11-2013

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Published in final edited form as:
Behav Processes. 2013 November ; 100: . doi:10.1016/j.beproc.2013.07.015.

An automatic recording system for the study of escape from fear in rats

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Abstract

Escape from fear (EFF) is an *active* response to a conditioned stimulus (CS) previously paired with an unconditioned fearful stimulus (US), which typically leads to the termination of the CS. In this paradigm, animals acquire two distinct associations: S-S [CS-US] and R-O [response-outcome] through Pavlovian and instrumental conditioning, respectively. The present study describes a computer controlled automatic recording system that captures the development of EFF and allows the determination of the respective roles of S-S and R-O associations in this process. We validated this system by showing that only rats subjected to a simultaneous CS-US conditioning (i.e., CS and US occur together at the beginning of each trial) acquired EFF, not those subjected to an unpaired CS-US conditioning. Paired rats had a progressively increased number of EFF and significantly shorter escape latencies than unpaired rats across the 5-trial blocks on the test day. However, during the conditioning phase, the unpaired rats emitted more 22 kHz ultrasonic vocalizations, a validated measure of conditioned *reactive* fear responses. Our results demonstrate that the acquisition of EFF is contingent upon pairing of the CS with the US, not simply the consequence of a high level of generalized fear. Because this commercially available system is capable of examining both conditioned active and reactive fear responses in a single setup, it could be used to determine the relative roles of S-S and R-O associations in EFF, the neurobiology of conditioned active fear response and neuropharmacology of psychotherapeutic drugs.

Keywords

Escape from fear; shuttle boxes; automatic recording; instrumental; 22 kHz USV

1. Introduction

Facing a dangerous situation, animals have two major responses in their defensive repertoire: freezing and fleeing (Bolles 1970; Fanselow 1997). These two species-specific defense reactions (SSDRs) can be conditioned to a neutral stimulus (conditioned stimulus, CS) through either Pavlovian fear conditioning or instrumental conditioning, respectively

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(Bolles 1972). After conditioning, the CS acquires the capacity to elicit responses that typically occur in the presence of danger. Responses such as conditioned freezing, passively avoiding a "shocked" environment, emitting 22 kHz ultrasonic vocalizations (USV) or potentiated startle responses to the CS, are innate, reflexive species-typical responses to threats and are expressed automatically in the presence of conditioned danger, thus they are classified as "*conditioned reactive fear responses*". Fleeing (active avoidance) in order to get away from the CS or terminate it requires animals to make an overt motor action and is a voluntary and intentional motor response to danger, thus, it is deemed as a "*conditioned active fear response"* (Amorapanth, LeDoux, and Nader 2000). Evidence suggests that conditioned reactive and active fear responses are mediated by distinct neural systems involving different sub-regions of the amygdala (Amorapanth, LeDoux, and Nader 2000), that they are sensitive to different classes of psychotherapeutic drugs (Li et al. 2004; Mead, Li, and Kapur 2008), and are best induced under different CS-US temporal relations (Esmoris-Arranz, Pardo-Vazquez, and Vazquez-Garcia 2003).

In recent decades, much conditioned fear work has almost exclusively focused on the *reactive* type (e.g. freezing, fear-potentiated startle, etc.), partially due to the simple setup and convenience of automatic data collection. Contemporary studies on the *active* fear responses and related neurobiology are scarce. One of the paradigms suitable for this endeavor is the escape from fear (EFF) (McAllister and McAllister 1971). In EFF, animals are first trained in a Pavlovian fear conditioning task (CS paired with an aversive unconditioned stimulus, US, e.g. footshock). They are then subjected to an instrumental conditioning procedure in which one of their motor acts (e.g. stepping into another compartment) leads to termination of the fear-provoking CS and is thus negatively reinforced. EFF allows a clean demonstration of the distinct roles of Pavlovian and instrumental conditioning processes in the mediation of various conditioned fear responses as they are conducted in separate phases. It is also an important construct in Mowrer's two-process theory (Mowrer and Lamoreaux 1946; Levis and Brewer 2001) and Denny's relaxation theory (Denny 1971) for the explanation of the instrumental component of avoidance conditioning and the transition of behavioral response patterns from reactive to active fear reactions (LeDoux and Gorman 2001).

Despite its importance, EFF has not been studied extensively due to various issues (Levis 1989; McAllister and McAllister 1991), including its reliability and reproducibility (Cain and LeDoux 2007). Unlike conditioned fear reactions, it is also difficult to be recorded automatically. Often times, an experimenter has to manually manipulate animals and count the number of EFF, which inevitably introduces variability among animals (Amorapanth, LeDoux, and Nader 2000; Crawford and Masterson 1982; Esmoris-Arranz, Pardo-Vazquez, and Vazquez-Garcia 2003). In the present study, we described a computer controlled automatic recording system that is suitable to the study of EFF. In addition, we also recorded the so called "22 kHz ultrasonic vocalization (USV)" throughout the EFF training and testing sessions. This type of vocalization is observed in rats that are exposed to fearful stimuli (Wohr, Borta, and Schwarting 2005) and was thus used as a measure of reactive fear.

2. Materials and methods

2.1. Subjects

Twenty male Sprague-Dawley rats (250–275g upon arrival, Charles River, Portage, MI) were used in this experiment which was approved by the University of Nebraska-Lincoln's Animal Care and Use Committee. Rats were maintained on a 12:12 light/dark schedule and allowed free access to food and water. All testing was conducted during the light phase.

2.2. Apparatus

Five identical two-way shuttle boxes custom designed and manufactured by Med Associates (St. Albans, VT) were used. Each box was housed in a ventilated, sound-insulated isolation cubicle (96.52 cm W35×56 cm D ×63.5 cm H). Each box was 64 cm long, 30 cm high (from grid floor) and 24 cm wide, and divided into two equal-sized compartments by a white PVC partition with an arch style doorway (15cm high × 9cm wide at base). The grid floor consisted of 40 stainless steel rods, spaced 1.6 cm apart, through which scrambled footshock (US, 0.5 mA) was delivered. Illumination was provided by two houselights (28 volts) mounted at the top of each compartment. An ultrasonic vocalization detector (ANL-937A) was situated on the right side wall of each box. The rat location and locomotor activity was detected by a set of 16 photobeams affixed at the bottom of the box (3.5 cm above the grid floor). A speaker was mounted on the ceiling of the cubicle, centered above the shuttle box. All the training and testing procedures were controlled by Med Associates programs running on a computer. Background noise (approximately 74 dB) was provided by a ventilation fan affixed at the top corner of each isolation cubicle.

2.3. Procedure

The basic procedure was adopted from Esmoris-Arranz et al. (2003) who showed that a simultaneous conditioning paradigm (CS and US occur simultaneously at the beginning of each conditioning trial) is better than a forward conditioning one (CS is followed by US) in inducing EFF. After 3 days of handling (2 min/day) and 4 days of habituation (20 min/day) to the shuttle boxes, rats were randomly assigned to two groups: paired and unpaired, and subjected to 4 days of fear conditioning. Unpaired controls were included to determine whether EFF learning was an aversively motivated and specific behavioral response to the CS.

On each conditioning day, rats first received a subcutaneous injection of sterile water (1.0 ml/kg, as a comparison to our antipsychotic drug work). Thirty minutes later, they were placed in either the left or right compartment of the shuttle boxes (the two compartments were blocked by an aluminum partition), alternating with each successive conditioning day and counterbalanced within and between groups. The fear conditioning commenced 3 min later. The CS was a compound stimulus consisting of the *onset* of an 85 dB 2800 Hz pure tone and *offset* of two houselights (tone+light off) for 15 s, similar to the compound CS used in Esmoris-Arranz et al. (2003). The US was a 3 s 0.5 mA electrical footshock. Rats in the *unpaired* group received 10 CS and US exposures, separated by 75 s with an intertrial interval of 180 s. Rats in the *paired* group received 10 trials of CS-US pairing with the 15 s CS and 3 s US occurring simultaneously at the beginning of each trial. Subsequent trials commenced 180 s after offset of the CS. Thus, during the conditioning phase, each rat received a total of 40 CS and US exposures (10 trial/day for 4 consecutive days). The number of 22 kHz USV (>20 ms, >50 dB in the 20–32 kHz range) was recorded for each session.

One day after the last conditioning day, all rats were tested for EFF. The metal partition was removed such that rats had access to both chambers of the shuttle boxes. After 3-min habituation, each rat was given 25 trials of CS presentation (no US) at a 30 s intertrial interval. Each CS was presented for 60 s. If a rat crossed over to the other chamber during the CS with its body and with or without its tail, the CS was terminated and the latency was recorded. The ITI started immediately after the CS termination. If it failed to cross over within 60 s, the trial was terminated and a latency of 60 s was recorded for that trial. In addition, the number of 22 kHz USV was recorded. Only those crossing with latency less than 10 s were deemed as an escape from fear response (EFF), consistent with the criterion used in our active avoidance response studies (Zhang and Li 2012; Swalve and Li 2012;

Sparkman and Li 2012; Li, Sun, and Mead 2012; Zhang, Fang, and Li 2011; Swalve and Li 2010; Sun, Zhao, et al. 2010; Mead and Li 2010; Li et al. 2010; Li, He, and Mead 2009; Li, Fletcher, and Kapur 2007; Li, He, and Mead 2009; Li et al. 2004). As the escape latency data are not normally distributed, group median escape latency was subjected to nonparametric tests, while the numbers of escape and 22 kHz USV were tested by parametric tests.

3. Results

Throughout the 4-day conditioning phase, rats receiving unpaired CS-US presentations appeared to emit more 22 kHz USV than rats presented with paired stimuli (Fig. 1A). A repeated measures analysis of variance (ANOVA) comparing the number of 22 kHz USV over four days of conditioning between the two groups revealed a significant interaction between the day and group, $F_{(3,54)} = 5.400$, $p = 0.003$. There was also a main effect of day, $F_{(3,54)} = 3.747$, $p = 0.016$, but no main effect of group, $p = 0.145$. Two group comparisons on each day showed that the unpaired group emitted significantly more 22 kHz USV than the paired group on day 3, $p = 0.029$. Paired-samples T tests revealed that the 22 kHz USV for paired animals were significantly higher on day 2 than on day 1, $p = 0.047$. Unpaired animals, however, emitted more USV on day 3 than on day 1, $p = 0.004$. They also emitted significantly more 22 kHz USV on day 3 and 4 than on day 2, all ps < 0.05. Thus, rats in the paired and unpaired groups all acquired fear reactions with the latter group appearing to experience a higher sustained fear reaction, possibly due to the stronger contextual fear experienced in the unpaired group.

On the test day, only the rats receiving paired CS-US presentations gradually developed EFF (Fig. 1B and 1C). Nonparametric Independent samples Mann-Whitney tests of the median latency revealed that the paired group had a significantly shorter escape latency than the unpaired group in blocks 4, $p = 0.049$ and 5, $p = 0.029$. Related samples Wilcoxon Signed Rank tests revealed that the escape latencies for paired animals were significantly shorter in blocks 4 and 5 than in block 1, $p = 0.049$ and 0.013, respectively, but no such changes were found in the unpaired animals. They demonstrated comparable low EFF in the first and last test blocks, $p > 0.05$. Similarly, with regard to the number of EFF, the paired and unpaired groups did not differ much in block 1 and 2, while the paired group had much higher number of EFF in the subsequent blocks. Repeated measures ANOVA on the last 3 blocks revealed a main effect of group, $F_{(1,18)} = 8.044$, $p = 0.011$. No such group effect was found in the first 2 blocks, $F_{(1,18)} = 0.010$, $p = 0.921$. Independent samples t tests revealed that the paired group had significantly higher numbers of EFF than the unpaired group on block 3, $t(18) = 2.305$, $p = 0.033$ and 4, $t(18) = 2.711$, $p = 0.014$. Paired-samples t tests revealed that the EFF for paired animals were significantly higher in blocks 3, 4 and 5 than in block 1, all $p < 0.017$. In contrast, the unpaired animals showed comparable EFF across the 5 blocks, all $p > 0.544$. Both groups emitted comparable numbers of 22 kHz USV, $p = 0.839$ (Fig. 1D). Therefore, only rats in the paired group acquired an active fear response to the CS, suggesting that the acquisition of the EFF is contingent on association of the CS with the US, not simply on the level of general fear.

4. Discussion

The present study introduced a novel paradigm to study both conditioned reactive fear (e.g. 22 kHz USV) and active fear responses (e.g. EFF). We showed that EFF resulted from a Pavlovian conditioning process in which animals acquire the CS-US association (S-S association) and from an instrumental conditioning process in which they utilize the S-S information to further acquire the Response-Outcome association (R-O). The role of Pavlovian conditioning in EFF is supported by the finding that only the paired rats (i.e.

acquired S-S association) acquired EFF (Rescorla and Solomon 1967), but not the unpaired ones. The role of instrumental conditioning in EFF is evidenced by the progressively increasing escape responses across 5-trial blocks, suggesting that termination of the CS served as a reinforcer to specifically target an active motor response (i.e. shuttling) (Fanselow 1997).

EFF has been a controversial paradigm as failures to demonstrate its existence are quite common (see Cain and LeDoux, 2007). One possible factor may be the arranged temporal relation between the CS and US in the Pavlovian conditioning phase. Typical EFF studies use a forward conditioning setup in which the CS is presented first, followed by the US. Esmoris-Arranz et al. (2003) clearly showed that this forward conditioning paradigm is best at causing conditioned freezing, but not conducive in inducing a reliable EFF. Rather, a simultaneous conditioning paradigm (CS and US occur simultaneously at the beginning of each conditioning trial) is better in this regard because it mimics the imminent appearance of the danger ecologically (the predatory imminence theory) (Fanselow and Lester 1988). Our result is consistent with the proposition. Another advantage with simultaneous conditioning is that it causes little freezing (Esmoris-Arranz, Pardo-Vazquez, and Vazquez-Garcia 2003), thus, the concern that the development of EFF was the result of a gradual and progressive decrease in freezing behavior does not apply in our paradigm (Cain and LeDoux 2007).

Our paradigm has applications for the study of the neurobiology of conditioned fear and psychopharmacology. As mentioned before, previous work suggests that conditioned reactive and active fear responses are mediated by distinct neural systems involving different sub-regions of the amygdala (Amorapanth, LeDoux, and Nader 2000). The central nucleus of the amygdala is suggested to be critical for the conditioned *reactive* fear responses, whereas the basal amygdala is critical for the *active* fear response, as lesions of the central nucleus impaired conditioned freezing while having no effect on the same animals' ability to actively escape the conditioned fear; on the contrary, lesions of the basal nucleus disrupted active response to the CS, but had no effect on conditioned freezing (Amorapanth, LeDoux, and Nader 2000). However, because EFF was done manually in that study, it is important to use an automatic system such as ours to validate their findings.

Conditioned reactive and active fear responses also differ in their sensitivity to different classes of psychiatric drugs, with the former more sensitive to anxiolytics such as chlordiazepoxide and diazepam (Fendt and Fanselow 1999), and the latter more sensitive to antipsychotic drugs (Sun, He, et al. 2010; Mead and Li 2010; Sparkman and Li 2012; Li et al. 2004; Li, Fletcher, and Kapur 2007; Li, He, and Mead 2009). For example, it has been shown that most benzodiazepines are capable of attenuating *conditioned freezing* (Fanselow and Helmstetter 1988; Harris and Westbrook 1999), decreasing *fear-potentiated startle* (Davis 1986; Joordens, Hijzen, and Olivier 1998), and disrupting *passive avoidance response* (Sanger and Joly 1985; Nabeshima et al. 1990). On the other hand, antipsychotics (both typical and atypical) are well known for their ability to inhibit conditioned *active* avoidance responding (Wadenberg and Hicks 1999; Li et al. 2004). Animals treated with low doses (non-cataleptic) of antipsychotics often fail to acquire or perform avoidance responses to the CS (Arnt 1982; Ader and Clink 1957). However, most avoidance conditioning studies could not determine which process is affected by antipsychotic drugs: the Pavlovian fear conditioning process or the instrumental conditioning because in avoidance conditioning, both the Pavlovian conditioning of fear and the learning of the instrumental response occur concurrently, and any drug effect could be attributed to either one of them or both. In contrast, EFF provides an uncontaminated index of fear as it separates the conditioning process of fear from the measurement of fear (McAllister and McAllister 1971). Therefore, it has an advantage over an avoidance conditioning paradigm

in isolating the drug effect on specific psychological processes by administering a drug at different stages of EFF.

Acknowledgments

Professor Ming Li was supported by a visiting professorship grant from the Key Laboratory of Cognition and Personality and Institute of Psychology at Southwest University, China and by National Institute of Mental Health grant R01MH085635. We thank the two anonymous reviewers for their insightful comments on the previous version of the manuscript.

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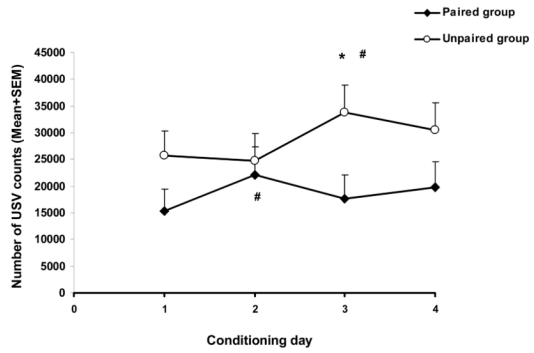
Research highlights

1. Escape from fear (EFF) is an *active* fear response to a conditioned stimulus (CS).
2. Rats received simultaneous CS-US conditioning developed EFF.
3. Rats received unpaired CS-US conditioning did not develop EFF.
4. The computer controlled automatic recording system is capable of tracking EFF development.
5. The paradigm described is useful in determining of the roles of S-S and R-O associations in EFF.

A



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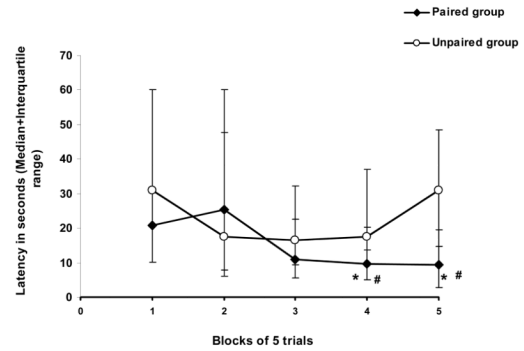

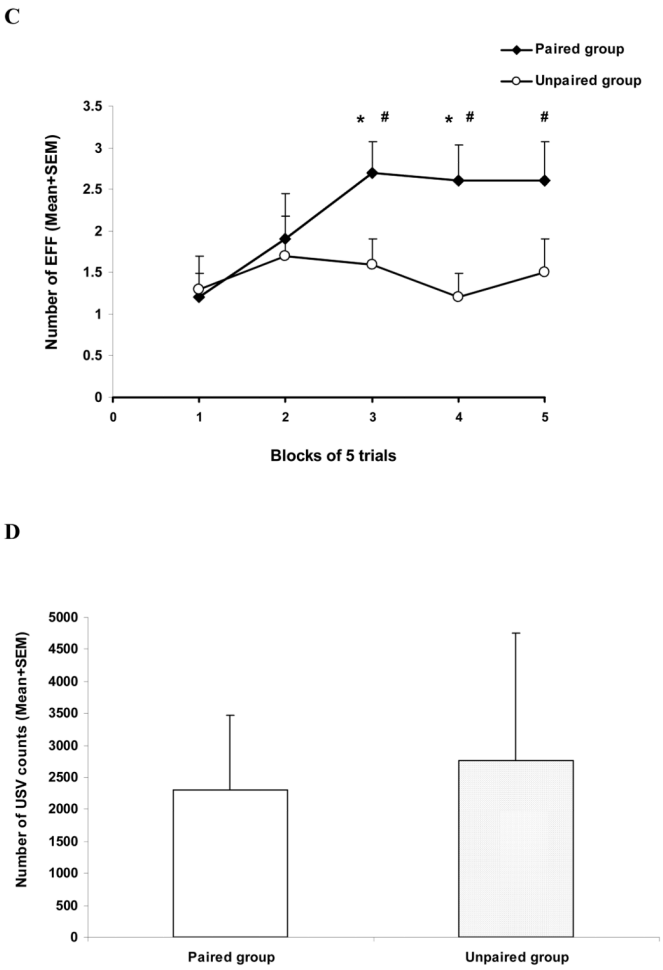

Fig 1.

Twenty-two kHz (22 kHz) USV as a measure of reactive fear responses recorded from rats that received a paired or unpaired CS-US conditioning over the four-day period (**A**). EFF performance as measured using escape latency (**B**) and number of escape responses (latency < 10s) (**C**) across the five 5-trial blocks on the test day. Mean number of 22 kHz USV from the paired and unpaired groups recorded on the test day (**D**).