

Apparatus and General Methods for Exposing Rats to Audiogenic Stress

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[Abstract] Most organisms react innately to the sudden onset of environmental stimulation. Audiogenic or loud noise in rodents provides an effective threatening signal to study the central nervous circuits responsible for the elaboration of various responses typically elicited by threatening/stressful environmental stimulation. Audiogenic stress offers many advantages over other environmental stimulation, including exquisite control over timing, intensity, and frequency, using off-the-shelf components that produce easily reproducible results. This protocol provides blueprints for the construction of sound attenuation chambers, the associated sound generation, amplification, and delivery equipment, and general procedures sufficient to elicit multimodal responses to loud noises in rodents.

[Background] For many years, audiogenic stress (loud noise) has been employed as an effective stimulus to activate multimodal responses traditionally associated with threatening situations and stressor exposures, including the neuroendocrine hypothalamo-pituitary-adrenocortical (HPA) axis (as indexed by the release of glucocorticoids and adrenocorticotropin hormone - ACTH; [Borrell *et al.*, 1980; Campeau and Watson, 1997; Henkin and Knigge, 1963; Segal *et al.*, 1989]), the autonomic system (as measured by peripheral catecholamine release, heart rate, blood pressure, or core body temperature measurements; [Bao *et al.*, 1999; De Boer *et al.*, 1989; Gamallo *et al.*, 1992; Masini *et al.*, 2008; Overton *et al.*, 1991; Saha *et al.*, 1996]), and a multiphase behavioral reaction which initially elicits forceful, quick evasive locomotor responses, followed within a few minutes by significant reduction or inhibition of locomotor activity, feeding, and drinking (Britton *et al.*, 1992; Campeau and Watson, 1997; Irwin *et al.*, 1989; Masini *et al.*, 2008; Segal *et al.*, 1989). These powerful effects of loud sounds likely arise from the fact that such high intensity auditory stimulation is frequently associated with environmental events occurring very close to the listener that require immediate, life-saving attention (predator pouncing, objects falling or traveling at a high rate of speed, etc.). The importance of these emergency responses is further suggested by their similarities across a wide array of species and environments (aquatic, terrestrial, airborne).

Compared to many stress protocols, audiogenic stress has a number of advantageous characteristics, and the current protocol has some advantages compared to previous audiogenic stress protocols (Siegel *et al.*, 1983; Boddle-Biber *et al.*, 1989). Perhaps the single most important advantage of audiogenic stress is its exquisite control over the amplitude of acoustic stimulation, providing one of the few procedures for which the intensity of the stressor can be controlled along a continuum of innocuous

auditory intensities to increasingly stressful exposures (Boadle-Biber *et al.*, 1989; Campeau and Watson, 1997; Burow *et al.*, 2005), as compared to other popular stressor protocols such as restraint, immobilization, tail suspension, social stress, and others. And whereas several previous loud noise protocols exposed multiple rats to loud noise in the same enclosure (Boadle-Biber *et al.*, 1989) or individually but in large rooms (Siegel *et al.*, 1983), the current protocol was developed to expose animals to noise independently, increasing the likelihood for reproducibility in exposed and control rats simultaneously. As described below, the procedures developed in our laboratory for exposure of rats to audiogenic stress allows the simultaneous measurement of multiple responses, which is necessary to study the integrated mechanisms necessary to understand the elaboration of multimodal responses to stress.

Materials and Reagents

1. Sprague-Dawley rats (6-12 weeks of age) from Envigo (Enrigo, catalog number: Sprague-Dawley® outbred rats)
2. Rat chow (Enrigo, catalog number: Teklad global 14% protein #2014)

Equipment

1. The sound attenuating enclosures (constructed from double wooden [2 cm plywood board] boxes)
 - a. Outer box [external dimensions: 85 (w) x 60 (d) x 72 (h) cm] lined internally with 2.5 cm insulation (Celotex™) (Figures 1A to 1D)
 - b. Inner box [internal dimensions: 60 (w) x 38 (d) x 38 (h) cm] (allows placement of a polycarbonate rat home cage inside, including food and water for overnight housing – Figures 1C, 1E, 1H, and 1I)
 - c. All these general construction materials can be procured in local hardware stores.
2. Cooling fans, 105 cm (General Wireless Operation, RadioShack, model: 2730241) (Figures 1A and 1B)
3. Car speakers, 15.25 x 22.85 cm (RadioShack Corporation, model: 12-1769 – 120 W RMS) (Figures 1E and 1H)
4. Fluorescent lamps (n: VISION, model: EDX0-14, 14 W, soft white; EcoSmart, catalog number: 423-599) (Figures 1E and 1H)
5. Noise generator (General Radio, model: 1381) (Figure 1F)
6. Band-pass filter (Krohn-Hite, model: 3100R)
7. Power amplifiers (PropertyRoom, model: Pyramid Studio Pro PA-600X) (Figure 1G)
8. Sound level meter (RadioShack, model: 33-2050 – A scale) (Figures 1I and 1J)

Notes:

- a. Some of the equipment discussed above can only be obtained from secondary sources. When possible, links to specifications sheets are provided to help in obtaining more recent equipment with similar characteristics.
- b. Animals are exposed to loud noise within individual sound attenuating chambers in an independent room, away from the general vivarium to ensure that only experimental animals experience the loud acoustic stimulation.
- c. The internal dimensions of individual sound attenuating chamber allows placement of a polycarbonate rat home cage inside, including food and water for overnight housing (see Figures 1E, 1H, and 1I).
- d. Each box is fitted with two 105 cm cooling fans, located in the lower back left (push air in), and upper front right side (draw air out) of the external box, respectively, to provide a constant flow of fresh air (65 CFM) inside each box (see Figures 1A and 1B).
- e. Each enclosure provides approximately 30 dBA (sound pressure level – SPL, A scale) of sound attenuation, which allows the testing of both loud noise and no noise experimental subjects simultaneously in adjacent enclosures.
- f. Each enclosure is fitted with a single Optimus speaker fixed in the middle of the ceiling of the internal enclosure (see Figures 1E and 1H). Speaker characteristics permit sound delivery between 20 and 27,000 Hz, with the intensity rolling off quickly (20 dB octave) at both ends of the frequency spectrum.
- g. Lighting is provided by a fluorescent lamp located in the upper left corner of the internal enclosure (see Figures 1E and 1H), which are kept on the same day-night cycle as the lighting of the main colony room.
- h. Noise is produced by a General Radio solid-state random-noise generator with the bandwidth set at 2-50,000 Hz for most of the experiments (see Figure 1F). Frequencies can be filtered through a Krohn-Hite filter to achieve different band-pass settings when necessary. The output of the noise generator is generally fed to power amplifiers (Pyramid Studio Pro [see Figure 1G]), the outputs of which are connected to the Optimus speakers.
- i. Noise intensity is measured by placing a Sound Level Meter in an empty rat's home cage at several locations and taking an average of the different readings (see Figure 1I). The noise level provided by the ventilating fans is approximately 57 dBA, which is defined as the 'no noise' or 'background/ambient noise' level. The noise level in the quiet animal colony averages approximately 55 dBA.



Figure 1. Representative images of equipment. Views of the external enclosure from the left angle (A) and right angle (B), showing the location of the fans (white arrows) to provide continuous fresh air internally. C. View of the open external enclosure and the internal closed enclosure. D. Detailed view of the external enclosure construction, with the 2-cm plywood panel (black arrows) lined internally with 2.5-cm celotex material, which also covers the hinged door (gray arrows). E. View of the open internal enclosure (white arrow) fitted with an empty home cage for demonstration purposes, and the location of the light and speaker (see also H below). The home cage sits directly under the speaker (see also H below). F. Front view of the General Radio #1381 noise generator employed to generate sound. G. Front view of the Pyramid Studio Pro PA-600X amplifier employed to amplify the sound from the noise generator. H. Additional view of the open internal enclosure fitted with Starr Life Sciences' ER4000 Receiver antenna (white arrow). A rat's home cage sits directly on top of the ER4000 to telemetrically provide heart rate, core body temperature, and general locomotor activity information when rats are implanted

with PDT 4000 HR Emitters. I. View of the RadioShack sound level meter (white arrow) employed to measure sound pressure levels inside a rat's home cage during sound delivery. The meter is moved in different home cage locations to verify the desired averaged SPL. J. Larger view of the sound pressure level display (95 dBA) during a sound test.

Procedure

A. Animal handling and preparation prior to audiogenic stress

1. Sprague-Dawley rats (males or females), weighing 150-325 g (6-12 weeks of age) upon arrival to the colony, are generally employed. They are housed in a dedicated vivarium facility and grouped four to five in clear polycarbonate cages (48 x 27 x 20 cm) containing floor wood shavings, and covered with wire lids providing food (rat chow) and water *ad libitum*. Rats are kept on a controlled light/dark cycle (lights on 7:00 AM - off at 7:00 PM), under constant humidity and temperature conditions. Animals are housed for a period of at least 7 days after arrival from the supplier, before any experimental manipulations are conducted.
2. Rats are then singly housed into smaller clear polycarbonate cages (43 x 22 x 20 cm) with similar floor wood shavings and wire lids for food and water. They are handled manually (picking rats up with hands and transferring from hand to hand repeatedly) for a few minutes, adapted to transportation from the vivarium to the experimental room, placed into the sound attenuating chambers for 30 min, and returned immediately to the vivarium upon removal, once a day for five days prior to the experimental manipulations. Procedures are generally performed between 9:00 AM and 12:00 PM to reduce variability due to normal circadian hormonal variations.

B. Behavioral procedures

1. The behavioral procedures generally consist of placing the entire polycarbonate cage of individual rats in the enclosures between 5:00 and 6:00 PM the afternoon prior to experimental noise exposure the next morning. This approach allows loud noise or background (no) noise treatments on the morning of the experiment without additional disturbances of the animals, as the noise is independently and remotely controlled for each chamber without opening the noise-attenuating chambers (see Video 1).
2. Loud noise can be administered to rats at intensities ranging from 57-110 dBA (routinely 95-105 dBA), for durations ranging from minutes to hours (routinely 30 min) (see Video 1).

Note: It is important to test noise amplitudes in each individual sound attenuating chambers as slight variations in speaker performance can lead to significant sound pressure level differences. These measurements should be performed both before placement and after removal of rats into the sound attenuating chambers (never during the presence of rats!) on a daily basis to ensure consistent sound pressure delivery across days and studies, as equipment failures happen. Ideally, a sound spectral analysis should be performed in individual sound attenuating chambers to ensure similarity of delivered frequency spectra. In our experience, inclusion of the lower

frequency spectra (20-2,000 Hz) is necessary to induce significant and reproducible noise-elicited HPA axis responses.

Video 1. Loud noise delivery. The video clip provides a demonstration of the main controls available during audiogenic stress exposure in rats. The video clip begins in a background state, without noise or internal lighting. Moderate white noise is then presented (95 dBA), with a demonstration of the lighting being turned on and off. The noise intensity is then increased slowly, and reduced at the end of the clip. A rat's home cage is present inside the internal enclosure for demonstration purposes.



3. Immediately upon termination of noise or background exposure, the cages are immediately removed from the sound-attenuating chambers and rats are typically either lightly restrained (rats are gently wrapped in a towel and held by an experimenter on a counter top) for collection of a blood sample from a small tail 'nick' that takes less than 2 min, or are euthanized via decapitation for extraction of brain, other organs, and collection of trunk blood.
4. Rats in repeated loud noise exposure studies are returned to the vivarium, and the above procedures are repeated until the end of the study.

Notes:

- a. *Although exposure of multiple animals to noise in the same housing cage could increase throughput and save time, we have noticed a reduction in HPA axis response and behavior when we have attempted such procedures (unpublished observations), suggesting significant effects of social factors when two or more animals are exposed to loud noise in the same housing cage.*
- b. *Adult female and male rats display similar HPA axis responses to audiogenic stress across multiple acute and repeated exposure protocols (Babb et al., 2013; 2014). Therefore, this stressor modality does not appear to provide an efficient approach to study sex differences in rats.*
- c. *It is good practice to verify the opening of the ear canal/external auditory meatus in all rats to be included in audiogenic stress studies, as we have encountered some rats from various*

vendors to have closed canals, and could therefore not be exposed appropriately to the intended sound pressure level. Simple visual inspection of the external auditory meatuses is easily performed during the initial handling of rats.

d. *Related to above, when animals need to undergo craniotomy using a stereotaxic apparatus, it is important to employ 'blunt' earbars (45° as opposed to the 18° angle typically provided) so as to keep the tympanic membranes intact; use of typical pointed earbars (18° angle) can easily rupture tympanic membranes and can induce significant variability in the sound pressure levels experienced by rats during loud noise exposure.*

Notes

1. All procedures have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Colorado and conformed to the United States of America NIH Guide for the Care and Use of Laboratory Animals.
2. Using procedures similar to the ones described above, we have reported dynamic time courses for the effects of noise on the release of hypothalamo-pituitary-adrenocortical axis hormones (adrenocorticotropin hormone and corticosterone) (Patz *et al.*, 2006), in addition to the effects of different noise intensities (Campeau and Watson, 1997; Burow *et al.*, 2005). We previously reported that the hearing acuity of rats, as determined by auditory-evoked brainstem potentials, does not change with repeated loud noise (105 dBA for 30-min/day) exposures for up to eight days (Campeau *et al.*, 2002). However, rats were tested under anesthesia, and with different frequencies than those used during repeated noise exposures, so it is conceivable that these modifications were enough to miss putative threshold shifts. In a follow-up study (Masini *et al.*, 2008), various modulation of the acoustic startle reflex (prepulse facilitation and inhibition, and gap detection) were found to be similar between acutely and chronically noise exposed rats, using prepulse and startle eliciting stimuli in the startle test that were characteristically similar to the loud noise stimulus, except for stimulus duration. These methods are routinely employed to assess several acoustic threshold functions in animals and humans (Bowen *et al.*, 2003; Ison *et al.*, 1997; Willott *et al.*, 1994; Young and Fechter, 1983), and therefore suggest that modifications of basic sensory function is not responsible for some of the response modifications observed to repeated loud noise exposures.
3. A slight variation of these procedures allows the measurement of multiple autonomic responses (heart rate, core body temperature) and general behavioral activity (Masini *et al.*, 2008; Nyhuis *et al.*, 2016), by employing implantation of Starr Life Sciences' PDT 4000 HR Emitters (Oakmont, PA - previously Mini Mitter/Respironics system) in the abdominal cavity of rats, and the presence of the associated ER4000 Receiver on the floor of the sound attenuation enclosures (see Figure 1H). This system remotely provides a relatively dynamic measure of autonomic and behavioral activation/inhibition induced by loud noise exposure.

Data analysis

Data analyses (standard analyses of variances, often combined with repeated measures mixed designs) are typically performed on the responses measured during and following loud noise and no noise exposure, and include the results of radioimmunoassays or enzyme-linked immunosorbent assays for adrenocorticotropic hormone and corticosterone (e.g., Burow *et al.*, 2005; Patz *et al.*, 2006), autonomic heart rate and core body temperature (e.g., Masini *et al.*, 2008; Nyhuis *et al.*, 2016), and general locomotor activity (Masini *et al.*, 2008). Individual datum exclusion is seldom necessary, and is typically based on values that fall outside of two standard deviations of calculated group means. Readers are directed to multiple additional published manuscripts from our laboratory to those mentioned above, for additional examples of specific data analyses in the context of audiogenic stress.

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