Defensive Responses to Predator Threat in the Rat and Mouse

Defensive responses include an array of specific behaviors, including flight, avoidance, freezing, risk assessment, and defensive threat/attack, that are elicited by unconditioned threat stimuli such as predators or predator odors. These behaviors, in rodents, are unconditioned and relatively resistant to habituation. They do not require pain or any previous experience of the predator in order to occur strongly and systematically upon encountering predator-threat stimuli. Determination of the specific defensive behaviors elicited depends on features of both the threat stimulus and the situation in which it is encountered. Highly structured threat situations such as the mouse defense test battery (MDTB; see Basic Protocol 1) involve manipulations of both the situation and the threat, such that individual defensive behaviors may be reliably elicited and measured at the instigation of the investigator, whereas less-structured tasks, such as the rat exposure test (RET; see Basic Protocol 2), allow the subject to display a pattern of defenses that may change over time, reflecting feedback from its own defensive behaviors in the situation. This unit describes these two tasks for mouse subjects, both utilizing rats as threat stimuli, but differing in the structure afforded by the protocol, as well as in the provision (in the RET) of a hiding area that promotes avoidance and risk assessment from this place of concealment. These tests have been used extensively to evaluate drugs that are potentially effective against anxiety disorders, and they both show strong sensitivity to different mouse strains (Blanchard et al., 2003a; Yang et al., 2004).

Predator odors provide an additional means of eliciting defensiveness and are more convenient and practical when testing rat subjects, due to the difficulty and expense of maintaining rat predators such as cats in a laboratory. However, partial predator stimuli such as odors are inherently more ambiguous, with reference to the possibility of predatory threat, than is confrontation with a living predator. Ambiguity of threat is an important factor in risk assessment, a pattern of behavior used for gathering information about the threat. Thus, predator odors tend to strongly elicit risk assessment behaviors while not producing defenses that rely on the presence of a corporeal threat, i.e., defensive threat and attack. Defensive behaviors to predator odors, like those elicited by predator exposure, respond selectively to anxiolytic drugs. However, predator odors are also coming to be used in studies aimed at analysis of rapid conditioning of defense to contextual stimuli. A single, relatively brief, experience of cat fur/skin odor in a particular context will produce conditioning of behaviors such as risk assessment and freezing, as well as avoidance of specific cues that previously exuded the predator odor. Stimuli that are less predictive of the immediate presence of the predator, such as cat feces, appear to be less capable of supporting this rapid conditioning phenomenon. Two predator odor tasks (see Basic Protocol 3 and Alternate Protocol 1), differing in the provision of a hiding area and in the specific method for obtaining cat fur/skin odor, are also presented. Both elicit defensive behaviors, and produce rapid conditioning. The most prominent behaviors shown on the conditioning test day are avoidance and risk assessment, which provide a useful counterpart to more traditional emotional conditioning tasks that strongly emphasize conditioned freezing.

Exposure to predators or predator odors is increasingly used in studies of brain systems potentially related to emotionality. The behaviors elicited in such tests provide important verification of the emotionality engendered by the procedure, providing an additional rationale for measurement of defensiveness, and raising the possibility that procedures

Contributed by D. Caroline Blanchard, Robert J. Blanchard, and Guy Griebel
Current Protocols in Neuroscience (2005) 8.19.1-8.19.20

selectively eliciting specific defensive behaviors may be analytically useful in determining specific brain systems underlying these individual behaviors. Another potential approach to such analysis is suggested by the fact that some inbred mouse strains show minimal levels of specific defensive behaviors, indicating genetic factors in the patterning and expression of defense to predator stimuli.

The four tasks presented in this unit utilize mouse or rat subjects, as indicated. They provide a variety of situations, threat stimuli, and degree of structuring to facilitate the attainment of different experimental goals. Each should involve standard groups of 8 to 12 subjects, and all should be performed by experimenters and/or raters blind to the experimental (e.g., drug) condition of the animals. Because drugs are often used in these tasks, the protocols are written to include references to drug conditions, but they are suitable for use with other (e.g., genetic, experiential) independent variables as well.

Strategic Planning

A number of choice-impacting factors are outlined under the various Basic Protocols and are not discussed here. However, one decision strongly impacting experimental design that is common to Basic Protocols 2 and 3, and Alternate Protocol 3, but not Basic Protocol 1, is the inclusion of experiential controls. For all except Basic Protocol 1, animals are tested in familiarized situations to which they should show relatively little defensiveness. As a control for factors associated with the presence of predator (rat) or of predator odor threat stimulus (a block covered with fabric or a cloth cat collar exuding cat fur/skin odor), a plush toy animal (see Basic Protocol 2) or the block or cat collar without fur/skin odor (see Basic Protocol 3 and Alternate Protocol 3), respectively, are used. Because cat fur/skin odor may cling to the test apparatus or other objects in the immediate vicinity and compromise behavior to the odorless controls of these three protocols, use of a single apparatus/testing room for experimental (predator or predator odor) groups and no-odor control groups is problematic. Thorough cleaning of the apparatus and its immediate environs (i.e., the bench or table holding the apparatus) at the end of an exposure day is sufficient to preclude odors from lingering to contaminate conditioning test day results. However, such thorough cleaning is generally too time-consuming to be done between trials, when odor and no-odor trials might be interspersed. Therefore, it is recommended that two apparatuses and two test rooms be employed if an experiential (no-odor) control is to be used. A possible alternative, if two similar spaces for two apparatuses are not available, might be to run odor and no-odor animals on separate days, with thorough cleaning at the end of each day; this option runs the risk of day effects. Another alternative, if the focus is on the effects of drugs or other manipulations on response to the threat stimulus only, might be to omit the experiential (no-odor) controls and concentrate only on drug-vehicle differences in response to threat. In this case, each of the three basic protocols could be modified to utilize only a single apparatus/testing room, and the number of groups can be reduced accordingly.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

BASIC PROTOCOL 1

USE OF MOUSE DEFENSE TEST BATTERY (MDTB) TO TEST DEFENSIVE BEHAVIORS OF MICE TO AN ANESTHETIZED RAT

This procedure elicits and measures reactions to both present and anticipated threat (a laboratory rat, under conditions of varying imminence). In an oval runway, animal subjects show an extremely precise delineation of defensive behaviors including flight, avoidance, risk assessment, sonic vocalization, defensive threat and attack, and escape attempts with each behavior controlled by distinctive characteristics of the threat stimulus

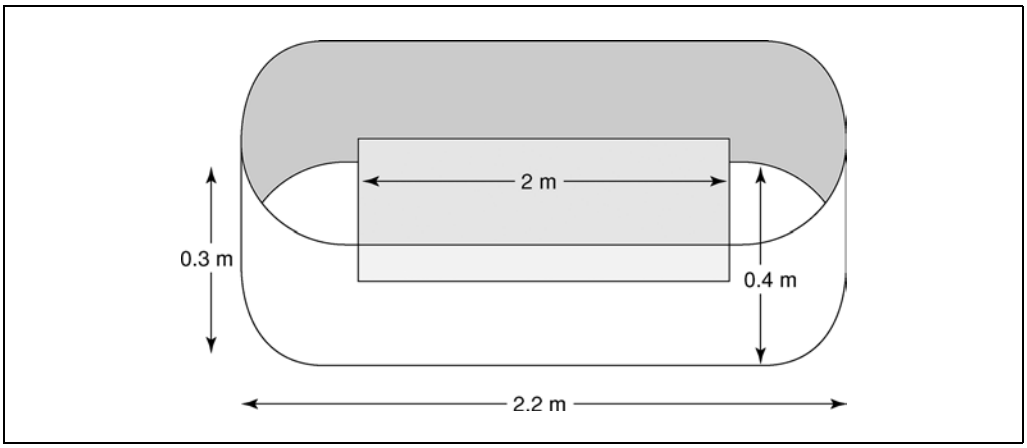

Figure 8.19.1 The runway apparatus is an oval runway made of black Plexiglas, 0.40 m wide, 0.30 m high, and 4.4 m in total length, consisting of two 2-m straight segments joined by two 0.4-m curved segments and separated by a median wall (2.0 × 0.30 × 0.06–m). The apparatus is elevated to a height of 0.80 m from the floor to enable the experimenter to easily hold the stimulus rat while minimizing visual contact with the mouse. The floor is marked every 20 cm to facilitate distance and activity measurement, and doors, 60 cm apart, (not shown on this sketch) are located near one end of the apparatus.

and situation. Anxiolytic drugs attenuate but do not completely suppress these defensive behaviors. These effects are not secondary to general motor impairment or associative learning deficits. Anxiogenic drugs increase defensive behaviors. Use of isolated male mice is recommended. The test can be used in a wide range of mouse strains, but distinct strain differences are reflected in baseline levels of defensiveness.

Materials

Adult male mice (e.g., Swiss strain)
Adult male rats
Drugs to be tested
CO_2
Laboratory detergent (mild)
Saline or other vehicle for control injections
Standard single mouse cages
Video camera (optional: television screen connected to video camera, located in an adjacent room)
Runway apparatus (Fig. 8.19.1; custom-made)
Quiet test room away from disturbance (run tests under red light)

Prepare animals

1. Singly house adult male mice for 7 days before testing. Provide food and water ad libitum.

   The Swiss strain of mouse is recommended because these animals have high levels of defensiveness.

2. Mount a video camera vertically above the runway apparatus.

3. Bring mice into a holding area immediately adjacent to the test room at least 1 hr before testing. Randomly allocate animals to the various treatment groups and administer drug at an interval appropriate to the drug and the route of administration.

   Test the mice during the light portion of the light/dark cycle. Standardize the time of testing to minimize diurnal variation.

4. Euthanize the stimulus rat by CO_2 inhalation and bring it into the test room.

5. Record all sessions on videotape.

   A time-saving step is to observe the mouse from a television screen in an adjacent room during the pre- and post-tests, and score pre- and post-rat activity and behaviors from the television screen as they occur.

Familiarize subjects with test arena

6. Pretest (3 min) by placing a mouse in the middle of the runway apparatus. Allow 3 min of free exploration and count line crossings, wall rears, wall climbs, and jump escapes.

   This may be done from a television monitor in the adjacent room.

Initiate and score defensive behaviors

7. Perform rat avoidance test (minutes 4 to 6). Immediately after the 3-min familiarization period, introduce the hand-held stimulus rat at one end of the runway, 2 m from the subject. Bring it up to the subject at a speed of ~0.5 m/sec, initiating approach only if the subject is at a standstill with its head oriented towards the hand-held rat. Consequently, intervals between trials are variable, but never exceed 15 sec. Terminate approach when contact with the subject is made or the subject runs away from the approaching rat. If the subject flees, record avoidance distance (the distance from the rat to the subject at the point of flight). Remove the rat from the apparatus between each trial so that there is no visual contact between the threat stimulus and the subject. Repeat for a total of five approaches.

8. Perform chase/flight test (minutes 7 to 8). Introduce hand-held rat at a distance of 2 m from the subject, and initiate chase only when subject is at a standstill with its head oriented toward the rat. Bring rat up to the subject at a speed of ~2 m/sec. Terminate chase when the subject has traveled a distance of 15 m. During chase, maintain a constant distance of 20 cm between the two animals. Consequently, if subject stops fleeing before traveling the full 15 m, stop the chase in order to avoid contact between the two animals; resume by moving the hand-held rat quickly from left to right in front of the subject to elicit flight. Record the following parameters: flight speed (measured when the subject is running straight; Fig. 8.19.2), number of stops (pauses in movement), orientations (subject stops, then orients the head toward the rat; see Fig. 8.19.3), and reversals (subject stops, then runs in the opposite direction). Remove the rat after the chase is completed.

9. Perform straight alley test (minutes 9 to 11). Convert the runway to a straight alley, in which the subject is constrained, by closing the two doors (60 cm distant from each other). Introduce the rat in one end of the straight alley, 40 cm from the mouse subject, and maintain hand-held rat where it was introduced during the entire test period (session is initiated when the subject faces the rat). During the next 30 sec, record the following measures: immobility time and the number of approaches/withdrawals (to be counted as an approach/withdrawal, the subject must move at least 20 cm forward from the closed door, then return to it). After this session, remove the rat from the straight alley area.

10. Perform forced contact test (minutes 12 to 13). Bring the rat up to contact the subject in the straight alley. Direct approaches quickly (within 1 sec) towards the head of the mouse subject. For each such contact, record vocalizations, upright postures, bites (Fig. 8.19.4), and jump attacks by the subject. Remove the rat from the apparatus if no defensive threat and/or attack responses are elicited within 15 sec. Repeat this test three times, with the time interval between each trial being ~5 ±1 sec.

Figure 8.19.2 This photograph shows flight, which is locomotion directed away from the oncoming threat source.

Figure 8.19.3 This photograph shows risk assessment behavior. During the chase, subject stops, then orients its head towards the hand-held rat.

11. Perform post-test (minutes 14 to 16). Remove the rat immediately after the forced contact test and open the doors to convert the straight alley back to an oval runway. Record line crossings, wall rears, wall climbs, and jump escape attempts (Fig. 8.19.5) during a 3-min session in the absence of the rat.

12. Remove any feces and wipe up urine with water after each trial. At the end of each day, wipe the apparatus with water and mild laboratory detergent.

Figure 8.19.4 This photograph shows defensive upright posture and biting. Upon forced contact with the hand-held rat, subject displays a typical terminal defense response, consisting of sonic vocalization, upright posture, and defensive attack behavior.

Figure 8.19.5 This photograph shows a typical escape attempt. Following the removal of the rat from the runway cage, subject attempts to escape from the place where it has been confronted with the threatening stimulus.

Analyze data

13. Compute average scores for all measures described under each test (steps 6 through 11).

    (a) Measure flight by: (1) avoidance distance (inversely related to flight) and (2) flight speed.
    (b) Measure risk assessment by: (1) stops, orientations, and reversals in the chase/flight test, and (2) approach/withdrawal in the straight alley test.
    (c) Measure freezing by the time the subject spends immobile in the straight alley test.
    (d) Measure defensive threat/attack by upright postures and vocalization, and by jump attack and biting, respectively, in the forced contact test.
    (e) Measure contextual defense by enhanced wall climbs and rears, and escape attempts in the post-test, compared to those measured in the pretest
    (f) Measure locomotion by the number of line crossings in the pretest.

USE OF RAT EXPOSURE TEST (RET) TO EVALUATE MOUSE DEFENSIVE RESPONSES TO A LIVE RAT

BASIC PROTOCOL 2

This protocol elicits and measures defensive responses of a mouse to a live rat presented in a familiar location in which the mouse subject can move between protected areas (the "home box" and "tunnel") to an open area in which the rat threat stimulus is located, but confined by a wire mesh screen. Features of this situation that differentiate it from the MDTB (see Basic Protocol 1) include the provision of a safe area from which the subject can control its own proximity to the threat stimulus; manipulable substrate such as wood chips or other rodent bedding material that can be used to display an additional defensive behavior, defensive burying (*UNIT 8.3*); and lack of experimenter manipulation of the environment, or of the threat and its distance from the subject, during the test session. This lack of experimenter manipulation enables the subject to show changes over the 10-min test period that may reflect the results of its own behaviors, notably risk assessment, rather than changes in the environment or the threat. The RET provides measures of avoidance and defensive burying, but its focus is on risk assessment. Contextual conditioning to the RET chamber is evaluated through an additional test session lacking the threat stimulus.

The activity of the threat stimulus, the rat, is increased and stabilized by administration of amphetamine to this animal prior to its use in the test situation.

Materials

Adult male mice
Adult rats (threat stimuli)
Control stimulus (plush toy animal about the same size as the rats to be used)
Drugs to be tested
Saline or other vehicle for control injections
5% alcohol
Mild laboratory detergent
D-amphetamine
Standard single mouse cages
Two identical RET apparatuses (custom-made; Fig. 8.19.6)
Two quiet, darkened rooms, of similar dimensions and construction, free from disturbances
Video camera

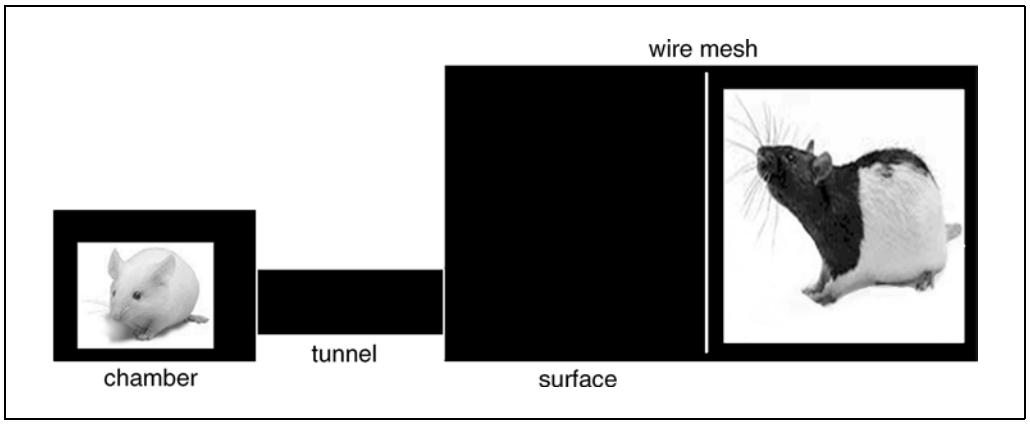

Figure 8.19.6 The RET apparatus apparatus is a 46.0 × 24.0 × 21.0–cm clear polycarbonate exposure cage covered with a metal lid. The exposure cage is divided into two equal sized compartments by a wall-to-wall 0.25- to 0.5-in. wire mesh screen. The home chamber is a 7 × 7 × 12–cm box constructed of black Plexiglas on three sides and clear Plexiglas on one side to facilitate videotaping. The home chamber is connected to the larger exposure cage by a clear Plexiglas tube tunnel 4.4 cm in diameter, 13 cm in length, and elevated 1.5 cm from the floor of the two chambers.

1. Singly house adult male mice for at least 4 days prior to use. Provide food and water ad libitum.
2. Set up an RET apparatus in each of two test rooms, one room and apparatus to be used for presenting the rat stimulus, one for the control (plush toy) stimulus. Mount a video camera horizontally, facing the clear Plexiglas wall of each RET apparatus.

   The second, identical, RET apparatus is needed to ensure that cat odor does not cling to the apparatus or the testing room and confound results for the control animals.
3. Randomly allocate mice to experimental (threat) and control (plush toy) conditions, and to treatment conditions (drug and dose conditions in this example) within the threat and control groups.

   If the drugs, doses, and mouse strain to be used have previously been investigated in the context of novel situations and there is evidence that these drug/dose combinations do not influence behavior of these mice in the absence of threat, it may be legitimate to omit the control (plush toy) condition, and focus only on comparisons of drug/dose combinations with a vehicle control group; all such groups having been tested with the rat stimulus. In this case, the second test room, the second apparatus, and the plush toy control stimulus may be omitted, and the following steps taken to apply only to mice confronted by the rat.
4. Just prior to the start of each habituation or test session for a given subject, place home cage bedding of that subject on the floor of the home chamber as well as on the mouse side of the surface (exposure) cage.
5. Habituate each mouse to its respective threat or control apparatus by placing it in the apparatus without a stimulus present for 10 min/day for 3 days.

   Sessions may be run under either low ambient room illumination or under 100-W red light. However, lighting should be consistent across all groups and sessions. All sessions, habituation, threat test session, and conditioning test session for a given animal should be conducted at approximately the same time of day on successive days with all animals tested during the same phase of the light/dark cycle.
6. Between trials, remove and dispose of bedding, clean each part of the apparatus with 5% alcohol, and dry with paper towels. At the end of each day, clean each apparatus thoroughly with water and a mild detergent, followed by extensive rinsing to remove possible detergent odor.

7. On day 4, 20 min prior to the first trial, administer D-amphetamine (5.0 mg/kg, i.p.) to the rat to be used as the threat stimulus.

   These D-amphetamine injections both produce an increase in activity accompanied by stereotyped movements, and standardize and reduce variation in activity from one mouse trial to the next. To avoid trial-to-trial changes in rat activity levels, a new rat should be used after every five trials. However, rats can be re-used on successive days.

8. Administer drug at the interval appropriate for the drug and for the route of administration.

9. As during each habituation day, place home cage bedding of each subject on the floor of the home chamber as well as on the mouse side of the surface (exposure) cage just prior to running that subject (discard bedding after this use).

10. Place a mouse subject in the subject side of the rat or control apparatus and immediately add the rat or the plush toy control stimulus, as is appropriate to its condition, to the threat stimulus/control side of the apparatus. Videotape each 10-min session. Clean the apparatus with 5% alcohol and wipe dry with paper towels between subjects.

Score defensive behaviors

11. From the videotape records, score the following defensive behaviors.

    (a) Measure spatiotemporal behavior by: (1) time in the home chamber, (2) time in the tunnel, (3) time in the exposure chamber, and (4) time in contact with the wire mesh screen.
    (b) Measure risk assessment by (1) stretch-attend (frequency and duration): animal faces stimulus (rat or plush toy) with fore- and hind-limbs far apart and body elongated with a low back; and (2) stretch-approach (frequency and duration): animal moves toward stimulus with an elongated body and low back.
    (c) Measure freezing (duration) by time spent immobile.

Analyze data

12. Compute average durations spent in each area. Evaluate avoidance as increased time in the home chamber and reduced time in the exposure chamber, or as contact with the screen. Evaluate risk assessment and freezing as described in step 11.

TESTING RAT DEFENSIVE RESPONSES TO CAT ODOR AND CONDITIONING TO ASSOCIATED CONTEXTUAL STIMULI

BASIC PROTOCOL 3

This protocol elicits and measures defensive responses of a rat to the fur/skin odor of a cat, presented in a familiar location. Cat fur/skin odor may elicit avoidance, risk assessment (investigation of the odor stimulus), and freezing. A single, 10-min cat fur/skin odor exposure is capable of producing defensive conditioning to the context in which the odor is presented.

Materials

Adult male rats
Adult cat (odor donor)
Drugs to be tested
Saline or other vehicle for control injections
9 × 9 × 2–cm Plexiglas blocks
Terry cloth
Single rat cages

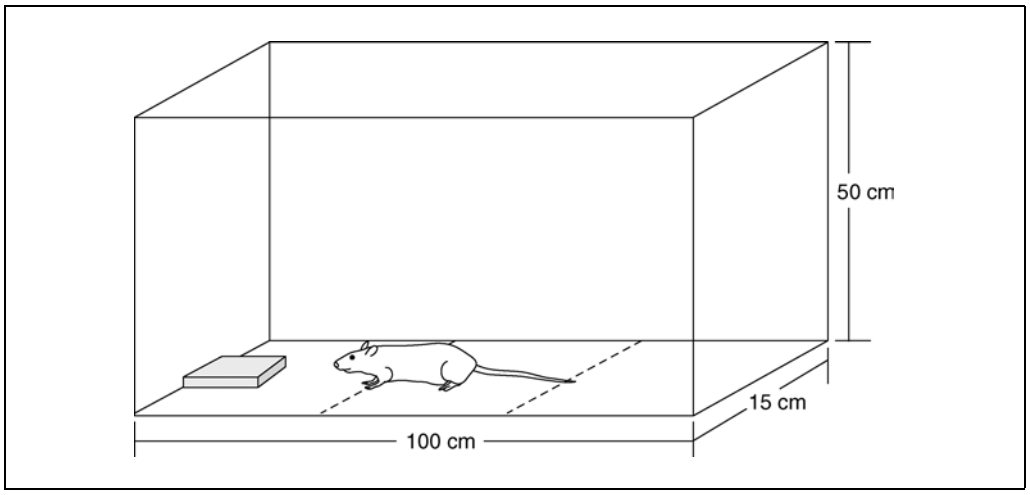

Figure 8.19.7 Each cat odor apparatus is a 100 × 15 × 50–cm white Plexiglas box, with the front wall of clear Plexiglas to allow videotaping. The compartment is divided by lines on the floor into three segments, each 33.3 cm in length.

Two quiet, darkened rooms of similar dimensions and construction, away from disturbances
Video camera
Two identical cat odor apparatuses (Fig. 8.19.7)

1. Prepare the odor stimuli by covering six 9 × 9 × 2–cm Plexiglas blocks with clean terry cloth, stapling or sewing the fabric so that it completely covers each block and adheres with no loose ends. Leave two blocks in the bed of the cat for a minimum of 2 days prior to testing. Reserve the additional four (no-odor) blocks in a sealed, labeled, plastic bag.
2. Singly house adult male rats for 4 days prior to use. Provide food and water ad libitum.
3. Set up a cat odor apparatus in each of two test rooms, one room and apparatus to be used for presenting odor stimuli and one for control, no-odor stimuli. Mount a video camera horizontally, facing the clear Plexiglas wall of each cat odor apparatus.
4. Randomly allocate rats to odor and no-odor conditions, and to manipulation conditions (drug and dose conditions in this example) within the odor and no-odor groups.
5. Habituate each rat to its respective cat-odor or no-odor apparatus by placing it in the apparatus without an odor block, for 10 min/day for 3 days. If drugs are to be given on the test day, administer saline just prior to each habituation session via the same route as the drug. Videotape these habituation sessions.

   Conduct all sessions, habituation, odor test session, and conditioning test session, under red light illumination. Run all sessions for a given animal at the same time of day ±1 hr, on successive days, with all animals tested during the same phase of the light/dark cycle.

6. On day 4, just prior to running the first experimental rats in an odor test, rub both of the odor blocks that had been left in the cat's bed, on the cat's fur, for a minimum of 3 min. Stroke each block along the sides and back of the cat and under its neck. Place one of these odor blocks in the cat-odor apparatus, adjacent to a short end of the alley. Place the other odor block in a sealed, labeled, plastic bag, for use if the initial block becomes soiled.
7. For controls, place one of the reserved no-odor blocks in the no-odor apparatus, in the same location as the cat-odor block in the odor apparatus.

8. Administer drug at the interval appropriate for the drug and for the route of administration.

9. Place a rat in the apparatus containing the odor block, or the no-odor block, as is appropriate to its condition, at the opposite end and facing away from the block. Videotape each 10-min session.

10. After each trial, remove any feces and wipe up urine with water. At the end of the test day clean each apparatus thoroughly with water and a mild detergent or an unscented soap. Rinse thoroughly to remove any residual detergent or soap scent.

11. At a time point 24 hr after the single-exposure trial, test each animal again using a procedure identical to that of the previous test day, except that no drugs are administered, and use a fresh, no-odor block at the beginning of the session.

Score defensive behaviors

12. From the videotape records, score the following defensive behaviors.

    (a) Measure avoidance by (1) location, which is the time (duration) spent in each of the three segments (far, middle, and near the cat odor/no-odor block). Location is scored when all four feet are in a particular segment. Location in the far segment is evaluated as avoidance of the stimulus block. (2) Contact (frequency and duration) with the odor/no-odor block is scored whenever any part of the body of the subject animal is within 1 cm of the block.

    (b) Measure risk assessment by (1) stretch-attend (frequency and duration), which is when the animal faces the stimulus block with fore- and hind-limbs far apart and body elongated with a low back, and (2) stretch-approach (frequency and duration), which is when the animal moves toward stimulus block, with an elongated body and low back.

    Stretch-attend and stretch-approach appear to reflect risk assessment to higher and lower magnitude of threat, respectively. To cat odor, a relatively mild threat stimulus, stretch-approach is particularly prominent, and changes in stretch-approach with this measure appear to be more common.

    (c) Measure freezing (duration) by total time spent immobile.

    (d) Measure activity by location changes (frequency); the number of times the animal moves from one segment to another with all four feet in the new location.

    Defensive response to the cat odor stimulus involves avoidance of the stimulus and risk assessment directed toward it; increased freezing, and reduced activity. These behaviors, seen on the following (no-odor stimulus) test indicate conditioning to the odor stimulus and cue.

USE OF CAT ODOR TO ELICIT A RANGE OF DEFENSIVE BEHAVIORS WHEN A HIDING AREA IS AVAILABLE

ALTERNATE PROTOCOL 1

This protocol enables the subject to avoid a cat odor threat stimulus by retreating to, and hiding in, a barrier structure within the testing arena. Because avoidance and risk assessment behaviors are strongly influenced by the ability of the subject to hide, the use of this apparatus, which is more complex than that of the basic cat-odor protocol (see Basic Protocol 3), may provide a superior measure of avoidance and risk assessment. Like Basic Protocol 3, this cat-odor with hide-box test elicits contextual conditioned defensive behaviors when animals are exposed to the context plus cue (the odor stimulus without odor) on the day following exposure.

An additional difference from Basic Protocol 3 is in the source of the cat odor threat stimulus. In this protocol, cat odor is provided by use of a cut section of a cat collar, worn by a domestic cat around its neck; the control stimulus is a similar section of an unworn cat collar.

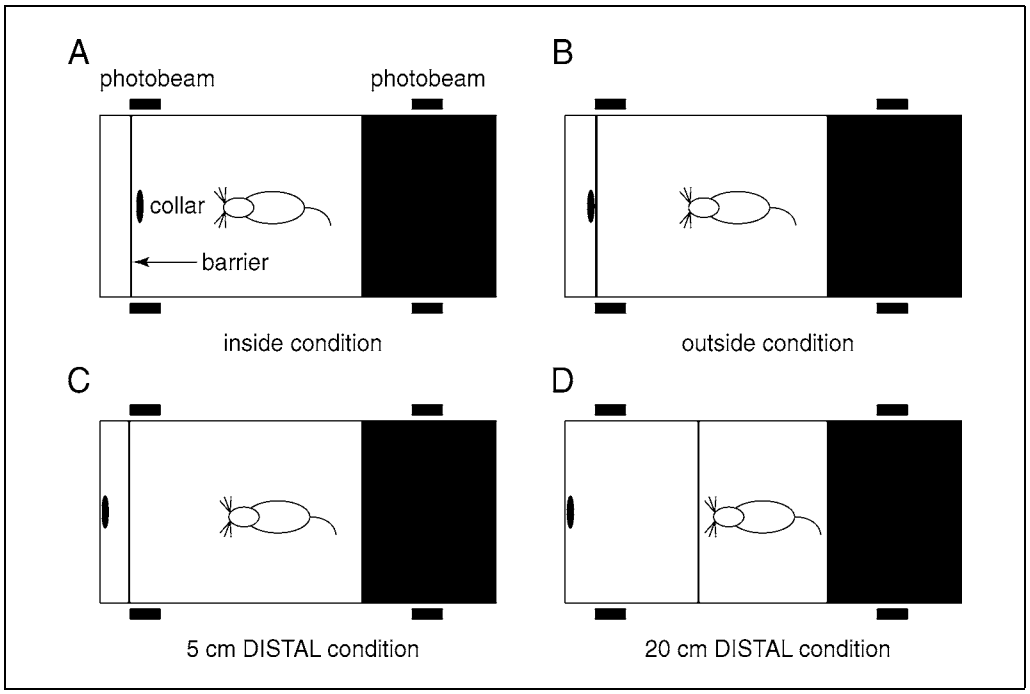

Figure 8.19.8 The apparatus for the cat odor test with hide box is a rectangular enclosure with Perspex or Plexiglas walls (60-cm length × 26-cm width × 36-cm height, with a metal grid or wire mesh floor, mounted above a tray containing absorbent material. Across one end of the chamber is a hide box or barrier delineating a 21-cm deep space, with a 6 × 6–cm square hole permitting the subject to enter the hiding space. On the opposite end of the chamber from the hide box, an alligator clip is positioned 4 cm above the floor. During testing this clip holds a portion of a cloth cat collar. Photobeam detectors are mounted to detect activity in the hiding area and approaches to the cat collar. An additional set of photobeams (not shown in figure) placed just in front of the hide box can be used to detect "head outs." Placement of the collar in front of, or behind a wire mesh barrier can be manipulated, as shown. A videocamera is mounted on the roof of the apparatus. As an alternative, photobeams and photobeam detectors may be omitted, and a side-mounted camera used to record behaviors.

Materials

Adult male rats
Access to an adult cat (odor donor, not brought into the lab)

Standard wool acrylic or synthetic nylon cat collars
Standard single rat cages
Two quiet, darkened rooms, of similar dimensions and construction, away from disturbances
Video camera
Two identical cat odor with hide box apparatuses (custom-made; Fig. 8.19.8)

1. Prepare the odor stimulus by placing a collar (standard wool acrylic or synthetic nylon cat collar) on a domestic indoor cat. Remove the collar 3 weeks later and cut into four segments.

   Collar segments may be used fresh or, for longer term storage, sealed in a non-porous plastic or glass container and kept in a freezer.

2. Singly house rats for 4 days prior to use. Provide food and water ad libitum.

3. Set up a cat odor apparatus in each of two test rooms, one room and apparatus to be used for presenting odor stimuli; one for control, no-odor stimuli. If photocells are used, mount the video camera overhead. If photocells are not to be used, mount the video camera horizontally, facing the clear Plexiglas wall of each cat odor apparatus.

4. Randomly allocate rats to odor and no-odor conditions, and to treatment conditions (drug and dose conditions in this example) within the odor and no-odor groups.

5. Habituate each rat to its respective cat-odor or no-odor apparatus by placing it in the apparatus without an odor source, for 20 min/day for 2 days. If drugs are to be given on the test day, administer saline just prior to each habituation session via the same route used for the drug.

   Conduct all sessions, habituation, odor test session, and conditioning test session, under red light illumination. Run all sessions for a given animal at the same time of day ±1 hr, on successive days, and test all animals during the same phase of the light/dark cycle.

6. Thirty minutes prior to testing the first experimental rats, place a segment of worn cat collar in the alligator clip of a cat odor apparatus. Place an unworn cat collar segment in the alligator clip of an otherwise identical apparatus to be used for control subjects.

7. Administer drug at the interval appropriate for the drug and for the route of administration.

8. Place a rat in the apparatus containing the worn cat collar, or the unworn cat collar, as is appropriate to its condition, at the opposite end and facing away from the collar. Videotape each 10-min session.

9. After each trial, remove any feces and rinse the tray under the floor of the apparatus with water. At the end of the test day, clean each apparatus thoroughly with water and a mild detergent or unscented soap, followed by extensive rinsing.

10. On the conditioning test day, 24 hr after the single-exposure trial, test each animal again, using a procedure identical to that of the previous test day without the administration of drugs and use a fresh, no-odor collar at the beginning of the session.

Score defensive behaviors

11. If photocells and photocell detectors are used, scoring involves the photocell detector output to a computer using data acquisition software and measures the following:

    (a) Approach time to the collar stimulus is detected by breaks in activation of the approach photocell detectors.
    (b) Hide time in the hide box is determined by breaks in activation of the hide box photocell detector without breaks in activation of the head-out detector.
    (c) Head-out time is evaluated through photocell breaks of the head-out detector.
    (d) In addition, the overhead video-camera records may be examined for stretch-attend and stretch-approach behaviors (see Basic Protocol 3, step 12).

12. Alternatively, defensive behaviors may be scored from the videotape records from the side-mounted video recorder, if photocells were not used. The following defensive behaviors are scored as follows:

    (a) Hide time is scored as the time in the hide box, with no part of the body outside the box.
    (b) Contact (frequency and duration) with the worn or unworn cat collar is scored whenever any part of the head of the animal is in contact with the collar.
    (c) Stretch-attend (frequency and duration) is scored when the animal faces the cat collar stimulus with fore- and hind-limbs far apart and body elongated with a low back.

(d) Stretch-approach (frequency and duration) is scored when the animal moves toward cat collar stimulus with an elongated body and low back.

(e) Head out (frequency and duration) is scored when an animal is inside the hide box and any part of its head protrudes through the opening.

(f) Freezing (frequency and duration) is measured as time spent immobile.

Avoidance is measured as increased hide time and reduced contact. Risk assessment is measured by stretch-attend, stretch-approach, and head out behaviors. Freezing also increases in response to odor stimuli. Conditioning to the context with odor stimulus is assessed by increases in these behaviors relative to no-odor controls on the conditioning test day.

COMMENTARY

Background Information

Mouse defense test battery

The mouse defense test battery (MDTB) was developed from tests of defensive behaviors in rats, reflecting earlier studies of responses of laboratory and wild rodents to threatening stimuli and situations. It was designed to examine anxiogenic- or anxiolytic-like properties of psychoactive drugs through effects on specific defensive behaviors (Griebel et al., 1995). Principal component analysis has suggested that the behaviors scored in this procedure may relate to different aspects of anxiety (Griebel et al., 1996). This analysis identified two main independent factors, which related either to the process of acquiring and analyzing information in the presence of threatening stimuli (e.g., risk assessment), or to more affective-orientated defense reactions (e.g., defensive threat and attack). The MDTB represents a significant improvement over other animal models for evaluating drugs active against emotional disorders such as generalized anxiety disorder and panic since it is capable of responding to and differentiating anxiolytic drugs of different classes through specific profiles of effect on different measures (Blanchard et al., 2003a).

Rat exposure test

The RET was specifically created as an extension of the MDTB in an attempt to expand the duration of risk assessment to predator stimuli and, through provision of relevant features such as a shelter (home cage) and substrate, to allow additional defensive behaviors to occur. When 10-week-old male BALB/c mice (Simonsen Laboratories) were used as subjects, the rat-exposed group exhibited significantly increased stretch-attend and stretch-approach behaviors, freezing, and time spent in the home chamber, but reduced contact time with the mesh screen, compared with toy-exposed mice (Yang et al., 2004). Administration of 10 mg/kg chlordiazepoxide i.p. significantly reduced stretch attend postures and enhanced contact time with the mesh screen in BALB/c mice, as did acidic astressin (Blanchard et al., unpub. observ.).

A comparison of defensive behaviors for two inbred mouse strains, C57BL/6 (Charles River Laboratories) and BALB/cAnN (Simonsen Laboratories), and two outbred strains, CD-1 (Charles River Laboratories) and Swiss-Webster (Simonsen Laboratories), found no significant differences between the two outbred strains, but several differences between the two inbred strains, and between each inbred strain and the two outbred strains (Yang et al., 2004). In addition, correlations between locations and activities strongly suggest an intrinsic organizational factor for defense that is strikingly expressed in both outbred strains, but less so in the inbred mice. When a place of concealment is available, risk assessment, freezing, and burying preferentially occur in this location. These findings suggest that the process of selection that has led to the creation of inbred mouse strains may have substantially altered some aspects of the organization of defense in these animals. However, the only finding of virtual absence of a particular defensive behavior in the RET was for BALB/c mice, which showed little or no defensive burying.

Cat odor test

Following analyses suggesting that ambiguous threat stimuli strongly elicit investigative (risk assessment) components of the antipredator defense pattern (Blanchard et al., 1989), cat fur/skin odor has been frequently used as a threat stimulus for studies of drug (e.g., Blanchard et al., 1990) and lesion (Blanchard et al., 2003b) effects on defense. It typically elicits both avoidance and risk assessment, as well as a moderate level of freezing; a single exposure to this unconditioned threat will support contextual defensive conditioning

in this test (Blanchard et al., 2001). Other noxious or predator-related stimuli, such as the synthetic fox feces product trimethylthiazoline (TMT) may elicit some defensive behaviors, particularly avoidance. However, neither TMT nor cat feces produces contextual conditioning under circumstances in which cat fur/skin odor serves as an effective unconditioned stimulus (Blanchard et al., 2003c).

Cat odor test with hide box

This test was devised by Dielenberg and McGregor (2001) and has been used to investigate behavioral response to cat odor and conditioning of cat odor effects. Findings from the cat odor test with hide box are similar to those of the cat odor test: cat odor exposure elicits a range of defensive behaviors (e.g., Dielenberg and McGregor, 1999) and contextual conditioning of defense will occur following a single-exposure trial (McGregor and Dielenberg, 1999; Dielenberg and McGregor, 2001; Dielenberg et al., 2001), but synthetic fox feces odor does not produce conditioning even though it elicits a number of defensive behaviors on initial presentation (McGregor et al., 2002). In this test, the benzodiazepine, midazolam, reduced avoidance of the cat odor stimulus (Dielenberg et al., 1999; McGregor and Dielenberg, 1999).

Critical Parameters

For all tests described in this unit, use animals only once. Repeated testing may change the nature of the defensive/anxiety response and hence will also change pharmacological responses.

Mouse defense test battery

Strain differences are particularly pronounced in the MDTB. Strains having low baseline levels on some (e.g., C57BL/6 or BALB/c) or all (e.g., CBA) defensive measures are poorly suited for the investigation of anxiolytics (Griebel et al., 1997). Strains showing high levels of defensiveness throughout the procedure and with a strong response to anxiolytics are Swiss-Webster from the Simonsen Laboratory and Swiss albino, regardless of the supplier. Although female mice have been used in a few experiments, little has been published by way of validation and the influence of the estrous cycle has not been investigated. Experiments must be performed under red light to minimize visual contact with the experimenter. The MDTB requires a well-trained experimenter capable of quickly evaluating multiple defensive behaviors, some of which may not be obvious at first glance. Scoring can be live or from tape and must be performed by an observer blind to the drug treatment and test condition.

Rat exposure test

Findings of differences between inbred and outbred mouse strains, and between inbred strains, suggest that the patterning of defensive behavior in the RET may be more variable in inbred strains (Yang et al., 2004). While this information may be important in analysis of the relationship between environmental features and the expression of individual defensive behaviors, it has not been demonstrated to be of importance in analysis of drug effects. However, BALB/c mice are not recommended for use in this (or other tests) if defensive burying is an important behavioral criterion.

The size of the rat stimulus appears to influence the magnitude of defensiveness of mice in the RET, and rats to be used within a series, or between series for which between series comparisons are of interest, should be of comparable size.

Cat odor test and cat odor with hide box test

This test is more suitable for rats than for mice, as mice show a highly variable response to cat odor (Blanchard et al., unpub. observ.). Although a number of outbred rat strains have been shown to respond strongly to cat odor stimuli, no direct comparisons of different rat strains have been made with reference to this specific protocol.

Troubleshooting

Mouse defense test battery

Inadequate levels of defensive behaviors. The main problem appears to be the use of strains that give rise to low levels of defensiveness. If this occurs, increase the period of individual housing from 7 to 14 days.

All defensive behaviors are reduced. This could be due to sedative drug effects. The pretest horizontal and vertical activities provide information on sedative or stimulant effects of a drug. These measures can be used to determine the specificity of any changes in defensiveness. If a compound has marked sedative effects, it is likely that all aspects of defensive behaviors will be reduced.

Fatigue of the experimenter. Performing the MDTB requires the experimenter to constantly move around the runway apparatus and from the holding area to the experimental room. To avoid fatigue and decrease in concentration, limit the testing to ~15 animals per day.

Rat exposure test

High baseline levels of defensive behavior. Both the habituation sessions and the placement of bedding from each subject's home cage into the RET apparatus should reduce defensiveness prior to the introduction of the rat threat stimulus.

Heightened defensive behaviors when no rat is present. This may be due to lingering rat odors in the apparatus from previous experiments. This problem can be addressed with thorough cleaning of the apparatus prior to retest. However, if experimental (rat exposure) tests and controls (toy exposure tests) are to be run in the same apparatus, the problem is more acute. Possible solutions include the following:

(1) Clean all areas of the apparatus with water and mild detergent or unscented soap, followed by thorough rinsing and drying with paper towels, between animals. This procedure is time consuming and possibly inadequate.

(2) Test rat-exposed and toy-exposed subjects on separate days, with thorough cleaning of the apparatus and room between days. This approach runs the risk of possible differences between days that may confound experimental-control differences.

Because neither of these measures provides certainty that lingering odors will not contaminate data, the authors recommend use of two identical RET apparatuses, in two separate but similar experimental rooms, for experimental (rat-exposed) and control (plush toy-exposed) animals. However, care must be taken to ensure that the apparatuses and experimental rooms are as similar as possible with reference to all relevant variables.

Cat odor test

Quantification of cat odor. As the effective constituents of cat fur/skin odor have not yet been analyzed, quantification of the cat odor stimulus remains a problem. However, there appears to be a clear dose-response relationship between the magnitude of cat fur-skin odor and the strength of defensive responding by rats (L. Takahashi, pers. comm.). Thus, if a particular stimulus proves ineffective, consideration should be given to increasing the magnitude of the odor captured. This might involve utilization of a larger portion of a cloth rubbed on the stimulus cat, or leaving the cloth in the cat's bed for a longer time. Also, informal observations (Dielenberg and McGregor, 2001) suggest that the effective secretions are enhanced by warm temperatures, such that cat odors may be more effective in summer months. As cat fur/skin odors appear to maintain potency well when sealed in a nonporous plastic or glass container and frozen, the solution is to obtain cat odors and freeze them during the summer months for use during colder periods.

All defensive behaviors are reduced. This may be due to sedative drug effects. The locomotor measures can be used to determine the activity specificity of any changes in defensiveness. If a compound has marked sedative effects, it is likely that all aspects of defensive behaviors will be reduced.

Heightened defensive behaviors when no source of cat odor is presented. Caveats under RET regarding lingering odors are also applicable here.

Cat odor with hide box test

Caveats with reference to quantification of cat odor are the same as for the cat odor test. In addition, the area under the neck of the cat appears to be the best source for cat collar odors (Iain McGregor, pers. comm.).

All defensive behaviors are reduced. This may be due to sedative drug effects. These may be less easily detected in the cat odor with hide box test than in the basic cat odor test, as animals sheltering in the hide box often show little locomotor activity, providing a low baseline for evaluation of potential sedative effects.

Anticipated Results

Mouse defense test battery

Anxiolytic compounds should decrease defensive behaviors, whereas anxiogenic drugs should show opposite effects. However, some responses may be specifically or mainly affected by certain drug classes. Thus, benzodiazepines have been shown to decrease risk assessment activities and defensive threat and attack responses, while 5-HT_{1A} anxiolytics mainly affect escape attempts and defensive threat and attack behaviors. In addition, panic-modulating drugs have a clearer impact on flight responses than on other defensive reactions. Data for the effects of a wide range of drugs on flight, risk assessment, and contextual defense behaviors in the MDTB are provided in Blanchard et al. (2003a).

Rat exposure test

Anticipated results of exposure to the rat include enhancement of avoidance, evidenced as increased time in the home cage and reduced

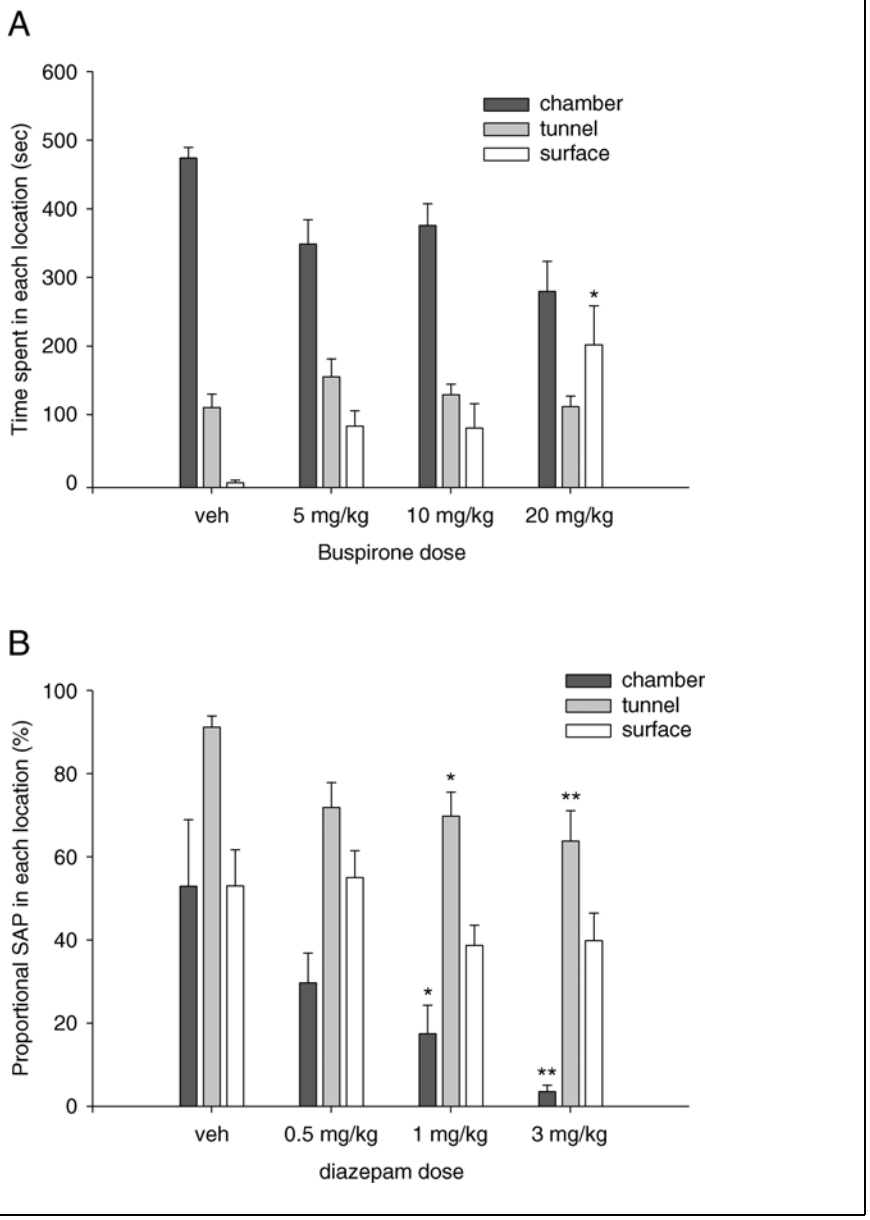

Figure 8.19.9 (**A**) Effect of buspirone (5.0, 10.0, and 20.0 mg/kg) on location within the RET: chamber, tunnel, or surface area. Enhanced time near the threat stimulus reflects reduced avoidance. (**B**) Effect of diazepam (0.5, 1.0, and 3.0 mg/kg) on the proportion of time spent in stretch approach while the animal is in each of three locations within the RET; chamber, tunnel, or surface area. An asterisk (*) indicates significant differences from controls ($p < .05$). Asterisks (**) represent $p < .01$ or less.

contact with the mesh screen; increases in risk assessment, particularly stretch approach; and increased freezing.

Anxiolytic compounds should decrease avoidance and also decrease risk assessment, particularly while animals are far from the threat stimulus (Fig. 8.19.9). Although anxiogenic drugs have not been used with this test, they are predicted to enhance avoidance. In the RET, risk assessment occurs most often in the tunnels. The high-level avoidance (enhanced time in the chamber) predicted to follow administration of anxiogenic drugs should therefore produce reduced risk assessment.

Cat odor test

Anticipated results of exposure to cat fur/skin odor include enhancement of avoidance, evidenced as increased time spent in the 'far' location from the odor stimulus and

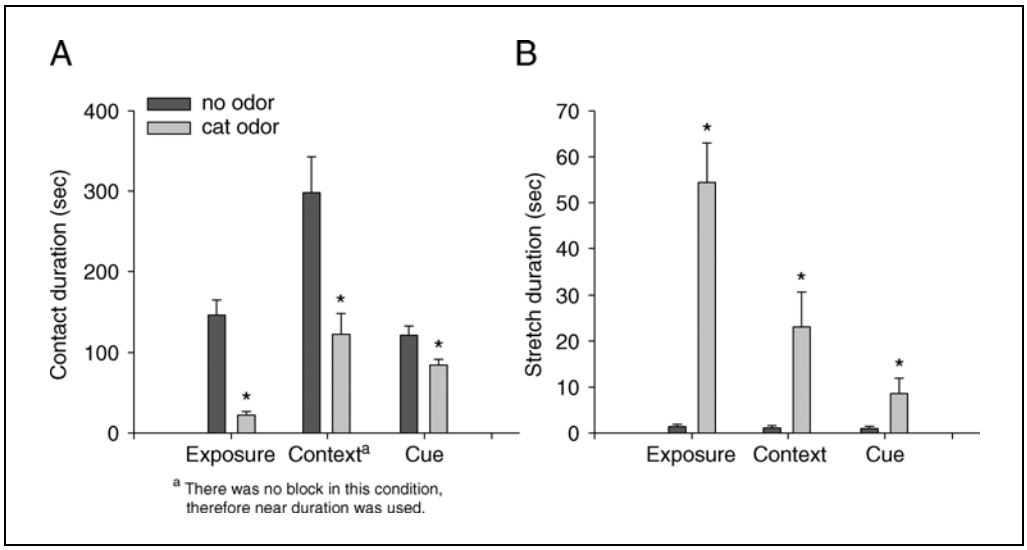

Figure 8.19.10 Cue and context conditioning to cat odor in the rat. (**A**) Contact with a block cue. (**B**) Duration of stretch behaviors (risk assessment). Each of these behaviors is shown during initial exposure to a cat odor block, or to a similar block without odor as control, and during subsequent tests to the context alone, or to the cue alone, presented in a different context. An asterisk (*) indicates significant cat-odor and control group differences ($p < 0.05$ or less).

decreased contact with the cat odor stimulus, and increases in risk assessment, particularly stretch approach. When re-exposed to contextual cues on the day following exposure (Fig. 8.19.10), again rats show avoidance, risk assessment, and sometimes enhanced freezing. Thus, the cat odor test can provide measures of contextual conditioning that do not involve freezing, in addition to freezing measures.

Anxiolytic compounds should decrease risk assessment and avoidance. Although anxiogenic drugs have not been used with this test, they are predicted to enhance avoidance. As noted under the RET, the effects of anxiogenic drugs on risk assessment are difficult to predict.

Cat odor with hide box test

Anticipated results of exposure to cat odor include increased time in hide box and decreased approach time (Figure 8.19.11). Risk assessment, including stretch attend/stretch approach and head out of the hide box, also increases. When re-exposed to contextual cues on the following day, rats show enhanced avoidance and risk assessment. Anxiolytic compounds should reduce avoidance and risk assessment. However, as noted under the RET, the effects of anxiogenic compounds on risk assessment are difficult to predict.

Time Considerations

MDTB

The MDTB requires ~1 week training for the pre- and post-test measures to be scored reliably, but longer for the defensive measures. A skilled experimenter may be able to perform the test in a satisfactory manner after 2 weeks of practice.

Rat exposure test

Habituation to the test situation to reduce defensiveness to the context requires 3 days, therefore adding time for the RET. Evaluation of the defensive behaviors measured in this test is relatively uncomplicated and training to score them reliably should take no more than 2 days.

Cat odor test

Although the odor test and the contextual conditioning tests are brief, 3 days of habituation to the test situation to reduce defensiveness to the context add to the time required for this test. Evaluation of the defensive behaviors measured in this test is relatively uncomplicated and training to score them reliably should take no more than 2 days.

Cat odor with box test

The testing time required, and training for evaluation of defensive behaviors, are virtually

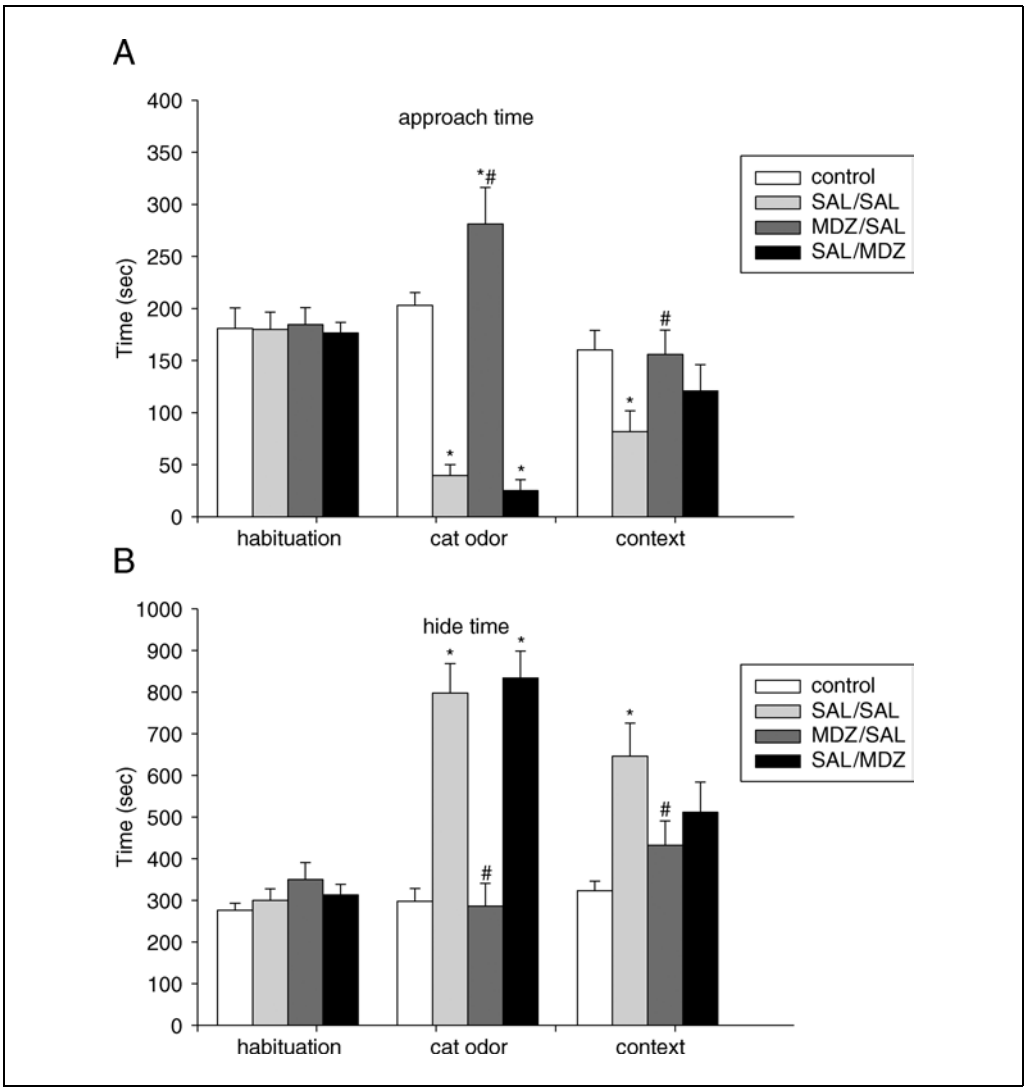

Figure 8.19.11 Effect of midazolam (0.375 mg/kg) on (**A**) approach time (time spent in close proximity to collar) and (**B**) hide time (time spent with most or all of the body inside the hide box). Data are from the habituation session (no collar present), cat odor session (worn or unworn collar present in chamber), and context conditioning test (rats were returned to the test box on the day after cat odor). Groups received either unworn cat collar in cat odor session and no drugs (control), saline in both cat odor exposure session and test for context conditioning (SAL/SAL), midazolam in cat odor session followed by saline in context test day (MDZ/SAL), or saline in cat collar session followed by midazolam in context test (SAL/MDZ). An asterisk (*) indicates significant differences from no-odor controls ($p < 0.05$ or less); # indicates significant differences from group SAL/SAL ($p < 0.05$).

identical to those of the basic cat odor test. However, the cat is required to wear the collar for ≥3 weeks, which substantially adds to the lead time required for this procedure.

Literature Cited

Blanchard, R.J. and Blanchard, D.C. 1989. Antipredator defensive behaviors in a visible burrow system. *J. Comp. Psychol.* 103:70-82.

Blanchard, D.C., Blanchard, R.J., Tom, P., and Rodgers, R.J. 1990. Diazepam alters risk assessment in an anxiety/defense test battery. *Psychopharmacology* 101:511-518.

Blanchard, D.C., Griebel, G., and Blanchard, R.J. 2003a. The mouse defense test battery: Pharmacological and behavioral assays for anxiety and panic. *Eur. J. Pharmacol.* 463:97-116.

Blanchard, D.C., Li, C.-I., Hubbard, D., Markham, C., Yang, M., Takahashi, L.K., and Blanchard, R.J. 2003b. Dorsal premammillary nucleus differentially modulates defensive behaviors induced by different threat stimuli. *Neurosci. Lett.* 345:145-148.

Blanchard, D.C., Markham, C., Yang, M., Hubbard, D., Madarang, E., and Blanchard, R.J. 2003c. Failure to produce conditioning with low-dose trimethylthiazoline or cat feces as unconditioned stimuli. *Behav. Neurosci.* 1172:360-368.

Blanchard, R.J., Yang, M., Li, C.-I., Garvacio, A., and Blanchard, D.C. 2001. Cue and context conditioning of defensive behaviors to cat odor stimuli. *Neurosci. Biobehav. Rev.* 26:587-595.

Dielenberg, R.A. and McGregor, I.S. 2001. Defensive behavior in rats towards predatory odors: A review. *Neurosci. Biobehav. Rev.* 25:597-609.

Dielenberg, R.A. and McGregor, I.S. 1999. Habituation of the hiding response to cat odor in rats *Rattus norvegicus*. *J. Comp. Psychol.* 113:376-387.

Dielenberg, R.A., Arnold, J.C., and McGregor, I.S. 1999. Low-dose midazolam attenuates predatory odor avoidance in rats. *Pharmacol. Biochem. Behav.* 62:197-201.

Dielenberg, R.A., Carrive, P., and McGregor, I.S. 2001. The cardiovascular and behavioral response to cat odor in rats: Unconditioned and conditioned effects. *Brain Res.* 897:228-237.

Griebel, G., Blanchard, D.C., and Blanchard, R.J. 1996. Evidence that the behaviors in the mouse defense test battery relate to different emotional states: A factor analytic study. *Physiol. Behav.* 60:1255-1260.

Griebel, G., Blanchard, D.C., Jung, A., and Blanchard, R.J. 1995. A model of 'antipredator' defense in Swiss-Webster mice: Effects of benzodiazepine receptor ligands with different intrinsic activities. *Behav. Pharmacol.* 6:732-745.

Griebel, G., Sanger, D.J., and Perrault, G. 1997. Genetic differences in the mouse defense test battery. *Aggress. Behav.* 23:19-31.

McGregor, I.S. and Dielenberg, R.A. 1999. Differential anxiolytic efficacy of a benzodiazepine on first versus second exposure to a predatory odor in rats. *Psychopharmacology Berl.* 147:174-181.

McGregor, I.S., Schrama, L., Ambermoon, P., and Dielenberg, R.A. 2002. Not all 'predator odours' are equal: Cat odour but not 2,4,5 trimethylthiazoline TMT; fox odour elicits specific defensive behaviors in rats. *Behav. Brain Res.* 129:1-16.

Yang, M., Augustsson, H., Markham, C.M., Hubbard, D.T., Webster, D., Wall, P.M., Blanchard, R.J., and Blanchard, D.C. 2004. The rat exposure test: A model of mouse defensive behaviors. *Physiol. Behav.* 8:465-473.

Key References

Blanchard et al., 2003a. See above.

Provides a general review of the development of the MDTB and reviews the effects of 70+ drugs in this procedure.

Dielenberg and McGregor, 2001. See above

Provides a general review of cat odor tests and findings, with particular attention to the development and use of the cat odor test with hide box.

Yang et al., 2004. See above

Describes procedures and measures of the RET and compares effects of two inbred and two outbred mouse strains in this test.

Blanchard et al., 2003b. See above

Describes procedures and measures for the cat odor test and compares conditioning for cat fur/skin odor, cat feces, and TMT in this test.

Contributed by D. Caroline Blanchard and Robert J. Blanchard
University of Hawaii
Honolulu, Hawaii

Guy Griebel
Sanofi-Synthelabo
Paris, France