



National Toxicology Program

U.S. Department of Health and Human Services

**NATIONAL TOXICOLOGY PROGRAM (NTP)
NEUROBEHAVIORAL TESTING SPECIFICATIONS**

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NATIONAL TOXICOLOGY PROGRAM (NTP) NEUROBEHAVIORAL TESTING SPECIFICATIONS

A. GENERAL CONSIDERATIONS FOR ALL NEUROBEHAVIORAL STUDIES

1. Housing
 - a. Pregnant and post-partum dams shall be housed as specified by NTP study design.
 - b. At each cage-change occurring between time of birth to time of weaning, cage bedding will be enhanced with a sampling (approx. 1/4th cup) of the original litter bedding to provide home-cage olfactory cues to minimize stress associated with cage-change.
 - c. Pups shall be weaned, and litter size and sex ratio standardized as specified by NTP study design.
 - d. Animals (rats) shall be group housed (by sex and dose group) upon weaning to maintain critical social dynamics in the home cage. Group housing of mice shall be considered in the absence of aggressive behavior. Male and females animals shall be house similarly. Scientific justification for deviation from group housing shall be provided by NTP.
 - e. Home cages shall be maintained and include items such as nesting material. Supplemental enrichment items shall be used as specified by NTP.
 - f. Animal room environmental conditions shall adhere to relevant specifications of NTP.
2. Animal Identification
 - a. Dams shall be marked for individual identification (e.g., transponder implant, tail tattoo, tail marking). Method of identification shall take into consideration stage of the dam and requirement to minimize stress as well as length of time on study and the need to individually identify animals over that interval.
 - b. Pups shall be marked for individual identification (e.g., paw tattoo, tail marking) in a manner to not cause damage that can compromise behavior

(e.g., toe/paw damage that may compromise strength or motor dependent behaviors). If damaged (as confirmed by veterinary staff), the animal should be excluded from behavioral assessments as the damage could interfere with performance. Prior to weaning, pups shall be uniquely identified with a more permanent marking system (e.g., tail tattoo, transponder implant) for long-term identification.

- c. Individual pup identification shall allow tracking of original dam/sire/litter. The ability to associate the pre-weaning pup number with the post-natal animal number shall be ensured.
 - d. The testing laboratory shall have in place a method to minimize experimenter bias (observational or handling) for each assessment in the study. Such methods shall be approved by NTP.
3. Animal Selection
- a. Any animal displaying clinical signs that significantly compromise activity or strength shall be noted and approval shall be obtained from NTP for continuation of animal in neurobehavioral assessment.
 - b. Unless specifically noted for identified endpoints, only one animal/sex/litter from litters standardized at PND4 to 8-10 pups (as determined by study design) shall be randomly selected for specific behavioral testing. One animal can undergo more than one type of assessment according to a testing schedule ensuring no confounding across tests (e.g., any test involving aversive stimuli (e.g., shock) shall be conducted at the end of the testing sequence; test are spaced at adequate intervals (days) to minimize influence of repeated testing). Testing history shall be consistent across animals at the time of any specific assessment. (e.g., all animals assessed for any one endpoint will have had identical testing history in that all will have experienced the same test sequence).

4. Expert Capability of Experimenters

Documentation shall be provided to demonstrate that investigators have knowledge of each test method and technical skills to conduct the tests as defined (e.g., publication record, recorded successful completion of such studies, hands on experience methods within the test laboratory).

5. Confirmation of Appropriateness of Testing Equipment and Procedures

- a. Selection of testing equipment shall be such to meet the testing parameters and data collection as defined for the species under study. Documentation shall be provided (e.g., published data, studies conducted within the testing facility) demonstrating the ability of the equipment and

testing paradigm to provide valid endpoints of normal behavior in the species under study and age at which test is conducted. (e.g., in control non-dosed animals: motor activity devices shall be capable of detecting 2D ambulatory activity, thigmotaxis, rearing, ambulatory activity path, acclimation to the novel activity environment). Male/female difference in total activity and habituation shall be detected. Startle apparatus shall be able to detect a reflex response at 120dB, >45% habituation over the test session, and a gradient of pre-pulse startle inhibition (PPI) over the different pre-pulse intensities; the Morris water maze (MWM) task shall demonstrate an acquisition curve over the training period with a minimum of 40% decrease in latency, and the probe test shall identify a preference for platform quadrant in the first time interval. The MWM data acquisition software shall have features allowing for tracking of the swim pattern and swimming speed of the animal).

- b. Documentation shall be provided supporting that each test, as conducted by the testing facility (animal handling, environment, testing procedures and conditions) generates data reflecting a normal expected (as per published literature) pattern of behavior of non-dosed age- and species-specific animals.
 - c. Documentation of either performance of specific tests within the year prior to the start of study animals or data obtained for relevant endpoints in a “confirmatory” pilot study of naïve animals shall be provided as requested by NTP.
6. Handling
- a. Animals shall be acclimated to handling as specific for all behavioral evaluations. For adult assessments, animals shall be individually handled for several minutes (approximately 2 min) each of 2 days prior to test including lifting the rat by whole body and holding the animal in a manner consistent with handling for the upcoming behavioral test.
 - b. Consistency shall be maintained across animals within any specific test with regard to technicians’ handling of animal, placing of animal within a test chamber, removal of animal from test chamber, and returning animal to home cage. If, for any reason, the animal is overly stressed or escapes during transfer from home cage to holding cage or test apparatus, this shall be documented and a minimum of 15 min shall be allowed for the animal to return to a quiet state.

7. Olfactory Stimuli

- a. To minimize influence of spurious olfactory cues, the test environment shall be devoid of all specific odors including, but not limited to, odors from chamber cleaning solutions and from the experimenter such as perfumes, tobacco smoke, hand sanitizer, or food-related odors.
- b. Due to sex-specific or stress-related urine odors, test chambers shall be wiped clean between subjects using a mild fragrant-free detergent with a disinfectant (e.g., Nolvasan (chlorhexidine diacetate) followed by distilled water rinse). At the end of the test day test chambers shall be cleaned with an excess of a mild fragrant-free detergent with a disinfectant, rinsed with distilled water, and wiped dry.
- c. In any one day, the influence of sex-specific odors on the behavior shall be minimized. This can be accomplished by having dedicated testing chambers for either males or females or testing all adult males prior to females within a morning or afternoon session. At the shift between sexes, the motor activity chamber (if not replaceable) and the startle animal holder shall be cleaned using an excess volume of a mild fragrant-free detergent with a disinfectant (e.g., Nolvasan (chlorhexidine diacetate) allowing the solution to remain on the apparatus for >1min prior to rinsing with distilled water and wiping dry. The experimenter shall change gloves if the shift between sexes occurs $\leq 2x$ a day or wash with a mild fragrant-free detergent if the shift occurs more frequently. Any urine deposited on the protective body coverall shall be wiped off with a mild fragrance free detergent prior to handling the next animal.

8. Environment

- a. Ambient noise level shall be minimized in the testing facility and testing room shall be maintained with white noise unless rooms provide sufficient noise-isolation such that background-masking noise is not necessary. Any occurrence of a loud noise occurring during testing shall be recorded.
- b. Decibel level shall fall between 62-70 dB at the general site of the placement of the animal (i.e., inside activity chamber, inside learning and memory apparatus). Whole room white noise is not required if the testing apparatus contains an individual background noise generator (e.g., startle apparatus) and the room provides sufficient noise-isolation.
- c. Conversation between experimenters within testing rooms shall be kept at a minimum.
- d. Vibration of testing units shall be minimized and controlled as applicable.

- e. Tests of motor activity shall be conducted under dim lighting condition (approximately 20 lumen). If video capture methods are to be used the lighting shall remain dim but optimized to ensure adequate image detection of the animal.
 - f. Only the test cohort undergoing assessment shall be in the test room at any one time (e.g., animals not within the startle apparatus during a test session shall not be within the test room; animals not being tested for motor activity on that specific day shall not be within the test room).
 - g. Quiet shall be maintained when placing animals into a test apparatus and during test session.
 - h. The testing room shall be monitored for light level as appropriate to the test at the start of a daily test session.
 - i. Environmental conditions of the animal testing rooms shall be maintained and monitored as specified by NTP.
9. Time of Testing
- a. Lighting of animal housing rooms will adhere to specifications of NTP including a 12-hr light/dark cycle. All behavioral testing shall be conducted at an interval within 2 hrs of that cycle. Thus, testing will not commence until 2 hrs after lights are turned on and end at the latest by 2 hrs prior to lights-off time. The time of day of testing for each animal will be recorded. This time restriction does not apply to transport of animals to/from the testing room.
 - b. Any endpoints of repeated measures should be conducted at approximately the same time of day for each animal and counterbalanced across exposure.
 - c. The impact of a direct dosing of a compound on the behavioral performance shall be considered and the schedule of dosing to testing shall be coordinated to minimize confounding of acute effects of the compound. As possible, behavioral testing shall be conducted prior to any direct dosing (e.g., gavage, injection, dermal application) for that day. This adjustment is not necessary for continuous dosing via feed or drinking water.
 - d. Studies involving inhalation exposure require the same consideration for time of testing as direct dosing and shall be conducted as recommended by NTP study design.

- e. Studies designed to examine the acute effects of a compound shall follow a recommended time to peak effect approach relevant to the compound under study and the test conducted.

10. Acclimation to Transport Prior to Testing

Animals shall be transferred to individual transport cages with coded identifier numbers and moved from their home cage room and placed in a holding area in close proximity to the testing room. Acclimation to transport shall occur (2x over a one-week period) prior to the initiation of each different behavioral test.

11. Transport on Day of Testing

Animals shall be transported and held in holding area for a minimum of 30 min prior to initiating of testing. Transport of animals is not restricted by the 2 hrs lights-on/2 hrs lights-off time interval required for behavioral assessments. Access to water shall be maintained over the holding period. Access to food over the time period shall be optional. Maintaining dosing over the period outside of the home cage shall be determined by NTP.

12. Location of Pre-Test Holding for Animals

- a. Animals shall not be held within the testing room for such tests that include a stimulus (e.g., startle), as non-specific exposure would occur to animals outside of the test chamber.
- b. Animals shall not be held within the testing room for such test that the light level is a factor (i.e., open field activity).
- c. Animals can be held within the testing room for Morris Water Maze.

13. Retrieval of Animals after Test

- a. Animals shall be removed from the test apparatus and placed into the transport cage prior to returning back to home cage. The “tested” animals shall not be returned to a cage containing animals that have not yet been tested but rather shall be placed in a holding cage until all animals in the cage have been tested for the day.
- b. Animals shall remain in the test apparatus (activity chamber, startle apparatus) until the test session times-out for all animals to minimize disturbance and distraction.
- c. For the Morris Water Maze, upon removal from water tank, animals shall be gently wiped off and placed on an absorbent material in a dry holding

cage. Care shall be taken to not subject wet animals to air drafts and to minimize potential for hypothermia.

14. Randomization

- a. All testing shall be conducted according to a counterbalanced randomization of dose groups. For each sex, dose groups shall be randomized across testing apparatus, time of day, and testing unit.
- b. If it is necessary for multiple technicians to perform any one task, the assignment of technicians shall be counterbalanced across dose groups to ensure an equal distribution of dose groups across technicians. For observational endpoints this requires technicians with >80% inter-rater reliability as statistically determined from the ratings of 2 or more technicians on a specific test.
- c. If an animal is terminated prior to testing, a secondary animal shall be identified as replacement and noted as such. However, testing history shall be maintained unless this represents a significant compromise to the group size for power. Decision shall be made in consultation with NTP.

15. Body Weights

Body weights shall be collected on the day of testing after the completion of testing to prevent interference with behavioral performance. For test requiring multiple sequential sessions (e.g., Morris water maze) body weights shall be collected on the 1st day of testing and then at weekly intervals or as defined by NTP study design.

16. Order of Behavioral Assessments

Behavioral assessments shall be performed in a sequential manner that shall not interfere with performance on subsequent tests (e.g., any test involving aversive stimuli (e.g., shock) shall be conducted at the end of the testing sequence).

B. DATA AND STATISTICAL REQUIREMENTS

1. Data Formatting Requirements

- a. Data sheets shall include individual animal identifiers: litter (dam) number, sire number, pup number, dose group, sex, age, test date, apparatus identifier, experimenter identifier. Dose group shall be coded in a manner to maintain experimenter blinding.

- b. Consolidated data sheets for observational endpoints shall be provided in a format compatible with SAS statistical package.
 - c. For all computer-assisted tests, all individual raw data files of all endpoints collected (not only those analyzed) shall be provided in a format compatible with SAS statistical package.
 - d. Video-capture images and/or pathway tracking and associated software files shall be provided in a format accessible by NTP.
 - e. Detailed physical descriptions and software algorithms shall be provided to describe how each measure is captured. This shall serve as a method to clearly describe variables.
2. Statistical Analysis Requirements
- a. For each endpoint, all raw data shall be provided.
 - b. For each endpoint, individual animal data, as well as summary descriptive statistics (mean, standard deviation, median, inter-quartile range, sample size), shall be provided in tabular and graphic format. Summary statistics for endpoints shall be stratified by relevant factors such as dose, time, sex, and session day, as applicable.
 - c. All analyses shall include a summary sheet providing a description of statistical methods used, sample sizes used for analysis, details of the analyses including, but not limited to, test statistic(s) used, statistical significance, and coefficients of variation.
 - d. Homogeneity of variance in dose groups shall be assessed to determine the method of analysis. If the assumptions of homogeneous and normally distributed model errors are not met, data transformations (e.g., log transformation for motor activity data) shall be applied and confirmed through analysis of model residuals. Non-parametric procedures shall be considered when transformations fail to ensure model error assumptions are met. Body weights shall be assumed as normally distributed.
 - e. Sensitivity analysis of modeling results to outliers and unusual observations shall be conducted by comparing modeling results with and without inclusion of these observations.
 - f. Endpoints shall be evaluated by appropriate statistical methods for two factors (t-tests or non-parametric alternatives) or multiple factors (analysis of variance (ANOVA) or non-parametric alternatives), with factors to include dose, time, sex, and session day, as applicable, as well as appropriate interactions. Where multiple measurements of the same

endpoint per animal are available, repeated measures ANOVA shall be used. Data transformations (e.g., motor activity: log transformation, startle response: square root transformation) shall be applied if required to meet model error assumptions. Data will be analyzed for each age and across ages for repeated assessment of animals at different ages.

- g. Litter shall be treated as a random variable in the design and statistical analysis in any case where more than one animal per litter is evaluated.
- h. Post-hoc multiple comparison procedures (e.g., Bonferroni, Dunnett) shall be applied and, where appropriate, confidence intervals shall be reported for significant differences in endpoints.

C. OBSERVATIONAL ASSESSMENTS

1. Maternal/Pup Interactions

- a. Maternal/pup interaction observational assessments will adhere to NTP study design for developmental studies and shall include but not be limited to:
 - 1) During the first 10 days post-partum, at the time of recording body weight and return of pups to the dam, the response of the dam to gather the pups to a nesting litter shall be recorded as absent (1) or present (2) at the end of an approx. 15-min period.
 - 2) During daily cage-side observations, abnormal behavior of the dam (i.e., aggression, hyperactivity, lack of nest building, lack of maintaining pups within the litter during the first 2 post-natal weeks) shall be identified and recorded for each as (1) absent or (2) present.
 - 3) Within the first postpartum week, pup shall be observed daily and the presence (1) or absence (2) of a milk band in the stomach shall be recorded.

2. Pups

a. Signs of General Health and Potential Pain/Distress

Pups shall be monitored for signs of general health effects including, but not limited to: physical features of skin/coat (color (e.g., pale), shriveled/smooth skin), stunting, body weight gain, body weight loss over two consecutive measurements (min 3-day interval), tremor (non-age related), hunched back, unkempt fur, lack of movement, body cold to touch, discharge around eye or nose. Indication of such signs shall be

recorded and reported to veterinary staff for consideration of pain/distress. NTP shall be notified of such conditions as they may influence study outcome.

b. Motor Ontogeny: (Pre-weaning: Recommended observations on 2 randomly assigned pups/sex/litter).

- 1) Righting reflex (scored as absent (1) or present (2) within 30 sec. On PND 4, pups shall be monitored for the ability to rotate to a prone position from a supine position. A pup is placed supine on a flat surface and the time it takes the animal to rotate 180° to a prone position with all four paws downward is recorded. A maximum time of 30 sec is allowed. If dosed animals show a deficit, the assessment shall be repeated at PND 8.
- 2) Straight line walking (scored as absent (1) or present (2)) - pup has developed past pivoting motion and is able to place one paw in front of the other for forward ambulation for 2 consecutive steps) – [Shift normally starts to occur PND 8 and prominent by PND 12] shall be measured up to day of presence and recorded as days until acquisition of a skill. Observational unit shall be until behavior occurs or maximum of 60 sec and can be conducted concurrently at time of body weight measurement. Statistical analysis shall involve a Cox proportional hazards model where days to acquisition is a function of dose and a random effect included for litter.
- 3) Gait – shall be scored as crawl on belly (1) or moving with abdomen off of the floor (2). Gait shall be scored after onset of straight line walking. Recommended at PND 17 (can be done concurrent with motor activity assessment) and to be included in scheduled out-of-home cage clinical observations until weaning.

c. Cage-side Observations in Juvenile Pups

During normal daily cage-side observations, behaviors of grouped juvenile animals between weaning and PND 35 shall be recorded for the entire litter as a whole. Activity occurring in 2 or more pups at time of observation shall be recorded as present.

1) Social Behaviors

- a) Normal interactive play behavior (tumble, pin) scored as present (1) or absent (2)
- b) Aggressive behavior (biting, pinning with associated verbalizations) scored as absent (1) or present (2)

- 2) Motor behaviors (grooming, rearing), each scored as present (1) or absent (2)

D. LOCOMOTOR ACTIVITY

Locomotor activity shall be evaluated using a computer assisted automated photocell apparatus or video tracking system designed to evaluate motor activity in a 2-dimensional (2-D) manner, allowing for detection of thigmotaxis and activity pathway tracking.

1. Configuration

The configuration of the system shall allow for data collection of activity within the entire chamber, immediately along the perimeter at the chamber wall, and within a defined smaller center arena. (See appendix for representative defined areas of a 40cm x 40cm x 20cm chamber: one photocell margin around the perimeter and a center arena of approximately 9cm square).

For photocell devices, the height placement of the photocell banks shall be appropriate for the age and species of animal under study. For rearing behavior, the detection limit (photocell height, video recording height) will be at a height equivalent to approximately $\frac{3}{4}$ full rearing height of animal to ensure accurate detection of rearing.

Parameters of the testing apparatus (e.g., size, shape, height location of the photocells, number of photocells in any specific plane, region definitions) shall be documented in the study file.

2. Testing Room Conditions

Tests of motor activity shall be conducted under dim lighting condition (approximately luminance of 20 lux as measured by Lux meter [equivalent to approximate room candle-lighting]). If video capture methods are to be used, the lighting shall remain dim but optimized to ensure adequate image detection of the animal.

3. Standardization and Calibration of the Apparatus

At the beginning of each test session, each apparatus shall be calibrated and test validated for photocell alignment and function as instructed in manufacturer's manual. In addition, each unit shall be tested to ensure accurate tracking by moving either the experimenter's hand or a controlled moving item (e.g., plastic ball) within the chamber and following the tracking pathway on the computer screen. The instrument shall provide a software diagnostic feature to be run prior to each session.

4. Decontamination of Chamber

After each animal, the test chamber shall be changed or the chamber shall be wiped-down with a fragrance-free mild detergent and a disinfectant (e.g., Nolvasan (chlorhexidine diacetate)) and rinsed with water. This shall be followed by a photocell alignment diagnostic check or a video camera placement check as the cleaning may shift the location of the test chamber.

5. Test-Time Collections and Duration

- a. Each apparatus shall be programmed for the software to automatically start the test session with the detection of first movement within the chamber. If this is not possible, then the computer test session shall be started manually by the experimenter upon entry of animal into the chamber and clearance of the experimenter's hand.
- b. All animals shall be placed into the chambers with minimal delay and with minimal excess movement or noise.
- c. Standard Test: Measurements shall be collected in 5 min epochs for a total of 30 min (pre-weanling/weanling < 35 days of age) or 60 min (pre-adult/adult).

Option: If the animals display habituation to the novel environment and full lack of movement (sleeping) at 60 min (pre-adult/adult) across 2 representatives of all treatment groups, consideration shall be made to shorten the session time to 45 min for remaining animals in the study. Any decision to alter test interval shall be made with approval from NTP.

6. Assignment

Each activity apparatus shall be uniquely and clearly identified in a manner that cannot be seen by the animal. Schedules shall be made in advance, noting which animals are to be tested in which apparatus and the computer system programmed accordingly. Where possible, an equal number of animals from each dose group shall be tested in each apparatus. The order of testing and assignment to apparatus shall be initially selected by randomization for counterbalancing to ensure that each session is representative of all dose groups. If the same animal is to be tested multiple times for activity, it shall be placed within the same apparatus for each test session.

7. Handling

Individual animals shall be placed in the center of the activity apparatus by holding the animal by the whole body for the rat and, for the mouse, the animal can be placed into the apparatus by lowering the animal into the chamber by holding the tail.

8. Endpoints

- a. Total activity (sum of all beam breaks), ambulatory activity (sum of consecutive beam breaks), resting time, ambulatory time (seconds spent breaking consecutive beams), distance traveled and activity time (seconds spent breaking beams) within the entire arena and within each of the defined arena regions allowing for calculation of thigmotaxis (preference along the margins of the arena) or arena area preference, and pathway tracking shall be recorded. Vertical beam breaks (i.e., rearing) shall be recorded for the arena. Detailed physical descriptions and software algorithms shall be provided to describe how each measure is taken as a method to clearly describe variables.
- b. Measurements of rearing within the full arena shall be recorded in addition to the 2-D configuration activity measurements.

9. Data Calculations

- a. Endpoints shall be evaluated as a total activity level over the entire test session as well as for each epoch (individual 5-min time periods of measurement) of the session.
- b. Habituation for each endpoint shall be calculated as the ratio of the endpoint measured at the final epoch to the endpoint measured at the first epoch.
- c. Data transformations, such as logarithms, shall be considered for analysis of endpoints over epochs to ensure model assumptions are met.

E. STARTLE REACTIVITY AND PRE-PULSE STARTLE INHIBITION

Animals shall undergo testing for pre-pulse startle inhibition using a computer assisted automated startle/PPI system. This system shall allow for the ability to view the continuous individual waveform responses and to post-hoc examine the data on the basis of shifting the msec time interval for recording a response.

1. Testing Units and Calibration

- a. All testing units shall be housed in individual sound-attenuated chambers with a continuous background noise level of 65 dB generated within each chamber. Background noise level shall be consistent across all chambers.
- b. Prior to the first test session at each age, each chamber shall be calibrated per equipment manufacturer's instructions for: 1) decibel level (calibrated at position normally occupied by animal within the closed experimental chamber) and, 2) sensitivity of the transducer platform (e.g., oscillation calibration device) at gain appropriately set for each age or species tested. All boxes shall be uniformly calibrated across the study.

2. Adaptation to Handling and Holding Enclosure

The startle apparatus requires that the animal be restricted for movement during the test session. The holding enclosure represents a novel environment for the animal that may require unique handling to place the animal into the holder to minimize stress and acclimate the animal to the holder to minimize influence on the animal's response during testing. The animal's age and weight will determine the appropriate size of the enclosure to restrict mobility as recommended by the startle apparatus manufacturer. As the novel environment can alter an initial startle response, acclimating the animals to holding and to the enclosure prior to any test session becomes of importance. Acclimation to handling of the animals shall be consistent with handling that is required to place the animal within the enclosure. For a box holder, placement of the rat by holding the body is recommended. For placement of a mouse, the animal can be lowered into the box by holding the tail. For a tube type holder, adaptation efforts are more complex. A recommended handling sequence and acclimation period involves conditioning the animal to being held in a manner for calmly placing the animal within the tube enclosure and may require additional handling sessions to accomplish (see Appendix; Geyer and Swerdlow, 2001).

3. Confirmation of Optimal Startle dB and PPI Intensities

Prior to examination of animals on study, confirmation of optimal startle intensities in naïve animals (5/sex) for specific test ages, species, and strain shall be conducted.

- a. An input/output function test shall be conducted. After a 5 min acclimation period under constant background white noise of 65 dB, startle stimuli (20 msec) shall be delivered on an inter-trial interval of 20 sec. Startle stimuli shall start at approximately 75 dB and increase by 5 dB until reaching 120-125 dB. Startle magnitudes shall be sampled each msec for 200 msec.

beginning at the onset of the startle stimulus. These data shall be used to determine the maximum startle response (largest response within 200 msec) and to average the response over the entire response window.

- b. PPI stimulus intensities shall be identified to elicit intermediate levels of PPI to allow for treatment-induced increases or decreases in PPI to be observed. Maximum startle response to each of the individual PPI intensities shall be determined. The required number and interval for PPI intensities shall be determined for the species, strain, and age of animal.
 - c. Once established, these settings shall be confirmed on an annual basis prior to initiating testing on study animals if the test has not been conducted on same species within a year period of time.
4. Establishment of Testing Schedule

A testing schedule shall be set up such that approximately equal numbers of each sex and treatment are tested in each startle box. If animals are pair-housed and both are to be tested, both animals in a cage shall be tested at the same time when possible. If concurrent testing is not possible, the first tested animal shall not be returned to a cage containing an animal not yet tested but rather maintained in a separate holding cage until both animals have been tested.

- a. The test session shall include the following parameters (See Appendix for startle protocol):
 - 1) Each session shall start with a 5-min period of acclimation to the holder and test chamber with continuous background noise (65 dB).
 - 2) Delivery of startle trials shall be under a fast rise-time (<2 msec) burst of noise presented for a 40-msec duration at an intensity of 120 dB.
 - 3) Startle amplitude shall be collected over a 100-msec sampling-window measured from startle stimulus onset.
 - 4) Intertrial interval (ITI) shall be set to an average of 15 sec (variable ITI 15 sec with a range 7-23 sec [7, 9, 11, 13, 15, 17, 19, 21, and 23] and stimuli shall be presented in pseudo-randomized block fashion.
 - 5) Pre-pulse stimuli [3, 6, 12, 15 dB above threshold, unless empirically changed based upon results of the naïve animal pilot (validation) study] shall be presented for 20 msec duration with inter-stimulus interval of 65 msec (mice), 80 msec (rat) gap prior to the onset of the 120 dB startle stimulus.

- 6) No-stimulus (NOSTIM) trials shall be recorded as a measure of activity of animal within the chamber.
 - 7) The testing schedule shall represent “blocks” as designated for statistical analysis. Excluding the initial 120dB pulse-only trial, each block shall be representative of 5 120dB pulse-only trials (See Appendix for startle protocol). Blocks 1, 4, 5, 6 shall contain only 120dB trials. Blocks 2 and 3 shall contain the addition of pre-pulse startle intensities and no-stimulus trials.
5. Animal Identifiers
 - a. Animal identifiers shall include dam (litter) number, animal number, sex, age, dose group, test chamber, order of test within day.
 6. Endpoints
 - a. Endpoints shall include
 - Peak response magnitude (V_{max}) for each trial
 - Time to maximum response (T_{max}) for each trial
 - b. Calculated endpoints
 - Response magnitude (V_{max}) on the first trial
 - For each “block” calculate mean T_{max} of the 120 dB pulse-only trials
 - For each “block” calculate mean V_{max} of the 120 dB pulse-only trials.
 - Habituation – 1) % change in mean V_{max} of block 6 compared to V_{max} of block 1 and 2) % change in mean V_{max} of 1st 120dB trial and 6th 120 dB trial.
 - For blocks 2 and 3 combined calculate the mean V_{max} of 120 dB responses to use for calculating PPI.
 - PPI for each intensity based upon the mean 120dB response of blocks 2 and 3
 - Mean response magnitude for each of the pre-pulse variations, overall and grouped by block.
 - An additional set of calculations will be conducted using a trimmed mean to reduce the effects of outliers on the average. A trimmed mean is an averaging method that eliminates a partial percentage of the greatest and smallest values before evaluating the standard mean of the giving data. This trimmed mean (approx. 20%) shall be used in for habituation and determining V_{max} 120dB means to calculate PPI.
 - c. Additional endpoints are options as available by the apparatus and software capabilities.

7. Special Considerations for Statistical Analyses

a. Startle Reactivity

- 1) Waveform data files generated from the startle computer software program shall be provided.
- 2) The response magnitude of the first trial shall be analyzed by ANOVA, with applicable factors including dose and sex and appropriate interactions.
- 3) The mean response 120 dB magnitudes for block 1 shall be analyzed by ANOVA, with applicable factors including dose and sex and appropriate interactions.

b. Habituation

- 1) For each animal, percent habituation shall be calculated (mean Vmax of 120 dB responses in block 1 minus mean Vmax of 120 dB responses in block 6 divided by mean Vmax of 120 dB responses in block 1. ANOVA models shall be fit to percent habituation as a function of applicable factors including dose and sex as well as appropriate interactions.
- 2) Repeated measures ANOVA models shall be fit for the mean response 120 dB magnitudes for blocks 1, 2, 3, 4, 5, and 6 with applicable factors including dose and sex as well as appropriate interactions.

c. Pre-pulse Startle Inhibition (PPI)

- 1) For each animal, the mean response amplitude (Vmax) of 120 dB pulse-only trials occurring in blocks 2 and 3 shall be used as the "120 dB Vmax" for each animal in calculating percent prepulse inhibition (% PPI).
- 2) For each animal, the Vmax for each response to a 120 dB stimulus preceded by a prepulse stimulus intensity shall be recorded. This shall be identified as the "prepulse Vmax" for a given prepulse trial.
- 3) For each animal and each prepulse trial, the % PPI shall be calculated as $[(120 \text{ dB Vmax} - \text{prepulse Vmax})/120 \text{ dB Vmax}] \times 100$. For each animal and each prepulse stimulus intensity, the average % PPI is then calculated across all trials.

- 4) Subject to confirmation of variance homogeneity assumptions outlined in Section A.14.c, ANOVA models shall be fit to calculated %PPI, with applicable factors including dose and sex and appropriate interactions.

F. MORRIS WATER MAZE

Animals will undergo a sequence of training tests to evaluate performance in a Morris Water Maze. On Days 1 and 2, animals shall be familiarized to the tank, water, and swimming requirements of the test and assessed for non-spatial cued learning. This shall be followed by acquisition of a spatial hidden platform task (3 training trials per day for 7 consecutive days. Twenty-four hrs later, spatial reference memory shall be assessed in a probe trial. Forty-eight hrs later, performance on a spatial reversal acquisition task shall be assessed (3 trials/day for 3 days).

1. Standardization of Testing Environment

a. Tank and Platform

- 1) Performance is dependent upon maze configuration. Standard circular tank interior diameter shall be approximately 180 cm [6 ft] (adult rats) or 130-150 cm [4-5 ft] (immature rats and mice) with non-reflective interior surfaces. A large tank can be modified to the smaller size by the insertion of a circular ring to decrease diameter.
- 2) When filled with water, the depth shall be sufficient such that the animal cannot touch the bottom (e.g., approximately 28-35 cm for 70 day old rat; 15-20 cm for mice) and with a surface to tank lip distance of approximately 10-12 cm to prevent the animal from jumping out of the tank but allowing for line of sight to visual cues.
- 3) The tank shall have no prominent interior features that might provide proximal cues, such as markings inside the tank (e.g., welded seams shall be smoothed and painted so as to blend with the surrounding walls). The sides of the tank shall be smooth to minimize attempts of the animals to climb walls and should be a non-reflective matte finish.
- 4) The maze shall be designated into 4 equal quadrants (randomly identified as North, West, East, and South orientations; see Appendix).
- 5) The tank shall have the capability for using a hidden platform and a visible platform
- 6) The goal platform shall be approximately 10 cm in diameter and covered by a non-reflective textured material to allow for gripping by the animal. It shall be submerged below the surface of the water

(approximately 1 cm for rats; approximately 0.5 cm for mice) and stabilized to prevent shifting.

- 7) The platform shall be positioned in the respective quadrant (Cued learning – NW; hidden platform – NE; reversal learning – SW) located approximately 36 cm (rat) or 30 cm (mice) from the interior wall of the tank (to minimize finding of the platform simply by swimming along the tank wall; yet a greater distance from center) and immobilized within the tank to prevent any shift over the day's testing. The platform shall remain within that quadrant for all animals during cued and hidden platform acquisition trials. During reversal learning trials the platform shall be placed in the SW quadrant.
- 8) The submerged platform should be "hidden" from view of the animal during hidden platform trials. For albino (or light coated) rodents, the tank shall be dark and the platform shall be dark or clear to prevent visualization from the surface of the water. For pigmented rodents, a light-colored tank with a similar colored or clear platform shall be used. If necessary, for animals with a dark coat, a white non-toxic water coloring (e.g., Crayola watercolor paint; tempera paint) can be used with a clear platform.
- 9) Water temperature shall be equilibrated to ambient room temperature (approximately 24-27°C). During training and testing, water temperature shall be recorded daily at the start and end of a test day to verify temperature within this range.
- 10) For tank configuration, all specific details of physical properties shall be documented and provided in study report.
- 11) Location markers shall be placed on the exterior of the tank that will line-up with markers on the floor to ensure identical location placement of the tank when returned from cleaning.

b. Room and External Spatial Cues

Visual cues within the testing room (enclosure) are a major defining factor in the ability of an animal to learn the location of the platform via spatial processing.

- 1) One specific cue room pattern shall be used across all animals and all studies and shall be maintained over the entire course of the study.
- 2) The room and walls shall be devoid of extraneous visual cues as much as possible and any items remaining shall be considered as part of the cue profile.

- 3) With a full-room configuration, a mark on the floor shall be provided to indicate exact location for experimenter to stand after placing animal into the water maze. The experimenter shall remain stationary in this constant location during the trial as they serve as a distal cue. If a curtain is placed around the tank, it shall be configured such that the experimenter can exit after placing the animal in the tank without disrupting the location of cues.
- 4) Walls shall be light in color. See Appendix for an example of test area dimensions and cues. Alternatively, curtains can be placed as walls around the tank to define a test area. The curtains shall be smooth and securely hung in a manner to minimize from testing room airflow. In this case, the curtains shall be at a sufficient distance from the tank to allow for experimenter mobility without movement of the curtain and disruption of the spatial cues. A permanent opening shall be maintained for the experimenter to enter and exit and to stand during the trial as to minimize any movement of the curtain and disruption of the spatial cues.
- 5) Easily visible spatial cues shall be located on the walls of the testing room (or curtains) such that they can be seen from the water level (see example in Appendix). Cues shall be placed between approximately 18-50 inches from the wall of the tank and shall be of sufficient size to discriminate as cues from wall.
- 6) All tanks shall have an identical cue profile. If multiple testing areas are to be used, the environment shall be as identical as possible with regard to defined cues and architectural features.
- 7) The rack of test animals within the test room is an odor and auditory cue. It can serve as a visual spatial cue as well and thus, localization shall be standardized across test sessions and test rooms as dictated by room configuration. Location marker shall be employed as needed to ensure uniform rack placement across sessions.
- 8) The final report shall include all dimensional details of the room, spatial defined area, and spatial cues in schematic format.
- 9) For non-spatial cued learning no spatial cues shall be visible. The experimenter shall remain at the start position during the trial to minimize movement or exit to the outside of a curtained wall depending on room configuration.

c. Room Lighting

Indirect diffuse lighting shall be used.

- 1) Lighting shall be bright enough to allow for visualization of spatial cues.
- 2) Lighting shall be bright enough to allow for the video camera to track the animal.
- 3) Lighting shall be arranged such to prevent reflection on the water or in the video image that will compromise video tracking as the software may confuse those reflections with the animal.
- 4) Lighting shall be arranged to prevent a shadow being cast into the tank interior from the experimenter standing by the tank.
- 5) Lighting shall be even such as obtained with a diffuse light source like a shaded fluorescent tube or globe-type incandescent bulb. Spotlights or uneven lighting shall not be used.
- 6) Lighting shall be indirect and not be in direct line-of-sight of the camera. [One way this can be accomplished by placing 4-6 globe bulbs around the pool below the level of the water surface outside of the line of sight of the camera lens.]
- 7) Details of lighting conditions shall be documented for each test cohort.

d. Camera Settings

- 1) The camera shall be positioned at a straight angle (perpendicular) to the plane in which the animal moves. This will be in a fixed position and anchored above the tank. If this requires a stationary arm attached to the tank, the arm will be considered one of the visual spatial cues and thus, will be maintained in one position. Any wiring for the camera shall also be considered as a spatial cue and be treated as such and included in the configuration schematic.
- 2) The camera zoom setting shall be adjusted and the lens focused to display the entire experimental arena in focus on the computer screen.
- 3) All camera automatic settings shall be disengaged.
- 4) The camera aperture shall be adjusted for maximal contrast of the image.

- 5) The entire setup shall be anchored during testing. The position of the arena, camera, and illumination shall be secured, relative to each other. If moved for tank cleaning, the system shall be recalibrated to original position prior to testing.
- 6) The camera zoom, focus control, and aperture setting shall be locked.
- 7) Confirmation of the settings and quality of detection shall be conducted each day prior to test of animals.
- 8) The camera shall have a polarizing lens filter to minimize reflection.
- 9) The visual field and lack of interference with capturing animal within that field (e.g., reflection) shall be confirmed each day prior to the start of the test session.
- 10) All details of the camera setting shall be confirmed at the start of each test day.

2. Animal Handling

a. Transport and Animal Handling

Within 2-4 days prior to testing, animals shall be transported to the test room in the transport cages and placed at a distance from the tank to minimize contribution to visual cues.

The animal shall be removed from the transport cage and handled in a manner to provide support (e.g., placed in crook of the arm; placed against body) for 90 secs to adapt the animal to the handling procedure. The animal shall be returned to the transport cage.

The procedure shall be conducted twice (1x per day)

b. Placement and Removal of Animal from Tank

- 1) The animal shall be removed from the transport cage and handled in a manner to provide support (e.g., placed in crook of the arm; placed against body). With the animal in the palm of the hand, the animal will be placed into the tank by gently lowering the hand into the water.
- 2) The animals shall be placed in the tank with nose facing the wall.
- 3) If the animal fails to find the platform within the maximum trial time, the experimenter will gently guide the animal toward the platform by placing the hand behind the animal and allowing the animal to maintain

a swimming action to reach the platform. The animal shall be allowed to escape the water by climbing onto the platform. This procedure will ensure that all animals learn to associate swimming and fully climbing onto the platform as the method of escape from the water as well as visual cues for spatial orientation to the platform.

- 4) Removal of animals from the platform shall be conducted by a whole body method and not by use of the tail (unless necessary). The animal shall be first allowed to climb into the experimenter's hand prior to picking up the animal from the platform. Upon removal, animals shall be held in absorbent towel and placed in a holding cage with an absorbent towel on the floor until animal coat is no longer wet at which time it can be transferred to the transport cage. The towel shall be changed between animals. Effort shall be taken to place the holding cage in an area without air drafts to minimize discomfort of the animal. A "tested" animal shall not be placed into a cage with animals waiting for testing.
- 5) If the animal cannot swim and sinks (not dives underwater), the animal shall be immediately removed from the tank and placed in the holding cage. For safety reasons, it is recommended that such retrieval be done with the use of a container rather than by hand. This behavior shall be documented. If this behavior occurs more than 2 times the animal shall be considered for removal from the study.

3. Start Location in the Tank

- a. A marking visible to the experimenter shall be placed on the outside of the tank to indicate starting location.
- b. Geographical nomenclature (N, NE, E, SE, S, SW, W, NW) is used to identify start locations (relative to cued/training goal/platform NE location) in the start location pattern provided. An alternative nomenclature can be used but must be consistent with the orientation. The following start-quadrant randomization shall be followed for the acquisition phase and the reversal-learning phase. Start positions using distal locations for which the goal (platform) is located in the NE quadrant during acquisition and in the SW quadrant during reversal. The sequences of starts are designed to balance the right/left goal location.

Morris water maze spatial start positions

Start locations for acquisition with NE quadrant as hidden platform location

Day	Trial 1	Trial 2	Trial 3
1	S	W	NW
2	NW	S	SE
3	SE	NW	W
4	W	S	SE

Repeat sequence as needed

Start locations for reversal learning with SW quadrant as hidden platform location

Day	Trial 1	Trial 2	Trial 3
1	N	E	SE
2	SE	N	NW
3	NW	SE	E
4	E	NW	N

Repeat sequence as needed

4. Cleaning of Tank
 - a. After each animal, feces shall be removed and water dispersed to minimize urine scent near platform.
 - b. The water shall be still prior to testing of any animal to minimize reflection that can interfere with video capture.
 - c. A clean tank shall be provided at the beginning of the animal's training sequence. At a minimum, the tank shall be drained and rinsed clean at the end of each 5-day period, refilled and allowed to equilibrate to temperature. Changing of the tank water can be conducted more often than each 5 day period but shall not occur at longer intervals. Partial changing of the tank water can be conducted within the 5-day period. The schedule of cleaning shall be constant across all test cohorts.
 - d. The tank shall be placed in original position and camera setting recalibrated to original settings.
5. Testing Schedule
 - a. For each day of testing, a test schedule shall be provided indicating the order that animals are to be tested ensuring a counterbalance across dose

groups. For any individual animal, this order shall be maintained across all test sessions.

- b. While animal performance will improve with training, the intertrial interval (ITI) between runs shall remain constant to maintain a uniform time for integration and learning.
6. Morris Water Maze (MWM) Assessment
- a. Familiarization to Tank, Swimming, and Cued Learning

On Days 1 and 2, animals shall be familiarized to the tank, water, and swimming requirements of the test and tested for non-spatial cued learning over 3 trials per day.

- 1) The task shall be performed under conditions to obscure visibility of room spatial cues (e.g., decreased room lighting, curtain around tank with no cues attached).
- 2) The platform shall be placed in the NW quadrant of the tank at a height approximately 1.5 cm above the surface of the water. A projection (6.4 cm ball or similar size flag) shall be attached to the platform and rise approximately 7 cm above the platform.
- 3) On day 1, the animal shall be placed on the platform for 30 sec prior to the initiation of the first trial.
- 4) For each trial, the animal shall be placed into the periphery of the tank, with nose facing the wall of the tank. The animal shall be allowed 90 sec to find the visible platform and to climb out of the water and have all 4 paws on the platform.
- 5) If the animal does not find the platform within the maximum time interval, the investigator will gently guide the animal to the platform by placing the palm of the hand behind the animal with slight pressure to guide the animal in the water directly toward the platform. This will allow the animal to maintain the normal spatial orientation during swimming to identify spatial cues. The animal shall not be removed from the water and placed on the platform, rather the animals shall be required to climb out of the water onto the platform to facilitate learning of the “escape” response. Each incident of “guidance” shall be recorded.
- 6) The animal shall be allowed to stay on the platform for ~20 sec prior to being removed. This process is to ensure that the animal learns that escape from the water will only occur from the platform. If the animal

jumps back into the water again, the experimenter shall gently guide the animal to the platform allowing the animal to stay on the platform for ~10 sec prior to removal.

- 7) The animal shall be removed from the tank to a holding cage within the test room. For rats, trials 2 and 3 shall be run in sequence for each individual rat after a defined intertrial interval (ITI). Mice are more prone to hypothermia-induced performance effects; therefore, each mouse shall be removed from the tank to a holding cage until all animals of the test cohort have completed each trial and then run in the subsequent trial.
 - 8) A relatively constant ITI will be maintained across the study for each animal in repeating the trial rotation for training. While this will be empirically determined by the study design, once identified, it shall be standardized across studies. The approximate ITI for each cohort shall be recorded and reported. The ITI length allows for integration of the learned event and minimizes the fatigue factor thus, decreasing variability in latency across trials. This sequence shall be repeated until testing of all scheduled animals of any one sex has been completed within a day.
 - 9) The start location sequence (SW, NE, SE) shall be followed in 3 sequential trials.
 - 10) If an animal “floats or “circles” in early sessions it may perform in later sessions and thus shall not be excluded. If an animal fails to swim (i.e., sink but not dive) across 3 trials this shall be noted. If this occurs on the 2nd day the animal shall be considered for removal from the study. Replacement of an animal at this point will result in lack of test history but shall be considered in discussions with NTP as an option to maintain sufficient n size.
 - 11) All animals shall reach criterion for performance in reaching the visible platform prior to the 90 sec cut-off before progressing to the hidden platform test. If animals do not reach criteria within the number of sessions designated a decision shall be made with regards to additional training sessions.
 - 12) Latency to find platform shall be recorded for each start location.
- b. Hidden Platform Test (Spatial Acquisition)
- 1) The platform shall be placed in the NE quadrant of the tank.

- 2) Each animal shall be placed in the maze facing the wall of the tank at a designated location (Table 1; Section G.3.b).
- 3) Immediately upon release of animal into the water, the timer will be started to record latency to find platform.
- 4) The animal shall be allowed 90 sec to locate the hidden platform after which it shall remain on the platform for approximately 20 sec before being removed. This approximate 20 sec interval shall be maintained during all sessions of the first day and in the first session of each subsequent day.
- 5) If the animal does not locate the platform within the 90 sec time interval, it shall be gently guided by hand through the water to the platform, allowed to climb onto the platform and remain on the platform for approximately 20 sec. Each such incident of experimenter guiding the animal shall be recorded as such on the data sheet.
- 6) A successful escape shall be recorded when the rodent places all 4 paws on the platform.
- 7) Latency to find the platform will be recorded to the nearest 0.1 sec.
- 8) The approximate ITI (interval of time between trials) for each cohort shall be maintained across trials.
- 9) A trial shall end when the animal escapes to the platform or reaches the 90 sec cut-off, followed by a 20 sec interval on the platform. At the end of trial, the animal shall be removed from the platform (see section F.2.b.4.).
- 10) For a mouse, at the end of each trial, the animal shall be removed to a holding cage (see section F.2.b.4) until all animals of the test cohort have completed the trial. After a defined ITI, the subsequent trial will be initiated and repeated for a total of 3 trials within a day.
- 11) For a rat, the sequence of training trials can follow the pattern used for mice. Alternatively, all 3 trials can run consecutively with an ITI as defined in the cued platform trials. At the end of trial 3 the rat shall be removed to a holding cage (see section F.2.b.4). The pattern will be maintained across all trials within a study.
- 12) A "tested" animal shall not be placed into a holding cage with animals waiting for testing.

13) Each animal shall receive 3 training trials per day for 7 consecutive days or until 80% of control animals reach a criteria of $\geq 50\%$ decrease in either latency to platform or swimming distance to platform.

c. Probe Trial (reference memory)

The probe trial allows for the confirmation and assessment of spatial reference memory in performance of the task. At approximately 24 hrs (+/- 3 hrs) following the last hidden platform test, each animal shall be assessed for spatial memory. A 24 hr time interval (24 hr ITI) between tasks shall be maintained for each animal.

- 1) The platform shall be removed from the tank.
- 2) Room cues and lighting conditions shall remain as for hidden platform task.
- 3) The animal shall be placed in the tank as described in the hidden platform testing at the SW start location.
- 4) The animal shall be allowed to freely swim for 90 sec with data collected for each 15 sec epoch.
- 5) The animals shall be removed from the tank at the end of 90 sec. The initial attempt at removal shall be to allow the animal to climb into hand or a container (see section F.2.b.4.).
- 6) If the apparatus allows for an automatic manipulation of the platform, at 90 sec into the probe trial, the platform shall be raised in the "hidden" platform location and height and the animal allowed to "escape" onto the platform rather than removing by hand.

d. Spatial Reversal Acquisition

Approximately 48 hrs (+/-4) after the probe trial [scheduled to maintain a constant time interval across all groups], animals shall be tested for the ability to learn a new platform location (reversal learning). Reversal learning reveals whether animals can extinguish their initial learning of the platform's position and acquire a path to the new goal location.

- 1) The equipment set-up and parameters shall be the same as for hidden platform testing with the platform moved to the opposite quadrant location (SW) relative to its position during acquisition.
- 2) Given that the animals have already learned the parameters of the task and are only shifting location, the animals shall be tested for 3

trials/day for 3 days. If performance fails to reach the level observed on the last day of the hidden platform acquisition (last day prior to probe trial), training shall continue for 2 additional days.

7. Endpoints

An automated video tracking system shall be used to capture MWM performance. To clearly describe variables, detailed physical descriptions and software algorithms shall be provided to describe how each measure is taken. The software tracking system shall provide documentation of testing protocol and animal assignment and allow for post-hoc analysis of video images.

a. Endpoints to be collected shall include:

1) Visible platform training (non-spatial learning tests)

- i. time to find platform (latency)
- ii. total distance (path length)
- iii. swim speeds
 - a. average swim speed
 - b. time spent in slow swimming (< 0.05 m/sec)
 - c. time spent floating (% trial duration)
- iv. Thigmotaxis
 - a. Percent thigmotaxis time (% trial duration when the subject was in the outer 10% of the pool diameter).
 - b. Thigmotaxic tendency (proportional distance travelled within the outer 10% of the pool relative to total distance travelled).
- v. Heading angle (average heading error) – deviation between the actual direction of the rat when leaving the start location and a straight line to the escape platform.

2) Hidden platform training (spatial learning tests)

- i. Time to reach platform (latency)
- ii. total distance (path length)

- iii. Gallagher's proximity (average distance to the platform during a trial).
 - iv. Swim speeds
 - a. average swim speed
 - b. time spent in slow swimming (< 0.05 m/sec)
 - c. time spent floating (% trial duration)
 - iv. Thigmotaxis
 - a. Percent thigmotaxis time as % trial duration when the subject was in the outer 10% of the pool diameter.
 - b. Thigmotaxic tendency (proportional distance travelled within the outer 10% of the pool relative to total distance travelled).
 - v. Heading angle (average heading error) – deviation between the actual direction of the rat when leaving the start location and a straight line to the escape platform.
 - vi. Pathway tracking as available by commercial video tracking and analysis software as instructed by manufacturer.
- 3) Probe trials – no platform (memory retention tests) –
- i. Latency – initial latency to swim to previous platform location.
 - ii. Quadrant time – the total time spent in each of the 4 quadrants individually during each of the 15 sec epochs (for a total of 6 epochs or 90 sec).
 - iii. Platform crossings - number of crossings over the previous escape platform location during each of the 15 sec epochs.
 - iv. Quadrant swimming distance - the total swimming distance (path length) within each quadrant during each 15 sec epoch.
 - v. Total swimming distance – the total swimming distance (path length) covered over the entire tank during each 15 sec epoch.
 - vi. Quadrant percentage – The percent of time spent in each quadrant during each of the 15 sec epochs.

- vii. Quadrant sequence - the sequence of swimming into each quadrant
 - viii. Number of crossovers from and to each quadrant.
 - ix. Proximity measure (Gallagher's measure) (Gallagher et al., 1993). Average distance from center of the original platform location across the probe test (Maei et al., 2009).
 - x. Heading angle (average heading error) – deviation between the actual direction of the rat when leaving the start location and a straight line to the previous escape platform location.
 - xi. Search strategy – (e.g., pathway analysis or tracing) as provided by instrument manufacturer software analysis.
- b. Each endpoint shall be averaged over each day to provide a mean daily response for statistical analysis.

G. NTP RELEVANT SOPs

The test facility shall have specific SOPs as required by NTP including, but not limited to, the activities listed below:

- a. Technician training (animal handling and specific for neurobehavioral testing tasks)
- b. Environmental conditions of testing rooms including lighting
- c. Cleaning and sanitization of equipment at start of experiment and between animals.
- d. Sanitization of equipment and study rooms during the study
- e. Randomization of animals to experimental groups defined by relevant experimental factors such as dose, experimenter, testing apparatus, apparatus location, time of day, and session.
- f. Identification of animals (e.g., microchip implant, tail marking, tail tattoo, paw tattoo) and tracking of individual animals between preweaning and postweaning evaluations
- g. Coding of animals to ensure experimenter blinding to dose groups of animals during testing

- h. Weighing of animals
- i. Observation of animals: Daily AM and PM check, detailed clinical observations
- j. Handling of pregnant and lactating dams
- k. Recording signs of parturition
- l. Handling, sexing, weighing, and conducting clinical observations of rodent pups
- m. Culling of animals
- n. Weaning pups for rodent toxicology studies
- o. Calibration of individual test units
- p. Handling of animals for acclimation
- q. Prepulse startle inhibition
- r. Motor activity
- s. Morris water maze (room configuration and test procedures)
- t. Pain and distress determination
- u. Environmental conditions for behavioral tests

H. SUPPLEMENTAL INFORMATION

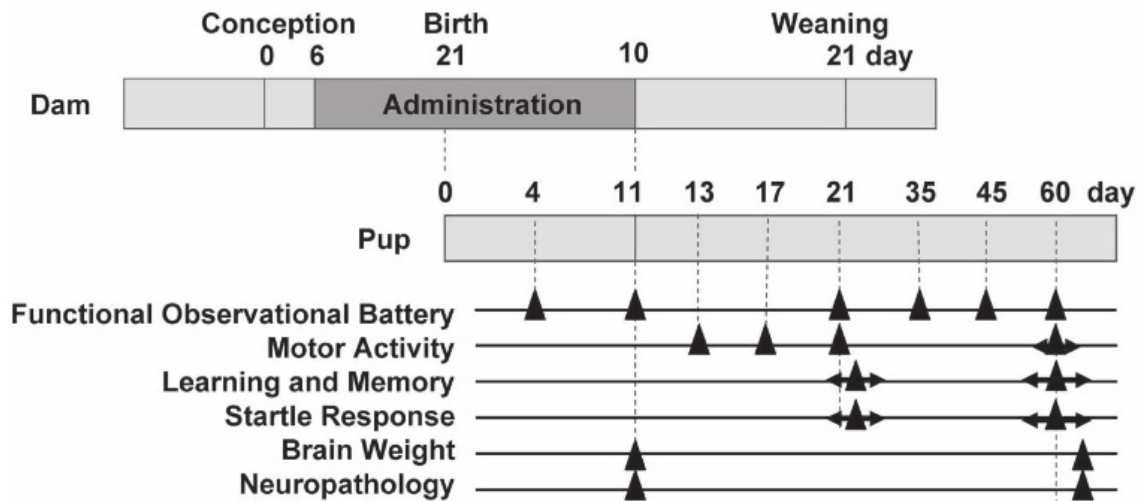
1. Age of Testing for a Developmental Neurotoxicity Study

The age of dosing, weaning, and testing varies from the US EPA (OPPTS 870.630) and the OECD (426) guideline studies (Fig. 1).

Figure 1. Schematic of EPA and OECD guideline studies.

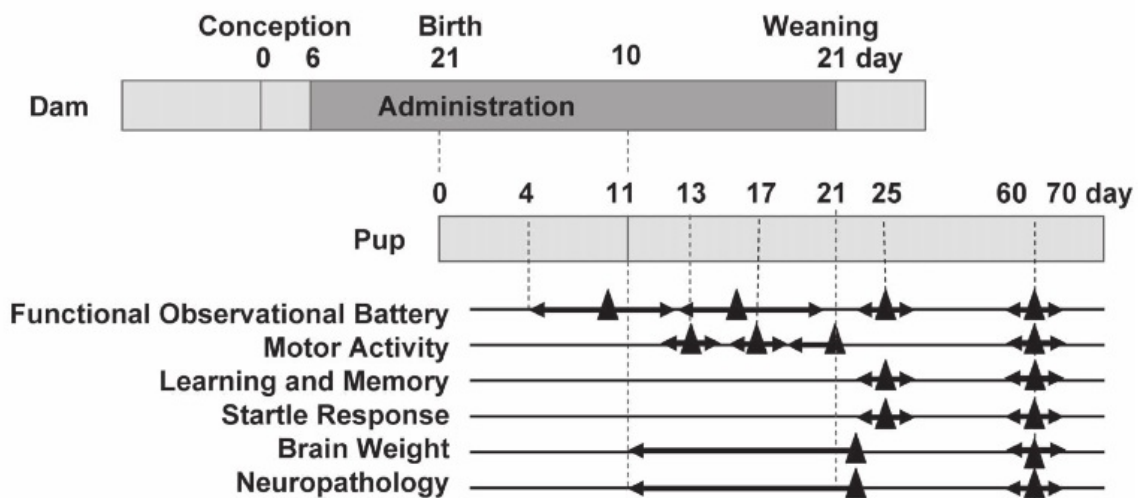
(a)

EPA guideline study (OPPTS 870. 630)



(b)

OECD guideline study (OECD 426)



Tsuji R, Crofton KM. *Congenit Anom (Kyoto)*. 2012 Sep;52(3):122-8.

a. Motor Activity

Ages for testing are selected to maintain continuity of developmental stage with EPA and OECD guideline studies. Rats shall be assessed on PND 17 and PND 21 for motor system ontogeny or on PND 21 for juvenile motor activity as determined by study design. For mice, juvenile motor activity shall be measured at PND 23 to maintain comparable developmental age to rats and avoid weaning as a confounder. A pre-adult assessment of open field activity shall be conducted on rats and mice.

1) Rats

- i. Motor System Ontogeny. To assess motor system ontogeny, open field locomotor activity measurements shall be collected in the same animal at PND 17 and 21 [1 pup/sex/litter].
- ii. Juvenile Open Field Activity. To assess juvenile open field locomotor activity, measurements shall be collected at PND 21 [1 pup/sex/litter].
- iii. Open field activity levels in pre-adult (approx. 50 (+/-3) days of age) shall be collected. Data can be collected from mice tested at an earlier age or from different animals randomly selected from each dose group [1 pup/sex/litter] as defined by specific study design.

2) Mice

- i. Juvenile open field activity measurements shall be collected at PND 23 (2 days post-weaning).
- ii. Open field activity levels in pre-adult (approx. 50 (+/-3) days of age) shall be collected. These can be the same animals examined at early ages or other randomly assigned animal from each litter to those use for pre-weaning activity levels, as determined by study design.

3) Recommended n size (sample size per group) ≥ 15 ; minimum of 10.

b. Startle and PPI

Both startle and PPI change across development. Startle and PPI regulatory circuitry (i.e., the forebrain circuits that descend to regulate the primary pontine startle and PPI mechanisms) develop into adolescence. Two issues for testing exist: the development of the primary circuitry and the development of the descending regulatory circuits. To capture this development, testing shall be conducted at PND 22 (rats) PND 23 (mice),

35 (+/-1), and 55 (+/-3) days of age within the same animal. This represents one additional age as compared to EPA and OECD DNT guidelines and captures critical developmental windows of circuitry formation. Recommended n size (sample size per group) ≥ 15 ; minimum of 10.

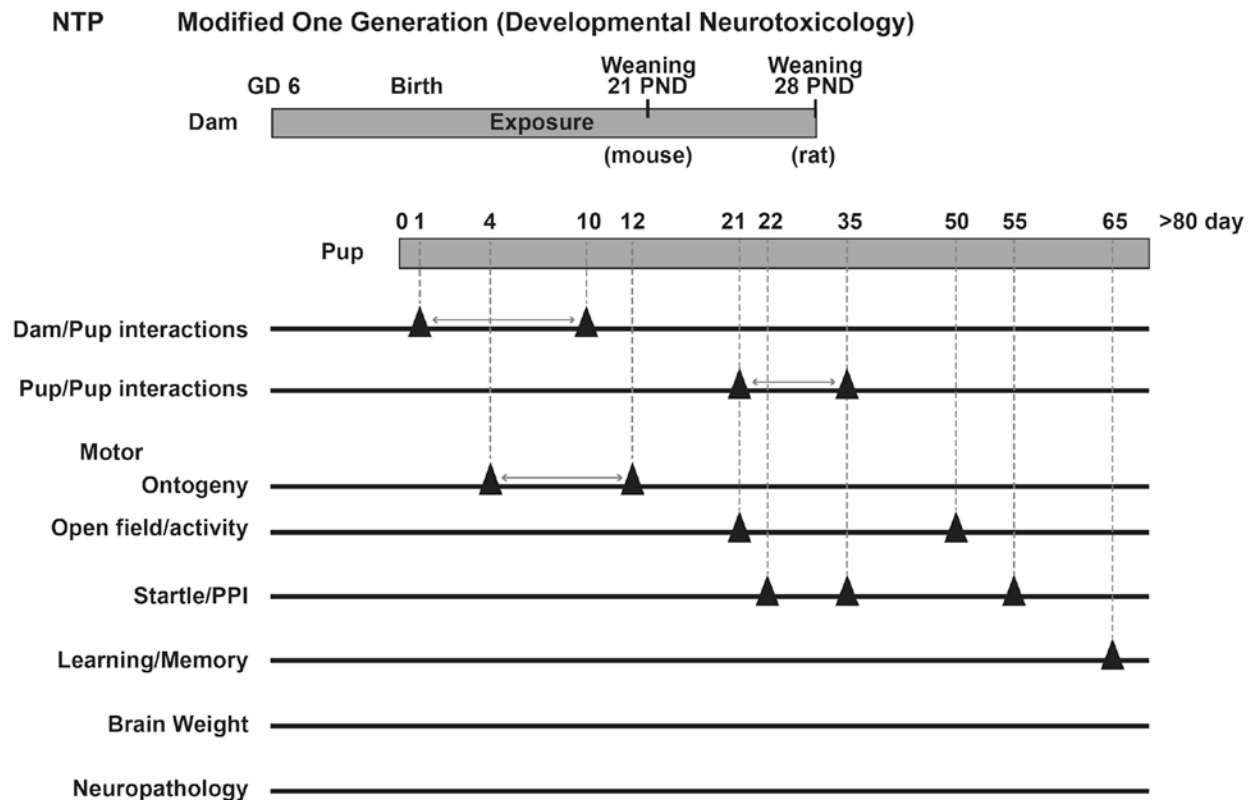
c. Morris Water Maze

Testing shall be conducted in young adult animals at approximately 65 (+/-5) days of age. Recommended n size (sample size per group) ≥ 15 ; minimum of 10.

2. Integration of Endpoints into a Modified One-Generation Study

Based upon the multiple endpoints collected within a modified one-generation (MOG) study design, consideration shall be given for restriction of the neuro-specific endpoints and time of assessment. At a minimum, clinical observations shall include those observations identified that record maternal/pup interactions (Section C1), pup health (Section C2a), pup motor ontogeny (Section 2Cb) and demonstration of normal interactive behavior between juvenile pups (Section C2c) as described. Open field activity (Section D) shall be measured at PND 21 and PND 50 (+/-3) for rats and PND 23 and PND 50 (+/-3) for mice. Startle response and PPI (Section E) shall be conducted at PND 22, 35 (+/-1), and 55 (+/-3) to allow for assessment of development of the startle sensory system. MWM (Section F) shall be conducted at PND 65 (+/-5). It is recommended that the additional observational endpoints listed in Section J1 be included in the standard observational assessments taken over the course of the study.

Figure 2. Schematic of Neurobehavioral Testing for NTP



Testing of open field motor activity in juvenile mice shall be conducted on PND 23

3. Time of Testing Relative to Dosing

The impact of a direct dosing of a compound on the behavioral performance shall be considered and the schedule of dosing to testing shall be coordinated to minimize confounding of acute effects of the compound. When possible, with direct bolus dosing, behavioral testing shall be conducted prior to any direct dosing for that day. This adjustment is not necessary for continuous dosing via feed or drinking water. Inhalation studies represent a unique type of study with regards to exposure requirements; however, all behavioral testing should be conducted at a time to minimize confounding from acute effects of exposure as identified by NTP.

4. Open-field Activity

Apparatus: Representative defined areas: a) within a (40 cm x 40 cm x 20 cm) photocell device using a two-dimensional (16 x 16) sensor array configuration, chamber margin (thigmotaxis; area within detectable range of the outer most infrared beam on each sensor [3.8 cm from wall]) and b)

center (area 8.9 cm from the wall encompassing a 10 x 10 photocell square). If allowed by computer software, the area exclusive of the margin and center shall be included.

5. Startle

Adaptation to Startle Animal Holding Chamber: Animals shall be handled for 3 sessions in a manner to facilitate placing into startle apparatus holder. For open box holders this shall include placing the animal into the chamber. For “tube” holders this shall include handling of the animal with covering the head to mimic insertion into holder, gently squeezing together the forepaws so they cross on the underside of the animal and holding of the hindquarters to prevent perambulation (Geyer MA, Swerdlow NR. 2001). On the 3rd handling session, the animal shall be placed into a simulation of the holder for 5 min.

6. Startle Protocol

Background level: 65dB

PPI intensities set as 3, 6, 12, and 15 dB above background

Trials to be delivered according to a variable interval 15 schedule

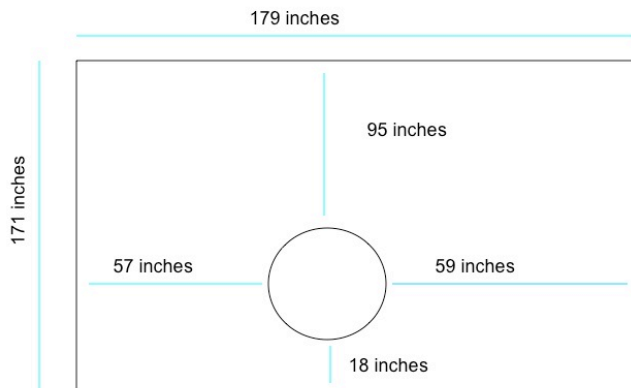
For Kinder Scientific Startle Unit the collection window shall be set for 500 msec with a sampling within that window of 100 msec for the startle response and sampling of 250 msec preceding the startle elicitation as a measure of baseline activity and to confirm absence of startle response elicited by pre-pulse intensities.

Trial #	Block #	Trial name	Prepulse level (dB)	Prepulse length (ms)	Inter-stimulus Interval (ms)	Noise level (dB)	Wait Length (ms)	Sampling Window (ms)
1	0	P120				120	40	100
2	1	P120				120	40	100
3	1	P120				120	40	100
4	1	P120				120	40	100
5	1	P120				120	40	100
6	1	P120				120	40	100
7	2	NOSTIM				65	40	100
8	2	PP77P120	77	20	80	120	40	100
9	2	PP68P120	68	20	80	120	40	100
10	2	PP80P120	80	20	80	120	40	100
11	2	P120				120	40	100
12	2	PP71P120	71	20	80	120	40	100
13	2	PP71P120	71	20	80	120	40	100
14	2	PP80P120	80	20	80	120	40	100
15	2	PP77P120	77	20	80	120	40	100
17	2	PP68P120	68	20	80	120	40	100
18	2	P120				120	40	100
19	2	P120				120	40	100
20	2	PP77P120	77	20	80	120	40	100
21	2	PP71P120	71	20	80	120	40	100
22	2	PP68P120	68	20	80	120	40	100
23	2	NOSTIM				65	40	100
24	2	PP80P120	80	20	80	120	40	100
25	2	PP80P120	80	20	80	120	40	100
26	2	P120				120	40	100
27	2	PP77P120	77	20	80	120	40	100
28	2	PP71P120	71	20	80	120	40	100
29	2	PP68P120	68	20	80	120	40	100
30	2	PP77P120	77	20	80	120	40	100
31	2	PP68P120	68	20	80	120	40	100
32	2	PP80P120	80	20	80	120	40	100
33	2	P120				120	40	100
35	2	PP71P120	71	20	80	120	40	100
36	3	P120				120	40	100
37	3	PP77P120	77	20	80	120	40	100
38	3	PP80P120	80	20	80	120	40	100
39	3	PP68P120	68	20	80	120	40	100
40	3	PP71P120	71	20	80	120	40	100
41	3	P120				120	40	100
42	3	PP80P120	80	20	80	120	40	100
43	3	PP71P120	71	20	80	120	40	100
44	3	NOSTIM				65	40	100
45	3	PP77P120	77	20	80	120	40	100
46	3	PP68P120	68	20	80	120	40	100
47	3	PP77P120	77	20	80	120	40	100

48	3	PP71P120	71	20	80	120	40	100
49	3	PP80P120	80	20	80	120	40	100
50	3	PP68P120	68	20	80	120	40	100
51	3	P120				120	40	100
52	3	NOSTIM				65	40	100
53	3	PP71P120	71	20	80	120	40	100
54	3	PP80P120	80	20	80	120	40	100
55	3	P120				120	40	100
56	3	PP68P120	68	20	80	120	40	100
57	3	PP77P120	77	20	80	120	40	100
58	3	PP77P120	77	20	80	120	40	100
59	3	P120				120	40	100
60	3	PP68P120	68	20	80	120	40	100
61	3	PP71P120	71	20	80	120	40	100
62	3	PP80P120	80	20	80	120	40	100
63	4	P120				120	40	100
64	4	P120				120	40	100
65	4	P120				120	40	100
66	4	P120				120	40	100
67	4	P120				120	40	100
68	5	P120				120	40	100
69	5	P120				120	40	100
70	5	NOSTIM				65	40	100
71	5	P120				120	40	100
72	5	P120				120	40	100
73	5	P120				120	40	100
74	6	P120				120	40	100
75	6	P120				120	40	100
76	6	P120				120	40	100
77	6	P120				120	40	100
78	6	P120				120	40	100

7. Morris Water Maze

a. A representative example for room arrangement and cues for guidance:



Within this area cues consist of:

- 1) Two dark vertical lines from floor to ceiling made (each line 20 cm wide with a space of approximately 15 cm between them),
- 2) A large dark circle approximately 32 cm in diameter,
- 3) A long dark horizontal line (20 cm in width) from wall edge to wall edge at a height easily seen by the animal from water level.

b. Use of curtains to restrict MWM spatial space

- 1) Cues utilized within a restricted curtained space shall be a minimum of 2 and maximum of 3. The size shall be proportional to the size identified for the represented area. The cues shall be large enough for the animal to detect but also small enough to be detected as cues relative to the background. The size, shape (circle, triangle, square, vertical rectangle), color, texture, and location shall be documented and a representative schematic of the test area and cues shall be provided in the report. The cues shall be placed within the line-of-sight of the animal as swimming in the maze.

- 2) Curtains shall be placed 2-3 ft. from the inner wall of the tank.

c. A schematic of the room configuration or curtain dimensions and cues shall be included in the testing protocol.

8. Controls for Validation and Proficiency

- a. Commercially available equipment based on well-established and validated methods for assessing the various neurobehavioral endpoints shall be used. Each procedure shall be shown to generate specific well-defined patterns of behavior as conducted in naïve animals (species, sex, age) by the testing laboratory. A cohort of naïve 5 males (and 5 females if females are to be assessed in the study) shall undergo each behavioral test at the defined age. This shall be repeated in 1 additional cohort of 5 animals/sex to demonstrate reproducibility of test data and provide a total n=10 with cohort as a statistical factor.

- b. Option: If there is a specific requirement to test for an acute (not developmental) effect of a compound on a specific behavior consideration shall be made for the need to include a pharmacological challenge for validation of the test paradigm relevant to the expected nature of effect. Expected results shall have been demonstrated in control animals (species, strain, and sex) within approximately 1 yr of start of NTP study animals.

For potential drug candidates, dose and time can vary depending upon the species, strain, and age of animal. Animals can receive more than one drug with a minimum of 1-week drug clearance period. This is not standard and not considered a required control for developmental exposure studies.

1) Locomotor Activity

- i. d-amphetamine (1 mg/kg sc)
- ii. MK-801 (0.2 mg/kg sc)

2) Startle Response and PPI

- i. Baseline startle response - risperidone (1 mg/kg body wt) dissolved in 1 N HCL and then titrated to final pH of 5 with 1N NaOH. Inject ip in a volume of 10 ml/kg body wt 30 min before the test.
- ii. Habituation – MK-801 (0.3 mg/kg in mice) ip 15 min before test
- iii. PPI increase - BP 897 (8 mg/kg) injected 30 min prior to test
- iv. PPI decrease - amphetamine (1 mg/kg ip) or dizocilpine (0.05 mg/kg ip)

3) Morris Water Maze

- i. Scopolamine (30 min before test)

9. Testing of Female Animals

The stage of the estrus cycle of the female rodent can significantly influence behavioral performance. Determining the stage of the estrus cycle in animals >35 days of age shall be considered on day of PPI and MWM Probe testing.

10. Additional Tests

a. Observational Endpoints

Prior to dosing for the day (if applicable), one male and one female rat randomly selected from each litter (n size as dictated by the specific NTP study design) shall undergo observations that include both open field observations (posture, activity, gait, hind-limb splay, tonic movements, clonic movements, ataxia, respiration, bizarre behavior), hand-held observations (ease of removal from cage, reactivity to handling, vocalization, fur appearance, lacrimation, salivation, piloerection,

exophthalmos, and palpebral closure) (each scored as absent (1) or present (2) or as according to a severity scale). Age of testing shall be determined by the study design. For developmental exposures, observations shall occur on the day prior to weaning and at later test times as determined by NTP study design. Observations shall not occur on the same day as any other behavioral assessment. Observations and ratings shall be standardized to minimize subjective nature of the assessment.

b. Forelimb and Hindlimb Grip Strength

- 1) Animals shall be assessed for fore- and hind-limb grip strength using a strain-gauge system appropriate for species and age of animal (screen, bar). The appropriate gauge strength size shall be used for each age and species. Control animals will measure mid-range of the meter and allow for detection of an increase or decrease in strength.
- 2) During handling acclimation, animals shall be handled in a manner that includes manipulation of fore- and hindlimbs similar to what would be required for placement on test apparatus.
- 3) The Grip Strength Meter shall be placed on a stable surface, away from drafts or vents that could disturb the measurement by the sensor.
- 4) Preference is given to equipment configuration that allows assessment of fore- and hindlimb grip strength in one pass and provides physical support for the animal.
- 5) The strain-gauge shall be set in the PULL mode for forelimb assessment and in the PUSH mode for hindlimb assessment.
- 6) A triangular bar shall be used for assessing forelimb and a T-bar for hindlimb in adult rats. A screen grid, sized appropriately for the animal, shall be used to assess forelimb strength for young rats and mice. A screen grid or T-bar of appropriate size shall be used to assess hindlimb strength for young rats and mice.
- 7) Contact with the forelimb apparatus shall require all four digits of both limbs.
- 8) Individual responses shall be recorded 1 per test session for a total of 2 sessions in a day. Within each session, all animals shall be assessed once. Animals are placed back in holding cage between trials 1 and 2. Aberrant high or low scores (failed tasks) reflective of experimenter error or a slip of the animal shall be recorded, noted as such, and the individual animal allowed a 3rd and final trial.

9) Testing of Grip Strength

a) For Forelimb/Hindlimb Grip Strength

- i. Confirm that the forelimb gauge is set in PULL mode and hindlimb is set in PUSH mode and that both gauges have been reset to zero.
- ii. Set the animal's forepaws on the screen (immature rats and mice) or triangular bar (adult rats) attached to a strain gauge. Alternatively, hold the rat until it grabs the screen/bar. Hold the rat by the base of the tail and pull the rat horizontally, smoothly and quickly, in one continuous motion until its grip is broken. A slight pause may be necessary before beginning the pulling motion, to assure that the rat's digits are properly curled around the screen or bar and that the paws are not crossed. If a platform is not present between the two gauges, the observer shall place their hand to lightly hold the ventral side of the body.
- iii. Immediately subsequent to this and within the one smooth, continuous pulling motion, the hindlimbs are allowed to grasp a bar (or screen) as the animal's body is quickly but smoothly pulled away. A slight pause may be necessary before beginning the pulling motion to assure that the rat's digits are properly curled around the screen or bar and that the paws are not crossed. Do not allow the front feet to touch the hindlimb screen or bar. Do not allow the experimenter's hand to touch the screen or bar as this can invalidate the reading.
- iv. Two trials each shall be conducted for fore- and hindlimb grip strengths, but these two trials are not to be consecutive. All rats to be tested that day are run through trial 1, all rats shall then be repeated for trial 2. A third trial shall be conducted if two valid measures are not obtained.
- v. The digital readouts on the gauge shall be recorded, and the gauges reset.

b) For Hindlimb Grip Strength Only

- i. Confirm that the strain gauge is set in the PUSH mode.
- ii. Hold the animal with its hind-feet close to the screen to T-bar attached to the strain gauge and follow relevant instructions as provided for forelimb/hindlimb grip strength.

c) Grip Strength Endpoints

- i. Average of 2 valid gauge readings for forelimb grip strength
- ii. Average of 2 valid gauge readings for hindlimb grip strength
- iii. Fatigue option: Grip strength measurements shall be collected over 6 sequential trials to assess differences that may reflect muscle rigidity or flaccidity.

c. Model Of Hyperactivity Disorder

If there is data to suggest that the developmental exposure may be associated with a hyperactivity/hyper-reactivity disorder a modification to the activity protocol may be considered. Following the protocol for motor activity, record activity measures in 5 min epochs for a total of 45 min each day for 3 days. Hyperactivity manifesting as the animals adapt to the novel environment is the targeted endpoint.

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