applied the day after eye opening around PD10 until PD15.

1. 1Suspend the pup by the tail.
2. Lower towards the tip of a swap without the vibrissae touching it.
3. The response is positive if it extended the paws to touch it.

PD11: Blast response.

This test can be used to test pups from PD 11-19.

1. Place the pup on a flat surface.
2. Apply near the pup a gentle puff of air.
3. Positive response is considered an exaggerated jumping or running behavior. Register the following score:

   (0) absence of response

   (1) response

   (2) jumping or running.

3. Neuromotor development: Locomotor activity has been used as a critical assay to establish the phenotypes for genetic manipulations in mice. It is arguable, though, that a delay in maturation of locomotor activity might influence adult locomotor behavior. The cranio-caudal maturation needed to reach to a correct neuromotor development takes place between PD7 to PD14. During this period, pup changes the pivoting locomotion produced by the movement of only forepaws towards straight-line walking. Neuromotor assessment is performed on PD7, 10 and 14.

1. Place pups in a empty mouse cage covered with a paper on which lines had been drawn to delineate four 90° quadrants.
2. Use gentle prodding by touching the pup's tail to motivate the pup to walk.

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3. Pivoting locomotion is assessed registering the total number of degrees turned by the pup during a 60-s period. The number of degrees is scored only in completed 90° quadrants. (Figure 3)

4. Walking activity appears when the immature locomotor pattern of pivoting disappears with the maturation of the neuromotor system. The latency for a mouse to lift up on all four legs and walk a distance exceeding its body length is measured.

4. General psychomotor development: Homing test: The test examined the general psychomotor development that requires proper integration of sensory, motor, associative and discriminative capabilities. It is based on the tendency of pups to maintain contact with the mother and the siblings. Pups have to recognize the mother's smell and be able to move towards the impregnated sawdust. From PD14 almost full sensory perception is available and mice are most of their time engaged in exploratory and investigatory behaviour.

1. On PD14 transfer individual pups to a cage containing 3/4 of new sawdust and 1/4 sawdust of the home litter ('nest arena').

2. Gently place the pups at the opposite side of the goal (nest) arena, near to the wall.

3. Register the time taken to reach the home litter sawdust (cut-off time 180 seconds).

Data analysis

The amount of time to achieve a developmental milestone (latency) and the presence or absence of a reflex was recorded and analyzed by a single experimenter who was blind to animal genotype and sex. Each test reveals the maturation of different functional domains thus data representation has to be done separately. In most of the cases, the statistical analysis is performed representing the first day of achieving a milestone with the maximum score. Number of pivotings at PD7, PD10 or PD14 is represented showing a decrease in this locomotion pattern according to the cranio-caudal maturation. Negative geotaxia, walking or homing tests, is represented as the time required to complete the task. The obtained values normally do not follow a normal distribution. Nonparametric Mann–Whitney and Wilcoxon signed-rank tests are used for single and repeated measures, respectively, to determine significant differences between groups. Fisher's exact test is used to determine differences between data points.

Representative Results

The timeline of the experimental design is represented in Figure 1. One of the most robust phenotypes described until now in the postnatal assessment of DS models is the reduced body weight and length, a feature that is maintained in the adult life. The comparison of the postnatal development of the three most used DS murine models (Ts65Dn, Ts1Cje and Dp(16)1/Yey mice) revels delays in the acquisition of neurological reflexes, as well as deficits in motor strength and coordination but at different ages among the models (Aziz et al 2018). Interestingly, Ts65Dn and Ts1Cje models, exhibited deficits in achieving early

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developmental milestones (Guedj et al., 2015; Aziz et al., 2018). Delays in body righting and coordination are observed as shown by the righting surface test in which euploid pups are able to turn over to rest in the prone position at PD7 whereas trisomic mice do it at PD9. In addition, vestibular nociception assessed by negative geotaxia is achieved in Ts65Dn and TsC1je mice around PD8–9 whereas in euploid mice is earlier (PD4-7). Motor strength delay is revealed in cliff aversion task where euploid mice complete the test at PD5-6 whereas trisomic mice at PD8-10. Similarly, deficits in forelimb grasping are observed in trisomic mice (PD7-10) compared to euploid mice (PD6-9). Trisomic mice exhibit also deficits in achieving late developmental milestones. As example, maturity of auditory system is impaired showing delayed startle response (at PD14 in euploid mice versus PD15-17 in DS models) or nociception in blast response test (at PD12-14 in euploid mice versus PD14-18 depending of the DS model). Interestingly, Dp(16)1/Yey mice present only deficits in late developmental milestone. These results suggest that the gene dosage hypothesis alone does not explain the postnatal DS phenotype since Dp(16)1/Yey mice have 13% more triplicated Hsa21 orthologs than Ts65Dn mice (Li et al., 2007). However, this model does not have a freely segregating extra chromosome, thus the disrupted genetic homeostasis produced by the aneuploidy or the altered physical state of triplicated chromatin, could be behind these differences (Pritchard & Kola, 1999).

Critical Parameters

A number of papers have described delays in achieving developmental milestones in DS murine models respect to their euploid littermates (Holtzman et al., 1996; Kazim et al., 2017; Aziz et al., 2018)(see Figure 1). However, the age at reaching the developmental milestone can differ between publications, not only in the trisomic mice but also in their respective euploid littermates. In view of this inconsistency, it is really important to standardize this protocol with the aim of minimizing the impact of the limited contact with the researcher. The low levels of noise, the experimentation period on the light-dark cycle, and time of pup manipulation are easily controllable factors, which will improve the reproducibility of the results.

Time Considerations

Neurodevelopmental assessment lasts 21 days, from PD1 to PD21. It is not recommended to separate the litter from the dams longer than 30 minutes, the longer the period the greater the probability of rejection afterwards. An experienced researcher can spend less than 4 minutes per pup applying the full battery.

Basic Protocol 2: Balance Beam

Alterations in the brain of DS mouse models can result in a loss of motor coordination and balance in mice. Deficits in limb coordination in rodents are associated with cerebellar structural deficits (Main & Kulesza, 2017; Gyengesi et al., 2019) and the ability to traverse beams of varying widths to a target destination is one of the manifestations of cerebellar dysfunction. Cerebellar structural changes and dysfunction have been replicated in DS mouse models (Baxter et al., 2000), making beam traversal a useful behavioral phenotype to include in these mouse models. Measures of traversal performance include the latency to cross a beam and the number of times the hind paws slip off of the beam while traversing the

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beam. The goal of this protocol is to assess and quantify the ability of a trisomic or normal mouse to traverse a beam of decreasing width to reach a home cage. The number of times the hind paws slip off the beam during traversal is scored from video records, with larger numbers of hind paw slips indicative of a greater motor coordination deficits, a potential behavioral correlate of cerebellar dysfunction. The test takes place over three consecutive days with two days of training and one day of testing. This procedure is based on similar protocols (Baxter et al., 2000; Carter et al., 2001; Luong et al., 2011) done using mice.

Materials

- Experimental animals [age, sex, strain, etc], are dependent on hypothesis to be tested. Performance may vary as a function of age and body size (mice at six weeks of age should be able to complete the task).

  In our case, we used male Ts65Dn and wild type littermate mice (Jackson Laboratories, stock number 001924) 50-60 days old].

- Wooden beams, square or rectangular, 1m in length, widths of 19mm, 12mm, 9 mm, and/or 6mm, painted with white or grey flat latex paint.
- Stand with metal poles, approximately 100 cm tall
- Metal clamps to attach beam to metal pole
- Goal box of ~20cm × 20cm × 20cm, made from black plexiglass
- Bedding from home cage including corncob or wood shavings and nestlet pieces
- Bedding (corncob or wood shavings, and nestlet pieces) that is unused
- 70% ethanol for cleaning
- Camera with tripod to record beam traversal on Day 3 of test

Preparation of the experimental setup

1. The mice should be acclimated to handling by an experimenter in the vivarium for several days before testing ensues.
2. Mice should be in the dark phase of the light/dark cycle (we used 12:12 reverse light cycle, so mice are housed in the dark when testing is done).
3. Room in which the testing is completed should be illuminated with red light (~ 700 nm) for an illumination of 8-10 lux.
4. The beam is attached to metal pole with clamps about 60 cm above the floor (Figure 4).
5. On the third session, camera with tripod is set up near the far end of the beam and facing the goal box to record the steps of the mouse as it traverses the beam toward the goal box, recording the performance of each mouse for all beam widths.

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Testing procedure—Before the start of testing, record identity (group, sex, age) of each mouse, as well as trial number, and label of the testing cage.

Day 1: 19 mm wide beam

1. Transfer a cohort of mice (2-5 mice, usually littermates) from their housing room to the testing room approximately 1 hour into the dark cycle (one hour after switching from light to dark in housing room).

   When transporting mice to the testing room, keep mice in the dark perhaps by covering the cage with a dark cloth.

2. Let the mice acclimate in the testing room illuminated with red light for 10 minutes.

3. Place a handful of bedding (familiar home cage bedding mixed with fresh bedding) into the black goal box.

4. Mouse is trained to traverse increasingly longer distances along the beam to reach the goal box (when fully trained, this should be ~1 m).

   a. Gently place the mouse ~40 cm from goal box in the first trial and then at increasing distances (in increments of ~20 cm) on subsequent trials, until they are able to traverse entire length of beam without stopping.

   b. If the mouse freezes or stalls on beam, gently touch the back of the mouse with a gloved finger or a small dowel to encourage progression on the beam.

   c. Gentle and patient training interactions by the experimenter are crucial for reaching the training criterion of a complete and uninterrupted traversal of the beam on the first day. The number of trials needed can vary, but the end result of a full traversal without hesitation provides a common level of performance on the first day.

5. After successfully traversing entire length of the 19 mm-wide beam without hesitation, allow the mouse to remain in the black box for 30s and return it to its home cage.

6. After the test, discard the bedding from black box, wipe the box and beam with 70% ethanol, and replace with a new mixture of fresh and home bedding for the next mouse.

7. Once the testing of all mice (2-5) has finished, return the test mice to their regular housing room.

Day 2: 12 mm wide beam

1. Follow the same steps as for Day 1 but this time with a 12 mm wide beam attached to the metal pole.

2. Mouse is trained to walk across the 12mm beam as in day 1 until mouse willingly crosses the beam without stopping on three consecutive trials.

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Note: Ending the training on Day 2 after a three consecutive unhesitating traversals of the full length of the 12mm beam limits the potential for overtraining, which may induce the mouse to stop and orient away from the task (and lose motivation to traverse the entire beam length without stopping).

Day 3: 12, 9, and 6 mm wide beam

1. Follow the same steps as for Day 1 but this time a 12, 9, or 6 mm wide beam is attached to the metal pole.
2. In this session, a camera is fastened to the pole that is opposite of the goal box to record trials.
3. Mice are given three trials on the 12 mm wide beam and results are recorded.
4. Mice are given three trials on the 9 mm wide beam and results are recorded.
5. Mice may also be given three trials on a 6 mm wide beam with results recorded.

   Note: A narrow beam introduced at this point may reduce overall sensitivity of the task if the control mice are beyond a competent level of performance (all mice making errors (Stringer et al., 2017)).

Data analysis:

1. Video records of beam traversal on final day are scored. Three trained independent scorers are used, blind to genotype or treatment.
2. Number of hind paw slips defined as either left or right hind paw entirely missing the beam and falling below the beam surface are quantified and average paw slips are reported for the genotype specific animals tested.
   a. As beam width decreases, it is expected for mice to show more paw slips.

Training Scorers

1. Scorers are trained by a person who is experienced and proficient in the task.
2. New scorers are given a written list of parameters to score and it is explained to them by a proficient scorer.
3. New scorers watch a proficient scorer enumerate the parameters in one session.
4. New scorers score a session while the proficient scorer explains the what they should see in the task.
5. New scorers score 3-4 sessions on their own.
6. New scorers ask questions to the proficient scorer.
7. New individuals score 3-4 sessions scored by the experienced scorer and values are compared.
8. If the scores of new individuals are not in sufficient agreement with those of the proficient scorer, repeat the training to increase inter-rater reliability.

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Note: A more sensitive quantification of paw slips count both paw contacts with the beam that slip off the beam and paw slips that entirely miss the beam.

Representative Results

We tested male Ts65Dn DS model and euploid mice with and without treatment of 20mg/kg or 50mg/kg EGCG in the drinking water. In one experiment using 19, 12, and 9 mm beams, we showed that trisomic (n = 9) as compared to euploid (n = 13) mice had more paw slips on the day of testing. The trisomic mice were significantly deficient on the 9 mm beam and displayed significantly more errors on the 9 mm than the 12 mm beam (Stringer et al., 2015). In a second experiment utilizing 19, 12, 9 and 6 mm beams, paw slips significantly increased for both trisomic (n = 11) and euploid (n = 19) mice as the beam width narrowed (Stringer et al., 2017). There was a trend for Ts65Dn as compared to euploid mice to commit more paw slips on the day of testing, consistent with a medium effect size of genotype with testing on all three beam widths (η^2_p=.065). The lack of statistical significance in this study in part may have been due to inclusion of the narrowest beam that resulted in increased paw slips in all groups, thereby reducing the overall effect size of genotype on the task. Our studies suggest the 9 mm beam may provide the most sensitivity to the impairment in the Ts65Dn mice, with the 9 mm beam yielding a medium effect size or better for genotype in both studies (Cohen's d=.73 in Stringer et al., 2015; d=.63 in Stringer et al., 2017)..

Basic Protocol 3: Multivariate Concentric Square Field Test (MCSF)

Cognitive deficits associated with DS are complex and many tests have been used in mouse models to establish the disease-relevant phenotypes associated with DS. There have been a number of differences and similarities in cognitive tests done on different DS mouse models and in different laboratories. When multiple tests are done on the same animal, the order of the tests may influence the outcomes such that the animal may respond differently to a particular test depending on when it occurred in a battery of tests as compared to a single test (Lundberg et al., 2019). A plausible solution to overcome some of the problems with performing multiple tests on a single animal is to use tests that measure multiple outcomes or multivariate combinations with sufficient independence to allow multiple behavioral dimensions to be evaluated simultaneously. Measuring multiple behavioral phenotypes simultaneously increases the ability to separate experimental groups of animals based on multivariate combinations of measures that align with biobehavioral dimensions that define individual differences.

The Multivariate Concentric Square Field (MCSF) is an integrated, complex, multicompartment environmental space to assess ecologically relevant behavioral activities. The MCSF can identify individual differences associated with differences in neurological or neurodevelopmental status that can alter different functional domains (locomotor, sensory, exploratory, emotional, cognitive, attentional) that are expressed through differences in behavioral patterns in the MCSF. Through a free-choice exploration of different defined environmental settings, differences in extent and distribution of patterns of locomotion, exploration, risk taking, risk assessment, and shelter seeking can be measured in DS mouse models as compared to euploid littermates. Originally developed by Meyerson and

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colleagues, the MCSF has identified distinct phenotypes in rodent models (Meyerson et al., 2006), has effectively identified other neurodevelopment disorders including effects of traumatic brain injury (Ekmark-Lewen et al., 2010), early life maternal separation stress (Daoura et al., 2010), and disparities in lines of rodents selectively bred for differences in voluntary alcohol drinking (Roman et al., 2012). In addition, repeated trials of MCSF on different days permits a test of the effects of experience (memory of the MCSF arena) and may define unique ways that the animal responds due to repeated exposure. The analysis of the test is done by the combination of ranked scores on different defined parameters in the test. The MCSF has been used recently to find differences between Ts65Dn and euploid littermate male mice given EGCG or control fluid. This protocol is based on similar studies using other rodent models (Ekmark-Lewen et al., 2010).

Materials

- Experimental Animals [age, sex, strain etc], are dependent on hypothesis to be tested.

  In our case we used male Ts65Dn and wild type littermate mice (Jackson Laboratories, stock number 001924) 45-46 days old.

- Development of the MCSF task was included in a larger collaboration between Dr. Erika Roman (Department of Pharmaceutical Biosciences. Uppsala University, Uppsala, Sweden) and faculty in the Department of Psychology at IUPUI (Roman et al., 2012). The MCSF apparatus scaled for mice (see Figure 5) was custom built at Uppsala University according to their previously published design specifications (Augustsson and Meyerson, 2004; Augustsson and Meyerson, 2005; Ekmark-Lewen et al, 2010) and shipped to IUPUI. The design consists of an outer square field [70 cm × 70 cm × 26 cm (high)] that encloses a smaller interior center square field [41 cm × 41 cm × 25 cm (high)] centered within the larger field. The center square field contains a demarcated circular zone [center circle: 16 cm diameter]. Three of the sides of the center square contain circular openings (8 cm) that permit entry into one of three corridors that makeup three sides of the perimeter of the MCSF: the north corridor (containing an entrance to the slope and bridge), the south corridor (containing an entrance to the dark corner room (DCR), and the west corridor (open sloped incline to the hurdle that contains two, 2.5-cm holes with photocells underneath the holes to measure nose pokes). The walls and three of the four sides were made of black plexiglass. The fourth side of the MCSF perimeter, made of clear plexiglass, is side-illuminated by a fluorescent light (approximately 320 lux) and contains an elevated bridge constructed of stainless steel (10 mm) wire mesh that is elevated 177 cm above the floor and is accessible from the north corridor. The DCR is enclosed and covered, and can only be accessed through the south corridor (Figure 5).

- 70% ethanol for cleaning

- Camera with Observer XT Version 15 software (Noldus Information Technology, Wageningen, The Netherlands)

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Preparation of the experimental setup

- Mouse should be in the dark phase of the light/dark cycle (we used 12:12 reverse light cycle, so mice are housed in the dark when testing is done).
- Testing room should be illuminated with only indirect dim light (8-10 lux) in the room, plus the bright light on the bridge.
- MCSF sessions are recorded with Ethovison system version 2.3 (Noldus Information Technology, Wageningen, Netherlands).
- Recorded sessions are scored offline by three independent observers (blind to treatment conditions) using Observer XT Version 15 software (Noldus Information Technology, Wageningen, The Netherlands.

Testing procedure—Before the start of testing, record identity (group, sex, age) of each mouse, as well as trial number, and label of the testing cage.

1. Transfer a cohort of mice (2-5 mice, usually littermates) from their housing room approximately 1 hour into the dark cycle (one hour after switching from light to dark in housing room) to the testing room.

   When transporting mice to the testing room, keep mice in the dark perhaps by covering the cage with a dark cloth.
2. Let the mice acclimate in a testing room illuminated with dim light for 45 minutes.
3. Make sure the apparatus is clean.
4. Set the ambient light in the center of the apparatus is ~10 lux.
5. Set the photocell counter is set to 0.
6. Ensure the light illuminating the bridge is on.
7. Gently pick up a mouse by the tail and place it in the center zone of the MCSF facing the wall with no openings.
8. Record activity for 20 minutes.
9. Note photocell counts of nose pokes and number of fecal boli after the session is complete.
10. MCSF is thoroughly cleaned between testing sessions with 70% ethanol.

Data analysis

1. Recorded sessions are scored offline by three independent and trained observers who demonstrate sufficiently high inter-rater reliability (See Training Scorers, above).
2. The three scorers' results are averaged to obtain the score value used for each animal.

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3. Outcome measures obtained from recordings include frequency of entries into defined areas, duration in areas, duration per visit, and latency to initial visit for each defined location (See Scores from MCSF).

Scores from MCSF

1. Number of entries into
   a. Corridors
   b. Hurdle
   c. Dark corner room
   d. Slope
   e. Bridge
   f. Center
   g. Center circle
2. Number of
   a. Nose poke counts
   b. Fecal boli
   c. Rearing events
3. Duration of time in
   a. Hurdle
   b. Dark corner room
   c. Slope
   d. Bridge
   e. Center
   f. Center circle
4. Latency to
   a. Initial slope visit
   b. Initial hurder visit
   c. Initial dark corner room visit
   d. Leave center circle
5. Time spent grooming

Notes: The animal has to cross into the defined zone with both hind legs to be scored as visiting that zone.

Small body size of some DS model mice (i.e. Ts65Dn) may allow them to hide in small regions (photocell count) for long periods of time and they should be eliminated from the analysis.

4. A mean of each of the individual parameter (e.g., latency to initial visit, duration, frequency, and duration per visit) for each zone of the MCSF is generated from the three independent observer scores.

Note: While total distance or velocity is available if we set the recording to generate it, it is practically meaningless because the animals disappear for long stretches in the DCR or in other nooks. We do not collect or report total distance or velocity for MCSR.

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5. Data for each contributing measure are first rank ordered across all subjects, then the ranks for the measures contributing to a given category are summed across the measures within a given animal to provide a single summed score for the animal for each category.

   Note: To assure that rank ordering reflected increasing amount of the target behavior in the category, the subject means of each measure are ranked in ascending order, except for the following which are ranked in descending order a) latency to leave the center (long latencies mean assessed risk is low), b) latency to initial slope visit (long latencies mean exploratory behavior is low), c) latency to initial hurdle visit (long latencies mean exploratory behavior is low), and d) latency to initial dark corner room visit (long latencies mean shelter seeking is low).

6. The measure of locomotor activity is derived from the total entries data (summed across the six included measures). This summed measure typically represents a continuous variable with an underlying normal distribution and may be analyzed with parametric statistics.

7. For the remaining categories (exploratory behavior, risk assessment, risk taking and shelter seeking), the rank-order analytic methodologies provide approaches to MCSF data that combine measures from different scales (frequency counts; durations) to identify multiple categories of distinctly different behavioral phenotypes that can be analyzed with distribution-free statistics. These combined measures reflect common underlying functions expressed in different behaviors or in different behavioral strategies across the complex environment of the MCSF. The five categories commonly reported are locomotor activity, exploratory behavior, risk assessment, risk taking and shelter seeking.

Different parameters included to determine behavioral properties in MSCF
Locomotor Activity = Total Entries = Frequency of all corridor entries + Frequency of hurdle entries + Frequency of DCR entries + Frequency of slope entries + Frequency of bridge entries + Frequency of center circle entries + Frequency of center entries
Exploratory Behavior = Total duration of hurdle visits + Hurdle duration per visit + Number of photocell counts + Latency to initial slope visit + Latency to initial hurdle visit + Center duration per visit
Risk Assessment = Latency to leave center arena + Slope duration per visit + (Latency to initial bridge visit)-(Latency to initial slope visit)
Risk Taking Behavior = Total duration of bridge visits + Bridge duration per visit + Total duration of center circle visits + Center circle duration per visit
Shelter Seeking = Frequency of dark corner room + Total duration of dark corner room + Duration per visit of dark corner room + Latency to initial dark corner room visit

Representative results

Male Ts65Dn (n=10) and control (n=19) littermate mice were given treatment either with ~50mg/kg/day EGCG via the drinking water or control fluid beginning on postnatal day 24, and tested for two consecutive days in the MCSF on postnatal days 45-46. As previously reported (Stringer et al., 2017), trisomic and euploid mice showed increased locomotor activity on the second day of testing compared to the first day, but the trisomic mice showed

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significantly greater increases as compared to controls across days [Fig 6, upper left panel: main effect of day ($p<.001$); day × genotype interaction $p=.004$)]. In contrast, our recent report (Goodlett et al., 2020) assessed male Ts65Dn (n=10) and euploid (n=12) mice chronically treated via daily gavage beginning on postnatal day 42 and tested in the MCSF on postnatal days 49 and 50. As shown in Fig 6 upper right panel, both groups given PBS control gavage treatments showed modest increases in activity on the second day [main effect of day ($p=.022$)] that did not approach levels seen in the fluid consumption study, Notably, the trisomic mice given PBS gavage treatments showed a significant reduction in risk taking behavior on both days relative to PBS-treated euploid mice [Fig. 6, lower right panel: main effect of genotype ($p<.001$)]. The coefficient of variation (CV) for the parametric measure of locomotor activity (total entries) was much higher in the fluid consumption study [42.9% (day 1) and 35.8% (day 2)] than in the gavage study [22,7% (day 1) and 30.2% (day 2)], suggesting the daily gavage treatments impacted the range of behavioral variation across subjects in the MCSF. In contrast, the genotype effect size for the activity measure of the two PBS-treated groups in the gavage study was relatively large (η^2_p =.144), yielding an observed power of .414. For the risk taking measure, the genotype effect size was very large (η^2_p =.487) yielding observed power of .985. The fluid consumption study yielded a relatively large effect sized for the interaction of day × genotype (η^2_p =.256) but a relatively low effect size for genotype for risk taking (η^2_p =.037). It is reasonable to hypothesize that the daily gavage treatment, in comparison with the fluid consumption treatment, was associated with chronic stress that likely diminished typical range of variation of activity and exploration on the second day in the MCSF in both groups, and the large reduction in risk-taking behavior in trisomic mice suggests that those mice are especially susceptible to behaviorally disruptive effects of the gavage treatments.

Commentary

The versatility of the set of protocols described here provides a comprehensive study of poorly explored phenotypes in DS murine models but with high incidence among the DS population. First, even individuals with DS achieve postnatal developmental milestones, but their onset is delayed respect to general population. Failure to reach key developmental milestones may at least partially correlate with cognitive challenges during adult life. There is evidence that early establishment of treatment could ameliorate cognitive and behavioral deficits in the adult life. The implementation of developmental batteries in DS murine models will help to determine the therapeutic window for the different functional domains. Second, individuals with DS are characterized by mild to severe intellectual disability that might be a limiting factor in their everyday life. However, several conditions that have been poorly studied in comparison with the cognitive impairment, are very relevant since they could lead to secondary physical pathologies.

Number of Mice Needed for Behavioral Phenotyping

One extreme individual can dramatically skew the results of a pilot experiment with a small number of animals. This is especially true for the DS mouse models where the number of outliers can be high due to the individual variation of phenotypes (Lathe, 2004; Freund et al., 2013). Larger numbers of DS mice are required for behavioral experiments than for many other phenotypic assays. To obtain statistically meaningful results, most behavioral

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experiments require 10 to 20 mice per experimental group. If sex differences are detected, N = 10–20 for each sex of each genotype is required. Ages of the mice must be approximately the same across treatment groups. Adult mice at ages 3 to 8 months are relatively homogeneous on most behavioral tasks. If large numbers of animals are not available simultaneously, experiments can be repeated with small groups as litters become available, which is quite often in DS mouse models due to reduced viability and perinatal loss of trisomic individuals. Wild-type and DS genotypes must be represented within each batch of experiments. Data across repeated experiments can be combined if no differences are detected between the wild-type controls across the dates of testing

Variation on testing between laboratories

Phenotypic variation is a hallmark of DS, but reasons behind variation in incidence and severity in phenotypes in humans have not been explained (Roper & Reeves, 2006; Zigman, 2013). Even without the genetic diversity found in humans, variation in traits also extends to DS mouse models (Fisher & Bannerman, 2019). Some variation in mouse models may be attributed to different laboratory environmental influences on genetically identical mice (Crabbe et al., 1999). In addition to some undefined laboratory variation, experiments may be performed in different ways. Even when similar tests are performed on DS model mice, there are significant differences on how these tests are performed and the outcomes of these tests. One well known example in DS model mouse testing is the Morris water maze (MWM) task (Stasko & Costa, 2004). There are a number of variations of the task including using a hidden platform, a visible platform target and reversal learning. Other variables include age of mice, the order of test administration, the number of learning acquisition days and how the animals are tested. Other secondary factors, beyond the primary hypotheses may influence the outcomes of the test. For example, both thigmotaxis and time spent floating in the pool may significantly affect the probe test results (Martinez-Cue et al., 2005). It is important to eliminate as many variables as possible from experiments and treatments with DS mice so that these experiments are reproducible between different laboratories.

Information to include in manuscripts and presentations with DS mouse models

Because of idiosyncrasies in DS mouse models, and the necessity to generate data that can be effectively reproduced in many laboratories, there is a need for adherence to information standards presented in the ARRIVE (Animal Research: Reporting of In Vivo Experiments) Guidelines (Kilkenny et al., 2010) with some additions specific for DS mouse models. Because of the high phenotypic variability displayed between individuals with DS and DS mouse models (Epstein, 2001; Prandini et al., 2007), information from ARRIVE guidelines 6-17 that encompass the presentation of methods and results is important to include in manuscripts and presentations utilizing DS mouse models. Specific to guidelines on **Study design and Experimental procedures**, numbers of animals in the experimental and control groups, precise details of how the experiments were carried out, how investigators were blinded to genotypes, and steps taken to reduce subjective bias to minimize variability between animals should be included.

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The ARRIVE guidelines on **Experimental animals** describing the source of the animals, strain, genetic modifications, sex, developmental stage and weight are important details to incorporate in work with DS mouse models.

Source and Strain: Different strains are available for Ts65Dn (Reeves et al., 1995; Costa et al., 2010) and Dp16 (Yu et al., 2010a; Lana-Elola et al., 2016) DS model mice that carry three copies of essentially the same genetic information but may have subtle differences between strains (Roper et al., 2020); therefore precise strain nomenclature and origin of animals is important. Additionally, because genetic drift may affect all mice including DS model mice that have been isolated for many generations (Bryant et al., 2008; Simon et al., 2013), information on when the mice were obtained from the source and the number of generations breeding within a particular colony need to be included.

Genetic modifications and Genotype: Some DS mouse models cannot be placed on an inbred background and often DS model mice are genetically modified by breeding to other mice. Because genetic background of DS model mice has been shown to influence DS-related phenotypes (Deitz & Roper, 2011; Li et al., 2016), it is important to state the genetic background of all mice utilized in the breeding schemes and how the crosses were generated. Sex: With the increasing knowledge of differences between the sexes of the DS model mice (Martinez-Cue et al., 2002; Block et al., 2015; Thomas et al., 2020), incorporation of how many of each sex of DS model and control mice are used is essential. Developmental stage: Subtle differences in development stages of DS model mice have been shown to influence phenotypes of mice (Roper et al., 2009; Aziz et al., 2018) and therefore the developmental stage should be precisely detailed. Age and weight: Often DS model and control mice differ in phenotypes according to their age and weight, but these variables may also be different between different mouse models of DS (Aziz et al., 2018). These factors may affect phenotypic variability, and timing and dosage of treatment. It is still not been established whether adjustments in age that consider developmental and aging differences seen in DS phenotypes should be adopted in DS mouse models.

DS mouse models are often difficult to generate in sufficient numbers of animals to use in experimental procedures (Roper et al., 2006) and may require specific animal husbandry. Because housing conditions, breeding methods and experimental conditions may influence murine phenotypes (Crabbe et al., 1999) including DS model mice (Martinez-Cue et al., 2005; Baamonde et al., 2011), it is important to follow the ARRIVE guidelines on **Housing and husbandry** including information about the origin of the trisomy (from male or female parent), number of mice housed together, environmental enrichment, and the times of the light/dark cycle (especially as correlates to experimental testing times). In accord with the **Sample size** ARRIVE guidelines, it is important to specify the exact number of animals (given as a number, not a range) used in each experiment group and condition, the number of replications in each experiment, and if any animals were eliminated from the experiment and why this was the case. An explanation of how the sample size was chosen to ensure adequate power to detect a predetermined effect size should be included. Within the sample size description, the average litter size and the numbers of litters should be included, largely because DS mouse models may have small litters and may require greater numbers of litters

to attain adequate numbers of mice needed for evaluation (Roper et al., 2006), and there may be bias in maternal behavioral differences between litters.

Because numerous experiments are often done on DS animals with multiple outcome measures, it is important to follow the ARRIVE guidelines on **Allocating animals to experimental groups**, including how animals were chosen for each group, the order of the tests, and whether the tests were performed in batches. Given the specific sensitivity of DS models to some types of stress (Martinez-Cue et al., 2005), possible stressful elements should be noted (number of mice per cage, how the mice were handled pre-experiment and during the experiment, control of hierarchical aggression, light intensity in the experimental arena, etc.). Another important factor for some learning and memory tests (e.g. those using footshock) is the different sensitivity to nociceptive stimuli. The use or not of specific measures to control for pain sensitivity needs to be specified. If using a stressful test, animals should be weighed after the experiments are finished to account for any weight loss. If treatment is chronic and experiments are done after treatment, the specific age at which experiment are done should be noted.

Additionally, it is important define the primary and secondary outcomes of each experiment as detailed in the **Experimental outcomes** of the ARRIVE guidelines. Sufficient details of **Statistical methods** should be provided, including whether the data met the assumptions of the statistical approach (e.g. normal distribution). Because there are often different numbers of DS model and control animal groups, and the variance may differ between these groups, often it is necessary to perform statistical tests to examine potential differences in variation including Bartlett’s and Levene’s tests.

Troubleshooting

Evidence shows that DS mouse models might be significantly more responsive to potential stressors. Classical behavioral mouse tests are susceptible to origin artifacts caused by the social isolation and forced handling stress by the experimenter. In the neurodevelopmental battery, the stress provoked by maternal separation and later manipulation could influence the behavioral outcomes not so much in the acquisition or loss of reflexes but in the psychomotor development. To minimize harm, it is relevant that the researcher impregnates thier gloves with nest sawdust and the handling time is as short as possible.

In general, automated devices for the high-throughput screening reduce the contact with experimenter with positive impact over the stress suffered by the mice. In the other two proposed protocols, intensive handling for several days prior to the tests may partially reduce the stress provoked by the presence of the manipulator. Routine laboratory animal handling has profound effects on their anxiety and stress responses. Many studies do not report key data for handling standardization like, if mice were handled, or the handling duration and frequency. Our experience suggests that five minutes per day of handling for at least three days, produce higher reliable data and raises standards of care in DS murine models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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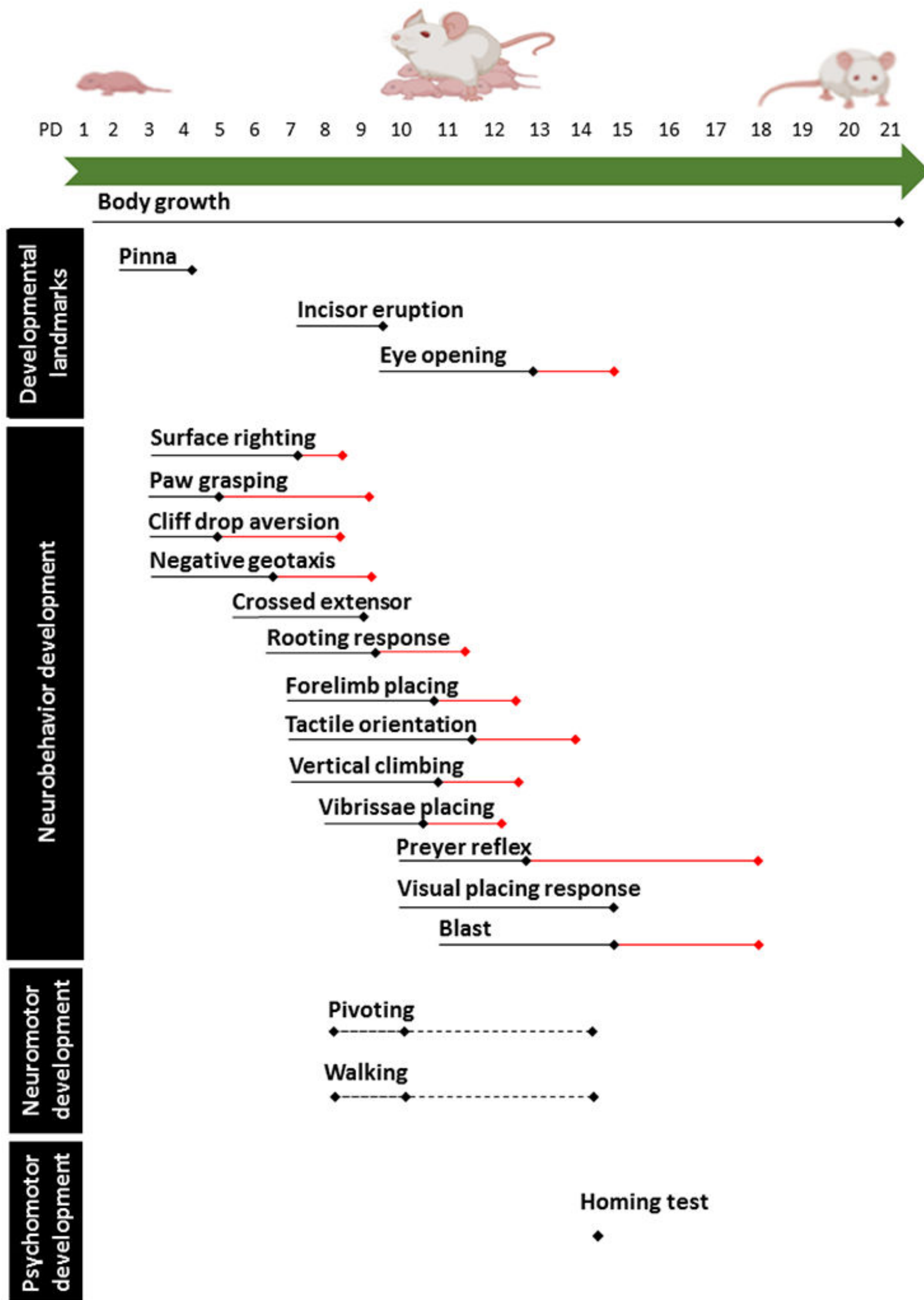

Figure 1. Representation of the postnatal neurodevelopmental battery.

Protocol lasts from postnatal day 1 (PD1) to day21 (PD21). For each milestone, the line represents the recommended age to start the evaluation and the age at 80% of mice achieve it. Prolonged line in red represents the delay in the achievement of some milestones by several DS models (Ts65Dn, Dp1Yeh, Ts1Cje).

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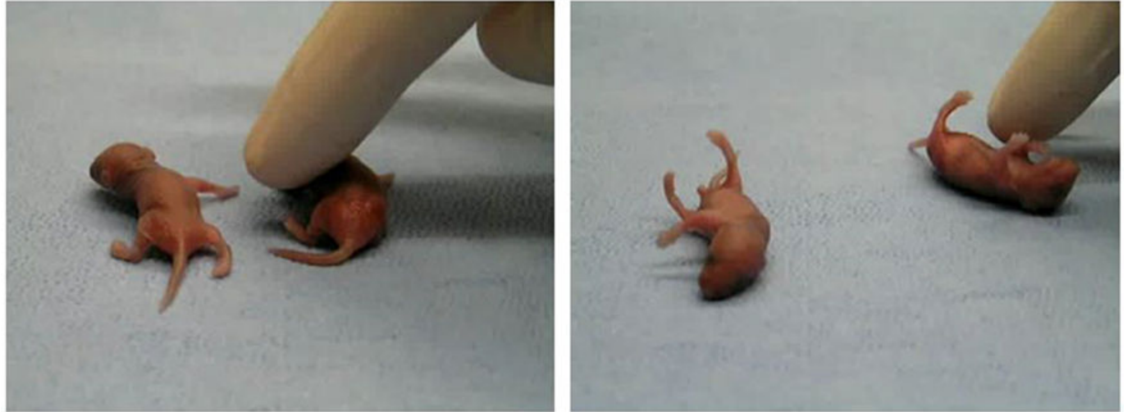

Figure 2. Surface righting reflex (see also Video 1).

The image represents the initial position of the pups on a paper towel (left) and the position of the researcher's finger to gently roll the pup to supine position (right).

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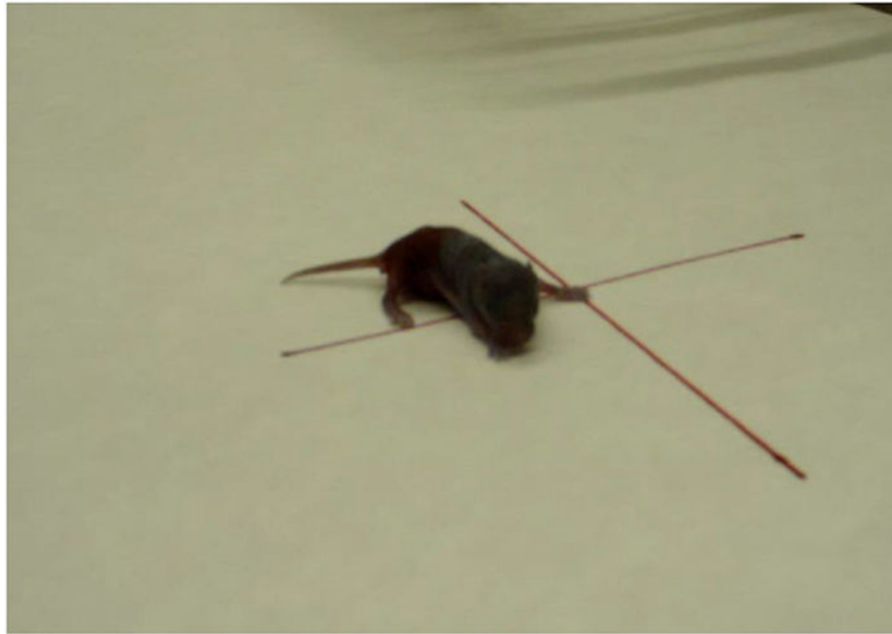

Figure 3. Pivoting test (see also Video 2).

The image represents the initial position of the pups on a paper on which lines had been drawn to delineate four 90° quadrants.

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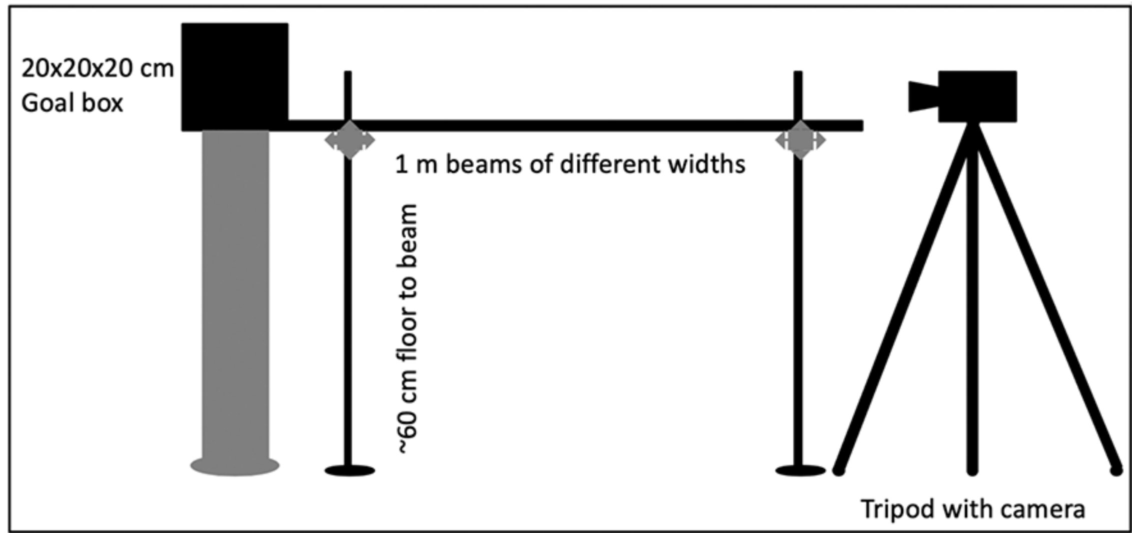

Figure 4. Schematic of the balance beam task set up

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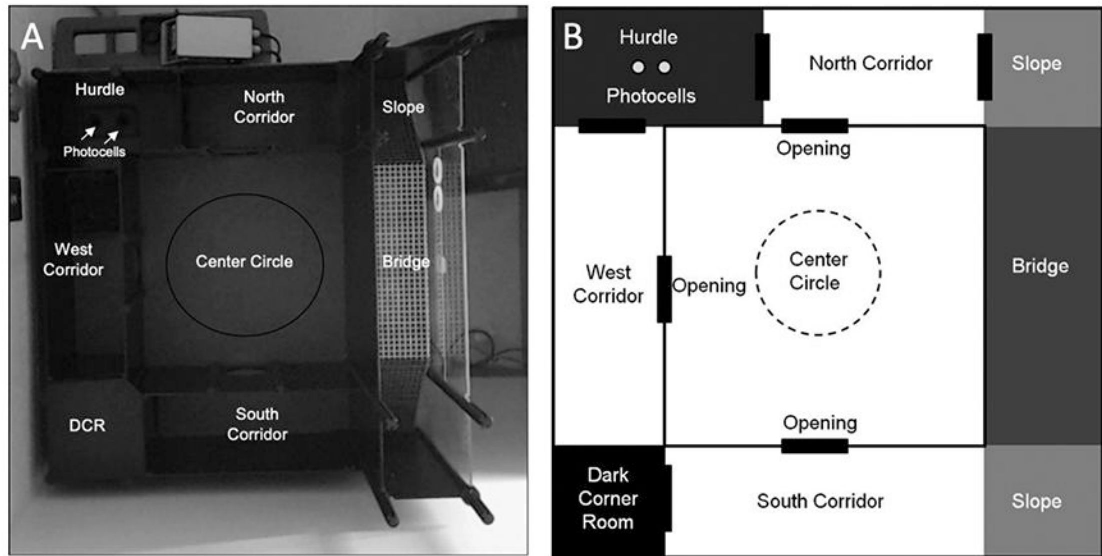

Figure 5.

Picture (A) and schematic (B) of Multivariate Concentric Square Field task for mice

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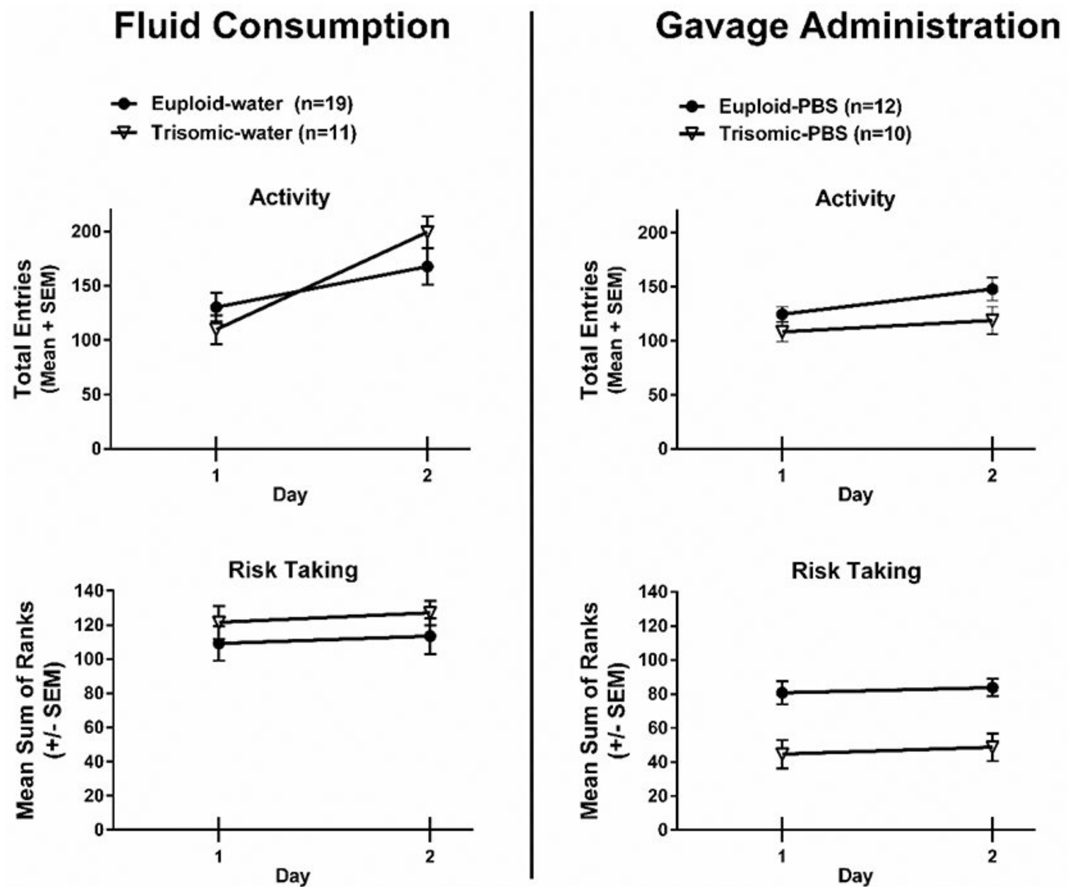

Figure 6. MCSF outcomes in Ts65Dn and euploid mice two different studies of the effects of EGCG, showing only the groups given vehicle-control treatment.

The left panels show data reported in Stringer et al. (2017) in which treatments were administered through the drinking water. The right panels show data reported in Goodlett et al. (2020) in which treatments were administered via daily gavage. Note the different patterns of outcomes in with the two different treatment conditions, particularly the more limited increase in activity on the second day in the gavage groups compared to fluid-consuming groups, and the significant reduction in risk taking behavior on both days in the trisomic mice given the control gavage treatments.