Assaying Fear Memory Discrimination and Generalization: Methods and Concepts

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Generalization describes the transfer of conditioned responding to stimuli that perceptually differ from the original conditioned stimulus. One arena in which discriminant and generalized responding is of particular relevance is when stimuli signal the potential for harm. Aversive (fear) conditioning is a leading behavioral model for studying associative learning and memory processes related to threatening stimuli. This article describes a step-by-step protocol for studying discrimination and generalization using cued fear conditioning in rodents. Alternate conditioning paradigms, including context generalization, differential generalization, discrimination training, and safety learning, are also described. The protocol contains instructions for constructing a cued fear memory generalization gradient and methods for isolating discrete cued-from-context cued conditioned responses (i.e., "the baseline issue"). The preclinical study of generalization is highly pertinent in the context of fear learning and memory because a lack of fear discrimination (overgeneralization) likely contributes to the etiology of anxiety-related disorders and post-traumatic stress disorder. © 2020 by John Wiley & Sons, Inc.

Basic Protocol 1: Tone cued fear generalization gradient
Basic Protocol 2: Quantification of freezing
Support Protocol: Alternate conditioning paradigms

Keywords: amygdala • discrimination • fear conditioning • generalization • prefrontal cortex • fear memory • fear learning

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INTRODUCTION

Generalization refers to the transfer of conditioned responding to stimuli that may only partially resemble a previously encoded conditioned stimulus (CS). Ivan Pavlov was one of the first to describe the phenomenon of generalization. He noted that his dogs salivated not only to an established auditory CS but also to other similar auditory tones (Pavlov, 1927). He also noticed that the effectiveness of novel stimuli to elicit conditioned responses declined in proportion to their resemblance to the CS (i.e., the generalization gradient, see below). Another famous laboratory demonstration of stimulus generalization was the case of "Little Albert." After John B. Watson and Rosalie Rayner conditioned a toddler to fear a white rat, he went on to mount defensive responses to perceptually similar stimuli, such as a rabbit, a fur coat, or Santa Claus's beard (Watson & Rayner, 1920). Generalization has been demonstrated across a wide range of species, in both classical

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and instrumental forms of conditioning (Pearce, 1987). Generalization is thought to be a central property of conditioning and has even been proposed as a universal law in psychology (Shepard, 1987).

The study of stimulus generalization is highly pertinent in the context of potential danger. All organisms have evolved a set of defensive systems that facilitate survival in the face of threat (Headley, Kanta, Kyriazi, & Pare, 2019). Some of these defensive responses are learned. Although learned defensive "fear" responses are thought to be among the most durable and persistent forms of memory known, they can also be flexible in the face of environmental change (Bergstrom, 2016). When confronted with potential danger, organisms must mobilize the appropriate defensive response. However, threatening stimuli do not always signal a clear and present danger. Sometimes threat cues are ambiguous or dissimilar from the original cue. Generalization allows for responding to cues that are unlike the original CS. From an evolutionary perspective, a false alarm is likely advantageous over a potentially fatal miss. Therefore, generalization represents an adaptive, flexible behavioral strategy in the face of continuously changing environmental stimuli. While generalization is clearly an important adaptive feature of the nervous system, discriminating between threatening and non-threatening (safe) signals is also important. Indeed, a finely tuned balance between generalization and discrimination is adaptive in an ever-changing world. While generalization is widely accepted as an adaptive behavioral response (Richards & Frankland, 2017), overgeneralization to non-threating or irrelevant signals is maladaptive and considered an etiological factor in the development of anxiety disorders and post-traumatic stress disorder (PTSD; Dunsmoor & Paz, 2015; Laufer, Israeli, & Paz, 2016). Therefore, the study of fundamental principles in how fear generalization and discrimination is processed in the brain may advance our understanding of anxiety disorders and PTSD (Headley et al., 2019)

Pavlovian "fear" conditioning is a leading model for studying the neuronal circuits underlying associative learning and memory mechanisms. In Pavlovian fear conditioning, a neutral stimulus acquires the ability to elicit a defensive response after being paired with a stimulus that naturally evokes a defensive response. Pavlovian fear conditioning is a useful behavioral paradigm for studying molecules, cells, and circuits involved in associative learning and memory because it is relatively simple to execute, reliable, and robust. For these reasons, Pavlovian fear conditioning is currently a leading model for studying circuity thought to be dysregulated in PTSD. Numerous brain systems have been demonstrated to contribute to fear discrimination and generalization processing, including the cingulate cortex (Ortiz et al., 2019), hippocampus (Besnard & Sahay, 2016), amygdala (Ciocchi et al., 2010; Ghosh & Chattarji, 2015; Grosso, Santoni, Manassero, Renna, & Sacchetti, 2018), thalamic and subthalamic nuclei (Ferrara, Cullen, Pullins, Rotondo, & Helmstetter, 2017; Ramanathan, Ressler, Jin, & Maren, 2018; Venkataraman et al., 2019; Xu & Sudhof, 2013), midbrain (Rozeske et al., 2018), and prefrontal cortices (Fitzgerald et al., 2014; Pollack et al., 2018; Rozeske et al., 2018; Scarlata et al., 2019). For a review on the neurobiology of fear generalization, see Asok, Kandel, and Rayman (2019) and Sangha, Diehl, Bergstrom, and Drew (2020).

Here, a basic protocol for fear discrimination and generalization is described. By following Basic Protocol 1, experimenters will be able to obtain measures of fear memory discrimination and generalization using basic fear conditioning procedures in rodents.

STRATEGIC PLANNING

When beginning a fear generalization experiment, it may be necessary to establish a stimulus generalization gradient (see Understanding Results for more detail). A

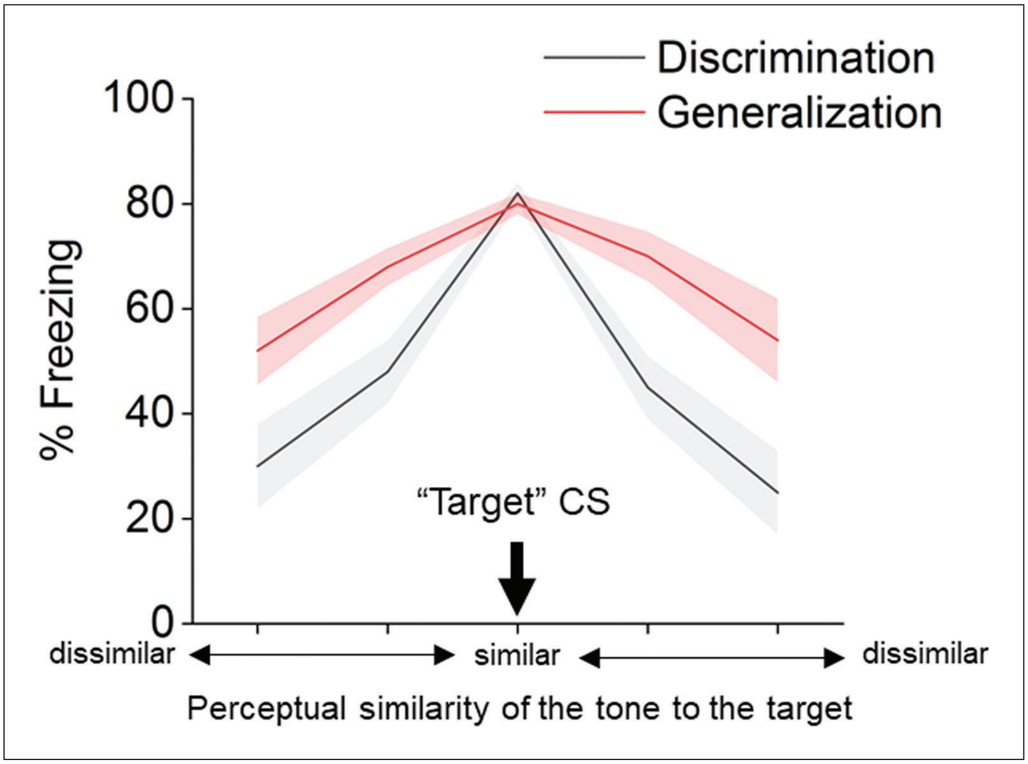

Figure 1 A hypothetical cued fear memory discrimination and generalization gradient. Gray line (discrimination) shows peak responding at the "target" stimulus and reduced freezing as the frequency of the stimulus progressively departs from the target. The red line (generalization) depicts a "widening" or "flattening" of the generalization gradient in relation to the gray line (recent) in which responding to alternative novel cues increases, especially at tone frequencies nearer in perceptual similarity to the "target" conditioned stimulus (CS).

stimulus generalization gradient is a method for systematically testing and visually representing the degree to which an array of stimuli are discriminated and generalized. To construct a generalization gradient, subjects are trained using a single stimulus and afterwards, subjects are tested using different values along the stimulus dimension in a between-subjects design (Mackintosh, 1974; Pearce, 1987; Pollack et al., 2018). The stimulus dimension is a physical variable such as frequency of tone or wavelength of light. Results are then presented as a response gradient along the stimulus dimension (Fig. 1). A steep sloping response gradient is formed when stimuli perceived or processed as unlike the CS elicit reduced conditioned responses (CRs) while stimuli perceived as perceptually like the CS elicit stronger CRs. Steeper shaped curves reflect a high degree of discrimination while flatter curves reflect more generalization (Fig. 1).

Auditory tones are a well-suited sensory stimulus for studying fear memory generalization in mice. Although olfactory stimuli are likely the most ethologically relevant stimulus in rodents, in a laboratory setting, it is challenging to present olfactory stimuli in temporal conjunction with the unconditioned stimulus (US; although there are protocols for olfactory conditioning in rodents, see Ross and Fletcher, 2018). For visual stimuli, the rodent visual pathways to the amygdala are intrinsically different from auditory pathways (Bergstrom, & Johnson, 2014) and it typically takes more visual CS pairings with the US to promote learning as compared with tones in mice (Heldt, Sundin, Willott, & Falls, 2000). Because the rodent auditory sensory system is relatively highly developed and the stimulus perceptual dimension (tone frequency in Hz) relatively easy to manipulate in laboratory settings, the use of cued tone fear conditioning is advantageous in the study of stimulus discrimination and generalization in rodent models. However, there are limitations in the ideal hearing frequency range in rodents (see Commentary, Critical Parameters section).

Context Shift

After pairing the CS with the US, freezing is elicited by both the cue (foreground) and context (background). Context freezing may occlude the ability to detect the cued response. To isolate the CS response, the background context in which the original CS and US were presented together can be changed. To isolate the CS response, researchers typically disguise the training from testing context. There are numerous procedures for masking the training from testing context, including altering olfactory, tactical, visual, and auditory cues. Even with a great degree of difference between the training and testing context, mice, especially C57BL/6 (B6) mice, tend to generalize the context (Balogh, Radcliffe, Logue, & Wehner, 2002; Huckleberry, Ferguson, & Drew, 2016; Laxmi, Stork, & Pape, 2003). Below are recommendations for shifting the training (hereafter referred to as Context A) and testing (hereafter referred to as Context B) environments. Additional details on addressing contextual fear in cued fear conditioning paradigms are addressed in the Troubleshooting section.

The training environment (Context A) can be an unmodified commercial fear conditioning chamber (i.e., exposed shock floor board and unmodified interior of the chamber) and 70% ethanol (EtOH) as a cleaning agent/oderant. For transporting mice from the holding room to the training room, an unmodified home cage carrier may be used. Mice may also be transferred on a cart with a sheet covering for transport from vivarium to training room. Mice are weighed in the same room as training.

The commercial chamber and training procedures can be modified for testing (Context B) in the following ways: 1% acetic acid, or alternate as described above, as a cleaning agent/odorant; black and white inserts applied to alter visual dimensions of the testing chamber (place on the outside of the chamber; Fig. 2). Importantly, insert a plexiglass floorboard to disguise the highly salient tactile features of the shock grid floor. Clean bedding may also be applied to the floor. In our hands (Bergstrom et al., 2013; Bergstrom, McDonald, Dey, Fernandez, & Johnson, 2013; Bergstrom, McDonald, & Johnson, 2011; Bergstrom, McDonald, & Smith, 2006), the addition of bedding reduces background freezing, although to the author's knowledge, an empirical investigation has not been formally undertaken to test this idea. In addition, an insert can be added to hide the geometry of the chamber. For instance, plastic sidewalls inserted at a 60° angle to the floor can form a triangle or a tube can be inserted to form a circle. However, keep in mind that the insert will also likely alter the color, lighting level, and acoustics of the chambers. Changes in acoustics of the chamber are one important consideration especially in the context of auditory CS experiments. Background white noise (60 to 65 dB) can be added by using a fan or HEPA filter in the room. The lux light level of the room can be reduced or red light added. A continuous cue light may also be added. The animals can also be carried to the holding room by hand (or dissimilar carrier from Context A). Mice are habituated in a separate room with different features from Context A (lighting on low or a red light; a fan for white noise is added). The use of white noise during the testing day is also helpful in masking putative exposure of the mice to the CS prior to the retrieval test. Mice can also be weighed first in the vivarium prior to transport to the testing room.

Note, of the variety of cues used to mask the environment, visual cues have been shown to be least important and tactile cues most important in shifting context (Huckleberry et al., 2016). For a more detailed discussion of which features are most salient in context discrimination, see Context Shift section above.

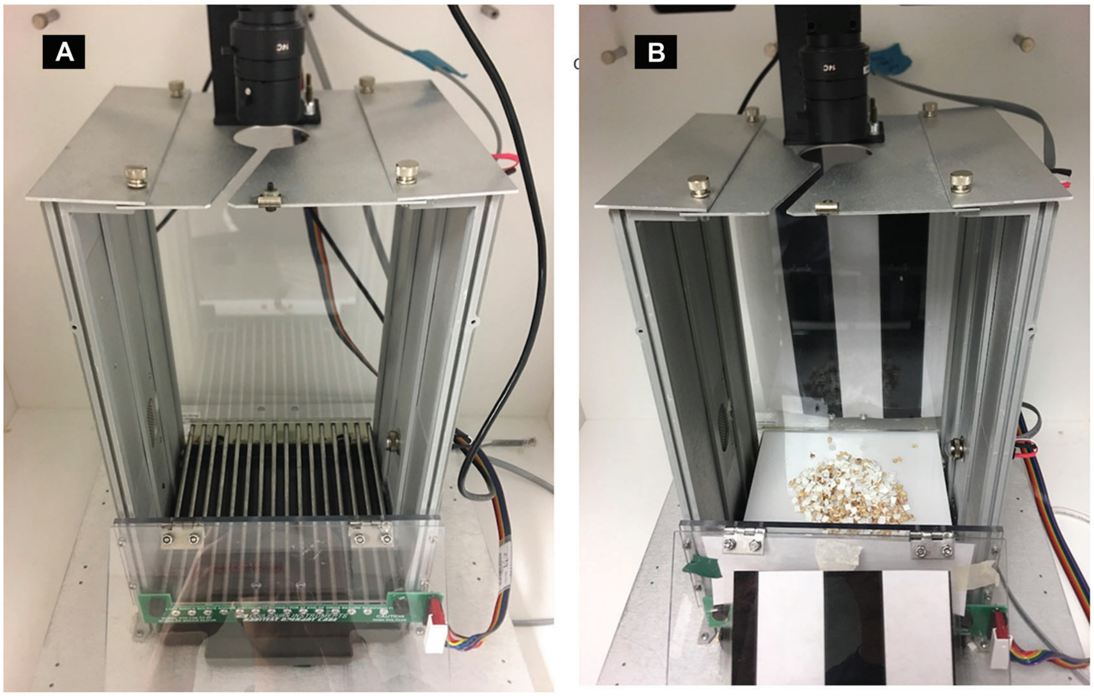

Figure 2 An example of typical ways in which the training context can be disguised from the testing context. (**A**) Unmodified commercial fear conditioning chamber, designated as Context A, for training. (**B**) Modified commercial chamber, designated as Context B, using a plastic floorboard and clean bedding to disguise the floorboard (somatosensory); black and white striped inserts provide a visual disguise. Additional lighting can also be added. Context B is cleaned using a novel odorant (1% acetic acid) compared with Context A (70% EtOH).

TONE CUED FEAR GENERALIZATION GRADIENT

BASIC PROTOCOL 1

Here, a step-by-step instructional guide for constructing a tone cued fear memory generalization gradient using a between-subjects design is provided. For additional, in-depth, guidance on basic fear conditioning parameters and procedures, see Wotjak (2019); Current Protocols article: Wehner and Radcliffe (2004); and Curzon, Rustay, and Browman (2009).

Materials

Male and female mice or rats (see Critical Parameters, Intrinsic factors section below for choosing strains), preferably <6 months of age

Fear conditioning system:
- Shock generator and grid floor for administering temporally precise electrical shocks at various intensities (0.1 to 3.0 mA; mice and rats require different spacing between rods: 0.5 cm for mice and 1.25 cm from center to center for rats recommended; Current Protocols article: Wehner & Radcliffe, 2004)
- Sound generator (that delivers a wide variety of pure tones at a wide range of varying frequencies, 500 Hz to 20 kHz, and dB sound pressure level; may also have capability to deliver white noise or clicker sounds)
- Computer (to run software that interfaces with the chamber to run various CS and US presentation schedules)
- Sound attenuating chambers (that house the fear conditioning chamber)
- Cleaning agents and oderants: Peppermint soap, 70% ethanol (EtOH), 4% acetic acid solution, 1% ammonium hydroxide solution, or isoamyl acetate (Wotjak, 2019)

Sound Level Meter (REED Instruments, cat. no. R8050, 30-130 dB, *www.microdaq.com*)
Meter to check stimulus intensity
Lux meter (to check light levels; 0.1-200,000 lux; Dr. Meter, cat. no. LX1330B, *www.drmeter.com*)
Ohaus Scout Balance Scale (2,200 g × 0.1 g)
Cameras and mounting brackets (for recording behavior; Sentech STC-MB33USB, USB 2.0 CCD, VGA, mono, cased)
Infrared lights and infrared cameras (optional)
Voltmeter (Extech EX330, *www.extech.com*)
Shock level tester (optional; Coulbourn instruments)

NOTE: There are numerous commercial fear conditioning systems available (e.g., Coulbourn Instruments; MazeEngineers; Med Associates; Stoelting Ugo Basile; Kinder Scientific; PanLab; CleverSys; Harvard Apparatus; Lafayette Instrument; San Diego Instruments). There are also open source systems available (see Junior et al., 2019).

Day 0 (habituation; optional)

1. Handle rodents on 2-3 consecutive days for 5 min each day prior to training.

   *While samples sizes should be determined a priori using a statistical power analysis (e.g., G*Power, Univeritat Dusseldorf), we have found that 12-15 animals/group produces reliable measures (Pollack et al., 2018).*

2. Move mice from vivarium to the testing room 30 min prior to testing (habituation). Use a distinct transport system (Context A).

3. Clean fear conditioning chambers with 70% ethanol solution (or chosen Context A solution).

4. Place mice into the fear conditioning chamber to explore for 30 min.

   An alternate habituation protocol is to expose rodents to the CS+ and/or Context B during the habituation period.

Day 1 (training)

5. Thirty minutes prior to training, transfer mice from vivarium to a holding room adjacent to the behavioral training area (Context A). Ensure holding room is sound attenuating.

6. Clean fear conditioning chambers with Context A cleaning solution/oderant using a paper towel.

7. Verify the auditory CS is generated; check tone frequencies and dB levels for each chamber. Record the background dB for each chamber for a comparison with the CS dB.

8. Ensure shock grid floor is delivering a shock and the shock is the appropriate level (mA) using a voltmeter.

9. Ensure cameras are in focus and working properly.

10. Transfer subjects to the training room in individual holding cages (Context A).

11. Weigh the subject.

12. Place subject into the first fear conditioning chamber (Context A). If using multiple chambers, record which chamber the subject was placed in.

13. Activate stimulus protocol.

The stimulus protocol is designed by the experimenter. For example, a stimulus protocol we have used for training in mice is: The subject freely explores the chamber for 3 min prior to the presentation of a 20-s, 5-kHz, 72-75 dB CS that co-terminates with a 0.5-s, 0.5-mA US. The CS/US pairing is presented three times. The intertrial interval (ITI) is variable (e.g., 20-80 s). The final ITI is 60 s. Total test time: 400 s.

14. When the training protocol is finished, remove subject from the chamber and return to the home cage.
15. Clean the chamber.
16. Repeat steps 11-15 for all subjects.

Days 2-3 (Context A and B extinction; optional)

17. Thirty minutes prior to training, transfer mice from vivarium to a holding room adjacent to the behavioral training area (Context A or B).
18. Ensure cameras are in focus and working properly.
19. Transfer subjects to the training room in individual holding cages (Context A or B).
20. Clean chambers with Context A or B cleaning solution/oderant using a paper towel.
21. Weigh the subject.
22. Place mouse into the first fear conditioning chamber (Context A or B). Repeat with additional chambers.
23. Activate the stimulus protocol.

    The stimulus protocol is designed by the experimenter.

    Run stimulus protocol 30 min without presentation of the CS or US.

24. When the protocol is finished, remove subject from the chamber and return it to the home cage.
25. Clean the chamber.
26. Repeat steps 23-25 for all subjects.

Day 4 (generalization test)

27. Thirty minutes prior to training, transfer subjects from vivarium to a holding room adjacent to the behavioral training area (Context B).
28. Verify the auditory CS is generated; check tone frequencies and dB levels for each chamber. Record background dB for each chamber for a comparison with the CS dB. Bear in mind that altering the frequency of the CS alters the tone level intensity (dB). Adjust accordingly.
29. Ensure cameras are focused and operating properly.
30. Transfer subjects to the training room in individual holding cages (Context B).
31. Clean chambers with Context B cleaning solution/oderant using a paper towel.
32. Weigh the subjects.
33. Place subject into the first fear conditioning chamber (Context B).
34. Activate stimulus protocol.

    The stimulus protocol is designed by the experimenter. For example, a stimulus protocol we have used for testing generalization in mice is: The subject freely explores the chamber *for 3 min prior to the presentation of a 20-s, variable-kHz, 75-db CS. The CS is presented three times. ITI is variable, from 20-80 s. Change the variability of the ITI from training. The final ITI is 60 s. Total test time: 400 s.*

35. When the training protocol is finished, remove subject from the chamber and return it to the home cage.
36. Clean chamber with Context B cleaning solution/oderant using a paper towel.
37. Repeat steps 31-36 for all subjects.

BASIC PROTOCOL 2

QUANTIFICATION OF FREEZING

The freezing response: There is a diverse behavioral repertoire of defensive responses available to rodents, including risk assessment, escape, defensive burying, rearing, startle, ultrasonic vocalization, and freezing. The most commonly indexed defensive response and behavioral readout for the cognitive concept of "fear" in behavioral neuroscience is the freezing response (Bergstrom, 2016). Freezing has been used most often because it is robust in classical laboratory-based fear conditioning setups and it is simple to observe and score. Freezing behavior in response to environmental stimuli represents an important variable to keep in mind when designing discrimination and generalization experiments.

Freezing is commonly tracked and analyzed using video tracking software. Several commercially available software packages for automatic scoring of freezing behavior are currently available, including FreezeFrame (Actimetrics), Smart (Panlab), Noldus Ethovision (Noldus), and AnyMaze (Stoelting). There are also a number of open source options available including OptiMouse (Ben-Shaul, 2017), ezTrack (Pennington et al., 2019), Phobos (Amorim, Moulin, & Amaral, 2019), and various other open source options (Patel et al., 2014; *www.mouse-phenotype.org/software.html*). Finally, photobeam-based systems are also available (San Diego Instruments). Whichever automated method for tracking freezing is chosen, it is advisable to first compare/correlate automatic versus manual scoring methods.

Freezing behavior can also be manually scored. Freezing behavior has been classically defined as the absence of movement, except for respiration (Blanchard & Blanchard, 1969). A freezing bout is typically recorded when it lasts 1 s or longer. Freezing is typically scored by dividing the seconds spent freezing during the presentation of the CS by the total duration of the CS and multiplying by 100 to yield a percentage time spent freezing. Freezing is also scored during the initial time period prior to the presentation of the first CS (habituation) and during the ITIs. The time spent freezing during the habituation (pre-CS) period is particularly important to quantify as it provides a proxy measure of contextual or background freezing. Another method for scoring freezing behavior is to directly observe the animal as either freezing or mobile per interval of time, such as every 5-10 s. Another method is to score freezing with a stopwatch using the same intervals as those described above (Bergstrom et al., 2011).

SUPPORT PROTOCOL

ALTERNATE CONDITIONING PARADIGMS

Context Discrimination

The context in which a US occurs can transform into a CS. Context conditioning requires both the amygdala and the hippocampus (Chaaya, Battle, & Johnson, 2018). In context generalization tests, mice are trained using one context and then tested in the same context or a different context. The next day, mice may be tested in the opposite context. A reduction in freezing in the novel context relative to freezing levels in response to the original context indicates discrimination. Methodology for shifting the context is highly variable across investigators. As in cued conditioning, alterations in the somatosensory (e.g., plexiglass covering the shock grid), auditory (continuous white noise versus fan), olfactory stimuli (70% EtOH versus Quatricide), and visual cues are typical. Several groups have tested the relative contribution of various contextual features to discrimination and generalization (González, Quinn, & Fanselow, 2003; Huckleberry et al., 2016). In one study using mice, altering only three elements of the physical testing environment (geometry of the chamber, flooring, and scent) was sufficient to promote discrimination (Huckleberry et al., 2016). When only the geometry of the chamber was changed, mice generalized. When only the floor configuration or olfactory cue was changed, mice discriminated. These data indicate that floor configuration and scent are the most salient features promoting discrimination of context in mice (Huckleberry et al., 2016).

Differential (Discriminative) Fear Conditioning

Rodents are placed into the training context and presented with a CS+ that is always paired with a US and a CS− that is never paired with a US. Rodents receive 5-10 CS+ and 5-10 CS− presentations interleaved randomly. The CS+ and CS− may differ by frequency. On the test day, rodents are presented with additional presentations of the CS+ or CS− alone. White noise or pure tone can be used as the CS+ and CS−. Generalization is inferred when responding to the CS− matches responding to the CS+. Discrimination is inferred when responding to the CS+ is greater than CS− responding. In this case, the CS− becomes what is known as a “safety signal.” This paradigm has been used successfully in both rat (Duvarci, Bauer, & Pare, 2009) and mouse models (Venkataraman et al., 2019).

Cued Safety Learning

Variations of the differential fear conditioning paradigm have been used to study “safety” learning and memory (Foilb, Flyer-Adams, Maier, & Christianson, 2016; Greiner, Müller, Norris, Ng, & Sangha, 2019; Müller, Brinkman, Sowinski, & Sangha, 2018; Sangha, Chadick, & Janak, 2013). While the various procedures for safety learning are outside of the scope of this methods article, there are several reviews on the topic (Christianson et al., 2012; Sangha et al., 2020). Outlined here are two methods for studying safety learning. In a between-subject design, one group of animals is presented with the CS− interleaved with the US. Another group is presented with the CS+ predicting the US. In the within-subjects design (differential conditioning), the subject is presented with both the CS+ and CS−. Increased responding to the CS− on the test day indicates increased generalization and/or safety learning.

Discrimination Training (Within-Subject Design)

Another way to test generalization is in a within-subjects design. In the within-subject design, all mice receive all of the non-target test stimuli in a session. This approach may be impossible when testing for conditioned freezing responses because they may carry over to other stimuli presented close in time. Therefore, in constructing cued fear memory generalization gradients, a between-subject design may be necessary. However, within-subject designs are possible when the presentation of non-target test stimuli is spread out over time. This approach may be useful when characterizing generalization and discrimination over time (see Extrinsic factors section below). For instance, a given non-target stimulus may be presented at both a recent and remote time point following learning. This design was shown to lead to increased discrimination at a remote time point when the same test stimulus was presented at a recent time point following learning (Pollack et al., 2018).

COMMENTARY

Background Information

The overgeneralization of defensive responses to otherwise harmless stimuli may contribute to the psychopathology of anxiety disorders and PTSD (Dunsmoor & Paz, 2015). The application of new behavioral paradigms in the preclinical study of fear generalization are needed. This protocol provides different methodologies for modeling fear generalization, including recommendations for designing the presentation of the conditioned and unconditioned stimuli, as well as dependent measures of conditioning. The advantage to the study of fear generalization in rodents using Pavlovian conditioning procedures is the tight control over the presentation and nature of the stimuli, which may lead to a more detailed understanding of the mechanisms of generalization and discrimination in the brain. The study of fear generalization using auditory cues may have particular relevance in the theater of war because combat acquired trauma likely contains a combination of both sound and contextual elements (Norrholm et al., 2014).

Critical Parameters

Points to consider before beginning

The physical stimulus

The intensity and frequency of the auditory tone are important considerations prior to beginning the experiment. The intensity (dB) of the auditory stimulus should be chosen based on the intensity of the background auditory stimuli. In addition, the dB should be "emotional neutral" and not elicit a response on its own. Therefore, staying below the threshold of a startle response is important. For instance, a startle response can be elicited by 90-dB intensity (Curzon, Zhang, Radek, & Fox, 2009). It is recommended that stimuli are presented in the 60- to 70-dB range. Behavioral responses to the tone alone can be incorporated into the experimental design by including a day 0 (habituation day) into the experimental design (see Basic Protocol 1). Measuring the intensity of the CS (dB) in each chamber before beginning both training and testing is highly recommended. Keep in mind that changing the frequency of the tone may change the dB. So, different dB levels may be required for different frequencies. The sound meter should be placed at the level of the animals and, most importantly, in a consistent position across cages. The dB measurements should be conducted with the chamber and sound-attenuating cabinet doors closed. The dB measurement can be read using the chamber cameras.

Hearing range in rodents

Another critical factor to consider prior to initiating experimentation is the perceptual ability of rodents. In mice, the range of frequencies that can be detected at 60-dB sound pressure level is 2.3 to 85.5 kHz (Heffner & Heffner, 2007). In rats, the range is 500 Hz to 64 kHz (Heffner & Heffner, 2007). Because frequencies at the higher end of the spectrum are not interpretable by humans, it has been proposed that the best frequency range for differential fear conditioning studies is 9 to 10 kHz (Wotjak, 2019). However, cued fear generalization experiments have been conducted using tone stimuli at lower frequencies. For instance, Pollack et al. (2018) tested a set of tone frequencies (2 to 12 kHz) after training at 5 kHz in mice. Results revealed discrimination at 2, 3, and 12 kHz (recent) and generalization at 2 and 3 kHz (remote). In another study, after training at 1 kHz, rats were presented with a set of stimuli with a range from 3 to 15 kHz. Rats generalized the 3 kHz but discriminated the 7- and 15-kHz CS (Grosso et al., 2018). Another consideration as it relates to the detection of different frequencies of tone is the age of the animal. It is well known that hearing performance gradually reduces over time. In particular, B6 mice show steady hearing decline over time. Therefore, it is advisable that B6 mice over 6 months of age not be used in cued tone fear generalization studies to avoid artifacts related to auditory sensory system decline (Ison et al., 2007).

Factors that interact with the degree of discrimination and generalization

Extrinsic factors

There are multiple extrinsic factors that increase generalization, including the intensity of the US, stress, alcohol exposure, and the passage of time. Increasing the intensity of the US is one of the most reliable parameters for promoting generalized responding. Numerous studies have shown that increasing the intensity of the US increases generalization (Baldi, Lorenzini, & Bucherelli, 2004; Ghosh & Chattarji, 2015; Venkataraman et al., 2019). For instance in mice, "weak" US intensities from 0.3 to 0.5 mA results in CS+ discrimination, while 0.8- to 1.0-mA US intensity promoted generalization (Ghosh & Chattarji, 2015). Another factor that has reliably been shown to increase generalization (reduce discrimination) is the passage of time. The passage of time has been shown to magnify both cued (Pollack et al., 2018) and contextual fear (Lynch, Cullen, Jasnow, & Riccio, 2013; Poulos et al., 2016; Sauerhöfer et al., 2012; Wiltgen & Silva, 2007). Approximately 30 days between training and testing has been consistently shown to increase both cued and contextual fear memory generalization, although shorter retention intervals have been reported (Lynch et al., 2013). There is also now a growing body of evidence to suggest that both chronic alcohol exposure and stress exposure promote generalized fear responses in rats and mice (Bender, Otamendi, Calfa, & Molina, 2018; Elliott & Richardson, 2019; Scarlata et al., 2019; Sillivan et al., 2017; Stephens et al., 2005). Finally, context and cued exposure before and after fear learning is another extrinsic factor that can determine the degree of generalization and discrimination. There is evidence showing that pre-exposure to a context that is similar, but not completely different, from the training context will enhance generalization, presumable via mechanisms akin to pattern completion (Sevenster, de Oliveira Alvares, & D'Hooge, 2018). Exposure to the original training context following learning (a reminder cue) has also been shown to promote discrimination (Wiltgen & Silva, 2007).

Intrinsic factors

There are also several intrinsic factors that promote generalization, including rodent strain, rodent age, and rodent sex. Animal strain is an important consideration when designing discrimination and generalization experiments. For instance, C57BL/6J mice were shown to exhibit overgeneralization as compared with the DBA/2J strain (Balogh et al., 2002) and the 129S1/SvImJ strain was shown to exhibit overgeneralization as compared with the C57BL/6J strain (Camp et al., 2012). To note, both the Wister (Pedraza et al., 2019) and Sprague-Dawley (Ghosh & Chattarji, 2015) rat inbred strains have been successfully used in fear discrimination and generalization experiments. Another point of consideration when designing fear generalization experiments are sex differences. There is now accumulating evidence to support a role for biological sex as an interacting variable in fear discriminant and generalized responses (Asok et al., 2019; Foilb, Bals, Sarlitto, & Christianson, 2017; Keiser et al., 2017; Lynch et al., 2013). As there is a renewed call for considering sex as a biological variable in behavioral neuroscience (Shansky, 2019; Shansky & Woolley, 2016), it is recommended that assays for fear generalization and discrimination are conducted using both male and female animals. There are also neurodevelopmental factors that influence generalized responses. For instance, adolescent mice exhibit more generalized fear responses relative to adults (Ito, Pan, Yang, Thakur, & Morozov, 2009).

Troubleshooting

A fundamental parameter in the study of how discrete cues are conditioned is the role of context (see Strategic Planning, Context Shift section). When a foot shock and tone cue are presented together in an environment, not only does the cue take on associative properties but so does the context. Context fear is typically tested by placing the subject back into the original training environment and testing for the freezing response. Cued fear, as discussed in detail above, is tested by placing the animals into a "novel" environment and testing for the freezing response to the cue. The isolation of cued versus context conditioned responding is a critical parameter in the study of cued fear memory discrimination and generalization. This parameter is of particular importance in the study of fear generalization because cued and contextual fear generalization may interact and/or differentially generalize.

There are several methods for attenuating contextual fear (Jacobs, Cushman, & Fanselow, 2010). The first option is to disguise the test chamber (see Strategic Planning, Context Shift section). If possible, separate testing chambers in different behavioral rooms is optimal. If separation of training and testing rooms is not possible, careful procedures using guidelines discussed above to mask the chambers is important (see Strategic Planning, Context Shift section). Another option is to extinguish contextual freezing by exposing the mice to chamber B, and A if necessary, prior to the cue test (Scarlata et al., 2019). A final option is to measure the freezing response immediately prior to the presentation of the CS for an equivalent time as the CS. Any elevation in freezing to the CS over the pre-CS values may be considered cue-specific freezing (Pollack et al., 2018).

For a comprehensive review on this topic, including recommendations for reducing background freezing, see Jacobs et al. (2010).

General locomotor behavior

As in most behavioral testing using rodents, it is prudent to measure the general locomotor abilities of the subjects prior to testing. For instance, locomotor measures in the open field test can provide a baseline level of activity. This is especially important when testing different mouse strains/mutants and when testing the effects of, for example, pharmacological agents, optogenetic/chemogenetic manipulations. If any of these variables interact with baseline locomotor activity, interpretation of the cued freezing response may be confounded.

Understanding Results

The generalization gradient

Following fear conditioning to the target stimulus (i.e., the CS), the extent to which novel stimuli elicit CRs is tested. The strength of the CR typically declines as the stimuli progressively do not resemble the original CS. By plotting conditioned responses to a range of frequencies, a bell-shaped curve with the peak of the curve at the CS can be depicted (Fig. 1). Flattening of the generalization gradient occurs when the presentation of non-target stimuli triggers greater conditioned responses (Fig. 1). One method for quantifying generalization is to calculate a discrimination index. A discrimination index may be generated by dividing the mean values for each animal in response to the target stimulus (T) by the sum of T and each non-target (N) stimulus:

$$\text{Discrimination index} = \mathrm{T}/(\mathrm{T} + \mathrm{N})$$

A value of 1 signifies discrimination while a value of 0.5 indicates complete generalization (Pollack et al., 2018; Scarlata et al., 2019).

Time Considerations

The basic experimental procedures for constructing a cued fear memory generalization gradient is not more than 5 days total. The number of sessions needed to complete the experiments is dependent on the number of conditioning chambers available to the researcher.

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Conflicts of Interest

The author has no conflict of interest to declare.

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