Standardized Pipeline for Metabolism and Cognition in GO-DS21 Mouse Model: Investigating Down Syndrome Comorbidities

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Down syndrome (DS) is the most prevalent form of intellectual disability (ID) globally, with an incidence rate of approximately 1 in 1000 births, affecting over 5 million individuals worldwide. DS is characterized by a genetic profile that predisposes individuals to a range of medical and cognitive conditions, including ID and obesity, which place considerable demands on healthcare systems and families alike. Mouse models carrying DS-related genetic mutations offer valuable tools for investigating the pathophysiological mechanisms underlying DS-associated behavioral and metabolic alterations. These models also allow for an evaluation of the impact of both intrinsic and extrinsic environmental factors, aiding in the development of biomarkers and personalized therapies for individuals with DS. In this article, we establish a detailed and comprehensive phenotyping pipeline designed to incorporate high-resolution assessments of cognitive, metabolic, and behavioral variables. Using advanced phenotyping techniques alongside standardized protocols in DS mouse models, this approach systematically captures the variability of DS-associated traits. Our phenotyping pipeline aims to cover the pathways leading to cognitive dysfunction and metabolic imbalances in DS, paving the way for targeted intervention strategies. © 2026 The Author(s). *Current Protocols* published by Wiley Periodicals LLC.

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INTRODUCTION

The European project GO-DS21 (Gene Overdosage and comorbidities during the early lifetime in Down syndrome) focuses on understanding the interplay of genetics, environment, and lifestyle factors in Down syndrome (DS). It aims to identify mechanisms driving the development of neuropsychiatric and associated comorbidities, like metabolic disorders. To achieve this, GO-DS21 adopts an innovative approach to systematic mouse phenotyping, aiming to explore the complex dimensions of genetic and environmental interactions that contribute to this condition and related health challenges in the context of neuropsychiatric and metabolic disorders.

The phenotyping pipeline described here was designed for DS mouse models. It includes a series of tests designed to assess a wide range of metabolic and behavioral functions, including the introduction of a high-fat diet (HFD) vs. a chow diet (CD) as a metabolic challenge to better understand and reveal the comorbidities associated with DS.

Some tests were adapted from standardized protocols used by the International Mouse Phenotyping Consortium (*https://www.mousephenotype.org/impress/pipelines*), ensuring consistency and comparability with broader research efforts (Ayadi et al., 2012; Brown et al., 2018). To ensure data robustness and the relevance of observed phenotypes, the tests are standardized and are conducted in a predefined order to minimize any potential impact of earlier procedures on subsequent analyses. Some tests are applied before and after the diet challenge to quantify the baseline and diet-induced phenotypic alterations.

However, given that the GO-DS21 project was conducted in two facilities with different pre-existing equipment and animal experimental licenses, some of the tests had to be adapted to the specific context of the facility. Therefore, alternate protocols are presented for some of the procedures.

The tests included in this pipeline evaluate key aspects such as body composition, energy metabolism, blood biomarkers, glucose homeostasis, microbiota composition, and histopathology as well as various behavioral and cognitive functions. Each test complements the others, contributing to a comprehensive understanding of the complex phenotypes associated with genetic mutations or environmental alterations in the GO-DS21 project.

A schematic representation of the chronological GO-DS21 pipeline used to assess metabolic, behavioral, and cognitive functions in the GO-DS21 project is shown in Figure 1. The pipeline includes 14 procedures from week 7 to week 29.

The basic protocols, with detailed procedures, include the following: diet challenge (CD vs. HFD; Basic Protocol 1), fecal or saliva microbiota analysis (Basic Protocol 2), body composition assessment by quantitative nuclear magnetic resonance (qNMR; Basic Protocol 3), indirect calorimetry (Basic Protocol 4), blood collection and processing (Basic Protocol 5), oral glucose tolerance test (Basic Protocol 6), long-term monitoring of social groups (Basic Protocol 7), intraperitoneal (IP) insulin sensitivity test (IPIST; Basic Protocol 8), Y-maze spontaneous alternation for evaluation of working memory (Basic Protocol 9), marble burying (Basic Protocol 10), object location memory (OLM)/object recognition for evaluation of recognition memory (Basic Protocol 11), nest- building test (Basic Protocol 12), sucrose preference analysis (Basic Protocol 13), and histopathology (Basic Protocol 14). The alternate protocols detail analysis of short-term dyadic interactions (Alternate Protocols 1 and 2), an alternative assessment of spontaneous alternation

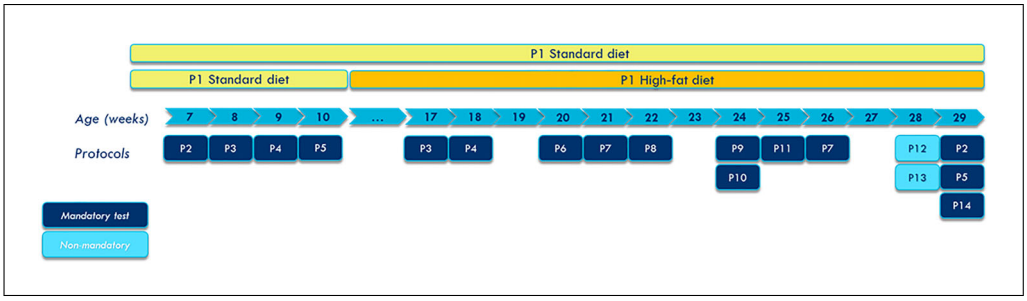

Figure 1 Overview of the GO-DS21 phenotyping pipeline for metabolism, physiology, and cognition. Starting at 7 week of age, the pipeline includes several basic protocols (P), including diet challenge (P1), feces collection for microbiota analysis (P2), quantitative nuclear magnetic resonance analysis of body composition (P3), indirect calorimetry (P4), blood collection and processing (P5), oral glucose tolerance testing (P6), Live Mouse Tracker testing (P7), intraperitoneal insulin sensitivity testing (P8), analysis of Y-maze spontaneous alternation (P9), marble-burying testing (P10), analysis of object location memory/novel object recognition (P11), nest-building testing (P12), sucrose preference assessment (P13), and histopathology (P14), with major sample collection for further OMICS analysis at week 29.

in the Y-maze (Alternate Protocol 3), an alternative analysis of marble burying (Alternate Protocol 4), and novel object recognition (NOR) in the Y-maze apparatus (Alternate Protocol 5).

NOTE: All protocols involving animals must be reviewed and approved by the appropriate Animal Care and Use Committee and must follow regulations for the care and use of laboratory animals. Here, all procedures were conducted under the following approved ethical licenses: France, #30859-2021040115336480 v5, and District Government of Upper Bavaria, Germany, #20-202. Drinking water and food were provided as appropriate.

BASIC PROTOCOL 1

DIET CHALLENGE (CHOW DIET VS. HIGH-FAT DIET)

The diet challenge is designed to assess the susceptibility of mice to metabolic stress induced by an HFD. In the GO-DS21 pipeline, this procedure helps reveal metabolic alterations associated with DS, including obesity, impaired glucose homeostasis, and changes in energy expenditure (EE). By comparing responses to a standard CD and an HFD, this protocol provides a controlled framework for evaluating diet-genotype interactions in DS mouse models.

Materials

Mice (<7 weeks of age)
Standard CD (e.g., Safe Diets D04/Altromin 1314; see Table 1)
HFD (e.g., Research Diets D12451; see Table 1)

Data sheets, for weekly monitoring
Precision scale (±0.1 g)

NOTE: Bedding and standard housing equipment should be provided for the mice, e.g., individually ventilated cages (IVCs) with standardized bedding material (Lignocel Select, SAFE), nesting material, and environmental enrichment (e.g., nesting material, igloos, tubes); with housing conditions controlled for temperature (22 ± 2°C), humidity (45% to 65%), and 12-hr light/dark cycle; and with *ad libitum* access to food (in feeder) and water.

General preparation

1. Allow mice to acclimate on standard CD prior to beginning the diet challenge.
2. Record baseline data for each animal on a data sheet: ID, genotype, sex, age, and body weight (measured with a precision scale).
3. Prepare sufficient quantities of standard CD and HFD for the entire study duration.

Table 1 Diet Composition According to Manufacturer

			Metabolized energy						
			Proteins		Fat		NFE[a]		Total
	Manufacturer	Ref. no.	kcal/kg	%	kcal/kg	%	kcal/kg	%	kcal/kg
CD[a]	Safe Diets	D04	604	18.1	279	8.4	2456	73.6	**3339**
	Altromin	1314	901	27	463	14	1976	59	**3340**
HFD[a]	Research Diets	D12451	946	20	2129	45	1656	35	**4730**

[a] CD, chow diet; HFD, high-fat diet; NFE, nitrogen-free extract.

Implementation of the diet challenge

4. For the CD Group (control condition), provide the CD *ad libitum* throughout the study.
5. For the HFD Group, replace CD with HFD, provided *ad libitum*.
6. Maintain identical environmental and handling conditions between groups. Change feeders regularly to prevent compaction of powdered diet.

Weekly monitoring

7. Record the following once per week, at the same time of day, on the data sheet: body weight, general health and behavior (e.g., coat condition, posture, activity), and any abnormal signs (e.g., more than 10% to 15% weight loss, dehydration).
8. At the end of challenge, record final body weight. Document any health issues or protocol deviations.

Expected Results

9. For mice on the CD, the following results are expected:
   a. Moderate weight gain.
   b. Balanced body composition.
   c. Respiratory exchange ratio (RER) of 0.9 to 1.0 (indirect calorimetry).
   d. Normal glucose tolerance.
10. For mice on the HFD (Research Diets D12451), the following results are expected:
    a. Accelerated weight gain.
    b. RER shift to 0.75 to 0.85.
    c. Elevated insulin and triglycerides.
    d. Impaired glucose tolerance and insulin sensitivity.
    e. Stronger metabolic dysregulation.

FECAL OR SALIVA MICROBIOTA ANALYSIS

BASIC PROTOCOL 2

Feces Collection for Microbiota Analysis

This protocol describes the standardized procedure for collecting and processing fresh fecal samples from laboratory mice to study the composition and diversity of the gut microbiota. The protocol emphasizes immediate sample collection and preservation to maintain the integrity of the microbial community structure. Fresh samples are collected during routine cage maintenance and immediately preserved by freezing in liquid nitrogen to arrest microbial activity and prevent compositional drift.

Saliva Collection for Microbiota Analysis

This protocol outlines the collection and processing of mouse saliva samples for analysis of buccal microbiota composition and diversity. To preserve microbiota stability and prevent degradation, fresh oral swabs are collected at a defined time of day (e.g., during bedding replacement) and immediately frozen in liquid nitrogen for subsequent analysis.

Materials

Mice
Liquid nitrogen
70% (v/v) ethanol

Personal protective equipment: gloves, mask, and lab coat
Collection tubes [SafeLock 2-ml microcentrifuge tubes (preferred) or standard 2-ml microcentrifuge tubes], sterile for saliva

Cleaned cage equipped with grid or platform and fresh bedding
Clean forceps
Oral swabs (breakpoint 100 mm), sterile, one per animal

Fecal microbiota analysis

1a. Put on appropriate personal protective equipment before starting the procedure.

2a. Prepare the collection tube by labeling it with the mouse ID number.

SafeLock 2-ml tubes are preferred over standard tubes, as their secure closure mechanism minimizes the risk of accidental opening when the plastic contracts at −196°C (see step 6a).

3a. Transfer a mouse from its original cage to a clean cage equipped with a grid or platform and fresh bedding.

4a. Wait briefly to allow the mouse to defecate. Collect freshly voided fecal pellets using clean forceps.

5a. Place the fecal sample into the labeled tube, removing as much bedding material as possible to limit contamination.

6a. Immediately snap-freeze the sample by immersing the tube in liquid nitrogen.

7a. Disinfect the forceps with 70% ethanol between animals to prevent cross-contamination.

8a. Store samples at −80°C until shipment or analysis.

Samples stored under this condition remain stable for at least 2 years.

9a. Record all environmental parameters and sample details in the designated metadata support file as indicated in **Metadata Records for Microbiota Analysis** section below.

Saliva microbiota analysis

1b. Wear appropriate personal protective equipment before starting the procedure.

2b. Prepare the sterile collection tube by labeling it with the mouse ID number.

SafeLock 2-ml tubes are preferred over standard tubes, as their secure closure mechanism minimizes the risk of accidental opening when the plastic contracts at −196°C (see step 10b).

3b. Prepare the workspace by arranging the cage containing the mice, sterile swabs, and labeled sterile collection tubes in an orderly and aseptic manner.

4b. Open the sterile swab packaging carefully, ensuring that the swab tip remains uncontaminated.

5b. Securely restrain the mouse to minimize stress and ensure safe and precise sampling.

6b. Insert the swab into the oral cavity:

i. Gently position the swab on the tongue and rotate it 4 to 5 times to collect oral microbiota.
ii. Move the swab to the inner left cheek and rotate it three times using gentle pressure.
iii. Repeat the procedure on the inner right cheek using the same rotational motion.

7b. Immediately transfer the swab into the labeled collection tube.

8b. Break the swab handle at the designated score line.

9b. Seal the tube tightly to prevent contamination or leakage.

10b. Store samples at −80°C until shipment or analysis.

Samples stored under this condition remain stable for at least 2 years.

11b. Record all environmental parameters and sample details in the designated metadata support file as indicated in the Metadata Records for Microbiota Analysis section below.

Metadata Records for Microbiota Analysis

Environmental data are essential for microbiota analysis, as they provide crucial context for interpreting biological findings, revealing how genetics, housing, diet, and management practices shape microbial communities. These metadata are required for all microbiome samples, regardless of collection source or analysis type. The following metadata should be collected:

1. Sample identification and biological context:
   a. Tube identification (unique ID): Enables accurate sample tracking and data linkage.
   b. Gene name: Documents genetic modifications (transgene or targeted gene, if any, of the line used, as it may impact microbiome phenotypes).
   c. Genotype: Identifies genetic determinants of microbial composition.
   d. Sample collection date (yyyy/mm/dd): Records temporal variation in microbiota dynamics.
   e. Strain including subline information (e.g., C57BL/6NJ, C57BL/6JCrl): Accounts for genetic differences affecting microbiota structure.
   f. Age in weeks: Captures age-dependent shifts in microbial diversity.
   g. Sex: Reveals sex-specific microbiome variations.
   h. Date or week of birth (yyyy/ww): Tracks cohort-level developmental effects.
   i. Sample type (e.g., feces, saliva, skin): Specifies origin, as microbiota composition varies dramatically by body site.
   j. Body weight of the individual.
2. Housing and environmental conditions:
   a. Room (animal sampled): Identifies room-specific effects on microbial composition.
   b. Room (parent housing location): Reveals maternal and parental environmental imprinting on the offspring microbiota.
   c. Cage ID: Accounts for cage-specific microbial variability.
   d. Type of cage (IVC, open): Influences ventilation-driven microbiota divergence.
   e. Sanitary status [specific pathogen free (SPF), specific opportunistic pathogen free (SOPF), germ free (GF) certificate or documents] can strongly impact the baseline microbial composition.
3. Diet and nutritional factors:
   a. Diet reference: Documents primary nutritional drivers of microbial metabolism.
   b. Diet treatment (autoclaved, irradiated): Reveals sterilization-induced changes in microbiota.
   c. Water treatment (e.g., chlorination): Identifies antimicrobial agents affecting microbial survival.
4. Husbandry practices:
   a. Bedding change frequency: Tracks microbial community disturbance and recovery.

b. Bedding type/material: Reveals bedding-sourced microbial inoculants.
c. Bedding treatment (autoclaved, irradiated): Identifies sterilization effects on cage microbiota.

5. Enrichment items: Documents the presence and type of cage enrichment (e.g., running wheels, nesting materials, mazes, tubes), as these reduce housing-associated stress and anxiety that may impact microbiome composition.

Intended Use of the Samples

These samples are adapted for downstream comprehensive metagenomic profiling of microbial communities, as in 16S rRNA gene or whole-genome shotgun sequencing. It provides high-quality material for profiling microbial communities across different body sites and developmental stages in mice. These samples can be used for robust alpha diversity analyses, including metrics of microbial richness (e.g., observed operational taxonomic units, or OTUs; amplicon sequence variants, or ASVs; and the CHAO1 index, an estimator of species richness that estimates the true number of species in a community based on the abundance pattern of rare species in your sample), evenness (e.g., Shannon and Simpson indices; Roswell et al., 2021), and phylogenetic diversity (e.g., Faith's PD; Faith, 1992), which quantify the complexity and evolutionary relationships of microbial communities within individual samples. They also allow for detailed beta diversity analyses, using distance metrics such as Bray-Curtis, Jaccard, and weighted/unweighted UniFrac, coupled with ordination approaches like principal coordinates analysis (PCoA) or non-metric multidimensional scaling (NMDS) to compare microbial community composition across samples or experimental groups. Statistical tests such as PERMANOVA or ANOSIM can be applied to determine whether observed differences between groups are significant.

Reporting of detailed metadata, as detailed in the Metadata Records for Microbiota Analysis section, allows one to account for confounding variables and distinguish biologically meaningful microbiota differences from technical or environmental artifacts.

BASIC PROTOCOL 3 BODY COMPOSITION ASSESSMENT BY QUANTITATIVE NUCLEAR MAGNETIC RESONANCE

The purpose of this protocol is to assess body composition using qNMR technology. This method provides precise, non-invasive measurements of body fat mass, lean mass, and free body fluids (extracellular fluids), offering a comprehensive analysis of the body composition of mice. Body composition is assessed using a Minispec NMR device (Bruker), which measures fat content, lean mass, and body fluid in live, awake mice. Furthermore, data on fat and lean mass are evaluated by linear regression including body weight. During the procedure, mice are gently placed in a cylindrical tube (∼50 mm in diameter) and inserted into the device. Each measurement cycle lasts ∼75 s. After the measurement, mice are returned to their home cages.

Body composition analysis is performed at two key time points in our GO-DS21 pipeline: first, at week 7, a total of 3 weeks before the dietary intervention with the HFD, and second, at week 16. This strategy enables us to assess the impact of the HFD on fat accumulation in our mouse models.

Materials

Mice
"Daily Check" sample (for calibration)

NMR analyzer (Minispec, model no. MQ10, Bruker)
Computer and Minispec Plus software

Precision scale (±0.1 g)
Data sheets, for weekly monitoring
Weighing box
Insert and piston

Preparation

1. Ensure that the NMR analyzer's front lights are yellow (right module) and green (left module).
2. Start the computer, log in, and open required Minispec Plus software.

Calibration

3. Perform daily calibration with the "Daily Check" sample and automated calibration, as instructed by the manufacturer. Check that the equipment confirms the correct calibration.

Measurement

4. Prepare mice by placing in the weighing box and collecting details (age and sex).

   No anesthesia is required, as the scanning lasts between 45 s and 60 s.

5. Weigh each mouse on a precision scale and record on the data sheet.
6. Input data into Minispec Plus software, including weight and animal ID.
7. Place the mouse head-first into the insert and secure it with the piston.
8. Begin measurement and follow prompts until completion.

   Each measurement cycle lasts ∼75 s.

Post-procedure

9. Return mice to their cages.
10. Shut down the software and log out, leaving the analyzer powered on.
11. If the insert is soiled, clean it promptly.

Data collection and export

12. Record measurements in the software log.
13. Export the data to a Microsoft Excel file and save it to the shared service area.

INDIRECT CALORIMETRY

BASIC PROTOCOL 4

Indirect calorimetry provides comprehensive insights into the energy metabolism of mutant mice. This technique evaluates EE by measuring oxygen consumption using an open-flow respirometric system. CO_2 and O_2 sensors detect differences in CO_2 and O_2 concentrations in the air flowing through control or animal cages (same as the home cage). By knowing the airflow rate through the cage, the amount of oxygen consumed over a specific period can be accurately calculated. An integrated activity and food/water intake monitoring system can be included in the setup to study circadian patterns and behavior. Using precise airflow rates and gas concentration data, oxygen consumption (VO_2) and carbon dioxide production (VCO_2) are determined. These measurements facilitate the calculation of the RER as VCO_2/VO_2, which provides insights into substrate utilization (e.g., fat vs. carbohydrate metabolism). EE or metabolic rate/heat production is calculated using the Weir equation (kcal/hr):

$$EE = (3.941 \times VO_2) + (1.106 \times VCO_2).$$

Simultaneously, locomotor activity is recorded using an infrared light beam system, capturing data on overall activity levels, distance traveled, and rearing behavior. Food and water intake is quantitatively monitored with high temporal resolution using a dedicated weighing system.

Indirect calorimetry is conducted at two distinct time points in our GO-DS21 pipeline: first, between weeks 8 and 9, which is 1 week prior to the dietary intervention with the HFD, and second, between weeks 17 and 18, which is ∼9 weeks after the initiation of HFD feeding. This approach allows us to evaluate the impact of the HFD on the energy metabolism of our mouse models.

During the procedure, mice are housed at regular room temperature with *ad libitum* access to food (either the CD or the HFD) and water. Prior to the start of the procedure, mice are weighed and then transferred from their home cages to individual calorimetric system cages, where they are acclimated to the new environment for ∼24 hr. After the acclimation period, energy metabolism parameters, including oxygen consumption (VO_2) and carbon dioxide production (VCO_2), are recorded continuously over two consecutive days. At the conclusion of the test, the mice are weighed again and returned to their home cages. Throughout the procedure, animals are monitored daily to ensure normal eating and drinking behavior. After the experiment, mice are returned to their home cages.

Data are presented as daily average values for each group, encompassing both the light phase (6 a.m. to 6 p.m.) and the dark phase (6 p.m. to 6 a.m.), expressed as mean ± SEM.

Materials

Mice
Dilute alcohol or other appropriate disinfectant

Calorimetric system equipped with cage, food hoppers, water bottles, and respirometer (PhenoMaster/LabMaster, TSE)
Precision scale (±0.1 g)
Computer with apparatus software and two monitors (ambulatory activity monitor and food and water intake monitor) installed

1. Optional: Allow mice to acclimatize to the phenotyping room, calorimetry cage, food hoppers, and water bottles for 24 hr before testing.
2. Prepare and calibrate the calorimetric system to confirm the accuracy of the gas sensors and flow meters. Specifically, do the following prior to each experiment:
    a. Apply known volumes of CO_2 and O_2 to determine the sensitivity of the gas sensors and flow meters.
    b. Run a complete calibration protocol according to the manufacturer's recommendations.
3. Provide each calorimetry cage with sufficient food and water for a period of ∼24 (or 48) hr.
4. Weigh each mouse with a precision scale.
5. Place the mouse into the calorimetry cage with food and water available *ad libitum*.
6. Label the chamber with the corresponding subject ID and close it, ensuring there is adequate air flow.
7. Initiate the calorimetric system for measurement using the computer with apparatus software and two monitors installed:

a. Set up a new experiment in accordance with the manual (or load a file from a previous experimental setting).
b. Start recording measurements 5 hr before lights off for a total duration of 21 hr at minimum.

Optionally, 24-hr acclimation can be applied, and the recording may continue for 48 hr.

The latency of CO_2 and O_2 activity transmitted and recorded is dependent on the number of chambers in use but will be logged periodically.

8. Remove each mouse from its chamber in turn at the end of the experimental session and record its weight. Return to the home cage.
9. Monitor the animals carefully to observe any abnormal behavior(s). Ensure that food and water are available *ad libitum*.
10. Wash and wipe clean the chamber with warm water and then dilute alcohol or other appropriate disinfectant.
11. Upload all data from the experiment, including the gas analysis VO_2 and VCO_2 (ml/hr/animal), heat production (kcal/hr/animal), and activity parameters.

The RER can be calculated using the VCO_2/VO_2 ratio.

The activity parameters recorded will depend on the specifications of the calorimetric system used:

Ambulatory activity can be derived from the number of beam splits during the session.

Total activity can be derived from the number of fine movements (e.g., grooming behaviors) as well as ambulatory activity.

An average of each of these parameters of activity is calculated hourly across the measurement period.

Water and food intake (cumulative, hourly, or total food and water intake) can also be determined.

BLOOD COLLECTION AND PROCESSING

BASIC PROTOCOL 5

This protocol provides a procedure for collecting and handling blood from mice using the retro-orbital or facial vein puncture method, in order to measure various blood biomarkers according to the specifics of the study. The retro-orbital method involves accessing the venous plexus behind the eye, whereas the facial vein puncture targets the veins located under the jaw (Fig. 2). Both approaches are effective for obtaining blood samples for various analyses, including biochemical and hormonal studies. This protocol outlines the necessary steps, considerations, and best practices to ensure successful and humane blood collection, aiding researchers in achieving accurate and reliable results in mouse phenotyping studies. To ensure the accuracy and reliability of these analyses, it is crucial to handle blood samples appropriately. This protocol details the procedures for preparing plasma samples for measuring various parameters, such as biochemical markers, lipid levels, and enzyme activities, and other specialized analyses.

Because blood volumes that can be collected from mice are quite limited in volume, analyses and samples have to be carefully planned beforehand to organize proper sample collection. For blood samples collected once within 2 weeks from a living mouse, it is recommended that the volume not exceed 7.5% of total blood volume (Li et al., 2025; see also the GV_SOLAS guidelines, *https://www.gv-solas.de/wp-content/uploads/2017/03/tie_blutentnahme17_e.pdf*), which is considered to make up 7.7 to 8 µl/g of body weight. During final blood collection, as much blood as possible can be collected.

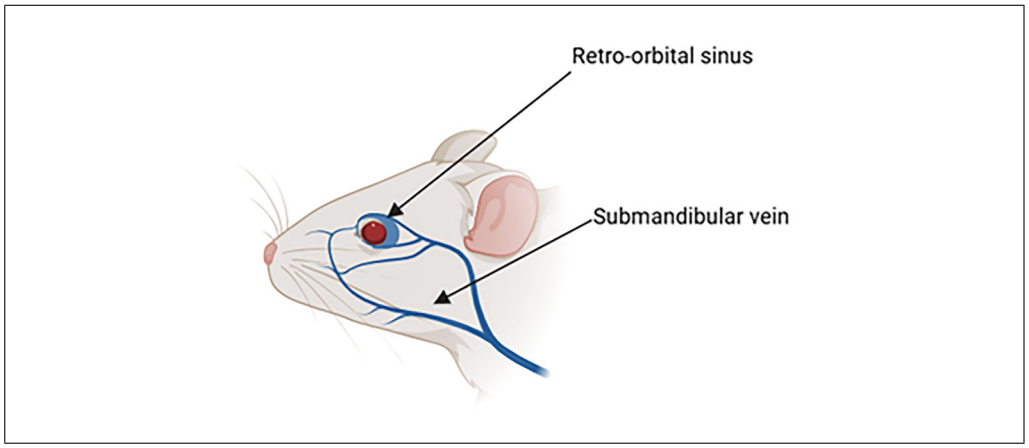

Figure 2 Schematic of the submandibular vein and retro-orbital sinus. Image generated using BioRender.

However, achieved volumes depend on the method chosen and the experience of the experimenter.

For each analysis, check beforehand about the required sample quality (e.g., Li-heparin plasma for clinical chemistry, EDTA–whole blood for hematology, whole blood collected in Qiagen RNAprotect Animal Blood Tubes for transcriptomics) and volumes to calculate, if all analyses can be conducted with the expected sample volumes. Prepare and label all sample collection tubes required for planned analyses for every animal (e.g., EDTA-coated tubes, Li-heparin-coated tubes, RNAprotect blood tubes). If plasma samples are to be used for a set of different analyses, prepare sample tubes (e.g., Eppendorf microtubes) or plates (e.g., microtiter plates for samples designated for ELISA) in order to freeze separate aliquots for all tests planned to avoid the necessity of repeated thawing and freezing of samples.

Materials

Isoflurane
Oxygen
Mice
0.9% (w/v) NaCl solution (for fluid replacement)

Collection tubes (determine appropriate type according to planned analysis):
- Li-heparin-coated tubes, for clinical chemistry and hormone measurements (Insulin, Corticosterone, T3/T4)
- EDTA-coated tubes, for proteomics and adrenocorticotropic hormone (ACTH) measurements
- RNAprotect Animal Blood Tubes (Qiagen), for blood samples used for transcriptomics

Microtubes (Eppendorf)
Anesthesia system
Precision scale (±0.1 g)
Autoclaved nose cones
Small glass capillary tubes (micro-hematocrit capillary tubes, max. 1-mm diameter, without heparin coating, e.g., Hirschmann) or Pasteur pipets (VWR, cat. no. HIRS92250101)
Soft absorbent paper
18G/19G needles
1-ml syringes
Refrigerated microcentrifuge (Biofuge Fresco, Heraeus), 8°C

Microtiter plates (optional)
Adhesive foil (optional)

Pre-sampling preparation

1. Determine sampling time based on experimental protocol and metabolic state (fed/fasted/fasting duration).
2. Label collection tubes and microtubes with mouse IDs.
3. Depending on the anesthesia system, set isoflurane concentration between 1% and 2% or 3% and 5% and adjust oxygen or compressed air flow rate to 0.3 or 0.5 L/min.
4. Weigh the animal beforehand on a precision scale to calculate the maximum blood volume that can be collected depending on the body weight.

Blood collection procedures

Retro-orbital sinus collection

5a. Anesthetize mouse using the gas anesthesia setup (isoflurane at 3% to 5% in compressed air or oxygen via a narcosis induction chamber) and autoclaved nose cone.

6a. Once the animal is properly narcotized, grab by the neck, tightening the skin around the neck in order to apply pressure on the jugular veins.

7a. Insert a small glass capillary tube (micro-hematocrit capillary tube, max. 1-mm diameter) at the medial/nasal corner of the eye between bulbus and eyelid with mild pressure, while rotating it between index finger and thumb to pierce the retrobulbar vein plexus.

8a. Dispense blood into the labeled collection tubes (see step 2), taking into account maximum recommended volume for blood withdrawal if the animal will be kept alive.

9a. Close the tubes and slowly invert 3 to 4 times immediately after blood has been added.

10a. Loosen the grip on the skin to relieve pressure on the jugular veins, remove the capillary tube, and press soft absorbent paper onto the mouse's closed eye for a few seconds to stop the bleeding if the animal will be kept alive. Check for bleeding afterward and observe mouse recovery.

11a. In the case of terminal blood collection, euthanize the animal immediately after blood collection by cervical dislocation, before it reaches consciousness.

Submandibular facial vein collection (should be preferred, as it is less traumatic)

5b. Anesthetize the mouse using the prepared gas anesthesia setup (isoflurane at 3% to 5% in compressed air or oxygen via a narcosis induction chamber) and autoclaved nose cone.

6b. Securely immobilize the anesthetized mouse's head.

7b. Puncture the submandibular vein with an 18G/19G needle.

8b. Collect blood as it flows from the vein using a Pasteur pipet.

9b. Prioritize filling hematological analysis tubes if needed.

10b. Distribute remaining blood into other collection tubes.

11b. Close tubes and gently invert immediately after filling.

12b. Apply gentle pressure to the mandible area with soft absorbent paper for 10 to 15 s.

13b. Monitor mouse for 5 to 10 min to ensure bleeding has stopped.

14b. Administer a subcutaneous injection of 0.9% NaCl solution, with a volume equivalent to collected blood volume, using a 1-ml syringe with 18G/19G needle.

15b. Observe the mouse until full recovery from anesthesia.

16b. Confirm absence of bleeding or distress.

Blood handling

17. After sampling, store blood samples at room temperature for 20 to 30 min up to 1 hr.

18. Centrifuge samples for 10 min at 5000 × *g*, 8°C, in a refrigerated microcentrifuge.

19. After centrifugation, collect the supernatant (plasma) with an adjustable pipettor. Avoid collecting red blood cells from the pellet.

20. Dispense plasma into the labeled microtubes (see step 2) and/or microtiter plates, taking care that the animal ID number on the initial sampling tube is unambiguously linked to each microtube and microtiter plate well for each aliquot of the sample. Close the tubes and seal microtiter plates carefully with adhesive foil to avoid evaporation. Prepare separate aliquots for each analysis planned.

21. Analyze blood samples and plasma aliquots immediately or store at −20° to −80°C until analysis is conducted.

    In the case of sample shipment, freeze plasma samples at −80°C. Send the samples in dry ice.

BASIC PROTOCOL 6

ORAL GLUCOSE TOLERANCE TEST

The glucose tolerance test measures the clearance of a glucose load, a primary energy source, from the body. This test enables detection of disturbances in glucose metabolism, which are often linked to conditions such as diabetes and metabolic syndrome.

To perform the test, animals are typically fasted. The fasting period ensures that their baseline blood glucose levels are measured accurately, without the influence of recent food intake. In our pipeline, we chose an overnight fasting protocol. Once fasting blood glucose levels are determined, a glucose solution is administered to the animals through oral gavage, a method that delivers the solution directly into the stomach. Following the administration of the glucose solution, blood glucose levels are monitored at various intervals over the next 2 hr. These measurements help track how efficiently the body clears the glucose from the bloodstream. In healthy individuals, blood glucose levels usually rise initially after the glucose is administered and then return to baseline levels within the 2-hr period. However, if the body struggles to regulate glucose effectively, the levels may remain elevated for a longer duration, indicating potential issues with insulin function or sensitivity.

We further include additional blood collection from the tail vein before glucose administration, after basal glucose levels are measured, and after glucose measurements at the time points 15 and 30 min after glucose administration for insulin level measurements. A volume of 30 µl whole blood is collected for each time point in Li-heparin-coated Microvette tubes (Microvette® 300 Lithium Heparin LH, 300 µl, Sarstedt, cat. no. 20.1309), centrifuged for 10 min at 10,000 rpm, 4°C (~5000 × *g*, Biofuge Fresco, Heraeus), and then 10 to 15 µl plasma is transferred into 96-well microtiter plates (Greiner Bio-One,

cat. no. 655101) placed on wet ice, until all samples from one group of mice (max. 12 animals tested in parallel) are collected. Afterward, microtiter plates are sealed with adhesive foil and stored at −20°C until analysis by ELISA (Ultra Mouse Insulin ELISA Kit, Crystal Chem, cat. no. 90080).

Materials

Mice
20% (w/v) glucose solution
Topical anesthetic cream (optional)

Clean cages
Experiment record sheet
Precision scale (±0.1 g)
1-ml syringes with gavage tubes
Restraining device (optional)
Fresh or sterilized scalpel blades or sharp sterilized scissors
Blood glucose meter and test strips
Li-heparin-coated or EDTA-coated capillary sample collection tubes
Soft absorbent paper

1. Fast mice overnight for ∼16 hr by transferring mice to clean cages with no food or feces in hopper or bottom of cage. Ensure that they have access to drinking water at all times.
2. Prepare an experiment record sheet for glucose measurement.
3. Weigh the mouse on a precision scale.
4. Calculate and record the volume of 20% glucose solution required (2 g glucose/kg body mass) for oral gavage injection and prepare an accordingly filled 1-ml syringe with a gavage tube for every mouse.
5. Optional: Apply topical anesthetic cream:
    a. Apply a small amount of topical anesthetic cream to the tail of the mouse, spreading over the tail evenly.
    b. Gently massage it in for ∼10 s to enhance the effect of the anesthetic cream, ensuring that the proposed incision site is fully covered.
    c. Allow an appropriate length of time for the local anesthetic to take effect.
6. Optional: Restrain the mouse in a restraining device with the tail exposed.
7. Score the tip of the tail using a fresh or sterilized scalpel blade or sharp sterilized scissors.
8. Discard first small drop of blood. Place another small drop of blood (3 µl) on the test strip of the blood glucose meter. For insulin level analysis, collect an additional sample of ∼30 µl in a Li-heparin-coated or EDTA-coated capillary sample collection tube by gently massaging the tail several times from the tail base to tail tip and collecting the blood drops. Apply gentle pressure to the puncture site using a clean soft absorbent paper to stop bleeding.

    The level measured with the test strip is the baseline glucose level ($t = 0$) and is recorded on the experiment record sheet from step 2.

9. Optional: Remove the mouse from the restraining device if this was used (see step 6).

10. Administer glucose solution to the mouse by oral gavage with the appropriate volume, as previously determined and prepared (step 4), and note the time point of injection.

11. Measure the blood glucose levels at 15, 30, 60, and 120 min ($t = 15$, $t = 30$, $t = 60$, and $t = 120$) after glucose injection by placing a small drop of blood on a new test strip and recording the measurements. Start the bleeding again by removing the clot from the first incision, massaging the tail if blood flow is inadequate. Record results on the experiment record sheet. Collect additional samples for insulin measurements at 15 and 30 min as described for the $t = 0$ blood collection (see step 8).

12. Ensure that further blood loss from the incision is minimal by briefly applying pressure to the incision with soft absorbent paper after each measurement.

13. At the end of the experimental session, place the mouse in a clean cage with water and food available *ad libitum*.

14. Monitor the animals carefully to observe any abnormal behavior(s).

BASIC PROTOCOL 7

LONG-TERM MONITORING OF SOCIAL GROUPS

The long-term monitoring of social groups assesses the daily activity patterns and social interactions expressed spontaneously by mice living in social groups over three consecutive days and nights. Mice are housed with their familiar cage mates, allowing the capture of spontaneous, undisturbed behaviors.

Materials

Mice (groups of four, with two mutant and two control mice of the same sex housed together for ≥3 weeks)
Isoflurane
Oxygen
Local analgesia (lidocaine)

RFID tags (APT 12 PIT tags, Biomark), disinfected with disinfectant compatible with internal application (Hibitane 5%)
Anesthesia system
Fresh bedding
Water bottle
Red Plexiglas house
Dental cotton rolls, as nesting material
Live Mouse Tracker system, including test arena, antenna floor, Kinect camera, computer, and environmental monitoring device connected through USB (de Chaumont et al., 2019; *https://micecraft.org/lmt/*)
Manipulation tube

Additional reagents and equipment for subcutaneous RFID tag insertion and local analgesia administration

1. Insert an RFID tag subcutaneously into each mouse (in the lower side of the flank) under gas anesthesia (with isoflurane and oxygen), with local analgesia injected subcutaneously, ≥1 week before the experiment.

2. Prepare the test arena on the day of the test, with fresh bedding, food *ad libitum* on the floor, and drinking water in a bottle fixed outside the arena. Place the red Plexiglas house in the left bottom quarter of the cage. Add 3 to 6 dental cotton rolls on the floor.

3. Launch the Live Mouse Tracker system and set the initial parameter "Max number of animals" to 4.
4. Gently introduce the four mice into the test arena using manipulation tube.
5. Once all the animals are in the setup, press "Start" to start the recording using Live Mouse Tracker. Check that each mouse has been identified through its RFID tag.
6. Leave the room and record the experiment for three consecutive days and nights.
7. After the 3 days of recording, stop the recording using the "Stop" button and place the mice back in their home cage using the manipulation tunnel.
8. Clean the test arena using soap and water and dry it with paper towels.
9. Repeat steps 2 to 8 until all groups of four mice are tested.
10. Process the files for the analyses following the LMT scripts found on GitHub (*https://github.com/fdechaumont/lmt-analysis*) or one of the dedicated applications (e.g., *https://github.com/ntorquet/lmt_toolkit_analysis*).

ANALYSIS OF SHORT-TERM DYADIC INTERACTIONS BETWEEN UNFAMILIAR MICE OF THE SAME GENOTYPE

ALTERNATE PROTOCOL 1

This short-term dyadic interaction testing assesses locomotor activity and the social contacts displayed by a pair of unfamiliar mice of the same genotype during a brief encounter.

Additional Materials *(also see Basic Protocol 7)*

Mice (groups of two of same sex and same genotype but housed separately)

Light meter

1. If the mice have not been equipped with RFID tags, follow step 1 of Basic Protocol 7 at $\geq$1 week before the experiment.
2. Transport animals from housing room to the holding room (ideally adjacent to the testing room) and leave them undisturbed for $\geq$15 min to acclimate.
3. Prepare the test arena by adding a thin layer of fresh bedding.
4. Adjust the light condition in the testing room to 50 to 100 Lux in the middle of the cage using a light meter.
5. Launch the Live Mouse Tracker system and set the initial parameter "Max number of animals" to 2.
6. Gently introduce two mice of the same sex and same genotype but from two different cages into the test arena using manipulation tube.
7. Press "Start" to start the recording using Live Mouse Tracker.
8. Leave the room to let the animals interact freely for 1 hr.
9. Stop the recording using the "Stop" button.
10. Place the animals back in their respective home cages.
11. Discard the bedding. Clean the test arena using soap and water and dry it with paper towels.
12. Repeat steps 3 to 11 until all mice have been tested.

13. Process the files to control the quality of the acquisition with LMT-toolkit (*https://github.com/ntorquet/lmt_toolkit_analysis*) and to analyze the data following the LMT-analysis scripts (*https://github.com/fdechaumont/lmt-analysis*).

ALTERNATE PROTOCOL 2

ANALYSIS OF SHORT-TERM DYADIC INTERACTIONS OF MICE FROM THE TESTED STRAIN WITH AN UNFAMILIAR C57BL/6J MOUSE

This short-term dyadic interaction testing assesses locomotor activity and social contacts during a brief encounter between one mouse of the tested strain and an unfamiliar mouse of a standard commercial strain (that remains standard across the genotypes of the tested strain).

Additional Materials *(also see Basic Protocol 7)*

Wild-type or mutant mice
C57BL/6J mice (of same age and sex as wild-type or mutant mice, housed in groups and referred to as newcomers)

1. If the mice have not been equipped with RFID tags, follow step 1 of Basic Protocol 7 at $\geq$1 week before the experiment.
2. Transport animals from housing room to the holding room (ideally adjacent to the testing room) and leave them undisturbed for $\geq$15 min to acclimate.
3. Prepare the test arena by adding a thin layer of fresh bedding.
4. Adjust the light condition in the testing room to 50 to 100 Lux in the middle of the cage.
5. Launch the Live Mouse Tracker system and set the initial parameter "Max number of animals" to 2.
6. Gently introduce the wild-type or mutant mouse to be tested into the test arena using manipulation tube.
7. Press "Start" to start the recording using Live Mouse Tracker. Leave the room.
8. Leave the tested mouse to explore the arena for 20 min to habituate the mouse to the environment.
9. At the end of the habituation period, press "Pause" to temporarily hold the recording.
10. Introduce the newcomer (C57BL/6J mouse of the same age and sex as the tested mouse) using the manipulation tube.
11. Press "Paused" to continue tracking and make sure the two animals are being identified through their RFID tags.
12. Leave the room to let the animals interact freely for 30 min.
13. Stop the recording using the "Stop" button.
14. Place the animals back in their respective home cages.
15. Discard the bedding. Clean the test arena using soap and water and dry it with paper towels.
16. Repeat steps 3 to 15 until all mice have been tested.
17. Process the files to control the quality of the acquisition with LMT-toolkit (*https://github.com/ntorquet/lmt_toolkit_analysis*) and to analyze the data following the LMT-analysis scripts (*https://github.com/fdechaumont/lmt-analysis*).

Critical Points

For long-term monitoring, make sure the light/dark circadian rhythm is working correctly in the room (light: 50 to 100 Lux in the middle of the arena during the day). Before introducing mice into the LMT arena, check that the test arena is centered on the antenna floor using the Calibration module in the Live Mouse Tracker application. Before launching the recording, adjust the "Experiment name:" keep the random number but add your desired name along with the date.

INTRAPERITONEAL INSULIN SENSITIVITY TEST

BASIC PROTOCOL 8

The IPIST is a method used for evaluating sensitivity to insulin by measuring the decrease in blood glucose levels in response to an insulin bolus. This test assesses how efficiently insulin facilitates glucose uptake and clearance, in the context of metabolic studies involving diabetes, obesity, and insulin resistance.

Animals are fasted for ∼4 hr to ensure a stable baseline blood glucose level. The basal blood glucose level is measured before insulin administration via IP injection. After insulin injection, blood glucose levels are monitored at regular intervals, typically at 15, 30, 45, 60, and 90 min post-injection, to track the glucose-lowering effect of insulin over time.

The results of the IPIST provide crucial insights into metabolic health. A rapid decline in blood glucose suggests normal or heightened insulin sensitivity, whereas a slower response indicates insulin resistance, a hallmark of metabolic disorders.

Materials

Bovine serum albumin (BSA)
Water, sterile
10 N HCl
0.9% (w/v) NaCl
10 IU/ml human insulin solution
Mice

Balance
Weighing boat
Bottle
pH indicator strips
Microtubes (Eppendorf)
Clean cages
Precision scale (±0.1 g)
Fresh or sterilized scalpel blades or sharp sterilized scissors
Blood glucose meter and test strips
Experiment record sheets
1-ml syringes
26G injection cannula
Soft absorbent paper

1. Prepare insulin diluent solution within 1 week before the experiment:
    a. Weigh 0.05 g BSA into a weighing boat and transfer quantitatively into a suitable bottle. Add 10 ml sterile water and then add 1 µl of 10 N HCl (0.5% final BSA concentration). Mix gently by manual inversion.
    b. Check that the pH of the solution is between 4 and 5 using pH indicator strips.

c. Add 52.6 ml of 0.9% NaCl and mix gently by manual inversion. Store solution at 4°C until use.

2. Prepare insulin injection solution the day before or day of use:

   a. Gently mix the human insulin solution (10 IU/ml) by inverting the container several times.
   b. Using a calibrated pipet, withdraw 10 µl insulin solution and transfer it into a clean microtube.
   c. Add 7.2 ml of the insulin diluent from step 1 to the tube.
   d. Mix the solution gently by inverting the tube several times to ensure homogeneity.

3. If the insulin injection solution from step 2 was prepared the day before use, store at 4°C overnight. Take out in the morning and let it warm to room temperature.

4. Fast mice for 3 hr in the morning by transferring them to clean cages without food supply. Ensure that they have drinking water at all times.

5. Weigh mice on precision scale to determine body weight and calculate volume of insulin solution to be injected.

6. Score the tip of the tail using a fresh or sterilized scalpel blade or sharp sterilized scissors. Discard first small drop of blood. Place another small drop of blood (3 µl) on the test strip of the blood glucose meter. Record the result on the experiment record sheet.

   The measured level is the baseline glucose level ($t = 0$).

7. Inject calculated volume of insulin solution (see step 5) using a 1-ml syringe with a 26G injection cannula.

8. Measure the blood glucose levels at 15, 30, 45, 60, and 90 min ($t = 15$, $t = 30$, $t = 45$, $t = 60$, and $t = 90$) after glucose injection, placing a small drop of blood on a new test strip and recording the measurement. Start the bleeding again by removing the clot from the first incision, massaging the tail if blood flow is inadequate. Record results on the experiment record sheet.

9. Ensure that further blood loss from the incision is minimal by briefly applying pressure to the incision with soft absorbent paper after each measurement. At the end of the experimental session, place the mouse in a clean cage with water and food available *ad libitum*.

10. Monitor the animals carefully to observe any abnormal behavior(s).

BASIC PROTOCOL 9

Y-MAZE SPONTANEOUS ALTERNATION TEST

The Y-maze spontaneous alternation test is a relatively simple and quick procedure to assess spatial working memory. It is based on the natural tendency of rodents to explore a novel environment. When placed in the Y-maze, normal mice prefer to explore the least recently visited arm and thus tend to alternate visits between the three arms. To explore the three arms successively, the mouse must maintain an ongoing record of the most recently visited arms and continuously update such records. A mouse with impaired working memory cannot remember which arm it just visited and thus shows decreased spontaneous alternation (Holcomb et al.,1998; Wall & Messier, 2002). This task also involves some aspects of attention related to active working memory (Wall & Messier, 2002). Scoring other behavioral parameters such as alternate arm returns (AARs) and same arm returns (SARs) can assess this. Finally, the novelty of the maze also generates

a state of anxiety, which can be assessed by scoring the time the mouse takes to exit the starting arm.

Materials

Mice (of desired strain, gender, and genotype depending on study objective)
50% (v/v) ethanol

Video-monitoring system (e.g., camera placed on ceiling of room, over apparatus, and connected to TV monitor or computer for observation; system should be placed out of animal's sight, ideally in an antechamber)
Light meter
Y-maze [homemade from Plexiglas, with three identical arms (40 × 9 × 16 cm) placed at 120° from each other, each with internal specific patterns allowing them to be distinguished from each other; for experiment, arms are identified as A, B, and C or 1, 2, and 3; see Fig. 3)
Stopwatch or timer
Video-tracking software (e.g., EthoVision XT, Noldus)

1. Transport mice from housing room to the holding room (ideally adjacent to the testing room) and leave undisturbed for ≥15 min to acclimate.
2. During the acclimation period, prepare the monitor and camera of the video-monitoring system to ensure a clear image will be obtained and place the monitor in an antechamber.
3. Using the light meter, adjust the illumination in the testing room to ∼100 Lux in the center of the Y-maze.
4. For testing, put the first mouse at the end of one arm of the Y-maze, with the head directed to the distal wall, and allow it to explore the apparatus freely for 5 min, with the operator out of the testing room.
5. Start the stopwatch or timer and observe the mouse's behavior via the monitor.
6. When the mouse leaves the starting arm (with all four paws), note this latency.
7. Note the pattern of entrance into the arms of the maze (e.g., A, B, A, etc.).

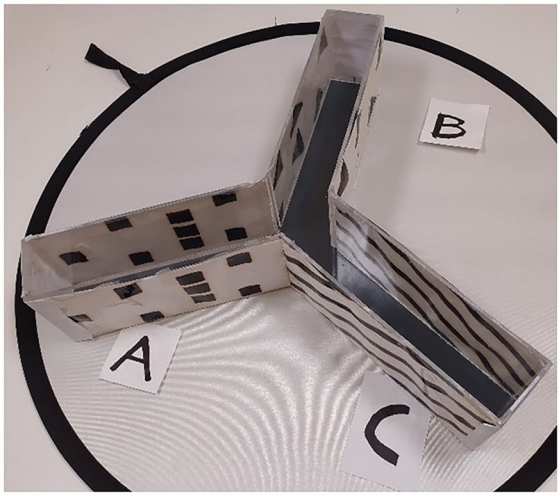

Figure 3 Y-maze with three distinct patterned walls providing intramaze cues.

8. At the end of the run (after 5 min), ensure identification of the mouse (if necessary) and replace it in its home cage.
9. After each mouse is tested, remove feces and clean the maze with tap water and then 50% ethanol.
10. Proceed the same way until all mice have been tested.
11. At the end of the experiment, return mice to their housing room.
12. Perform appropriate data analysis with video-tracking software:
    a. Operationally define alternations as successive entry into each of the three arms as in overlapping triplet sets (e.g., ABC, BCA, etc.).
    b. Determine spontaneous alternation performance percentage (% SPA), defined as the ratio of actual (total alternations) to possible alternations (total arm entries – 2) × 100.
    c. Score the number and percentage of AARs and SARs for each animal to assess aspects of attention within spontaneous working memory.

    Not taking SARs into account means that % SPA can be compared with 50% (chance).

    d. Score total entries and latency to exit the starting arm as indices of ambulatory activity and emotionality, respectively in the Y-maze.

ALTERNATE PROTOCOL 3

Y-MAZE SPONTANEOUS ALTERNATION TEST VERSION 2

The Y-maze spontaneous alternation test is a measure of short-term working memory and executive function, relying on the mouse's innate tendency to explore novel environments (Prieur & Jadavji, 2019). In this alternate version (compare to Basic Protocol 9) for testing DS-relevant models, all arms are identical and devoid of intramaze cues to reduce reliance on spatial landmarks. Working memory impairments are a well-established feature of DS and have been linked to hippocampal and prefrontal cortex dysfunction in DS mouse models. This assay is therefore a valuable tool in DS-focused pipelines. As outlined in Alternate Protocol 4, the Y-maze can also be adapted to assess NOR, further extending its relevance in cognitive phenotyping for DS.

Additional Materials *(also see Basic Protocol 9)*

Y-maze (arm length: 29.5 cm; width: 8 cm; height: 15 cm) with homogenous bare walls (see Fig. 4)
Sound-attenuated testing room
Mounted ring light
Precision scale (±0.1 g)
Clean cages

1. Place the Y-maze with homogeneous bare walls in a sound-attenuated testing room and ensure uniform illumination using a mounted ring light with 80 Lux in the center of the maze.

   Avoid stress and noise during testing, as this will affect the spontaneous behavior of the mouse.

2. Allow mice ≥30 min to acclimatize to the testing room before commencing.

   Testing can start at the beginning of the light phase, but give 1 hr after lights on.

3. Place the Y-maze on the floor so one arm is pointing to the observer's position during the test and label each arm using a card (Fig. 4).

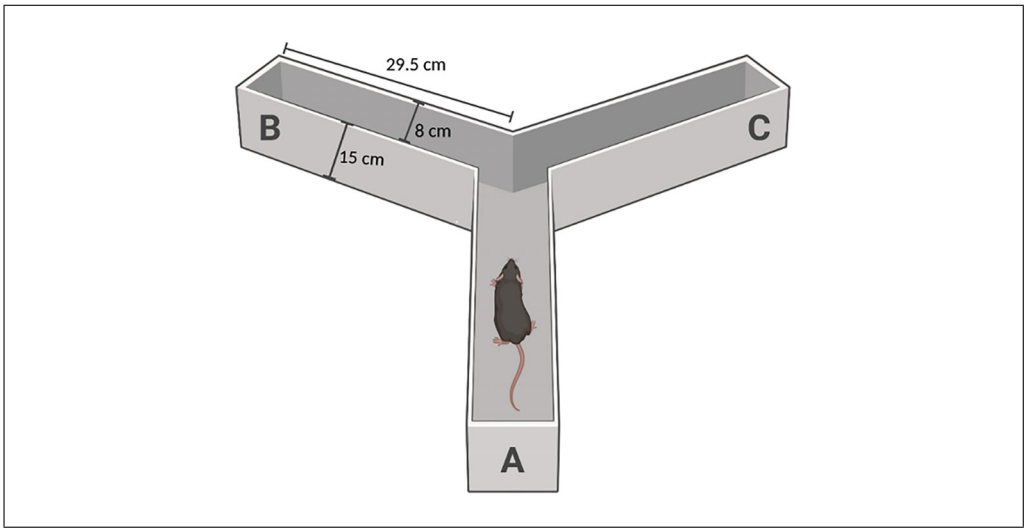

Figure 4 Y-shaped maze for conducting Y-maze testing of spontaneous alternation without intramaze cues. The dimensions are highlighted, and each arm of the maze is labeled either A, B, or C. Image generated using BioRender.

4. Lay a mouse gently in the starting arm, facing the short wall, and begin recording movement.
5. Record the latency for the mouse to exit the starting arm with all four paws.
6. After the 5- to 8-min run, use a precision scale and record the body weight of the mouse before transferring to a clean cage.

   Keep mouse in the clean cage until all cage mates have been tested. This measure will avoid exposing an untested mouse to any transmitted unease or conflicts.

7. Clean the maze with tap water and then 50% ethanol to remove any urine, fecal boli, and olfactory cues that may remain and then dry it.
8. For subsequent testing of mice in a cohort, repeat steps 4 to 7. Distribute mice with different genotypes to different starting arms (e.g., mutant 1: arm A, control 1: arm A; mutant 2: arm B, control 2: arm B; etc.)
9. Take the following (see Data Analysis section) into consideration for the data analysis, whether conducted manually or with the video-tracking software.

Data Analysis

The following should be considered in the data analysis:

1. The total number of arm entries: this is all the arm entries over the course of the testing period (excluding the starting arm). Animals with fewer than five arm entries are omitted from further data analysis.
2. Count the triplets of the different alternations as shown in the example below:

	A	**C**	A	B	C	A	C	A	B	A	C	A	C	B	A	B
1st triplet:	A	**C**	A													
2nd triplet:		**C**	A	B												
3rd triplet:			A	B	C											
4th triplet:				B	C	A										

SPA (spontaneous alternation) is a triplet of three different arms, such as ABC, BCA, or CBA. AAR is a triplet where the first and third arms are the same, such as ABA, BAB,

and CBC. In SAR, the first and second arms are the same, such as AAB, BBC, and CCA. Be sure to just count SARs once, as shown below:

Example:	…	B	A	**A**	C	B	…	
- Count:		B	A	**A**				
				A	C	B		
BUT								
Example:	…	B	A	**A**	A	C	B	…
- Count:		B	A	**A**				
				A	A	C		

Use a spreadsheet template to record all data, as follows: latency, entry number, triplet number and SPA/AAR/SAR number, and % SPA/AAR/SAR. Determine the average and the standard deviation per genotype and sex.

Analyze each parameter by comparing the control and experimental groups using statistical methods appropriate to the study design (e.g., *t*-tests or ANOVA for normally distributed data or non-parametric alternatives if assumptions are not met).

BASIC PROTOCOL 10

MARBLE-BURYING TEST

Burying behavior in rodents often refers to the displacement of bedding material using the snout and forepaws in an effort to cover an object. The marble-burying assay was developed to take advantage of this inherent burying behavior to evaluate how many novel but innocuous glass marbles a rodent would bury.

The marble-burying test is used to assess anxiety-related and obsessive/compulsive/repetitive behaviors in mice. The experiment is designed for 15 min of testing, under dim light (~25 Lux), and is conducted between approximately 8:00 a.m. and 6:00 p.m. depending on the size of the mouse groups.

Materials

Mice (of desired strain, sex, and genotype depending on study objective)
50% (v/v) ethanol

Video-recording system (e.g., camera mounted on ceiling of room, above test cage, and connected to TV monitor or computer located in testing room antechamber)
Light meter
Test cages (standard mouse cages, dimensions: 35 × 17.7 × 12.5 cm, Tecniplast)
Fresh bedding (poplar wood particles, grain size: 3.5 to 4.5 mm)
Plastic pattern, for marble location (see Fig. 5)
20 black glass marbles (1-cm diameter)
Transparent Plexiglas cover

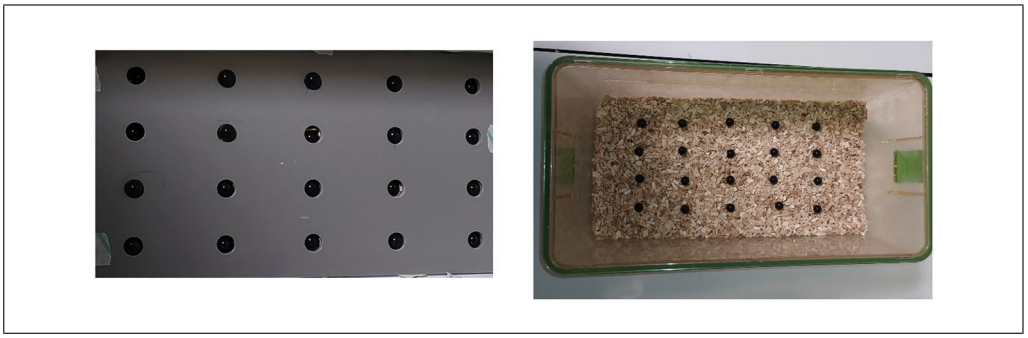

Figure 5 Pattern in which to place the marbles (left) and the results obtained (right).

Colander
Tissue paper

1. Transport mice from the housing room to the holding room (ideally adjacent to the testing room) and leave undisturbed for ≥15 min to acclimate.
2. During the acclimation period, prepare the monitor and camera of video-recording system to ensure a clear image will be obtained.
3. Using the light meter, adjust the illumination in the testing room to ∼25 Lux in the test cage.
4. Fill the test cage with fresh bedding to a height of 4 to 5 cm to prevent any animal odor.
5. Place the plastic pattern on the bedding to smooth it out.
6. Place the 20 black glass marbles according to the pattern (Fig. 5, left).
7. Remove pattern (Fig. 5, right).
8. Place a mouse in the test cage and place the transparent Plexiglas cover over the cage.
9. Leave the room and start recording.
10. After 15 min, stop recording, remove the animal, and return it to its home cage.
11. Empty the test cage and collect the marbles using the colander, rinse with tap water and 50% ethanol, and dry with tissue paper. Dispose of bedding (use fresh bedding for the next trial) and clean the cage with 50% ethanol and allow it to dry.
12. Repeat steps 4 to 11 until all animals have been tested.
13. At the end of the experiment, return mice to their housing room.
14. Collect appropriate data:
    a. Play the videos and count the number of marbles buried for each minute between the fifth and fifteenth minutes of testing.

       A marble is considered buried when >75% of the visible surface of the marble on the screen is covered.

    b. Express results as the number of buried marbles or as the percentage of buried marbles in relation to the total number of marbles.
15. Perform appropriate data analysis (e.g., examine distribution, perform statistical test, transform data).

MARBLE-BURYING TEST VERSION 2

ALTERNATE PROTOCOL 4

The marble-burying test is an ethologically grounded test of repetitive and anxiety-related behavior in mice (Angoa-Pérez et al., 2013). Whereas Basic Protocol 10 offers a robust and widely used approach to assess repetitive and anxiety-related behaviors, this alternate version introduces minor procedural refinements. These include a shorter test duration (10 min) and use of blue marbles to improve contrast and photo-based scoring. These changes further streamline analysis and support reproducibility without compromising the core behavioral readout.

Additional Materials *(also see Basic Protocol 10)*

Sound-attenuated testing room with adjustable light source (optional but preferable)

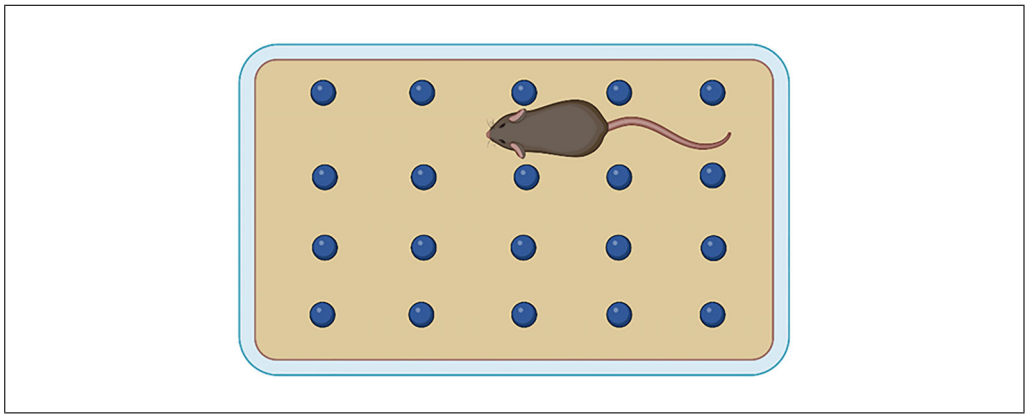

Figure 6 Marble-burying test, which takes place in a standard mouse cage with four rows of five blue marbles evenly spaced and placed on top of bedding material. Image generated using BioRender.

Fresh bedding (e.g., wood chips, sawdust)
20 blue glass marbles (1-cm diameter)

1. Position the mouse cage in a sound-attenuated testing room with adjustable light source if possible and add fresh bedding to a depth of 4 to 5 cm, which can be leveled using the base of another cage.
2. Place the 20 blue glass marbles in an even configuration of four rows of five marbles (Fig. 6).
3. Set up video-tracking system or video camera above the test cage to record the burying and digging bouts over the course of the test.
4. Bring the mice to the testing room 30 min before testing to enable sufficient time for habituation.
5. Start testing at the beginning of the light phase but give 1 hr after lights on.
6. Begin video recording and gently place the individual mouse in the corner of the cage, being careful not to disturb the marbles.
7. Allow the mouse to behave freely for 10 min.
8. On completion, carefully remove the mouse and place it in a fresh cage, taking care not to disrupt the bedding or dislodge the marbles.
9. Take a top-down image of the final appearance of the marbles.
10. Collect all the marbles from the cage and disinfect them with 50% ethanol before using them for subsequent trials (see Basic Protocol 10, step 11). Dispose of bedding (use fresh bedding for the next trial) and clean the cage with disinfectant and allow it to dry.
11. Repeat steps 5 to 10 until all animals have been tested.
12. At the end of the experiment, return mice to their housing room.
13. Have an observer blind to the experimental groups score the proportion of each of the following categories of marbles for each animal: not buried, >50% buried, >75% buried, and fully buried.
14. Analyze the percentage of each parameter by comparing the control and experimental groups using statistical methods appropriate to the study design (e.g., *t*-tests or ANOVA for normally distributed data or non-parametric alternatives if assumptions are not met).

OBJECT LOCATION MEMORY/NOVEL OBJECT RECOGNITION

The OLM and NOR tasks have been widely used in the study of the neurobiological mechanisms underlying memory formation. Both tasks can be performed in the same experimental group. Animals that remember the original training experience will preferentially explore the displaced object relative to the nondisplaced object (OLM) or the novel object relative to the familiar object (NOR).

Mice are tested in a square arena (44-cm square and 40-cm high) with a 10-cm-wide vertical white stripe in the middle of one wall (Fig. 7). Locomotor activity is recorded with the EthoVision XT video-tracking system (Noldus). The arena is illuminated at 40 Lux. Animals are first habituated to the arena for 15 min on two consecutive days. Each mouse is placed in the periphery of the arena and allowed to explore freely the apparatus, with the experimenter out of the animal's sight. The distance traveled is recorded during the test session.

The next day, mice are tested for the OLM task in the same arena. They are submitted to a 10-min acquisition trial during which they are placed in the arena in the presence of two identical objects (2.5-cm-diameter marble or 2-cm-edge plastic dice). Typically, the objects used should be too small for adult mice to sit atop. Exploration is strictly defined as active sniffing with the nose directed toward the object at $<$1-cm distance, explicitly excluding any climbing behavior per standard OLM/NOR protocols. The two objects used were validated in a pilot study to confirm no spontaneous preference, as detailed in the Troubleshooting section of the Commentary. Larger objects are deliberately avoided, as they reduce overall sniffing contacts, induce neophobia (aversion to unfamiliar large items that confounds memory discrimination), and alter spatial perception in standard arenas (44 $\times$ 44 cm), thereby lowering test sensitivity.

The time the animal takes to explore the objects (sniffing) is manually recorded. A 10-min retention trial is performed 24 hr later. During this trial, one of the identical objects explored the day before is displaced, and the times the animal took to explore the two objects are recorded. The OLM index is defined as follows:

$$OLM\ index = \frac{time\ to\ explore\ displaced\ object}{time\ to\ explore\ displaced\ object + time\ to\ explore\ nondisplaced\ object} \times 100$$

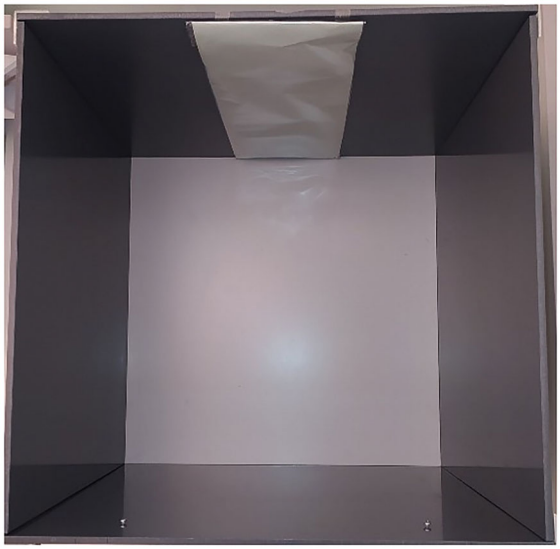

Figure 7 Square arena for the object location and novel object tests.

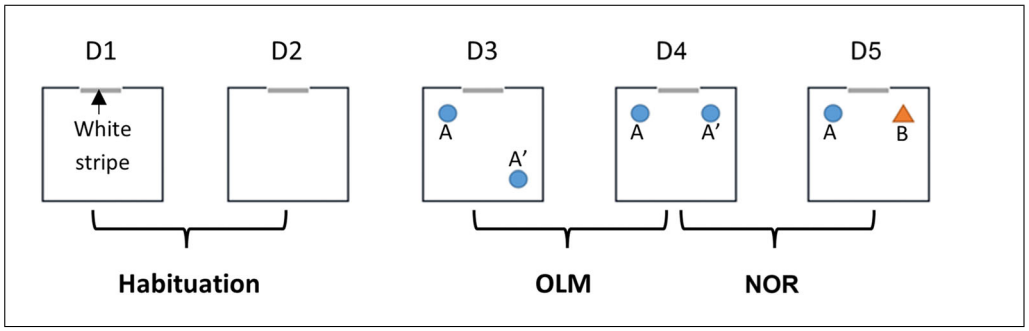

Figure 8 Complete protocol for combining habituation (days 1 to 2), object location memory (OLM; days 3 to 4), and novel object recognition (NOR; days 4 to 5).

Twenty-four hours later, mice are tested for object recognition in the same arena. A 10-min retention trial is performed. During this 10-min trial, one of the identical objects explored the day before is replaced by a novel object (marble or dice, depending on acquisition), and the times the animal takes to explore the two objects are manually recorded. The NOR index is defined as follows:

$$NOR\ index = \frac{time\ to\ explore\ novel\ object}{time\ to\ explore\ familiar\ object + time\ to\ explore\ novel\ object} \times 100$$

See Figure 8 for a schematic of the complete protocol.

Materials

Mice (of desired strain, gender, and genotype depending on study objective)
50% (v/v) ethanol

Light meter
Video-tracking system (e.g., EthoVision XT, Noldus), with software, monitor, and camera (e.g., mounted on ceiling of room, above test arena, and connected to tracking system located in testing room antechamber)
Square arena (44-cm square and 40-cm high, with 15-cm-wide vertical white stripe in middle of one wall; see Fig. 7)
Patafix glue pads (from any supermarket or other provider, e.g., Amazon)
Two identical objects to be discriminated: glass marbles (2.5-cm diameter) or plastic dice (2-cm square)
Two stopwatches

Days 1 to 2: Habituation

1. Transport mice from the housing room to the holding room (ideally adjacent to the testing room) and leave undisturbed for ≥15 min to acclimate.
2. During acclimation period, using a light meter, adjust the light in the testing room at 40 Lux in the center of the arena. Prepare the monitor and camera of video-recording system to ensure a clear image will be obtained.
3. Open the video-tracking software and check that all tracking parameters (arena settings, detection threshold, trial duration, and subject list) are correctly set.
4. Launch the experiment.
5. Place the first mouse in the periphery of the square arena, with the head directed to the proximal wall, and allow the mouse to explore for 15 min.

   Once a mouse is detected, recording will start immediately.
6. At the end of the run, return the mouse to its home cage.

7. After each run, remove feces and clean the arena with tap water and then 50% ethanol.
8. Proceed the same way as in steps 5 to 7 until all mice are tested.
9. Repeat the same procedure the following day.

Day 3: Acquisition trial (OLM)

10. Using Patafix glue pads, fix two identical objects to be discriminated (glass marbles or plastic dice) in the arena. Place the first object (A) in the corner of the arena, ∼10 cm from the wall where the vertical white stripe is located and ∼10 cm from the lateral wall. Place the second object (A') symmetrically in relation to the center of the arena (Fig. 8).
11. Launch the experiment.
12. Place the mouse in a corner of the arena, away from objects and white stripe, and let it explore for 10 min.

    Once a mouse is detected, recording will start immediately.

13. Using the stopwatch, record the time the animal takes to explore the two identical objects (when the animal's snout is directed toward the object at a distance $\leq$1 cm).
14. At the end of the run, return the mouse to its home cage.
15. After each run, remove feces and thoroughly clean the arena with tap water and then 50% ethanol. Clean objects with ethanol and let evaporate before use.
16. Repeat steps 10 to 15 for each mouse.

Day 4: Retention trial (OLM)/acquisition trial (NOR)

17. Use the same pair of objects as the day before. Place object A in the same position as the day before. Place object A' ∼10 cm from the wall with the vertical white stripe and ∼10 cm from the lateral wall, in the opposite corner as object A in relation to the white stripe (Fig. 8).
18. Launch the experiment.
19. Put the mouse in the periphery of the arena, with its head facing the wall opposite the white stripe, and allow it to explore for 10 min.

    Once a mouse is detected, recording will start immediately.

20. Using stopwatches, record the time the animal takes to explore objects A and A'.
21. At the end of the run, repeat steps 15 and 18 to 20.

Day 5: Retention trial (NOR)

22. Use the same object position as the previous day. Replace object A or object A' with a new object B of a different shape (dice or marble; Fig. 8).
23. Launch the experiment.
24. Put the mouse in the periphery of the arena, with its head facing the wall opposite the vertical white stripe, and allow it to explore for 10 min.

    Once a mouse is detected, recording will start immediately.

25. Using stopwatches, record the time the animal takes to explore the former and novel objects.
26. At the end of the run, repeat steps 15 and 23 to 25.

Data analysis

27. Perform appropriate data analysis:

    a. Assess parameters indicating OLM performance:
    ° Times tA and tA' that the animal spends exploring objects A and A' during OLM retention trial.
    ° OLM index $= 100 \times t\text{B}/(t\text{A} + t\text{B})$; an index of 50% corresponds to the chance level, and an index significantly above chance reflects good memory performance.
    b. Assess parameters indicating NOR performance:
    ° Times tA and tB that the animal spends exploring objects A and B during NOR retention trial.
    ° NOR index $= 100 \times t\text{B}/(t\text{A} + t\text{B})$; an index of 50% corresponds to the chance level, and an index significantly above chance reflects good memory performance.
    b. Assess parameters indicating the level of activity and exploration:
    ° Total distance traveled in the whole arena during habituation, acquisition, and retention trials.
    ° Time of exploration of both objects during the acquisition trials.
    ° Time of exploration of both objects during the retention trials.

ALTERNATE PROTOCOL 5

NOVEL OBJECT RECOGNITION IN THE Y-MAZE APPARATUS

The NOR task (Basic Protocol 11) assesses recognition memory, a cognitive domain often impaired in DS [2]. It involves parts of the hippocampus including the cornu ammonis (CA)1 subregion, but it is predominantly mediated by the peri-rhinal cortex [3]. This alternative version of the NOR test was developed to streamline behavioral testing in DS mouse models by reducing test duration and handling while maintaining core components of recognition memory assessment. The protocol spans 3 days and focuses solely on NOR, with Y-maze testing (Alternate Protocol 3) incorporated on day 1 to maximize throughput and efficiency within a multi-assay behavioral pipeline (Fig. 9). Behavioral data are collected via an overhead video camera and scored using event-logging software with a handheld recorder to capture the duration of object exploration. Distinct objects are used to facilitate clear discrimination. This streamlined approach reduces the testing burden while maintaining relevance for assessing recognition memory in the context of DS-related neurodevelopmental alterations.

The objects are selected for NOR based on the size relative to the arena in question and to ensure consistency of this as well as the weight and material across trials. It is also important to select objects with sufficiently different characteristics in terms of smell

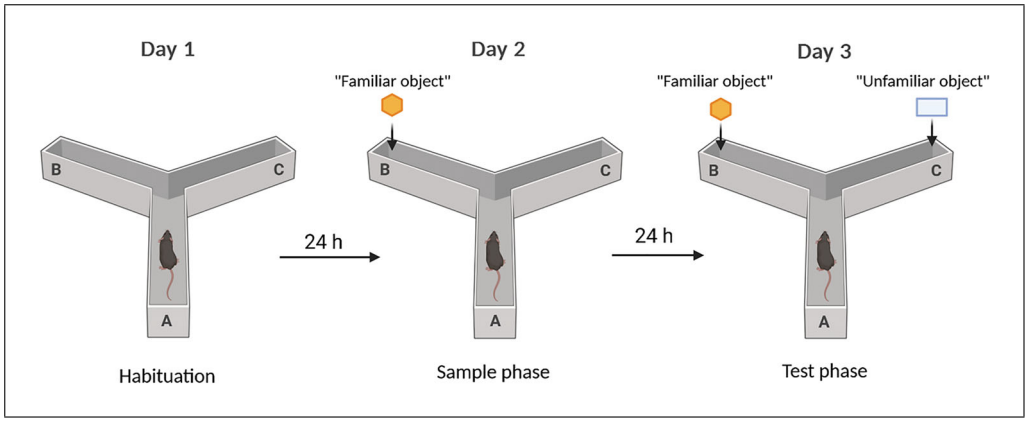

Figure 9 Novel object recognition testing was performed in the Y-maze apparatus from Alternate Protocol 3 over the course of 3 days. Image generated using BioRender.

and texture so that the mice can make the distinction between novel and familiar objects and complete the test within the designated time. The objects are firmly affixed to the base of the arena and are heavy enough to prevent displacement by the animals, thereby minimizing variability due to movement of the object. All objects are thoroughly cleaned between trials to eliminate olfactory cues.

Regarding climbing behavior, although mice can occasionally interact with the objects by touching or briefly mounting them, this is rare and should not interfere with the scoring criteria. Importantly, exploration is defined strictly as active investigation directed toward the object, such as sniffing in proximity, and passive contact, such as sitting and climbing, is not scored as exploratory behavior (see step 9).

The size and shape of the objects are selected to strike a balance between stability and exploratory engagement, in line with commonly used protocols. Even using larger objects that prevent climbing can introduce other confounders, such as reduced investigation behavior or altered anxiety-related responses. Our design is therefore the middle ground that is widely adopted in standard NOR tests.

In any case, object properties are kept consistent across all groups and conditions to ensure that any potential rare interaction effects do not influence comparisons between the control and experimental groups.

Additional Materials *(also see Alternate Protocol 3)*

Digital video recorder (top mounted, HD recommended)
Two different objects: plastic toy brick (3.2 × 1.6 cm) and metal weight (2.1-cm diameter)
Patafix glue pads (from any supermarket or other provider, e.g., Amazon)
Handheld event recorder (e.g., Psion, used in conjunction with Observer XT)
Behavioral event–logging software

1. Place the Y-maze in a sound-attenuated testing room and ensure uniform illumination using a mounted ring light, with 80 Lux in the center of the maze, with the video camera of the video-monitoring system centered above.

   Avoid stress and noise during testing, as this will affect the spontaneous behavior of the mouse.

2. Allow animals ≥30 min to acclimatize to the testing room before commencing.

   Testing can start at the beginning of the light phase, but give 1 hr after lights on.

3. Place the Y-maze on the floor so one arm is pointing to the observer's position during the test and label each arm using a card (as in Fig. 4).

4. Start the video recording using a digital video recorder prior to placing the mouse in the apparatus.

Day 1: Habituation

5. Place the mouse in the starting arm of the Y-maze and allow the animal to explore freely for 8 min. After this period, remove the mouse and return to its home cage.

   To fit into the DS testing pipeline, this first habituation session can be analyzed as the Y-maze test of spontaneous alternation (see Alternate Protocol 3).

Day 2: Sample phase

6. After a 24-hr retention interval, affix the first of the two different objects at the end of one of the arms of the Y-maze using a Patafix glue pad (Fig. 9).

To ensure that mice can execute this task successfully within the designated timeline, the objects used must be as distinct as possible, e.g., with different shapes, sizes, and materials.

7. Remove the mouse gently from the home cage, place it in the starting arm, allow the mouse to explore freely for 8 min, and track using the video tracker. At the end of this period, remove the mouse, return it to the home cage, and disinfect and dry the apparatus.

   The animal is considered to be investigating the object when its nose makes contact or is directed at the object within a 2-cm distance. Standing, sitting, or leaning on the object is not considered an investigation by the mouse.

Day 3: Test phase

8. After a 24-hr retention interval, affix the second of the two different objects to the end of another arm of the Y-maze while the first object remains in place (Fig. 9). Allow the mouse to explore the maze freely for 8 min. On completion, remove the mouse and return to the home cage.

9. Use the handheld event recorder and the behavioral event–logging software to record the duration of investigation of the familiar and unfamiliar objects by the mouse.

Data analysis

10. Calculate the total duration of investigation of familiar and unfamiliar objects during the sample and test phases. Use these values to calculate the NOR and discrimination indices as follows:

$$\textit{NOR index} = \frac{\textit{time to explore unfamiliar object}}{\textit{time to explore unfamiliar object} + \textit{time to explore familiar object}}$$

$$\textit{Discrimination index} = \frac{\textit{time to explore unfamiliar object} - \textit{time to explore familiar object}}{\textit{time to explore unfamiliar object} + \textit{time to explore familiar object}}$$

11. Analyze the NOR and discrimination indices by comparing the control and experimental groups using statistical methods appropriate to the study design (e.g., *t*-tests or ANOVA for normally distributed data or non-parametric alternatives if assumptions are not met).

BASIC PROTOCOL 12

NEST-BUILDING TEST

For small rodents, nests are important for keeping warm and providing shelter. Nesting is sensitive to brain lesions, pharmacological agents, and genetic mutations.

On the day of the test, mice are singly transferred to a housing cage for the duration of nest-building measurement. A block of nesting material (5 × 5–cm hemp square, Happi Mats, Utopia) is placed in the cage. Pictures are taken, and visual scoring occurs at 2, 5, and 24 hr without disturbing the animals. The room temperature is noted when the nest is scored, as nest building has a thermoregulatory function and therefore may be influenced by ambient temperature. We use a 0 to 5 scale described by Gaskill et al. (2013): 0 = undisturbed nesting material; 1 = disturbed nesting material but no nest site; 2 = a flat nest without walls; 3 = a cup nest with a wall less than $\frac{1}{2}$ the height of a dome that would cover a mouse; 4 = an incomplete dome with a wall $\frac{1}{2}$ the height of a dome; and 5 = a complete dome with walls taller than $\frac{1}{2}$ the height of a dome, which may or may not fully enclose the nest.

Materials

Mice (of desired strain, gender, and genotype depending on study objective)

New housing cages (36 × 17 × 12 cm)
Fresh bedding
Unshredded hemp nesting material (5 × 5–cm hemp square, Happi Mats, Utopia)

1. Before starting the test, transfer each mouse individually to a new housing cage with fresh bedding, food, and drinking water.

   The test takes place in the room where the animals are housed.

2. At 10 a.m., place a square of unshredded hemp nesting material in each cage.
3. At 12 p.m., observe the hemp square in each cage without disturbing the mice and assess the level of nest construction according to the rating scale indicated in the protocol introduction.
4. At 3 p.m., observe the hemp square in each cage without disturbing the mice and assess the level of nest construction according to the rating scale indicated in the protocol introduction.
5. At 10 a.m. the next day, observe the hemp square in each cage without disturbing the mice and assess the level of nest construction according to the rating scale indicated in the protocol introduction.
6. At the end of this evaluation, return the animals to their original cages.
7. Analyze and compare nesting scores of each experimental group.

SUCROSE PREFERENCE ANALYSIS

BASIC PROTOCOL 13

Anhedonia, or reduced sensitivity to pleasure, is one of the core symptoms of depression that can be modeled in animals. Hedonic behavior can be evaluated by measuring the preference for sucrose when the animal has free access to two bottles containing either a sucrose solution or water. Mice are first habituated to sucrose overnight, and then sucrose preference is evaluated on three consecutive days mainly during the dark phase (repeated measurements). On the first day, mice are habituated overnight to sucrose in their home cages, where water is replaced with 0.8% sucrose solution (from 5 p.m. to 9 a.m. the following day). From the second to the fifth days, mice are individually transferred to the testing cages 2 hr before lights off (5 p.m.), with food available *ad libitum*. One hour later (6 p.m.), a bottle of water and a bottle of 0.8% sucrose are provided for 15 hr (from 6 p.m. to 9 a.m. the following day). The second-day measurement is designed to reduce the potential confounding effect of anxiety when mice are put in a novel environment, in addition to sucrose habituation. All 3 days of measurements are used for evaluation of sucrose preference. For each day, water and sucrose consumption over 15 hr is measured by weighing bottles before and after the test (at 5 p.m. and 9 a.m.).

Materials

Sucrose
Mice (of desired strain, gender, and genotype depending on study objective)

Bottles (one per animal)
Test cages (housing cages with two places for drinking bottles; one per animal)
Scale

Day 1: Sucrose habituation

1. Prepare 0.8% (w/v) sucrose solution in drinking water.

2. From 5 p.m. to 9 a.m. the following day, replace the water bottles in the home cages of the mice with bottles of 0.8% sucrose (one bottle per cage).

Days 2 to 5: Sucrose preference test

3. Prepare 0.8% sucrose solution in drinking water.
4. Fill half the test bottles with sucrose solution and the others with drinking water.
5. Each day, transfer mice to individual test cages 2 hr before lights out (5 p.m.), with food *ad libitum*. One hour later (6 p.m.), provide each animal with a bottle of drinking water and a bottle of sucrose solution until 9 a.m. the following day.
6. Between 9 a.m. and 5 p.m., return animals to their housing cages.
7. Every day, measure water and sucrose consumption by weighing bottles before and after the test (at 5 p.m. and 9 a.m.).
8. Calculate the sucrose preference percentage for each test day:

$$Sucrose\ preference\ \% = \frac{sucrose\ consumed\ \ (g)}{sucrose\ consumed\ \ (g) + water\ consumed\ \ (g)} \times 100$$

BASIC PROTOCOL 14

HISTOPATHOLOGY

Mice are euthanized, and tissues are fixed in formalin overnight. Following fixation, tissues are embedded in paraffin, sectioned at 3 µm, and stained with hematoxylin and eosin. Slides are scanned for histopathological evaluation.

Materials

Mice, euthanized
4% (w/v) formaldehyde, ready-to-use, phosphate buffered, pH 7 (~10% formalin; ROTI Histofix, Carl ROTH, cat. no. P087.2)
Formalin
70% (v/v), 96% (v/v), and 100% ethanol
Xylene (VWR, cat. no. 28973363)
Paraffin (ROTI Plast, Carl ROTH, cat. no. 6642.6)
Hematoxylin Gill II (hematoxylin solution acc. to Gill II, Carl ROTH, cat. no. T864.3)
Eosin G (solution 0.5% in water, Carl ROTH, cat. no. X883.2)
Mounting medium (Cytoseal, Epredia, cat. no. 11932365)

Dissection instruments: scissors (FST, cat. no. 91460-11), scalpel (Carl ROTH, cat. no. CE18,1), and short forceps (Braun/Aesculap, cat. no. BD130R)
Histological cassettes (KABE Labortechnik, cat. no. 54625) and lids (KABE, cat. no. 53830), pre-labeled (using cassette printer with output collection system; Epredia Printmate AS 150)
Tissue processor (Epredia Excelsior AS)
Metal embedding molds (Carl ROTH, cat. no. TT29.1)
Embedding system (Leica HistoCore Arcadia H) with cold plate (Leica HistoCore Arcadia C)
Paraffin wax trimmer (Biozol, cat. no. XH-90)
Histology water bath (W20, VWR, cat. no. 720-2423)
Slide printer (Signature XTRA Force, Primera, cat. no. 078101, with output Sig78101-3)
Glass slides (KP Plus printer slides with ground edges, VWR, cat. no. KLINPR-001)
Microtome (Epredia HM 340E)
Microtome blades (A35, PFM, cat. no. 207500011)

Figure 10 Tissues trimmed into seven histological cassettes: 1a – brain; 1bF – reproductive tissues, perigonadal white adipose tissue, mammary gland, and urinary bladder; 2 – esophagus, trachea, thyroid, and parathyroid; 3a – liver, kidneys, lung, spleen, and thymus; H_Mus – heart and skeletal muscle; 4 – salivary glands, lymph nodes, and adrenal glands; 5 – stomach, intestine, pancreas, and skin.

Light microscope (Primostar 3, Zeiss SF 20)
Slide rack
Incubator (Binder BD 23), 62°C
Automated stainer (Epredia Gemini AS)
Automated coverslipper (Epredia CTM6)
Coverslips (thickness 1, 24 × 50 mm, Carl ROTH, cat. no. 1871.2)
Slide scanner (Hamamatsu NanoZoomer S60, cat. no. C13210-04), with computer and NDPview2 software

1. Dissect and trim mouse tissues with dissection instruments, place in pre-labeled histological cassettes (Fig. 10), and fix by immersion in 4% formaldehyde overnight.

   A standardized high-throughput schema is used according to the International Mouse Phenotyping Consortium's standard operating procedures (https://www.mousephenotype.org/impress/ProcedureInfo?action=list&procID=613&pipeID=14).

2. Process histological cassettes overnight in a tissue processor (steps during the 18-hr cycle: 2 × 4% formaldehyde, 3 hr; 2 × 70% ethanol, 1 hr; 2 × 96% ethanol, 1 hr; 2 × 100% ethanol, 50 min; 3 × xylene, 50 min; and 3 × paraffin, 62°C, 1 hr).

3. After paraffin infiltration, place tissues in metal embedding molds and top up molds with paraffin. Place molds on a cold plate for paraffin solidification. Remove excess paraffin by rubbing the cassette on a paraffin wax trimmer.

4. Set the histology water bath to a temperature of 52°C.

5. Place paraffin blocks on a humid cold plate before sectioning.

6. Using a slide printer, print glass slides with a 2D code containing mouse ID number, slide ID number, and block type.

7. Cut paraffin sections at a thickness of 3 µm using a microtome with an appropriate blade, place on the surface of the water bath (see step 4) to remove wrinkles and distortions in paraffin, and then transfer to glass slides. Examine sections under a light microscope to verify tissue representation.

8. Place slides in a slide rack and dry overnight in an incubator at 62°C.

9. Stain slides with hematoxylin and eosin in an automated stainer (steps of staining cycle: 2 × xylene, 5 min; 100% ethanol, 1 min; 96% ethanol, 1 min; 70% ethanol, 1 min; distilled water, 1 min; hematoxylin Gill II, 1 min; running tap water, 4 min;

eosin G, 2 min; running tap water, 30 s; 70% ethanol, 20 s; 2 × 96% ethanol, 30 s; 2 × 100% ethanol, 1 min; xylene, 1 min; xylene, 90 s; and xylene as a final step).

10. Transfer slides to an automated coverslipper to add mounting medium and coverslips. Check slides for air bubbles and then leave to dry at room temperature.

11. Clean slides with stained sections, load into racks, and scan in brightfield profile (20× lens) using a slide scanner.

The slide ID number is automatically read from the 2D code and used for file naming in NDPI format. Images are viewed using NDPview2 software.

COMMENTARY

Critical Parameters

Body composition assessment by quantitative nuclear magnetic resonance (Basic Protocol 3)

To ensure accuracy of the qNMR, calibration should be done daily. Mouse identification by tattooing is necessary. The NMR equipment used is highly sensitive, and any additional metallic objects, such as ear tags, can disrupt the magnetic field and lead to inaccurate readings. Mice should be handled carefully to prevent injuries or contamination of samples.

Indirect calorimetry (Basic Protocol 4)

Data are reported as daily averages for each group, covering both the light phase and the dark phase, expressed as mean ± SEM. This approach ensures robust, reproducible insights into the metabolic and behavioral phenotypes of the tested mice.

The following are important for data quality control:

1. The RER is between 0.7 and 1.00.
2. Mice show normal feeding and drinking behavior.
3. Mice show stable weight before and after calorimetry.
4. Correct calibration of gases is performed according to the manufacturer's manual.

The system requires periodic calibration of the gas sensors and flow meters to ensure precise measurements. The calibration procedure consists of the application of a gas of known composition and adjustment of the control knobs in the front of the oxygen and carbon dioxide sensors to obtain readings that reflect the contents of the calibration gas. System calibration is recommended to be done prior to each experiment. The analyzers should not be shut down unless urgently required for maintenance. If this has to be done, a warmup time of ≥90 min is required for the gas sensors for calibration (refer to the manufacturer's manual). Calibrations and shutdowns should be recorded in the laboratory record.

Blood collection (Basic Protocol 5)

In terms of volume limits, do not exceed 200 µl per submandibular bleed for single sampling.

For repeat sampling, for sequential bleeds, either remove the scab gently or puncture a distal site.

Sample quality is crucial for reliability of measurements. Especially significant hemolysis and clot formation in samples can affect the results obtained from a number clinical chemistry and hematology measurements. Clot formation occurs due to slow blood flow or too-late or inadequate mixture of blood with anticoagulant. Hemolysis occurs due to clotting during collection or incorrect blood handling, such as vigorous shaking, inadequate centrifugation, or too-cold (near 0°C) or prolonged cooling of whole blood samples.

Oral glucose tolerance test/intraperitoneal insulin sensitivity test (Basic Protocols 6 and 8)

The outcome of tests applied to assess regulation of glucose metabolism is significantly affected by the stress response of the animals, as corticosterone significantly affects gluconeogenesis. Therefore, it is important that all persons involved in testing are well trained and calm during the testing procedure. Loud noises have to be avoided during testing. If anything unexpected happens during the testing procedure, like a loud noise, the time and duration have to be recorded in the protocol for the test.

Y-maze testing (Basic Protocol 9 and Alternate Protocols 3 and 5)

Evaluation of performance in the Y-maze requires that mice explore correctly. The number of entries is a critical parameter: it is necessary that each mouse displays at least five arm entries, and otherwise, the mouse should be

excluded from the data analysis. Environmental factors that may contribute to the levels of anxiety, including noise intensity and lighting intensity, must be maintained at levels appropriate for mice; anxious mice, for the reasons described above, tend to show higher latencies to exit the starting arm and thus might have a reduced number of arm entries.

Marble burying (Basic Protocol 10 and Alternate Protocol 4)

The size and weight of the marbles and the grain size of the bedding are decisive factors in this test. In fact, marbles that are too heavy (e.g., metal) or a grain size of the litter that is too small could artificially increase the number of marbles buried. Similarly, if the marbles are too large or the grain size too coarse, the number of marbles buried could be reduced (see Basic Protocol 11). To be able to detect a decrease or increase in the number of marbles buried in a group of mice of interest compared to a group of control mice, it is important that the marbles are not too easy or too difficult to bury.

Object location memory/novel object recognition (Basic Protocol 11 and Alternate Protocol 5)

The choice of objects is crucial. Indeed, if the animals have a spontaneous preference for one of the two objects presented during the NOR retention session, this will confound the results.

Accurate evaluation of object recognition performance requires that mice explore correctly during the acquisition and retention trials. Mice with reduced exploration time (<3 s) should be excluded.

Nest building (Basic Protocol 12)

As one of the main reasons for nesting is to conserve heat, the temperature of the housing room is an important parameter for this test and must be controlled. In addition, nesting material can also have an impact on results and must be carefully chosen. The quantity of material should also be considered for this test.

Sucrose preference (Basic Protocol 13)

Place preference can potentially bias the test with an animal that will always drink from the same bottle without trying to drink from the second bottle. It is also crucial to check that none of the bottles is blocked.

Troubleshooting

Y-maze testing (Basic Protocol 9 and Alternate Protocols 3 and 5)

For reasons discussed in the Critical Parameters section, anxious or hypoactive strains should be avoided for this test. In the case of a study using mutants with hypoactive or anxious phenotypes or bred on a genetic background with such traits, it is advisable to use an alternative test to evaluate the same aspects of learning processes measured in the Y-maze. In this regard, while validating this test in four mouse strains, we found that although all C57BL/6J individuals tested (12/12) displayed several arm entries above the criterion, with an average of 20 entries, only 4/12 mice from the 129SvPass strain had more than five entries. Alternative strains with a sufficient tendency toward exploration should be used if needed for, e.g., pharmacological studies. For phenotyping mutant lines, using alternative tests or backcrossing onto an appropriately explorative background is recommended.

Marble burying (Basic Protocol 10 and Alternate Protocol 4)

Please see Table 2 for a troubleshooting guide for marble burying.

Object location memory/novel object recognition (Basic Protocol 11 and Alternate Protocol 5)

To ensure that there is no preference for one of the two objects used in the NOR retention session, a validation must first be carried out with wild-type animals. For this validation, the animals are directly exposed to two objects of different shapes, and the exploration time for the two objects is measured. If the animals show a preference for one of the two objects, change the objects and repeat the validation test. To ensure that most animals explore the objects sufficiently, it is advisable to avoid using low-activity strains such as 129Sv mice.

Nest building (Basic Protocol 12)

To avoid the mice nesting too quickly, it is best to use pressed cotton or hemp squares (nestlets), which are calibrated and help with the shredding action. It is advisable to avoid strands of Kraft paper, as it is more difficult to estimate the quantity available to the animal, and the volume allows the animal to make its nest very quickly.

Table 2 Troubleshooting Guide for Marble Burying

Problem	Possible cause	Solution
Almost all marbles are buried in the control group at the end of the test	- Bedding grain size too small - Marbles too small - Marbles too heavy	- Increase grain size - Increase marble diameter - Choose glass marbles rather than metal ones
Hardly any marbles are buried at the end of the test	- Bedding grain size too coarse - Marbles too large	- Decrease grain size - Decrease marble diameter

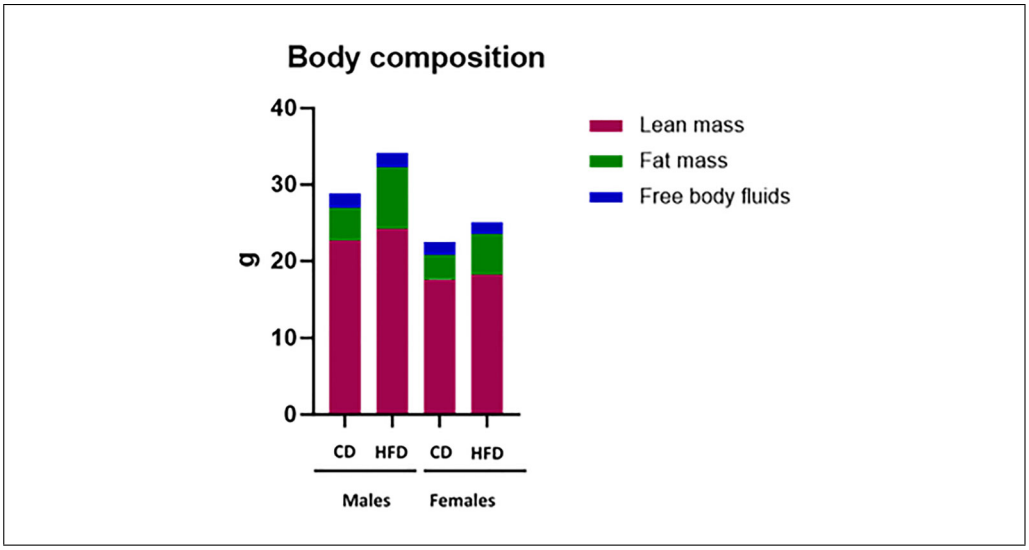

Figure 11 Body composition ranges (lean mass, fat mass, and free body fluids) for C57BL/6J mice, as measured by quantitative nuclear magnetic resonance, based on age (14 weeks old), sex (males and females), and dietary conditions [normal chow diet (CD): Safe Diets D04; high-fat diet (HFD): Research Diets D12451].

Sucrose preference (Basic Protocol 13)

To avoid place preference, we recommend alternating the position of the sucrose bottle and the water bottle on each test day. If the bottle is blocked, clean the nipple with a bottle brush or change the bottle.

Understanding Results

Figure 11 shows some indicative body composition ranges (lean mass, fat mass, and free body fluids) for C57BL/6J mice as measured by qNMR, based on age (14 weeks old), sex (males and females), and dietary conditions (normal CD: Safe Diets D04; HFD: Research Diets D12451).

In the Y-maze protocol (Basic Protocol 9 and Alternate Protocols 3 and 5), the % SPA in normal (i.e., wild-type or vehicle-treated) mice is generally >60%. A decrease in % SPA indicates deficits in working memory performance. This decrease can be observed with amnestic treatments, such as the anticholinergic scopolamine (see Current Protocols article: Riet et al., 2022), or in a DS mouse model (Fig. 12). If AARs are not considered, it is also possible to compare the % SPA of each group with chance (50%) using the one-group *t*-test (Faizi et al., 2012).

In the marble-burying test (Basic Protocol 10 and Alternate Protocol 4), the average percentage of buried marbles is generally between 50% and 70% at the end of the 15-min test in the C57BL/6N and C57BL/6J mouse groups. In mouse models of autism, the percentage of buried marbles is increased (Veeraragavan et al., 2012). This increased percentage of buried marbles can also be observed in a DS mouse model (Fig. 13).

For the OLM/NOR test (Basic Protocol 11 and Alternate Protocol 5), the main variables are recognition indices. A recognition index that is not statistically different from the chance level (50%) indicates memory impairment. In a DS mouse model, mutant male mice show a recognition index close to chance for OLM as well as for NOR (Fig. 14).

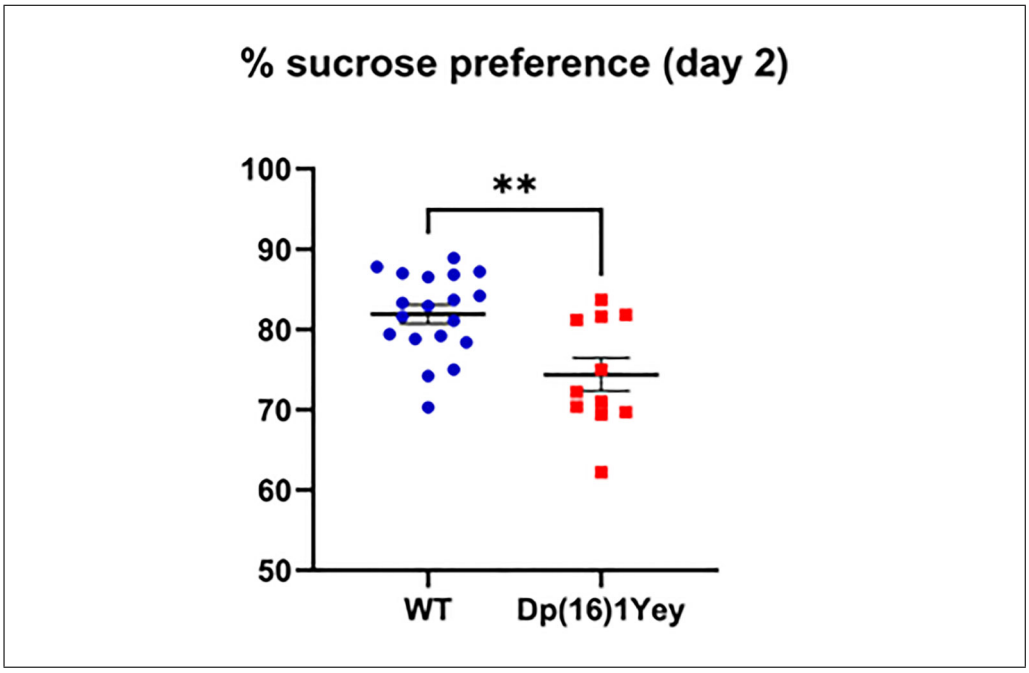

Figure 12 Example of results from the Y-maze test. Dp(16)1Yey male and female mice show a significantly decreased spontaneous alternation performance percentage (% SPA) compared with WT mice. Data are expressed as the mean ± SEM and were analyzed using two-way ANOVA followed by Sidak's multiple comparisons test. *$p < 0.05$, **$p < 0.01$.

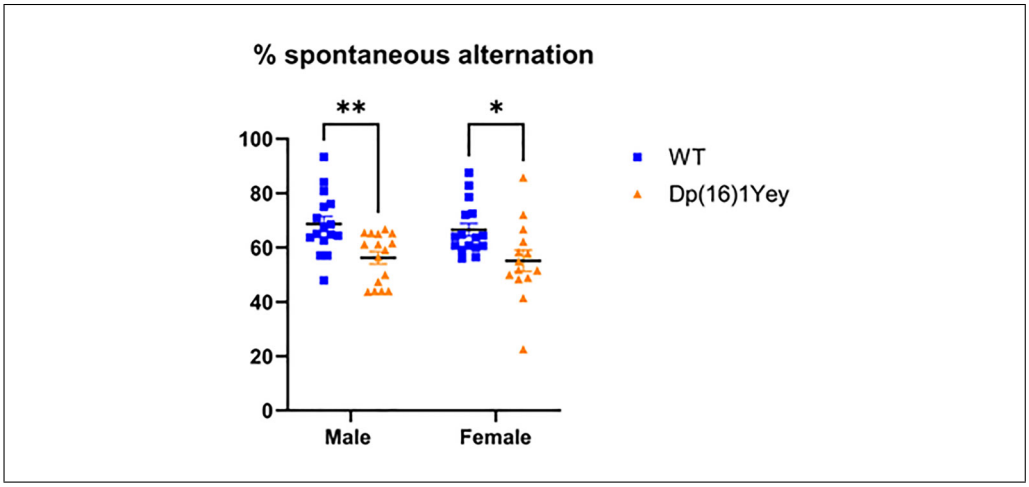

Figure 13 Example of results from the marble-burying test. Dp(16)1Yey male mice show a significantly increased percentage of buried marbles compared with WT mice. Data are expressed as the mean ± SEM and were analyzed using the Mann-Whitney test. *$p < 0.05$.

In the nest-building test (Basic Protocol 12), the average nesting score is around 2 in C57BL/6J mice after 2 hr of testing. The score is 4 to 5 after 5 and 24 hr of testing, respectively. In an autism mouse model, we observed a significant decrease in the nesting score 5 and 24 hr after the start of the test. This nesting deficit was reversed by AAV treatment (Habbas et al., 2022).

The percentage of sucrose preference (Basic Protocol 13) is generally >75% in C57BL/6N and C57BL/6J. A percentage not significantly higher than chance (50%) indicates anhedonia. A significant decrease in the percentage of sucrose preference could be observed in a DS mouse model compared with wild-type (Fig. 15).

Time Considerations

Y-maze testing (Basic Protocol 9 and Alternate Protocols 3 and 5)

The test is short (5 min), and cleaning the maze takes ∼3 min. It therefore takes ∼8 min to test a mouse.

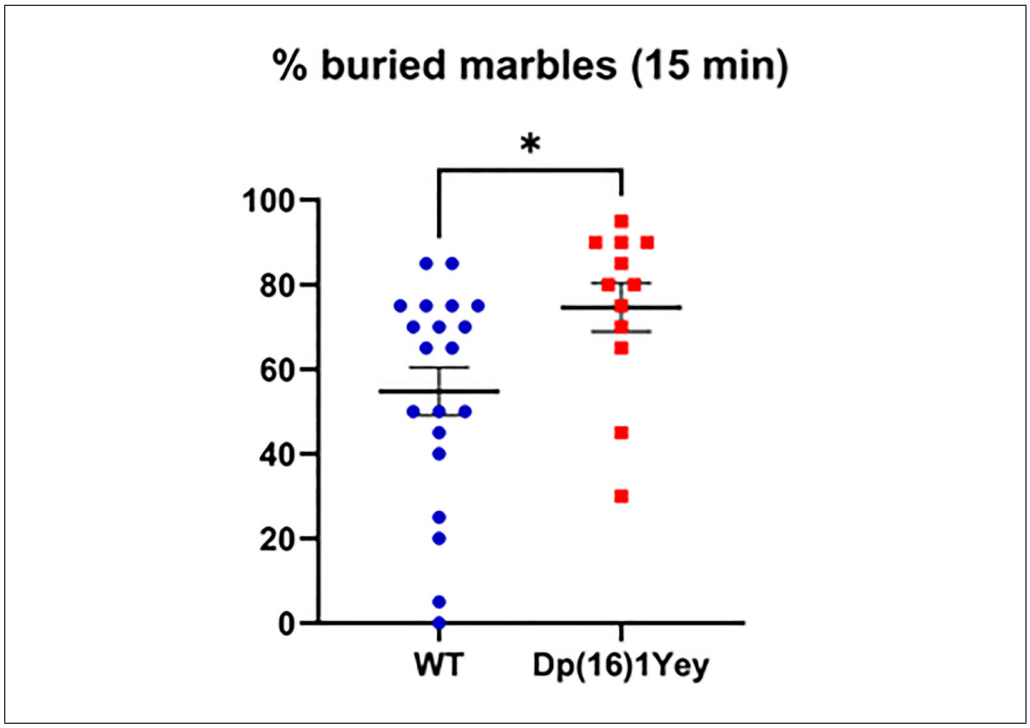

Figure 14 Example of results from the OLM/NOR test based on the recognition index. Dp(16)1Yey male mice show recognition indices not different from chance, whereas in WT mice, recognition indices are significantly higher than chance. Data are expressed as the mean ± SEM and were analyzed using a one-sample *t*-test. *$p < 0.01$ versus chance (50%).

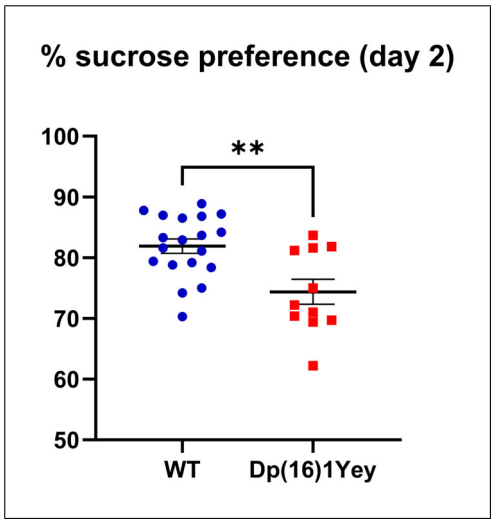

Figure 15 Example of results for the sucrose preference test. Dp(16)1Yey male mice show a significantly decreased percentage of sucrose preference compared with WT. Data are expressed as the mean ± SEM and were analyzed using a one-sample *t*-test. **$p < 0.01$.

Marble burying (Basic Protocol 10 and Alternate Protocol 4)

A total of 20 to 25 min are required to test a mouse (15 min testing + 5 to 10 min preparation and cleaning). Analysis time is ~10 min per mouse.

Object location memory/novel object recognition (Basic Protocol 11 and Alternate Protocol 5)

For each mouse, 20 min are required for each habituation session (15 min testing + 5 min preparation and cleaning). Fifteen minutes are required per mouse for each acquisition or retention session (10 min testing + 5 min preparation and cleaning). The whole protocol can be performed on 24 mice in 1 week.

Nest building (Basic Protocol 12)

Preparing test cages for 24 animals takes ~10 min. Each scoring phase lasts ~15 min. Overall, it takes 1 hr of work to test 24 animals.

Sucrose preference (Basic Protocol 13)

It takes ~10 min to prepare the sucrose solution, i.e., 20 min for the two preparations. Preparing and cleaning the test cages take ~1 hr 10 min for 24 animals. A total of 15 min are needed for weighing one bottle during the test days, i.e., 1 hr 30 min for the six weighings. In all, 3 hr are needed to carry out this test on 24 animals.

Other time considerations

Basic Protocols 1 to 3 and 5 to 13 should be performed in the morning unless otherwise specified (e.g., see the introduction to Basic Protocol 4). Basic Protocol 14 requires tissue

collection in the morning; after fixation, processing may occur at any available time.

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Conflict of Interest

The authors have no conflict of interest to disclose.

Data Availability Statement

All the data and metadata from the GO-DS21 pipeline will be made available through the FAIR3R resource portal. The data that support the protocols are openly available in the FAIR3R resource portal at *https://www.fair3r.fr/*.

Literature Cited

Angoa-Pérez, M., Kane, M. J., Briggs, D. I., Francescutti, D. M., & Kuhn, D. M. (2013). Marble burying and nestlet shredding as tests of repetitive, compulsive-like behaviors in mice. *Journal of Visualized Experiments*, (82), 50978. https://doi.org/10.3791/50978

Ayadi, A., Birling, M.-C., Bottomley, J., Bussell, J., Fuchs, H., Fray, M., Gailus-Durner, V., Greenaway, S., Houghton, R., Karp, N., Leblanc, S., Lengger, C., Maier, H., Mallon, A.-M., Marschall, S., Melvin, D., Morgan, H., Pavlovic, G., Ryder, E., … Herault, Y. (2012). Mouse large-scale phenotyping initiatives: Overview of the European Mouse Disease Clinic (EUMODIC) and of the Wellcome Trust Sanger Institute Mouse Genetics Project. *Mammalian Genome*, *23*(9–10), 600–610. https://doi.org/10.1007/s00335-012-9418-y

Brown, S. D. M., Holmes, C. C., Mallon, A.-M., Meehan, T. F., Smedley, D., & Wells, S. (2018). High-throughput mouse phenomics for characterizing mammalian gene function. *Nature Reviews Genetics*, *9*(6), 357–370. https://doi.org/10.1038/s41576-018-0005-2

de Chaumont, F., Ey, E., Torquet, N., Lagache, T., Dallongeville, S., Imbert, A., Legou, T., le Sourd, A.-M., Faure, P., Bourgeron, T., & Olivo-Marin, J.-C. (2019). Real-time analysis of the behaviour of groups of mice via a depth-sensing camera and machine learning. *Nature Biomedical Engineering*, *3*(11), 930–942. https://doi.org/10.1038/s41551-019-0396-1

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biological Conservation*, *1*(1), 1–10. https://doi.org/10.1016/0006-3207(92)91201-3

Faizi, M., Bader, P. L., Saw, N., Nguyen, T.-V. V., Beraki, S., Wyss-Coray, T., Longo, F. M., & Shamloo, M. (2012). Thy1-hAPPLond/Swe+ mouse model of Alzheimer's disease displays broad behavioral deficits in sensorimotor, cognitive and social function. *Brain and Behavior*, *2*, 142–154. https://doi.org/10.1002/brb3.41

Gaskill, B. N., Karas, A. Z., Garner, J. P., & Pritchett-Corning, K. R. (2013). Nest building as an indicator of health and welfare in laboratory mice. *Journal of Visualized Experiments*, (82), 51012. https://doi.org/10.3791/51012

Habbas, K., Cakil, O., Zámbó, B., Tabet, R., Riet, F., Dembele, D., Mandel, J.-L., Hocquemiller, M., Laufer, R., Piguet, F., & Moine, H. (2022). AAV-delivered diacylglycerol kinase DGKk achieves long-term rescue of fragile X syndrome mouse model. *EMBO Molecular Medicine*, *14*(5), e14649. https://doi.org/10.15252/emmm.202114649

Holcomb, L., Gordon, M., McGowan, E., Yu, X., Benkovic, S., Jantzen, P., Wright, K., Saad, I., Mueller, R., Morgan, D., Sanders, S., Zehr, C., O'Campo, K., Hardy, J., Prada, C.-M., Eckman, C., Younkin, S., Hsiao, K., & Duff, K. (1998).

Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. *Nature Medicine*, *4*, 97–100. https://doi.org/10.1038/nm0198-097

Li, Z., LoBue, A., Heuser, S. K., Li, J., Engelhardt, E., Papapetropoulos, A., Patel, H. H., Lilley, E., Ferdinandy, P., Schulz, R., & Cortese-Krott, M. M. (2025). Best practices for blood collection and anaesthesia in mice: Selection, application and reporting. *British Journal of Pharmacology*, *2*(11), 2337–2353. https://doi.org/10.1111/bph.70029

Prieur, E. A. K., & Jadavji, N. M. (2019). Assessing spatial working memory using the spontaneous alternation Y-maze test in aged male mice. *Bio-Protocol*, *9*(3), e3162. https://doi.org/10.21769/BioProtoc.3162

Riet, F., Mittelhaeuser, C., Lux, A., Bour, R., Selloum, M., Sorg, T., Herault, Y., & Meziane, H. (2022). Behavioral testing design for evaluation of cognitive disabilities. *Current Protocols*, *2*, e382. https://doi.org/10.1002/cpz1.382

Roswell, M., Dushoff, J., & Winfree, R. (2021). A conceptual guide to measuring species diversity. *Oikos*, *130*(3), 321–338. https://doi.org/10.1111/oik.07202

Veeraragavan, S., Graham, D., Bui, N., Yuva-Paylor, L. A., Wess, J., & Paylor, R. (2012). Genetic reduction of muscarinic M4 receptor modulates analgesic response and acoustic startle response in a mouse model of fragile X syndrome (FXS). *Behavioural Brain Research*, *228*(1), 1–8. https://doi.org/10.1016/j.bbr.2011.11.018

Wall, P. M., & Messier, C. (2002). Infralimbic kappa opioid and muscarinic M1 receptor interactions in the concurrent modulation of anxiety and memory. *Psychopharmacology*, *160*(3), 233–244. https://doi.org/10.1007/s00213-001-0979-9