# Assessing Healthspan and Lifespan Measures in Aging Mice: Optimization of Testing Protocols, Replicability, and Rater Reliability

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The relationship between chronological age (lifespan) and biological age (healthspan) varies amongst individuals. Understanding the normal trajectory and characteristic traits of aging mice throughout their lifespan is important for selecting the most reliable and reproducible measures to test hypotheses. The protocols herein describe assays used for aging studies at The Jackson Laboratory's Mouse Neurobehavioral Phenotyping Facility and include assessments of frailty, cognition, and sensory (hearing, vision, olfaction), motor, and fine motor function that can be used for assessing phenotypes in aged mice across their lifespan as well as provide guidance for setting up and validating these behavioral measures. Researchers aiming to study aging phenotypes require access to aged mice as a reference when initiating these types of studies in order to observe normal aging characteristics that cannot be observed in young adult mouse populations. 

Keywords: activity • aging • cognition • episodic memory • frailty • hearing • mice • olfaction • vision

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## INTRODUCTION

The relationship of biological age and chronological age as measures of healthspan and lifespan vary amongst individuals, including both mice and men. This variance includes differences in susceptibility and resiliency to age-related physical and cognitive decline and disease. Mice are the most common laboratory animal used to model human disease, including diseases of aging (Rosenthal & Brown, 2007). Several studies have assessed behavior of aging mice (Fahlström, Yu, & Ulfhake, 2011; Shoji, Takao, Hattori, & Miyakawa, 2016). Mouse frailty indices have been developed but are invasive, cannot be conducted longitudinally (Parks et al., 2012), or are limited primarily to motor and neuromuscular changes (Whitehead et al., 2014). Just as changes in motor function occur with age, other aging-related changes (e.g., in vision, hearing, and ability to smell) that become impaired in humans with age or certain aging-related disorders are also evident in aging mice and may be the most translational measures of aging pre-clinically.



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A longitudinal assessment of a battery of phenotyping tests provides the opportunity for studying cohorts of mice as they age, taking advantage of the dynamic range of aging and behavior. Age-related decline is not limited to physical frailty of an individual. In addition to a frailty assessment (Whitehead et al., 2014), a battery of non-invasive behavioral tests can be used to assess motor and fine-motor function, vision, hearing, olfaction, and memory to understand the normal aging process. Not only are these measures characteristic traits of aging, but impairments may confound the interpretation of other outcome measures. For example, cognitive tests that use visual or audio cues would not be appropriate for mice that have aging-related visual or hearing impairments. Thus, their validity can only be determined if vision and hearing tests are included in the testing battery.

The protocols herein describe a comprehensive non-invasive behavioral testing battery that can be administered longitudinally in order to assess mouse sensory, motor, sensory, and cognitive aging as markers of healthspan. Detailed protocols are given for each task along with examples of data in order to increase the replicability of each task. It is particularly important that laboratories planning to conduct aging studies validate these assays in their laboratory; in some cases, validation studies must be conducted with young versus aged mice. Specifically, there are certain aspects of frailty that cannot be observed in young mice, so aged mice must be used in order to properly train the user to identify the spectrum of aging characteristics, many of which are not observed in mice $<$18 months of age. In cases where aged mice may not be available but there is a desire to have the assay validated in preparation for studying aged mice, optional information and reference data for known standards are provided for many of the protocols (e.g., administration of scopolamine to demonstrate cognitive impairment is detectable under the assay conditions). The example data also allow for comparison against results from future replications. It should be noted that subtle effects of phenotypes may not be evident due to differences in testing environments, although it has been established that there are significant interactions between testing environment and task performance across inbred mouse strains and that large performance differences between strains are generally preserved across testing environments (Crabbe, Wahlsten, & Dudek, 1999; Wahlsten et al., 2003). Nevertheless, it is critical that researchers wishing to run these experiments in their own laboratories conduct validation experiments that include a positive control for each assay, such as data reported herein, in order to ensure that the assay being set up is reliable and robust in the laboratory environment (Sukoff Rizzo & Silverman, 2016). These data can also be used as reference data for training staff to ensure that they are proficient at replicating these results under blinded conditions prior to running experimental cohorts.

## STRATEGIC PLANNING

### General Considerations

All experiments with animals should be approved by the Internal Animal Care and Use Committee and local governance boards. Subject information except for sex should be blinded by a researcher familiar with the study design but not directly involved with the testing or data analysis. Subject information can be coded as A, B, C . . . or similar to facilitate randomization of treatment groups across order of testing and instrumentation.

Subjects should be acclimated to the testing facility light cycle for at least 5 days prior to testing. A worksheet for each day's testing should be prepared in advance that provides testing order, planned timing, and an area for notes, comments, or protocol deviations. Typically, testing should be scheduled with at least a 1-day rest period between tests and no testing on the same day of a cage change.

All testing described here is performed during the light phase of a 12:12 light/dark cycle, with initiation at least 60 min after lights on and conclusion at least 60 min before lights off. On each test day, subjects are transported to the procedure room after the environment (e.g., lighting levels) has been set and all environmental variables for lighting, temperature, humidity, and noise levels have been recorded. For all protocols, testing is conducted in a purpose-built behavioral testing facility optimized for conducting noise-sensitive tests.

Before the first subject and between subjects, all testing arenas are sanitized with 70% ethanol/water solution and dried prior to introducing the next subject. Testing equipment is always cleaned prior to the first mouse to ensure adequate removal of previous odors and to expose the first mouse in the test to the same conditions as all subsequent subjects.

### Housing Conditions

Mice can be group-housed (within sex; $n$ = 2-4 per pen) or individually housed. Depending on strain, male mice may require separation due to fighting. This should be planned for prior to starting the testing battery, as opposed waiting until separation is required during the testing battery. Importantly, aging mice should not be re-housed with new cage mates once social groups have been established. Mice are housed in ventilated cages with a temperature range of 22°-23°C (72°-74°F) and a humidity range of 55% to 65%. Food and water should be available *ad libitum* except during testing.

### Subject Identification

A form of permanent identification for individual mice is necessary for longitudinal studies but should also be considered for cross-sectional studies. Ear notching is a common, low-cost, and acceptable method for permanent identification, whereas toe notching is not preferred because some tests require intact toes (e.g., grip strength). Ear tags can be inadvertently lost throughout lifespan and commercially available microchips may dislodge from original placement. Irrespective of the method, it is recommended that permanent identification be completed at least one week prior to phenotyping. A method commonly used in our laboratory in addition to any permanent identification is a non-invasive tail-labeling procedure that facilitates easy visual identification of individual mice and minimizes the need to restrain the subject each time it is handled to confirm its permanent identification. Using a non-toxic marker (e.g., Sharpie brand), the technician writes the subject's identification number on the tail in red or blue ink for odd or even identification numbers, respectively, and further spaces the number placement on the tail in a systematic configuration from lowest number at the base of the tail to higher numbers midway or lower on the tail. For example, in a group-housed cage of four mice (assigned as #1-4), #1 is marked with a red 1 at the base of the tail, #2 is marked with a blue 2 at the base of the tail, #3 is marked with a red 3 midway on tail, and #4 is marked with a blue 4 midway on tail (Fig. 1). Such markings are typically decipherable for several days.

## FRAILTY ASSESSMENT WITH CORE BODY TEMPERATURE

***BASIC PROTOCOL 1***

Frailty is defined as a decline and deterioration of normal physiological functioning with chronological aging that leads to increasing vulnerability to disease and mortality (Fedarko, 2011; Rockwood, Fox, Stolee, Robertson, & Lynn, 1994; Xue, 2011). Frailty manifests in individuals at different rates and is generally characterized by weakness, weight loss, low activity, and functional deficits across multiple physiological symptoms (Clegg & Young, 2011). Frailty can be thought of as a spectrum of aging-related changes that manifest differently in different individuals and at different rates of change in both mice and men. For example, two individuals may be of the same chronological age but one may demonstrate alopecia, urinary incontinence, and gait deficits while the other may

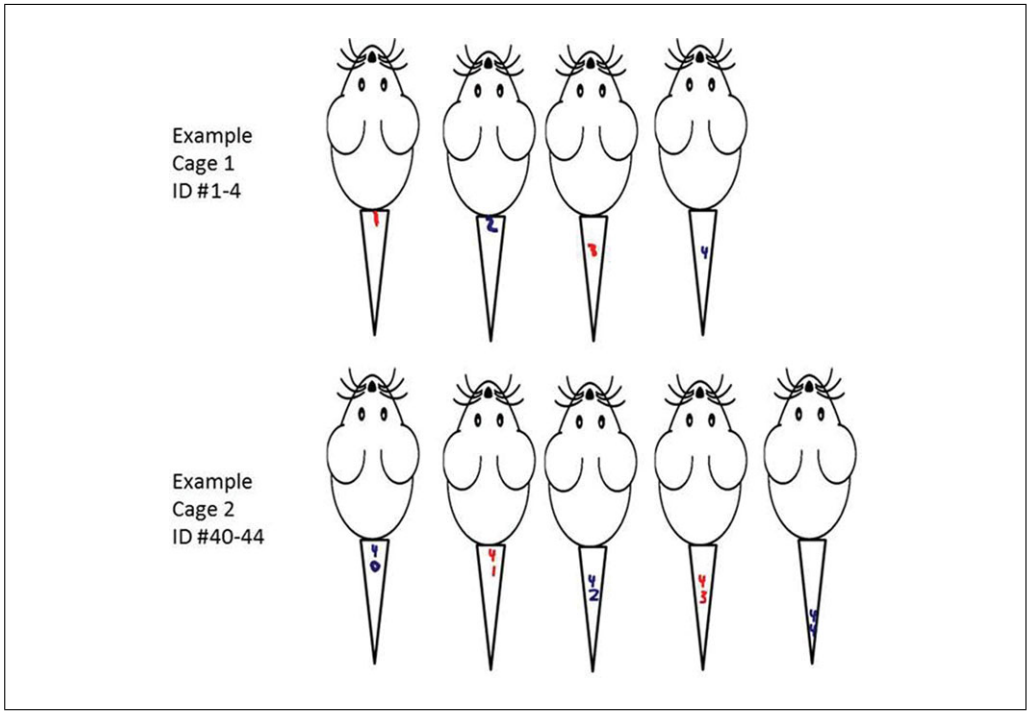


**Figure 1** Example of tail-labeling procedures. A visual means of identification that can be easily viewed can facilitate visual confirmation of the correct mouse without inducing the stress of restraint needed to read permanent ID tags (e.g., ear tags, notches, microchips). Non-toxic markers can be used and typically remain visible for several days. Odd numbers are labeled in red and even numbers are labeled in blue. Examples of two separate cages with $n = 4$ per cage or $n = 5$ per cage are presented with IDs of #1–4 and #40–44, respectively.

manifest sarcopenia (loss of muscle mass and strength), deafness, and impaired vision. The frailty assessment used in the current protocol is a modification and extension of the methods of Whitehead et al. (2014) and is designed as a non-invasive characterization of aging-related changes in physical characteristics and reflexes aimed at assessing the onset of aging traits in individual mice. In this protocol, mice are individually observed and evaluated for the absence or presence and severity of 27 different characteristics each scored as 0, 0.5, or 1 based on level of severity. A frailty index score is calculated as the cumulative score of all measures with a maximum score of 27 (Fig. 2). As a final measurement of frailty, body temperature is recorded.

### *Materials*

Subjects: young adult male or female mice 2–6 months of age (e.g., C57BL/6J, The Jackson Laboratory, no. 000664) and sex-matched mice >10 months of age
Glycerol with container (e.g., weigh boat or small beaker)
70% (v/v) ethanol solution

Animal scale (0.1-g precision)
Blue and red non-toxic permanent markers
Cotton-tipped swabs
Observation arena: empty cage without food, water, bedding, or lid
Standard wire bar lid
Thermometer and rectal probe (e.g., Braintree Scientific TH5 Thermalert digital thermometer and RET 3 rectal probe, ¾ in. long, 0.028 in. diameter, 0.065 in. tip)
Bedding arena: clean cage filled with bedding for a soft landing surface
Paper towels
Clean, empty cages for transition cages

| Parameter | Score | #1 | #2 | #3 | #4 | #5 | #6 | #7 | #8 | #9 | #10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Weight | Actual body weight (g) | | | | | | | | | | |
| Alopecia | 0 = normal fur density | | | | | | | | | | |
| | 0.5 = < 25% fur loss | | | | | | | | | | |
| | 1 = >25% fur loss | | | | | | | | | | |
| Loss of fur colour | 0 = normal colour | | | | | | | | | | |
| | 0.5 = focal grey/brown changes | | | | | | | | | | |
| | 1 = fur color changes throughout body | | | | | | | | | | |
| Dermatitis | 0 = absent | | | | | | | | | | |
| | 0.5 = focal lesions (*e.g.* neck, flanks, under chin) | | | | | | | | | | |
| | 1 = widespread or multifocal lesions | | | | | | | | | | |
| Loss of whiskers | 0 = no loss | | | | | | | | | | |
| | 0.5 = reduced number of whiskers | | | | | | | | | | |
| | 1 = absence of whiskers | | | | | | | | | | |
| Coat condition | 0 = smooth, sleek, shiny coat | | | | | | | | | | |
| | 0.5 = coat is slightly ruffled | | | | | | | | | | |
| | 1 = unkempt and un-groomed, matted appearance | | | | | | | | | | |
| Piloerection | 0 = no piloerection | | | | | | | | | | |
| | 0.5 = involves fur at base of neck only | | | | | | | | | | |
| | 1 = widespread piloerection | | | | | | | | | | |
| Cataracts | 0 = no cataract | | | | | | | | | | |
| | 0.5 = small opaque spot on one eye | | | | | | | | | | |
| | 1 = clear evidence of spots on both eyes | | | | | | | | | | |
| Eye discharge/swelling | 0 = normal | | | | | | | | | | |
| | 0.5 = slight swelling and/or secretions | | | | | | | | | | |
| | 1 = obvious bulging and/or secretions | | | | | | | | | | |
| Microphthalmia | 0 = normal size | | | | | | | | | | |
| | 0.5 = one or both eyes slightly small or sunken | | | | | | | | | | |
| | 1 = one or both eyes very small or sunken | | | | | | | | | | |
| Corneal opacity | 0 = normal | | | | | | | | | | |
| | 0.5 = minimal clouding on one eye | | | | | | | | | | |
| | 1 = marked clouding on both eyes | | | | | | | | | | |
| Nasal discharge | 0 = no discharge | | | | | | | | | | |
| | 0.5 = small amount of discharge | | | | | | | | | | |
| | 1 = obvious discharge, both nares | | | | | | | | | | |
| Rectal prolapse | 0 = no prolapse | | | | | | | | | | |
| | 0.5 = small amount of rectum tissue visible below tail | | | | | | | | | | |
| | 1 = rectum clearly visible below tail. | | | | | | | | | | |
| Penile prolapse (male) | 0 = no prolapse | | | | | | | | | | |
| Vaginal prolapse (female) | 0.5 = small amount of prolapsed tissue visible | | | | | | | | | | |
| | 1 = prolapsed tissue clearly visible | | | | | | | | | | |
| Diarrhea | 0 = none | | | | | | | | | | |
| | 0.5 = softened/smeared stool or bedding near rectum | | | | | | | | | | |
| | 1 = feces plus blood and bedding near rectum | | | | | | | | | | |
| Body condition score | 0 = bones palpable, not prominent | | | | | | | | | | |
| | 0.5 = bones prominent | | | | | | | | | | |
| | 1 = bones very prominent | | | | | | | | | | |
| Tumors | 0 = absent | | | | | | | | | | |
| | 0.5 = <1.0 cm | | | | | | | | | | |
| | 1 = >1.0 cm or multiple smaller tumours | | | | | | | | | | |
| Kyphosis | 0 = absent | | | | | | | | | | |
| | 0.5 = mild curvature | | | | | | | | | | |
| | 1 = clear evidence of hunched posture | | | | | | | | | | |
| Gait disorders | 0 = no abnormality | | | | | | | | | | |
| | 0.5 = abnormal gait but animal can still walk | | | | | | | | | | |
| | 1 = marked abnormality, impairs ability to move | | | | | | | | | | |
| Tremor | 0 = no tremor | | | | | | | | | | |
| | 0.5 = slight tremor | | | | | | | | | | |
| | 1 = marked tremor; animal cannot rear | | | | | | | | | | |
| Breathing rate/depth | 0 = normal | | | | | | | | | | |
| | 0.5 = modest change in breathing rate and/or depth | | | | | | | | | | |
| | 1 = marked changes in rate/depth, gasping | | | | | | | | | | |
| Menace reflex | 0 = always responds | | | | | | | | | | |
| | 0.5 = no response to 1 or 2 approaches | | | | | | | | | | |
| | 1 = no response to 3 approaches | | | | | | | | | | |
| Tail stiffening | 0 = no stiffening, tail curls around finger | | | | | | | | | | |
| | 0.5 = tail responsive but does not curl | | | | | | | | | | |
| | 1 = tail completely unresponsive | | | | | | | | | | |
| Vestibular disturbance | 0 = absent | | | | | | | | | | |
| | 0.5 = mild head tilt and/or slight spin when lowered | | | | | | | | | | |
| | 1 = severe disequilibrium | | | | | | | | | | |
| Vision loss (Visual Placing) | 0 = before vibrasse contact | | | | | | | | | | |
| | 0.5 = upon vibrasse contact | | | | | | | | | | |
| | 1 = does not reach (nose in contact with surface) | | | | | | | | | | |
| Distended abdomen | 0 = absent | | | | | | | | | | |
| | 0.5 = slight bulge | | | | | | | | | | |
| | 1 = abdomen clearly distended | | | | | | | | | | |
| Malocclusions | 0 = mandibular longer than maxillary incisors | | | | | | | | | | |
| | 0.5 = teeth slightly uneven | | | | | | | | | | |
| | 1 = teeth very uneven and overgrown | | | | | | | | | | |
| Temperature | record rectal body temperature °C | | | | | | | | | | |
| Righting Reflex | 0 = normal | | | | | | | | | | |
| | 0.5 = lands on side or back but rights itself immediately | | | | | | | | | | |
| | 1 = lands on back, fails to right itself | | | | | | | | | | |
| | Comments | | | | | | | | | | |

**Figure 2** Template for recording frailty assessment scores. The template can be printed or pasted into an Excel spreadsheet and used to record the scores for each observation as well as body weight and temperature.

### *Setup and habituation*

1. Set up the testing room in preparation for the experiment, ensuring that the testing environment has lighting, temperature, and humidity levels comparable to the colony. Place instrumentation and consumables on a clean, flat surface.

2. Prepare a treatment sheet (either electronic or paper) including mice to be tested, testing order, and scoring template for each mouse in which the data will be entered (Fig. 2).
3. Bring mice to the testing room. Weigh each mouse and label for easy visual identification. Allow mice to habituate undisturbed to the testing environment for a minimum of 1 hr.

***Testing***

4. Remove the first subject from the home cage, restrain gently with the middle of the tail between the thumb and forefinger, and place on a solid surface at eye level to enable visual inspection.
5. Inspect for signs of alopecia (fur loss) excluding barbering around the snout. On the data sheet, enter a value of 0 for no sign of fur loss, 0.5 for fur loss $<25\%$ of normal fur density, or 1 for fur loss encompassing $>25\%$ of normal fur density.
6. Inspect for loss of fur color. Enter a value of 0 if fur color is typical for adult mice, 0.5 for any sporadic focal changes (black to grey/white or brown; brown or agouti to tan or grey), or 1 for fur color changes throughout the body.
7. Inspect for dermatitis or presence of skin lesions. Enter a value of 0 for no observed dermatitis or skin lesions including bite wounds, 0.5 for presence of a single small lesion ($<1$ cm), or 1 for multiple lesions or widespread dermatitis.
8. Inspect whiskers. Enter a value of 0 if whiskers are present and have even density on both sides, 0.5 for reduced number of whiskers including inconsistent whisker density on both sides of snout, or 1 for whiskers absent.
9. Inspect the mouse's coat condition. Enter a value of 0 for a sleek shiny coat, 0.5 for ruffled or matted fur in a single location, or 1 for ruffled or matted fur throughout body, indicative of poor grooming.
10. Inspect the coat for signs of piloerection, defined as erection of the hairs. Enter a value of 0 for no sign of piloerection, 0.5 for piloerection involving only the area around the nape of the neck, or 1 for piloerection throughout.
11. Inspect the eyes for cataracts indicated by opaque spots. Enter a value of 0 if eyes are clear and bright without any spots, 0.5 for an opaque or white spot in one eye, or 1 for opaque or white spots in both eyes.
12. Inspect the eyes for discharge or swelling. Enter a value of 0 for no discharge or swelling, 0.5 for discharge or swelling/bulging in one eye, or 1 for discharge or swelling/bulging in both eyes.
13. Inspect the size of the eyes for microphthalmia. Enter a value of 0 if both eyes are normal size, 0.5 if one eye is abnormally small, or 1 if both eyes are abnormally small.
14. Inspect the eyes for cataracts as indicated by clouding. Enter a value of 0 for clear and bright eyes with no evidence of clouding, 0.5 for clouding in one eye, or 1 for clouding in both eyes.
15. Inspect the nares and snout for nasal discharge. Enter a value of 0 for no discharge observed, 0.5 for a small amount of discharge in one nare, or 1 for discharge in both nares.
16. While continuing to grasp the tail, lift the tail upward to inspect the rectal area for prolapse. Enter a value of 0 for no prolapse (normal), 0.5 if a small amount of

rectal tissue is observed (pink to red color), or 1 if a rectal prolapse is indicated by clear observation of internal flesh that does not retract with manipulation of the tail.

17. While continuing to grasp the tail, inspect for penile (male) or vaginal (female) prolapse. Enter a value of 0 if no prolapse is observed, 0.5 if a small amount of tissue (pink to red color) is observed and retracts upon movement, or 1 if prolapsed tissue is pronounced and does not retract upon movement.

18. While continuing to grasp the tail, lift the tail upward to inspect the rectal area for diarrhea. Enter a value of 0 for no evidence of diarrhea or soft stool, 0.5 for evidence of soft or smeared stool, or 1 for observation of diarrhea.

19. Inspect body for the presence of tumors. Enter a value of 0 for no tumors observed, 0.5 for a visible tumor $<1$ mm, or 1 for a visible/palpable tumor $>1$ mm.

20. Inspect for kyphosis as indicated by hunched posture due to curvature of the spine. This can also be observed during assessment of gait in the observation arena. Enter a value of 0 if absent, 0.5 for mild curvature of the spine, or 1 for prominent curvature of the spine resulting in hunched posture.

21. Manually palpate the sacroiliac region (back and pubic bones) to evaluate body condition. Enter a value of 0 for normal palpable flesh without prominence of bone, 0.5 for bones slightly palpable, or 1 for bones palpable and prominent.

22. Place the subject in the observation arena and immediately observe gait. Enter a value of 0 if gait is normal and fluid, 0.5 if there is any mild abnormality in gait (including wide stance, asymmetrical cadence, wobbling, or weakness) but animal can still walk and rear, or 1 for a marked abnormality that impairs the animals' ability to walk.

23. Observe the mouse while freely moving and while stationary in the arena for sign of tremor. Enter a value of 0 for no tremor, 0.5 for mild tremor, or 1 for tremor that impairs the animal's ability to walk or rear.

24. While the mouse is freely moving, observe breathing rate and depth. Enter a value of 0 for normal breathing rate and depth, 0.5 for a modest change in breathing rate or depth, including any crackling or squeaking noises while freely moving or stationary, or 1 for labored breathing.

25. To assess the menace reflex, while the mouse is freely moving, approach the subject's face with a cotton-tipped swab without touching the subject or making contact with its face or whiskers. The cotton swab serves as the stimulus for the menace reflex assessment. Present the stimulus three consecutive times towards the face, between the eyes. The menace reflex is intact if the mouse responds with a blink or squint of the eye. Enter a value of 0 if the mouse responds to all three stimulus presentations, 0.5 if the mouse responds to only one or two presentations, or 1 if the mouse does not respond to any presentations.

26. Grasp the base of the tail with one hand and stroke the underside of the tail with a finger of the opposite hand to assess tail stiffening. Importantly, ensure that the mouse is still and not pulling when the stimulus is presented. The normal response to the stimulus is a curling of the tail. Enter a value of 0 if the tail curls in response to the stimulus, 0.5 if the tail responds but does not curl, or 1 if the tail is limp and does not respond.

27. Pick up the subject by the middle of the tail and slowly lower the subject. Observe its head position to assess for vestibular disturbance. Enter a value of 0 if the head

is straight (the normal expected position), 0.5 if the head is tilted, or 1 if the animal spins upon being lowered.

28. Slowly lower the mouse by its tail towards the wire bar lid and observe the extension of the forelimbs for visual placing. Enter a value of 0 if the mouse extends the forelimbs and hindlimbs before the whiskers touch the wire bars, 0.5 if it extends the forelimbs upon contact of the whiskers with the bars, or 1 if the mouse fails to extend its forelimbs prior to nose contact with the wire bar lid.

29. Restrain the mouse in the supine position and evaluate for distended abdomen. Enter a value of 0 if the abdomen is not extended over the hip bone area, 0.5 if the abdomen is slightly distended, or 1 if the abdomen is clearly distended beyond the hip bone area.

30. While the subject is restrained in the supine position, use the bare end of a cotton-tipped swab to gently expose the teeth by retracting the lower lip and evaluate the teeth for uneven position or malocclusion. Enter a value of 0 if teeth are even and symmetrical and the mandibular teeth are longer than the maxillary incisors, 0.5 if teeth are slightly uneven, or 1 for very abnormal, uneven, or overgrown teeth.

31. While the subject is restrained in the supine position, lubricate the rectal probe of a digital thermometer with glycerol and insert it ~1 cm into the rectum for ~10 sec or until the digital reading stabilizes. Record the value to the nearest 0.1°C.

32. While the subject is restrained in the supine position with the belly up, place the restraint hand ~25 cm over the bedding arena and gently but swiftly release the restraint while simultaneously retracting the restraining hand to allow the mouse to fall freely onto the landing surface. Record the righting reflex response as 0 if normal (indicated by the mouse landing on all four paws), 0.5 if the subject lands on its side or back but immediately rights itself, or 1 if the subject fails to right itself immediately upon contact with the surface.

33. Return the mouse to the transition cage until all subjects from the home cage have completed testing.

34. Sanitize the testing environment, observation arena, rectal probe, and wire bar lid with 70% ethanol.

35. Repeat testing for all mice.

36. Return mice to the colony.

***Data QC and analysis***

37. Prior to data analysis and while still blinded, exclude any subject for which any of the frailty values could not be obtained or the technician failed to enter any single value.

    *This is important since the cumulative score of all values is used for data analysis.*

38. Sum the score for all 27 measures to calculate each mouse's cumulative frailty index score. Do not include body weight or temperature. Analyze by one-way ANOVA (within sex) with appropriate post-hoc test versus young controls or by *t*-test if comparing only two age groups (Fig. 3A).

39. Separately analyze body temperature (Fig. 3B) and body weight by one-way ANOVA (within sex) with appropriate post-hoc test versus young controls or *t*-test if comparing only two age groups.

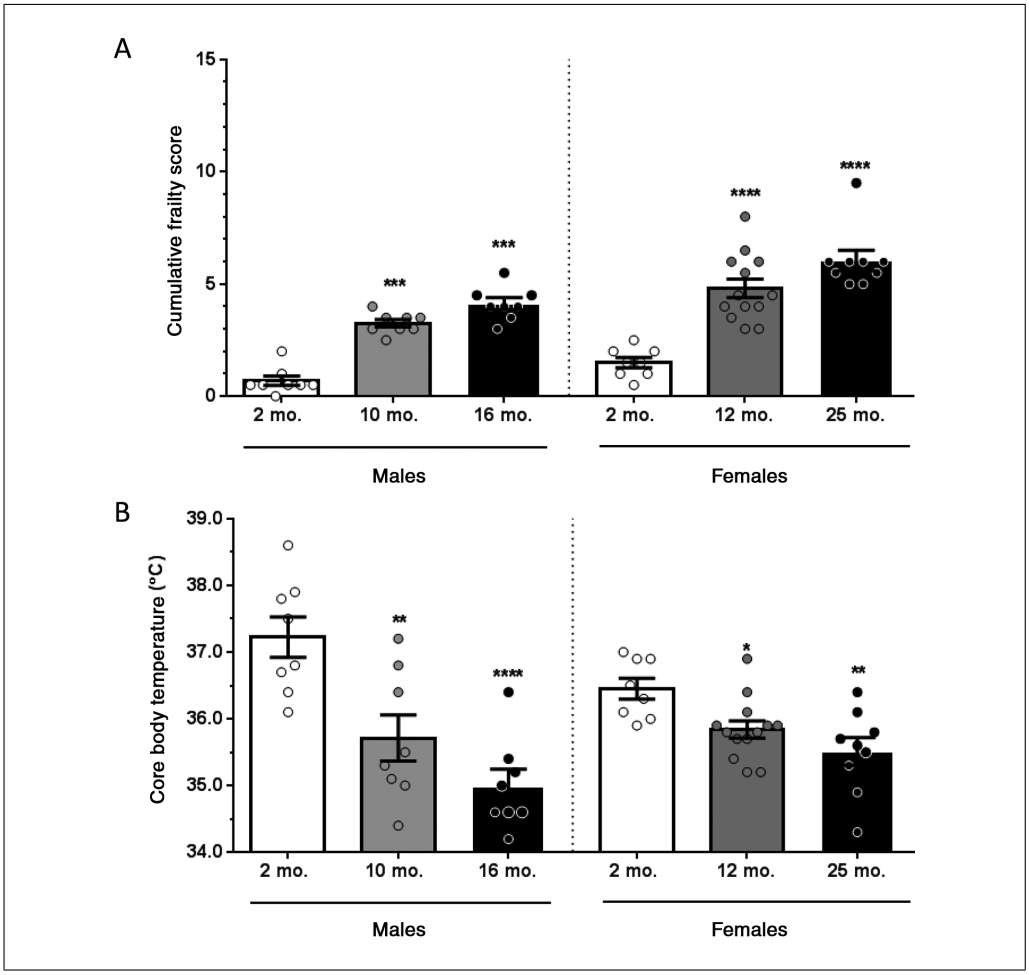


**Figure 3** Age-dependent changes in frailty index and core body temperature. Whereas some measures may represent 'state vs. trait' phenomena and the spectrum of aging characteristics may be manifested differently among animals, the cumulative frailty score demonstrates reliable measures of healthspan in aging mice and is inversely correlated with age-dependent reductions in core body temperature. Mice were behaviorally naïve at time of testing and age groups were independent groups of aging mice tested on the same day (males on one test day, females on a separate day). Data are presented as group mean ± s.e.m. within age and sex for (**A**) cumulative frailty scores and (**B**) core body temperature in males and females. All data were analyzed by one-way ANOVA (within sex) with Dunnett's post-hoc comparisons versus young controls.

## SPONTANEOUS OPEN-FIELD ACTIVITY

***BASIC PROTOCOL 2***

An open field is used to measure spontaneous exploratory activity. This task can be used to assess locomotor activity and general exploratory behaviors, as well as anxiety-related phenotypes (e.g., thigmotaxis behavior). Several different commercially available open-field arenas in a variety of sizes are available with various methods for tracking individual subjects, including infrared beams or video cameras. Researchers need to be aware that when using video tracking that is not infrared, variations in coat colors can make tracking problematic (i.e., for white mice against a white background). The background color of the arena can be changed to enable contrast against variations in coat colors; however, using different environments for different mouse coat colors may contribute to variations in behavioral responses and is therefore not recommended. The current protocol employs an infrared beam system with a clear arena housed in an environmentally controlled chamber that is white in color. Mice are habituated to the testing room undisturbed for 60 min prior to the test, then placed in individual open-field chambers to freely explore the arena without interruption.

Each standard-size, square arena (40 × 40 × 40 cm) is placed within a sound-attenuated and ventilated cabinet (the environmental control chamber) with a dimmable light affixed over the center of the arena, ∼50 cm from the arena floor. Lighting levels should be consistent with typical housing room light levels (∼500 lux). The choice of arena lighting is an important component of this test. Lighting should be consistent across arenas and should not emit heat (e.g., halogen lights), as mice may gravitate to a heat source, which could influence the test. Mounted externally along the perimeter, on opposite walls of the arena and at the level of the arena floor, are two horizontal 16 × 16 arrays of infrared photobeam sensors. These automatically track the location and movements of the mouse. An identical pair of infrared photobeam sensors is positioned on the remaining two walls in opposition to each other and elevated ∼7.6 cm above the floor to monitor vertical activity (rearing).

### *Materials*

Subjects: young adult male or female mice 2-6 months of age (e.g., C57BL/6J, The Jackson Laboratory, no. 000664) and sex-matched mice >16 months of age
70% (v/v) ethanol solution

Open-field arena (40 × 40 × 40 cm; Omnitech Electronics) with removable aerated lid
Environmental control chamber (60 × 64 × 60 cm; Omnitech Electronics) with dimmable xenon light
Four horizontal 16 × 16 arrays of infrared photobeam sensors
Behavioral tracking software (Fusion, Omnitech Electronics)
Red and blue non-toxic permanent markers
Paper towels

### *Setup and habituation*

1. Set up the testing room in preparation for the experiment, ensuring that the testing environment has lighting, temperature, and humidity levels comparable to the colony.

2. Power on the open field hardware and software and ensure that the system is operational. Ensure that infrared beams are not blocked and are functioning by waving your hand in the chamber. In addition, confirm that the arena is connected and assigned as intended (i.e., arena #1 is reading as arena #1).

   *If hardware or software needs to be repaired, it should be done prior to habituation of the mice to the testing environment.*

3. Prepare a treatment sheet (electronic or paper) including the mice to be tested, testing order, and arena ID the mouse will be tested within. Ensure treatment groups are randomized and counterbalanced across different instruments and for testing order.

   *Pay careful attention to counterbalancing representative samples of each treatment group or age group across multiple instruments to prevent any one treatment group from always being placed first or all controls, for example, always being in chamber 1.*

   *If multiple mice are housed in a single cage, it is best practice to test all mice within a cage in a single session and avoid exposing mice that have completed testing to cagemates that are still habituating and have not yet been tested. If the number of mice in a cage exceeds the available number of open-field arenas, do not return tested mice to their home cage with untested mice. These mice will interact with the untested mice and may influence their performance. Instead, move tested mice to a temporary transition cage until all mice from a cage have been tested and return all mice to their home cage at the same time.*

   *Allocate mice evenly across multiple batches of testing. For example, if 20 open-field arenas are available and 24 mice are scheduled to be tested, do not test 20 mice in the*

*first batch and 4 mice in the second. Instead, test 12 mice in each batch, ensuring for counterbalancing across arenas with representative samples from each treatment group or age group.*

4. Bring mice into the testing room and label each mouse for easy visual identification. Leave the testing room and let the mice habituate to the testing environment for a minimum of 1 hr.

### *Testing*

5. Before placing mice in any open-field arena, sanitize the instrumentation with 70% ethanol. For each arena, generously spray the walls and floor and wipe the floor with a clean paper towel, removing all feces and urine. Next, re-spray the arena and wipe the walls first, moving in a downward motion, followed by the floor. Then, wipe the walls followed by the floor using a clean, dry paper towel. Finally, repeat wiping the walls followed by the floor with another clean, dry paper towel to ensure that the arena is dry and free of ethanol.

6. Pick up the first mouse by the tail and gently lower it to the center of the first open-field arena. Immediately turn on the infrared tracking beams and gently close the chamber door.

   *It is not necessary to use sound-attenuating chambers to conduct open-field studies; however, in addition to minimizing noise transfer between chambers, sound-attenuating chambers minimize extra-maze cues around the arenas, which can result in unexpected responses of the mice, particularly if multiple chambers in a room are exposed to an environment with variable visual cues (e.g., doors, lights, computer screens). If sound-attenuating chambers are not an option, it is recommended that variations in visual cues be minimized as much as possible. Furthermore, when loading mice into multiple chambers, the technician should plan a traffic pattern that avoids randomly crossing in front of an arena in which a mouse has already placed. This can be facilitated by planning to place the first mouse in the arena at the far back of the room, placing subsequent mice in chambers progressively closer to the door where the technician will exit until the testing has completed.*

7. Repeat for each mouse until all mice are loaded into their testing arenas.

8. Quietly exit the testing room after testing has started for all mice. Do not re-enter until the last mouse has completed testing.

   *It is recommended to not enter the testing room or begin taking mice out of arenas until testing is completed for all subjects in the session.*

9. Enter the room, open the first chamber, turn off the software tracking, and return the subject to its home cage or a clean transition cage. Repeat for each mouse.

10. Clean the arenas as in step 5.

11. If testing additional batches of mice, repeat steps 6–10.

12. Export the data and turn off the instrumentation.

    *Do not turn off testing room lights until mice have been removed from the procedure room to avoid exposing them to the dark cycle.*

13. Return mice to the colony.

### *Data QC and analysis*

14. Prior to data analysis and while still blinded, confirm that the instrument has reported values for each subject for each sample period. Exclude data from any mouse that escaped the arena at any point during testing or where the instrument failed to report values.

15. Evaluate the primary measures of interest that demonstrate age-dependent changes as illustrated in Figure 4. For each measure, look at response in 5-min bins and over the entire 60-min session.

    a. Total distance traveled in cm (Fig. 4A,B).
    b. Total number of vertical beam breaks (rearing activity; Fig. 4C,D).
    c. Time (in sec) spent along the margin (perimeter) of the environment in close proximity to the walls (Fig. 4E,F).

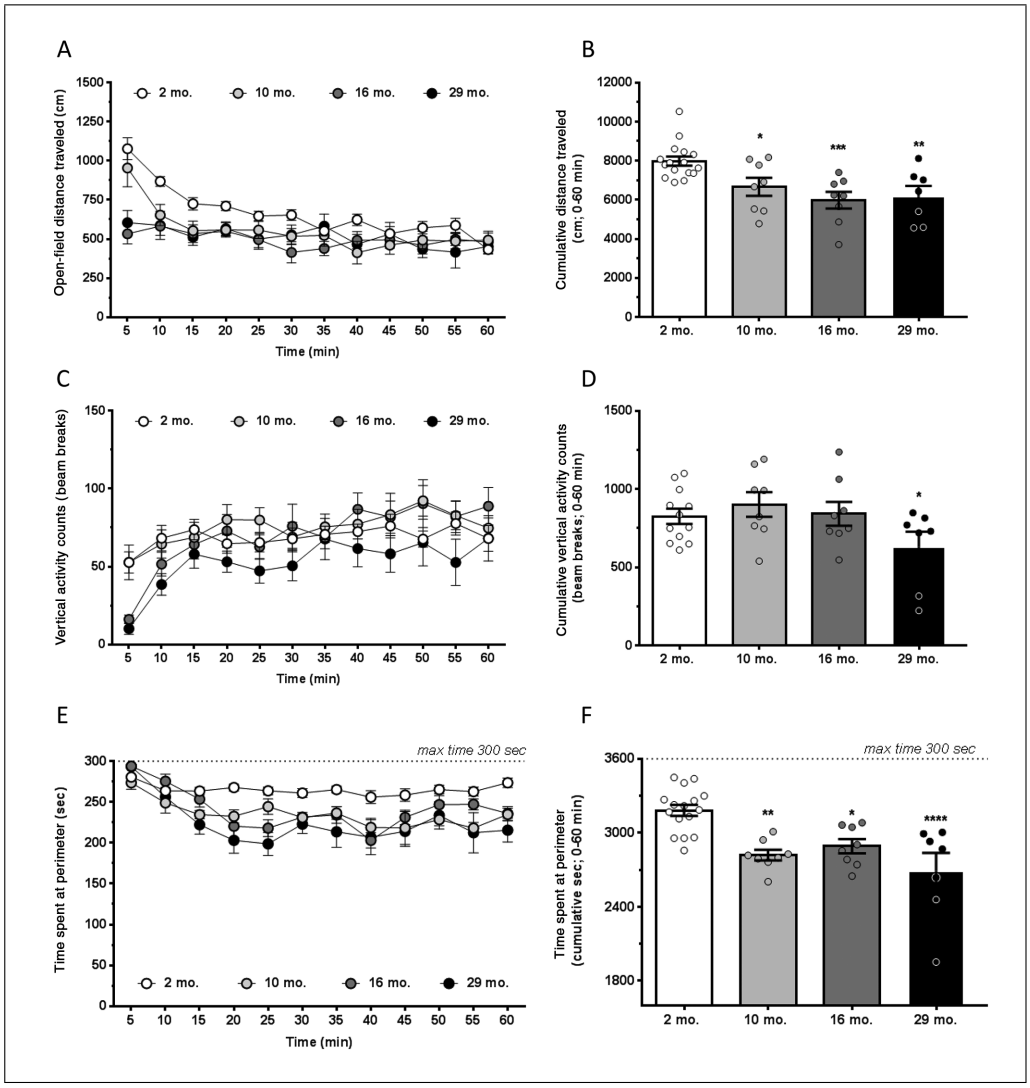


**Figure 4** Sensitivity of open-field measures for detecting aging-related changes. Data are analyzed by two-way repeated measures ANOVA (age versus time, within sex) over time bins (left) or as a one-way ANOVA for cumulative measure over the course of the entire open-field session (right). (**A**,**B**) Male C57BL/6J mice demonstrated reductions in locomotor activity as measured by total distance traveled in the open field over time (A) and cumulative distance traveled (B). (**C**,**D**) Male C57BL/6J mice also demonstrated age-dependent reductions in rearing behavior as measured by vertical activity counts over time (C) and cumulative vertical activity counts (D). (**E**,**F**) Thigmotaxic behavior, an indicator of anxiety-related behavior, was significantly reduced with age as demonstrated by age-dependent reductions of time spent at the perimeter of the open field in male C57BL/6J mice over time (E) and cumulatively (F). Test subjects were independent groups of aging mice evaluated on a single test day, with males and females on separate days.

## GRIP STRENGTH

Grip strength is used to study neuromuscular function by measuring the maximum force a mouse can exert. The task is performed by allowing a mouse to grab on a wire mesh grid with either the forepaws or all four paws, then pulling the mouse gently but firmly by the tail until it releases its grip from the grid. A force transducer is used to measure maximum force (measured in grams), which is then normalized to body weight. Given that aging mice demonstrate dynamic changes in body weight over lifespan; it is important to normalize force to body weight (measured in kg). Each mouse is tested for six consecutive trials, three with the forepaws only, followed by three with all four paws. In the absence of aged mice to validate the grip assay and demonstrate the expected aging-related impairments, validation of the protocol and proficiency testing of the technician can be achieved by demonstrating the ability to observe dose-dependent impairments of subataxic ethanol treatment on grip strength under blinded conditions.

### *Materials*

Subjects:
Young adult male or female mice 2-6 months of age (e.g., C57BL/6J, The Jackson Laboratory, no. 000664) and sex-matched mice >12 months of age
OR
Adult male C57BL/6J mice 8-12 weeks of age ($n = 8$ per dose level)
0.2, 0.15, and 0.1 g/ml ethanol (*optional*; see recipe)
70% (v/v) ethanol solution

Grip strength meter (Bioseb BIO-GS3) with mouse-specific wire grid (100 × 80 mm, angled 20°)
Computer for data collection
Red and blue non-toxic permanent markers
Animal scale (0.1-g precision)
1-cc syringes with 26-G needles (*optional*)
Paper towels
BIO-CIS software (*optional*; for exporting data to Microsoft Excel)
Force gauge/spring meter (*optional*; VWR, cat. no. 470017-446, or similar; for calibrating manual pulled force in grams with meter's digital reading)

### *Setup and habituation*

1. Set up the testing room in preparation for the experiment, ensuring that the testing environment has lighting, temperature, and humidity levels comparable to the colony room. Turn on computer and set up software for collecting force values.

2. Turn on the grip meter and set to collect a series of measures. Ensure that the software is set to collect the desired number of samples for each mouse per trial (six samples per trial, three for forepaws and three for all paws).

3. Attach the grid to the grip meter and connect the grip meter to the computer for data collection. Confirm precision of the grip meter reading by attaching the force gauge/spring meter to the center of the grid and pull back to confirm digital reading is consistent with pulled force in grams.

4. Prepare a treatment sheet (electronic or paper) indicating order of testing, dose time, and test time for each mouse, as well as body weight and dose volume. Assign each mouse to a treatment group (A, B, C, or D) in a randomized order of testing.

   *If mice are group housed, the subjects within a single cage should not be administered the same dose. Instead, each subject within a cage should be assigned a different treatment of either (A, B, C, or D), and subsequent cages of group-housed animals should also have randomized and counterbalanced representation of each treatment or dose level.*

5. Bring mice to the testing room. Weigh each mouse and label for easy visual identification. Leave the testing room to allow mice to habituate undisturbed to the testing environment for a minimum of 1 hr.

6. *Optional (for drug treatment):* While the mice are habituating, in a separate area not in the testing room, formulate the test compound (three doses of ethanol) and vehicle control ($dH_2O$), and code the vials as A, B, C, or D to keep the experimenter blinded to treatment.

   *A technician familiar with the experiment, but not conducting the testing or performing the data analysis, should be responsible for coding the vials and maintaining the blind.*

   *It is recommended that the technician pre-label and pre-load the syringes to the accurate injection volume (10 ml/kg) prior to starting the test to minimize the time between tests. In addition, a separate syringe and needle should be used for each subject.*

***Testing***

7. *Optional (for drug treatment):* Pretreat mice 20 min prior to testing using an intraperitoneal (i.p.) dose of 10 ml/kg.

   *At 0.1, 0.15, and 0.2 g/ml ethanol dilutions, this gives total doses of 1, 1.5, and 2 g/kg.*

   *Mice can be dosed 2 min apart, resulting in testing time 2 min apart. The timing of dosing and testing should be planned carefully to avoid dosing at the same time as testing. Dosing times can be planned to occur immediately before or after the next test cohort.*

8. Start the grip meter and computer software data acquisition. Tare the instrument to zero the value on the meter.

9. Remove a mouse from the home cage by gently grabbing the middle of the tail.

10. Lower the mouse towards the grid at the middle of the junction of the cross bar in the grid.

11. Once the mouse grasps the grid with forepaws only, gently and steadily pull the mouse in a horizontal manner, parallel to the bench.

12. If the value is valid, press enter on the meter. If the placement of the mouse was outside of the center or the mouse grasped with all paws when assessing forepaws only, exclude that value as a technical error.

13. Let the mouse rest briefly on the table to enable time to tare the instrument (~10 sec), then repeat until all trials (three with forepaws only followed by three with all four paws) have been completed.

14. Return mouse to the home cage. Wipe the table with 70% ethanol. Do not press on the grid while it is attached to the meter.

15. Repeat steps 7–14 for each mouse.

    *The internal memory of the Bioseb grip meter stores up to 100 measures. Choose a break point between subjects, not during measurements for any single mouse, to export or transcribe the data and clear the memory.*

16. At the end of testing, reset the meter's memory and power off the unit. Wipe the table and meter with 70% ethanol. Remove the grid from the meter and store.

17. Return mice to the colony.

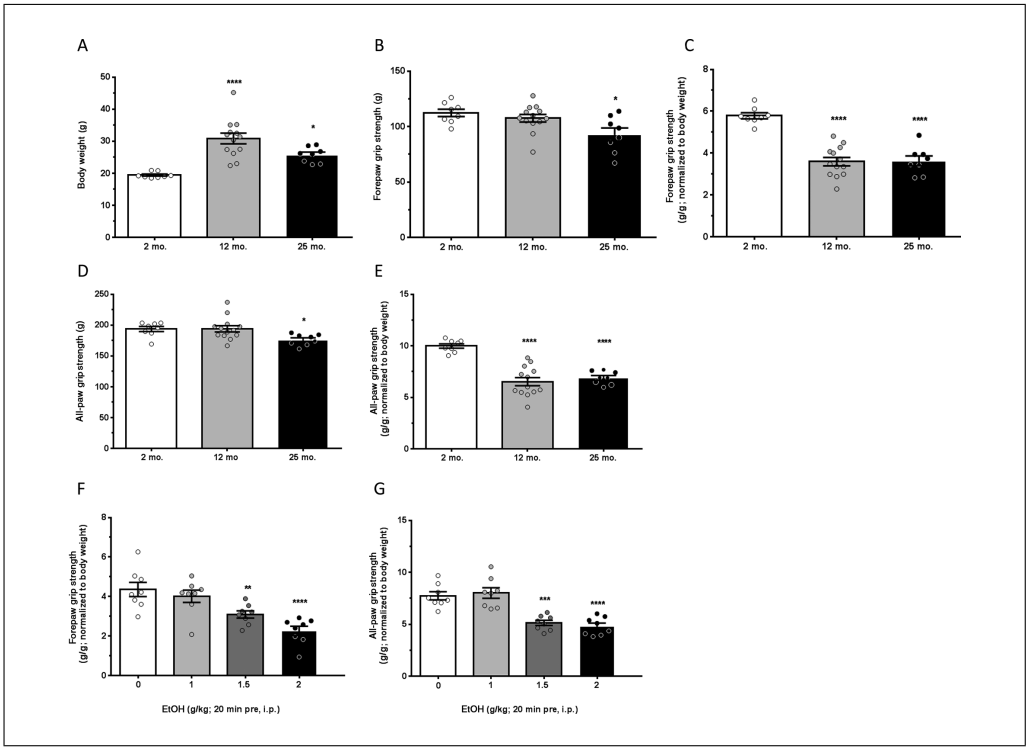


**Figure 5** Grip strength analysis and test validation data. C57BL/6J mice demonstrate age-related impairments in strength. This test can also be validated with administration of subataxic doses of ethanol to demonstrate a dose-dependent grip strength impairment. (**A**) Dynamic changes in body weight with age support the requirement to normalize grip strength force measurements to body weight. (**B**) Grip strength in female C57BL/6J mice presented as group means for age and analyzed by one-way ANOVA. Non-normalized forepaw grip strength is measured in force (grams). (**C**) Forepaw grip strength normalized to body weight. (**D**) Non-normalized grip strength for all paws. (**D**) All-paw grip strength normalized to body weight. (**E**,**F**) C57BL/6J mice treated with ethanol demonstrate dose-dependent reductions in forepaw and all-paw grip strength. For aging studies, test subjects were independent groups of aging mice evaluated on a single test day, with males and females on separate test days. For ethanol testing, subjects were drug- and behaviorally naïve. These reference data can be used to demonstrate a technician's proficiency for conducting the assay, when the technician remains blinded to testing, and to demonstrate that the laboratory environment is sensitive to measuring the expected outcome measure.

### *Data QC and analysis*

18. Prior to data analysis and while still blinded, exclude data from mice for which values were not entered/recorded or from mice that failed to grip during testing. Also exclude subjects that were mis-dosed or did not receive the full volume upon administration.

19. Calculate the average value of the three forepaw measures and the average value of the three all-paw measures. Normalize force values to body weight and analyze forepaw data and all-paw data separately. For aging studies, analyze data as one-way ANOVA with an appropriate post-hoc test versus young controls (Fig. 5A–E). For drug treatment, analyze data as one-way ANOVA with a Dunnett's post-hoc test versus vehicle control (Fig. 5F–G).

## ROTAROD TEST FOR MOTOR COORDINATION

*BASIC PROTOCOL 4*

The rotarod assay is performed by placing the mouse on a rotating rod and measuring how long it can maintain its balance. The rotarod consists of a computer-controlled, motorized horizontal rotating rod with five individual lanes separated by evenly spaced dividers for testing up to five mice simultaneously. Below each lane is a trip-plate that

records the latency for each mouse to fall. The rod begins rotating at four revolutions per minute (rpm) and accelerates over 5 min to a maximum of 40 rpm. Each mouse is assessed in three consecutive trials with only a brief rest period between trials to provide enough time to clean the instrument and reset the timers. In the absence of aged mice to validate the assay and demonstrate the expected aging-related impairments, validation of the protocol and proficiency testing of the technician can be achieved by demonstrating the ability to observe dose-dependent impairments of subataxic ethanol treatment on motor coordination under blinded conditions.

### *Materials*

Subjects:
Young adult male or female mice 2–6 months of age (e.g., C57BL/6J, The Jackson Laboratory, no. 000664) and sex-matched mice >18 months of age
OR
Adult male C57BL/6J mice 8–12 weeks of age ($n = 8$ per dose level)
0.2, 0.15, and 0.1 g/ml ethanol (*optional*; see recipe)
70% (v/v) ethanol solution

Rotarod (Ugo Basile)
Animal scale (0.1-g precision)
Red and blue non-toxic permanent markers
1-cc syringes with 26-G needles (*optional*)
Paper towels

### *Setup and habituation*

1. Set up the testing room in preparation for the experiment, ensuring that the testing environment has lighting, temperature, and humidity levels comparable to the colony room.
2. Turn on the computer first and open the software, then turn on the rotarod.
3. Set up the software for the desired testing protocol. Set the rotation for a starting speed of 4 rpm accelerating over the course of 5 min to a maximum of 40 rpm.
4. Start the rotarod rotating at 4 rpm. Leave it rotating throughout the entire habituation period to allow the subjects to acclimate to the noise of the rotating rod.
5. Prepare a treatment sheet (electronic or paper) indicating order of testing, assigned rotarod lane, dose time (for drug treatment), and test time for each mouse, as well as body weight and dose volume. Assign each mouse to a treatment group (A, B, C, or D) in a randomized order of testing.

   *Up to five mice can be tested at one time. If mice are housed in groups of five or less, run an entire cage of mice at the same time. If possible, do not split mice from one cage into separate runs. If more than five mice are housed together, it is recommended to apply a systematic sampling across cages, where upon testing subjects are placed in a temporary transition cage until all mice from a cage have completed testing. Then return all mice from a cage to their home cage at the same time.*

   *Importantly for drug treatment, if mice are group-housed, mice within the same cage should not be administered the same dose. Instead a representative sample of each dose should be represented within a cage.*

6. Bring mice to the testing room. Weigh each mouse and label for easy visual identification. Leave the testing room to allow mice to habituate undisturbed to the testing environment for a minimum of 1 hr.

7. *Optional (for drug treatment):* While the mice are habituating, in a separate area not in the testing room, formulate the test compound (three doses of ethanol) and vehicle control ($dH_2O$), and code the vials as A, B, C, or D to keep the experimenter blinded to treatment.

   *A technician familiar with the experiment, but not conducting the testing or performing the data analysis, should be responsible for coding the vials and maintaining the blind.*

   *It is recommended that the technician pre-label and pre-load the syringes to the accurate injection volume (10 ml/kg) prior to starting the test to minimize the time between tests. In addition, a separate syringe and needle should be used for each subject.*

***Testing***

8. *Optional (for drug treatment):* Pretreat mice 20 min prior to testing using an intraperitoneal (i.p.) dose of 10 ml/kg. Dose all mice for a single session immediately one after the other (up to five if all five lanes will be used in each session).

   *At 0.1, 0.15, and 0.2 g/ml ethanol dilutions, this gives total doses of 1, 1.5, and 2 g/kg.*

   *The timing of dosing and testing should be planned carefully to avoid dosing at the same time as testing. Dosing times can be planned to occur immediately before or after the next test cohort.*

9. Move the cage of test mice from the rack to the counter beside the rotarod to facilitate loading of the mice.

10. With the rotarod rotating at 4 rpm, position the trip-plate so that it clicks into place and the handle end rises ~¾ in. from base.

    *The digital panel on the instrument should show a 0 for that lane if the trip-plate is properly set.*

11. Gently but swiftly place mice on rotor in preassigned lanes. Load mice in sequential order from left to right (lane 1 to 5) every time.

    *If any mice fall or jump off before the program is started, do not re-load these mice for a re-trial, but continue to load all mice. After starting the software, move the mice that jumped or fell back to their home cage and manually record a latency of 0 sec for that subject's trial. In the notes section of the treatment sheet, record whether 0 was for falling or for jumping, as these denote divergent phenotypes. Mice that fall or jump upon placement do not receive a second attempt (e.g., until they stay on) for any trial.*

12. When all subjects are loaded, start the program to measure latency to fall.

    *As the mice fall, they will activate the trip-plate, and data will be sent in real-time to the software. It is possible that a mouse will gently lower itself off or fall off at close range, so that the plate does not drop. In this case, manually push down on the trip-plate to break the connection and record latency.*

13. As each mouse falls, return it to the home cage.

14. End the trial at 300 sec or when all mice have fallen off the rod. Remove any mice that remain after 300 sec and return to the home cage.

    *In the notes section of the treatment sheet, record whether mice met the maximum time of 300 sec and were removed by the experimenter or if they fell at 300 sec.*

15. After each trial (when all mice have fallen or have been removed), reset the rod to the constant start speed of 4 rpm and reset the trip plates to 0.

16. For repeated trials of the same mice, gently wipe the rod of gross urine and feces. For trials between groups of mice, also sanitize the rod, plates, and dividers with 70% ethanol.

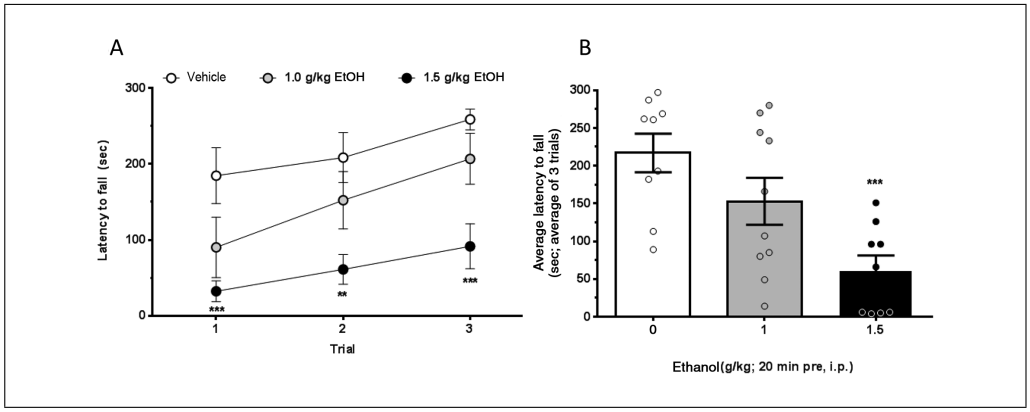


**Figure 6** Rotarod validation data. Dose-dependent reductions in motor coordination in the presence of acute administration of ethanol to adult male C57BL/6J mice as analyzed by two-way repeated measures ANOVA (treatment × trial; **A**) or as the average of the three consecutive trials via one-way ANOVA with Dunnett's post-hoc test versus vehicle-treated controls (**B**). These reference data can be used to demonstrate a technician's proficiency for conducting the assay, when the technician remains blinded to testing, and to demonstrate that the laboratory environment is sensitive to measuring the expected outcome measure. Subjects were drug- and behaviorally naïve for testing.

17. To start another group of mice, reset the software protocol and repeat steps 6–16.
18. At the end testing, clear the memory from the rotarod and turn off the instrument. Thoroughly wipe down the equipment with 70% ethanol, ensuring that the machine is cleaned behind and underneath.
19. Export the data.
20. Return mice to the colony.

### *Data QC and analysis*

21. Prior to data analysis and while still blinded, ensure all values for each subject and each trial have been entered. Exclude subjects that escaped during testing for which a value could not be obtained for any of the three trials. Also exclude subjects that were mis-dosed or did not receive the full volume upon administration.

    *Importantly, mice that fell off or jumped off upon placement on the rod, prior to pressing the start button, receive a data entry value of 0 for that trial and should not be excluded.*

22. Present latency to fall for each trial (Fig. 6A) and analyze as a two-way repeated measures ANOVA (age × trial or treatment × trial) and as a one-way ANOVA as an average of the latencies across the three trials (Fig. 6B).

## *BASIC PROTOCOL 5* ACOUSTIC STARTLE RESPONSE AS AN INDICATOR OF HEARING

Acoustic startle is used to assess auditory sensory capabilities and the startle reflex of a mouse to a sudden auditory tone. Previous data have demonstrated age-related hearing loss in mice by this non-invasive procedure (Ouagazzal, Reiss, & Romand, 2006). While there are alternative procedures for assessing hearing in mice, such as auditory brainstem response, these require anesthesia, which may not be desirable for studies assessing lifespan in aging mice or if there is any concern of interaction with a drug intervention being evaluated. For the current protocol, mice are placed in an enclosure affixed to a piezoelectric accelerometer that measures transduced movement. During the assessment, the subject is presented with 5 min of background noise (65 dB) followed by six different auditory tones (70, 80, 90, 100, 110, 120 dB) with ten repetitions each, pseudo-randomly presented throughout the session. Critically, subjects should be acclimated in an anteroom outside of or away from the testing room to avoid being exposed to the startle stimuli

prior to testing. Intact hearing in this assay is indicated by a decibel-dependent increase in the startle response. Startle amplitude values below the level of the background noise for each stimulus value may be indicative of deafness at that decibel level.

This protocol utilizes a commercial startle response system (SR-LAB, San Diego Instruments) that consists of a plastic cylindrical animal enclosure affixed with a piezoelectric accelerometer and contained within an isolation cabinet for sound attenuation that is also equipped with a speaker for delivery of auditory stimuli. It is highly recommended that chambers be placed in an area not subjected to random vibration and not near sources of vibration such as an elevator, cage wash, or air vent. Due to the sensitivity of the equipment for detecting subtle movements, a marble slab or other vibration-attenuating materials can be used to minimize unintended response.

### *Materials*

Subjects: young adult male or female mice 2-6 months of age (e.g., C57BL/6J, The Jackson Laboratory, no. 000664) and sex-matched mice >10 months of age
70% (v/v) ethanol

Startle response system (SR-LAB, San Diego Instruments)
Sound meter capable of measuring up to 120 dB, with a "Fast A" weighting and maximum value option (e.g., Extech Instruments sound level meter 407330)
Standardization unit (San Diego Instruments) for calibrating motor output of each chamber
Red and blue non-toxic permanent markers
Paper towels

### *Calibration and setup of audio output signals*

1. Using the instrument's diagnostic software, test the audio output stimuli at varying values to determine the arbitrary unit that corresponds with the intended decibel value on the sound meter.

   a. Place the sound meter in the center of the chamber, on the maximum hold setting, and close the chamber door.
   b. Turn on the light for the chamber such that the value on the sound meter can be visualized through the peephole in the chamber.
   c. For each decibel level desired, adjust the software's output audio value until the desired decibel reading is achieved and record the corresponding arbitrary unit from the software.

      CAUTION: *It is highly recommended that ear protection be worn as a safety precaution, especially when exposing oneself to levels >90 dB. In addition, the manufacturer's recommendation for long-term care of the speaker in the instrument is to not maintain decibel levels >110 dB on a constant output for more than 1 sec duration. It is recommended to follow the manufacturer's recommendations.*

   d. If multiple chambers are being used, repeat for each chamber. Note that output signals are never isolated for a single chamber, since the hardware is daisy-chained together, but are synchronized across all multiples of chambers.

      *It is critically important to not attempt to run two independent systems simultaneously that are not configured together on the same interface, as audio outputs cannot be time-locked and synchronized together unless they are physically connected and daisy-chained together.*

   e. If the chambers are outfitted with a dial to adjust speaker output level that can easily be accessed, adjust each chamber so they are comparable to each other. If this is not possible, calculate the average of the arbitrary unit values for each

decibel level across all chambers and program this average value as the output value for that decibel level.

*Once the arbitrary units that correspond to the desired audio output (decibel) stimuli are determined, the testing script can be written.*

2. Program the startle protocol testing script.

   a. Start the testing script with 5 min exposure to background noise (65 dB).

      *The background noise used for this test is dependent upon the ambient background noise in the testing room. For example, if the ambient background noise is 70 dB, then 70 dB should be used as background noise in the program.*

   b. At the conclusion of the exposure to background noise, program the first trial to consist of the arbitrary unit that corresponds to the desired audio stimulus (dB level) as the output stimulus for a duration of 40 msec, followed by a recording window during which the animal's response is recorded. Program the recording window for each audio stimulus as 100 samples collected over 100 msec.

      *The program returns to background noise at the conclusion of each 40-msec audio stimulus presentation.*

   c. Between all audio stimulus presentations, program an intertrial interval that ranges from 9 to 25 sec. This can be done using an automated feature in the software or manually programmed.

      *During the intertrial interval, only background noise is presented and there is no additional audio stimulus or recording.*

   d. Pseudo-randomly program ten repetitions of the presentation of each of the audio stimuli (70, 80, 90, 100, 110, and 120 dB) as in step 2b. Include ten pseudo-randomly presented presentations of "no stim" trials for which recordings are collected but only background noise is generated.

      *The "no stim" trials serve to normalize ambient movement of the subject in the chamber (background noise) that is not in response to the intended stimuli as part of the data analysis. All stimuli including "no stim" trials should be followed by intertrial intervals as in step 2c.*

***Setup and habituation***

3. Set up the testing room and anteroom in preparation for the experiment, ensuring that the testing environment has lighting, temperature, and humidity levels comparable to the colony.

4. Power on the chambers and software. Confirm that the fan is on and functioning and that the light is off.

   *The light remains off and the fan on throughout the experiment.*

5. Calibrate each startle chamber prior to habituating the mice to confirm function.

   a. Connect the standardization unit to the first chamber, check that it is functioning by touching to confirm a vibrating movement, and allow it to reach steady state for 15 min prior to calibration.

   b. For each chamber, ensure that the standardization unit is firmly in place by tightening the screws, then close the chamber door.

      *Minimize any movement to the chamber during calibration by stepping away from the chamber and avoiding leaning on the chamber or bench.*

   c. View the responses on the diagnostic program that corresponds to the motor output being generated by the standardization unit. In the current protocol, the boxes are calibrated to a reading that is within 700-710 mV for the average

response value (mV_AVG) and not the maximum response value. Ensure that the response is within this range and is consistent for at least five to eight consecutive responses before moving the standardization unit to the next chamber.

*If the value is outside the indicated range, adjust the sensitivity of the box using the dial on the side of the chamber until a 700–710 mV_AVG range can be achieved for at least five to eight consecutive readings. In addition, ensure that there is no crosstalk from the other chambers, which should have values reading <10 mV_AVG. Once the chamber is calibrated as defined, the next chamber in the series should be calibrated.*

6. Set up the software for the desired testing protocol as per the script above.
7. Prepare a treatment sheet (electronic or paper) of the mice to be tested and testing order. Ensure that treatment groups are randomized and counterbalanced across different chambers and for testing order.

   *Pay careful attention to counterbalancing representative samples of each treatment group or age group across multiple instruments to prevent any one treatment group from always being placed first or all controls, for example, always being in chamber 1.*

   *Once a mouse is tested, do not return it to its home cage with untested mice. Instead, place it in a temporary transition cage. Group-housed mice placed in a transition cage should only share a transition cage with other mice from their home cage. Mice should remain in these transition cages until all mice from the same home cage have completed testing, and then returned to their home cage at the same time. If multiple startle chambers are used, all mice from a cage should be tested at the same time and should not be split from the same cage into separate batches unless this cannot be avoided. A temporary transition cage should be used if all mice from a single cage cannot be tested at the same time.*

8. Bring mice into the anteroom space. Label each mouse for easy visual identification. Leave to acclimate for a minimum of 1 hr.

   *Mice should not be habituated or left in testing room while other mice are being tested, so that they are not exposed to the auditory stimuli from the startle chambers prior to the test.*

9. Run the protocol in the set of empty chambers while the mice are habituating. During the 5 min of background noise, briefly open the chamber to confirm that the background noise is audible.

   *The empty chamber run serves multiple purposes. The first is to confirm the function of each box before mice are placed in the chambers to test, the second is to ensure that no responses other than ambient recordings are being generated as there are no mice in the chamber, and the third is to expose the first group of mice to potentially any audio transfer from the anteroom to the testing room that may be audible to the mice while habituating, as would subsequent groups of mice while waiting to be tested.*

***Testing***

10. Move the first batch of mice to be tested from the habituation rack onto a rolling cart and bring them to the testing room.
11. Place each mouse into the animal enclosure within its pre-assigned chamber.
12. Start the testing protocol and quietly exit the testing room.

    *Once the testing protocol has completed, the data will automatically be saved.*

13. When the test is complete, return mice to their home cage or a temporary transition cage. Clean the startle chambers and animal enclosures with 70% ethanol and wipe dry.

    *It is not necessary to re-calibrate the output motor response with the standardization unit between groups of mice unless the enclosure that contains the piezoelectric accelerometer has been displaced or disconnected. It is important, however, to calibrate at the beginning*

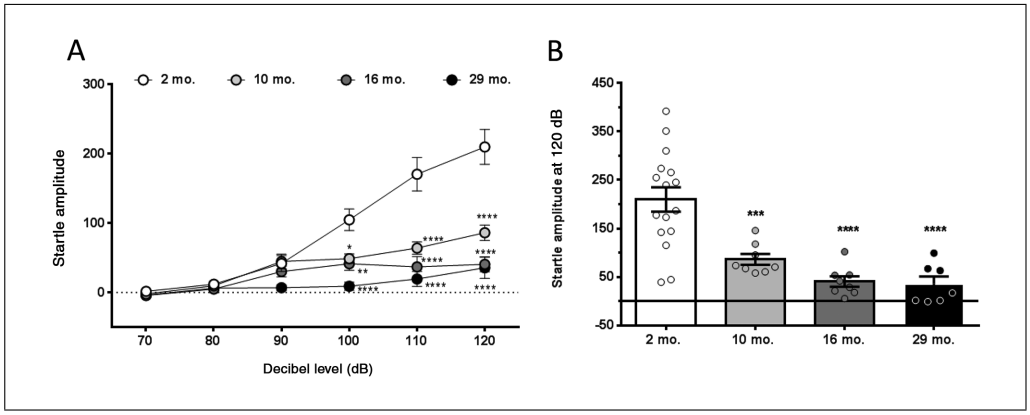


**Figure 7** Acoustic startle response. Adult male C57BL/6J mice demonstrate age-dependent reductions in hearing as indicated by decibel-dependent reductions in the startle response. (**A**) Acoustic startle decibel response curves reveal that the expected decibel-dependent increase in startle response in young mice is attenuated with age. (**B**) Individual responses to a 120-dB stimulus reveal statistically significant hearing impairment in aging mice as indicated by attenuated startle amplitudes relative to young controls, with deafness indicated by a startle amplitude at or below 0 (horizontal line). Test subjects were independent groups of aging mice evaluated on a single test day, with males and females on separate test days.

*of each test day, as the chambers and procedure room cleaning may result in equipment being inadvertently moved, which could result in significant changes to the output signal.*

14. Repeat steps 10–13 for each batch of mice.
15. Export the data.
16. Return mice to the colony.

### *Data QC and analysis*

17. Prior to data analysis and while still blinded, ensure that all values are reported for each trial for each subject. Exclude mice for which there are missing values, mice that escaped during testing, or mice for which there were any technical issues (e.g., equipment malfunction). Use only average response values (mV_AVG) for analysis.
18. While still blinded, calculate for each subject an average value of the ten repetitions of each stimulus (70, 80, 90, 100, 110, 120 dB) and a value for the "no stim" recording.

    *This should result in a total of seven values per subject.*

19. For each subject, subtract the "no stim" value from each of the six different stimulus values.

    *This serves to normalize the ambient background noise or non-stimulated response levels produced by the animal due to normal movement in the chamber.*

20. Calculate group means for each stimulus type to generate the acoustic startle decibel response curve. Analyze by two-way repeated measures ANOVA with appropriate post-hoc comparisons (Fig. 7).

## *BASIC PROTOCOL 6* OPTOKINETIC FUNCTION TEST FOR VISUAL ACUITY

This task is a simple and rapid method for the assessment of functional visual capabilities of a mouse and has been used to demonstrate age-related changes in mice (Prusky, Alam, Beekman, & Douglas, 2004). Mice are tested in a virtual environment consisting of an elevated platform surrounded by four monitors. A mouse is placed on the platform and black-and-white vertical gradients are presented moving either clockwise or

counterclockwise. The system is based on the concept that mice perceive the moving gradient and will exhibit a tracking response resulting in the mouse's head moving in the same direction and at approximately the same speed as the gradient. Importantly, this test should be used to confirm whether impairments in functioning in assays that require visual stimuli (e.g., tests for learning and memory that use visual cues) are confounded by visual impairments or blindness.

The OptoMotry virtual environment (CerebralMechanics) contains an elevated platform (5.3 cm diameter, 13 cm above the floor) surrounded by four 17-inch LCD monitors. The floor of the apparatus is mirrored. The top has a central access hole for placing the mouse on the platform, and a hinged lid with an overhead video camera for continuous monitoring of the mouse's head movements during optokinetic stimulation.

***Materials***

Subjects:
Young adult male or female mice 2-6 months of age (e.g., C57BL/6J, The Jackson Laboratory, no. 000664) and sex-matched mice >10 months of age
OR
Blind mouse strain (e.g., C3H3B/FeJ, The Jackson Laboratory, no. 000658) with age-, sex-, and coat-color-matched sighted controls (e.g., CBA/CaJ, no. 000654)
70% (v/v) ethanol

OptoMotry virtual environment (CerebralMechanics)
Red and blue non-toxic permanent markers
Paper towels
90 dB audio cue (Starmark Pro-training Clicker; Starmark Pet Products)

***Setup and habituation***

1. Set up the testing room in preparation for the experiment, ensuring that the testing environment has lighting, temperature, and humidity levels comparable to the colony.
2. Turn on the OptoMotry environment and data acquisition computer and software. Ensure that the platform is visible and aligned to the center of the video display. Set up the software for the desired testing protocol.

   *Always test under "Blind Testing" conditions, meaning the visual gradient should be blanked out by the software so the experimenter cannot see which visual gradient is currently being displayed, only the direction of movement.*
3. Ensure that the platform is precisely in the center of the camera window. If necessary, adjust the placement of the video camera on the optokinetic drum by loosening/tightening the screws to alter the tilt. If the live video is clear and in focus, the video camera settings do not need to be adjusted.
4. In the Camera window, select the crosshair icon and click the center of the platform in the drum. Drag the circle outwards to align with the black circle at the edge of the platform. When finished, click the crosshair a second time.
5. Select the mouse head icon and the gratings icon to activate.
6. Select "Blind Testing" under the dropdown menu. A black overlay will appear that will hide the drum's video screen gratings from view of the experimenter. The green overlay will still be present to show the direction that the gratings are moving, but not the distance between the stripes. If any of the video screen's gratings are visible, select the "T" button on the video camera to zoom in until gratings are no longer visible; conversely, to zoom out select the "W" button.

7. Move the Camera window to the bottom of the computer screen so that the bottom bar showing grating distance is hidden from view.

8. In the OptoMotry controller window, only the settings listed below in **bold** will need to be changed. All other values are defaulted as follows:

   a. Stimulus

      *Gratings:*
      Spatial frequency = 0.061 c/d (cycles/degree)
      Contrast = 100%
      Drift speed = 12 d/sec

      *Calibration*
      Screen width = 40.6 cm
      Screen distance = 23.5 cm
      Max frequency = 0.75 c/d

   b. Camera

      *Video in*:
      Frame rate = Max
      Display = Auto
      Magnification = 100%

      *Adjust*:
      iSight dropdown selected
      Camera Focus = 58
      Shutter = 0.284

      *Calibration*:
      Camera calibration = 37.08 pixels/cm
      Camera y = ~50% (depends on how well camera is centered)
      Camera x = ~50% (depends on how well camera is centered)

      *Overlays (recommended settings; set as desired by technician)*:
      Compass size = 100%
      Cursor size = 70%
      Tick (grating) spacing = 45 degrees
      Tick (grating) size = 22 degrees
      Color = Green
      Blind mask = 230%

   c. Testing

      *Psychophysics*:
      Psychophysical method = Simple staircase
      Threshold = Acuity (frequency)
      Directions = Randomize/separate

      *Options*:
      **Feedback = check "End of Run" only**
      **Responses = Yes/No buttons**
      Max of 7 reversals
      Check "Reserved" only
      Terminate at 1%

      *Blanking*:
      **Check "Blank on Tracking" only**
      Tracking blank = Gray
      Ref frequency = 0.100 c/d
      Trial duration = 5.0 sec

      *Presets*:
      Preset set = Mouse (Prusky et al., 2004)
      # of frequencies = 6
      Highest frequency = 0.606 c/d
      Lowest frequency = 0.061 c/d

9. Prepare a treatment sheet (electronic or paper) of the mice to be tested and testing order.
10. Bring mice to the testing room and label each mouse for easy visual identification. Leave the testing room to allow mice to habituate undisturbed for a minimum of 1 hr.

***Testing***

11. Set the display of the OptoMotry environment to show an even grey screen.
12. Wipe the platform with 70% ethanol prior to testing each mouse.
13. Place the mouse on the central platform and close the lid.
14. Observe via the closed-circuit camera the space between the subject's eyes and click with the computer mouse in that space to start the tracking software. This will initiate the testing protocol and a visual gradient will appear to the mouse. Monitor the mouse's head closely for tracking of the visual gradient. A mouse is considered tracking when its head moves in the same direction and at approximately the same speed as the gradient.
15. If tracking is observed, select Yes. If no tracking is observed, select No.
16. If the mouse moves on the platform and the location of the head has changed, click with the computer mouse in the space between the subject's eyes again to orient the display for the current location.

    *It is critical that the software has the position between the eyes accurately placed, because head location is used to correctly display the gradient, taking into consideration the distance from the mouse's head to the individual monitors.*

17. Do not allow the mouse to acclimate to the visual gradient. If the mouse is hyperactive or appears to not focus on the task, turn the monitor display gray and do not present the visual gradient until the mouse appears to have settled down.

    *Tracking movements are a reflex. If the mouse is focused on the task, tracking should occur shortly after the visual stimulus is presented.*

18. If the mouse begins to groom or rear, use a clicker to elicit a loud sound and startle the mouse to disrupt these behaviors.

    *The clicker is used to produce a consistent auditory stimulus to disrupt behaviors that interfere with task performance. The use of a clicker is recommended in place of tapping on the instrument.*

19. If the mouse falls from the platform, turn the display gray and return the mouse to the platform before continuing to test.
20. Once the testing protocol is complete, the software will display the results for the current mouse. Record these values.
21. Return the mouse to its home cage. Clean the platform and floor of the arena with 70% ethanol.
22. Repeat steps 11–21 for each mouse.
23. Export the data.
24. Return mice to the colony.

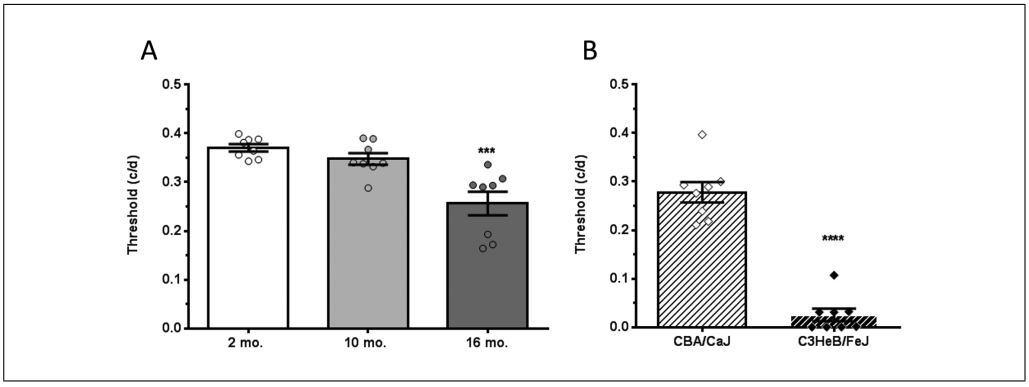


**Figure 8** Age-related impairments in visual function in mice. (**A**) Adult male C57BL/6J mice demonstrate age-dependent reductions in visual acuity in an optokinetic function test as measured by the threshold at which they fail to demonstrate the ability to track a visual stimulus (cycles/degree). In the absence of availability of aging mice, this protocol can be validated by using sex-, age-, and coat-color-matched mice with a technician blind to genotype/strain. (**B**) Adult male C3HeB/FeJ mice with retinal degeneration demonstrate significant impairments in threshold relative to sighted adult male CBA/CaJ mice. For aging studies, test subjects were independent groups of aging mice evaluated on a single test day, with males and females on separate test days.

### *Data QC and analysis*

25. Prior to data analysis and while still blinded, ensure that all values are reported for each trial for each subject. Exclude mice for which there are missing values, mice that escaped during testing, or mice for which there were any technical issues (e.g., equipment malfunction).
26. Visual acuity is defined as the mean clockwise/counterclockwise response to visual cycles/degree (Fig. 8).

## *BASIC PROTOCOL 7* OLFACTORY DISCRIMINATION

The ability to detect and discriminate odors declines with healthy aging in both mice and men and may manifest as an early onset phenotype with pre-prodromal neurodegenerative diseases (Mobley, Rodriguez-Gil, Imamura, & Greer, 2014; reviewed in Doty, 2012). The present protocol uses a novel-versus-familiar scent discrimination task in which the mouse is housed with two wooden blocks overnight and then during testing evaluated for time spent sniffing a block from its own cage or a block from a same-sex, unfamiliar mouse. Intact olfaction in this assay is indicated by preference for spending more time sniffing the novel scented block relative to time spent sniffing the familiar block.

The mouse's own home cage is used for testing in this protocol. Cages must be clear to allow for front-facing recording of the mouse's behavior. Cage tops that contain water and food are removed during testing and the cage is instead covered with a flat lid or filter top. A video camera is placed adjacent to the home cage to provide a front-facing view for easy visualization of the mouse and the wooden block in the home cage. The video camera needs to output the video signal to a recording for offline video analysis. Multiple mice can be tested simultaneously in this protocol, provided each mouse's behavior and interaction with the wood block can be clearly visualized once recorded. The rate-limiting factor for increasing the throughput of this protocol is the number of video cameras or the ability to record multiple video feeds simultaneously.

### *Materials*

Subjects: young adult male or female mice 2–6 months of age (e.g., C57BL/6J, The Jackson Laboratory, no. 000664) and sex-matched mice >10 months of age

70% (v/v) ethanol

Detergent for soaking and cleaning wooden blocks (e.g., Haemosol)
Olfactory cage: clean, clear home cage
Wooden blocks: two unfinished, autoclaved wooden cubes (2.54 × 2.54 × 2.54 cm) per test subject, marked N for novel and F for familiar using black non-toxic permanent marker
Video camera and recording device
Red and blue non-toxic permanent markers
Re-sealable sandwich-size plastic bags (16.5 × 15 cm or similar)
Paper towels

***Setup and habituation***

1. One day prior to testing, house mice individually overnight with two autoclaved wooden blocks, one each marked N and F, in the home cage.

   *If mice are already individually housed, they will need to be placed into a clean cage. If duplex cages are used for standard housing, each mouse to be tested should be placed in its own duplex cage, leaving the opposite side empty to prevent odor transmission between mice.*

2. On testing day, set up the testing room in preparation for the experiment, ensuring that the testing environment has lighting, temperature, and humidity levels comparable to the colony room.
3. Turn on the video camera and recording device (e.g., computer software or DVD recorder).
4. Prepare a treatment sheet (either electronic or paper print out) of the mice to be tested and testing order.

   *The testing order of subjects and treatment groups should be counterbalanced and randomized, as well as the order of presentation of the novel and familiar blocks. In addition, it is important to plan so that the familiar block is the block from the animal's own home cage and the assigned novel block is from a mouse that is the same sex but was not previously a cagemate with the test subject.*

5. Bring mice into the testing room and label each mouse for easy visual identification.
6. Remove the wooden blocks from each mouse's cage one at a time and place each block, with a small amount of the bedding material, in a separate plastic bag labeled on the outside with the subject's identification. Seal each bag and generously spray gloves with 70% ethanol after touching each block to prevent odor transmission between blocks.
7. Leave the testing room and allow mice habituate to the testing environment undisturbed for a minimum of 1 hr.

***Testing***

8. Remove the cage top that holds food and water, place the home cage in front of the camera, and place a flat, aerated lid on the home cage.
9. Start trial 1: Place a wooden block, either familiar or novel, ~3 cm from the front of the home cage with the letter (N or F) facing the camera and record the mouse's behavior for 10 min. Spray gloves with 70% ethanol after touching each block upon placement.
10. At the conclusion of trial 1, remove the wooden block and discard until washed and autoclaved. Do not re-use blocks that were already used for testing. Spray gloves with 70% ethanol after touching each block.

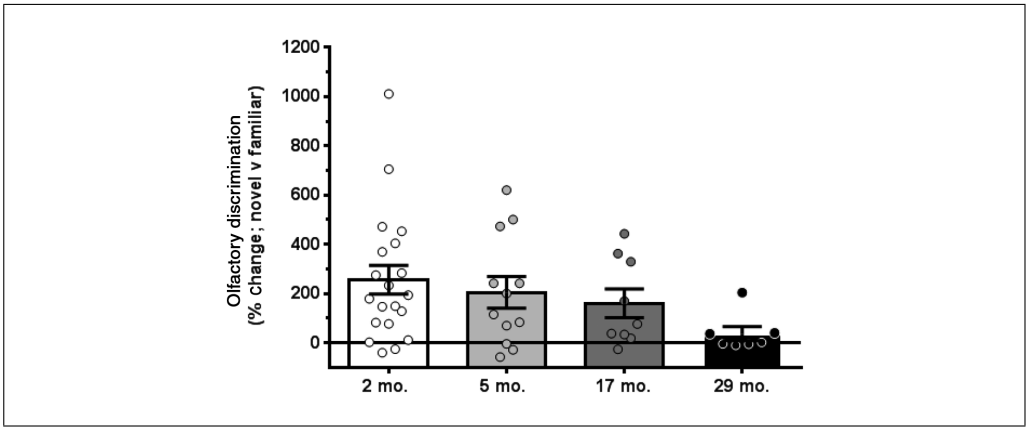


**Figure 9** Age-related impairments in olfaction in mice. Adult male C57BL/6J mice demonstrate age-dependent reductions in the ability to discriminate a novel mouse odor from their own familiar odor, indicative of impaired olfaction. Test subjects were independent groups of aging mice evaluated on a single test day.

11. Start trial 2: Place the second wooden block (novel or familiar, whichever was not used in trial 1) into the cage in the same location as was in trial 1. Record the mouse's behavior for 10 min. Spray gloves with 70% ethanol after touching each block.
12. At the conclusion of trial 2, remove the wooden block and discard until washed and autoclaved. Do not re-use wooden blocks that were already used for testing. Spray gloves with 70% ethanol after touching each block.
13. Return mice to the colony room.
14. If another session of mice is to be tested, repeat steps 8–13 with blocks that were not previously used in the recording sessions.
15. At the conclusion of the test day, clean and autoclave the wooden blocks for future test sessions unless there is obvious damage (e.g., excessive chewing). Soak the blocks in a mixture of warm water and detergent for 1 hr, then scrub and rinse each block. Let blocks dry overnight and autoclave the next day.

***Data QC and analysis***

16. Prior to data analysis and while still blinded, score the videos for cumulative time spent exploring each block.

    *Exploration time is defined as the mouse's nose in contact with or within 1 cm of each block and does not include climbing or rearing upon, digging, burying, or chewing the block.*

17. Calculate a percent change score for the time spent sniffing the novel block ($t_N$) relative to the familiar block ($t_F$):

$$\% \text{ change} = [(t_N - t_F)/t_F] \times 100$$

    *See example in Figure 9.*

## SPONTANEOUS ALTERNATION

***BASIC PROTOCOL 8***

The spontaneous alternation protocol is used to assess spatial working memory. Performance is dependent on an intact hippocampus and various interconnected structures (Lalonde, 2002). When placed within a maze with multiple arms, mice exhibit the spontaneous behavior of alternating between arm choices with a greater frequency than re-entering the same arm most recently visited (Hughes, 2004). For this task, mice are

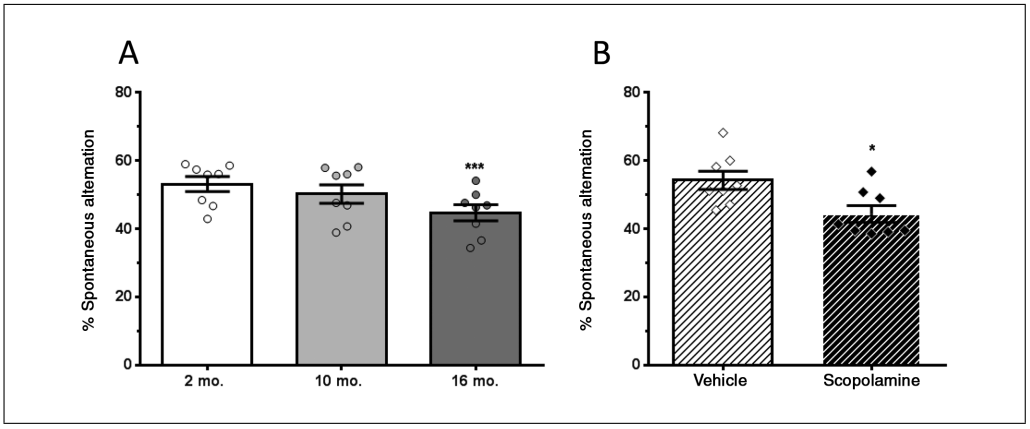


**Figure 10** Spontaneous alternation validation data. (**A**) Adult male C57BL/6J mice demonstrate age-dependent reductions in % alternation behavior. Data calculated as group mean for % alternation as the ratio of correct, unrepeated sequences of three arm entries (triads) in the Y-maze prior to re-entry, relative to the number of alternation opportunities [(correct triads/opportunities) × 100]. Significant reductions in % alternation were observed as early as 16 months of age relative to 2 months of age. (**B**) In the absence of aging mice, the protocol can be validated in young adult male C57BL/6J mice pretreated with scopolamine (1 mg/kg, 30 min pre, i.p.) with the technician demonstrating the ability to observe a significant deficit with treatment relative to vehicle-treated control under blinded conditions. For aging studies, test subjects were independent groups of aging mice evaluated on a single test day. For scopolamine testing, subjects were drug- and behaviorally naïve.

placed within a three-arm maze (typically, Y- or T-shaped) with no intended visual cues and a perimeter curtain to minimize extra-maze visual cues. The subject is evaluated for alternation behavior during an 8-min observation session and its behavior is recorded and tracked to provide the number and sequence of arm entries. Interestingly, this is one of the few cognitive tests that can be conducted in blind mice, as there are no intended visual cues required for subjects to perform this test, which makes it an excellent assay for testing aged mice that may have visual impairments (Fig. 10). In the absence of aged mice to validate this assay and demonstrate the expected aging-related deficits in alternation behavior, validation of the protocol and proficiency testing of the technician can be achieved by demonstrating a scopolamine-induced impairment in alternation behavior relative to vehicle-treated controls under blinded conditions.

The Y-maze is a clear polycarbonate arena in the shape of a Y with each of the three arms (assigned as A, B, and C) having dimensions of 33.65 cm length, 6 cm width, and 15 cm height. It has a removable clear aerated lid. A floor-to-ceiling length solid black curtain (the perimeter curtain) is mounted from the ceiling on a track to surround the perimeter of the maze in order to minimize extra-maze visual cues and the location of the tester. There should be no intended intra-maze or extra-maze visual cues within or external to the maze or within the curtain perimeter. The maze is placed on top of a white tabletop with infrared backlighting that does not emit heat or visible light. When paired with an infrared camera, the base minimizes glare and makes any mouse appear as black on white, independent of coat color. This serves to minimize or eliminate the environmental modification (e.g., changing the table top color dependent upon mouse coat color) necessary for accurate video tracking of automated behavioral tracking software, making the task more uniform across animals. An infrared camera is mounted overhead in a position to include the entire maze within the frame, preferably nearly filling the frame to maximize recording quality. Behavioral tracking software is used to automatically track the behavior of the center point of the mouse's body to determine the order of arm entries. Finally, the lighting in the testing room is dimmed to provide a light level in the center of the testing room of ∼50 lux. A lamp with a single incandescent 40-watt light bulb is clamped to the ceiling-mounted camera bracket in each arena and positioned with the light aimed

towards the ceiling of the center of the arena to produce light levels at the level of the maze of ~50 lux.

### *Materials*

Subjects:
Young adult male or female mice 2–6 months of age (e.g., C57BL/6J, The Jackson Laboratory, no. 000664) and sex-matched mice >16 months of age
OR
Adult male C57BL/6J mice 8–12 weeks of age ($n = 8$ per dose level)
0.1 mg/ml scopolamine (*optional*; see recipe)

0.9% NaCl solution (*optional*)
70% (v/v) ethanol
Y-maze (arm dimensions: 33.65 cm L × 6 cm W × 15 cm H) with a removable clear aerated lid
Infrared backlit base (Noldus Information Technology)
Perimeter curtain on ceiling track
Lamp with a single incandescent 40-watt light bulb
Infrared camera
Computer with behavioral tracking software (Noldus Ethovision XT)
Animal scale (0.1-g precision)
Red and blue non-toxic permanent markers
1-cc syringes and 26-G needles (*optional*)
Paper towels

### *Setup and habituation*

1. Set up the testing room in preparation for the experiment, ensuring that the testing environment has temperature and humidity levels comparable to the colony. Set testing room lighting so that light levels are ~50 lux inside and outside the curtain surrounding the Y-maze.

2. Turn on the infrared backlit base, camera, and data acquisition computer and software. Set up the software for the desired testing protocol. The current protocol uses 8-min recording sessions for each mouse. Ensure that the Y-maze is clearly visible and free of major obstructions that can impede tracking accuracy (e.g., lighting glare, part of the maze off the video frame, etc.). If using live-tracking, set up the maze to have four zones defined as arm A, arm B, arm C, and center. Each arm is defined as the area from the end of the arm to ~3 cm from the center of the Y-maze. The center is defined as the area in the center of the maze connecting each arm.

   *Either live or offline tracking of the mouse's behavior can be done, but a video record of the behavior should be saved. The video can be reviewed for anomalous tracking results and re-tracked if better tracking accuracy is needed. Importantly, the determination of arm entries should be based on the center point of the animal and not the noise/head of the animal, as mice demonstrate stretch-attend posture, which may result in false entries into an arm.*

3. Prepare a treatment sheet (electronic or paper) of the mice, indicating order of testing, dose time, and test time for each mouse, as well as body weight and dose volume. Assign each mouse to treatment group A or B in a randomized order of testing.

   *If mice are group-housed, the entire cage should not be administered the same dose. Instead, a representative sample of each dose should be represented within a cage.*

4. Bring mice to the testing room. Weigh each mouse and label for easy visual identification. Leave the testing room to allow mice to habituate undisturbed to the testing environment for a minimum of 1 hr.

5. *Optional (for drug treatment):* While the mice are habituating, in a separate area not in the testing room, formulate the test compound (scopolamine) and vehicle control (0.9% NaCl), and code the vials as A or B to keep the experimenter blinded to treatment.

   *A technician familiar with the experiment, but not conducting the testing or performing the data analysis, should be responsible for coding the vials and maintaining the blind.*

   *It is recommended that the technician pre-label and pre-load the syringes to the accurate injection volume (10 ml/kg) prior to starting the test to minimize the time between tests. In addition, a separate syringe and needle should be used for each subject.*

### *Testing*

6. *Optional (for drug treatment):* Pretreat mice 30 min prior to testing using an intraperitoneal (i.p.) dose of 10 ml/kg.

   *At 0.1 mg/ml scopolamine, this gives a total dose of 1 mg/kg.*

   *The timing of dosing and testing should be planned carefully to avoid dosing at the same time mice are being tested in the maze.*

7. Before the first mouse is placed in the maze, clean the maze thoroughly with 70% ethanol and wipe the maze dry.

8. Pick up the first mouse from its home cage by the tail and place it through the curtain opening into the designated start arm closest arm to the entrance of the curtain facing the center of the maze. Immediately place the lid on top of the maze and quietly close the curtain.

   *Arm names A, B, and C are arbitrary designations and only used for tracking the order a mouse enters the arms across the trial. The start arm should be consistent across mice and should be selected for ease of loading the mice without excessive handling or transport.*

9. Record the mouse's behavior during free exploration of the Y-maze for 8 min.

10. Return the mouse to its home cage. Clean the Y-maze and lid thoroughly with 70% ethanol and wipe dry.

11. Repeat steps 6–10 for each mouse.

12. Export the data.

13. Turn off the instrumentation and return mice to the colony.

### *Data QC and analysis*

14. Prior to data analysis and while still blinded, confirm that data have been collected for each subject. Exclude subjects that failed to explore all three arms during the test or escaped during testing. Also exclude subjects that were mis-dosed or did not receive the full dose volume.

15. Export the sequence of arm entries across the trial and the timestamp of entry into each arm with a precision of 0.1 sec.

    *Many commercially available automated tracking software programs can calculate the percent of spontaneous alternations completed by a mouse, but the algorithm can vary across software and produce different results. It is advised to confirm manual scoring with automated results prior to using any automated calculations.*

*Re-entry into the same arm (i.e., a mouse enters arm A two times in a row) is only considered a true re-entry if there is greater than 1 sec delay between the sequential entries. This minimum cutoff is used to exclude instances where the mouse is sitting at the edge of the zone and the tracking software detects the mouse as repeatedly entering/exiting the same zone when it is actually not moving.*

*Mice demonstrate typical stretch-attend postures during exploration of the maze. Using a center body point for automated tracking minimizes false entries in this manner; however, false entries are still possible if video tracking observes the mouse on the division where an arm ends/starts. Therefore, the above criteria are used to confirm if a re-entry is true or false.*

16. From the sequence of arm entries, calculate the total arm entries as the total number of times a subject enters a new arm (including re-entry into the same arm with $>1.0$ sec between entries.

17. From the sequence of arm entries, calculate the number of correct triads, defined as entries into all three arms in three sequential arm entries without repeating an arm and independent of arm order (i.e., A-B-C, A-C-B, C-A-B, etc.). Calculate triads for every possible set of three arms, moving one arm at a time through the sequence (i.e., set 1 = entries 1, 2, 3; set 2 = entries 2, 3, 4; set 3 = 3, 4, 5; etc.).

18. From the sequence of arm entries, calculate the number of alternation opportunities as the total number of correct and incorrect triads or the number of total arm entries minus two. Then calculate the percent alternation as the ratio of correct triads to the number of alternation opportunities:

$$\% \text{ alternation} = (\text{correct triads}/\text{opportunities}) \times 100$$

*See example in Figure 10.*

***BASIC PROTOCOL 9***

## NOVEL SPATIAL RECOGNITION

The novel spatial recognition protocol is used to assess short-term recognition memory and is similar to novel object recognition but replaces the use of objects with visual cues. This protocol is a modification of the methods reported by Paumier et al. (2013). During the first trial (T1), mice are placed within a three-arm, Y-shaped maze but allowed to access only two arms, with the third arm inaccessible. Mice are allowed to freely explore for 10 minutes and are then returned to their home cage. Mice are given a delay period ranging from 5 to 60 min prior to the start of trial 2. During trial 2, mice are placed back in the three-arm maze with the barricade removed, giving access to all three arms. Mice are allowed to freely explore for 5 minutes. Behavior is recorded and tracked to provide the amount of time spent in each arm. Intact short-term recognition memory in this procedure is indicated by a preference to spend more time in the novel arm relative to the familiar or start arms.

In this protocol, the Y-maze is a clear polycarbonate arena in the shape of a Y with identical arm dimensions of 27 cm length, 6 cm width, and 15 cm height. It has a removable clear aerated lid. The three arms are defined as start arm, familiar arm, and novel arm. The start arm is at the entrance of the perimeter curtain, the familiar arm is assigned as the left arm of the maze, and the novel arm is assigned as the right arm of the maze. A solid black polycarbonate wall (6 cm width, 15 cm height) is placed at the entrance to the novel arm, from the threshold of the center, to prevent access to that arm during the first trial. At the distal end of each arm is a distinct visual cue: a black-and-white diagonal striped cue for the start arm, a white plus sign on a black background for the familiar arm, and a black circle on a white background for the novel arm (Fig. 11). The rest of the setup—including the perimeter curtain, infrared backlit base, lighting, infrared

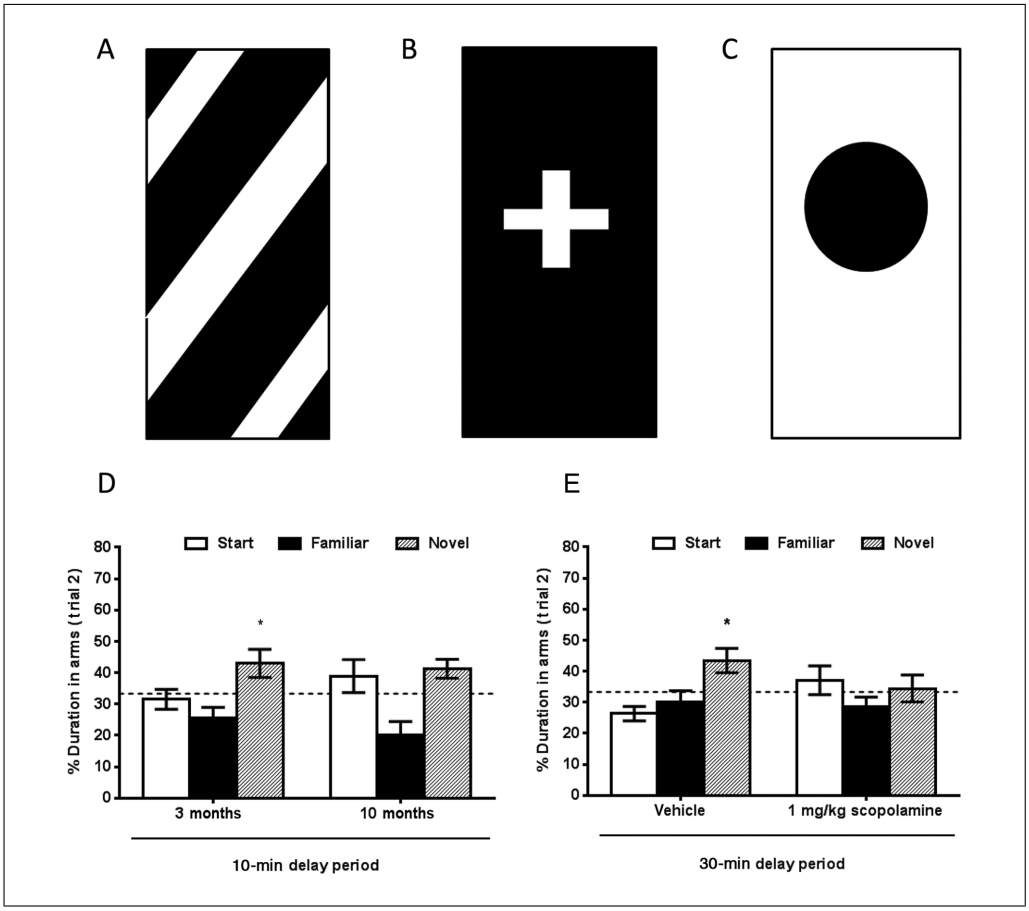


**Figure 11** Novel spatial recognition task. (**A-C**) Visual cues are placed at the distal end of each arm of the Y-maze and include a diagonal striped cue for the start arm (A), a white plus sign on a black background for the familiar arm (B), and a black circle on a white background for the novel arm (C). (**D**) Aging mice demonstrate delay-dependent impairments in this task. In a 10-min delay procedure, 3-month-old male C57BL/6J mice demonstrate intact short-term memory as indicated by a significant preference to spend time in the novel arm relative to the start or familiar arms, whereas sex-matched 10-month-old mice show a lack of preference for the novel arm. (**E**) In the absence of aging mice, this protocol can be validated in young adult male C57BL/6J mice pretreated with scopolamine (1 mg/kg, 30 min pre, i.p.) with the technician demonstrating the ability to observe the expected preference for the novel arm in vehicle-treated mice and a lack of preference for the novel arm in scopolamine-treated mice under blinded conditions. For aging studies, test subjects were independent groups of aging mice evaluated on a single test day. For scopolamine testing, subjects were drug- and behaviorally naïve.

camera, computer, and tracking software—is the same as described for the spontaneous alternation test (see Basic Protocol 8).

### *Materials*

Subjects:
Young adult male or female mice 2–6 months of age (e.g., C57BL/6J, The Jackson Laboratory, no. 000664) and sex-matched mice >10 months of age
OR
Adult male C57BL/6J mice 8–12 weeks of age ($n = 8$ per dose level)
0.1 mg/ml scopolamine (*optional*; see recipe)
0.9% NaCl solution (*optional*)
70% (v/v) ethanol

Y-maze (arm dimensions: 27 L × 6 cm W × 15 cm H) with removable clear aerated lid and solid black polycarbonate wall (6 cm W × 15 cm H) for novel arm

Visual cues (Fig. 11)
Infrared backlit base (Noldus Information Technology)
Perimeter curtain on ceiling track
Lamp with a single incandescent 40-watt light bulb
Infrared camera
Computer with behavioral tracking software (Noldus Ethovision XT)
Animal scale (0.1-g precision)
Red and blue non-toxic permanent markers
1-cc syringes with 26-G needles (*optional*)
Paper towels

***Setup and habituation***

1. Set up the testing room in preparation for the experiment, ensuring that the testing environment has temperature and humidity levels comparable to the colony. Set testing room lighting so that light levels are ~50 lux inside and outside the curtain surrounding the Y-maze. Place the visual cues at the distal end of each arm as follows:

   Start arm: black-and-white diagonal striped cue
   Familiar arm: white plus sign on a black background
   Novel arm: black circle on a white background

2. Turn on the infrared backlit base, camera, and data acquisition computer and software. Set up the software for the desired testing protocol with the appropriate number of trials required. This protocol is comprised of two trials with a 30-min delay between trials (for drug treatment) or a 10-min delay between trials (for testing aging effects on recognition memory). Trial 1 is a 10-min recording session in which the mice have access to the start and familiar arms but not the novel arm. Trial 2 is a 5-min recording session in which the mice have access to all arms. Ensure that the Y-maze is clearly visible and free of major obstructions that can impede tracking accuracy (e.g., lighting glare, part of the maze off the video frame, etc.). If using live-tracking, set up the maze to have four zones defined as start arm, familiar arm, novel arm, and center. Each arm is defined as the area from the end of the arm to ~3 cm from the center of the Y-maze. The center is defined as the area in the center of the maze connecting each arm.

   *Either live or offline tracking of the mouse's behavior can be done, but a video record of the behavior should be saved. The video can be reviewed for anomalous tracking results and re-tracked if better tracking accuracy is needed.*

3. Prepare a treatment sheet (electronic or paper) of the mice, indicating order of testing, dose time, and test time for each mouse and each trial, as well as body weight and dose volume. Assign each mouse to a treatment group A or B in a randomized order of testing.

   *If mice are group-housed, the entire cage should not be administered the same dose. Instead, a representative sample of each dose should be represented within a cage.*

4. Bring mice to the testing room. Label each mouse for easy visual identification and weigh mice if required (as necessary for dosing). Leave the testing room to allow mice to habituate undisturbed to the testing environment for a minimum of 1 hr.

5. *Optional (for drug treatment):* While the mice are habituating, in a separate area not in the testing room, formulate the test compound (scopolamine) and vehicle control (0.9% NaCl), and code the vials as A or B to keep the experimenter blinded to treatment.

*A technician familiar with the experiment, but not conducting the testing or performing the data analysis, should be responsible for coding the vials and maintaining the blind.*

*It is recommended that the technician pre-label and pre-load the syringes to the accurate injection volume (10 ml/kg) prior to starting the test to minimize the time between tests. In addition, a separate syringe and needle should be used for each subject.*

***Testing***

6. *Optional (for drug treatment):* Pretreat mice 30 min prior to the start of trial 1 using an intraperitoneal (i.p.) dose of 10 ml/kg.

   *At 0.1 mg/ml scopolamine, this gives a total dose of 1 mg/kg.*

   *The timing of dosing and testing should be planned carefully to avoid dosing at the same time mice are being tested in the maze.*

7. Ensure that the visual cues and the door blocking the novel arm are in place.

8. Before placing the first mouse in the maze, clean the maze thoroughly with 70% ethanol and wipe the maze dry.

9. Start trial 1: Pick up the first mouse from its home cage by the base of the tail and place it through the curtain opening into the start arm facing the center of the maze. Immediately place the lid on top of the maze and quietly close the curtain.

   *If desired, two identical mazes with identical visual cues in independent arenas, each with a perimeter curtain, can be located adjacent to each other, allowing two subjects to be tested during the same trial periods.*

10. Record the mouse's behavior during free exploration of the Y-maze for 10 min.

    *When running multiple subjects simultaneously, the technician should wait until the trials have completed for both subjects in order to avoid disrupting the last seconds of the test period for a subject whose trial has not yet completed.*

11. Return the mouse to its home cage for the pre-determined delay period before the start of trial 2. Clean the Y-maze and lid thoroughly with 70% ethanol and wipe dry.

12. Prior to starting trial 2 remove the door blocking the novel arm to provide access to all three arms.

13. Start trial 2: After the pre-determined delay period, pick up the mouse from its home cage by the base of the tail and place it through the curtain opening into the start arm facing the center of the maze. Immediately place the lid on top of the maze and quietly close the curtain.

    *If multiple mazes are running simultaneously, ensure the test subject is placed in the same maze for both trials.*

14. Record the mouse's behavior during free exploration of the Y-maze for 5 min.

15. Return the mouse to its home cage. Clean the Y-maze and lid with 70% ethanol and wipe dry.

16. Repeat steps 6–15 for testing of subsequent mice. Be sure to replace the door that blocks the novel arm before starting trial 1 for the next mouse.

17. Export the data.

18. Turn off the instrumentation and return mice to the colony.

***Data QC and analysis***

19. Prior to data analysis and while still blinded, confirm that data have been collected for each subject. Exclude subjects that failed to explore both start and familiar arms during trial 1 or subjects that escaped during any of the trials. Also exclude subjects that were mis-dosed or did not receive the full dose volume.
20. Export the cumulative time spent in each arm and the number of entries into each arm for trial 2.
21. Sum the number of entries into each arm to calculate the total arm entries per mouse.
22. From the cumulative time spent in each arm, calculate the percent duration in each arm as:

$$\%\ \text{duration in arm } x = (\text{time spent in arm } x / \text{sum of time spent in all arms}) \times 100$$

*Note that this formula divides by the sum of time spent in all arms, not the total trial duration of 5 min, so that the amount of dwell time in the center zone is not included in the time in arms calculation.*

***BASIC PROTOCOL 10***

## EPISODIC MEMORY TASK

Episodic memory is the memory of a specific past event that is recalled in the context of a particular place at a particular time and in association with contextual information, such as emotional responses, semantic knowledge, and olfactory, auditory, and/or visual cues (Dere, Kartteke, Huston, & De Souza Silva, 2006; Eacott & Easton, 2010; Eichenbaum, Sauvage, Fortin, Komorowski, & Lipton, 2012; Kim et al., 2015). Episodic memory function has been reported to decline with healthy aging, similar to other cognitive processes. It has also been reported that some of the earliest impairments observed in Alzheimer's disease patients are episodic memory deficits. Several methodologies for assessing episodic-like memory in mouse models have been published, including "what, where, which" (WWW) tasks in which the subject is required to discriminate an object (what) and its location (where) from an associated context (which). However, given the well-described issue of neophobia to novel objects in mice, this protocol uses a novel WWW episodic-like memory paradigm employing a three-trial Y-maze procedure that requires subjects to discriminate incongruency of familiar visual cues, in a specific location/arm of the maze, in relation to a specific visual-tactile context. In trial 1 of this task, the subject is initially placed in one context with two distinct visual cues in two different locations (left and right arms) for a 5-min period. During trial 2, the subject is placed into a different context with the same two visual cues, which are now familiar but are located in opposite arms relative to their previous location in the context used in trial 1. After a pre-determined delay period (5-30 min) during which mice are returned to their home cage, the mice are then evaluated in trial 3, in which they are returned to their initial context (from trial 1) but with both visual cues identical in both arms. Intact episodic-like memory in this assay is indicated by a preference for spending time in the arm with the incongruent pairing (of cue, context, and location) relative to the arm with the familiar pairing of the cue (what) with its original context (which) and location (where).

This protocol uses two Y-mazes (clear polycarbonate, arm dimensions 27 cm length, 6 cm width, and 15 cm height, with removable clear aerated lid). The three arms are defined as start, right, and left. A solid black polycarbonate wall (6 cm width, 15 cm height) is placed at the entrance to the start arm. For contextual cues, one maze contains a textured floor consisting of a clear rubberized bathmat (suction cup side facing down; Fig. 12A). In the second maze, the walls of each arm contain diagonal lines created using black electrical tape (Fig. 12B). At the distal end of the right and left arms

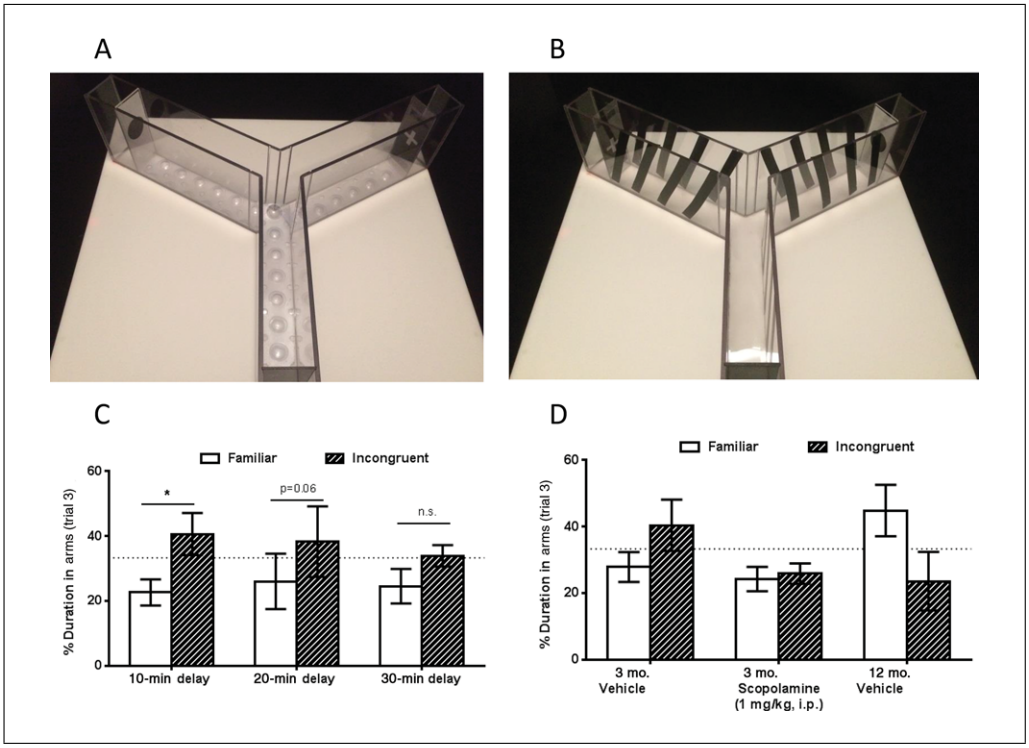


**Figure 12** Episodic memory task. (**A**,**B**) Mice are exposed to two different contexts for trials 1 and 2, respectively, and then tested in trial 3 in a familiar context with two identical familiar cues. Episodic-like memory in this assay is indicated by a preference for spending time in the arm with the incongruent pairing (of cue, context, and location) relative to the arm with the familiar pairing of the cue (what) with its original context (which) and location (where). (**C**) Young adult C57BL/6J mice (8–16 weeks of age) demonstrate the expected delay-dependent impairment in episodic-like memory. (**D**) Treatment with scopolamine in young mice (3 months) and aged mice (12 months) demonstrates impairment in episodic-like memory in this task as indicated by a lack of preference to spend time in the arm of the maze with the incongruent pairing relative to spending time with the familiar pairing of context, location, and cue. For time delay studies (C), test subjects were independent groups of mice evaluated on separate test days. For scopolamine testing (D), vehicle and scopolamine treated subjects were drug- and behaviorally naïve.

is a distinct visual cue: a white plus sign on a black background and a black circle on a white background (Fig. 11B,C). The cues are pre-assigned to the left and right arms and are placed in opposite locations in the two contexts. Specifically, in context 1, the plus sign is placed on the right and the circle on the left, while in context 2 the circle is placed on the right and the plus sign on the left. For testing, all visual cues are the same in both arms for both contexts. The rest of the setup—including the perimeter curtain, infrared backlit base, lighting, infrared camera, computer, and tracking software—is the same as described for the spontaneous alternation test (see Basic Protocol 8).

### *Materials*

Subjects:
Young adult male or female mice 2–6 months of age (e.g., C57BL/6J, The Jackson Laboratory, no. 000664) and sex-matched mice >12 months of age
OR
Adult male C57BL/6J mice 8-12 weeks of age ($n = 8$ per dose level)
0.1 mg/ml scopolamine (*optional*; see recipe)
0.9% NaCl solution (*optional*)
70% (v/v) ethanol

Two Y-mazes (arm dimensions: 27 L × 6 cm W × 15 cm H) with removable clear aerated lid and solid black polycarbonate wall (6 cm W × 15 cm H) for start arm, plus:
*For context 1:* clear rubberized bathmat cut to fit arm width
*For context 2:* diagonal lines created using black electrical tape (3/4-in. width) spaced ~1 in. apart and applied to the internal walls of the maze
Visual cues (Fig. 11B,C)
Infrared backlit base (Noldus Information Technology)
Perimeter curtain on ceiling track
Lamp with a single incandescent 40-watt light bulb
Infrared camera
Computer with behavioral tracking software (Noldus Ethovision XT)
Animal scale (0.1-g precision)
Red and blue non-toxic permanent markers
1-cc syringes and 26-G needles (*optional*)
Paper towels
Clean cages with lid and bedding only (one per test subject)

***Setup and habituation***

1. Set up the testing room in preparation for the experiment, ensuring that the testing environment has temperature and humidity levels comparable to the colony. Set testing room lighting so that light levels are ~50 lux inside and outside the curtain surrounding the Y-maze. Place the visual cues at the distal end of the right and left arms as follows:

   For arena 1 (context 1 = textured floor): plus sign in left arm and circle in right arm
   For arena 2 (context 2 = stripes): circle in left arm and plus sign in right arm

2. Turn on the infrared backlit base, camera, and data acquisition computer and software. Set up the software for the desired testing protocol with the appropriate number of trials required. This protocol is comprised of three trials per subject. Trials 1 and 2 are 5 min in duration, with mice briefly returned to their home cage between trials (~2 min) to allow the maze to be sanitized. Trial 2 is followed by a pre-determined delay period appropriate for the study (see step 12) and then trial 3, which is 3 min in duration. Ensure that the Y-maze is clearly visible and free of major obstructions that can impede tracking accuracy (e.g., lighting glare, part of the maze off the video frame, etc.). For video tracking, subjects should be tracked via the animal's center point of the body and the maze should be divided into four zones defined as start arm, left arm, right arm, and center. Each arm is defined as the area from the end of the arm to ~3 cm from the center of the Y-maze. The center is defined as the area in the center of the maze connecting each arm.

   *Either live or offline tracking of the mouse's behavior can be done, but a video record of the behavior should be saved. The video can be reviewed for anomalous tracking results and re-tracked if better tracking accuracy is needed.*

3. Prepare a treatment sheet (electronic or paper) of the mice, indicating order of testing, which arena/maze the subject is assigned to, dose time, and test time for each mouse and for each of the three trials per mouse, as well as body weight and dose volume. Assign each mouse to treatment group A or B in a randomized order of testing.

   *Two separate mazes with independent curtains and cameras are situated adjacent to each other, such that two subjects can be tested simultaneously. In this paradigm, instead of changing out the context and the cues for each trial, the two mazes are reserved as context*

*1 and context 2 and the mice are assigned to context 1 or context 2 in arena 1 or arena 2, respectively, and then swapped to the opposite context in the opposite arena during trial 2. For the test trial (trial 3), the subject is always placed in the initial context/arena used in trial 1.*

4. Bring mice to the testing room and individually house into new clean cages (with bedding only). Label each mouse for easy visual identification and weigh mice if required (as necessary for dosing). Leave the testing room to allow the mice to habituate to the testing environment undisturbed for a minimum of 1 hr.

   *For group-housed mice that are switched to individual housing at the time of this test, a 90-min habituation period is recommended. For mice previously housed individually but moved to a clean cage, a 60-min habituation period is acceptable.*

5. *Optional (for drug treatment):* While the mice are habituating, in a separate area not in the testing room, formulate the test compound (scopolamine) and vehicle control (0.9% NaCl), and code the vials as A or B to keep the experimenter blinded to treatment.

   *A technician familiar with the experiment, but not conducting the testing or performing the data analysis, should be responsible for coding the vials and maintaining the blind.*

   *It is recommended that the technician pre-label and pre-load the syringes to the accurate injection volume (10 ml/kg) prior to starting the test to minimize the time between tests. In addition, a separate syringe and needle should be used for each subject.*

***Testing***

6. *Optional (for drug treatment):* Pretreat mice 30 min prior to the start of trial 1 using an intraperitoneal (i.p.) dose of 10 ml/kg.

   *At 0.1 mg/ml scopolamine, this gives a total dose of 1 mg/kg.*

   *The timing of dosing and testing should be planned carefully to avoid dosing at the same time mice are being tested in the maze.*

7. Confirm that visual cues are in the correct location within each context for trial 1.

8. Before placing the first mice in the mazes, clean the mazes thoroughly with 70% ethanol and wipe them dry.

9. Start trial 1: Place subject 1 in context 1 and subject 2 in context 2. Immediately upon placement into each maze, place the lid on the maze, close the curtains, and record behavior for 5 min.

10. At the conclusion of trial 1, once both mice have completed testing, remove them to their home cages briefly to sanitize the mazes. Sanitize mazes with 70% ethanol and wipe dry.

    *When running multiple subjects simultaneously, the technician should wait until the trials have completed for both subjects in order to avoid disrupting the last seconds of the test period for a subject whose trial has not yet completed.*

11. Start trial 2: Place subject 1 in context 2 and subject 2 in context 1. Immediately upon placement into each maze, place the lid on the maze, close the curtain, and record behavior for 5 min.

12. At the conclusion of trial 2, once both mice have completed testing, remove them to their home cages for the pre-determined delay period. Sanitize the mazes with 70% ethanol and wipe dry.

    *Relatively young, healthy mice (8-16 weeks of age) demonstrate the expected delay-dependent impairment in memory (Fig. 12C). Therefore, it is important to consider the appropriate delay period that can be used when planning studies. In aging studies where*

*mice were tested longitudinally at 2, 6, 9, and 12 months of age, a 5-min delay period was employed (Fig. 12D).*

13. During the delay period, set up for trial 3, ensuring that a circle is used as the visual cue for both arms in both contexts.

    *For additional information, see Critical Parameters.*

14. Start trial 3: Place subject 1 in context 1 and subject 2 in context 2. Immediately upon placement into each maze, place the lid on the maze, close the curtain, and record behavior for 3 min.

15. At the conclusion of trial 3, once both mice have completed testing, remove mice to their home cages.

16. Sanitize mazes with 70% ethanol, wipe dry, and set up cues for trial 1 for the next set of subjects.

17. Repeat steps 6–16 for testing of subsequent mice.

18. Export the data.

19. Turn off the instrumentation and return mice to the colony.

***Data QC and analysis***

20. Prior to data analysis and while still blinded, confirm that all data have been collected for each trial for each subject. Exclude subjects that failed to explore all arms in both trials 1 and 2 or escaped from the maze during any of the trials. Also exclude subjects in which the technician failed to place the appropriate visual cues for that trial, and subjects that were mis-dosed or did not receive the full dose volume.

21. Analyze data as the amount of time spent in the incongruent arm relative to the cumulative time spent in all arms of the maze (minus center time) and calculate as a %.

    *Episodic-like memory is intact if time spent in the incongruent arm is >33% (chance levels in a three-arm assay) and significantly different from time spent in the familiar arm (within group mean t-test of incongruent vs. familiar; Fig. 12C,D).*

***BASIC PROTOCOL 11***

## WHEEL RUNNING

Wheel running behavior in mice is a simple, easily quantifiable measure of behavior that can be assessed in the home cage and with little interruption (Sherwin, 1998). As previously reported by several laboratories, mice demonstrate age-dependent reductions in both distance and speed (Kopp, Ressel, Wigger, & Tobler, 2006; Kohman et al., 2011; Cheng et al., 2013; Soffe, Radley-Crabb, McMahon, Grounds, & Shavlakadze, 2016) and also time spent running, which may be an indicator of motivation (Rhodes, 2005; Coyle, Strand, & Good, 2008). It is also well reported that wheel running behavior is sensitive to genetic alterations (Lightfoot, Turner, Pomp, Kleeberger, & Leamy, 2008) and may be a more sensitive indicator of subtle phenotypes in activity levels that may not be detectable in the standard spontaneous open-field test (Mandillo et al., 2014). In this protocol, mice are individually housed, as data cannot be precisely quantified for individual mice when mice are housed together.

The mouse's home cage may be used for the wheel running cage if it is large enough to accommodate the running wheel without impeding free movement or access to the wheel, food, or water. In some cases, it is necessary to remove the food hopper so that it does not block seamless movement of the wheel; in this case, food can be placed on

the cage floor. If the home cage cannot accommodate the wheel, disposable cages offer an alternative, again with the food hopper removed and food placed on the cage floor. The low-profile wireless running wheel consists of a 15.5-cm-diameter plastic disc with a hole in the center of the bottom for mounting on an electronic base. The disc also has a small magnet embedded in the underside, so the electronic base can track each revolution. The base holds the wheel at an angle, so the front is 3.3 cm from the cage floor and the back is 10.25 cm from the floor. It is battery powered for continuous monitoring of wheel revolutions. Data are wirelessly transmitted every 30 sec to the data acquisition software on a local computer. The electronic base connects to a clear plastic platform by two metal pegs that serve to hold the wheel and base in place and provide stability.

### *Materials*

Subjects: young adult male or female mice 2–6 months of age (e.g., C57BL/6J, The Jackson Laboratory, no. 000664) and sex-matched mice >10 months of age
Detergent for soaking and cleaning non-electronic wheel components
70% (v/v) ethanol

Wheel running cages: home cages or disposable cages (e.g., Innovive, product no. M-BTM, with static cage lid)
Low-profile wireless running wheels (Med Associates, cat. no. ENV-047), including:
- 15.5-cm-diameter wheel
- Electronic base with AAA batteries
- Platform (15.25 × 13.7 cm)
- Data acquisition software

Red and blue non-toxic permanent markers
Paper towels

### *Setup and habituation*

1. Set up the testing room in preparation for the experiment, ensuring that the testing environment has lighting, temperature, and humidity levels comparable to the colony. To demonstrate age-related reductions in wheel running activity, use a 12:12 light/dark cycle with lights on at 6:00 am and lights off at 6:00 pm.

   *The mice will be housed in the testing room overnight and across multiple days, so it is critical that there is proper ventilation and that the light/dark cycle is set accordingly for the test. The lights should be checked to ensure that they go on and off as planned prior to bringing in mice for testing.*

   *If desired, the light cycle can be altered during testing to evaluate changes in circadian cycle (e.g., phase shift).*

2. Prior to turning on the computer and data acquisition software, ensure that none of the wheels are transmitting data by confirming that the on/off toggle switch is in the OFF position.

3. Turn on computer and set up the data acquisition software for the desired testing protocol. For this protocol, monitor the number of wheel revolutions every 60 sec for four nights (6:00:00 pm to 5:59:00 am) and three days (6:00:00am to 5:59:00pm).

   *If any software wheel IDs populate once the data acquisition software is turned on, this would indicate that a wheel base was left on and transmitting data. Find the wheel base and turn it off and delete all wheel data and IDs from the acquisition software.*

   *It is recommended that the data acquisition computer be removed from the internet for the duration of testing to prevent unscheduled software updates from installing and restarting the computer, which would exit out of the data acquisition software and result in lost data.*

4. Set up the number of running wheel cages needed (one per mouse). Clearly label each cage with the ID of the mouse that will be housed in the cage. Place an electronic wheel base and platform in each cage, and note the wheel base ID associated with each mouse ID. Do not place the running wheel on the base, because this will cover up the on/off switch. Fill each cage with enough bedding to cover the floor and platform the wheel sits upon. Provide water and food.

5. Prepare a treatment sheet (electronic or paper) of each mouse ID, electronic wheel base ID, and data acquisition software wheel ID.

   *The hardware and software used in this protocol can associate up to 40 unique wheels with a single hub used to acquire the wireless wheel signal and transmit the data to the data acquisition software. Multiple hubs can be used to expand the number of wheels in batches of 40. Each wheel needs to be clearly identified to be able to track hardware problems if they arise. The data acquisition software maintains wheel identification for the duration of an experiment but does not save this identification across experiments. Wheels are identified in the software 1–40 and by hub name, and these identifications are assigned in the order that wheels are turned on. As a result, mouse IDs need to be associated with electronic wheel base IDs and software wheel IDs. In addition, the wheel ID and mouse ID can also be placed onto the cage card as a secondary piece of information to avoid discrepancies.*

6. Bring mice to the testing room.

***Testing***

7. For the first cage, turn the wheel switch to the ON position and rotate the wheel two to three times to confirm the software accurately records the number of rotations and that the wheel can rotate without being impeded by the cage lid.

   *As the wheel is turned ON, it will begin transmitting data and will automatically populate in the data acquisition software with an ID. Software IDs are assigned as wheels are turned on. If the steps are followed correctly, the first mouse software ID should be wheel 1 and subsequently the tenth mouse should be wheel 10. Make note on the treatment sheet of the software wheel ID associated with the mouse ID.*

   *The data acquisition software will also indicate the current battery power of the electronic wheel base. If the power is below 3 V, replace the batteries for that wheel prior to turning on the next wheel.*

8. Place the first subject in the cage with the first wheel.

9. Repeat step 7–8 for each mouse, making sure to note the software ID for each mouse and replacing batteries as needed.

10. Double-check that every cage has food, water, and a mouse.

11. Start the data acquisition software to record wheel revolutions. Turn off the monitor to prevent disruption of circadian rhythms during the dark cycle by the illumination of the monitor screen. Turn off only the monitor and not the computer hardware and software, which should be left on at all times.

12. Leave the testing room and log the time.

13. Each day, check that the equipment is functioning and check the welfare of each mouse:

    a. During welfare checks, ensure that all mice have sufficient food and water.
    b. During equipment checks, turn on the computer monitor and view the software to make sure each wheel has power and has registered revolutions. If a wheel has registered $<10$ revolutions, find the wheel and confirm it is not stuck. Rotate the wheel manually to ensure the software is accurately tracking wheel revolutions.

c. Note on the treatment sheet any aberrations, including if the technician opened a cage to test the wheel.
d. Log the entry and exit time into the testing room.

14. After completion of testing, return all mice to their home cages.
15. Export the data in 1-min bins for full nights and days. Data will begin at the time of lights off on the first day and end at the last minute before lights on for the final day of testing.
16. Turn off the data acquisition software and computer.
17. Return mice to the colony.
18. Remove all running wheels from their bases, turn off all wheel bases, and remove wheel bases and platforms from the cages.
19. Soak non-electrical components (wheels and platforms) in warm water with detergent for 1 hr, scrub with a brush, then rinse and set out to dry. Wipe outside surfaces of the wheel bases that contain electronic components and batteries with 70% ethanol.

***Data QC and analysis***

20. Prior to data analysis and while still blinded, confirm that all values have been entered for all subjects and exclude data for technical issues (e.g., wheel malfunction).
21. Calculate the time spent running for each 30-min time bin (Fig. 13A). First, transform revolutions per minute (rpm) into binary data: if there were any revolutions for a 1-min time bin, that bin will be a 1; if there were no revolutions it will be a 0. Then, sum the values for each 30 min to calculate the number of minutes spent running per bin.
22. Calculate the total time spent running for each night/day by summing the values for each night/day from the binary data (Fig. 13B).
23. Calculate the speed as the average rpm for each 30-min time bin (Fig. 13C).
24. Calculate the total distance traveled for each night/day by multiplying the sum of revolutions per night/day by the circumference of the running wheel (0.3768 m; Fig. 13D).

## ADHESIVE REMOVAL

***BASIC PROTOCOL 12***

Adhesive removal is used to assess sensory and fine motor ability and has previously been reported to be sensitive to fine motor deficits in healthy aging mice as well as models of neurodegenerative diseases associated with fine motor deficits (Bouet et al., 2009; Fleming, Ekhator, & Ghisays, 2013). Mice are individually habituated to an observation arena and then a small adhesive sticker is placed on the fur in the center of the mouse's forehead. The latency to initiate removal of the sticker and cumulative time spent removing the sticker are both recorded.

***Materials***

Subjects: young adult male or female mice 2–6 months of age (e.g., C57BL/6J, The Jackson Laboratory, no. 000664) and sex-matched mice >12 months of age
70% (v/v) ethanol

Small observation arena (e.g., Ugo Basile modular enclosure, model 3700-006, w/covers)
Forceps (e.g., Roboz RS-5137)

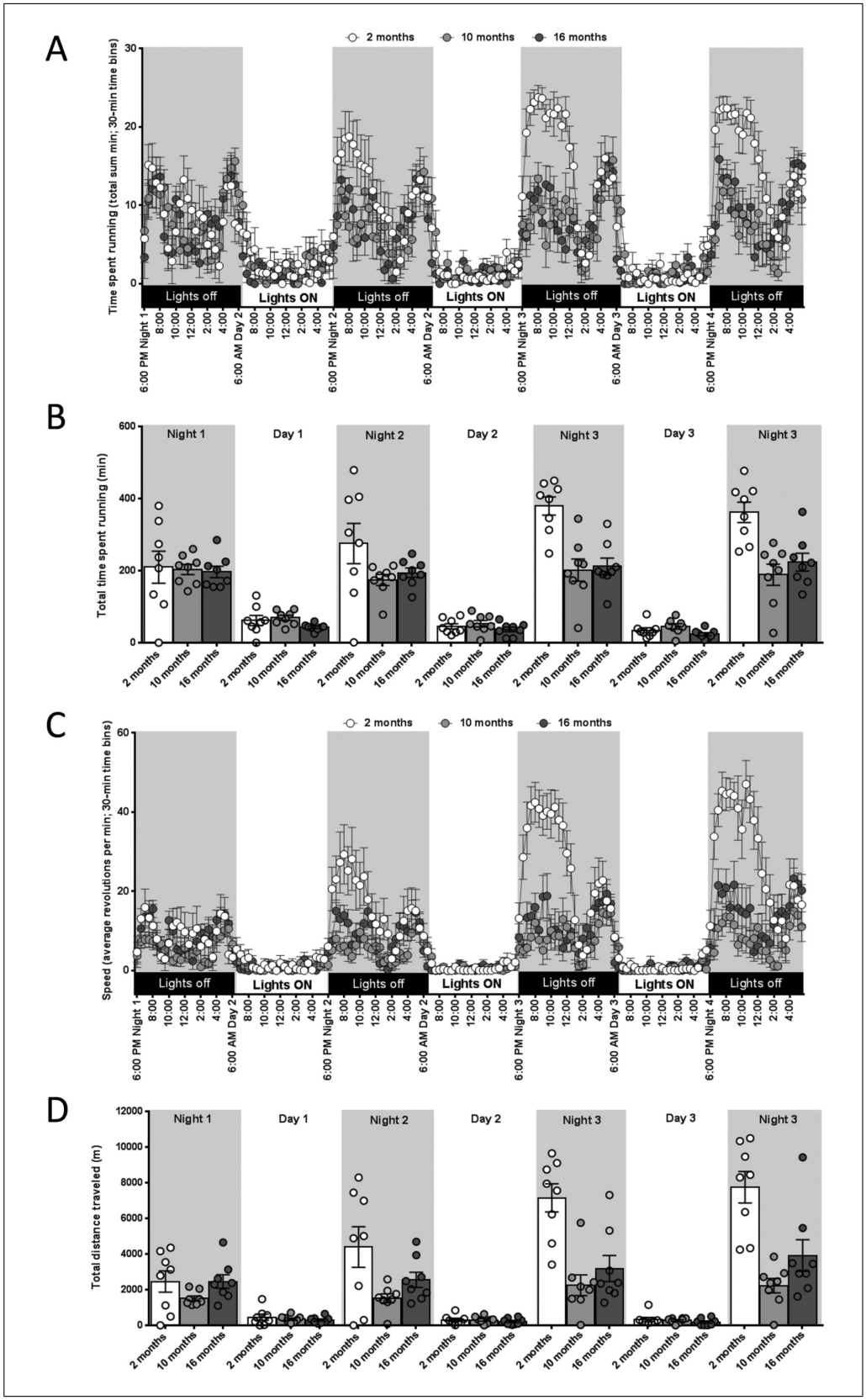


**Figure 13** Wheel running in mice is sensitive for detecting aging-dependent alterations. As presented, young adult male C57BL/6J mice demonstrate time-related increases in wheel running activity over the course of multiple days and nights. Aging mice demonstrate reductions in total time spent running (**A**,**B**), speed (**C**), and total distance, defined as cumulative meters for each 12-hr day and night period (**D**), relative to young (2-month) controls. For aging studies, test subjects were independent groups of aging mice evaluated during a single test session.

Digital stop watches, silenced
Paper towels
Red and blue non-toxic permanent markers
Round adhesive labels, ¼-inch-diameter (Avery, cat. no. 5796), yellow preferred

***Setup and habituation***

1. Set up the testing room in preparation for the experiment, ensuring that the testing environment has lighting, temperature, and humidity levels comparable to the colony.
2. Set up six arenas (one per mouse to be tested) so that they are aligned with the technician directly facing in front of them.
3. Prepare a treatment sheet (electronic or paper) of the mice, indicating order of testing and test time for each mouse.
4. Bring six mice to the testing room and label each mouse for easy visual identification. Leave testing room to allow mice to habituate undisturbed for a minimum of 1 hr.

***Testing***

5. Thoroughly clean the testing arena of each enclosure and its cover with 70% ethanol and wipe the arenas dry.
6. Place the first subject into its testing arena for a 10-min acclimation period.

   *When testing multiple mice in a single session, subjects are acclimated in individual arenas with acclimation periods beginning 3 min apart, which is the maximum test time for each trial.*

7. At the conclusion of the 10-min acclimation period, manually scruff the mouse to restrain.
8. While the animal is restrained, use forceps to gently place a sticker centrally on its forehead (above the eyes and below the ears).
9. Immediately return the mouse to the testing arena and start two stop watches simultaneously. Use one stop watch for timing latency (sec) to begin to remove the sticker and the other for cumulative latency (sec) required to remove the sticker.
10. Record both times. End the trial at 3 min (180 sec).
11. Repeat steps 7–10 for the remaining habituated mice, moving to the next enclosure in sequential order.
12. When all mice within a group have completed testing, return mice to their home cages. Ensure that the sticker is not on the mouse prior to returning to the home cage.
13. Clean enclosures, covers, and benchtop with 70% ethanol and allow to dry.
14. Repeat steps 6–13 for the remaining mice in the testing cohort.

***Data QC and analysis***

15. Prior to data analysis and while still blinded, confirm that all values have been entered for all subjects and exclude data from subjects where the sticker did not effectively stick to the fur or the placement was not accurate.
16. Analyze latency to initiate sticker removal and cumulative time for sticker removal (Fig. 14).

    *Latency for sticker removal is defined as the amount of time (sec) required for the subject to initiate removal by swiping with its forepaws or hind paws near the forehead, which is*

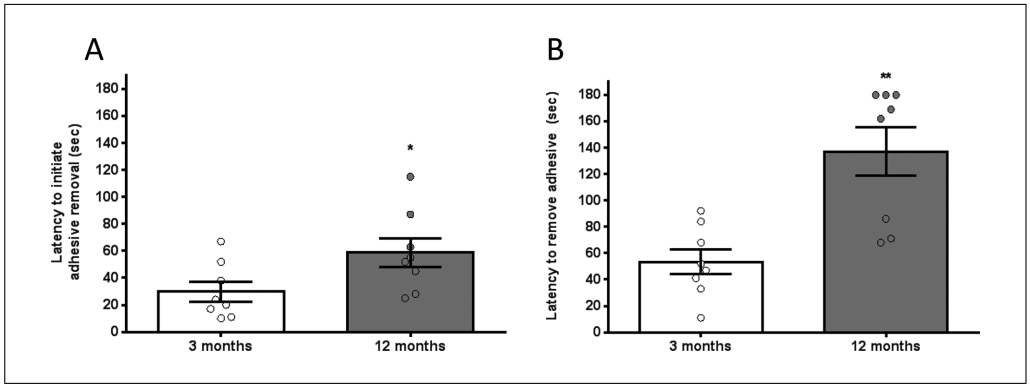


**Figure 14** The adhesive tape removal task is sensitive for detecting fine motor deficits in aging mice. Adult male C57BL/6J mice demonstrate age-dependent impairments in the latency to initiate removal of an adhesive placed on the forehead (**A**) as well as an increase in the time required to remove the adhesive (**B**). Test subjects were independent groups of aging mice evaluated in a single test day.

*defined as above the snout. Cumulative time for sticker removal is defined as the amount of time (sec) required for the subject to completely remove the sticker from its forehead.*

## REAGENTS AND SOLUTIONS

### *Ethanol, 0.2, 0.15, and 0.1 g/ml*

Dilute laboratory-grade ethanol (200 proof; 0.794 g/ml) in $dH_2O$ vehicle as follows:

Dilute 5 ml of 0.794 g/ml EtOH into 14.85 ml $dH_2O$ (final 0.2 g/ml)
Dilute 10 ml of 0.2 g/ml EtOH into 3.33 ml $dH_2O$ (final 0.15 g/ml)
Dilute 5 ml of 0.15 g/ml EtOH into 2.50 ml $dH_2O$ (final 0.1 g/ml)

Store undiluted ethanol at room temperature and prepare dilutions fresh daily.

*At a 10 ml/kg dose volume, this gives total doses of 1, 1.5, and 2 g/kg.*

### *Scopolamine, 0.1 mg/ml*

Weigh an appropriate amount of (−)-scopolamine hydrochloride (Sigma, cat. no. S1013) in an amber glass vial. Dilute to 0.1 mg/ml by adding 0.9% NaCl solution to the vial and sonicating for ∼1 min. Prepare fresh daily.

*At a 10 ml/kg dose volume, this gives a total dose of 1 mg/kg.*

*Scopolamine is hygroscopic and should be stored at room temperature in a desiccator.*

## COMMENTARY

### Background Information

Careful consideration and strategic planning are required when setting up behavioral assays. If the space is being retrofitted, an understanding of the dynamic laboratory activities around the intended space should be critically considered, as most behavioral assays are sensitive to intermittent noise and vibration. As a means to ensure the environment is optimized for conducting behavioral experiments, the technician should be able to reproduce published data for known standards under blinded conditions. This also provides confidence in the ability of the technician to perform the technique prior to testing experimental cohorts (Sukoff Rizzo & Silverman, 2016). Experiments aimed to study healthspan and lifespan in aging cohorts of mice are unique in that tests optimized in young, otherwise healthy mice may not be sensitive for detecting subtle aging deficits.

The methods provide here have demonstrated aging-related phenotypes across different background strains of mice and have also provided data sets as a reference for validating these procedures as well as insight into challenges and limitations of executing these protocols. It is not being suggested that the exact instrumentation be purchased in order to reproduce these data sets. Rather, whatever

instrumentation is sourced, in order to ensure the assay is optimized and sensitive to detecting the expected behavioral outcome measures as currently set up, then validation experiments should be conducted and data should reproduce known standards such as the reference data provided herein.

When developing a testing battery, the choice of assay and order of testing should be piloted prior to running experimental cohorts (McIlwain, Merriweather, Yuva-Paylor, & Paylor, 2001; Paylor, Spencer, Yuva-Paylor, & Piekedahl, 2006). In the current battery, the testing order was generally as follows: frailty assessment with core body temperature, open field, grip strength, rotarod, spontaneous alternation, visual acuity, episodic memory, acoustic startle, novel spatial recognition, wheel running, olfactory discrimination, and adhesive removal, typically with at least a 1- to 2-day rest period between assays and at least 1 week between re-exposure to the Y-maze for the spontaneous alternation, episodic memory, and novel spatial recognition tasks. This comprehensive battery therefore requires ~6-8 weeks to complete, which may not be ideal for some laboratories. It is recommended that whatever tasks are chosen, pilot data with the assays and in the proposed testing order should be conducted to ensure the data meet the expected outcome measures. Further, it is critical that the experimenter remain blinded to treatment group/age during the test and that the blinding code be maintained until the data have been quality controlled and analyzed.

## Critical Parameters and Troubleshooting

As with all behavioral testing protocols, intermittent noise and vibration can contribute to variability in responses and should be minimized when conducting this assay (Sukoff Rizzo & Silverman, 2016). It is also recommended that assays not be conducted on the same day after a cage change.

Prior to transporting the mice to the procedure room for testing, the equipment should be checked for proper functioning to avoid starting a test (e.g., dosing) and learning after the fact that the instrumentation requires maintenance or repair. Investing in a basic light meter (e.g., Sper Scientific Light Meter 840020) and sound meter (e.g., Extech Instruments sound level meter 407730, Flir Commercial Systems) is recommended to record light and background noise and ensure they are maintained as indicated in the protocol for each test.

Subjects should be observed for any health or welfare issues that would exclude them from testing prior to starting the test. This is recommended prior to starting the acclimation period that precedes the test and at the point when the mice are handled for tail labeling. If a subject escapes before or during the test, this may exclude the mouse from the testing. Importantly, this should be noted in the lab notebook or prepared run sheet. If the technician cannot capture the mouse within 30 sec or without disrupting the testing environment or delaying the timing of the test, the technician should wait to attempt capture after the end of the testing period to avoid compromising the testing of other subjects. Irrespective of whether the mouse is caught and a decision is made to test the mouse, it is important to annotate this aberration, as spurious data may result from a mouse that escaped prior to testing.

When both sexes are being tested, it is recommended that males and females be tested separately and data analyzed within sex, as many behaviors are sexually dimorphic. Females in estrus in the testing room may impact the behavior of males performing the tests. Cleaning of the testing arenas is also critical to eliminate odors, particularly that of estrus urine. Performance in the tasks can be negatively impacted if careful cleaning between subjects is not achieved, resulting in scent marking or excessive sniffing instead of attending to the task.

### *Frailty and core body temperature*

In order to be capable of identifying some of the characteristic traits of aging noted here, it is critical that the experimenter have access to an aging colony to learn and be able to recognize these traits prior to testing the experimental cohort, as many of these phenotypes are not observed in young mice (<24 months in some cases). In addition, it is recommended that this assay, particularly the recording of body temperature, be completed within 6 hr of lights on in the facility, due to the reported increase in diurnal corticosterone levels, which may contribute to changes in core body temperature. Importantly, if collecting baseline body temperatures in group-housed mice, cagemates should not be returned to the home cage until all subjects within the home cage have been tested. If cagemates are tested from the same cage consecutively for body temperature, careful attention should be paid to determine if body temperatures are rising with each cagemate tested. If this is the case, it is recommended that mice be individually

housed prior to testing to avoid the stress-induced hyperthermic response phenomenon (Zethof, Heyden, Tolboom, & Olivier, 1994).

***Spontaneous open-field activity***

It is important to counterbalance treatment groups across multiple instruments to avoid any one treatment group being always placed first or all controls, for example, always in chamber 1. If multiple mice are housed in a single cage, it is best practice to test all mice within a cage in a single session and avoid re-exposing cagemates that completed testing back to their cagemates that are habituating and have not yet been tested. If the number of mice in a cage exceeds the available number of open-field arenas, do not return tested mice to their home cage with untested mice. These mice will interact with the untested mice and may influence the performance of the subsequent mice. Instead, upon completion of testing, move mice to a temporary transition cage until all mice from a cage have been tested and then return all mice to their home cage at the same time. It is also important to allocate mice evenly across multiple batches of testing. For example, if 20 open-field arenas are available and 24 mice are scheduled to be tested, do not test 20 mice in the first batch and 4 mice in the second. Instead, test 12 mice in each batch, ensuring counterbalancing across arenas with representative samples from each treatment group or age group.

***Grip strength***

This assay requires a high level of experimenter proficiency to reduce inter-rater variability. Frequent mistakes in mouse placement on the grid (i.e., allowing the subject to grip near the edges of the grid and measurements to be excluded from the meter before attempting to let the subject grip again) will result in lower values over time as the mouse tires from repeated exertion. The mouse-specific grid must be used for testing mice due to gauge size differences in the wires comprising the grid. For longitudinal assessments, it is recommended that the same technician be assigned in order to minimize inter-rater variability. Training of staff should include practicing with a force gauge meter attached to the grid to achieve consistent responses of 400 g of pull force.

***Rotarod***

There are many variations in rotarod protocols across laboratories, including inter-trial times, number and duration of trials, start speed, and acceleration rate. Whatever protocol is used, it is important that young, otherwise healthy mice demonstrate a trial-dependent increase in ability to maintain balance on the rod with the chosen parameters. If a software package is used to automatically record fall latencies, it is recommended that the technician use the software as backup and the treatment sheet as the primary record, because many software packages do not capture mice that fall upon placement on the rod before the start button is activated. This is a very important phenotype that is not captured by the software. Importantly, mice that fall or jump upon placement on the rod should not be administered a "re-attempt" until they walk on the rod, but instead should be given a data value of 0 and the behavior (jumping or falling) noted as the phenotype for that trial. In addition, it is recommended that the experimenter maintain their position in front of the rotarod throughout the test as opposed to moving away or sitting down. The tester needs to be available to move the mice immediately back to their home cages upon falling, and the noise from moving to sit on a chair can be distracting to the performing mice.

***Acoustic startle response***

Mice for this assay should not be exposed to the audio stimuli prior to being tested. To minimize this, mice should be habituated to an anteroom or alternative area in the laboratory with a comparable environment (lighting, noise, temperature, and humidity) to the procedure room. It is critically important to not attempt to run two independent systems simultaneously that are not configured together on the same interface, because audio outputs cannot be time-locked and synchronized together unless they are physically connected and daisy-chained together. In addition, a daily calibration of the boxes with a standardization unit to achieve similar background levels of responses is important to ensure function of the testing equipment and that the unit is sensitive enough to detect responses from mice.

***Optokinetic function***

If the mouse is focused on the task, tracking should occur shortly after the visual stimulus is presented. To facilitate mouse attending to the visual stimuli when a competing behavior occurs (e.g., grooming), a 90-dB audio stimulus (clicker) can be used. Importantly, the tester must remain blind to treatment/age and to the visual stimulus presented. The tracking algorithm will not complete, and a threshold cannot be determined, if no positive responses are

made. A cutoff of 10 min is imposed for mice that are not tracking the grating at all (no positive responses). This threshold allows for a standard test time for blind mice. In addition, an overall time cutoff of 20 min is imposed to establish a clear stopping point for mice that are not testable, e.g., due to hyperactivity (subject jumps off platform repeatedly) or vestibular deficits that prevent the subject from maintaining balance on the platform.

***Olfactory discrimination***

To minimize confounds with general locomotor activity and to normalize to within-subject baseline exploratory behavior as with aging mice, raw exploration time cannot be directly compared across ages. Instead a within-subject value (% change) for the time spent sniffing the novel block should be normalized relative to the time spent sniffing the familiar block. To avoid odor transmission, it is important that a block identified as the novel stimulus come from the cage of a mouse that was never housed with the test subject, and that gloves be sanitized generously with 70% ethanol between blocks.

***Spontaneous alternation***

It is important that the baseline range for % alternation in young, healthy controls of the background strain be established and consistent with intra-laboratory studies and across experimenters prior to running experimental cohorts. Chance levels in a three-arm spontaneous alternation assay equate to ~22%, and it is rare that mice fall below chance levels given their robust endogenous exploratory drive. However, when the assay is optimized, young control mice should demonstrate fairly consistent % alternation levels across multiple studies. Therefore, reductions in % alternation by only 5% may be significant, both physiologically and statistically. Although aging mice may have decreased exploration as indicated in this assay by reductions in total arm entries, hypoactivity or motor impairments in this assay are not necessarily a confound of the subject's ability to demonstrate alternation behavior in this task. However, if mice fail to perform as indicated by *a priori* criteria (e.g., mice fail to enter each arm of the maze at least once), those animals require exclusion. Mice should be tracked via the center point of the body to avoid false entries from only a head dip due to an animal exhibiting stretch-attend postures. For non-automated tracking, an entry can be defined as placement of all four paws into that arm. It is important to QC data, even when an automated program is used. False entries may occur on automated tracking programs when the animal sits in a space for a period of time (e.g., grooming) and that space was designated as a threshold area for transitions between the arm and the center. In this case, the data will appear to show repeated entries and exits within the same arm that would not be true visits to that arm. To minimize this, 1-sec rule is employed that defines a re-entry within the same arm as only a true occurrence when the time difference from exit to re-entry is >1 sec.

***Novel spatial recognition***

Critical parameters for this task include selection of salient and unbiased visual cues and choosing an appropriate delay period between trials 1 and 2 that control mice can perform. We initially employed a 30-min delay period for young mice in validation and training experiments with scopolamine, but determined through an iterative process that delays >10 min were too challenging in aged mice. It is therefore important to perform pilot studies in the background strain at the intended age to determine the optimal choice of delay period and visual cues. The visual cues in Figure 11 have been determined to be salient and unbiased in this task and can be printed for use. Importantly, it is critical that some type of visual task, such as the optokinetic function reported herein, be conducted to determine whether mice have impaired performance in this task due to deficits in cognition and not confounded by visual impairments. In addition, if the subjects fail to explore both the start and familiar arms in trial 1, it should be excluded for testing in trial 2.

***Episodic memory***

As with all cognition tasks that employ visual cues, mice must be sighted to perform this task and intact vision should be confirmed either prior to the test or after a perceived cognitive deficit is performed. The selection of visual cues and contexts is critical, as is the salience of the pairings of cue with context. Initial studies evaluated whether the order of context, side bias of cue placement, or choice of context for the test phase (trial 3) affected performance. While there was no evidence of bias of cue, context, or cue location in trial 1 or 2, or an effect of order of context presented, there was an increased variability in recognition of the incongruent pairing when the plus sign was used as the cue in either context for trial 3 that was not observed when

the circle was used. This type of troubleshooting is important when optimizing and setting up this experiment. The ability to demonstrate the preference for the incongruent pairing in vehicle-treated controls and a lack of preference in age- and sex-matched scopolamine-treated subjects will provide confidence in the optimization of the testing paradigm. Importantly, if mice do not explore both arms in trial 1 and/or 2 (the consolidation phase), they should be excluded for testing in trial 3.

***Wheel running***

Mice must be individually housed in this paradigm and the wheel must fit into the cage and rotate freely without any obstacles (e.g., hitting the cage lid). Considerations should be taken for identifying appropriate space to conduct wheel-running studies. While it may be convenient to house the running wheels in the housing room, competing activities in the housing room (such as regular husbandry procedures) may impact performance levels (e.g., mice may start to run excessively during the light cycle in response to noise and activity). It is therefore recommended that a separate procedure room with identical environmental conditions to the housing room (e.g., light/dark cycle, temperature, humidity, lighting levels) be used for these tests. When setting up the wheels, they should all be disconnected or turned off before they are placed into cages. Then, with careful attention and working one wheel at a time, each wheel is switched, the wheel's ID number is recorded as it populates into the software, and the mouse being paired with the wheel ID number is also recorded. It is important not assume that a wheel labeled as "wheel 1" will be the first wheel unless it is the first wheel turned on and populates in the software as such. In addition, mice should be checked at least once daily and the monitor should be shut off through the dark phase, as the illumination may impact the dark cycle.

***Adhesive removal***

This test can be confounded if alopecia or barbering is present at the location of sticker placement. Although the protocol requires only a single session with a maximum of 3 min to observe significant differences in aging mice, some mouse models may require a lengthier observation period (5 min or longer) or multiple trials within subject. It is also important to be consistent with the type and color of sticker used for testing. Initial studies using different colors of stickers demonstrated that the adhesive properties varied across different colored stickers. Therefore, this protocol uses the yellow stickers, which demonstrated the best adhesive properties.

## Anticipated Results

***Frailty and core body temperature***

As mice age, they are expected to demonstrate an increase in the cumulative frailty score and a corresponding reduction in core body temperature relative to young (2–6 months) sex-matched controls (Fig. 3). Body weight changes dynamically over the course of lifespan, increasing through middle-age and decreasing beginning ~24 months. This varies across strain and sex.

***Spontaneous open-field activity***

Reductions in locomotor activity and exploratory behaviors are expected with age (Fig. 4). Aging mice may also demonstrate reduced anxiety-like behavior, which can be measured by analyzing time spent at the perimeter of the open field. Young mice tend to exhibit thigmotaxis, which is attenuated with age (Fig. 4E,F).

***Grip strength***

When there are significant differences in body weight as with aging studies, force should be normalized to body weight (Fig. 5). As mice age, they are expected to demonstrate reductions in grip strength relative to young controls (Fig. 5A-E). Acute treatment with ethanol should result in dose-dependent impairments in grip strength for both forepaw and all-paw measures (Fig. 5F,G).

***Rotarod***

Young mice are expected to demonstrate trial-dependent increases in the ability to maintain their balance on the rotarod as illustrated for vehicle treated mice (Fig. 6A). Acute administration of ethanol should result in dose-dependent impairments in latency measures (Fig. 6A,B). Motor coordination deficits in aging mice in this protocol are subtle and are not robustly observed until mice are advanced in age (>30 months).

***Acoustic startle response***

Young mice (2-6 months) are expected to demonstrate a decibel-dependent increase in startle amplitudes that are attenuated with age (Fig. 7). Significant hearing impairment is indicated by a startle response value at or below the background noise level for that subject (the 'no stim' value).

***Optokinetic function***

Threshold values in young, healthy C57BL/6J mice (2-6 months) typically range from 0.3-0.4 cycles/degree. This may vary across different background strains. As mice age, a reduction in threshold values is expected (Fig. 8A). Mice with known retinal degeneration and blindness are expected to demonstrate threshold values below 0.1 cycles/degree (Fig. 8B).

***Olfactory discrimination***

Intact olfaction in this assay is indicated by preference for spending more time sniffing the novel block relative to time spent sniffing the familiar block (Fig. 9).

***Spontaneous alternation***

Impaired hippocampal working memory in this assay is indicated by a reduction in % alternation level relative to control. Chance levels in a three-arm spontaneous alternation assay equate to ~22%, and it is rare that mice fall below chance levels given their robust endogenous exploratory drive, even with advanced age or treatment with an amnestic agent such as scopolamine (Fig. 10). However, when the assay is optimized, young control mice should demonstrate fairly consistent % alternation levels across multiple studies and reductions in % alternation by only 5% may be significant, both physiologically and statistically (Fig. 10). Differences in locomotor activity as measured by total arm entries or distance traveled should be reported but do not necessarily confound the behavior unless the subject does not explore all arms of the maze or does not meet the *a priori* exclusion criteria.

***Novel spatial recognition***

Intact short-term recognition memory in this procedure is indicated by a preference to spend time (>33%) in the novel arm relative to the other arms of the maze (Fig. 11). If young mice cannot perform the task, piloting shorter delay periods or different visual cues may be required. Under optimized conditions, it is expected that acute treatment with the amnestic agent scopolamine (1 mg/kg, i.p. 30 min prior to the start of trial 1) will produce impairments in short-term recognition memory as indicated by a lack of preference to spend time in the novel arm (Fig. 11E).

***Episodic memory***

Intact episodic-like memory in this assay is indicated by a preference for spending time in the arm with the incongruent pairing (of cue, context, and location) relative to the arm with the familiar pairing of the cue (what) with its original context (which) and original location (where) (Fig. 12). If validation experiments to optimize conditions do not result in the expected preference of young healthy mice to spend a greater duration of time in the arm with the incongruent pairing, piloting of different visual cues and different contexts may be required. Under optimized conditions, it is expected that acute treatment with scopolamine (1 mg/kg, i.p., 30 min prior to the start of trial 1) will produce impairments in episodic-like memory as indicated by a lack of preference to spend time in the arm with the incongruent pairing (Fig. 12D).

***Wheel running***

Healthy young mice previously not exposed to a running wheel will nearly immediately begin running upon lights off. Over the course of the dark cycle it is expected that periods of running will be interspersed with periods of non-running behaviors (feeding, resting, grooming). Wheel-running activity should be significantly reduced during lights on. Young mice typically increase running activity levels (e.g., distance and time spent running) over the course of consecutive nights of exposure to the wheel (Fig. 13). As expected, mice will demonstrate aging-related attenuations in running wheel measures, including locomotor components of speed and distance, as well as time spent running, which may be an indicator of motivation.

***Adhesive removal***

Fine motor deficits are expected with age. Aging mice demonstrate increased latency to initiate the removal of the adhesive (Fig. 14A) and increased total time to remove the adhesive (Fig. 14B).

## Time Considerations

***Frailty and core body temperature***

A highly trained and proficient technician can complete the frailty assessment for each mouse in ~3-4 min inclusive of body temperature. An experiment with 40 mice, inclusive of time to weigh and label mice (~1 min/mouse) and acclimate for 60 min to the procedure room prior to starting the test, requires ~5-6 hr total to complete as follows: 10 min set-up time, 40 min weigh and label time, 60 min undisturbed habituation time, 160 min test time (40 mice × 4 min/mouse), and 10 min clean-up time.

***Spontaneous open-field activity***

The following times assume a setup with 20 identical open-field chambers running simultaneously. Setting up the software and performing a system check requires ~10 min. Labeling mice prior to habituation requires ~30 sec per mouse. Placing mice in each arena at the start requires ~30 sec per mouse. Cleaning at the end of each session requires ~1.5–2 min per arena. Therefore, inclusive of the 60-min habituation time that precedes the 60-min test, for a cohort of 20 mice, a total of 2.5 hr is needed. For each additional session of up to 20 mice, where all mice are habituated to the testing room at the same time, an additional 1 hr (run time) and 40 min (cleaning) should be estimated. It should be noted that, when working with wild-derived or hyperactive mice, it may take several extra minutes to catch and place mice in the arenas and retrieve them from the arena and return them to their home cages.

***Grip strength***

A highly trained and proficient technician can complete six consecutive measurements per mouse (three forepaw, three all paws) in 1 min. Three minutes per mouse is estimated inclusive of rest time between trials to tare the instrument, confirm the data entry with each trial on the system, and clean between mice. Additional time should include weighing and labeling of mice (~1 min per mouse), 60 min habituation time to the procedure room prior to testing, and 10 min post-testing cleaning time and data export. Therefore, for 40 mice, 3 hr should be planned. In the optional dosing paradigm, a 20-min pretreatment period needs to be allotted for and timed accordingly.

***Rotarod***

It is convenient to run only four mice per session, leaving the fifth lane blank and use that timer as a backup. Each group of four mice with three consecutive trials takes 20 min per session, inclusive of cleaning time between trials and between mice. Additional time should be planned for labeling mice (30 sec per mouse), 60 min habituation time to the procedure room prior to testing, 10 min for pre-testing setup, and 10 min of post-testing cleanup and data export. In the optional dosing paradigm, a 20-min pretreatment period needs to be allotted for and timed accordingly.

***Acoustic startle response***

This protocol is ~28 min in duration, inclusive of the 5-min habituation period to background noise. Additional time should include labeling of mice (30 sec per mouse) and 60 min habituation time to the anteroom prior to testing. With an eight-chamber setup, pre-testing set-up time for calibration and ~2 min per chamber to clean between subjects is allocated. For a more efficient time allocation, the instrument calibration and empty chamber test run can be conducted during the 60-min habituation period.

***Optokinetic function***

A highly proficient technician requires 6–20 min per mouse for testing time. The variability in time is dependent upon the subject's rate of response. A maximum of 20 mice can be planned for each test day with time estimates as follows: 10 min set-up time; 20 min labeling time; 60 min undisturbed habituation time; 6–20 min test time per mouse; 15 min cleaning and data export after testing.

***Olfactory discrimination***

A 1-hr set-up time should be allotted on the day prior to the test day for individually housing 20 mice and labeling and placing blocks into the home cages. In the current laboratory, four camera setups are used to record four subjects simultaneously. For each session, a 30-min window of time should be considered for two 10-min recording sessions (trials 1 and 2) inclusive of the time to place the blocks between trials and clean between mice. Additional time should include labeling of mice and re-sealable bags (1 min per mouse) and 60 min habituation time of the subjects to the testing room prior to the test.

***Spontaneous alternation***

This protocol is 8 min in duration, with 2 min for cleaning time per maze, giving an estimate of 12 min per two mice when two mazes are used simultaneously. Additional time should include labeling of mice (30 sec per mouse), 60 min habituation time prior to testing, 15 min post-testing cleanup and data export. When facilitated by a sequence tracker algorithm that automatically timestamps entry and exit of each arm, a time estimate of 3 min per mouse is allotted for data QC and calculating sequences for each subject. Manual scoring of videos will require 8 min per subject and additional calculation time.

***Novel spatial recognition***

The time requirements for this task are dependent on the delay period between trials. Trial 1 is 10 min in duration and trial 2 is 5 min in duration. The delay period is

**Table 1** Novel Spatial Recognition Assay Template with 30-min Pretreatment Period and 30-min Delay Period

| Subject | Body weight | Treatment | Maze | Pretreatment (30 min pre) | Trial 1 (10 min) | Delay (30 min) | Trial 2 (5 min) |
|---|---|---|---|---|---|---|---|
| 1 | | A | 1 | 7:30 | 8:00–8:10 | 8:10–8:40 | 8:40–8:45 |
| 2 | | B | 2 | | | | |
| 3 | | B | 1 | 7:43 | 8:13–8:23 | 8:23–8:53 | 8:53–8:58 |
| 4 | | A | 2 | | | | |
| 5 | | B | 1 | 7:56 | 8:26–8:36 | 8:36–9:06 | 9:06–9:11 |
| 6 | | A | 2 | | | | |
| 7 | | A | 1 | 8:45 | 9:15–9:25 | 9:25–9:55 | 9:55–10:00 |
| 8 | | B | 2 | | | | |
| 9 | | A | 1 | 8:58 | 9:28–9:38 | 9:38–10:08 | 10:08–10:13 |
| 10 | | B | 2 | | | | |
| 11 | | A | 1 | 9:11 | 9:41–9:51 | 9:51–10:21 | 10:21–10:26 |
| 12 | | B | 2 | | | | |
| 13 | | B | 1 | 10:00 | 10:30–10:40 | 10:40–11:10 | 11:10–11:15 |
| 14 | | A | 2 | | | | |
| 15 | | B | 1 | 10:13 | 10:43–10:53 | 10:53–11:23 | 11:23–11:28 |
| 16 | | A | 2 | | | | |

initiated when the mouse returns to its home cage after the completion of trial 1. With a 30-min delay, three mice can be tested consecutively for trial 1 before the first mouse needs to be returned to the maze for trial 2. In this respect, with one maze, three mice can be tested in 45 min, or six mice can be tested in the same time frame when two independent mazes are set up adjacent to each other (Table 1). When a 10-min delay period or shorter is selected, the same mouse is run consecutively in the maze and the time requirement is 28 min per mouse (or per two mice when running two mazes simultaneously), inclusive of cleaning time between trials. Additional time should include pre-habituation tail labeling and weighing, if required for drug treatment studies, and 60 min habituation time to the testing room prior to the start of the test.

***Episodic memory***

The time requirements for this task are dependent on the delay period between trials. Trials 1 and 2 are each 5 min in duration, with brief 2–3 min cleaning time between trials, and trial 3 is 3 min in duration. With a 5-min delay period between trials 2 and 3, a time allocation of 22–23 min per mouse is required inclusive of cleaning time between trials. Two mice can be tested in the same time frame when two independent mazes are set up adjacent to each other (Table 2). Additional time should include pre-habituation tail labeling and weighing, if required for drug treatment studies, and 60 min habituation time to the testing room prior to the start of the test.

***Wheel running***

On set-up day, for an experiment with 40 mice, the time required is ~40 min to set up clean boxes, 20 min to place running wheels in boxes, 40 min to label each mouse tail and place mice in the boxes. On each full day of testing, 10 min is required to check all water bottles, verify adequate grain remains in boxes, look at each wheel to verify that it has not shifted position within the box, and verify that software is running correctly. On breakdown day, the time required is ~40 min to remove mice while verifying that subject number matches wheel number, 5 min to export data, and 60 min to break down boxes, wipe running wheel bases, and wash running wheel tops and platforms. This is a total of 3.5–4 hr over the course of the testing period.

***Adhesive removal***

The protocol is 3 min in duration (maximum time) preceded by a 10-min acclimation period. A single technician can only observe a single subject per 3 min observation period when scoring both latency to initiate removal

**Table 2** Episodic Memory Assay Template with a 30-min Pretreatment Period and 5-min Delay Period

| Subject | Body weight | Treatment | Maze | Pretreatment (30 min) | Trial 1 (5 min) | Interval (2 min)[a] | Trial 2 (5 min) | Delay (5 min) | Trial 3 (5 min) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | A | 1 | 10:20 | 10:50–10:55 | 10:55–10:57 | 10:57–11:02 | 11:02–11:07 | 11:07–11:10 |
| 2 | | B | 2 | | | | | | |
| 3 | | B | 1 | 10:44 | 11:14–11:19 | 11:19–11:21 | 11:21–11:26 | 11:26–11:31 | 11:31–11:34 |
| 4 | | A | 2 | | | | | | |
| 5 | | B | 1 | 11:10 | 11:40–11:45 | 11:45–11:47 | 11:47–11:52 | 11:52–11:57 | 11:57–12:00 |
| 6 | | A | 2 | | | | | | |
| 7 | | A | 1 | 11:34 | 12:04–12:09 | 12:04–12:11 | 12:11–12:16 | 12:16–12:21 | 12:21–12:24 |
| 8 | | B | 2 | | | | | | |
| 9 | | A | 1 | 12:00 | 12:30–12:35 | 12:35–12:37 | 12:37–12:42 | 12:42–12:47 | 12:47–12:50 |
| 10 | | B | 2 | | | | | | |
| 11 | | A | 1 | 12:24 | 12:54–12:59 | 12:59–1:01 | 1:01–1:06 | 1:06–1:11 | 1:11–1:14 |
| 12 | | B | 2 | | | | | | |
| 13 | | B | 1 | 12:50 | 1:20–1:25 | 1:25–1:27 | 1:27–1:32 | 1:32–1:37 | 1:37–1:40 |
| 14 | | A | 2 | | | | | | |
| 15 | | B | 1 | 1:14 | 1:44–1:49 | 1:49–1:51 | 1:51–1:56 | 1:56–2:01 | 2:01–2:04 |
| 16 | | A | 2 | | | | | | |

[a]During the interval, subjects are returned to holding cages and mazes are cleaned.

with one stop watch and cumulative latency to remove with the other stop watch. To maximize technician time, six mice with staggered acclimation and start times, 3 min apart, can be set up to be viewed one after another, provided six individual observation arenas are available. In this arrangement, six mice can be completed in 30 min. For a cohort of $n = 30$, the time requirement would be 2.5 hr, inclusive of cleaning of the arenas between subjects, plus additional time for habituation to the procedure room (60 min) and labeling the mice prior to habituation.

### *Statistical analyses*

To understand variability of behavioral responses with lifespan, it is important that no subjects be excluded based on mathematical determination (e.g., 2 standard deviations from the mean), but rather only based on technical errors (e.g., escaped mouse, instrument failure) or failure to meet the *a priori* inclusion criteria of the specific assay. *A priori* exclusion criteria for each assay are provided in the protocols, where applicable. In addition, since many behaviors are sexually dimorphic, it is recommended that sexes be analyzed separately.

If repeated observations are used, repeated measures should be included within the statistical analysis. In general, for all experiments, data are analyzed with one- or two-way ANOVA as appropriate. Appropriate post-hoc comparisons should be relative to young controls or, for longitudinal testing, within subject from baseline results. For drug treatment, appropriate statistical comparison is versus vehicle-treated control.

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