

**WVU ACUC STANDARD OPERATING PROCEDURE (SOP):
Use of the Rodent Behavior Core (RBC)
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1. Background

The Rodent Behavior Core (RBC) provides validated and optimized animal behavioral tests to West Virginia University (WVU) researchers and their collaborators. The RBC is conveniently located within the WVU Health Sciences Center (HSC) building in the Office of Laboratory Animal Resources (OLAR) vivarium rooms 201, 201a, 202, and 203 a-c. The RBC is managed by the RBC Director (Dr. Engler-Chiurazzi; post-doctoral fellow under the mentorship of Dr. Simpkins). Behavioral equipment is available for use by WVU investigators free of charge. This use includes access to expert consultation regarding experimental design, task selection, training, data analysis, and dissemination of results. Because most behavioral testing involves working directly with animals with minimal engineering controls outside their cages and change stations, full personal protective equipment (PPE) is typically required.

Although two animal species (mouse and rat) are used, the basic procedures for handling the animals will be the same. All animals used in the RBC are from various investigators at WVU. The animals remain on the individual investigator's protocol, which references this SOP. The investigator's protocol indicates rationale for requesting experimental animals, experimental groups, numbers of animals needed, behavioral tests to be conducted, assurances or justifications regarding duplication of previous work, and/or length of the study (as appropriate for the tests being utilized). All appropriate RBC staff members must be included in each animal protocol in order to provide any hands-on service.

Animal models have and will continue to play a vital role in the study of mechanism(s) of human disease stroke, as well as in the assessment of potential drug and alternative therapies for prevention, treatment and recovery from stroke. The goal of the RBC is to support the translational research projects of investigators at WVU.

Note: Non-RBC personnel who improperly use the RBC equipment, damage apparatus, leave messes for others, do not follow procedures as outlined here, or do not follow instructions as detailed by the RBC Director may be banned from RBC equipment use, can have their old HSC vivarium access removed by OLAR, or can be charged for cleaning by OLAR, as needed.

1. Pain and distress category:

Some behavioral tests will cause animal distress. The distress is a normal progression of the behavioral tests and will not be treated. Therefore, the pain and distress category is defined as **E***. We use the "Recognition and Alleviation of Pain and Distress in Laboratory Animals, NRC 1992" signs of acute pain or distress in mice and rats. This information will be conveyed to the PIs who will use the RBC. Animal welfare scoring pain and distress categorization will also be determined in the PI's protocol and may reflect the procedures requested in this SOP. For example, forced swim testing is a category E procedure.

2. General Behavioral Test Procedures

2.1 Behavior Testing Issues:

A wide range of behavior tests can be used for the assessment of functional outcomes. The SOP details herein are limited to testing procedures as individual experiments are described in each investigator's protocol. These

assessments, instruments and expertise are freely available within RBC. General procedures and training information for each behavioral test are listed below and citations for each to aid in manuscript preparation can be made available at the request of the investigator. Detailed step-by-step procedure documents and training information for each behavioral test are available from the RBC Director and are accessible in RBC testing rooms.

Timelines suggested by the WVU RBC Director are recommendations based on the typical required time for successful data collection for the tasks described (days and trials, trial durations, heat setting, shock voltage, etc). However, there may be a scientific need to modify these timetables (EX: a memory impairing treatment is being administered) or these procedures (EX: utilizing multiple probe trials) and these modifications should be described in the individual PI's protocol whenever possible. It is advisable to analyze behavior data on a daily basis to ascertain performance changes across time. As well, in case of unforeseen delays in testing and to allow for rooms/equipment to be properly cleaned, it is advisable to allow for a 3-day flexibility window between unique users for a given behavior task/apparatus when scheduling room/equipment use.

For all testing, proper signage indicating that animal experimentation is underway must be placed on the door and in designated locations in the halls. This is done to alert OLAR staff and other investigators that the room is in use, that noise in the area is to be limited, and that entry into the room is not allowed. As well, these signs indicate that proper PPE must be utilized if entry into the room is required (for urgent needs only).

For all tests, care will be taken to gently place and remove the animal from the testing apparatus and between animals the apparatus will be cleaned of debris (bedding, fecal boli, urine, etc.) with an anti-bacterial spray and if needed, alcohol for dry land tests. For some tests, food restriction may be needed to motivate behavior; the implementation of food restriction will vary from study to study and should be reflected in the specific laboratory animal protocol. A daily log should be kept of body weights and food amounts supplied while animals are undergoing food restriction.

For all testing involving aversive stimuli including shocks, animal are never left unattended. For shock treatments, the PI must stay at or below 1 mA, the animal must be able to escape the shock, the shock delivery system must be calibrated with values recorded in a log to show this has occurred at least monthly or at the beginning of a study, (whichever occur first). All parameters of shock delivery must be detailed in the PI's protocol if different from this SOP, including the following: milliamperage, voltage, duration, AC or DC, and maximum frequency possible and any safety procedures against forced continued shocking.

For anxiety/depression behavior testing, an acclimation period between transport of animals to the facility and the testing of animals may be necessary to ensure accurate data collection.

For water testing: all animals are tested in water that has the following characteristics: it is cleaned of debris between animals using a fishnet or other device, it has a temperature of between 20 and 25 °C (as appropriate for the specific test), the water is changed as appropriate for the specific test and species used (EX: Forced swim test = after every animal, water radial arm maze = after every 7 days), animals are monitored continuously while in the water, and escapable heat is supplied to animals after each swimming session. Animals will never be left unattended while swimming. An experimenter must always be present for water-based tests.

No animals containing vectors or other potentially infectious agents (ABSL-2 studies) will be used without attention to stricter requirements for disinfection (e. g. non-alcohol disinfectants), PPE and clean-up procedures as approved by the IBC and in collaboration with OLAR. An IBC protocol has to be in place for such studies as connected to the PI's protocol that describes precautions taken, with careful consideration of adjustments to the SOP as described here. Proper signage needs to be in place during these times.

The following categories of tests are included within the core's expertise and are described below: **1) Neurological Assessment:** Neurological score, Modified neurological severity score; Health/sickness behavior screen; **2) Sensori/locomotor:** Adhesive dot removal, Cylinder test; Forepaw inhibition(aka: swim test); Foot fault ladder test; Grip strength test; Rotarod; Sunflower test; **3) Learning and memory:** Active avoidance; Delayed match to sample (repeated reinforcement and two-trial versions); Delayed non-match to sample; Fear conditioning; Inhibitory (passive) avoidance; Morris water maze; Morris water maze reversal; Novel object (visual and place) recognition; Radial arm maze (land and water versions); **4) Anxiety-related:** Elevated plus maze; Hole board; Light/dark transition; Marble burying; Open field; Social investigation **5) Depression-related:** Differential reinforcement of low rate schedule; Forced swim; Novelty suppressed feeding; Sucrose preference; Tail suspension; **6) Visual and Locomotor Competence:** Visible platform; Spontaneous locomotion; **7) Pain:** Hot plate; Tail flick.

2.1.1 Neurological Assessment

2.1.1.1 Neurological Score

Rodents are assessed for neurological deficits according to a 6-point scale (0 = no deficit, 1 = failure to extend left forepaw fully, 2 = circling to the left, 3 = falling to the left, 4 = no spontaneous walking with a depressed level of consciousness, and 5 = moribund or dead). In all cases, the earliest endpoint to avoid unneeded pain and distress will be used for humane intervention.

2.1.1.2 Modified Neurological Severity Score

The modified Neurological Severity Score is collected from mice and rats. This is a commonly used diagnostic scale for detecting multiple domains of functional deficits following ischemic stroke. Neurologic function is graded on a scale of 0 to 14 for mice and 0-18 for rats (normal score 0; maximal deficit score 14 for mice and 18 for rats). This score is based on rodent performance on a series of motor, sensory, balance, and reflex tests. All animal behavior will be recorded with a video camera. Each sub-test may need to be repeated several times to obtain an accurate performance measure. This scale has the benefit of being rapid to administer and able to be re-evaluated at multiple time points pre- and post-stroke/brain injury to document a trajectory of impairment and recovery of function. Between animals, the surface will be cleaned with an appropriate disinfecting antibacterial spray, and if needed, alcohol (EX: isopropyl or ethanol).

Motor tests include the forelimb/hindlimb flexion and locomotor tests. The forelimb/hindlimb flexion tests assess forelimb and hindlimb flexion towards the ground while the animal is briefly hung inverted by the tail. For this test, the experimenter gently inverts the rodent by gently but firmly holding the base of the animal's tail and suspending the animal in the air above a tabletop. The extension or flexion of the limbs is noted. As well, body turn direction is noted.

The locomotor test assesses walking behavior along a level surface. The animal is gently placed on a flat surface such as a tabletop and allowed to walk to explore the environment. Depending on experimental manipulations the use of a salient reward (EX: a darkened cage with bedding) may be required to motivate animals to walk.

Sensory tests include forepaw placement following visual, tactile, vibrissae, and proprioceptive stimulation and are typically done only in rats. The animal is held around the torso near the edge of a table and the proficiency of paw placements induced by each stimuli is measured. For each placing test, animals are gently but securely held by their torso by the experimenter, allowing their front paws to hang freely below them. The experimenter then moves the animal slowly toward the edge of a tabletop. For the visual placing test, the experimenter moves the animal to just short of the table edge without allowing the vibrissae to touch the edge. For the vibrissae-induced placing test, the experimenter moves the animal to just short of the table edge but allows the vibrissae

to touch edge. For the tactile-induced placing test, the experimenter gently brushes the animal's paws against the table edge while slightly elevating the head. For the proprioceptive placing test, the experimenter gently pushes the animal's paws against the table edge while slightly elevating the head.

Balance is assessed using the beam test and measures the ability to balance on and possibly navigate the length of the square beam without mistakes in paw placement or falling. The beam is elevated above a padded cushion so that if an animal falls, it will not be injured. Beams of various thicknesses will be utilized as needed based on the performance of the particular cohort of animals. For instance, stroke animals may be unable to balance on a beam 1.5 cm thick but can perform on a thicker beam. Beam thicknesses will be selected based on those used in published papers and will include beams of 4-35 mm thick for mice and 2 to 13 cm thick for rats. For a particular trial, an animal will be placed on the beam and allowed to balance/navigate. Its performance will be scored on a 0-6 scale, with a score of 6 indicating that the animal fell immediately, and a score of 0 indicating that the animal either balanced for trial duration and/or traversed the length of the beam.

Reflexes assessed include pinna, corneal, and startle reflex.

2.1.1.3 Health/Sickness Behavior Screen

To assess general health status and quantify and track changes in sickness behavior post-stroke/infection/brain injury, the following test series can be completed at multiple time points during recovery. Tests in the series are: body weight monitoring, body temperature, food consumption, respiration rodent self-care quantification (body position, posture, fur coat quality, presence of porphyrin staining around the eyes), home cage activity index, dehydration, pain sensitivity, and social interaction. These measures will take place in the home cage and involve minimal disturbing of the animal, with the exception of dehydration, body weights and temperatures assessments. (Significant porphyrin staining can lead to dermatitis and in such cases, OLAR veterinary staff may need to be notified).

2.1.2 Sensorimotor Tests

2.1.2.1 Adhesive Dot Removal

An adhesive removal test assesses contralateral lesion neglect and ipsilateral lesion bias by recording latency to contact and remove labels and the order of label contact and removal. A paper tape will be used in the test. A test of the forepaw contact response will be scored as 0 if the response is immediate and normal, 1 if the response is within a short time range (ie: 2 s), and 2 if the response is greater than the pre-set criteria. In addition, latency to contact and latency to remove the tape will be recorded. Any uncontacted paper tape will be removed after each trial. The tape removal will be done carefully and if needed, using warm water, soap and gentle pressure on the tape edges, and will not leave a residue. Care will be taken to not harm the paw or fur.

2.1.2.2 Cylinder Test

The Cylinder test assesses spontaneous forelimb use. Animals are placed at the bottom of a glass, plastic, or Plexiglas cylinder. The cylinder is placed on a surface with an angled mirror on the ventral side of the box to aid in observation. The exploratory behavior of each animal in the transparent cylinder is video-recorded. Forepaw use (unique paw placements for each limb and both limbs simultaneously) on the vertical sides of the apparatus is quantified.

2.1.2.3 Forepaw Inhibition Swim Test

The Swim test assesses forelimb inhibition as intact animals do not utilize forepaws during swimming. Animals are placed in a rectangular aquarium tank and must swim from end to end. Forepaw use during the straight swim is video-recorded and the number of forepaw strokes taken, the dependent variable, is documented. Water temperature will be maintained at approximately 25 degrees Celsius. Animals are trained to swim to a visible

escape platform. On the final day, researchers acquire several sessions (defined as a straight swim with no interruption in direction) and evaluate forepaw use as an average of the sessions. Between trials, animals will be removed from the maze and placed in a heated test cage (heat lamps or pads) for a designated interval while the remainder of the squad is tested; water will be replaced at the beginning of each trial.

2.1.2.4 Foot Fault Test

The foot fault test assesses deficits in fore- and hind limb placing during locomotion. Animals are placed on an elevated horizontal enclosed ladder with a plastic barrier at the start end (to facilitate the animal to move across the ladder) and home cage or a darkened box with a small opening on the completion end. If animals that do not initiate movement across the grid, the experimenter will clap their hands or make a snapping noise above the animal. A trial is terminated when the subject traverses the ladder or when the maximum trial duration is reached, whichever comes first. A video camera positioned below the ladder will record the animals' paw placement and the number of slips and misses are quantified.

2.1.2.5 Grip Strength Test

The grip strength test assesses differences in forelimb/hindlimb strength of animals. The apparatus consists of a grip bar/rod/grid attached to a force meter. Animals are held firmly but gently by the torso in the air above the platform. The rodent is encouraged to grasp the bar/rod/grid. Once held, the experimenter gently but consistently pulls the animal away from the force meter (a readout on the software program associated with the force meter ensures that consistent gentle force is applied by the experimenter). The force at which the animal lets go of the bar/rod/grid is recorded. Test can be repeated at multiple timepoints during recovery.

2.1.2.6 Rotarod

The rotarod has been used extensively in the behavioral neurosciences. This test is carried out in mice and rats. The apparatus consists of a horizontal rotatable bar with vertically placed circles to designate individual lanes. Rodents are placed in a lane and allowed to balance. The device begins to rotate in the direction opposite of forward movement of the rodent. The rodent must walk forward in order to maintain its position on the bar. The procedure used by the core allows determination of maximal performance capacity and motor learning independently. Each animal will be placed on the cylinder and the latency to fall will be recorded. Testing is conducted until a criterion of stability is reached (i.e., until the average latency to fall fails to increase by more than 15% over the last three sessions). If using multiple sessions per test day, sessions may be separated by a pre-set interval but may vary from experiment to experiment depending on the experimental manipulations (severity of injury, pharmacological agent used, etc). Experimenters can choose to test animals in a static speed condition (speed does not change across duration of the trial) or in an accelerating speed condition (in which the speed increases across the duration of the trial). The final rotational speed and acceleration rate will vary by experiment depending on experimental factors but may range from very slow (4 to 44 rotations per minute over 300 seconds) to very rapid (4 to 44 rotations per minute in 60 seconds).

2.1.2.7 Sunflower Seed Opening Test

The sunflower seed opening test assesses goal-directed forelimb and digit use. The testing apparatus consists of a clear square box with an angled mirror placed on the ventral side of the box to aid in observation. Each animal is given a set number of sunflower seeds and they are observed (and video recorded) while they open each. Time spent opening and consuming each seed is quantified as well as the number of broken off shell pieces needed to open the seed.

2.1.3 Learning and Memory Tests

2.1.3.1 One- or Two-way Active Avoidance

For this test, animals learn to avoid a shock associated with the presentation of a light and tone stimulus by moving from the shock compartment into the unshocked compartment. An experimenter will be present at all times during avoidance testing. On the first day all animals are habituated to the shuttle box. After a predetermined delay period, animals will begin avoidance testing. Before testing, the shuttle box will be calibrated; the calibration check is necessary to verify that the appropriate shock is delivered at the beginning of the experiment.

One-way: During each daily session, animals first will receive an acclimation period inside the shuttle. Animals will receive a series of test trials separated by a fixed inter-trial interval. For each trial, the mouse will be presented with a short duration light and/or tone stimulus, indicating the impending shock. After a predetermined delay interval, a shock of pre-determined intensity (0.05-0.8mA) will be administered. The animal must move into the adjoining compartment to avoid receiving the footshock. Latency to move is recorded for each trial. Following the completion of a trial, the animal is replaced in the start location and subsequent trials are completed. Between animals, the apparatus is completely cleaned with disinfectant and/or alcohol spray. Trials will be terminated should an animal display continued signs of pain or distress such as excessive fecal boli/urine excretion, excessive and sustained vocalizations, sustained limping, etc. Daily testing will continue until the animals demonstrate successful learning; absolute number of test days may vary depending on experimental manipulations. Retention tests (single test day) using the same daily procedure will be instilled as soon as a few hours and as long as a month following training completion.

Two-way: all test procedures for two-way active avoidance are similar to one-way passive avoidance with the exception that the animal is tested for a pre-set number trials per day separated by a variable inter-trial interval. The animal is not replaced in the start location between trials and thus must learn to escape the shock into a location that may have previously been associated with foot shock on another trial. Daily testing generally continues as needed and may vary depending on experimental manipulations.

2.1.3.1 Delayed Match to Sample-Repeated Reinforcement Version

The delayed match to sample test assesses spatial navigation memory. With slight modifications (inserts added to block available arms) but similar procedural components, the following apparatus may be used to carry out this test: Y-maze, T-Maze, plus maze, radial arm maze. At the beginning of a test session, animals will be gently placed in the start arm and allowed to freely select arms to visit. A session continues until the food pellets are collected (land version) or the platform is located (water version) or maximal trial time has elapsed, whichever occurs first. If an animal fails to find a platform during the allotted time, it is gently guided to the location of the nearest cache/platform using a plastic stick. The location of the cache/platform remains fixed within a day but changes across days. The first trial is the information trial, whereby the animal learns the location of the reward. The second trial is the working memory trial, which assessed whether an animal can remember the just located reward. Trials three to six are the recent memory trials in which the animal is repeatedly reinforced for returning to the same place in space for additional rewards.

Once learning is demonstrated, a temporal challenge can be instilled to assess delayed memory retention performance. After an animal completes Trial 1 (information trial), it will be housed in the colony room or testing room on the testing cart with access to food and water bottles for a temporal delay. Following this delay, animals will be returned to the maze for one additional trial.

Land version: In this test, animals are trained to collect a hidden cache of food pellets from the end of one maze arm. Animals will be food-restricted to 85-90% of their free feeding body weights according to the procedure detailed in the PI's animal protocol. Once all animals achieve a reduction to 85-90% of free feeding weight, testing will begin. A daily log will be maintained to monitor the quantity of food given and body

weights during food restriction.

Water Version: In this version, animals swim to locate a hidden platform at the end of one of the maze arms. At the beginning of a test session, animals will be gently placed in one of the maze arms and allowed to swim to visit additional maze arms. A session continues until a hidden platform is located at the end of a designated arm. A maximum trial time is allowed for an animal to locate a hidden escape platform within a session. Once on the hidden platform, the animal remains there to orient to its location in the room. After this, the animal is placed in a testing cage that is heated with escapable heat from heating lamps/pads for a consistent inter-trial interval. During this time, the experimenter cleans the maze of debris (bedding, feces, etc) using a small net. The animal is replaced in the maze and swims to locate the hidden escape platform. An assigned researcher will perform observations throughout; such as period of time of each mouse in the water, proper water temperature, water depth and drying and care of the animal after the maze is completed.

2.1.3.2 Delayed Match to Sample- Two Trial Version

The delayed match to sample test assesses spatial navigation memory. This version assesses long-term memory for information that stays consistent across time (reference memory). With slight modifications (inserts added to block available arms) but similar procedural components, the following apparatus may be used to carry out this test: Y-maze, T-Maze, plus maze, radial arm maze. The key is that the apparatus consists of 3 arms available for entry arranged in the shape of a 'T' or 'Y'. For trial 1, one of the arms is blocked such that only two arms are accessible. An animal is placed in the maze at the end of one of the two arms and allowed to freely explore. A hidden food cache/platform is located at the end of the non-start arm. A session continues until the food pellets are collected (land version) or the platform is located (water version) or when the maximal trial time is reached, whichever occurs first. Should an animal fail to find the reward, it will be gently guided to the location. For the second trial, all arms are made accessible (the arm blocker is removed) and animals are again allowed to freely choose arms in which to enter. Animals must return to the same place in space to receive an additional reward. Additional trial pairs may be given within a single test day.

Once learning is demonstrated, a temporal challenge will be instilled to assess delayed memory retention performance. After an animal completes Trial 1, it will be housed in the colony room or in the testing room on the testing cart with access to food and water bottles for a temporal delay. Following this delay, animals will be returned to the maze for one additional trial.

Land version: In this test, animals are trained to collect a hidden cache of food pellets from the end of one maze arm. Animals will be food-restricted to 85-90% of their free feeding body weights as described in the PI's protocol. Once all animals achieve a reduction to 85-90% of free feeding weight, testing will begin. A daily log will be maintained to monitor the quantity of food given and body weights during food restriction.

Water Version: In this version, animals swim to locate a hidden platform at the end of one of the maze arms. At the beginning of a test session, animals will be gently placed in one of the maze arms and allowed to swim to visit additional maze arms. A session continues until a hidden platform is located at the end of a designated arm. A maximum trial time is allowed for an animal to locate a hidden escape platform within a session. Once on the hidden platform, the animal remains on it to orient to its location in the room. After this, the animal is placed in a testing cage that is heated with escapable heat from heating lamps/pads for a consistent inter-trial interval. During this time, the experimenter cleans the maze of debris (bedding, feces, etc) using a small net. The animal is replaced in the maze and swims to locate the hidden escape platform.

2.1.3.3 Delayed Non-match to Sample (aka delayed alternation)

The delayed match to sample test assesses spatial navigation memory. This version assesses short-term memory for information that changes across time (working memory). With slight modifications (inserts added to block available arms) but similar procedural components, the following apparatus may be used to carry out this test: Y-maze, T-Maze, plus maze, radial arm maze. The key is that the apparatus consists of 3 arms available for entry arranged in the shape of a 'T' or 'Y'. For trial 1, one of the arms is blocked such that only two arms are

accessible. An animal is placed in the maze at the end of one of the two arms and allowed to freely explore. Food or water restriction is not always necessary and minimal training is required. For motivated behavioral versions of this task, a hidden food cache/platform is located at the end of the non-start arm. A session continues until the food pellets are collected (land version) or the platform is located (water version) or when the maximal trial time is reached, whichever occurs first. Should an animal fail to alternate, it will be gently guided to the location. For the second trial, all arms are made accessible (the arm blocker is removed) and animals are again allowed to freely choose arms in which to enter. To successfully complete the task, the animal must shift to the previously inaccessible arm.

Once learning is demonstrated, a temporal challenge will be instilled to assess delayed memory retention performance. After an animal completes Trial 1, it will be housed in the colony room or the testing room on the testing cart with access to food and water bottles for a temporal delay. Following this delay, animals will be returned to the maze for one additional trial.

Land version: In this test, animals are trained to collect a hidden cache of food pellets from the end of one maze arm. Animals will be food-restricted to 85-90% of their free feeding body weights according to the procedure outlined in the PI's protocol. Once all animals achieve a reduction to 85-90% of free feeding weight, testing will begin. A daily log will be maintained to monitor the quantity of food given and body weights during food restriction.

Water Version: In this version, animals swim to locate a hidden platform at the end of one of the maze arms. At the beginning of a test session, animals will be gently placed in one of the maze arms and allowed to swim to visit additional maze arms. A session continues until a hidden platform is located at the end of a designated arm. A maximum trial time is allowed for an animal to locate a hidden escape platform within a session. Once on the hidden platform, the animal remains on it to orient to its location in the room. After this, the animal is placed in a testing cage that is heated with escapable heat from heating lamps/pads for a consistent inter-trial interval. During this time, the experimenter cleans the maze of debris (bedding, feces, etc) using a small net. The animal is replaced in the maze and swims to locate the hidden escape platform.

2.1.3.4 Fear Conditioning

Fear Conditioning Procedure:

This is performed in rats and mice. The instruments must be calibrated before use and a log will be kept along the instruments. The calibration check is necessary to verify that the shock is delivering at the beginning of the experiments based on Standard Operation Procedure.

To distinguish the effects of drugs/treatments on fear learning versus consolidation, the freezing levels during tone presentation over the conditioning session are analyzed. The first day, animals are habituated to the training and tone testing chambers. The Plexiglas training chamber has a metal grid floor, is dimly lit, scented (Ex: with 70% ethanol), and ventilated with a small fan. The tone testing chamber contains a soft plastic floor, is brightly lit, and scented differently than the other chamber (Ex: with 1% acetic acid). Animals are then briefly acclimated to the conditioning chamber immediately before behavioral training. For fear conditioning, animals are presented with a set number of pairings of a tone conditioned stimulus (CS) that co-terminate with a foot shock unconditioned stimulus (US). Animals remain in their conditioning boxes for a pre-set amount of time (several minutes) after the last conditioning trial.

Testing of Conditioned Fear Responses:

This is to test whether drugs/treatments impair or improve the auditory and contextual fear conditioning in mice and rats. The instruments are calibrated before use and a log is kept along the instruments. After conditioning, long-term fear memories to the conditioning apparatus (context) and to the tone are tested separately. Testing for contextual conditioning occurs in the same environment as fear conditioning. Rats or mice are placed in the chamber, and freezing responses are recorded after animals are given an acclimation period to recognize the

context. At some time point later, responses conditioned to the tone CS are measured in the novel context (described in the preceding text). Rats or mice are acclimated, after which freezing responses are recorded while subjects are presented with a series of test tones.

2.1.3.5 Inhibitory (Passive) Avoidance

This is performed in rats and mice. The instruments must be calibrated before use and a log will be kept along the instruments. The calibration check is necessary to verify that the shock is delivering at the beginning of the experiments based on Standard Operation Procedure.

Step Through:

This is performed in rats or animals using a two-compartment chamber, which consists of illuminated and darkened compartments connected by a guillotine door. During training, each rat is placed in the illuminated compartment and receives an electric shock when it enters the darkened side. This procedure is repeated immediately (to verify animals have learned) after the initial training. At a specified timepoint(s) post-training, the rat is again placed in the illuminated compartment and the retention latency to enter the darkened compartment is recorded (a pre-set max trial time should be set in the event that the animal does not move). For all retention tests, each animal is placed on the platform and the step-down latency is recorded, with an upper cut-off time; no shocks are administered.

Step Down:

This test is carried out in mice and rats using a chamber containing a wooden platform on one side of the grid floor; electric shocks are delivered to the grid using an isolated pulse stimulator. During the training, animals are individually placed on the platform and subjected to a footshock when they completely descend to the grid floor. This procedure is repeated at designated points after the initial training. Animals that stay on the platform for longer than a designated time point are considered to have learned the task and are removed to their home cages, without being given further shocks. Retention tests are carried out at pre-set timepoints after the last training session. For all retention tests, each animal is placed on the platform and the step-down latency is recorded, with an upper cut-off time; no shocks are administered.

2.1.3.6 Morris Water Maze

This test is carried out in mice and rats. The testing for animals can involve three phases: (i) a place discrimination acquisition phase in which the subject must learn and remember the location of the platform in space, (ii) a retention phase in which the animal is tested for retention of the learned behavior after several days of no exposure to apparatus, and iii) a reversal test in which the location of the escape platform is relocated to the opposite side of the maze to test for striatal-dependent learning, to match our current protocol used in animals. The animals are placed in water of a somewhat cool but comfortable temperature to motivate swimming, which has been made opaque with non-toxic coloring. The animals are trained to locate a platform just below the surface of the water, and are allowed to search while swimming until they have found it or until the maximal trial duration has elapsed. If they do not find the platform in the allotted time, they are guided to it or placed on it, remain on the platform for a pre-set amount of time (will vary depending on experimental manipulations), and are then removed from the tank. The speed with which they locate the platform and their path lengths are both monitored. The animal must learn the location of the platform relative to spatial cues located outside the maze in the testing room; this learning is referred to as place learning. A probe trial, in which the platform is removed, should be given immediately following the last training trial, to test the utilization of a spatial search strategy. However, additional probe trials may be given throughout the training period to assess platform localization. Retention tests, in which animals are placed back into the maze after a long duration (ranging from several days to several weeks). Following each trial, animals are placed in [escapable] heated testing cages (heat pads and/or heat lamps) and will have their body temperature monitored to prevent hypothermia. Should an animal fail to groom itself, it will be dried with paper towels. The

advantages of this task include the lack of a need for the traditional motivational constraints of shock or food deprivation, and the rapid acquisition of the task. This task is particularly sensitive to manipulations of the hippocampus, which is important to spatial learning in animals and to memory consolidation in humans. Because animals are good swimmers and are monitored while in the water, they never drown and do not suffer significant adverse effects from this test. During the course of this testing, an experimenter is present to monitor the animals while performing the task. Animals that cannot perform swimming or exhibit an inability to groom will be removed from the experiment.

2.1.3.7 Morris Water Maze Reversal

Following Morris water maze training, the platform will be relocated to the quadrant opposite the original training location, to test striatal-dependent re-learning/perseveration. Testing procedures will be identical to those used in the learning phase of the Morris water maze.

2.1.3.8 Object Location Test and Novel Object Recognition Test

This test is carried out in mice and rats and relies on the natural proclivity of rodents to explore novelty. No training, food restriction, nor aversive stimuli are needed to motivate behavior on this task. Animals are acclimated to the room, the apparatus and the task for several days. Once acclimation days are complete, testing days will begin. A testing session comprises two trials for object recognition and object location tests. During T1, the apparatus contains two identical objects (samples). An animal is always gently placed in the apparatus. After the first exploration period, the animal is put back into its home cage. Subsequently, after a delay interval, the animal is put back into the apparatus for T2, but while one object remains in its previous position, the other is placed in a novel location. In the object recognition test, instead of moving an object to a novel location in T2, the original object is replaced by a novel object. In addition, all combinations and locations of objects are used in a balanced design to reduce potential bias due to any preferences for particular locations or objects. The time spent exploring each object during T1 and T2 are recorded. Exploration is defined as follows: directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object is not considered exploratory behavior. To avoid the presence of olfactory trails, the objects are thoroughly cleaned after each trial with antibacterial disinfectant and alcohol. Moreover, each object is available in triplicate, so neither of the two objects from the T1 has to be reused as the familiar object in T2.

2.1.3.9 Radial Arm Maze- Land Version

This test is carried out in food restricted mice and rats (85-90% free feeding weight). A daily log is maintained to ensure close monitors of food and body weights. The test is performed in animals using an eight-arm radial maze. For the land version, animals are trained to collect food pellets from four of the eight arms in the radial maze. For one week prior to training, food rewards are placed in the home cage. Before training, the animal is placed in the maze with food rewards scattered about the arms for a habituation period and allowed to freely explore. On the first test day, arms are baited with a food reward, the animal is placed in the maze and allowed to explore freely; an experimenter records the sequence of arms entered. A trial continues until all food pellets are collected or until the maximal trial time elapses, whichever occurs first. Errors of working and reference memory (i.e., re-entries into the baited arms and entries into the unbaited arms, respectively), total arm entries, and the test duration (i.e. the time spent in the completion of all the food pellets in the maze) are recorded. Frequency of working or reference memory errors, an index of memory, is calculated as the number of working or reference memory errors, respectively, divided by the number of total arm entries; the average exploration time, a measure of general locomotor activity, is calculated as the test duration divided by the number of total arm entries. Variations of this task include rewards located in 4 of the 8 arms (which assesses working and reference memory) or rewards located in 8 of the 8 arms (which assesses working memory). After animals have demonstrated successful learning of this task, a temporal challenge is instilled to assess performance as working memory load increases. When an animal locates half of the possible food rewards, it will be removed from the maze for a delay (with access to water). Following this delay, animals will be returned to the maze to continue

locating the remaining rewards, as done on other test sessions.

2.1.3.10 Radial Arm Maze- Water Version

The water radial arm maze is a complex cognitive water-escape task that assesses spatial navigation memory, as well as memory performance under an increasing cognitive load. The maze is filled with room temperature water and the water is tinted with a non-toxic dye (food coloring or non-toxic paint powder) to obscure escape platform locations. The apparatus consists of a central arena and eight arms radiating outward. Slightly submerged hidden water escape platforms are located at the ends of the arms. At the beginning of a test session, animals will be gently placed in one of the maze arms (the start arm) and allowed to swim to visit additional maze arms. A session continues until a hidden platform is located at the end of a designated arm or until the maximum trial time elapses. If an animal fails to find a platform during the allotted time, it is gently guided to the location of the nearest platform using a plastic stick. Once on the hidden platform, the animal remains on it to orient to its location in the room. After this, the animal is placed in a testing cage that is heated with escapable heat from heating lamps/pads for a consistent inter-trial interval. During this time, the experimenter removes the just located platform and cleans the maze of debris (bedding, feces, etc) using a small net. The animal is replaced in the maze and swims to locate the next hidden escape platform. The above described process continues until all platforms have been located. Variations of this task include hidden escape platforms located in 4 of the 8 arms (which assesses working and reference memory) or hidden escape platforms located in 7 of the 8 arms (which assesses working memory). After animals have demonstrated successful learning of this task, a temporal challenge should be instilled to assess performance as working memory load increases. When an animal locates half of the possible platforms, it will be removed from the maze for a delay (with access to water). Following this delay, animals will be returned to the maze to continue locating the remaining platforms, as done on other test sessions.

2.1.4 Anxiety-related tests

2.1.4.1 Hole board test

Rats and mice are used in this test, which is carried out in an open box with four holes in the floor. Each animal is placed individually in the center of the floor and allowed to explore. The number of head-dips and the time spent in head-dipping are recorded.

2.1.4.2 Elevated Plus Maze

This test is carried out in mice and rats. The plus maze is elevated above the floor in a dimly or brightly illuminated test room (depending on study goals) and consists of two arms that are open to the room and two that are closed such that the floor is not visible. Cushioning is placed below the open arms in case an animal should fall from the ledge. Shifts in preference for the closed versus open arms under different lighting conditions will be used to detect anxiolytic and anxiogenic effects. For testing, the animal is quickly and carefully lowered into the center choice zone where the arm pairs intersect. (A derivation is to place the animal at the end of one of the open arms). Animals are allowed to freely explore the apparatus. The animals' path is tracked by tracking software. Between animals, the maze is cleaned of debris with anti-bacterial spray and alcohol, to disrupt any odors. Dependent variables include distance and duration in open vs closed arms, frequency of entry into open and closed arms, and the number of fecal boli excreted.

2.1.4.3 Light-dark transition test

Rats or mice are individually placed in the dark compartment of a light-dark chamber. The ability of treatment prior to the test to alter anxiety-like behavior is determined by the following measures: the latency to cross

through the hole into the light compartment, the time spent in the light compartment, and transitions, defined as the number of crossings from the dark compartment to the light side.

2.1.4.4 Marble Burying

This is carried out in mice. Mice are placed in a standard mouse cage containing bedding and a pre-set number of marbles, spaced equally along the edge of the cage. A wire-mesh lid is placed on the cage. Number of marbles buried at the end of the session is quantified. A marble is considered buried if it is at least 2/3 beneath the surface of the bedding.

2.1.4.5 Open Field (see also spontaneous locomotion)

The open field test assesses locomotor and anxiety-like behavior in a novel environment. Mice or rats are placed into an open arena and allowed to freely explore. Depending on experimental goals, lighting conditions may be dimmed or a bright light above the center of the maze. Between animals, the apparatus is cleaned of debris and wiped down with antibacterial spray and alcohol to disrupt any odors. Data are gathered using a tracking system.

2.1.4.6 Social Investigation

Following an acclimation period to the testing room, a single test animal will be placed in a rectangular open field for a set time period to determine locomotion. For the first trial, the test animal will then be temporarily removed from the box and an ‘interaction animal’ (a new animal with whom the test animal has never had contact) will be placed in a small containment box on one side of the open field. The box will have slats in it to limit physical interaction between animals while still allowing for olfactory interaction. Another small containment box (not containing another animal) will be placed on the opposite side of the open field. The test animal will then be replaced in the open field and the duration of time spent investigating each containment box will be recorded. For the second trial, the test animal will be briefly removed again and a novel animal will be placed in the previously empty containment box such that both containment boxes will contain ‘interaction rats’ (one novel and one familiar) and exploration of each box by the test animal will be measured. Between test animals, the apparatus will be thoroughly cleaned. An experimenter will be present at all times to observe animals undergoing the test.

2.1.5 Depression-related tests

2.1.5.1 Differential reinforcement of low rate (DRL) operant schedule (likely Category C)

This is carried out in mice and rats. Water is restricted to one-hour periods following each daily DRL session, whereas food is freely available in the home cage. A daily log is maintained to ensure the experimenter closely monitors of water consumption and body weight. The animals are trained to press a lever in order to obtain access to water. Once lever pressing is established, the schedule requirements are changed so that ultimately only those responses that follow the previous response by a minimum interval are reinforced, requiring the animals to make a temporal discrimination. The minimum interval for rats is 72 sec (DRL72) and for mice is 36 sec (DRL36).

2.1.5.2 Forced swim test (FST) (likely Category E)

The forced swim test (for mice and rats) is a commonly used animal model for drug screening of antidepressant-like activity, which induces a depressed state in mice or rats by forcing them to swim in a narrow cylinder from which they cannot escape. Animals are individually placed in a cylinder filled with water (25°C at least), allowing for free swimming. For rats, there is a training followed by a test. Mice are only tested once. The duration of immobility, diving, swimming and climbing will be recorded. Immobility is defined as floating in an upright position without additional moving other than that necessary for the animal to keep its head above water. Swimming is defined as movement throughout the swim chamber and the climbing is upward-directed movements of the forepaws along the side of the swim chamber. Animals are then removed from the water,

dried with paper towel, and placed into plastic cages with a heating pad/lamp for at least 5 min. Animals are also monitored until the risk of hypothermia is gone (until their hairs are completely dry).

2.1.5.3 Novelty suppressed hypophagia (likely category C or E, depending on feeding schedule)

This test is carried out in mice and rats. As an index of emotionality, the latency to start eating a food pellet is monitored in food-restricted or non-restricted animals in a brightly illuminated chamber/new rodent cage.

Food restricted method: Briefly, animals are singly housed and are deprived of food at least 24 h prior to the test. A daily log is maintained to ensure close monitors of food and body weights. On the day of test, each animal is placed in the corner of a white, plastic, open chamber. A food (regular chow; sucrose solution, etc) is placed in the novel environment. The latency to begin to consume the food and the total amount consumed is recorded. Exposure durations will vary.

Non-food restricted method: On the first day of this test, animals will be individually housed in order to accurately assess food consumption. For habituation, in the home cage, animals will be presented the highly palatable food, (EX: sweetened condensed milk diluted with sterile water at a 1:3 sucrose to water ratio) for a set period of time each day. On another test day, food consumption will be measured in the home cage environment under normal lighting conditions in a testing or home room. On a separate test day, novel environment testing will occur by placing animals in a new cage with no bedding and in a brightly lit environment (testing room). For each test day, animals will be exposed to the highly palatable food and the latency to drink and total quantity consumed will be recorded. For this version using a highly palatable food, no food restriction is necessary. Exposure durations will vary.

2.1.5.4 Repeated open-space forced swim procedure (likely category E)

This test is carried out in mice and rats. The repeated open-space forced swim model produces a mildly stressful stimulus and an activation of neural changes that are similar to those occurring in human depression. Swimming is carried out in tub cages, containing tepid tap water. Animals are individually forced to swim for an extended pre-set duration min/d on several days. Animals are removed from the water, dried with paper towel, and placed into plastic cages with a heating pad (underneath the cage) for at least 5 minutes. Animals are also monitored until the risk of hypothermia is gone (i.e., until their hair is completely dry).

2.1.5.5 Sucrose consumption test (likely category C)

This method is carried out in rats and mice. Sucrose is available in a standard drinking bottle or similar apparatus and intake is measured by weighing the bottle before and after the test. Duration of exposure will vary

2.1.5.6 Tail suspension test (likely category E)

This test is carried out in mice. Tail suspension test was based on the method of Steru et al. (1985), which is a non-escapable stressful situation and is widely used for measuring antidepressant-like activity in drug discovery research in rodents. Each animal is suspended using adhesive tape placed from the tip of its tail. The duration of immobility (i.e. passive hanging and complete motionlessness) is recorded during the test period. The ability of treatment to alter immobility is determined. Care will taken to remove tape from tail and if needed, tape will be removed using warm water and soap. Lightweight, plastic cylinders will be placed around the tails of mice that have a tendency to climb their tails during this test.

2.1.6 Visual and locomotor competence/control tasks

2.1.6.1 Non-spatial Cued Visible Platform

This is a widely used control task to confirm visual and motoric capacity among experimental subjects. This is a task that requires animals to swim to a non-hidden visible platform that is placed in a tub filled with clear, room temperature water. Each animal is gently placed in the maze from one of several possible start locations. If the

animal does not find the platform within the allotted trial time, it is gently led to the platform by the experimenter. Latency to escape, speed and distance swum are dependent variables. Once an animal locates a platform, it is removed from the maze, placed in a dry testing cage under escapable heat provided by heating lamps/pads to prevent hypothermia and monitored by the experimenter until fur is dried.

2.1.6.2 Spontaneous Locomotion

This test is carried out in mice and rats. This assessment will involve a short session in which different components of spontaneous locomotion will be recorded after placement of animals in an automated photocell apparatus. Each animal will be placed in the test chamber and allowed to freely explore. Movements in the horizontal plane as well as a vertical plane will be detected by the photocells and processed by software to yield locomotor information.

2.1.7 Pain

2.1.7.1 Hot Plate

The hot plate test detects differences in pain sensitivity. For this test, the hot plate is heated to a pre-set temperature. Animals are placed on the hot plate and prevented from escape by a tall plastic cylinder or rectangle. The latency to contact and licking of a hindlimb, flinching, jumping, or other first sign of nociception is recorded. If an animal does not demonstrate nociception, it will be removed from the test at the pre-set cutoff time (a standard time point used in other studies assessing pain. An experimenter will be present at all times to observe animals undergoing the test.

2.1.7.2 Tail Flick

This test detects differences in pain sensitivity. The apparatus emits a high intensity beam of light, under which the tail is placed. The animal flicks its tail out from under the beam when the pain is detected and becomes uncomfortable. Maximum trial durations can be set as a safety feature, to prevent prolonged beam exposure and reduce the chance of tail injury. As well, pilot data should be collected to determine the intensity level needed so that untreated animals flick their tails between 3-5 seconds from start of beam exposure. An experimenter will be present at all times to observe animals undergoing the test.

3. Housing of animals during studies

Animals will be housed in a room designated for holding animals within OLAR involved in behavior studies so they can be transported to testing rooms in the RBC.

4. Transport of Animals to RBC

Wherever possible, to reduce the allergen load, animals should be transferred to new clean cages (sometimes reused if repeated trips in a relatively short period is required) for transport to RBC testing. In addition, for transport of animals to the RBC testing rooms, on a given testing day, if required by the experiment (EX: water maze testing) so as not to contaminate the home cage environment, animals will be removed from their home cages in the colony room and placed individually into testing cages. Animals will then be transported from the colony room to the testing room. Testing cages will be changed to coincide with that experiment, (EX: when completed), or as needed (should the cage bedding become sufficiently soiled). Testing cages will not typically have food and water; therefore, should testing be prolonged by more than 4 hours so food is withheld this long, as during a single test day (EX: a delay challenge between trials), food and water will be provided.

5. Personal Protective Equipment

Full PPE is required for use of the behavioral testing facilities; individual needs may exceed these minimal requirements as determined on a case by case basis by Occupational Medicine. A disposable gown, mask and hair net are put on in the hallway. Booties will be put on using the step-in method. Gloves are put on before, at or immediately after room entry. In animal holding rooms, the gloves are kept wet with disinfectant when handling cages or animals. In the testing areas this practice should be continued whenever it will not interfere with testing. In addition, N-95 respirator and safety glasses are available upon request. Proper use of N-95 respirators requires fit testing to assure proper function. When exiting, all PPE except booties is removed at the point of exit. Booties are removed using the step-out method. The investigator has the option of wearing medical scrubs under the above listed garments and these scrubs should be washed in the OLAR washing machine before use with animals. For water maze testing, experimenters have the option to wear a plastic apron atop the gown supplied by RBC personnel.

6. RBC Cleaning Procedures

All surfaces that come into contact with an animal must be cleaned thoroughly. RBC uses an OLAR approved disinfectant (Virkon, etc.) on the behavioral instruments and procedure tables and surfaces unless the selected agents interfere with the equipment's proper use. In such cases OLAR may allow other agents to be used, with the proviso that high level disinfectant application be applied between studies. Surfaces will be cleaned prior to beginning a behavior test, between cages of animals and after the completion of a behavior test. Isopropyl alcohol or ethanol can be used between animals from the same cage in addition to antibacterial spray to disrupt odors. Because Virkon is potentially corrosive, its use should be followed with cleaning with a water-dampened paper towel on devices that can corrode (computer keyboards, stainless steel countertops, etc). Multiple users can utilize the same apparatus within a day (with the exception of water-based tasks) but the below listed cleaning procedures must be observed.

Daily cleaning of testing rooms in the RBC is described below. Inspections will be conducted following the completion of any behavioral experiment and regularly on the first Monday of every month by the RBC director. Any issues will be detailed in an email report. At the beginning of each test day, countertops or surfaces upon which testing cages will be placed will be wiped down with antibacterial spray and if needed, alcohol. Any maze apparatus (except for water mazes) which the animal will come into contact with will also be wiped down with antibacterial spray and/or alcohol. On an as needed basis (approximately once per week) the floors will be mopped and walls will be cleaned by OLAR staff or researchers, as dictated by the experimental timeline. Daily upon completion of behavioral testing, the following steps will be taken to clean the apparatus and room as appropriate for the given test:

- Countertops and surfaces wiped down with antibacterial spray and if needed, alcohol
- Maze apparatus wiped down with antibacterial spray and, if needed alcohol
- Flooring swept to remove bedding, excreted fecal boli, etc

- 1-2 buckets full of water in water mazes removed and fresh water replaced
- Water maze bottoms swept with nets to remove bedding, fecal boli, etc
- Trash removed to large trash receptacles near cage wash area

In the event that ABSL-2 animals will be tested in the RBC, all of the above cleaning procedures will be utilized. In addition, the room will be mopped daily. As well, in the event of multiple users of a given room/apparatus, ABSL-2 animals will be the final cohort of animals tested in the apparatus in a given day; no additional animal testing can be conducted following ABSL-2 animals.

7. Behavior and Experimental Procedure Documentation

After behavioral testing has been completed daily or at the end of studies, the procedures and any observations will be documented. Examples of documentation include testing sheets, calendars, study notebooks, OLAR cage cards, etc. Documentation should include test dates, procedures, observations and identification of experimenter. It is the responsibility of the PI to ensure the proper storage of data collected in the RBC. The RBC is not responsible for maintaining backups of data long-term.

8. Injections and Exogenous Treatment Administration

No medications, pharmaceutical agents, chemical compounds or other treatments will be administered in the RBC testing rooms, unless experimentally necessary and prior approval from the RBC director is granted. Any treatment administration should be done in qualified personnel will inject medications, anesthetics, and/or experimental substances via one or more of the following routes as dictated by study needs. Any and all materials/agents must be removed from the RBC rooms at the completion of each test day.

9. Subject Identification

The PI is responsible for identifying animals used in RBC studies and for informing any RBC staff completing testing of the identification method. The PI may choose to identify animals with tail tattooing and/or ear clipping. The tail tattooing, ear tags and ear clipping (etc) methods of identification involve only momentary restraint as well as momentary pain; No anesthesia is required with skilled application of the method of identification. Such methods, depending on their intensity may have to be detailed in the PI's protocol.

10. RBC Contacts

If you have any questions or comments, please contact:

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