Neuroscience Letters 505 (2011) 134–139

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Interdependence of measures in Pavlovian conditioned freezing

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ARTICLE INFO

Article history:
Received 14 June 2011
Received in revised form
21 September 2011
Accepted 3 October 2011

Keywords:
Pavlovian fear conditioning
Classical conditioning
Learning
Memory
Mice

ABSTRACT

Pavlovian conditioned freezing is an intensively utilized paradigm that has become a standard model of memory and cognition. Despite its widespread use, the interdependence among each measure commonly reported in fear conditioning studies has not been described. Using mice, we examine the relationship of each common freezing measure (Training Baseline, Post-Shock freezing, Contextual Fear, Tone Baseline, and Tone Fear), as well as baseline locomotor activity measures, to better understand the significance of each. Of particular interest, Post-Shock freezing appears to be a good measure of immediate contextual memory. In contrast, Tone Baseline freezing, as typically measured in a novel context, appears to be contaminated with multiple sources of fear. Finally, Contextual and Tone Fear show a weak interdependence.

1. Introduction

Pavlovian fear conditioning is being used with increasing frequency as a measure of learning and memory in a variety of settings, including large-scale pharmacological and genetic screens [17,25,27]. The robustness and efficiency of the paradigm has yielded a wealth of findings in recent years, greatly expanding the number of laboratories assessing memory. Fear conditioning uses a rapid procedure that can produce enduring, life-long memory [16]. Training consists of an initially neutral conditioned stimulus (CS), usually a pure tone, paired with an unconditioned stimulus (US), usually a mild footshock. As a result of this pairing, when presented alone, the CS comes to elicit fear, the conditioned response (CR). Aside from fear of the discrete CS, subjects come to fear the environmental conditions surrounding the fear conditioning episode, a phenomenon known as context conditioning. Contextual Fear is measured simply by returning the subject to the training context. Finally, cued fear is assessed by placing the subject in a new context, which is varied on a number of dimensions (e.g. appearance, odor, sounds) from the training context, and, after a baseline period to ensure little generalization, playing the tone from the training day [28].

Both contextual and cued fear are dependent upon the amygdala, whereas Contextual Fear is further dependent on the hippocampus, in a time-limited fashion [1,19]. In contrast, cued fear, as typically performed, is usually found to be hippocampus-independent (e.g. [3]), but can depend on the hippocampus in certain conditions, especially when a trace conditioning procedure is utilized [11,21,24,26]. Fear conditioning, therefore, is a very well-characterized paradigm, utilized as a model of both explicit memory and pathological fear (e.g. specific phobias) in humans [1,14,30].

Freezing, or the lack of movement aside from respiration, is the measure most commonly used to quantify memory in Pavlovian fear conditioning. Freezing is a species-specific defensive reaction, thought to be adaptive for rodents in the face of predators such as snakes and cats [8,9]. Locomotor activity can also be measured during any of the training or testing measures, but is generally used to assess any sort of movement disturbance due to a genetic or pharmacological manipulation of the subjects being tested, and typically during the baseline periods of the training day, prior to any fear conditioning [2,5,7,23].

This paradigm generates several freezing measures. First, Training Baseline is the period during which the animal is initially exposed to the training environment, and is free to explore the box with no tone or shock present. This period is usually at least 2 min because animals may fail to learn contextual cues when the placement-to-shock interval is too short [12,15]. Training then occurs, with single or multiple presentations of a tone

Abbreviations: A.Train.BL, Training Baseline period activity; A.Tone.BL, Tone Baseline period activity; CR, conditioned response; CS, conditioned stimulus; PS, Post-Shock freezing; Tone.BL, Tone Baseline period; Train.BL, Training Baseline period; UR, unconditioned response; US, unconditioned stimulus.

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doi:10.1016/j.neulet.2011.10.006

co-terminating with a footshock. The unconditioned response (UR) to the shock is usually an activity burst, but mice and rats quickly settle into Post-Shock freezing that Fanselow [13] has argued is a CR to the context, and not an extended UR as it has sometimes been interpreted [31]. In either case, freezing during the period of tone-shock pairings or immediately thereafter is known as Post-Shock freezing (PS). Contextual Fear can be examined by measuring freezing when the animal is returned to the training context at a later time, usually one day later. Finally, the tone test, performed an additional day later, is typically divided into the Tone Baseline period, during which the animal explores the novel environment, and a Tone Fear period, during which the animal is exposed to the tone CS, often repeatedly [2,28].

While these measures are commonly reported, their interdependence (e.g. correlational structure) has not been described. Significantly, Fanselow has argued that Post-Shock freezing reflects Contextual Fear [13], but it is sometimes still considered a shock UR, or more often, altogether ignored. If Post-Shock freezing is a measure of Contextual Fear, it is an important opportunity to collect the status of short-term memory. Also, while the tone test is designed to measure fear that is independent from Contextual Fear, there can still be much variability in Tone Baseline freezing, especially in mice [29,32,33]. A recent paper has argued that the standard methods for accounting for differences in the Tone Baseline period (usually subtraction of the baseline from the tone average) is inadequate [18]. Context and Tone Fear can be dissociated in lesion or mutant studies of the hippocampus, but little attention is paid as to whether the two measures are independent in the intact animal [3,19].

The current study explores the interdependence of the different measures of a typical fear conditioning study. While, generally, studies of fear conditioning use sample sizes adequate to obtain good power to detect the effects of most independent variables, these sample sizes are inadequate to analyze the correlational structure of the dependent measures. Here, a large group of mice were run under the identical fear conditioning protocol, and correlations between the different measures were performed. Moreover, we examined the predictive power of the Post-Shock freezing measure, using a median split. If Post-Shock freezing truly reflects Contextual Fear, as Fanselow (1980) argued, an arbitrary split of Post-Shock freezing should affect only Contextual Fear.

2. Material and methods

2.1. Subjects

Forty-eight (24 females) Hybrid C57Bl/6Jx129T2SvEms/J (129B6, stock from the Jackson Laboratory, West Sacramento, CA) mice were used. This strain was chosen because it performs in a manner comparable to rats and is widely used in mutant and behavioral studies of fear conditioning [10]. Mice were weaned 19 days after birth and were between 25 and 48 weeks old at the time of testing. Mice were group housed (2–5 per cage) with unrestricted access to food and water under a 14:10-h light:dark cycle. Experiments were conducted during the light phase. All animal care and experimental procedures were approved by the University of California, San Diego Institutional Animal Care and Use Committee and were in accordance with the National Research Council *Guide for the Care and Use of Laboratory Animals*.

2.2. Apparatus

2.2.1. Conditioning context

Four conditioning chambers (Med-Associates, Inc., St. Albans, VT) were located in a windowless room, allowing four mice to be run concurrently. A HEPA air cleaner provided background noise (~65 dBA), and two 100-W bulbs provided bright white light. Each chamber (32 cm wide, 25 cm high, 25 cm deep) was made of three white acrylic sidewalls, and a clear polycarbonate front wall to allow for viewing. Stainless steel drop-pans were scented with 7% isopropyl alcohol to provide background odor. Each chamber contained a stainless steel grid floor (36 rods, each rod 2 mm in diameter, 8 mm center to center), connected to a solid-state scrambler, providing AC constant current shock. A speaker in a sidewall of each chamber was connected to an audio stimulus generator located in an adjacent room. A single color video camera, mounted to the wall facing the conditioning chambers, fed video of the mice to a computer also in the adjacent room. The shock and tone administrations were controlled via an interface connected to a Windows computer running Med-PC (Med-Associates, Inc., St. Albans, VT). Freezing was scored by a custom-designed software adaptation of NIH Image running on an Apple Macintosh G4 as previously described [2].

2.2.2. Alternate context

Four chambers located in a room separate from the training context were used to measure Tone Fear. The chambers (30 cm wide, 25 cm high, 24 cm deep) consisted of solid white walls, floors, and ceilings, with a clear Plexiglas front wall to allow for observation via a wall-mounted infrared video camera, connected to the same computer described above. Each chamber contained a speaker in the sidewall, also connected to the computer. The alternate context was different from the conditioning context along several dimensions: a white acrylic, triangular tent (23 cm each side) formed a tee-pee in each box, the chambers were cleaned with 5% white vinegar between trials, and the room was lit only with dim red light.

2.3. Procedure

2.3.1. Training

Training consisted of a 2 min baseline period (Train.BL), followed by one tone-shock pairing. The tone was 30 s (2.8 kHz, 85 dBA), co-terminating with a scrambled, constant current AC footshock (2 s, 0.75 mA, RMS). Immediate Post-Shock freezing was measured for another 2.5 min (PS), resulting in a 5 min, total, exposure to the training context.

2.3.2. Testing

Contextual Fear (Context) was measured by returning the mice to the conditioning context 24 h after training for 5 min. Tone (cued) fear was measured 48 h after training, in the alternate context. Testing consisted of a 2 min baseline period (Tone.BL), followed by a 3 min tone identical to the tone used in training (Tone). Freezing was used as the dependent measure for both tests.

3. Results

Two primary sets of statistics were run to address the questions posed above. First, a median split was performed on Post-Shock freezing to determine if this measure relates to context freezing, uniquely, as has been previously postulated [13]. ANOVA was used to determine significant differences between measures. Second, to determine the degree to which different aspects of fear conditioning are related, a correlation matrix was generated for all fear conditioning measures. *P* values were generated using Fisher's *r*-to-*z*.

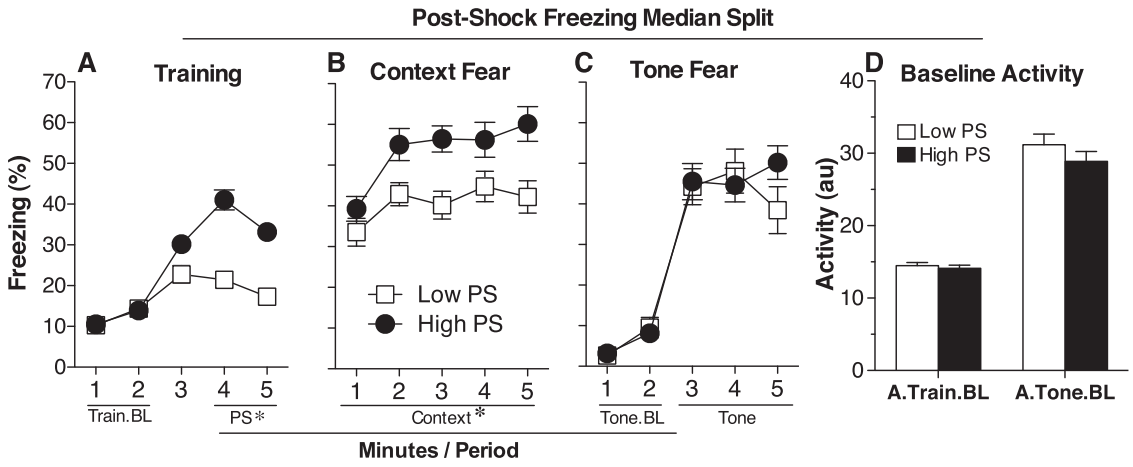

Fig. 1. *Post-Shock freezing median split.* Subjects were divided into two groups based on their fear during the PS period. This division created two statistically different groups for the PS period (A; *$p < .05$), but not for the Train.BL period. Likewise, the two groups continued to display fear at significantly different levels during the Context Test (B), but not the Tone.BL or Tone periods of the tone test (C). The two groups also showed no difference in baseline activity, during training or the tone test (D).

3.1. Training and testing

Freezing increased from the Training Baseline period, Train.BL (average, 12.3 ± 0.8%) to the Post-Shock period, PS [29.0 ± 1.6%; ANOVA, $F(1, 47) = 81.6$, $p < 0.0001$]. Mice also showed high levels of freezing when returned to the training context 24 h later, Context (average, 47.6 ± 2.1%). Finally, subjects displayed little freezing when initially introduced to the alternate context, during Tone.BL (average, 5.9 ± 0.8%), but demonstrated significantly higher levels of freezing when the tone was presented, Tone [45.3 ± 2.7%; $F(1, 47) = 230.1$, $p < 0.0001$].

3.2. Post-Shock freezing median split

To explore the relationship of Post-Shock freezing to other measures, subjects were split into two groups, based upon their performance during PS (min 4 and 5 of training; see Fig. 1). Mice in the "High PS" ($n = 23$) group froze during 27% of PS or more, while the "Low PS" ($n = 25$) group froze less than 27% of PS. Remarkable selectivity in the effect of this median split was found. Mice in the two groups showed no difference in baseline freezing (min 1 and 2) during Train.BL [$F(1, 46) = 0.01$, *n.s.*], but froze at significantly different levels during PS [Fig. 1A; $F(1, 46) = 73.2$, $p < 0.0001$]. Most significantly, the "High PS" group froze more than the "Low PS" group during Context [Fig. 1B; $F(1, 46) = 11.3$, $p < 0.01$]. No difference was seen between the two groups during either Tone.BL [$F(1, 46) = 0.07$, *n.s.*] or during Tone [Fig. 1C; $F(1, 46) = 0.33$, *n.s.*]. Finally, differences in Post-Shock Freezing were not attributable to differences in activity [2] as there were no differences found in terms of locomotor activity during either the training [A.Train.BL; $F(1, 46) = 0.39$, *n.s.*] or Tone Baseline [A.Tone.BL; $F(1, 46) = 1.26$, *n.s.*] periods (Fig. 1D). Thus, the median split on Post-Shock freezing showed remarkable selectivity for Contextual Fear, indicating it is indeed a measure of Contextual Fear. This is in agreement with Fanselow [13], who, using an entirely different approach, reached the same conclusion (see Section 4).

3.3. Post-Shock freezing correlations

The interdependence of the measures was examined first by correlating PS with the other freezing measures. PS freezing was highly correlated with freezing during Context (Fisher's *r*-to-*z*; $r = 0.65$, $p < 0.0001$), but was largely unrelated to Tone ($r = 0.23$, *n.s.*), Tone.BL ($r = 0.09$, *n.s.*), or Train.BL ($r = -0.04$, *n.s.*) freezing (Fig. 2A–D).

3.4. Overall correlations – freezing

Fig. 3A depicts the correlations among all periods of freezing during testing and training, in descending strength of their *r* values of each correlation. PS freezing was highly correlated with Context ($r = 0.65$, $p < 0.0001$). Freezing during Context was also weakly correlated with freezing during Tone.BL ($r = 0.34$, $p < 0.05$), as well as with freezing during Tone ($r = 0.29$, $p = 0.05$). Tone.BL and Tone freezing were also correlated ($r = 0.31$, $p < 0.05$). Overall, the only strong correlation was between Post-Shock freezing and Contextual Fear. The only disturbing finding is that Tone.BL did not entirely reflect generalized Contextual Fear as most investigators, including us, would implicitly assume. Tone Baseline freezing was a highly contaminated measure, correlated with both Contextual and Tone Fear (see also [18]).

3.5. Overall correlations – baseline activity

Periods of locomotor activity during the training and tone test baselines were compared to all freezing measures in Fig. 3B. The activity during Tone Baseline (A.Tone.BL) was highly negatively correlated with Tone.BL freezing ($r = -0.68$, $p < 0.0001$). While this may seem like a necessary relationship, it is worth noting that activity and freezing during Train.BL were not significantly correlated ($r = -0.21$, *n.s.*), and Train.BL measures were not significantly related to any other measures. This suggests that even though there was more overall activity in the Tone.BL period [as it was performed in the dark; $F(1,47) = 31.5$, $p < 0.0001$; Fig. 2D], the activity during the Train.BL is less dominated by a fear reaction as it occurs prior to any shock. Activity during Tone.BL was also correlated negatively with Tone freezing ($r = -0.40$, $p < .01$) and Context freezing ($r = -0.39$, $p < .01$), reflecting similar contamination to Tone.BL freezing. Finally, activity during Train.BL and Tone.BL were unrelated ($r = -0.03$, *n.s.*).

4. Discussion

The findings presented above provide researchers today with a better understanding of the importance and meaning of each fear conditioning measure commonly reported. Most significantly, and as previously concluded by Fanselow [13], our analyses suggest that Post-Shock freezing is short-term memory for Contextual Fear. It is known that when the placement-to-shock interval is very short (e.g. 5 s) animals fail to acquire Contextual Fear, as measured on a typical test one day later, a phenomenon known as the

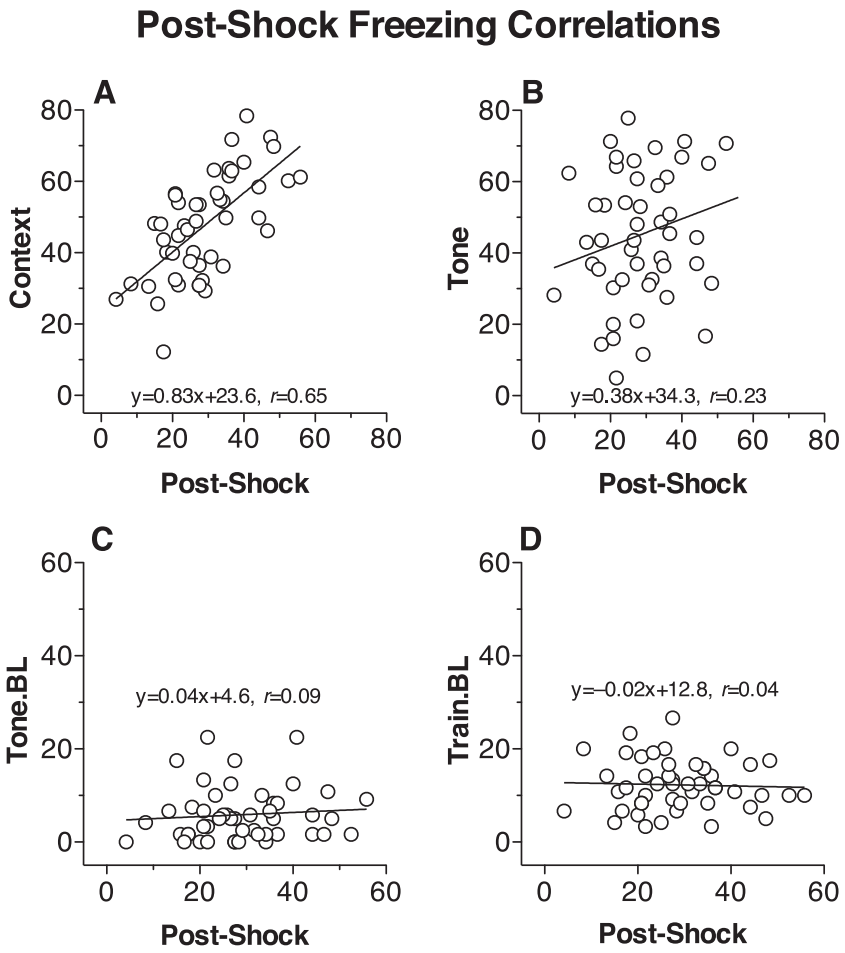

Fig. 2. Post-Shock *freezing correlations.* Post-Shock freezing was significantly correlated with context fear (A), but not tone (B), Tone Baseline (C) or Training Baseline (D).

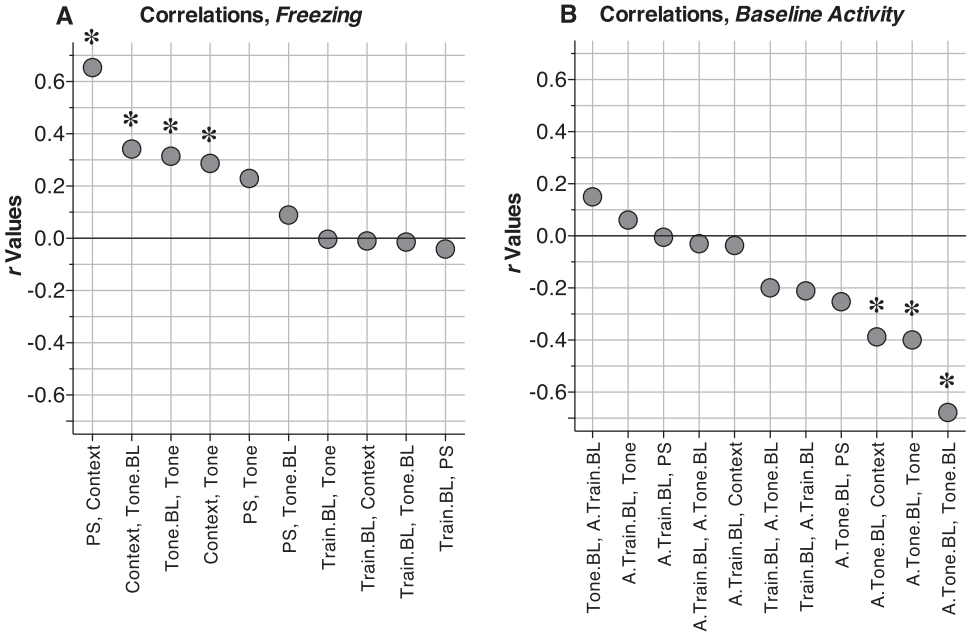

Fig. 3. *Correlations.* Correlations among all the freezing measures are presented (A), with PS-Context, Context-Tone.BL, Tone.BL-Tone, and Context-Tone all showing significant, positive correlations. Correlations among baseline activity measures and freezing measures are also shown (B). The strongest relationship was found between the A.Tone.BL (A. designating measures of activity) and Tone.BL, with A.Tone.BL having significant correlations with both Tone and Context freezing measures.

"immediate shock deficit" [12,15]. Interestingly, those same animals show a normal activity burst UR, but fail to show Post-Shock freezing. Moreover, Fanselow [13] also found that Post-Shock freezing was eliminated if animals were moved to a different context immediately following the shock. Taken together, these data suggest that Post-Shock freezing can be used as a measure of "immediate," or "short-term memory" for Contextual Fear (see, also, discussion in [4]). We provide the caveat, however, that when utilizing fear conditioning for pharmacological studies, care in interpreting the results must be taken if the drug leads to altered levels of activity while administered for the training period [34,35].

Contextual and Tone Fear are weakly correlated, as well, which is not altogether surprising. The two fear measures are generally treated as dissociable in the literature, as seen in studies using lesions, for example [14,20]. Perhaps, in intact animals, some may have perceived the shock as more painful, leading to higher freezing scores in both conditions.

We also found that Tone Baseline is a problematic area for the standard paradigm. Although rats typically do not usually show much freezing during this period, and it usually does not increase with the passage of many days (e.g. [3,19]), mice often show some freezing during this period that can even increase with the passage of weeks [32]. Unfortunately, we found that Tone Baseline freezing is equally correlated with both Contextual Fear and Tone Fear. If Tone Baseline freezing were to reflect solely the animal's memory of the training context, it would only correlate with Contextual Fear. However, as this measure also correlates with Tone Fear, it must be concluded that it does not purely reflect contextual generalization. Moreover, locomotor activity during the Tone Baseline is negatively correlated with both Contextual and Tone Fear. In contrast, Activity during the Training Baseline was unrelated to any other measure, including activity during Tone Baseline. Overall, these findings suggest that subtracting Tone Baseline freezing from tone freezing, as is often performed, is problematic. Jacobs et al. [18], by manipulating extinction and training to the two contexts, reached the same conclusion, and found that the relationship between Tone Baseline and Tone Fear is not strictly linear. They suggest, and we concur, that the greatest effort must be made to eliminate Tone Baseline freezing. We further suggest it may be possible to solve this problem in mice by further enhancing the differences between the two contexts. For example, one might not use a Skinner-type chamber at all, such as modified home cage, for the alternate context (see, for e.g. [6]) or, simplifying the design of Jacobs et al. [18], extinguish only the alternate context prior to tone testing.

5. Conclusions

Overall, these findings help the interpretation of Pavlovian fear conditioning data being produced today in increasing numbers. The Post-Shock period of the training day is a good indication of short-term Contextual Fear memory, because it correlates strongly only with Contextual Fear measured later. Fear exhibited on the tone test day is more difficult to interpret, with generalization from the Context Test accounting for only some, but not all of the fear exhibited during the baseline period. These data indicate why it is important to standardize the Pavlovian conditioned freezing procedure as it develops into a standard assay of memory in various settings [22]. Despite the robustness of the paradigm, the data suggest some further improvement could occur in the area of discrete cue testing [18].

Acknowledgements

We thank Angela Smith and Melissa Guaderrama for technical assistance, and Jennifer Sage for thoughtful comments on this manuscript. This research was supported by a Hellman Fellowship (SGA). SCW was supported by an NIH NRSA (DA026259) and NSF Graduate Research Fellowship.

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