

# The separate and combined effects of a dangerous context and an epinephrine injection on sensory preconditioning in rats

Katarina Kikas, R. Frederick Westbrook, and Nathan M. Holmes

*School of Psychology, University of New South Wales Sydney, New South Wales 2052, Australia*

Four experiments examined the effects of a dangerous context and a systemic epinephrine injection on sensory preconditioning in rats. In each experiment, rats were exposed to presentations of a tone and light in stage 1, light-shock pairings in stage 2, and test presentations of the tone alone and light alone in stage 3. Presentations of the tone and light in stage 1 occurred in either a safe or a previously shocked context, and/or under a systemic injection of epinephrine. Experiment 1 showed that a trace interval of 20 sec between presentations of the tone and light produced sensory preconditioning of the tone in a previously shocked context but not in a safe context, while experiment 2 provided evidence that this trace preconditioning was associative, due to the formation of a tone-light association. Experiment 3 showed that, in a safe context, exposure to the trace protocol under the influence of an epinephrine injection also produced sensory preconditioning of the tone, while experiment 4 provided evidence that a shocked context and an epinephrine injection have additive effects on trace preconditioning. These findings are discussed in relation to theories of trace conditioning. They suggest that the release of epinephrine by danger enhances attention and/or working memory processes, and thereby associative formation across a trace interval.

Animals learn about stimuli that signal motivationally significant events such as the availability of food or imminence of danger and use this information to guide food-seeking or defensive behaviors (Pearce and Bouton 2001). Animals also learn about the relations between affectively neutral stimuli, but do not always express this learning in behavior; presumably because the events lack motivational significance. A protocol used to reveal this learning is what Brogden (1939) termed sensory preconditioning. This protocol consists in three stages. In stage 1, subjects (e.g., rats) are exposed to a signaling relation between two neutral stimuli, such as a sound whose presentations are followed by a light. In stage 2, rats are exposed to a signaling relation between one of these stimuli, for example, the light, and a motivationally significant event, such as aversive shock unconditioned stimulus (US). In stage 3, rats exhibit defensive responses (e.g., freezing [Holmes et al. 2013; Wong et al. 2019] or suppression of appetitively rewarded lever pressing [Rescorla 1980]) when tested with the conditioned visual stimulus (CS) and when tested with the sensory preconditioned auditory stimulus, even though the latter stimulus was never paired with the aversive US. Control groups have confirmed that the defensive responses elicited by the sensory preconditioned sound are due to its association with the light in stage 1 rather than to generalization of such responses from the light, and to the association between the light and the aversive US in stage 2 rather than to any unconditioned ability of the light to imbue the tone with fear-eliciting properties (Rizley and Rescorla 1972).

Our previous work used the sensory preconditioning protocol to examine the substrates of the association produced by pairing two affectively neutral, auditory and visual stimuli, specifically focusing on the roles of two regions in the medial temporal lobe, the perirhinal cortex (PRh), and basolateral amygdala complex (BLA). This work showed that the nature of the experience in the context

before the pairings determines whether the PRh or BLA is selected for forming the association between the paired stimuli. When rats are exposed to the pairings in a familiar, safe context, formation of the association requires neuronal activity, including activation of N-methyl-D-aspartate (NMDA) receptors, in the PRh but not the BLA (Holmes et al. 2013). In contrast, when rats are exposed to the pairings in an equally familiar but previously shocked (and hence dangerous) context, formation of the association requires neuronal activity, including NMDA receptor activation, in the BLA but not the PRh (Holmes et al. 2013).

Our previous work using the sensory preconditioning protocol also showed that the circumstances of associative formation differed when the auditory and visual stimuli (a pure tone sound and flashing light, respectively) were presented in a safe or dangerous context (Holmes and Westbrook 2017). Rats were placed into a safe or a previously shocked context and exposed to presentations of a sound and a light. For half of the rats in each group, every presentation of the sound was followed immediately by presentation of the light, whereas for the remaining rats in each group, every presentation of the sound was followed 20 sec later by presentation of the light. The 20-sec trace interval was selected to reduce the likelihood of associative formation among rats exposed to tone-light pairings in the safe context, and thereby, maximize our capacity to detect any effect of the dangerous context on associative formation. All rats then received pairings of the light and foot shock, and, finally, test presentations of both the sound and the conditioned light. There were no differences among the four groups in the levels of defensive/fear responses (freezing) elicited by the conditioned light, but there were such differences in the levels of freezing elicited by the sound. Specifically, rats that had been exposed to the 20-sec trace interval between the sound and light in a dangerous

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**Corresponding author:** [n.holmes@unsw.edu.au](mailto:n.holmes@unsw.edu.au)

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context froze more to the sound than rats that had been exposed to the trace interval in a safe context, and critically, just as much as rats exposed to the contiguous relation between the sound and light in either the safe or dangerous context (which did not differ).

A potential mechanism by which the dangerous context might have enabled associative formation across the trace interval rests in its activation of peripheral and central adrenergic systems. There is considerable evidence that exposure to foot shock in a distinctive context increases levels of epinephrine in the periphery, and via its effects on systems that respond to stress, norepinephrine in the amygdala (Hatfield et al. 1999; McIntyre et al. 2002; McGaugh 2004). Increased epinephrine and norepinephrine levels are thought to enhance learning about cues that signal danger, including auditory and visual stimuli paired with foot shock (e.g., Rodrigues et al. 2009). Increases in these levels have also been identified with enhanced memory for affectively neutral experiences. For example, object recognition memory is enhanced by BLA infusions of norepinephrine, and this enhancement is blocked by co-administration of the  $\beta$ -adrenergic receptor antagonist, propranolol (Roosendaal et al. 2006, 2008). Such evidence suggests that a systemic injection of epinephrine may influence sensory preconditioning in the same way as a dangerous context; and hence that a 20-sec trace interval between the auditory and visual stimuli under a systemic injection of epinephrine will support sensory preconditioning in a safe context, as does such an interval in a dangerous context.

The present study tested this suggestion. It had two specific aims. The first aim was to replicate our previous finding that test presentations of the tone elicit freezing among rats exposed to the trace interval between tone and light presentations in a dangerous, but not in a safe context (experiment 1). We additionally sought to show that danger acts directly on associative formation between the tone and the light in the trace protocol, thereby enabling the tone to elicit freezing at test (experiment 2). The second aim was to determine whether a systemic injection of epinephrine prior to placement in a safe context functions like a dangerous context to enable associative formation in the trace protocol, as in-

dexed by the test levels of freezing to the tone (experiment 3). We additionally sought to determine whether an epinephrine injection and a dangerous context have additive effects on associative formation in the trace protocol (experiment 4).

### Experiment 1

The aim of this experiment was to replicate our previous finding that rats exposed to a trace interval between presentations of a tone and light in a dangerous context (but not in a safe context) freeze when tested with the tone following light-shock pairings (Holmes and Westbrook 2017). The design is shown in Table 1. Four groups of rats were exposed to presentations of a tone and a light in stage 1, pairings of the light and shock in stage 2, and test presentations of the tone alone and light alone in stage 3. The groups differed with respect to their experiences in the context prior to stage 1 and in the temporal relation between the tone and the light across stage 1. Two groups had been previously shocked in the context, thereby rendering it dangerous when rats were exposed to presentations of the tone and light (Groups Danger). The remaining two groups had not been shocked, and hence the context was safe across the presentations of the tone and light (Groups Safe). The groups in each pair differed with respect to the interval between tone offsets and light onsets in stage 1. For one group, this interval was 0 sec (Groups Danger-0 and Safe-0), and for the other, it was 20 sec (Groups Danger-20 and Safe-20). We expected that rats exposed to the contiguous relation between the tone and light (Groups Danger-0 and Safe-0) would freeze when tested with the tone in stage 3. We also expected that rats exposed to the trace interval between the tone and light in a safe context (Group Safe-20) would fail to associate the tone and light in stage 1, and hence exhibit little or no freezing when tested with the tone in stage 3. The question of interest concerned rats that were exposed to the trace interval between the tone and light in a dangerous context (Group Danger-20). We expected that these rats would freeze more than rats in Group Safe-20, and just as much as those in Groups Danger-0 and Safe-0.

**Table 1.** Design of experiments 1–4

Group	Stage 0	Stage 1	Stage 2	Test 1	Test 2
<b>Experiment 1</b>					
Safe-0	Cxt-nothing	Tone-light	Light-shock	Tone	Light
Safe-20	Cxt-nothing	Tone----light			
Danger-0	Cxt-shock	Tone-light			
Danger-20	Cxt-shock	Tone----light			
<b>Experiment 2</b>					
Group Trace	Cxt-shock	Tone----light	Light-shock	Tone	Light
Group L/T		Light/tone			
<b>Experiment 3</b>					
Veh-0	Cxt-nothing	(Veh) tone-light	Light-shock	Tone	Light
Veh-20		(Veh) tone----light			
Epi-0		(Epi) tone-light			
Epi-20		(Epi) tone----light			
<b>Experiment 4</b>					
Safe-Veh	Cxt-nothing	(Veh) tone----light	Light-shock	Tone	Light
Safe-Epi		(Epi) tone----light			
Danger-Veh		(Veh) tone----light			
Danger-Epi		(Epi) tone----light			

Multiple dashes (----) denotes a 20-sec delay interval between presentations of the tone and light, a single dash (-) denotes a zero-sec delay between presentations of the tone and light, and the forward slash (/) denotes eight presentations of the light followed by eight presentations of the tone (explicitly unpaired arrangement).

Results

Rats in Groups Danger-0 and danger-20 acquired fear of the context prior to sensory preconditioning. In the final minute of the session that preceded sensory preconditioning, rats in these groups froze more than those in Groups Safe-0 and Safe-20,  $F_{(1,28)} = 25.07$ ,  $P < 0.05$ , 95% CI = [1.05, 2.49]. Figure 1 shows the mean (+SEM) levels of freezing to the light across its pairings with shock in stage 2. All rats acquired fear of the light, as evidenced by a significant linear increase in freezing across the four light-shock pairings,  $F_{(1,28)} = 196.90$ ,  $P < 0.05$ , 95% CI = [2.08, 2.79]. The light elicited significantly less freezing among rats in Groups Danger than in Groups Safe  $F_{(1,28)} = 10.47$ ,  $P < 0.05$ , 95% CI = [0.28, 1.23], perhaps reflecting a partial blocking of the light-shock association by the already conditioned context among rats in Groups Danger (Kamin 1969). However, all groups showed similar levels of freezing on the final light-shock pairing, indicating that each had learned the relationship between the light and shock, and there were no significant between-group differences in the rates at which freezing increased across presentations of the light,  $F_s < 4.02$ . The overall levels of freezing to the light among groups exposed to the contiguous or the trace relation between the tone and light

were similar, and there was no significant interaction between the context in preconditioning (Danger vs. Safe) and the interval between tone and light presentations in preconditioning (zero vs. 20 sec),  $F_s < 1$ .

Figure 2, A and B, shows the test levels of freezing to the preconditioned tone averaged across blocks of two trials and averaged across all trials, respectively. The baseline levels of freezing before test presentations of the tone were moderate (<10%) and did not differ between the four groups,  $F_s < 3.5$ . Overall, the tone elicited significantly less freezing in Group Safe-20 than in the three other groups,  $F_{(1,28)} = 6.14$ ,  $P < 0.05$ , 95% CI = [0.13, 1.34]. Critically, however, the overall level of freezing to the tone in Group Danger-20 was not significantly different from that in Groups Safe-0 and Danger-0, and there were no significant differences between the latter two groups,  $F_s < 1.2$ . Finally, averaged across groups, there was no significant change in freezing to the tone across blocks of test trials, and no differences between groups in the rate that freezing changed across blocks of trials,  $F_s < 1.72$ , indicating that the difference between Group Safe-20 and the remaining groups persisted across the test session.

Figure 2, C and D, shows the test levels of freezing to the conditioned light across blocks of two trials and averaged across all

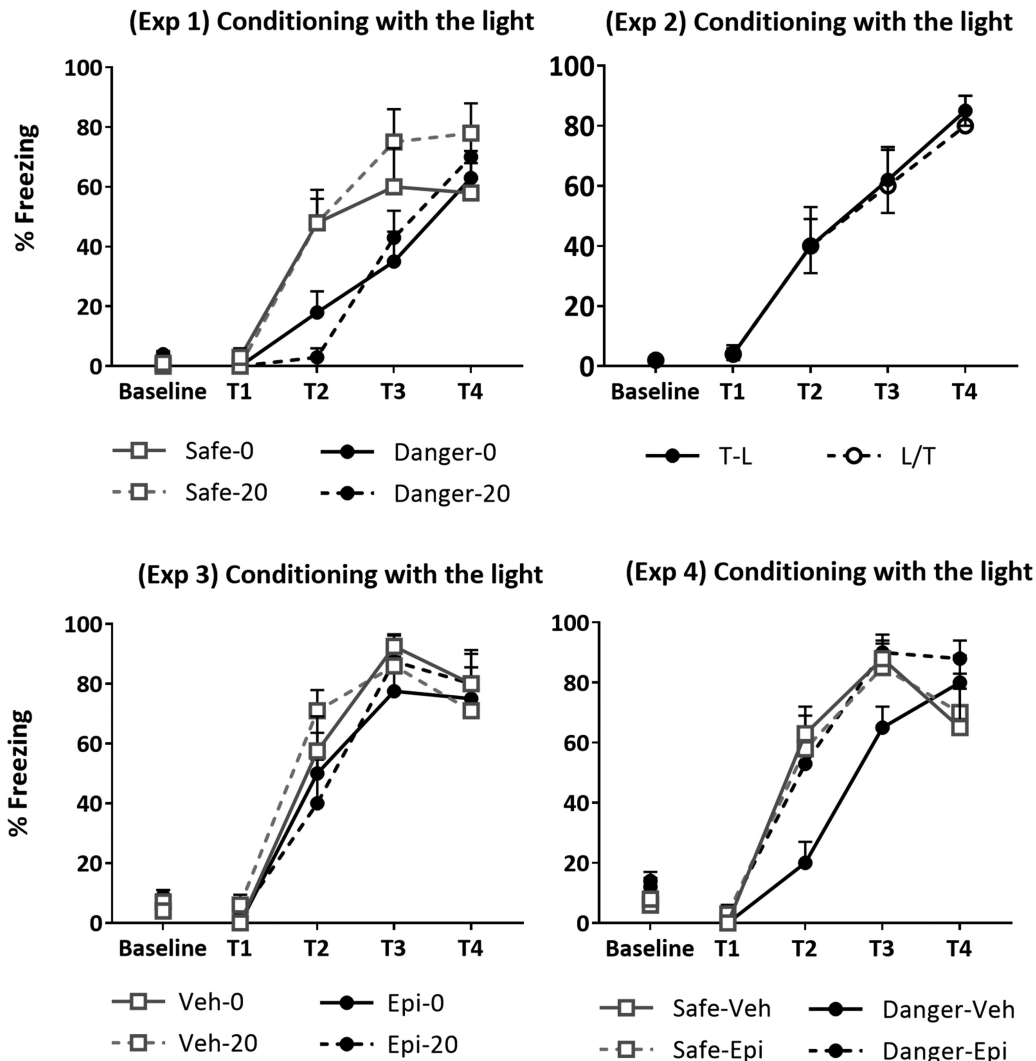
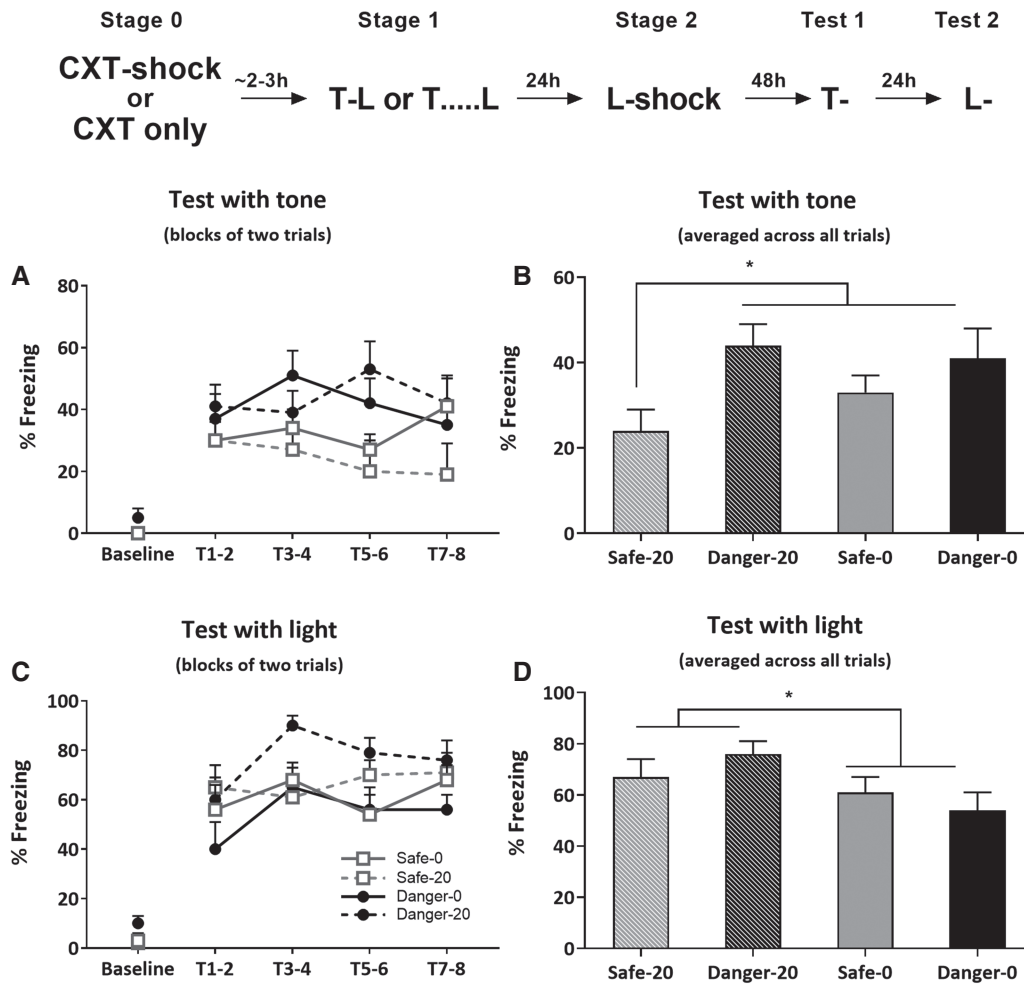


Figure 1. The mean (+SEM) level of freezing across pairings of the light and shock for each group in experiments 1–4.



**Figure 2.** Mean (+SEM) levels freezing to the tone (A) and light (C) across blocks of two trials and mean (+SEM) levels of freezing to the tone (B) and light (D) averaged across all trials at test in experiment 1. (T) Trial block.

trials, respectively. The baseline levels of freezing before test presentations of the light were low (<10%) and did not differ between the four groups,  $F_s < 1.7$ . There were no statistically significant differences between freezing to the light in Groups Danger (Danger-0 and Danger-20) and Groups Safe (Safe-0 and Safe-20),  $F < 1$ , and there was no significant interaction between the type of context (Danger vs. Safe) and type of preconditioning (contiguous vs. trace),  $F_s < 1.6$ . However, unexpectedly, there was a small but statistically significant between-group difference such that groups that had been exposed to the trace interval between tone and light presentations (Safe-20 and Danger-20) froze more to the light than groups that had been exposed to the contiguous (i.e., no delay) tone and light presentations (Safe-0 and Danger-0),  $F_{(1,28)} = 4.58$ ,  $P < 0.05$ , 95% CI = [-1.2, -0.03]. Finally, averaged across groups, the change in freezing to the light across blocks of test trials was significant,  $F_{(1,28)} = 4.67$ ,  $P < 0.05$ , 95% CI = [0.02, 0.68]. Observation suggested that the initial trials elicited orienting and increased activity that was then replaced by freezing across the subsequent trials. There were no significant differences between the groups in the rate at which freezing changed across trials,  $F_s < 1$ .

This experiment confirmed that danger alters what rats learn when exposed to a trace interval between presentations of a tone and a light (Holmes and Westbrook 2017). Rats exposed to a 20 sec interval between the offset of each tone presentation and the onset of each light presentation in a shocked context (Group

Danger-20) froze when tested with the sensory preconditioned tone just as much as rats exposed to a contiguous relation between tone offset and light onset in either a shocked (Group Danger-0) or nonshocked context (Group Safe-0). In contrast, rats exposed to the 20-sec interval in a nonshocked context (Group Safe-20) froze significantly less when tested with the tone but just as much to the conditioned light as those in the other three groups. However, the nature of what rats learn when exposed to the trace protocol in a dangerous context remains to be determined. This is addressed in the next experiment.

### Experiment 2

The aim of experiment 2 was to determine the basis of freezing to the sensory preconditioned tone among rats exposed to the trace interval between tone and light presentations in a dangerous context. There are at least three explanations for this freezing. The first is that the dangerous context enhances processing of the stimuli in working memory, enabling the formation of a long-delay tone-light association in stage 1. This association is subsequently integrated with the light-shock association formed in stage 2 such that test presentations of the tone elicit freezing. The second appeals to second-order conditioning whereby associations are formed between the tone (and the light) and the shocked context in stage

1. Presentations of the tone at test retrieves the memory of its association with the dangerous context, leading to freezing. The third is that the dangerous context reduces processing of the stimuli in stage 1, effectively minimizing habituation to the tone (and the light). Fear conditioning to the light then sensitizes the rats, leading to freezing when they are tested with the effectively novel tone.

The design of this experiment is shown in Table 1. In stage 1, two groups of rats were exposed to eight tone presentations and eight light presentations in a dangerous context. For rats in Group Trace, the sequence and timing of these tone and light presentations were identical to those received by rats in Group Danger-20 in experiment 1: The offset of each presentation of the tone was followed 20-sec later by the onset of each presentation of the light. In contrast, rats in Group L/T were exposed to the tone and light presentations in a way that was intended to preclude the formation of any tone-light association. This was done by exposing these rats to eight presentations of the light followed by eight presentations of the tone. All rats were then exposed to light-shock pairings in stage 2, and finally, tested with presentations of the tone alone and light alone in stage 3. If the test level of responding to the tone is due to its association with the shocked context in stage 1, then both groups should retrieve this memory and exhibit similar levels of freezing. Likewise, if the shocked context impaired familiarization of the tone (and the light) in stage 1, then both groups should be equally sensitized by the light-shock pairings and exhibit similar levels of freezing to the effectively novel tone at test. In contrast, if the test level of responding to the tone results from formation of a long-delay tone-light association in stage 1, test presentations of the tone will elicit more freezing in Group Trace than in Group L/T.

## Results

All rats learned that the shocked context was dangerous and there were no differences between Groups Trace and L/T in their levels of freezing during the last minute of the shocked context exposure,  $F_s < 1$ . Figure 1 shows the mean (+SEM) levels of freezing in both groups across pairings of the light and shock. Conditioning of the light in stage 2 was successful. Freezing to the light increased across its pairings with the shock,  $F_{(1,19)} = 228.63$ ,  $P < 0.05$ , 95% CI = [1.91, 2.52]. There were no differences between the groups in the rate at which freezing developed to the light across its pairings with shock, or in their overall levels of freezing to the light,  $F_s < 1$ .

Figure 3, A and B, show the test levels of freezing to the tone averaged across blocks of two trials and all trials, respectively. The baseline levels of freezing before test presentations of the tone were moderate (<10%) and did not differ between the two groups,  $F_s < 2.1$ . When tested with the tone, rats in Group Trace froze significantly more than rats in Group L/T,  $F_{(1,19)} = 5.98$ ,  $P < 0.05$ , 95% CI = [0.11, 1.47]. The level of freezing declined across test presentations of the tone,  $F_{(1,19)} = 9.37$ ,  $P < 0.05$ , 95% CI = [0.25, 1.30], indicating extinction. However, there were no significant differences between Groups Trace and L/T in the rate at which freezing declined across the tone presentations,  $F_s < 1.5$ , showing that the between-group difference in freezing was maintained across the tone alone presentations.

Figure 3, C and D, shows the test levels of freezing to the conditioned light across blocks of two trials and all trials, respectively. The baseline levels of freezing before test presentations of the light were again moderate (<11%) and did not differ between the two groups,  $F_s < 2.5$ . There was no significant difference in the overall level of freezing to the light between Groups Trace and L/T,  $F_s < 1$ , confirming that both groups had conditioned equally to the light. Averaged across groups, freezing to the light extinguished across the test session,  $F_{(1,19)} = 26.29$ ,  $P < 0.05$ , 95% CI = [0.71, 1.69]. There were no differences between the two groups in the

rate at which freezing declined across the test presentations of the conditioned light,  $F_s < 1$ .

This experiment has shown that freezing to the tone among rats exposed to a 20 sec trace interval between presentations of the tone and light in a dangerous context is associatively mediated. Rats that had been exposed to the tone and light in such a way as to prevent formation of a tone-light association froze less when tested with the tone than rats given equivalent exposures to the tone and light but in such a way that the former could predict the latter. Hence, freezing to the tone at test is not due to its retrieval of the dangerous context where it had been presented in stage 1 nor to the shocked context having prevented familiarization of the tone in stage 1, and thereby, eliciting novelty induced freezing after fear conditioning of the light. If either had been the case, the two groups would have exhibited equivalent freezing when tested with the tone. Instead, freezing to the tone in Group T-L is due to the integration of the tone-light association formed in stage 1 and the light-shock association formed in stage 2.

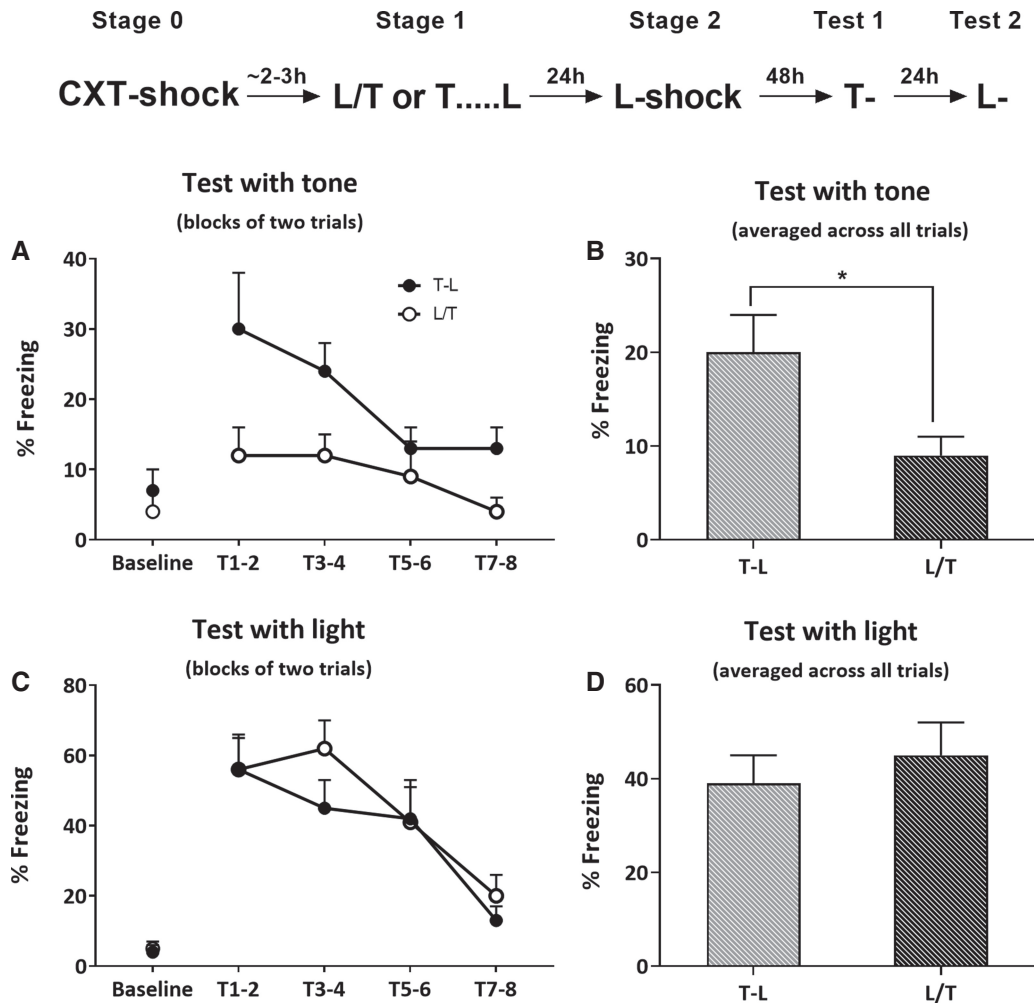
## Experiment 3

The previous experiments have shown that rats can form long-delay associations between two neutral stimuli in a context where shock has occurred. The present experiment examined whether rats injected systemically with epinephrine can likewise form such long delay associations between two neutral stimuli. The design is shown in Table 1. In stage 1, rats in two groups were exposed to a contiguous relation between the two stimuli such that the offset of each tone presentation cooccurred with the onset of each light presentation (Groups Veh-0 and Epi-0). Rats in two other groups were exposed to a trace relation such that the offset of each tone presentation was followed 20 sec later by the onset of each light presentation (Groups Veh-20 and Epi-20). Exposure to these relations occurred under a systemic (intraperitoneal [i.p.]) injection of either epinephrine (0.05 mg/kg; Groups Epi-0 and Epi-20) or saline (Groups Veh-0 and Veh-20). The dose of epinephrine was selected based on its capacity to reinstate extinguished fear responses in a previous study by our laboratory (Morris et al. 2005). All Groups were then exposed to light-shock pairings in stage 2, and finally, tested with presentations of the tone alone and light alone in stage 3. If an injection of epinephrine functions like a dangerous context to permit the formation of a long-delay association between two neutral stimuli, rats in Group Epi-20 will freeze more when tested with the tone than rats in Group Veh-20, and just as much as rats in Groups Veh-0 and Epi-0.

## Results

Figure 1 shows the mean (+SEM) levels of freezing across pairings of the light and shock in each group. One rat in Group Veh-20 was excluded from the statistical analysis because it did not receive any foot-shocks during stage 2 (due to an equipment failure), and thus, did not acquire freezing to the light. All groups learned about the relationship between the light and shock in stage 2, as evidenced by a significant linear increase in freezing across the four light-shock pairings,  $F_{(1,27)} = 205.32$ ,  $P < 0.05$ , 95% CI = [2.07, 2.76]. There were no overall differences in freezing to the light among the groups, and no significant interaction between drug (epinephrine vs. vehicle) and the type of preconditioning (contiguous vs. trace),  $F_s < 1$ . Finally, there were no between group differences in the rates at which freezing increased across the light presentations,  $F_s < 1.64$ .

Figure 4, A and B, shows the test levels of freezing to the tone averaged across blocks of two trials and across all trials, respectively. The baseline levels of freezing before test presentations of the tone were low (<5%) and did not differ between the groups,  $F_s <$



**Figure 3.** Mean (+SEM) levels freezing to the tone (A) and light (C) across blocks of two trials and mean (+SEM) levels of freezing to the tone (B) and light (D) averaged across all trials at test in experiment 2. Note that the test levels of freezing in this experiment were lower than in the previous experiment, and that the scales on the Y-axes have been adjusted to reflect these lower levels. (T) Trial block.

1. The statistical analysis confirmed what is clear from inspection of the figure: Rats in Group Veh-20 froze significantly less than those in the other three groups,  $F_{(1,27)}=7.47, P<0.05, 95\% CI=[0.22, 1.52]$ ; rats in Group Epi-20 did not differ in their levels of freezing from rats in Groups Epi-0 and Veh-0,  $F<1$ , and rats in the latter two groups did not differ from each other,  $F<1$ . Averaged across groups, there was no significant change in freezing across blocks of test trials, and no differences between the groups in the rate that freezing changed across blocks of trials,  $F_s<2.08$ .

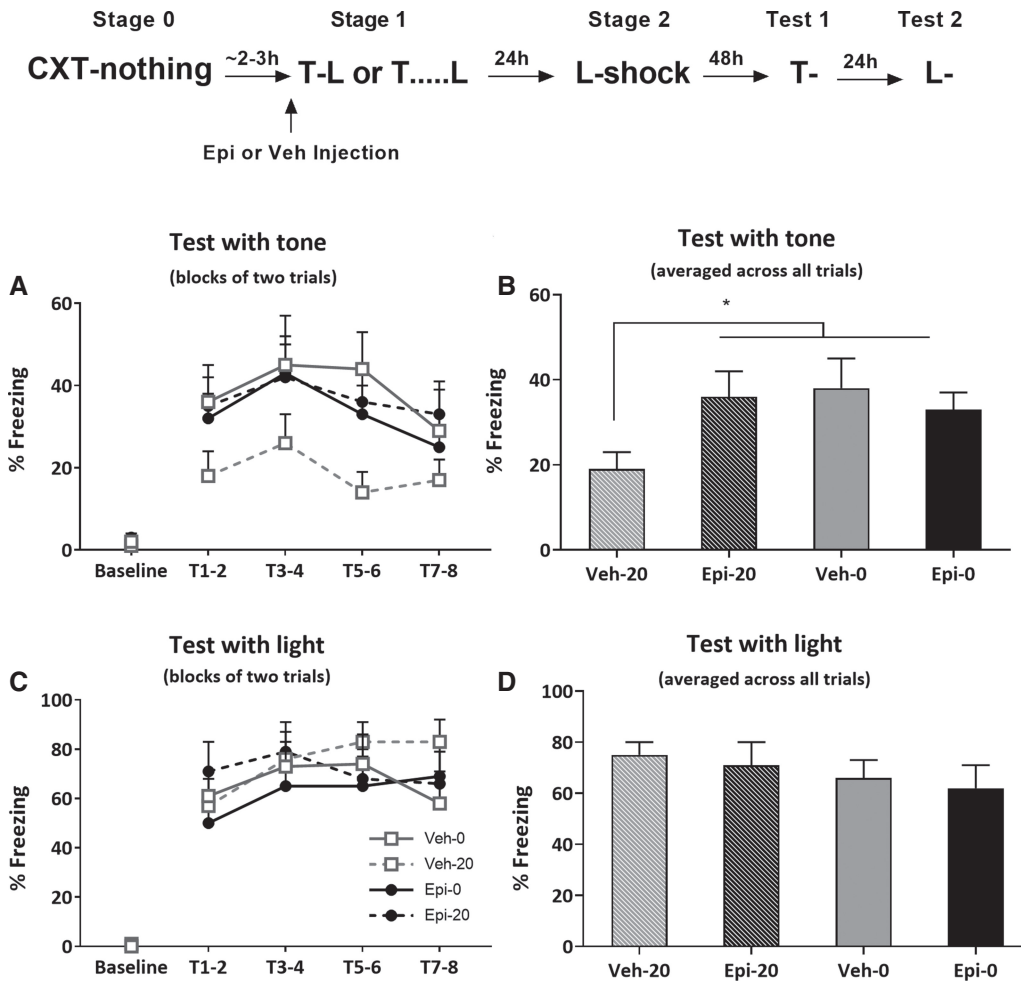
Figure 4, C and D, shows the test levels of freezing to the conditioned light averaged across two trials and across all trials, respectively. The baseline levels of freezing before test presentations of the light were low (<5%) and did not differ between the four groups,  $F_s<2.1$ . There were no significant differences in the levels of freezing between any of the groups, or interactions between drug (Epi vs. Veh) and type of preconditioning (Contiguous vs. Trace),  $F_s<1.27$ . Averaged across groups, there was no significant change in freezing across blocks of test trials, nor a significant between-group difference in this change of freezing,  $F_s<2.5$ . However, there was a significant three-way interaction between drug (Epi vs. Veh), type of preconditioning (Contiguous vs. Trace) and trial block,  $F_{(1,27)}=7.27, P<0.05, 95\% CI=[-1.45, -0.20]$ . From inspection of the figure, this was due to a persistence

of freezing across the test session in Group Veh-20, and a slight decline in freezing across the test session in Group Veh-0.

This experiment has shown that a systemic injection of epinephrine influences trace sensory preconditioning. Rats that had been exposed to the 20-sec trace interval between the tone and light under vehicle (Veh-20) froze less when tested with the tone than rats exposed to this interval under epinephrine or exposed to a contiguous relation between the tone and light. Critically, rats exposed to the 20-sec trace interval under epinephrine (Epi-20) froze just as much as rats that had been exposed to the contiguous relation under vehicle (Veh-0) or epinephrine (Epi-0). Moreover, preconditioning in stage 1 under epinephrine did not alter conditioning in stage 2, as rats in the four groups froze at an equivalent level when tested with the conditioned light. These results show that epinephrine functions like a dangerous context to permit the formation of an association between the tone and light under temporal conditions where it does not otherwise occur, and to do so without altering the levels of conditioning to the light.

### Experiment 4

The finding that epinephrine functions like a dangerous context raises the question as to whether these manipulations have



**Figure 4.** Mean (+SEM) levels freezing to the tone (A) and light (C) across blocks of two trials and mean (+SEM) levels of freezing to the tone (B) and light (D) averaged across all trials at test in experiment 3. (T) Trial block.

additive effects on formation of the long-delay association between the tone and light; specifically, whether the trace protocol would produce a greater level of sensory preconditioned fear in rats subjected to both manipulations than in rats exposed to either manipulation in isolation. The present experiment examined this question. The design is shown in Table 1. Four groups of rats were exposed to presentations of the tone followed 20 sec later by presentations of the light in stage 1, light-shock pairings in stage 2, and tests of the preconditioned tone and the conditioned light in stage 3. The groups differed in their treatment prior to the session in which they received the trace protocol in stage 1. Two groups were shocked in the context 2 h prior to the trace preconditioning session (Groups Danger), whereas the remaining two groups were not shocked (Groups Safe). Additionally, one group in each of these pairs was injected with epinephrine immediately prior to the trace preconditioning session (Groups Danger-Epi and Safe-Epi), while the other group in each pair was injected with vehicle (Groups Danger-Veh and Safe-Veh). Based on the previous results, we expected that rats injected with vehicle before trace preconditioning in a dangerous context (Group Danger-Veh) and those injected with epinephrine before trace preconditioning in a safe context (Group Safe-Epi) would freeze more when tested with the preconditioned tone than rats that had been injected with vehicle prior to trace preconditioning in a safe context (Group Safe-Veh). The question of interest was whether rats

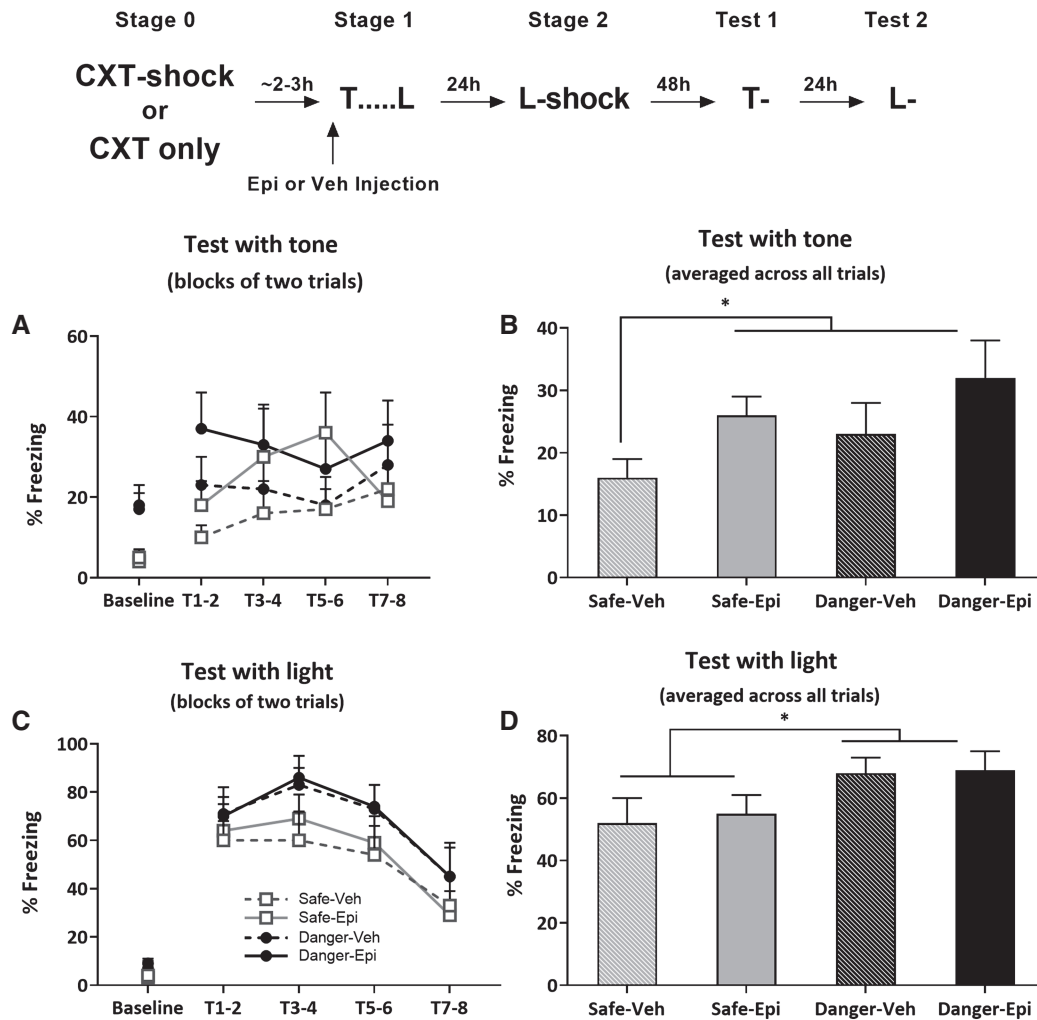
that were injected with epinephrine and preconditioned in a dangerous context (Groups Danger-Epi) would freeze even more when tested with the tone than rats in Groups Danger-Veh and Safe-Epi.

## Results

The shocked exposures rendered the context dangerous. In the final minute of the context conditioning session that preceded trace preconditioning, rats in Groups Danger (Danger-Veh and Danger-Epi) froze significantly more than rats in Groups Safe (Safe-Veh and Safe-Epi),  $F_{(1,28)}=57.60, P<0.05, 95\% \text{ CI } [1.96, 3.41]$ . Figure 1 shows the mean (+SEM) levels of freezing across pairings of the light and shock in each group. All groups successfully acquired freezing to the light, as evidenced by a significant linear increase in freezing across the four light-shock pairings,  $F_{(1,28)}=227.61, P<0.05, 95\% \text{ CI } [2.43, 3.20]$ .

There were no between-group differences in the rates at which freezing increased across the light-shock pairings or in the overall levels of freezing to the light,  $F_s < 3.51$

Figure 5, A and B, shows the test levels of freezing to the preconditioned tone averaged across blocks of two trials and across all trials, respectively. The baseline levels of freezing before test presentations of the tone were high (~20%), and were significantly greater by rats in Groups Danger (Danger-Veh and Danger-Epi)



**Figure 5.** Mean (+SEM) levels freezing to the tone (A) and light (C) across blocks of two trials and mean (+SEM) levels of freezing to the tone (B) and light (D) averaged across all trials at test in experiment 4. (T) Trial block.

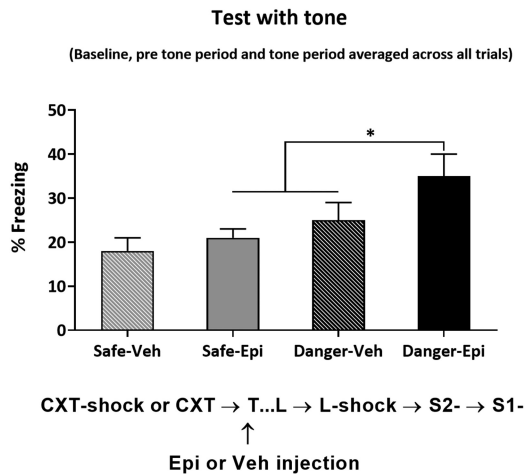
than Groups Safe (Safe-Veh and Safe-Epi),  $F_{(1,28)} = 14.8$ ,  $P < 0.05$ , 95% CI [0.66, 2.09]. Overall, the tone evoked less freezing in Group Safe-Veh than in the other three groups combined,  $F_{(1,28)} = 4.55$ ,  $P < 0.05$ , 95% CI [-1.15, -0.02]. There were, however, no significant differences in overall levels of freezing to the tone between Group Danger-Epi and the weighted average of Groups Safe-Epi and Danger-Veh, or between the latter groups,  $F_s < 2.26$ . Finally, averaged across groups, there was no significant change in freezing across blocks of test trials, and no differences between groups in the rate that freezing changed across blocks of trials,  $F_s < 1.52$ .

Figure 5, C and D, shows the test levels of freezing to the conditioned light averaged across blocks of two trials and across all trials, respectively. The baseline levels of freezing before test presentations of the light were low (<10%), and were again significantly greater by rats in Groups Danger (Danger-Veh and Danger-Epi) than Groups Safe (Safe-Veh and Safe-Epi),  $F_{(1,28)} = 5.5$ ,  $P < 0.05$ , 95% CI [0.11, 1.56]. The statistical analysis revealed that rats in Groups Danger (Group Danger-Veh and Danger-Epi) froze significantly more than those in Groups Safe (Groups Safe-Veh and Safe-Epi),  $F_{(1,28)} = 6.09$ ,  $P < 0.05$ , 95% CI [-1.20, -0.11]. However, freezing to the conditioned light in Groups Vehicle (Groups Safe-Veh and Danger-Veh) was not significantly

different from that in Groups Epinephrine (Groups Safe-Epi and Danger-Epi), and there was no significant interaction between the context (Danger and Safe) and drug conditions (Epinephrine and Vehicle),  $F_s < 1$ . Finally, averaged across groups, freezing extinguished across test presentations of the conditioned light,  $F_{(1,28)} = 38.48$ ,  $P < 0.05$ , 95% CI [-1.37, -0.69]. However, there were no significant differences between the groups in the rate at which freezing declined across presentations,  $F_s < 1$ .

The analysis of freezing to the tone failed to reveal evidence that a dangerous context and epinephrine injection have additive effects on trace preconditioning. In order to provide a further assessment of any such additivity, we calculated the total amount of freezing that each rat exhibited across the test session (i.e., in the baseline period before the first tone presentation, the 30-sec periods before onset of each tone, and during each tone presentation) (see Fig. 6) and compared the level of freezing in Group Danger-Epi versus the combined level of freezing in Groups Danger-Veh and Safe-Epi. This comparison revealed that rats in Group Danger-Epi froze significantly more than those in Groups Safe-Epi and Danger-Veh,  $F_{(1,28)} = 7.44$ ,  $P < 0.05$ , 95% CI [0.17, 1.19]. There were, however, no significant differences in the total amount of freezing across the test session with the light between Group Danger-Epi and the weighted average of Groups Danger-Veh and





**Figure 6.** The mean (+SEM) levels of freezing across the entire tone test session in experiment 4, averaged across the baseline period, the 30-sec periods prior to onset of each tone presentation, and each 30-sec presentation of the tone.

Safe Epi,  $F_s < 1.23$  (Danger-Epi:  $M = 42$ ,  $SEM = 6$ ; Danger-Veh:  $M = 45$ ,  $SEM = 5$ ; Safe-Epi:  $M = 56$ ,  $SEM = 4$ ; Safe-Veh:  $M = 57$ ,  $SEM = 5$ ).

This experiment has replicated the main findings from experiments 1 and 3, that a dangerous context and epinephrine injection enhance associative formation between the tone and light in trace sensory preconditioning. Rats that received one, the other or both of these manipulations prior to the trace protocol in stage 1 froze more when tested with the tone than control rats injected with vehicle and exposed to the trace protocol in a safe context. It also provided some support for the hypothesis that a dangerous context and epinephrine injection have additive effects on trace preconditioning. Rats subjected to both of these manipulations froze more across the entire test session with the preconditioned tone than rats exposed to either manipulation administered alone. The implication of this result is that a dangerous context and epinephrine injection have additive effects on freezing that is determined by the context-evoked expectancy of the preconditioned tone (baseline plus pretone) and the tone-evoked expectancy of the directly conditioned light. This result is considered further in the Discussion.

## Discussion

The present series of experiments tested the hypothesis that a dangerous context influences sensory preconditioning by increasing levels of epinephrine in the periphery and/or norepinephrine in the BLA. It had two specific aims. The first was to replicate our previous finding that a dangerous context affects what rats learn when exposed to a trace interval between presentations of a tone and an innocuous but to-be conditioned light; specifically, that these rats, but not those exposed to the trace protocol in a safe context would freeze when finally tested with the tone. The second aim was to determine whether artificially increasing epinephrine in the periphery (via a systemic epinephrine injection) would function like a dangerous context to enable trace preconditioning. In each experiment, rats were exposed to presentations of a tone and light in stage 1, light-shock pairings in stage 2, and test presentations of the tone alone and light alone in stage 3. The experiments differed with respect to the treatment afforded the rats prior to stage 1 as well as the sequencing and/or timing of tone and light presentations in stage 1.

In experiment 1, we replicated the effect of a dangerous context on trace preconditioning: Rats that had been exposed to the trace relation between the tone and light in a previously shocked context exhibited more freezing than rats that had been exposed to that relation in a safe context, and just as much freezing as rats that had been exposed to contiguous relation between the tone and light in either context. These results are consistent with the view that a shocked context promotes formation of a tone-light association under conditions where it otherwise fails to occur, specifically, when the interval between presentations of the two stimuli is 20 sec or more (Holmes and Westbrook 2017).

However, there are at least two alternative explanations for these results. The first is that rats exposed to the trace relation between the tone and the light in a dangerous context formed associations between the tone and that context. Thus, test presentations of the tone activated this memory of the dangerous context, eliciting freezing. The second alternative explanation is that the dangerous context prevented familiarization with the tone and light across the trace protocol. Thus, test presentations of the tone elicited freezing because the tone was relatively novel, and rats had been sensitized by the light-shock pairings.

In experiment 2, we distinguished between the various explanations of the results for experiment 1 by showing that rats exposed to the trace relation in a dangerous context again froze when tested with the tone, and critically, that this level of freezing was greater than that exhibited by rats exposed to explicitly unpaired presentations of the tone and light in the dangerous context. This difference is inconsistent with both alternative explanations for freezing to the tone noted above. If the basis of that freezing was due to an association between the tone and the shocked context or to the elicitation of a sensitized freezing response by the effectively novel tone, it should have been independent of the sequencing and timing of the tone and light presentations. In other words, the two groups should have exhibited equivalent levels of freezing to the tone at test. Instead, the difference in freezing to the tone between the groups in this experiment confirms that the trace protocol in a dangerous context results in the formation of a tone-light association in stage 1, which is then integrated with the light-shock association formed in stage 2 to generate freezing to test presentations of the tone in stage 3.

Experiment 3 confirmed that artificially increasing the level of epinephrine in the periphery (via a systemic epinephrine injection) also permits associative formation in the trace protocol and hence sensory preconditioned fear of the tone. Rats injected with epinephrine prior to the trace protocol froze more when tested with the tone than rats that had received a saline injection prior to this protocol, and just as much as rats exposed to a contiguous tone-light relation after an injection of epinephrine or vehicle. Experiment 4 replicated the effects of a dangerous context (experiment 1) and epinephrine injection (experiment 3) on trace sensory preconditioning, while additionally providing evidence that these manipulations have additive effects on trace preconditioning. Here, rats exposed to the trace protocol under the influence of an epinephrine injection, in a dangerous context, or under epinephrine in the dangerous context froze more when tested with the tone than rats injected with vehicle and exposed to the trace protocol in a safe context. There was no evidence that the combination of the two treatments produced additive effects on freezing to the tone relative to either treatment in isolation. However, there was such evidence when the total levels of freezing across the test session with the tone was used: Rats injected with epinephrine and exposed to the trace protocol in a dangerous context froze significantly more across the test session (baseline, pretone, and tone) than rats subjected to one or the other manipulation.

Overall, the present results are consistent with the hypothesis that a dangerous context enables associative formation across the trace interval by increasing epinephrine levels in the periphery. However, they leave open the question of how increased epinephrine levels achieve this effect. There is considerable evidence that epinephrine injections increase subjective reports of arousal and physiological responses indicative of arousal in people, including heart rate and the electrodermal skin response (Mezzacappa 1999; Cahill and Alkire 2003). This increase in arousal may have enhanced the functional salience of the tone and light or the amount of attention that they commanded across their presentations. Such an increase in attention is typically thought to enhance associability (e.g., Mackintosh 1975; Pearce and Hall 1980), including the formation of associations across delays (Mather 2007). The present experiments, however, failed to detect evidence for enhanced associative formation when rats were exposed to a contiguous relation between the stimuli under the drug, in a dangerous context, or under both manipulations. This failure could be due to the learning produced by that relation being at asymptotic levels, and thereby unable to reveal the enhancement in associative formation detected when the trace relation was used.

In addition to the trace preconditioning effect described here, a dangerous context has been shown to alter the neural substrates of the tone-light association that forms in sensory preconditioning (Holmes et al. 2013, 2018). When this association forms in a safe context, its encoding and consolidation requires neuronal activity in the PRh, but not the BLA; and conversely, when it forms in a dangerous context, its encoding and consolidation requires neuronal activity in the BLA, but not the PRh. While the mechanisms that determine this shift in processing of the tone-light association are unknown, the present findings suggest that it may be related to increased levels of epinephrine in the periphery. Accordingly, future work could examine whether an epinephrine injection reproduces the effect of a dangerous context on processing of the tone-light association in stage 1 of sensory preconditioning; specifically, whether it shifts the encoding and consolidation of this association from the PRh to the BLA. Additionally, given that the BLA is a critical locus of epinephrine-induced enhancements of learning and memory, including memory for innocuous objects (Roosendaal et al. 2008), future work could also examine whether the effect of a systemic epinephrine injection on trace preconditioning is due to its mechanisms of action in the BLA; for example, whether an intra-BLA infusion of norepinephrine prior to trace tone-light pairings in stage 1 would permit trace sensory preconditioning. Finally, future work might also examine the limit on trace preconditioning in a dangerous context (i.e., the maximum interval between presentations of the tone and light in stage 1 that is conducive to formation of the tone-light association) and the generality of trace preconditioning to other stimulus arrangements and types of contexts; for example, whether the same results would be obtained if the light was the preconditioned CS and the tone the first-order CS, or if trace preconditioning was conducted in an appetitive context rather than a dangerous context.

In summary, the present study has shown that a dangerous context and a systemic epinephrine injection both facilitate trace preconditioning and provided some evidence to suggest that their combination has an additive effect on formation of the trace preconditioned association. These findings are generally consistent with the view that a dangerous context facilitates trace preconditioning by increasing the level of epinephrine in the periphery, and thereby, arousal-induced enhancements in attention to and learning about environmental stimuli. They imply that a dangerous context may influence other aspects of sensory preconditioning, such as the neural substrates of a preconditioned association, by increasing epinephrine levels in the periphery; and that the effects of a dangerous context may be blocked by

drugs that reduce peripheral epinephrine levels (e.g., sotalol or propranolol).

## Materials and Methods

### Subjects

Subjects were experimentally naive, female, Long-Evans rats (195–370 g) obtained from the Randwick Breeding Facility maintained by the School of Psychology at the University of New South Wales. The rats were housed in plastic tubs (67 cm length  $\times$  40 cm width  $\times$  22 cm height) with continuous access to food and water. The tubs were located in a temperature-controlled colony room (20°C–22°C) kept on a 12 h light–dark cycle (lights on 0700 and lights off 1900 each day). Rats were handled for at least 5 d prior to the start of the experiment. The Animal Care and Ethics Committee at the University of New South Wales approved all experimental procedures. As noted in the text, one rat was excluded from experiment 3 as it did not receive any foot-shocks during day 4 conditioning and did not acquire freezing to the light. The final number of subjects in each experiment were 32 in experiment 1 ( $n=8$  per group), 21 in experiment 2 ( $n=11$  in Group T-L and  $n=10$  in Group L/T), 31 in experiment 3 ( $n=7$  in Group Veh-20 and  $n=8$  in the remaining groups), and 32 in experiment 4 ( $n=8$  per group).

### Apparatus

All experiments were conducted in a set of four identical chambers, each measuring 31 cm (length)  $\times$  26 cm (width)  $\times$  33 cm (height). The chambers were located in separate compartments of a sound- and light-attenuating wooden cabinet whose walls, floor and ceilings were painted black. The back and front walls of each chamber were made of clear Perspex, and the sidewalls and ceiling were made of aluminum. The floor of each chamber consisted of stainless-steel rods, 5 mm in diameter, spaced 10 mm apart (center to center). A tray containing bedding material was located below the floor. The floor of each chamber was cleaned with a small amount of water after each rat was removed, and the bedding material changed. A speaker mounted to the back wall of each cabinet was used to present the auditory stimulus (1000-Hz tone at 72-db intensity). A set of LEDs mounted on that wall was used to present the visual stimulus: a flashing light (3 Hz at 57 lux measured at the center of the chamber). A constant-current shock generator, which delivered unscrambled 50-Hz AC electricity to the grid floor of the conditioning chamber, was used to deliver a 0.5-sec foot-shock (at either 0.5- or 0.8-mA intensity). An infrared light (940  $\pm$  25 nm) and camera were also located on the back wall of each cabinet. The camera was connected to a monitor and DVD recorder located in another room of the laboratory. Together the infrared light and camera permitted the behavior of each rat to be recorded for later scoring. All stimulus presentations were controlled by Matlab (MathWorks) software.

### Drugs

Epinephrine (Sigma-Aldrich) was dissolved in 0.9% (w/v) nonpyrogenic saline to obtain a concentration of 0.05 mg/mL (Morris et al. 2005). The 0.9% saline was also used for control (saline) injections. All injections were given i.p. in a volume of 1.0 mL/kg. The rats were injected i.p. with vehicle (saline) or epinephrine (0.05 mg/kg) 6 min before the placement in the conditioning chamber.

## Experiment 1

### Procedure

On each of days 1 and 2, rats received two exposures to the context in the absence of any scheduled events. Each exposure lasted for 20 min, and the two daily sessions were separated by at least 3 h. These sessions were intended to familiarize the rats with the context, and thereby reduce any neophobic reactions that could obscure effects of the manipulations.

On day 3, rats were randomly allocated to one of four groups, and each received two sessions of training. In the first session, all rats were placed in the conditioning chambers for 5-min. During this session, half the rats were exposed to two 0.5-mA, 0.5-sec foot shocks (Groups Danger-0 and Danger-20). The first shock occurred 3 min after placement in the chamber, and the second shock occurred 1 min after the first. The remaining rats were not exposed to shock during this 5-min session (Groups Safe-0 and Safe-20). The second session occurred ~2 h later. All rats were returned to the context for sensory preconditioning, which consisted of eight presentations of the tone and eight of the light. Each presentation of the tone lasted for 30 sec, and each presentation of the light lasted for 10 sec. The first presentation of the tone occurred 5 min after rats had been placed in the chamber, and the interval between tone presentations was fixed at 5 min. For rats in Groups Safe-0 and Danger-0, the interval between offset of the tone and onset of the light was 0 sec, and for those in Groups Safe-20 and Danger-20, this interval was 20 sec. Rats remained in the context for 2 min after the final light presentation and were then returned to their home tubs in the colony room.

On day 4, all rats received conditioning. This consisted in four pairings of the light and shock (0.8 mA  $\times$  0.5 sec). Each presentation of the light lasted for 10 sec and coterminated with the shock. The first light presentation occurred 5 min after rats had been placed in the chamber, and the interval between each light-shock pairing was 5 min. Rats remained in the context for 1 min after the final light-shock pairing and were then returned to the colony room.

On day 5, all rats received two sessions of context extinction. This was done to eliminate any context-elicited freezing that might otherwise obscure the levels of freezing elicited by the tone and light in the subsequent test sessions. The context extinction sessions were identical to the context exposure sessions on days 1 and 2. On the morning of day 6, all rats received an additional 10-min session of context extinction in order to reduce any spontaneous recovery of extinguished context-elicited freezing. Approximately 3 h later, all rats were tested across eight presentations of the tone alone. Each tone presentation lasted for 30 sec, the first occurred 2 min after placement in the chamber, and the interval between tone presentations was 3 min. Rats remained in the context for 1 min after the final tone presentation and were then returned to the colony room. On day 7, rats were tested across eight presentations of the light alone. The onset of the first light presentation occurred 2 min after placement in the chamber. Each presentation of the light lasted 10 sec, and the interval between the light presentations was 3 min.

It should be noted that all rats were trained and tested in the same context in each experiment in this series. This was done to simplify interpretation of the test data on days 6 and 7. For example, if we had only tested the rats in a different context and observed that the tone failed to elicit freezing, this might have meant that the rats had failed to encode the trace association in stage 1, or alternatively, that the trace association that formed in stage 1 did not readily transfer across contexts. As we would not be able to distinguish between these two possible explanations of the results (were they to eventuate), we decided to test the animals in the same context used in training subsequent to extinction of context-elicited freezing. With the use of appropriate controls, such as the group exposed to explicitly unpaired presentations of the tone and light in experiment 2, this method of testing permits stronger inferences to be drawn regarding the impact of different manipulations on the strength of trace sensory preconditioning. It would, of course, be interesting to assess the impact of a context shift on test levels of freezing to the tone among rats exposed to either standard or trace sensory preconditioning. This remains to be addressed in future research.

### Scoring and statistics

Freezing was defined as the absence of all movements except those related to breathing (Fanselow 1980). Rats were observed

every 2 sec and scored as either freezing or not by two observers, one of whom was naïve to the purposes of the experiment. The correlation between the scores of the two observers was high, with a Pearson product-moment correlation  $>0.9$ , and any discrepancies between the two scores were resolved in favor of the naïve observer. Freezing was scored for the 30-sec duration of each tone presentation and 10-sec duration of each light presentation. The number of 2-sec samples scored as freezing were expressed as a percentage of the total numbers of observations during tone and light periods. The test data on day 6 were analyzed using a set of planned orthogonal contrasts with repeated measures in ANOVA (Hays 1963). These contrasts were derived from our hypotheses regarding group differences in freezing to the tone. We had no such hypotheses regarding freezing to the light, and thus, took an agnostic approach to the analysis of these freezing levels. That is, the data across light-shock pairings on day 4 and testing of the light on day 7 were analyzed using a mixed model ANOVA with two between subject-factors (experiment 1: context and interval; experiment 3: drug and interval; experiment 4: drug and context), and a within subject factor of trial-block (average of two trials). The criterion for rejection of the null hypothesis ( $\alpha$ ) was set at 0.05. This corresponded to a critical  $F$  statistic of 4.2 in experiment 1, 4.4 in experiment 2, 4.2 in experiment 3, and 4.2 in experiment 4. 95% standardized confidence intervals (CI) were constructed and reported for all statistically significant differences.

## Experiment 2

### Procedure

On days 1 and 2, rats were exposed to the context in the manner described for experiment 1. On day 3, each rat was placed in the conditioning chamber for 5 min and exposed to two foot shocks. The timing and intensity of the shock was the same as that described in experiment 1. Approximately 2 h later, rats were returned to the context for the sensory preconditioning session that consisted of eight presentations of a tone and eight of the light. Rats in Group T-L were exposed to a 20-sec delay between presentations of the tone and light in the manner described for Group Danger-20 in experiment 1. Rats in Group L/T were exposed to series of eight presentations of the light alone followed by a series of eight presentations of the tone alone. For all rats, the first presentation of the stimulus occurred 5 min after rats had been placed in the chamber, and the interval between all stimulus presentations was fixed at two and a half min. All rats remained in the context for an additional 2 min after the final stimulus presentation and were then returned to the colony room. On day 4, all rats received light-shock conditioning, on day 5, two sessions of context extinction, testing of the tone on day 6 and of the light on day 7, all in the manner described previously.

## Experiment 3

### Procedure

Rats were randomly allocated to one of four groups. On days 1 and 2, rats were familiarized with the context. On day 3, rats were injected i.p. with epinephrine or vehicle, and 6 min later, placed into the context where they were exposed to eight 30-sec presentations of the tone and eight 10 sec presentations of the light. For rats in Groups Veh-0 and Epi-0, termination of each tone presentation cooccurred with the onset of the light, while for rats in Groups Veh-20 and Epi-20, termination of the tone was followed 20 sec later by presentation of the light. The interval between tone presentations was 5 min. Rats remained in the context for ~2 min after the final light presentation. On day 4, all rats received light-shock conditioning, on day 5, two sessions of context extinction, on day 6, testing of the tone and on day 6, testing of the light, all in the manner described previously.

## Experiment 4

### Procedure

Rats were randomly allocated to one of four groups. On days 1 and 2, rats were familiarized with the context. On the morning of day 3, all rats were placed in the conditioning chambers for 5-min. During this session, half the rats were exposed to two-foot shocks (Groups Danger) in the manner described in experiment 1. The remaining rats were not exposed to shock across the 5 min session (Groups Safe). Approximately 2 h later, rats were injected i.p. with epinephrine (Groups Epi) or vehicle (Groups Veh), and 6 min later, placed into the context where they were exposed to eight 30-sec presentations of the tone and eight 10-sec presentations of the light. Termination of each tone was followed 20 sec later by presentation of the light. The interval between tone presentations was 5 min, and rats remained in the context for ~2 min after the final light presentation. On day 4, all rats received light-shock conditioning, on day 5, two sessions of context extinction, on day 6, testing of the tone, and on day 7, testing of the light, all in the manner described previously.

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