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Incubation of conditioned fear in the conditioned suppression model in rats: role of food-restriction conditions, length of conditioned stimulus, and generality to conditioned freezing

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Abstract

We recently adapted the conditioned suppression of operant responding method to study fear incubation. We found that food-restricted rats show low fear 2 days after extended (10 d; 100 30-sec tone-shock pairings) fear training and high fear after 1–2 months. Here, we studied a potential mechanism of fear incubation: extended food-restriction stress. We also studied whether fear incubation is observed after fear training with a prolonged-duration (6-min) tone conditioned stimulus (CS), and whether conditioned freezing incubates after extended training in rats with or without a concurrent operant task. Conditioned fear was assessed 2 days and 1 month after training.

In the conditioned suppression method, fear incubation was reliably observed in rats under moderate food-restriction conditions (18–20 g food/day) that allowed for weight gain, and after extended (10 d), but not limited (1 d), fear training with the 6-min CS. Incubation of conditioned freezing was observed after extended fear training in rats lever-pressing for food and, to a lesser degree, in rats not performing an operant task.

Results indicate that prolonged hunger-related stress does not account for fear incubation in the conditioned suppression method, and that fear incubation occurs to a longer-duration (6-min) fear CS. Extended training also leads to robust fear incubation of conditioned freezing in rats performing an operant task and weaker fear incubation in rats not performing an operant task.

Keywords

Fear conditioning; anxiety; stress; incubation; post-traumatic stress disorder; animal models; conditioned freezing; conditioned suppression

Introduction

In fear conditioning studies, an initially neutral stimulus (e.g., tone or context) elicits fear responses after pairing with a noxious stimulus (e.g., electric shock). Under certain conditions, responses to these conditioned stimuli (CSs) increase over time in the absence of

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further stress exposure, a phenomenon termed “fear incubation” (McAllister and McAllister, 1967). Fear incubation has been demonstrated in humans (Diven, 1937, Golin, 1961) and laboratory animals (McMichael, 1966, Houston et al., 1999, Balogh et al., 2002), but most fear incubation studies have examined intervals of 24 h or less (McAllister and McAllister, 1967). Beyond this interval, fear responses typically remain stable over many weeks (Gleitman and Holmes, 1967, Hendersen, 1978, Gale et al., 2004).

Recently, we developed a fear incubation procedure in which conditioned fear increases over several weeks (Pickens et al., 2009b). We trained food-restricted rats to lever-press for food. We then gave each rat 100 30-sec tones co-terminating with footshock over 10 days (10 pairings/day). We found that rats trained using this procedure showed low fear responses to the tone 2 days after the end of conditioned fear training, moderate fear after 15 days, and high fear after 31 or 61 days. In control experiments, we found that time-dependent increases in conditioned suppression to tones are not observed in rats given random tone and shock presentations or tone-only during training. We also found that rats given 1 day of fear conditioning (10 tone-shock pairings) showed high fear responses to the tone 2 days later that did not incubate over time (Pickens et al., 2009b). Thus, under our experimental conditions, fear incubation is dependent on contingent tone-shock exposure during training and on the amount of training.

Here, we further characterize the phenomenon of fear incubation by addressing three issues. The first issue deals with a potential alternative mechanism of fear incubation under our experimental conditions: the level of food restriction. The second issue is the generality of our fear incubation findings, obtained using training conditions with short-duration 30-sec tone-shock pairings, to training conditions in which a prolonged-duration (6-min) tone CS is employed. Finally, we examined the generality of our fear incubation findings to fear measured by conditioned freezing. Below, we provide the rationale for addressing these issues.

One potential alternative explanation for fear incubation is that it is due to a stress-like state induced by extended food-restriction conditions (~14 g/day to keep body weight stable) used in our previous studies (Pickens et al., 2009a, Pickens et al., 2009b), which may have led to increased responsiveness to the fear CS over time. Male rats increase their body weight over time under free feeding conditions, and may experience increased food-restriction stress over time if kept at a constant body weight. Food-restriction is regarded as an environmental stressor because, like other stressors, it activates the hypothalamic-pituitary-adrenal (HPA) stress system (Kant et al., 1988, Marinelli et al., 1996, Heiderstadt et al., 2000, Toth and Gardiner, 2000). Additionally, stress exposure either prior to fear conditioning (Rodrigues et al., 2009) or between training and fear testing (Akirav and Maroun, 2007, Baratta et al., 2007) can increase conditioned fear expression. Thus, under conditions of extended food restriction, incubation of fear may be due to a non-associative time-dependent increase in reactivity to conditioned stressors.

A second issue is that, in our previous experiments, we used 30-sec tones co-terminating with shocks. Thus, it is unclear whether fear incubation occurs only in rats trained with these parameters, or whether fear incubation is a robust phenomenon that can occur under other training conditions. In this regard, Bouton and colleagues (Waddell et al., 2006) and Davis and colleagues (Walker and Davis, 2008, Walker et al., 2009) have suggested that prolonged-duration CSs (> 1 min) paired with predictable or unpredictable shocks cause an emotional state similar to anxiety, with different neuronal substrates than those observed with the short-duration fear CSs with predictable shocks used in our previous studies (Pickens et al., 2009a, Pickens et al., 2009b). From this perspective, it would seem important

to determine whether fear incubation would be observed after extended fear training with longer-duration CSs.

The third issue is that all of our previous experiments have measured fear using conditioned suppression of lever-pressing, as opposed to other measures such as conditioned freezing or fear potentiated startle (Davis, 1990, Fendt and Fanselow, 1999, LeDoux, 2000). Thus, it is unknown whether fear incubation is specific to the conditioned suppression response or whether it is a more general phenomenon observed after extended fear conditioning. Thus, we determined whether fear incubation is observed in the commonly used conditioned freezing method that has been used in numerous studies (LeDoux, 2000, Maren, 2001, Fanselow and Gale, 2003).

Materials and methods

Subjects

Male Long-Evans rats (total $n=148$, Charles River, Raleigh, NC, 280–475 g on first day of deprivation) were individually housed in a colony room under a reverse 12-h:12-h light-dark cycle with lights off at 9 am. We excluded 4 rats due to a problem with the conditioning chambers. For all experiments, food given per day was the total of pellets earned in training and home-cage food chow (weight of food pellets earned in the operant chambers was subtracted from their daily food ration). All procedures followed the guidelines outlined in the “Principles of Laboratory Animal Care” (NIH publication no. 85-23) and were approved by the local Animal Care and Use Committee.

Apparatus

Experiments were conducted in 12 operant chambers (Med Associates, St Albans, VT). Each chamber had two levers 9 cm above the floor, but only one lever (“active,” retractable lever) activated the pellet dispenser, delivering 45-mg food pellets (# F00021, 5.5% fat, 60% carbohydrate, 4.5% fiber; Bioserv, Frenchtown, NJ). The chambers’ grid floors were connected to electric shock generators. A video camera for closed captioned viewing (Panasonic Canada Inc., Mississauga, ON) was mounted to the top of each chamber, extending 2 cm into the chamber with software to record freezing responses time-locked to tone presentations (Coulbourn Instruments, Allentown, PA).

Exp. 1–2: Conditioned suppression

Procedures—We used a fear incubation protocol consisting of 6 phases: food-cup training (1 session), operant training (5 sessions), fear conditioning (1 or 10 sessions), incubation period (2 days or 1 month), baseline session (1 session), and test for tone-induced conditioned fear (1 session). Rats were trained during the dark cycle. Sessions began with extension of the active lever and illumination of a red houselight. Rats were weighed and fed after the daily sessions.

Exp. 1: Effect of feeding regimen on fear incubation

Two groups were used to assess incubation of fear using within-subjects comparisons in a mixed-factor design. These rats were weighed and one group was given 4 g/d of food for 4–7 days until they reached 85% of their weight on day 1, and then received 20 g/d thereafter. A second group was weighed and given 20 g/d of food without any initial food-deprivation. In addition, rats in a third group were restricted to 85% of the initial weight and then given 20 g per day; they were tested for the first time after 1 month (between-subjects comparison). A comparison group with free food access was weighed daily, but these rats were not run in the operant chambers.

Twelve days (± 2) after the initial day of deprivation, the rats were given 60-min food-cup training (pellet delivery every 125 sec). The following day, 2 sessions on a fixed-interval-1 (FI-1) reinforcement schedule (lever-presses could earn a pellet each sec) were run 2–4 h apart. These sessions ended when rats received 50 pellets (up to 1 h). A third session of up to 3 h was run immediately after the second session for rats that did not earn 50 pellets in the second session. All rats earned 50 pellets in this session. The rats were then given one 90-min session in which pellets were earned on a variable-interval-30 (VI-30) reinforcement schedule (pellet availability for lever-presses ranging from 1 to 59 sec), and 2 daily 90-min sessions on a VI-60 schedule (pellet availability ranging from 1 to 119 sec). Rats were maintained on the VI-60 schedule for the rest of the experiment.

Fear conditioning training occurred over 10 90-min sessions. During each session, the rats were given ten 30-sec tones (2900 Hz, 20 dB above background), ranging from 3 to 14 min apart and co-terminating with an electric shock (0.5-sec, 0.5-mA, scrambled, shock intensity adjusted for inter-chamber variability) while earning pellets on a VI-60 schedule. Conditioned suppression of lever-pressing for food pellets was our measure of fear (Estes and Skinner, 1941). Lever-presses were recorded during the 30 sec prior to tone presentation (PreCS) and during the 30-sec tone presentation (CS). The lever-press rates in these 2 periods (lever presses/min) were converted into a suppression ratio: $\text{Suppression ratio} = ((\text{PreCS} - \text{CS}) / (\text{PreCS} + \text{CS}))$. This suppression ratio normalizes lever-pressing during the tone for baseline preCS responding (Armony et al., 1997). A value of 1 indicates total conditioned suppression of lever-pressing during tone presentation (high fear). A value of 0 reflects no lever-press suppression during tone presentation (no fear). The rats in the different groups were matched for their preCS lever-pressing and suppression ratios during training.

During the incubation period, the food-restricted rats were weighed and handled 5–7 times per week. On the last day of each incubation period, lever-pressing was re-stabilized in a 90-min baseline session with no tones or shocks. The following day, conditioned fear to the tone CS was tested. Four 30-sec tones were presented, without shock, over 35 min. The first tone occurred after 6.5 min and subsequent tones occurred after inter-trial intervals of 4, 7, and 11 min. The two primary groups ($n=8$ –12/group) were tested on day 2 and retested on day 33 ± 1 (designated as 1 month). An additional group ($n=8$) was deprived to 85% then given 20 g/d throughout training and was tested for the first time on day 32 (designated as 1 month) to compare the within-subjects and between-subjects incubation effects. In this experiment, we assessed both conditioned suppression and conditioned freezing during testing. During testing, freezing, defined as immobility except for movement related to breathing (Fanselow, 1980), was measured during the preCS and CS periods every 2 sec (according to a metronome). A blind primary rater scored the data for both feeding groups (85% then 20 g and 20 g). A second rater scored the 85% then 20 g group for calculating inter-rater reliability ($r=0.92$).

Exp. 2: Fear incubation with a prolonged-duration tone CS

There were 4 groups: Extended Training-Day 2 Test, Extended Training-1 Month Test, Limited Training-Day 2 Test, and Limited Training-1 Month Test (12–16 rats per group). The rats were weighed and given 4 g/day of food until they reached 85% of their initial weight, and then received 19 ± 1 g/d thereafter. Fifteen days (± 2) after the initial deprivation day, the rats were trained to lever-press for food as described in Exp. 1. Half of the rats received 1 day of fear conditioning with the 6-min tones (Limited Training, 2 shocks per CS during exposure to 5 tones). The other half of the rats received 10 days of conditioning with the 6-min tones (Extended Training, 2 shocks per CS during exposure to 50 tones). During each session, the rats were given five 360-sec tones (2900 Hz, 20 dB above background), ranging from 6 to 18 min apart and each containing 2 electric shocks (0.5-sec, 0.5-mA) at

random times during the tones while earning pellets on a VI-60 schedule. Each tone contained 1 shock in the first 180 sec and one shock in the second 180 sec. Lever presses were recorded during the 2 min prior to the tone (PreCS) and during the 6-min tone presentation (CS). The rats assigned to the different treatments were matched for their preCS lever pressing and suppression ratios during training.

During the incubation period, the food-restricted rats were weighed and handled 5–7 times per week. On the last day of each incubation period, lever-pressing was re-stabilized in a 90-min baseline session with no tones or shocks. The following day, conditioned fear to the tone was tested. Two 360-sec tones were presented, without shock, over 35 min. The first tone occurred after 9 min and the second tone occurred after an inter-trial interval of 11 min. Within each training condition, half of the rats were tested on day 2 and then retested on day 32±1 (designated as 1 month). The other rats were tested for the first time on day 32±1 (designated as 1 month).

Exp. 3: Conditioned freezing

Procedures—In Exp. 3, we used a fear incubation protocol consisting of 5 phases: experimental chamber habituation (1 session), fear conditioning (10 sessions), incubation period (2 days or 1 month), experimental chamber “re-habituation” (1 session), and test for tone-induced conditioned freezing (1 session). There were 4 groups: Restricted Feeding-Day 2 Test, Restricted Feeding-1 Month Test, Free Feeding-Day 2 Test and Free Feeding-1 Month Test (n=12 per group). The rats were weighed and one group was given 4 g/d of food for 4–6 days until they reached 85% of their initial weight, and received 19±1 g/d thereafter. A second group was weighed and given unlimited food throughout training. Eighteen days (±3) after the initial day of deprivation the rats were placed in the experimental chambers for 90-min with no levers or food pellets available.

The rats were then given 10 sessions of fear conditioning with each session containing ten 30-sec tones (2900 Hz, 20 dB above background), ranging from 3 to 14 min apart and co-terminating with an electric shock (0.5-sec, 0.5-mA) in the absence of the lever or food pellets. The rats assigned to the different treatments were matched for freezing to the tones during training.

Half of the rats in each feeding condition were tested on day 2 and then retested on day 35±1 (designated as 1 month). The other half of the rats were tested for the first time on day 35±1 (designated as 1 month). All rats had a 90-min chamber habituation session (with no active lever, pellets, tones or shocks) on the last day of the incubation interval, which also served as a contextual fear test. In the tone test, four 30-sec tones were presented, without shock, over 35 min. The first tone occurred after 6.5 min and subsequent tones occurred after inter-trial intervals of 4, 7, and 11 min. Freezing across the 4 extinction trials was used as our measure of conditioned fear. For behavioral assessment during training, each rat was rated for freezing once during each 5 sec during the 30-sec CS period. During the first 5 min of the chamber habituation session on Day 1 or Day 34 (the day before the tone test) and during fear testing 24 h later, freezing was measured every 2 sec. Freezing to the training context (day 1 and day 34 sessions) and tone tests (day 2 and 1 month) were scored by the blind primary rater. A second rater scored these sessions as well for purposes of inter-rater reliability (r=0.96 for the chamber habituation context test, r=0.97 for the tone test).

Statistical analyses—Data were analyzed by Statistica 5.1 software (Tulsa, OK). The main dependent measure in Exp. 1–2 was suppression ratio that is defined as: ((PreCS-CS)/(PreCS+CS)). The main dependent measure in Exp. 3 was freezing. The factors used in the statistical analyses are described in the Results section and significant effects (p<0.05) in the different ANOVAs were followed by post-hoc Fisher PLSD tests.

Results

Exp. 1: Effect of feeding regimen on fear incubation

Body weight—The high food-restriction rats (85% then 20 g/day; $n=12$), the low food-restriction rats (20 g/day; $n=8$), and the free-feeding rats ($n=12$) gained 64 ± 2 g, 58 ± 3 g and 110 ± 7 g, respectively, during the 1 month incubation period of the food-restricted (experimental) rats.

Training—The rats showed increased conditioned suppression across training with no significant decrease as training progressed (Training Session: $F_{(9,162)}=3.1$, $p<0.01$) (Fig. 1A). There was a significant difference between the two feeding conditions on preCS lever-press/min across training ($p<0.01$) and on the last day of training (85% then 20 g: 22.1 ± 4.2 ; 20 g: 9.9 ± 2.2 ; $p<0.05$), but no significant difference in suppression ratios across training or on the last day ($p>0.05$).

Conditioned suppression testing—The rats exhibited more fear, as measured by conditioned suppression, after one month than after 2 days, and fear incubation was stronger in rats deprived to 85% before receiving 20 g/day of food (Fig. 1B,D). The statistical analysis included the between-subjects factor of Feeding Regimen (85% then 20 g and 20 g) and within-subjects factors of Test Day (day 2 and 1 month) and Test Trial (the 4 tone presentations). There were significant effects of Feeding Regimen ($F_{(1,18)}=6.6$, $p<0.05$), Test Day ($F_{(1,18)}=50.5$, $p<0.01$), Test Trial ($F_{(3,54)}=21.3$, $p<0.01$), Feeding Regimen X Test Day ($F_{(1,18)}=8.4$, $p<0.01$), and Test Day X Test Trial ($F_{(3,54)}=4.6$, $p<0.01$). Post-hoc group differences within each feeding condition are indicated in Fig. 1B and 1D. There were no differences in preCS response rates (all p values >0.05) (Table 1).

We also fear conditioned a comparison group that was deprived to 85% and then given 20 g/d and was tested for the first time after 1 month. This group showed significant fear incubation (mean test suppression ratios: Day 2 test group: 0.17 ± 0.07 ; 1 Month test group: 0.72 ± 0.09). The rats showed a similar pattern of results to the rats retested after 1 month, with increased conditioned suppression on all 4 trials. The statistical analysis revealed significant effects of Test Day ($F_{(1,18)}=34.9$, $p<0.01$) and Test Trial ($F_{(3,54)}=6.5$, $p<0.01$), but no interaction ($p>0.05$). There was no group difference in preCS response rates ($p>0.05$).

Conditioned freezing testing—Both feeding groups also showed significant fear incubation, as assessed by freezing scores (Fig. 1C,E). There were significant effects of Feeding Regimen ($F_{(1,18)}=5.1$, $p<0.05$), Test Day ($F_{(1,18)}=37.1$, $p<0.01$), Test Trial ($F_{(3,54)}=18.5$, $p<0.01$), and Test Day X Test Trial ($F_{(3,54)}=3.9$, $p<0.05$). Post-hoc group differences within each feeding condition are indicated in Fig. 1C and 1E. There was a significant interaction of Feeding Regimen X Test Day in the preCS freezing rates, but no other effects were significant (all p values >0.05) (Table 2). Additionally, we found significant correlations between conditioned suppression of lever pressing and percent time freezing during the tone for both feeding condition groups ($r=0.76$ and 0.89 , $p<0.01$ for 20 g/d with and without initial 85% deprivation, respectively).

Exp. 2: Fear incubation with a prolonged-duration tone CS

Training—During training, the rats in the 10-day training condition showed an initial increase in conditioned suppression that was followed by a decrease as training continued (Training Session: $F_{(9,270)}=19.6$, $p<0.01$); the suppression ratio increased from day 1 to day 2 and then declined. This is indicated by a lower suppression ratio on day 10 than on days 1–6 (post-hoc comparisons are indicated in Fig. 2A). There was no significant difference in

suppression ratios between the extended (10 d) training and limited (1 d) training groups on the first training day ($p>0.05$).

Conditioned fear testing—Fear incubation was seen in rats given 10 days, but not 1 day, of conditioned fear training (Fig. 2B–E). The fear response decreased during the first tone presentation and was not observed during the second tone presentation. The statistical analysis for the between-subjects comparison included the between-subjects factors of Training Duration (1 or 10 training days) and Test Day (the day 2 or 1 month test) and the within-subjects factor of Test Trial (the 2 tone presentations). This analysis revealed significant effects of Test Trial ($F_{(1,52)}=32.7$, $p<0.01$) and Incubation Day X Test Trial ($F_{(1,52)}=6.0$, $p<0.05$); the interaction reflects that the increased fear response after 1 month was only observed in the first but not second trial. Post-hoc comparisons are indicated in Fig. 2. An ANOVA examining preCS response rates found a significant effect of Training Duration ($F_{(1,52)}=9.6$, $p<0.01$) (Table 1), with the limited-training rats showing lower preCS lever-pressing. However, there was no effect of Test Day or an interaction (p values < 0.05). Thus, it is unlikely that changes in preCS lever-pressing over time affected fear incubation.

Analysis of the within-subjects comparison revealed significant effects of Test Trial ($F_{(1,26)}=15.7$, $p<0.01$) and Training Duration X Test Day ($F_{(1,26)}=5.5$, $p<0.05$); the interaction reflects that the increased fear response after 1 month was observed in the extended training rats, but not in the limited training rats. Post-hoc comparisons are indicated in Fig. 2. An ANOVA examining preCS response rates found a significant effect of Training Duration ($F_{(1,52)}=4.6$, $p<0.01$) (Table 1), with the limited-training rats showing lower preCS lever-pressing. However, there was no effect of Test Day or an interaction (p values > 0.05).

Exp. 3: Fear incubation with conditioned freezing

Training—The rats showed high conditioned freezing that increased during training (Training Session: $F_{(9,396)}=2.9$, $p<0.01$) (Fig. 3A). There was greater conditioned freezing in the free-feeding rats than in the restricted-feeding rats (Feeding Regimen: $F_{(1,44)}=23.2$, $p<0.01$). No other effects or interactions were significant.

Conditioned tone fear testing—There was a significant increase in freezing to the tone over 1 month, which was mostly caused by the restricted-feeding rats (Fig. 3B–E). The statistical analysis for the between-subjects comparison included the between-subjects factors of Feeding Regimen and Incubation Day and the within-subjects factor of Test Trial. This analysis revealed significant effects of Feeding Regimen ($F_{(1,44)}=11.9$, $p<0.01$), Incubation Day ($F_{(1,44)}=4.3$, $p<0.05$) and Incubation Day X Test Trial ($F_{(3,132)}=4.5$, $p<0.01$). Post-hoc group differences within each feeding condition are indicated in Fig. 3B and 3D. Analysis of the within-subjects comparison revealed significant effects of Feeding Regimen ($F_{(1,22)}=4.5$, $p<0.05$), Test Trial ($F_{(3,66)}=3.5$, $p<0.05$), and Incubation Day X Test Trial ($F_{(3,66)}=4.4$, $p<0.01$). Post-hoc group differences within each feeding condition are indicated in Fig. 3C and 3E.

Context fear testing—There was a significant increase in freezing to the context over 1 month in the free-fed rats in the between-subjects comparison, but not in the within-subjects comparison or in the food-restricted rats. The freezing scores (percent time freezing during the first 5 min of the context habituation session) for the between-subjects comparison were: Free Feeding-Day 2: 2.7 ± 0.6 ; Free Feeding-1 Month: 11.4 ± 3.1 ; Restricted Feeding-Day 2: 1.7 ± 0.7 ; Restricted Feeding-1 Month: 2.8 ± 0.9 . The statistical analysis for the between-subjects comparison included the between-subjects factors of Feeding Regimen and Incubation Day. This analysis revealed significant effects of Feeding Regimen: ($F_{(1,44)}=9.0$,

$p < 0.01$), Incubation Day ($F_{(1,44)} = 9.4$, $p < 0.01$) and Incubation Day X Feeding Regimen ($F_{(1,44)} = 5.7$, $p < 0.05$). Separate analyses of the Free Feeding and Restricted Feeding groups alone revealed a significant effect of Incubation Day in the Free Feeding rats ($F_{(1,22)} = 8.5$, $p < 0.01$), but not in the Restricted Feeding rats ($p > 0.05$). The freezing scores (percent time freezing during the first 5 min of the context rehabilitation session) for the within-subjects comparison were: Free Feeding-Day 2: 2.7 ± 0.6 ; Free Feeding-1 Month: 5.7 ± 1.7 ; Restricted Feeding-Day 2: 1.7 ± 0.7 ; Restricted Feeding-1 Month: 1.5 ± 0.3 . The analysis revealed a significant effect of Feeding Regimen ($F_{(1,22)} = 7.7$, $p < 0.05$), but no effect of Incubation Day or interaction (all p values > 0.05).

Discussion

There are 3 main findings in our experiments. First, we found that fear incubation occurred under moderate food-restriction conditions (18–20 g food/d) that are used in many learning and memory and behavioral pharmacology studies. This finding suggests that a potential prolonged stress-like state induced by the more restrictive feeding conditions (~14 g/d) in our previous studies (Pickens et al., 2009a, Pickens et al., 2009b) does not account for fear incubation. Second, we found that fear incubation occurred after pairing prolonged-duration (6-min) tones with shocks randomly occurring within each tone. As is the case with short-duration 30-sec tones, fear incubation only occurred after extended (10 day), but not limited (1 day), training. Third, fear incubation, as measured by conditioned freezing, was observed in rats while performing an operant task and, in some cases, in rats not performing an operant task. Below, we discuss our findings.

Role of feeding regimen in fear incubation

Our goal in Exp. 1 was to determine whether fear would incubate under several food-restriction conditions that are more moderate than those used in our previous studies (Pickens et al., 2009a, Pickens et al., 2009b). We studied this issue, because a potential alternative account of fear incubation in our previous studies is that it is due to a stress state induced by extended food-restriction to keep rats at a stable body weight (Heiderstadt et al., 2000), which led to increased responsiveness to the fear CS over time. Thus, under conditions of extended food-restriction at a constant weight, incubation of fear could be due to non-associative time-dependent increases in stress reactivity to conditioned stressors, such as CSs previously paired with shock.

We found no evidence that our previous, more severe, food-restriction conditions (decreasing body weight to 85% of normal weight over several days and maintaining rats during training and testing at this weight) can account for fear incubation. We demonstrated reliable fear incubation in rats allowed to gain weight over time in Exp. 1–2. Additionally, we observed that conditioned suppression on day 2 and conditioned freezing on day 30 was higher under the least restrictive feeding condition (20 g of feed without initially decreasing body weight to 85% of normal weight, see Fig. 1) than in our more restrictive feeding conditions. Such results do not support the notion that increasing food-restriction stress can account for fear incubation observed in our previous studies (Pickens et al., 2009a, Pickens et al., 2009b).

Fear incubation with sustained prolonged-duration tones

We found that conditioned suppression to prolonged-duration (6-min) CSs increased over time after 10 days of training, extending our incubation findings with short-duration 30-sec CSs. We also extended our previous results on the lack of fear incubation in rats given a single day of conditioning, by showing that rats given a single day of conditioning with prolonged-duration CSs containing unpredictable shocks showed no increase in fear over

time. Waddell et al. (2006) and Walker and Davis (2008) have suggested that fear conditioning using prolonged-duration CSs with shocks occurring either predictably or unpredictably during CS exposure causes an anxiety-like state. Our findings suggest that the emotional response that prolonged-duration CSs evoke undergoes incubation similar to the response evoked by shorter-duration CSs that are hypothesized to primarily evoke adaptive fear rather than maladaptive anxiety states (Waddell et al., 2006, Davis et al., 2009).

One complication with our findings is that we found lower baseline lever-pressing in rats given one day of conditioning, compared with those trained for ten days. However, the comparison between the extended and limited training groups is less important to our incubation findings than the comparison between the two time-points within the limited training group, because it is the change in suppression ratio between the two time-points that constitutes incubation. There was no difference in preCS lever-pressing between the two time-points in the limited-training rats, suggesting that the lack of fear incubation after limited training is not caused by changes in baseline lever-pressing.

One explanation for the differential baseline lever-press results between rats trained for 10 days versus rats trained for 1 day is that contextual conditioning suppressed baseline lever-pressing, particularly in the limited-training rats. Contextual fear conditioning could have occurred on the first day of conditioning and then been extinguished during the nonreinforced intertrial intervals over the subsequent 9 days in the extended training condition, but not in the limited-training condition, in which rats were only trained for that one day (Rescorla and Wagner, 1972). Given a constant session length (90 min in our experiments), longer duration CSs do not condition as rapidly as shorter duration CSs (Gibbon and Balsam, 1981). Thus, rats trained with prolonged-duration CSs could demonstrate increased contextual conditioning, since the prolonged (6-min) CSs provide less competition for learning than our short duration (30-sec) CSs.

Fear incubation in the conditioned freezing procedure

We found that conditioned freezing increased across a 1-month incubation interval in rats that were lever-pressing for food. Conditioned suppression of lever pressing has been shown to correlate with conditioned freezing (Bouton and Bolles, 1980, Mast et al., 1982). In agreement with these reports, we found significant correlations between conditioned suppression of lever pressing and conditioned freezing during tone presentations (see Results). However, the degree to which incubation of conditioned suppression and incubation of conditioned freezing are mediated by the same neurobiological substrates is currently unknown. As can be seen in the first trial in Fig. 1, rats that showed complete cessation of lever responding in response to the tone after one month do not show complete freezing to the same CS (see also Mast (1982)). Additionally, several investigators (Amorapanth et al., 1999, McDannald, 2010) reported that periaqueductal gray lesions that decrease conditioned freezing have no effect on the expression of conditioned suppression.

Fear incubation of conditioned freezing also occurred in rats that were not lever-pressing for food, both in the context and the tone tests. However, the effects were less robust than the fear incubation seen in rats that were lever pressing for food. For instance, there was an effect of the incubation interval on contextual freezing, in accord with findings from other laboratories (Houston et al., 1999, Balogh et al., 2002, Balogh and Wehner, 2003), but only in the between-subjects comparison where rats were tested for the first time after 1 month. Even within the between-subjects comparison, only the free-feeding rats, but not the restricted feeding rats, showed fear incubation.

Likewise, there was incubation of conditioned freezing to the tones. However, the overall effect was significant only in the between-subjects comparison where rats were tested for the

first time after 1 month. The restricted-feeding rats, but not the free-feeding rats, showed fear incubation in both the between- and within-subjects comparison. The reasons for this small effect of fear incubation in the absence of an operant task are not known.

The time course of fear responding during training and the development of fear incubation

In Exp. 2, and in our previous work (Pickens et al., 2009a, Pickens et al., 2009b), incubation of fear was accompanied by time-dependent decreases in conditioned suppression during acquisition of fear conditioning. Based on this finding, and the observation that the magnitude of renewal of fear conditioning after a context switch is correlated with time-dependent decreases in fear during extended training (Bouton et al., 2008), we suggested that decreased conditioned suppression during training is the cause of subsequent fear incubation (Pickens et al., 2009a). We speculated that the underlying mechanism of fear incubation is a process akin to spontaneous recovery; the resumption of the extinguished conditioned response that occurs after time has passed following the conclusion of extinction training (Pavlov, 1927, Bouton and Swartzentruber, 1991). Specifically, when rats become less sensitive to shocks after repeated exposure, the repeated pairing of the tone with these less effective shocks causes reduced responding to the CS, similar to that which occurs during extinction when rats are exposed to tone without shock. This results in decreased responding to the tone late in training, as well as during day 2 fear testing. After one month, however, the fear response spontaneously recovered (similar to resumption of fear in traditional spontaneous recovery after extinction), leading to the emergence of fear incubation. However, as can be seen in Fig. 1, Exp. 1 results indicate that incubation of fear can be observed without decreases in conditioned suppression during training. This finding questions the validity of our putative spontaneous recovery-like behavioral mechanism of fear incubation.

Conclusion

We found that fear incubation after extended training is a robust phenomenon that can be seen under several experimental conditions, including different food deprivation states, CS lengths and CS-shock pairing parameters, and methods of fear measurement. We also extended our previous finding that fear incubation requires extended fear conditioning training. Our data suggest that prolonged hunger-related stress does not account for fear incubation. Importantly, fear incubation generalizes to prolonged-duration fear CSs, hypothesized to more closely model human anxiety disorders than short-duration CSs (Davis et al., 2009). Fear incubation can also be demonstrated using the common conditioned freezing measure. Finally, we propose that incubation of fear after extended fear training can be used to model both fear and anxiety responses that grow over time, as in the case of delayed-onset posttraumatic stress disorders (Andrews et al., 2007, Andrews et al., 2009).

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Figure 1.

Fear incubation in rats maintained on mild food-restriction conditions of 20 g per day that lead to daily weight gain either with or without initial deprivation to 85% of free feeding weight. Data are mean $\pm$ sem test suppression ratios and percent conditioned freezing of rats given 100 30-sec tone-shock pairings and tested 2 days and 1 month later. (A) Fear conditioning acquisition. * Different from Session 1, $p<0.05$. (B) Mean suppression ratios across the 4 tone trials. (C) Mean conditioned freezing across the 4 tone trials. (D) Trial-by-trial suppression ratios. (E) Trial-by-trial conditioned freezing. Left columns represent the rats whose body weight was decreased to 85% free feeding weight and then maintained on 20 g food per day ($n=12$). Right columns represent rats maintained on 20 g per day without initially decreasing their body weight to 85% ($n=8$). * Different from Day 2, $p<0.05$.

Figure 2.

Fear incubation after limited (1 d) or extended (10 d) training with prolonged (6 min) tones: Mean ($\pm$ sem) test suppression ratios of rats given 5 (1 session) or 50 (10 sessions) 6-min tones paired with shock and tested 2 days or 1 month later. (A) Fear conditioning acquisition. * and # Different from Session 1 or Session 10, respectively, $p < 0.05$. (B) Mean suppression ratios across the first test trial. (C) Mean suppression ratios across the second test trial. (D) Minute-by-minute suppression ratios across the two test trials for the limited-training rats ($n=24$, 12 per test day). (E) Minute-by-minute suppression ratios across the two test trials for the extended-training rats ($n=32$, 16 per test day). Left columns represent between-subjects comparisons. Right columns represent within-subjects comparisons. * Different from Day 2, $p < 0.05$; # different from the corresponding limited training group, $p < 0.05$. For the minute by minute comparisons, asterisks represent significant differences found with Tukey's HSD post hoc tests.

Figure 3.

Fear incubation in a conditioned freezing procedure: Mean ($\pm$ sem) conditioned freezing to 30-sec tones in rats that were not trained to lever press for food and were given 100 30-sec tone-shock pairings in the chambers. (A) Fear conditioning acquisition, 30-sec tone. * and # Different from Session 1 or Session 10, respectively, $p < 0.05$. (B) Mean conditioned freezing across the 4 tone trials for the between-subjects comparisons. (C) Mean conditioned freezing across the 4 tone trials for the within-subjects comparisons. (D) Trial-by-trial conditioned freezing for the between-subjects comparison. (E) Trial-by-trial conditioned freezing for the within-subjects comparison. Left column represents rats maintained on unlimited food ($n=24$, 12 per test day). Right column represents the rats whose body weight was decreased to 85% free feeding weight and then maintained on 18–20 g food per day ($n=24$, 12 per test day). * Different from Day 2, $p < 0.05$.

Table 1

PreCS lever-presses per min during the fear conditioning tests in Exp. 1 and 2.

<u>Exp. 1:</u> 85% then 20 g	<u>Day 2</u> 22.0±4.5	<u>1 Month Retest</u> 17.5±2.2	
<u>Exp. 1:</u> 20 g	<u>Day 2</u> 12.4±2.1	<u>1 Month Retest</u> 12.9±2.3	
<u>Exp. 2:</u> Limited Training	<u>Day 2</u> 14.4±1.9	<u>1 Month</u> 13.3±1.2	<u>1 Month Retest</u> 14.1±2.1
<u>Exp. 2:</u> Extended Training	<u>Day 2</u> 20.5±1.7	<u>1 Month</u> 19.3±2.6	<u>1 Month Retest</u> 17.7±1.6

1 Month: Between-subjects tested for first time

1 Month Retest: Within-subjects tested for second time

Table 2

Percent freezing during the preCS period of the fear conditioning tests in Exp. 1. (* Different from day 2, $p < 0.05$).

Exp. 1: 85% then 20 g	Day 2 9.0±3.1	1 Month Retest 3.5±1.5*
Exp. 1: 20 g	Day 2 9.8±2.6	1 Month Retest 11.3±5.1

1 Month Retest: Within-subjects tested for second time