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Temporally Massed CS Presentations Generate More Fear Extinction Than Spaced Presentations

Christopher K. Cain, Ashley M. Blouin, and Mark Barad
University of California, Los Angeles

Rodent fear conditioning models both excitatory learning and the pathogenesis of human anxiety, whereas extinction of conditional fear is a paradigm of inhibitory learning and the explicit model for behavior therapy. Many studies support a general learning rule for acquisition: Temporally spaced training is more effective than massed training. The authors asked whether this rule applies to extinction of conditional fear in mice. The authors find that both short- and long-term fear extinction are greater with temporally massed presentations of the conditional stimulus (CS). The data also indicate that once CS presentations are sufficiently massed to initiate, or "induce," extinction learning, further CS presentations are more effective when spaced.

Pairing an initially neutral stimulus, such as a noise (conditional stimulus, CS), with an intrinsically aversive stimulus, such as a mild footshock (unconditional stimulus, US), generates robust conditional fear in rodents. Such Pavlovian fear conditioning has served as an important model for the study of molecular mechanisms underlying learning and memory for many decades (Davis, 1986; Fendt & Fanselow, 1999; LeDoux, 1992; McGaugh, 2000). In addition, fear conditioning has long been an important experimental model for the pathogenesis of human anxiety disorders (Eysenck, 1979). Before the institution of modern ethical standards, early experimenters even used it to generate an experimental phobia of a lab rat and other small furry animals in a human infant, "Little Albert" (Watson & Rayner, 1920).

It is of even greater clinical relevance that extinction, the attenuation of Pavlovian fear conditioning by repeated presentations of the CS in the absence of the US, is the explicit model for behavior therapy (Wolpe, 1969). Behavior therapy is of experimentally proven efficacy in a variety of human anxiety disorders, including not only specific phobias but also panic disorder, social phobia, posttraumatic stress disorder, and obsessive compulsive disorder (Craske, 1999). Extinction can eliminate all fearful responding with enough unpaired CS presentations, but it does not reflect an erasure of the original fear memory. The retention of the original association can be uncovered by a variety of maneuvers including changing the test context (renewal; Bouton & Bolles, 1979), presenting unsignaled USs (reinstatement; Rescorla & Heth, 1975), or simply allowing time to pass (spontaneous recovery; Baum, 1988). Thus, rather than erasure, extinction appears to be new learning that acts to inhibit or compete with the original association (Bouton, 1993; R. R. Miller & Matzel, 1988; Pavlov, 1927; Wagner, 1981).

Despite its importance as a model of both inhibitory learning and psychotherapy, far less is known about the mechanisms underlying extinction than about those underlying acquisition learning. To study those mechanisms, we set out to find an efficient protocol to generate extinction. Extinction of fear often takes more trials than acquisition. For example, although a single CS–US pairing is enough to generate substantial conditional fear, extinction usually requires many CS presentations, sometimes spread over days. Although it is unclear why extinction requires more extensive training, optimizing CS presentation patterns may improve our ability to examine the mechanisms of extinction and even provide some insight into these mechanisms. Given the consistent advantage of temporally spaced training trials in learning (Barela, 1999; Carew & Kandel, 1973; Ebbinghaus, 1885/1913; Fanselow, DeCola, & Young, 1993; Fanselow & Tighe, 1988; Freudenthal et al., 1998; Gibbon, 1977; Humphreys, 1940; Jenkins, Barnes, & Barrera, 1981; Josselyn et al., 2001; Kogan et al., 1997; Scharf et al., 2002; Terrace, Gibbon, Farrell, & Baldock, 1975; Tully, Preat, Boynton, & Del Vecchio, 1994), we hypothesized that extinction learning would be more efficient with spaced than with massed CS presentations. Previous studies have been inconsistent on this issue, as we describe in the General Discussion. We therefore decided to test this hypothesis using extinction of Pavlovian fear as reflected by behavioral freezing in mice.

Experiment 1

Experiment 1 examined the contribution of trial spacing to extinction of Pavlovian conditional freezing in mice. To equalize

Christopher K. Cain and Ashley M. Blouin, Interdepartmental Program in Neuroscience, University of California, Los Angeles; Mark Barad, Department of Psychiatry and Biobehavioral Sciences, Neuropsychiatric Institute, and Brain Research Institute, University of California, Los Angeles.

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Correspondence concerning this article should be addressed to Mark Barad, Department of Psychiatry and Biobehavioral Sciences, University of California, Los Angeles, 695 Charles Young Drive South, Los Angeles, California 90095-1761. E-mail: mbarad@mednet.ucla.edu

all experimental factors except for inter-CS interval during extinction (intertrial interval, ITI), we trained all animals in Context A, then extinguished and tested them in Context B. All groups spent the same total time in each context during each phase of testing. During each phase of the experiment, all groups had equivalent total exposure to the US, contexts, and CS (except for the retention control [RC] group). For experimental groups, only the ITI of CS presentations varied, whereas RC mice were placed in the extinction context during Phase 2 but received no CS presentations. We scored freezing both during the extinction phase and a day later during a single CS presentation. Thus, the design allowed for examination of short-term extinction rates as well as long-term extinction (spontaneous recovery). Fear levels during the final test for extinction groups compared with the RC group provided a measure of long-term extinction. Comparisons between the various extinction groups provided a measure of the relative efficacy of massed versus spaced extinction training.

Method

Subjects. All experiments were conducted on naive 12–20-week-old C57/bl6 male mice (Taconic, Germantown, NY) and were approved by the University of California, Los Angeles' Institutional Animal Care and Use Committee. Mice were maintained on a 12:12-hr light–dark schedule and allowed free access to food and water. All testing was conducted during the light phase in illuminated testing rooms.

Apparatus. Two contexts (A and B), in separate rooms, were used for all behavioral testing. Shuttle box compartments (Med Associates, St. Albans, VT, Model ENV-010MC) measuring 20.3 cm × 15.9 cm × 21.3 cm served as Context A, and larger conditioning boxes (Med Associates, Model ENV-008) measuring 30.5 cm × 24.1 cm × 21.0 cm served as Context B. Both contexts had transparent front and back walls and stainless steel grid floors (3.2 mm diameter, 8.0 mm centers). Context A was wiped down before testing with 10% ethanol, and Context B was wiped down with 10% methanol. In Experiments 2 and 5, flat white acrylic inserts covered the grid floors in Context B to minimize context generalization. Individual video cameras were mounted in the ceiling of each chamber and were connected by a quad processor to a standard VCR and monitor for videotaping and scoring of freezing. Grid floors were connected to a scrambled shock source (Med Associates, Models ENV-412 and ENV-413). Auditory stimuli (Med Associates, Model ANL-926) were delivered by a speaker in the chamber wall. Delivery of stimuli was controlled with a PC and Med-PC software through a SmartCTL Interface System (Med Associates, Model DIG-716). Background white noise was maintained at 62 dB throughout behavioral testing.

Procedure. The experiment included three phases, each separated by 1 day: (a) fear acquisition, (b) CS presentations (i.e., extinction treatment), and (c) testing. Acquisition occurred in Context A, whereas CS presentation and testing occurred in Context B. All mice were preexposed to Context B for 10 min 1 hr before fear acquisition to minimize the contribution of context generalization to CS-elicited freezing during CS presentations. Mice received two pairings (2-min ITI) of the CS (80 dB, 2-min white noise) with coterminating USs (0.4 mA × 2-s footshocks) during cue fear acquisition. Additional 2-min stimulus-free periods preceded and followed the pairings. Mice were then matched into equivalent treatment groups on the basis of postshock freezing. The following day, all treatment groups received twenty 2-min CS presentations; only the ITI was varied: 6 s (massed), 60 s (intermediate), or 600 s (spaced). ITI refers to the period from CS termination to onset of the next CS throughout this article. CS presentations began 2 min after entry into the box, and mice remained in the box between and following CS presentations (total session length: 232 min for all groups). RC mice were also placed in the chamber for an equivalent duration, but they received no CS presentations. The final test consisted of a 2-min acclimation period followed by one continuous 2-min 80-dB white noise presentation for all mice.

Data analyses. Behavioral freezing, the absence of all nonrespiratory movements, was rated as present or absent every 5th second by an experienced investigator during each phase of the experiment. Freezing was rated only during pre-CS and CS periods as indicated in each experiment. Such scores during long sessions with CS presentations are hereafter called *immobility* scores to acknowledge a contribution from context habituation. The investigator was blind to treatment classification while rating the final tests in all of the experiments; however, it was not possible to maintain blindness while rating massed and spaced CS presentation sessions, because the pattern of presentations varied in each experiment. Percentage of freezing or immobility scores were calculated for each mouse, and data represent mean freezing or immobility percentages ($\pm SEM$) for groups of mice during specified time bins. To obtain a measure of interrater reliability, two blinded investigators scored the pre-CS and CS freezing for the final test in Experiment 2 ($r = .90$). Freezing during single CS presentations (or single equivalent pre-CS periods) were analyzed with a one-way analysis of variance (ANOVA). Post hoc contrasts were conducted with Dunnett's or Newman–Kuels tests. The Dunnett's test was used in instances where an RC was present, and the Newman–Kuels test was used in instances where there was no RC. When necessary, the difference between two variations of the same group (i.e., massed group where half of the mice start early vs. late in the session) or two conditions in the same group (pre-CS vs. CS) was analyzed with an unpaired two-tailed student's t test. Multiple trial data (CS presentation sessions) were analyzed with a mixed two-way (Group × Trial) ANOVA using the SAS procedure for general linear models with repeated measures. Planned comparisons between groups were conducted after collapsing over trials. Differences were considered significant if p was less than .05.

Results

The data from Experiment 1 are shown in Figure 1. One day following the extinction phase, all groups were tested for retained fear during a single 2-min CS presentation in Context B, $F(3, 28) = 3.72$, $p < .05$. Mice that received spaced CS presentations did not show any long-term extinction compared with RCs ($p > .05$), whereas the massed group showed significant long-term extinction compared with both RC and spaced groups ($p < .05$ compared with each); the intermediate group showed an intermediate level of freezing, though this was not significantly different from any of the other groups (ps $> .05$; Figure 1C).

We also scored immobility during the 20 CS presentations on Day 2 to determine the effect of CS spacing on responding to the CS (i.e., short-term extinction), main effect for group: $F(2, 19) = 38.65$, $p < .01$; for trials: $F(19, 361) = 9.73$, $p < .01$; for the Group × Trials interaction: $F(38, 361) = 4.21$, $p < .01$. Prior to the first CS, immobility was very low for all groups. All groups that received CS presentations started the extinction session with similar levels of conditional immobility during the first CS, $F(2, 21) = 0.67$, $p > .05$. Immobility during CS presentations decreased in both the massed and intermediate groups, however, the massed group showed greater short-term extinction, $F(1, 19) = 5.00$, $p < .05$. The spaced group showed less short-term extinction than the other groups (vs. massed group: $F[1, 19] = 68.16$, $p < .01$; vs. intermediate group: $F[1, 19] = 42.27$, $p < .01$; Figure 1B). Pre-CS immobility was also observed to increase throughout the session for the spaced group.

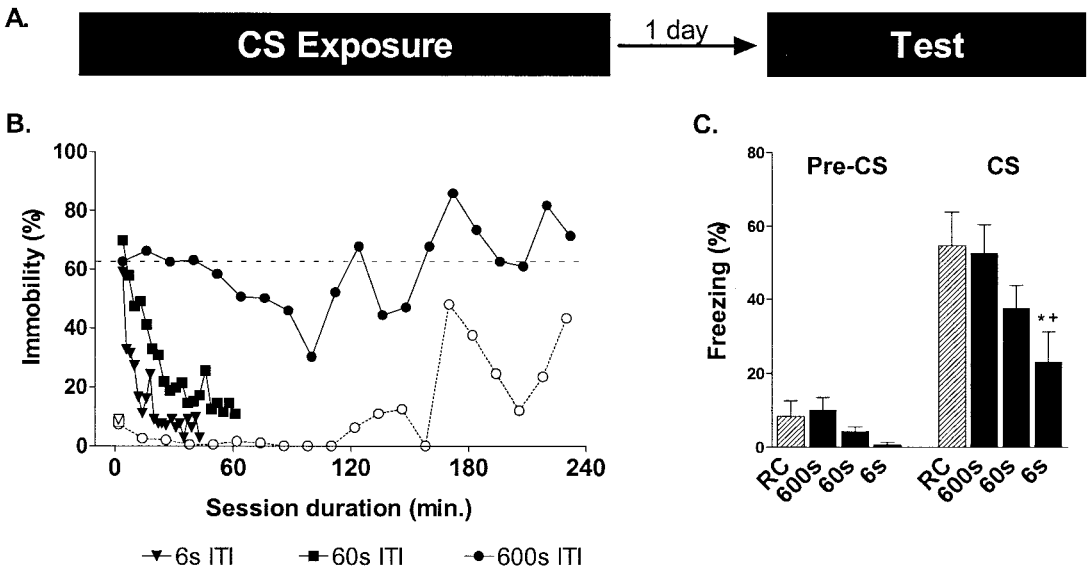

Figure 1. Temporally massed conditioned stimulus (CS) presentations produce greater cue fear extinction than spaced presentations. Panel A: Experimental design (*n*s = 6–8). Panel B: Immobility during 2 min prior to the CS (pre-CS) and the 2-min CS presentations 1 day after cue fear acquisition in a different context. All CS presentation groups were presented with 20 CSs; only the intertrial interval (ITI) varied: 6 s (massed; filled triangles), 60 s (intermediate; filled squares), or 600 s (spaced; filled circles). Open symbols represent immobility during equivalent pre-CS periods. Retention control (RC) mice were placed in the chambers for an equivalent period of time, but were not presented with any CSs. Panel C: Freezing during a single 2-min CS presentation in the extinction context 1 day after presentations. Error bars represent standard errors. $*p < .05$ versus RC. $+p < .05$ versus spaced.

Discussion

These data indicate that temporally massed CS presentations produce greater short- and long-term extinction of conditional fear than spaced presentations. The extinction groups showed equivalent immobility during the first CS presentation of the session, indicating that different levels of initial fear were not responsible for the extinction differences. By the final test, all treatment groups had equivalent exposure to the CS, US, and context, suggesting that differential total exposure to experimental stimuli also cannot account for the freezing differences. However, the final test indicates that the temporal pattern of CS presentation during the extinction session determined the amount of long-term extinction retained by each group. Temporal massing produced more long-term extinction than spacing and was graded such that the shortest ITI generated the greatest extinction; the longest, the least extinction; and the intermediate ITI, intermediate extinction.

At least four possibilities could explain the pattern of results in this long session: (a) CS presentations may simply generate more of the inhibitory learning underlying extinction when massed; (b) CS presentations may initiate two opposing processes, inhibitory extinction learning, and another mechanism that prevents extinction and, possibly, strengthens fear, through rehearsal or incubation (Eysenck, 1968; Silvestri, Rohrbaugh, & Riccio, 1970); (c) mice in the spaced group habituate to the context and are therefore less active later in the session despite extinction of their fear; or (d) mice in the spaced group habituate to the degree they are less alert or even asleep late in the session and so effectively perceive fewer of the CS presentations. Our results are consistent with all of these possibilities, however, if the second possibility is true, then trial spacing appears to preferentially recruit the fear-enhancing process. Although our experimental paradigm does not allow us to differentiate between the first and second possibilities, we can begin to address the third and fourth possibilities (i.e., that increased immobility in the spaced group reflects context habituation).

Behavioral freezing may be the net result of competing tendencies to remain immobile in presence of danger and to explore the unfamiliar environment. Although the extinction session duration was the same for all groups, animals in the massed group completed their CS presentations long before those in the spaced group. Because rodent exploration of a novel context habituates with time (Danzinger & Mainland, 1954), late increases of freezing in the spaced group may simply reflect a reduction in the tendency to explore as the animals habituate to the chamber, even though their fear is effectively extinguished. This interpretation is suggested by the observation of greater pre-CS immobility for the spaced group later in the session and led us to use the term *immobility* over *freezing* for the long Day 2 sessions. Such a habituation effect, however, should not carry over to the final, Day 3, test, when mice that received spaced CS presentations showed no long-term extinction at all. Although the animals were clearly not asleep late in the sessions, as pre-CS immobility remained well below CS immobility and CS immobility remained below 100%, it remains possible that the animals were less atten-

tive to the CS during late presentations, effectively reducing the total CS exposure they perceived, and that this could contribute to their lack of long-term extinction. We therefore designed a second experiment to control for this possibility.

Experiment 2

Experiment 2 was designed to replicate the basic finding of Experiment 1 and to address several shortcomings of that experiment. First, to control for the possibility that mice in the spaced group were inattentive or asleep at the end of their long period in the extinction context, the experiment included two massed extinction groups: one with CS presentations starting at the beginning of the session and one starting at the end of the session. Were the mice less alert or asleep toward the end of the long extinction session, then massed CS presentations beginning late in the session would generate less long-term extinction than the same number and pattern of presentations beginning early in the session. We also rated pre-CS immobility throughout the experiment to determine whether immobility increases during the spaced protocol were CS specific. Second, an unpaired control group was also subjected to the spaced CS-presentation protocol to determine whether immobility increases observed with this protocol depend on the conditional relationship of the CS and US. If the immobility increases observed with the spaced protocol in Experiment 1 reflected a real strengthening of the excitatory CS–US association, then spaced CS presentations should not increase immobility in the unpaired group. Lastly, massed CS presentations might produce greater short-term extinction, but less long-term extinction (Davis, 1970; Wagner, 1978). Therefore, in the present experiment, 8 days separated the CS presentation session and the final test session.

Method

Subjects and apparatus. Subjects and apparatus were as described for Experiment 1.

Procedure. The experiment included three phases: (a) fear acquisition, (b) CS presentations, and (c) testing. One day separated Phases 1 and 2, whereas 8 days separated Phases 2 and 3. Acquisition occurred in Context A, whereas CS presentations and testing occurred in Context B. Three groups of mice received three pairings (5-min ITI) of the CS (80-dB, 2-min white noise) with coterminating USs (0.4 mA $\times$ 2s-footshocks) during cue fear acquisition. A 5-min acclimation period preceded the pairings, and a 2-min period followed the last pairing. A fourth group of mice received the same stimuli but explicitly unpaired (CS presentations occurred exactly as in the paired condition, but US presentations preceded them by ~90 s). Paired mice were then matched into equivalent treatment groups based on freezing during the third CS of acquisition. The following day, all treatment groups received 15 CS presentations in different patterns and remained in the chambers for an equivalent period (total session length: 317 min for all groups). CS presentations in the massed-early (ME) group began 5 min after entry to the chamber and were separated by a 5-s ITI. The massed-late (ML) group received the same treatment, but the presentations began 284 min after entry to the chamber. CS presentations in the spaced-paired (SP) and spaced-unpaired (SU) groups began 5 min after entry to the chambers and were separated by a 20-min ITI. The final test was conducted 8 days after the CS presentations and consisted of a 5-min acclimation period followed by one continuous 2-min, 80-dB white-noise presentation for all mice.

Data analyses. Data analyses were identical to those in Experiment 1.

Results

The data from Experiment 2 are shown in Figure 2. In the final test of this experiment, during a single CS presentation in the extinction context 8 days after the extinction phase, CS-elicited freezing differed between the groups, $F(3, 28) = 5.20$, $p < .01$ (for data in both Figures 2C and 2E). Pre-CS freezing was absent in all groups. CS-elicited freezing was greatest in the SP group, followed by ME, SU, and finally ML. The SP group froze significantly more than all of the other groups (ps $< .05$). There were no significant differences in freezing during this final test for the ME, ML, and SU groups (ps $> .05$).

During the Day 2 CS presentation session, we did not observe any significant generalization between contexts that caused freezing in the extinction context alone; immobility scores prior to the first CS in the ME, SP, and SU groups were all less than 5%. The higher immobility score prior to the first CS for the ML group reflects the reduced tendency to explore the chamber as time passes. Immobility scored during the first CS presentation of the extinction session revealed differences between the groups, $F(3, 28) = 9.10$, $p < .01$ (Figure 2B). This difference was primarily due to the very low immobility in the SU group, because the SU group differed from the ME, ML, and SP groups (ps $< .05$), but the ME, ML, and SP groups did not differ from each other (ps $> .05$). Nevertheless, immobility during the first CS in the SU group was greater than prior to that CS, though not quite significantly so, suggesting that the unpaired protocol might generate some CS–US association, $t(14) = 2.1$, $p = .056$.

As in Experiment 1, we also scored immobility during all 15 CS presentations on Day 2, main effect for group: $F(3, 28) = 15.2$, $p < .01$; for trials: $F(14, 392) = 1.90$, $p < .05$; for Group $\times$ Trials: $F(42, 392) = 3.14$, $p < .01$. Examination of the pattern of CS-elicited immobility revealed that mice in the ME group showed a progressive reduction in immobility during subsequent CS presentations, however, immobility gradually increased during subsequent CS presentations for both the SP and SU groups. Planned comparisons among groups (collapsing over trials) indicated that the ME group differed from both the SP, $F(1, 28) = 31.51$, $p < .01$, and SU, $F(1, 28) = 7.87$, $p < .01$, groups. Though the SP and SU groups both showed evidence of increased immobility with repeated CS presentations, the groups differed in their overall immobility behavior during the session, $F(1, 28) = 7.89$, $p < .01$. To determine whether the increase in CS-elicited immobility was statistically significant in the spaced groups, we conducted another two-way ANOVA including only the SP and SU groups. Though these groups differed in overall immobility, $F(1, 14) = 6.72$, $p < .05$, the increase with trials was significant, $F(14, 196) = 7.53$, $p < .01$, and the Group $\times$ Trial interaction was not, $F(14, 196) = 1.26$, $p = .23$. Immobility during the 15 CSs followed a more erratic pattern for the ML group, possibly reflecting the complex interaction of fear extinction with context habituation in this group, ML versus ME: $F(1, 28) = 34.56$, $p < .01$; ML versus SP: $F(1, 28) = 0.07$, $p = .79$; ML versus SU: $F(1, 28) = 9.45$, $p < .01$.

Immobility rated prior to the first CS presentation did not differ among the four groups, $F(3, 28) = 2.55$, $p = .08$. Because it was only possible to repeatedly rate pre-CS immobility for the two spaced groups, a separate two-way ANOVA was conducted for these groups. The analysis revealed that although pre-CS immo-

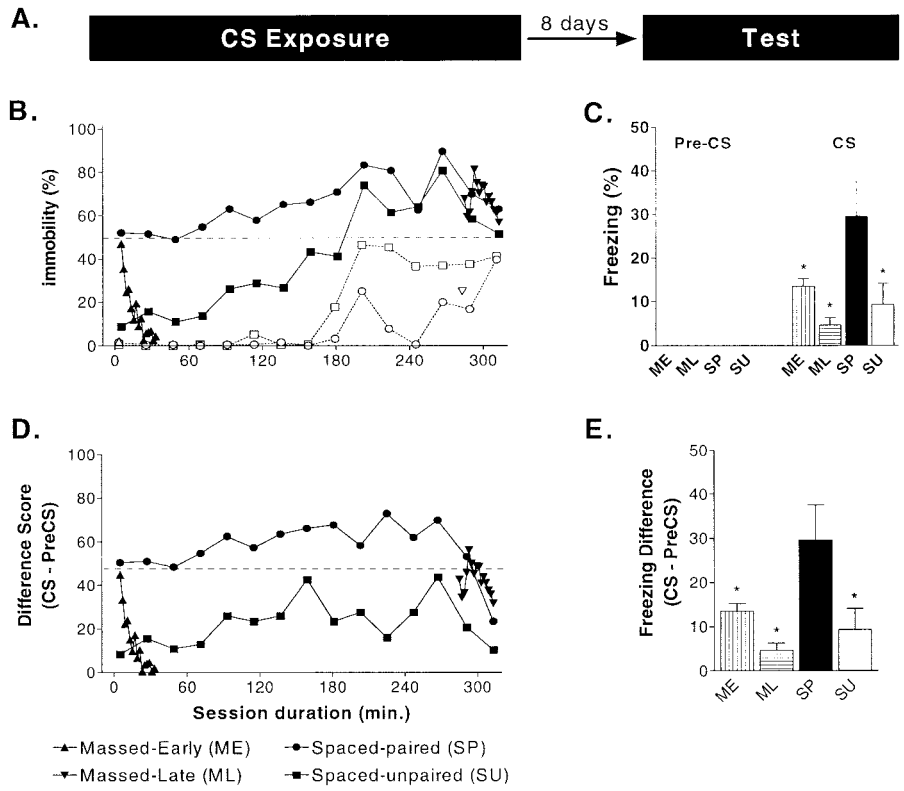

Figure 2. Massed presentation produces greater extinction than spaced even when conditioned stimulus (CS) presentations begin late in the session and fear is assessed a week later. Panel A: Experimental design (*n*s = 8). Panel B: Pre-CS and CS immobility during the extinction session in a different context 1 day after training. Panel C: Pre-CS and CS freezing scores at the final test 8 days later. Panel D: CS immobility scores after subtraction of pre-CS scores during the extinction session 1 day after training. Panel E: Final freezing scores after subtraction of pre-CS freezing. Filled symbols represent CS immobility; open symbols represent pre-CS immobility. Error bars represent standard errors. *$p < .05$ versus SP.

bility increased with trials, $F(14, 196) = 4.85$, $p < .01$, there was an overall difference between the SP and SU groups, $F(1, 14) = 5.97$, $p < .05$. However, the SP and SU groups did not differ in the pattern of pre-CS immobility change with trials, Group × Trials: $F(14, 196) = 1.01$, $p = .44$.

In an attempt to isolate the contribution of fear to CS immobility, we subtracted pre-CS immobility from CS immobility and plotted this difference score (Figure 2D). Immobility prior to the first CS was used for all calculations for the ME and ML groups because of the short ITI used in these conditions, so these data patterns did not change for the massed groups. However, immobility was rated for the 2 min preceding each of the 15 CSs in both spaced conditions, and a separate two-way ANOVA was again performed on the SP and SU groups. Fear increases and group differences were less pronounced once pre-CS immobility was subtracted out for both spaced groups, but statistical significance did not change, group: $F(1, 14) = 15.59$, $p < .01$; trials: $F(14, 196) = 1.92$, $p < .05$; Group × Trials: $F(14, 196) = 0.46$, $p = .95$.

Discussion

The results of Experiment 2 confirm the basic finding of the first experiment. Massed CS presentations produce greater long-term extinction of conditional fear than spaced presentations, whether the massed presentations occurred early or late during the time in the extinction context. During the final test, both the ME and ML groups froze significantly less than the SP group despite receiving the same number of CS presentations and the same total context exposure on Day 2. The absence of pre-CS freezing in any group on that day indicates that there was no detectable second-order conditioning to the context by the Day 2 CS presentations that might have contributed to the final CS freezing differences.

Very low initial pre-CS immobility during the Day 2 presentations indicated that there was almost no generalization between the acquisition and extinction contexts. Mice did appear to habituate to the extinction chamber during the long session. Pre-CS immobility in the SP, SU, and ML groups increased significantly later in the session. This context habituation clearly influenced immobility scores during the CS, most convincingly in the comparison between the ME and ML groups. Relative to the ME group, the ML group was more immobile during the first CS and showed little evidence of short-term extinction. Nevertheless, although immobility performance was affected during the extinction session, the mice were neither asleep nor inattentive to the CS presentations. The ML group extinguished well, showing the lowest freezing of

any group in the final test and significantly less than the SP group. Mice in the SP, SU, and ML groups also consistently startled at CS onsets late in this session, and during the later CS presentations mice were rarely immobile throughout, but rather were observed to cycle between movement and immobility, as reflected by immobility percentages well below 100%. All of these observations strongly suggest that the weaker long-term extinction in the spaced group is not due to reduced alertness or sleeping late in the extinction session and is therefore likely due to a difference in the extinction learning generated by massed and spaced CS presentations.

This experiment also indicates that the greater relative efficacy we observed for temporally massed CS presentations was not reversed in the long term. The final freezing assay occurred 8 days after the extinction session, well after any short-term effects would have dissipated. Despite this long delay, massed CS presentations produced greater extinction than spaced presentations, just as after the 24-hr delay in Experiment 1.

Our results do suggest that freezing is not a pure measure of fear but reflects, to some degree, a balance between tendencies to explore and to remain immobile. Pre-CS immobility scores demonstrated that habituation to the context does occur and influences the amount of freezing during CSs given late in the session. This habituation may account for much of the increased immobility observed with repeated CS presentations in the spaced conditions. Nevertheless, the difference between CS and pre-CS immobility increased with trials for the spaced groups (Figure 2D), suggesting that greater spacing of CS presentations may slightly increase fear and not just habituation of the tendency to explore.

Notably, spaced CS presentations also increased immobility scores in mice that had received explicitly unpaired CSs and USs during acquisition. This SU group did show some immobility to the first CS, consistent with previous observations that some excitatory CS–US association is formed during backward pairing (Heth, 1976). As with the SP group, the difference between CS and pre-CS immobility increased with subsequent trials. This increase therefore appeared to also be the result of a strengthening of the weak excitatory association between the CS and US, although our data cannot rule out a nonassociative mechanism.

Whatever the cause of increased immobility in spaced groups, its effects are short lived. Freezing during the final test did not reflect the short-term increase in immobility during Day 2 presentations. SP mice simply showed a failure of long-term extinction, whereas SU mice froze very little, just as they did at the outset of the extinction session.

Experiments 1 and 2 both indicate that temporal massing effectively generates long-term extinction and that temporal spacing results in less, or no, extinction. One hypothesis to explain these results is that the fundamental mechanisms of extinction learning are simply different from other mechanisms of learning and obey a rule opposite to that consistently observed for other forms of learning. Another hypothesis is that CS presentations elicit two opposing processes: one favored by CS spacing that somehow prevents the initiation of extinction learning, possibly by strengthening fear, and one favored by massing, which promotes extinction learning.

Experiment 3

The results of Experiments 1 and 2 provide strong evidence that temporal massing of individual CS presentations (of the length used in training) enhances extinction of conditional fear. The result was surprising, as a long history of research in excitatory learning paradigms supports the notion that temporal spacing of training trials enhances learning. A closer examination of our data suggested that the massed protocols effectively initiated extinction learning, whereas the spaced protocols did not. Thus, it could still be the case that massing is necessary to initiate, or "induce," extinction learning, but, once induced, spaced extinction learning trials would increase the amount and retention of this extinction, as it does for other forms of learning. We therefore examined the effectiveness of purely massed protocols compared with a protocol that used several blocks of massed CSs spaced across several days. If massing were generally more effective than spaced training for extinction learning, a single long massed session should produce more extinction than several shorter massed sessions (comprising equivalent total CS presentations) spaced over several days. To do this, we tested the effect of 80 2-min CS presentations given either in a single massed session on a single day (5-s ITI) or in four massed sessions of 20 CS presentations each (5-s ITI) daily over 4 consecutive days. To control for the effects of time since training in the single-session groups, we exposed separate groups to 80 CSs either on the day after training (4 days before testing) or on the day before testing (the 4th day after training). Each experimental group was matched to a control group that received no CS presentations but spent equal time in the extinction context in the same pattern as their respective experimental group. On the final (6th) day of the experiment, all mice were exposed to a single 2-min CS.

Method

Subjects and apparatus. Subjects and apparatus were as described for Experiment 1.

Procedure. The experiment included three phases conducted over 6 days: (a) fear acquisition, (b) extinction CS presentations, and (c) testing. Acquisition occurred in Context A, whereas CS presentations and testing occurred in Context B. We used a stronger training protocol to yield greater initial freezing before the numerous CS presentations of this experiment and to allow us to distinguish among groups that all were expected to show significant extinction. Mice received five pairings (2-min ITI) of the CS (80-dB, 2-min white noise) with coterminating USs (0.7 mA $\times$ 2-s footshocks) during cue fear acquisition on the first day. Additional 2-min stimulus-free periods preceded and followed the pairings. Mice were then assigned to matched treatment groups based on postshock freezing. The treatment groups were as follows: 4 $\times$ 20, RC1, 1 $\times$ 80 early (E), RC2, 1 $\times$ 80 late (L), and RC3. Mice in the 4 $\times$ 20 group were presented with twenty 2-min CS presentations (5-s ITI) after a 2-min acclimation on Days 2, 3, 4, and 5. RC1 mice were also placed in the chambers on Days 2, 3, 4, and 5 for an equivalent duration, but were never presented with any CSs. Mice in the 1 $\times$ 80E group were presented with eighty 2-min CSs (5-s ITI) after a 2-min acclimation on Day 2. RC2 mice were placed in the chambers for an equivalent duration, but were not presented with any CS. Mice in the 1 $\times$ 80L and RC3 groups received the same treatment, but on Day 5 of the experiment (4 days postacquisition). Freezing was assessed for all mice on Day 6 during a single 2-min CS presentation after a 2-min acclimation period.

Data analyses. Freezing was scored as in Experiment 1. Results of final test scores were analyzed by a one-way ANOVA with Dunnett's post hoc tests.

Results

None of the three RC groups differed in their freezing levels during the final test, 79% versus 79% versus 77%, $F(2, 9) = 0.06$, $p > .05$, and all three were combined for the final analysis. Similarly, there was no difference in freezing levels between the two groups receiving all 80 CSs in a single session, 45% versus 44%, $t(14) = 0.1$, $p > .05$, and these groups were also combined. The groups differed in their CS-elicited freezing during this final test, $F(2, 45) = 44.63$, $p < .01$. Both presentation patterns generated significant long-term extinction relative to RCs ($ps < .01$). However, 4 days of 20 massed CS presentations generated significantly more long-term extinction than 80 CS presentations in a single session ($p < .01$; Figure 3).

Discussion

Although our previous data indicated that massed CS presentations were more effective than spaced presentations for generating extinction learning in a single day, we wondered whether this unusual learning rule would extend to longer periods and spacing across several days. The present experiment examined this question by giving 80 massed CS presentations on a single day or giving one block of 20 massed CS presentations on each of 4 days. All experimental groups received the same total number of CS presentations and time in the extinction context. Additionally, RC groups that received no CS presentations were included in the design and also received equivalent total exposure to the extinction context. All of the extinction protocols included massed presentations of individual CSs and all generated significant fear extinction relative to RC mice. However, separating the massed CS presentations into four spaced blocks (one per day) resulted in greater long-term extinction than simply massing all of the CS presentations in a single session. This finding supports the notion that temporal spacing of individual CS presentations results in less extinction because this pattern is insufficient to induce extinction learning. However, once induced, extinction appears to obey the common learning rule that temporal spacing of training improves learning.

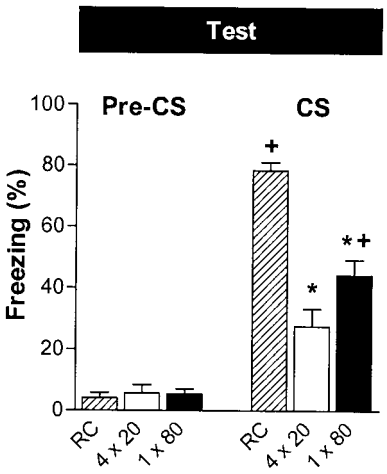

Figure 3. With total conditioned stimulus (CS) presentations held constant, multiple short massed sessions produce more extinction than single long massed sessions. Fear acquisition was conducted for all mice on Day 1. On each of the next 4 days, mice in the 4 × 20 group received 20 presentations of the 2-min CS (5-s ITI; $n = 8$). Two separate groups of mice were presented with 80 CSs (1 × 80, 5-s ITI; $ns = 8$) in a single session on either Day 2 or Day 5. Three separate retention control groups ($ns = 4$) were placed in the chambers, but not presented with CSs, on the same days and for the same durations as their respective treatment groups. On Day 6, all mice were presented with a single 2-min CS in the extinction context. Freezing was identical for the 1 × 80 groups and the retention control groups, and the data were combined for the final analyses. Error bars represent standard errors. ITI = intertrial interval; RC = retention control. $*p < .05$ versus RC group. $+p < .05$ versus 4 × 20 group.

Experiment 4

The results of Experiment 3 refined our hypotheses of the contribution that massed and spaced CS presentations make to the extinction learning process. Massing is necessary for the induction of extinction, whereas spacing appears to enhance the retention of this learning, once it is induced. Our next experiment sought to replicate and extend this finding. In this experiment, we examined the same question, but rather than separating massed presentation blocks by a day, we gave several of these blocks within a single session separated by 20 min. Massed groups received all of their CS presentations in a single block beginning either early or late in the session. This design also addressed a potential flaw in the previous experiment, where we exposed the 4 × 20 group to early session cues four times, whereas we exposed the 1 × 80 groups only once. A strong facilitating effect of such early session cues on long-term extinction might account for the deeper extinction in the spaced group (Brooks & Bouton, 1993). We eliminated this confounding factor in the present experiment, because all groups of mice were placed in the extinction context only once before all patterns of CS presentation.

Method

*Subjects and a*pparatus. Subjects and apparatus were as described for Experiment 1.

Procedure. The experiment comprised three phases: (a) fear acquisition, (b) CS presentations, and (c) testing. Acquisition occurred in Context A, whereas CS presentation and testing occurred in Context B. For acquisition, after a 5-min acclimation, mice received three pairings (2-min ITI) of the CS (80-dB, 2-min white noise) with coterminating USs (0.7 mA × 2-s footshocks). An additional 2-min period followed the pairings. Mice were then assigned to matched treatment groups based on postshock freezing. The treatment groups were as follows: 4 × 5, RC, 1 × 20E, and 1 × 20L. Mice in the 4 × 5 group were presented with four blocks of five 2-min CS presentations (5-s ITI) after a 5-min acclimation. The ITI between blocks was 20 min. Mice in the 1 × 20E group were presented with twenty 2-min CS presentations (5-s ITI) after a 5-min acclimation. Mice in the 1 × 20L groups received an identical protocol that began 65 min after entry to the chamber. RC mice were placed in the chambers for an equivalent duration (106 min), but were not presented with any CS. Freezing was assessed for all mice on Day 3 during a single 2-min CS presentation after a 5-min acclimation period.

Data analyses. Data analyses were as described for Experiment 3.

Results

The data for Experiment 4 are shown in Figure 4. One day following extinction all groups of mice were tested during a single CS presentation in the extinction context. Pre-CS freezing was low for all groups (< 5%). As in the previous experiment, there was no difference in freezing scores during the final test for mice receiving all 20 massed CS presentations in a single block (37% and 25% for 1 × 20E and 1 × 20L, respectively), $t(14) = 1.0$, $p = .33$, and these groups were combined for the final analysis. The groups differed in their freezing during the CS, $F(2, 45) = 14.40$, $p < .01$ (Figure 4). Relative to RC mice, both extinction treatments resulted in significant fear reduction as assessed by freezing in the final test (1 × 20 vs. RC, $p < .05$; 4 × 5 vs. RC, $p < .01$). However, greater extinction occurred with 4 blocks of 5 massed CS presentations than with a single 20 CS massed block (1 × 20 vs. 4 × 5, $p < .05$).

Discussion

This experiment again examined the relative efficacy for long-term extinction of a single longer massed block of CS presentations versus multiple shorter massed blocks separated in time. As in Experiment 3, multiple shorter massed blocks (4 × 5) produced greater long-term extinction than a single longer massed block (1 × 20). This finding adds to the evidence that, although massed presentations more effectively induce extinction, once induced, extinction learning is more effective when training is spaced. In this sense extinction obeys the general learning rule for acquisition: Spaced training produces greater learning. Because this experiment also controlled for exposure to early session cues and an identical pattern of results was observed in Experiments 3 and 4, such cues do not account for the greater efficacy of multiple massed CS blocks in generating extinction over a single massed block of equivalent length. The results of this study also indicate that the facilitatory effect of this type of spacing is not limited to the day-to-day intervals used in the previous study, but can be observed by varying CS presentation patterns over less than 2 hr.

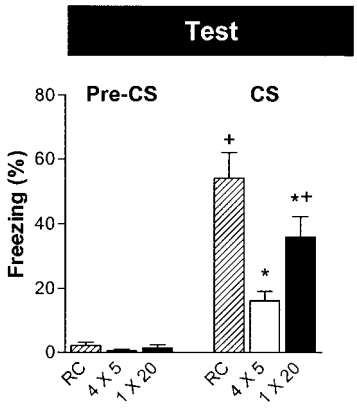

Figure 4. In a single session with total conditioned stimulus (CS) presentations held constant, spaced blocks of massed CSs produce more extinction than purely massed CS presentations. Fear acquisition was conducted for all mice on Day 1. Twenty-four hours later, mice received 20 CS presentations in different patterns: 1 × 20 early (E; 5-s ITI, beginning 5 min after entry to the chambers; $n = 8$), 1 × 20 late (L; 5-s ITI, beginning 65 min after entry to the chambers; $n = 8$), or 4 × 5 (4 blocks of 5 CSs, beginning 5 min after entry to the chambers, 5-s inter-CS interval, 20-min interblock interval; $n = 8$). Retention control (RC) mice ($n = 8$) were placed in the chambers for an equivalent period of time but not exposed to any CSs. On Day 3, all mice were returned to the chambers and presented with a single 2-min CS. Freezing was similar for the 1 × 20E and 1 × 20L groups, and the data were combined for the final analysis. Error bars represent standard errors. ITI = intertrial interval. *$p < .05$ versus RC group. +$p < .05$ versus 4 × 5 group.

General Discussion

The primary goal of our laboratory is to elucidate the molecular mechanisms underlying extinction of fear. We initially undertook the experiments in this article to discover an efficient means of generating rapid and lasting extinction as an assay for future molecular dissection. Contrary to our expectation, the experiments revealed that the dependence of extinction on the ITI of CS presentations was a complicated one. Specifically, individual presentations were more effective in generating both short-term and long-term extinction when they were temporally massed (Figures 1 and 2), but temporally spaced blocks of massed CS presentations produced the greatest long-term extinction (Figures 3 and 4). These results provide important guidelines to the design of extinction training and perhaps of behavior therapy exposures. They also suggest that fear-conditioned animals may show a compound of opposing responses to CS presentation that challenges simple theoretical explanation.

The greater efficacy of spaced than massed training is one of the most consistent observations in excitatory learning (see citations at the beginning of this article). Our data indicate that this rule does not apply, in its simplest form, to inhibitory extinction learning. Our findings may also explain why the relationship between ITI and extinction has been ambiguous in the literature. Although some studies report that spaced training is better in extinction (Baum, Andrus, & Jacobs, 1990; Sheffield, 1949; Westbrook, Smith, & Charnock, 1985), others report that massed training is better (Oler & Baum, 1968; Pavlov, 1927; Reynolds, 1945), and still others that there is no difference (Birch, 1965; Martasian, Smith, Neill, & Rieg, 1992; Schiff, Smith, & Prochaska, 1972; Shipley, 1974; Stanley, 1952). In each category, some studies focus only on short-term extinction (Oler & Baum, 1968; Pavlov, 1927; Reynolds, 1945; Schiff et al., 1972; Sheffield, 1949; Shipley, 1974; Stanley, 1952). Other experiments only examine long-term extinction (Baum et al., 1990; Martasian et al., 1992; Westbrook et al., 1985). Discrepant results may be due to other variables between studies. Some deal with appetitive rather than aversive conditioning (Birch, 1965; Pavlov, 1927; Sheffield, 1949; Stanley, 1952). Others deal with instrumental rather than Pavlovian conditioning, often allowing the animals' response latency to alter the interval between CS presentations (Birch, 1965; Sheffield, 1949; Stanley, 1952). In particular, in aversive studies such as active avoidance, animals control their own CS and US exposures (Martasian et al., 1992; Oler & Baum, 1968). Interpretation of these findings is further complicated by the suggestion that the

apparent extinction of the avoidance response results from the animals learning to freeze; a response incompatible with avoidance but not an indication of reduced fear (Coulter, Riccio, & Page, 1969; Page, 1955). Few studies varied only trial spacing and not CS duration (Reynolds, 1945; Westbrook et al., 1985). Because our objective was to develop a robust fear-extinction protocol for pharmacological dissection, we decided to collect our own data using a simple model of Pavlovian conditioning.

In our first experiments, massed CS presentations nearly eliminated conditional freezing acutely and significantly reduced freezing when assessed a day or a week later (Figures 1 and 2). In contrast, purely spaced presentations of an identical number of nonreinforced CSs led to increased immobility acutely and little to no fear reduction in the long term. The design of our experiments allows us to rule out differential handling, context exposure, CS exposure, or US exposure as contributing factors to this robust effect. Several observations also allow us to rule out the possibility that spaced protocols produce less extinction because the animals are less alert, or sleeping, late in the session. Mice were rarely completely immobile during the 2-min period preceding each CS, or during the CS presentation itself, and locomotion is inconsistent with sleeping. Mice were also observed to startle at the onset of each CS. Lastly, massed CS presentations at the end of the long session produced robust long-term extinction. Thus, every effort was made to isolate the effect of ITI during extinction, and in each case shorter intervals produced greater extinction.

Although massed CS presentations resulted in rapid and progressive elimination of conditional immobility, spaced CS presentations generated no short-term extinction and even small paradoxical increases in immobility acutely. One must ask whether this increased immobility reflects enhanced fear through associative or nonassociative mechanisms, or rather reflects a simple habituation to the context. Freezing is a useful behavioral measure precisely because rodents have a strong tendency to explore their environment. However, this tendency to explore habituates with time, making it difficult to draw solid conclusions from freezing behavior observed during long sessions. Increases in freezing could simply reflect a reduction in a competing tendency to explore. Our results suggest that the increased freezing observed with spaced CS presentations is partly due to context habituation. With time, mice in the spaced conditions displayed more immobility in the period preceding each CS. Perhaps most convincingly, mice given massed CS presentations at the end of the long session also showed elevated immobility prior to the first CS. Thus, interpretation of freezing scores during long sessions should include consideration of context habituation effects.

Though context habituation does affect freezing, it is important to note that immobility scores increased over baseline during spaced CS presentations even after subtraction of pre-CS immobility (Figure 2). Thus, it remains possible that spaced CS presentations cause some increase in fear. Although interpretation of freezing behavior during the long CS presentation sessions is admittedly difficult, the results from the long-term tests are unambiguous. Whether spaced presentations of nonreinforced CSs generated any true fear increase, spacing clearly prevented the induction of extinction; massed presentations generated robust and long-lasting fear extinction, whereas an equivalent number of spaced presentations generated significantly less, or even no, extinction (Figures 1 and 2).

Experiments 3 and 4 included blocks of massed CS presentations. In these experiments, spaced blocks of massed presentations were consistently more effective in generating extinction than massed blocks of massed presentations. This was true for four blocks of 20 CSs distributed over 4 days compared with 80 CS presentations on a single day (Experiment 3) and for four blocks of five CS presentations delivered with a 20-min ITI, compared with 20 CS presentations in a single massed block (Experiment 4). Again, it didn't matter whether the massed blocks were administered at the beginning or at the end of the period of extinction training, and this effect could not be attributed to differential exposure to early session cues (Experiment 4).

Thus extinction appears to obey two opposing learning rules. First, for individual CSs, massed presentations were more efficient than spaced presentations in generating both short- and long-term extinction. Second, once blocks of CS presentations were sufficient to induce extinction, further extinction was more effective when the blocks were spaced than when they were massed. These complex effects of ITI on extinction present a particular challenge to theoretical explanations. Few learning models make explicit predictions about the effect of ITI on extinction. Rate expectancy theory takes as a "salient empirical fact about extinction" that there is no effect of the inter-CS interval on the rate of extinction (Gallistel & Gibbon, 2000, p. 305), even though this contradicts an explicit earlier statement by one of the theory's authors: "Long ITIs generate greater resistance to extinction just as they generate more rapid conditioning" (Gibbon, Farrell, Locurto, Duncan, & Terrace, 1980, p. 49). The sometimes opponent process (SOP) model has been used by Wagner (1978) to explain why short-term habituation may be more profound with massed stimulus presentations, whereas long-term habituation is more profound with spaced presentations (Davis, 1970; Wagner, 1978). Westbrook et al. (1985) applied similar logic to explain their data in taste- and odor-aversion learning, finding that two CS presentations generated more long-term (24 hr) extinction when spaced 24 hr apart than when spaced from 0.5 to 6 hr apart. They argued that with massing more of the CS representation is in the A2 (secondary activity) state of SOP and therefore unavailable to be represented in the A1 (primary activity) state (according to SOP, representational elements of memory can only move from A1 to A2 to inactivity; they cannot move from A2 to A1). Because CS in A1 must be paired with the retrieved US in A2 to generate an inhibitory link (long-term extinction), CS massing should reduce long-term extinction, even though it may cause less conditioned responding in the short term. This interpretation of the predictions of SOP are clearly inconsistent with our data, which show that massed CS presentations are more effective than spaced in generating both acute extinction, during CS presentations, and long-term extinction lasting at least 8 days.

We believe that our data are most consistent with a model in which CS presentations elicit two competing learning responses in the subject animal, the first being a reminder effect that causes rehearsal of the CS–US association and perhaps even strengthens it, whereas the second is a reevaluation of the contingency of US presentation in the presence of the CS, which leads to extinction. Because we do not see day-to-day strengthening of fear with

spaced CS presentations (although we have seen such an increase in combination with a pharmacological manipulation; Cain & Barad, 2001) and because even the acute increases we see cannot be surely called an increase in fear, we here only infer such a fear-strengthening mechanism from the absence of extinction. This interpretation is perhaps most consistent with Eysenck's (1968) theory of fear incubation, though with some caveats. Eysenck (1968) proposed that following conditioning, CS-alone presentations provoke increments and decrements in conditional response (CR) strength, so that "the observed CR is the resultant of two opposing tendencies; *extinction* will be observed if the decrementing tendencies are greater than the incrementing ones, while *incubation* will be observed if the incrementing tendencies are greater than the decrementing ones" (p. 312). Eysenck (1968) went on to describe parameters that may favor incubation over extinction; namely strong USs, CRs, or differences in innate reactivity (in animals) or personality (in humans). Our results indicate that temporal spacing is also a relevant parameter affecting the balance between incubation and extinction, consistent with observations of others that brief CS presentations may lead to the strengthening of fear memory through rehearsal (Gordon, Taylor, & Mowrer, 1981; B. V. Miller & Levis, 1971; Rohrbaugh & Riccio, 1970; Silvestri et al., 1970) and can even mitigate extinction (Stone & Borkovec, 1975). Interestingly, it has also been suggested that for extinction to occur clinically, CS exposures must be long enough to allow for substantial anxiety to build and subside (Stampfl & Levis, 1967). Such an idea is consistent with our hypothesis that CS massing is necessary for the induction of extinction and may suggest that massing serves to reduce the perception that the CSs are independent events.

In summary, we are not proposing that extinction learning itself follows a different learning rule than acquisition learning, but rather that extinction induction requires massed CS presentations, because spaced CS presentations evoke a second mechanism that prevents the effective induction of extinction in the first place. That mechanism may be a simple resistance to extinction due to failure to gather information that contradicts the predictive validity of the CS, or it may be incubation due to rehearsal and strengthening of the CS–US association. The net effect of either mechanism is to prevent the induction of extinction. Once extinction is induced by a sufficient number of massed CS presentations, the usual learning rule takes over, and spacing of further presentations generates more extinction than further massing. Although this suggests that the fundamental mechanism of extinction learning obeys the general learning rule, it nevertheless indicates that CS presentations evoke a complex response in the animal. Practically, this means that massed CS presentations are a more effective and efficient way to generate extinction than temporally spaced presentations, with implications for experimental design and perhaps for behavior therapy. Neuroscientists studying extinction mechanisms with time-constrained techniques can take advantage of the CS-massing benefit for extinction to shorten experimental protocols. Also, therapists may wish to incorporate some massing of anxiogenic stimuli into exposure therapy sessions to more quickly reduce the aversiveness of therapy and increase the patient's willingness to continue with treatment.

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