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Temporal Behavior in Auditory Fear Conditioning: Stimulus Property Matters

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The accuracy of time judgments depends upon many factors, including the sensory properties of the to-be-timed stimulus. In auditory Pavlovian fear conditioning, an initially neutral tone (conditioned stimulus, CS) predicts the arrival of an aversive event (unconditioned stimulus, US) at a fixed time interval. The temporal relation between the CS and US events is encoded, leading to the development of a temporal pattern of responding. Little attention has been paid to the potential impact of the characteristics of the tone-CS on the development of this temporal pattern. Here we compared the acquisition of the temporal pattern of conditioned responses of rats to different tone-CS frequencies. Rats were first conditioned to lever press for food. Then, while lever pressing for food, they were presented with 60-s tones of two widely separated frequencies, 1 kHz or 11 kHz, each paired with a foot-shock given 30 s after tone onset. This fear conditioning led to the appearance of CS-induced conditioned suppression of lever pressing, with no observed difference in conditioning to the shock. On probe trials, the tone duration was 60 s, and the reinforcer was omitted. With training, a pattern of suppression evolved during the probe trials, showing a maximum of suppression near the programmed time of the shock-US. However the 11-kHz tone-CS yielded better temporal control than did the 1-kHz tone. A second experiment investigated rats' abilities to discriminate between two times of shock arrival (10 s or 30 s) predicted by the different tone frequencies (1 kHz or 11 kHz). In this experiment, rats showed poorer discriminative timing performance when the lower frequency (1 kHz) was associated with the longer duration (30 s). Our results suggest a strong impact of the CS sensory properties on the expression of temporal learning within the context of auditory fear conditioning in rats.

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The ability to estimate, memorize, and compare durations of time intervals is critical for the interpretation of present events and anticipation of future ones. Yet, no sensory organ specifically dedicated to the perception of time has been identified. The time we measure and memorize corresponds to intervals between events we perceive through our physical senses (Wittmann, 2009). These events can be as diverse as a tone, a light flash, or tactile stimulation (Pavlov, 1927; Wearden, Edwards, Fakhri, & Percival, 1998). The quality of the to-be-timed stimulus, in turn, seems to influence the temporal performance controlled by it. For example, studies on humans have shown that auditory stimuli can be timed with better precision than visual ones (Stauffer, Haldemann, Troche, & Rammsayer, 2012; Wearden et al., 1998). Moreover, it has also been suggested that within a single sense, characteristics of the stimuli can impact time perception. For example, in the auditory modality, previous studies have demonstrated that whether the to-be-timed interval is filled or empty affects temporal perception (Grondin, 1993; Rammsayer & Lima, 1991; Santi, Miki, Hornyak, & Eidse, 2006). In acoustically marked empty intervals, only the onset and the offset of the interval are marked by clicks, whereas in filled intervals, a tone or noise burst is presented continuously throughout the interval. It has been found that filled intervals are discriminated more accurately than empty intervals (MacInnis, 2007; Santi et al., 2007). Interestingly, in humans, temporal perception for filled or empty intervals of short durations is also affected by pitch, an effect that might disappear in the supra-second range (Brigner, 1988; Cohen, Hansel, & Sylvester, 1954; Lake, LaBar, & Meck, 2014). Whether pitch affects temporal behavior in animals is not known.

In animals, associative learning protocols have been used extensively to investigate timing behavior. A commonly used paradigm to investigate associative learning in animals is Pavlovian conditioning in which a conditioned stimulus (CS) is paired with an unconditioned stimulus (US) (Pavlov, 1927). Some theories of associative learning imply that time plays a fundamental role in this acquisition process. For instance, knowledge about specific delays to US is learned very rapidly from the start of training (Arcediano, Escobar, & Miller, 2003; Boulanger Bertolus, Hegoburu, Ahers, Londen, Rousselot, Szyba, et al. 2014; Davis, Schlesinger, & Sorenson, 1989; Drew, Zupan, Cooke, Couvillon, & Balsam, 2005; Dìaz-Mataix, Ruiz Martinez, Schafe, LeDoux, & Doyère, 2013; Shionoya, Hegoburu, Brown, Sullivan, Doyère, & Mouly, 2013). Some authors have suggested that the learning of specific duration may be a necessary precursor for associative learning to occur, thus placing the appreciation of event durations at the core of learning processes (Balsam & Gallistel, 2009; Balsam, Drew, & Gallistel, 2010). In animals, this very early learning of the CS-US interval is critical for adaptive behavior as it leads to the development of appropriate anticipatory responding.

Interestingly, it has been shown that, in Pavlovian conditioning, the sensory characteristics of the CS can modulate conditioned responding, and, therefore, possibly the CS-US association learning. For example, it has been acknowledged in the literature that the intensity of the stimulus can influence the speed of acquisition and the level of the conditioned response (e.g., Beck, 1963; Zielinski & Walasek, 1977). Moreover, it has been shown that the frequency of an auditory stimulus can impact associative learning or its expression (Andrews Freeman, Carter, & Stanton 1995; Bang, Allen, Jones, Boguszewski, & Brown, 2008). In these latter studies, two tone frequencies (a low frequency and a high frequency, 2.8 *vs.* 9 kHz; and 4 *vs.* 19 kHz, respectively) were presented to rats, one being reinforced by an aversive electric shock (peri-ocular shock or foot-shock), the other tone being unreinforced. The authors described an asymmetry in performance: the frequencies were better discriminated when the high frequency was reinforced and the low one was not than in the reverse condition. As the learning of the CS-US association may be related to the learning of the CS-US time interval, one might ask whether temporal learning could also be affected by CS properties.

To our knowledge, no study has investigated whether characteristics of a tone CS, such as its frequency, may have an impact on the acquisition of the temporal pattern of behavioral responses in animals. The present study was thus exploratory, and sought to assess to what extent the frequency of a tone CS could influence the ability of rats to learn and discriminate CS-US time intervals in a Pavlovian conditioned suppression paradigm (Estes & Skinner, 1941). We chose two very different tone frequencies: a low frequency (1 kHz) often used in neurophysiological studies of fear conditioning but close to the lower limit of the rat's auditory spectrum, and another frequency (11 kHz) within the middle range of the rat's auditory spectrum (Kelly & Masterton, 1977; Zheng, Ito, Minami, Horikawa, & Murata, 1992). For both frequencies we used an intensity (80 dB) well above the animal's auditory detection threshold. We investigated the impact of these tone CS frequencies on the learning of a single duration (Experiment 1), and on the discrimination between two durations (Experiment 2). For this purpose, we analyzed the progressive development of a temporal pattern of behavioral suppression elicited by the CS by introducing non-reinforced probe trials as in peak interval timing tasks (Catania, 1970; Roberts, 1981). Our data highlight the fact that, although often overlooked in behavioral experimental designs, stimulus properties other than its sensory modality may also have a significant impact on the development of temporal control of behavior.

Method

Subjects

Twenty-three male Sprague-Dawley rats (Charles River, France) were used for this experiment. They weighed 300 g on average when they arrived at the laboratory, and were housed in pairs in Plexiglas cages (355 x 235 x 191 mm) with *ad libitum* access to water and food, under a 12/12 h circadian cycle (7:30 am light on). After 1 week of adaptation, daily food rations were progressively reduced before the start of training, and the rats were subsequently maintained at 85% of normal free-feeding weight. The experiment was run during the light phase of the cycle. Animals were weighed before each experimental session, and fed immediately after each session. All experiments were carried out in accordance with the recommendations of the EEC (86/609/EEC) and the French National Committee (87/848) for the care and use of laboratory animals.

Apparatus

Seven operant chambers (Rat Test Cage, Coulbourn Instruments, Harvard Apparatus, USA, 30 x 25 x 30 cm, see also Figure 1A) were used for behavioral training. They were equipped with a grid floor (16 bars with a diameter of 0.47 cm, 0.95 cm apart) connected to a constant current scrambler for foot-shock delivery. A food magazine (4 x 3 x 3 cm) connected to a food pellet dispenser (45 mg, Rodent Grain-Based Diet, Bioserve, France) was located on the left wall of the cage. A 4-cm retractable lever was placed on the right side of the food magazine, 2.5 cm above the grid floor. A white light was located 10 cm above the magazine. An audio speaker delivering 80 dB tones was located adjacent to the light. Each chamber was lit during conditioning sessions with a red houselight. Bedding was placed under the grid floor; after each session it was replaced and the grid was cleaned. Each operant chamber was placed in a soundproof chamber equipped with an exhaust fan providing a 65 dB background noise inside the chamber. All programs and behavior recordings were controlled by Graphic State software (Coulbourn Instruments, Harvard Apparatus, USA).

Procedure

We used a conditioned suppression paradigm (Estes & Skinner, 1941) in order to assess the animal's temporal expectation of the US arrival (see Figure 1C for the phases of conditioning). The rats were first trained to lever press for food in an *operant conditioning* task until reliable lever pressing was observed. Then, tone *fear conditioning* (tone CS–shock-US pairing) was implemented in the same chamber, leading to a suppression of lever pressing during the CS. Finally, the temporal behavior of the rats was studied by introducing probe trials in which the CS was presented without shock. The temporal pattern of response suppression observed during these probe trials presented an inverted U-shaped curve with a maximum (peak) of conditioned suppression at about the time of US arrival. These sessions will be referred to as *peak interval* sessions in the present study.

Operant lever press training. Lever press training consisted of two phases. The pre-training phase started with a magazine training session in which 30 pellets were delivered with a mean inter-pellet interval of 60 s (range 40 to 80 s), with the lever retracted. The next day the animals were trained to press the lever under continuous reinforcement until 50 lever presses were made. This was followed by six sessions during which the reinforcer was delivered in response to lever presses on a variable interval 30.5s schedule (VI, intervals ranging from 1 to 60 s with a uniform distribution). At the end of VI training, the lever pressing rate was stable over the session for all the rats.

Fear conditioning (10 sessions). Rats were then assigned to four groups matched according to their mean lever pressing rates (two groups for Experiment 1 and two others for Experiment 2). A fear conditioning protocol was implemented, in which a 60-s tone CS was paired with a shock-US while the animals were lever pressing for food under the VI schedule. The US consisted of a white light flash (1 s) co-terminating with a mild foot-shock (0.5 s). The intensity of the US was initially set at 0.3 mA (except for two rats for which the shock was lowered at 0.2 mA for one session). To ensure a stable and equivalent average suppression ratio between rats (see Method, Behavioral Analysis), the foot-shock intensity was raised to 0.4 mA for all rats from the end of the third week of Peak Interval, and then individually adjusted to 0.5 mA from the beginning of the fifth week of Peak Interval (one animal in the Simple 11kHz group, two in the 30s@1kHz Discrimination group and three in the 30s@11kHz Discrimination group). The mean level of shock intensity remained equivalent between experimental groups after the individual adjustment, $F(3,19) = 1.62$, $p = 0.22$.

In Experiment 1 (Figure 1B), the CS was a 60-s continuous tone and the CS-US interval was 30 s, with either a 1-kHz or an 11-kHz tone CS (Simple conditioning Groups 1 kHz, $n = 6$, and 11 kHz, $n = 6$).

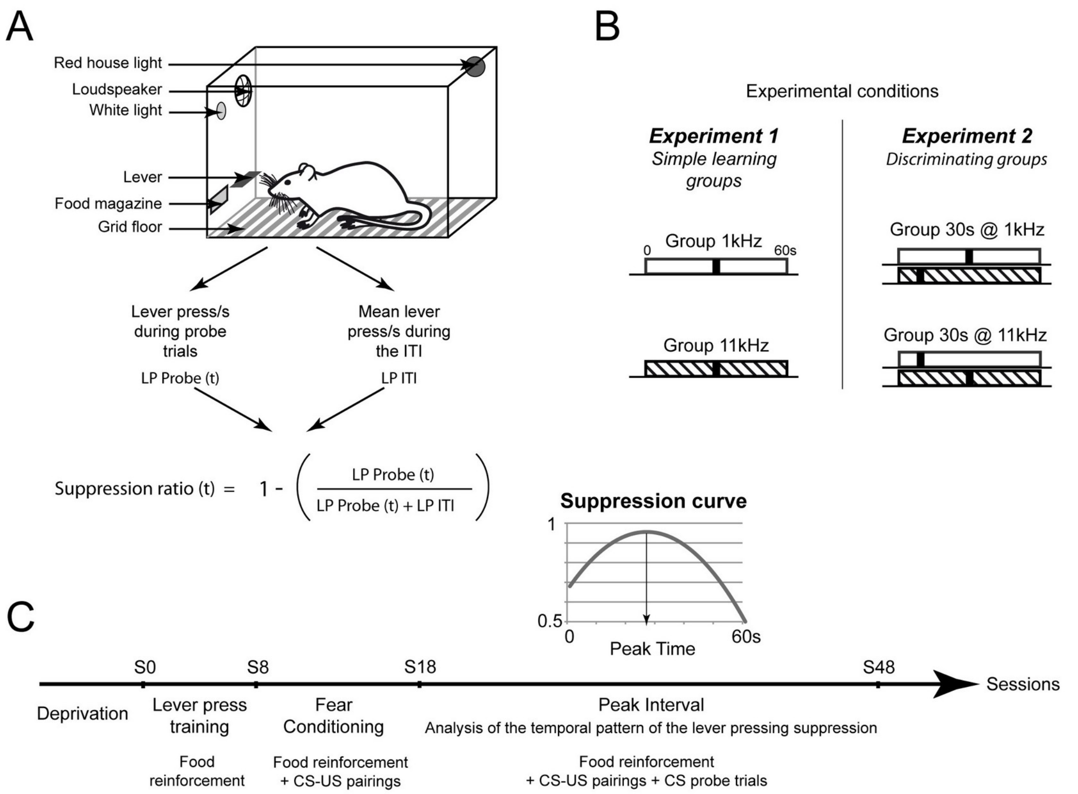

Figure 1. Experimental apparatus and paradigm. A. Schematic view of the experimental set up. Lever presses are monitored and used to calculate the suppression ratio at each time bin (t) of the probe trials. B. Training conditions for the two groups in the two experiments. The rectangles represent the 60-s tone CS with a frequency of either 1 kHz (empty) or 11 kHz (hatched). The time of the US arrival is represented by the black vertical mark and can be at either 10 s or 30 s after CS onset. C. Outline of the experimental phases. The insert represents a theoretical suppression curve during a probe trial, with the position of the peak time.

In Experiment 2 (Figure 1B), a discrimination procedure was employed in which two CS-US intervals (10 s or 30 s) were each associated with a given tone frequency (1 or 11 kHz), with duration and frequency balanced across groups. The CS was a 60-s continuous tone of either 1 kHz or 11 kHz, and the shock-US was delivered either 10 s or 30 s after tone onset. This arrangement yielded a difference in CS-US interval, with total CS duration held constant. For Group 30s@11kHz (n = 6), the 11-kHz tone signaled US arrival at 30 s while the 1-kHz tone signaled US arrival at 10 s. The reverse arrangement was applied for Group 30s@1kHz (n = 5).

Rats were first given five sessions with 8 presentations of each CS-US type (i.e., 8 trials in Experiment 1 and 16 trials in Experiment 2), with an intertrial interval (ITI) of 120 s on average (60 s, 90 s, 120 s, 150 s, or 180 s). In the next five sessions, in order to increase the number of exposures to the intervals to be learned without increasing the number of foot-shocks delivered, CS-Light trials, in which no shock and only the light flash was delivered, were introduced. Four CS-Light trials for each CS-US type trial (i.e. 4 trials in Experiment 1 and 8 trials in Experiment 2) were added to the previous protocol. CS-Light and CS-US trials were presented in quasi-random order (with a maximum of 2 CS-Light and 4 CS-US consecutive trials).

Peak interval sessions: Introduction of the probe trials (30 sessions). Four probe trials for each CS type were added in quasi-random order to the previous protocol (maximum of 2 successive probe trials). In those trials, the tone CS was presented alone to permit the assessment of the temporal pattern of behavior in the absence of shock delivery. Rats were given a total of 30 Peak Interval (PI) sessions, analyzed as six weekly blocks of five sessions.

Behavioral Analysis

In order to characterize the temporal pattern of conditioned suppression on probe trials, the following behavioral measures were recorded for each rat on each session: the total number of lever presses during the ITI, and the time of occurrence of each lever press during each probe trial (Figure 1A). The mean lever press rate (responses per second) during the ITI served as a baseline measure. The second measure was the number of lever presses in each second (bin), for each probe trial type. The response rate for each bin was used to calculate a suppression ratio for each second of a trial with the formula indicated in Figure 1A. According to this formula, suppression ratio ranges from 0 (maximum facilitation) to 0.5 (no facilitation or suppression) to 1 (maximum suppression). The suppression ratio was then averaged over 5 sessions (one week) for each rat in each experimental group.

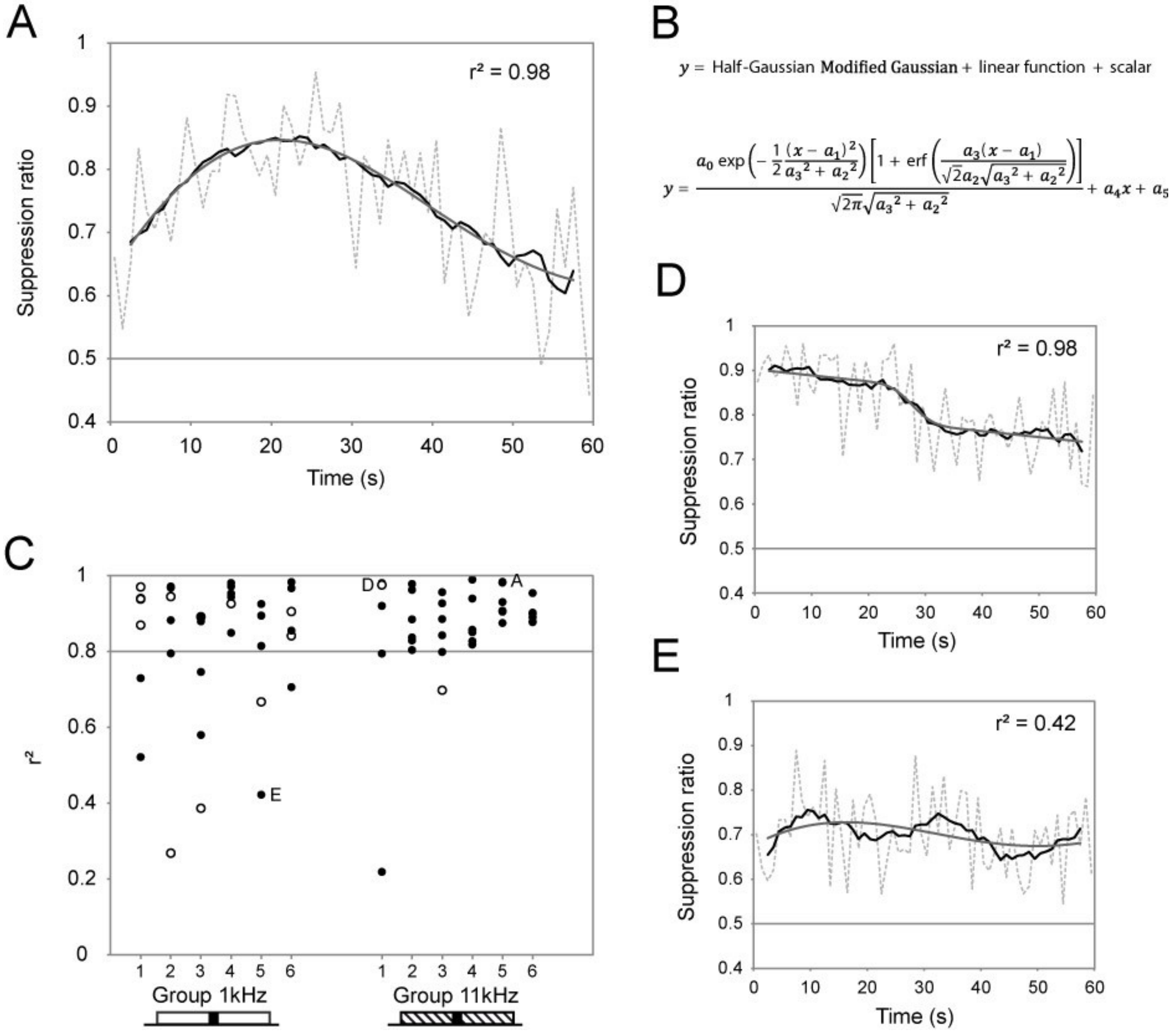

Figure 2. Method used to determine the peak time of the suppression curves of each rat each week. A. Raw curves of the measured suppression during probe trials (dotted line) are smoothed (black line) and fitted (grey line) using PeakFit software. The r² is noted, and the abscissa value corresponding to the peak of the fitted curve is taken as the peak time. The horizontal line at 0.5 represents no suppression. B. Equation used to fit the suppression

curves (formula of the half-Gaussian modified Gaussian: PeakFit User's Manual, p.7-31). C. r^2 obtained for all rats over the 6 weeks for the two Simple groups. Filled dots represent fits exhibiting a peak, empty ones those that do not. Letters correspond to the curves selected as examples in panels A, D and E. The criterion to consider a curve fitted was set at $r^2 = 0.8$. D, E. Examples of curves rejected because of the absence of a peak (D) or a $r^2 < 0.8$ (E).

Data Analysis

Experiment 1. The suppression ratio was normalized for each rat by dividing the value at each bin by the mean value over the 60 bins, and was compared between groups using a mixed ANOVA, with tone frequency as a Group factor, and Bin and Week as repeated factors.

To analyze the speed at which the two groups reached their final performance level, a superposition index was calculated for each rat between each PI week and the final PI week (week 6), both normalized to their mean. This superposition index (η^2, min = 0, max = 1) represents the proportion of variance accounted for by the mean of the data points from a suppression curve of a given week and those from the curve of the final week (week 6, Brown, Hemmes, & Cabeza de Vaca, 1992). This measure has the advantage that it does not presuppose of the shape of the final suppression curve and, therefore, takes into account the fact that the final curve of each subject is unique (Gallistel & Gibbon, 2000). It yielded a learning curve for the group mean superposition index.

In order to objectively determine the peak time, corresponding to the temporal locus of maximum suppression (see Figure 1C), we fitted the non-normalized suppression curve of each week for each rat using a fitting formula that allowed us to accommodate as many curves as possible. The fitting was achieved by smoothing the suppression curves using the mean of a sliding window of 11 successive data points, and fitting the smoothed curves using PeakFit (Systat Software Inc., USA, Figure 2A), which employs the Marquardt-Levenberg algorithm, a nonlinear curve fitting procedure. In our case, the shape of a suppression curve was asymmetrical, and was better fitted with a half-Gaussian modified Gaussian (GMG) curve to which a linear function and a constant were added (Figure 2B). This function allowed the fitting of 58 curves out of 72 (Figure 2C). Only the curves with a coefficient of fit (r^2) greater than 0.8 and showing a maximum later than 3 s (thus excluding curves showing a decreasing slope) were selected for the peak time estimation (examples of rejected curves Figure 2D, 2E). Therefore, the peak times for each week were not obtained on the same pool of animals and statistical analyses were not performed on these data.

Experiment 2. The suppression ratio was normalized by its average value over the 60 bins, and analyzed with a mixed ANOVA with experimental group as a Group factor, and Bin, Week and Duration (the duration of the CS-US interval, either 10 s or 30 s) as repeated-measures factors. The non-normalized suppression curves of the last week of PI training (week 6) were also fitted using the procedure described for Experiment 1. The rats for which a peak time could be determined for both the 10-s and the 30-s durations were selected (3 rats for Group 30s@1kHz and 4 rats for Group 30s@11kHz). For each group, the difference between peak times for the 10-s and the 30-s trials of those rats were then compared using a paired Student's *t*-test. The alpha level was 0.05 for all analyses in both experiments.

Results

Experiment 1

The acquisition of the CS-US association does not differ depending on the tone-CS frequency. The influence of the frequency of the tone CS on the acquisition of the suppressed responding was investigated by comparing the suppression based on the average response rate over the first 30 s of tone CS presentation during the first two sessions of fear conditioning. Doing so permitted the investigation of the unconditioned response of the rats to the tone-CS, and response levels early in training.

One rat from Group 11kHz did not press the lever during the first conditioning session but did press normally during the following sessions. It was thus excluded from the session-by-session analysis, but retained for the week-by-week analysis, with suppression for week 1 being averaged over only four sessions for this rat.

Both groups (1-kHz and 11-kHz tone-CS) exhibited suppression during the first presentation of the tone prior to any shock delivery (Figure 3A, one-sample *t*-test comparison with the no-suppression level, i.e. ratio of 0.5: Group 1kHz: $t(5) = 3.62$, $p = 0.02$; Group 11kHz: $t(4) = 3.42$, $p = 0.03$). This unconditioned response was likely due to an orienting response evoked by the novel stimulus. However, this level of unconditioned suppression did not differ significantly between the two groups (two-sample *t*-test: $t < 1$). Therefore, the two tones triggered unconditioned responses that could not be differentiated in suppression ratio levels. Moreover, there was no evidence that the development of conditioned suppression responses with repeated pairings between the tone-CS and the shock-US differed between groups. This was shown by a significant increase in the amount of suppression between the first two conditioning sessions (Figure 3B, $F(1,10) = 20.32$, $p = 0.001$), with no difference between groups and no Group x Session interaction (*F*s < 1). In addition, rats in both groups exhibited no difference in level of suppression ($F < 1$), with an average suppression ratio (± SEM) of 0.74 ± 0.02 during the first two weeks of fear conditioning under a fixed interval training schedule. Thus, the data suggest non-differential acquisition of the conditioned suppression, independent of the tone-CS frequency.

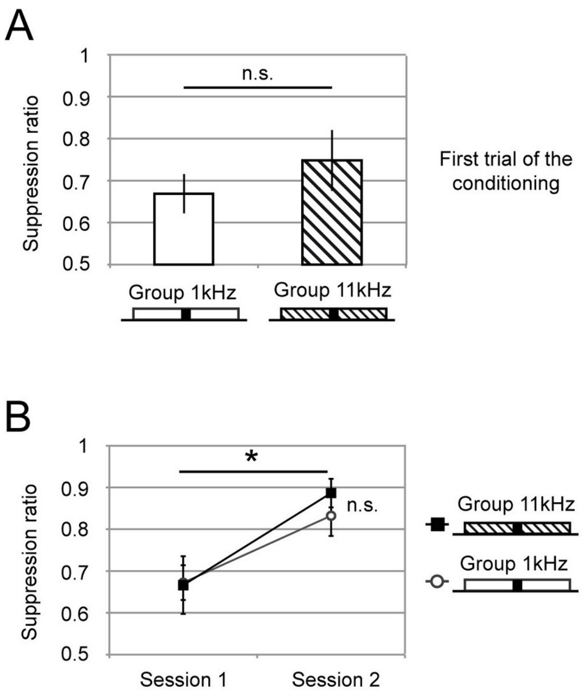

Figure 3. Suppression level at the beginning of conditioning. A. The suppression ratio was calculated for each rat and averaged (mean ± SEM) for each group during the initial 30 s of the tone-CS (before the US) for the first conditioning trial, showing the unconditioned response to the CS. B. Evolution of the group mean (± SEM) suppression ratio during the first 30 s of CS presentation for the first two conditioning sessions for each group. $*p < 0.05$.

The dynamics of temporal behavior depends on the frequency of the tone-CS. With repetition of training, a temporal behavior pattern progressively emerged, as shown by an inverted U-shaped suppression curve obtained during probe trials, allowing the determination of a peak time (Figure 4). In both groups, the suppression ratio curve evolved over the course of training, the peak emerging progressively. However, while a peak was observed as early as the first week of PI training in Group 11kHz (Figure 4B), it was only evident from week 3 or 4 in Group 1kHz (Figure 4A). The 2 (Group) x 60 (Bin) x 6 (Week) mixed ANOVA confirmed that the pattern of suppression evolved over training (Bin x Week: $F(295, 2950) = 1.48$, $p < 0.001$). Moreover, a Group x Bin x Week interaction just reached significance, $F(295, 2950) = 1.14$, $p = 0.05$, suggesting that the suppression curves evolved differentially in the two groups. This possible difference in the evolution of the groups' suppression curve was further supported by the fact that for week 1, the curves of the two groups were not statistically different (Group x Bin for week 1: $F(59, 590) = 1.08$, $p = 0.33$) whereas they differed significantly in week 6 (Group x Bin for week 6: $F(59, 590) = 1.97$, $p < 0.001$).

The acquisition of the final pattern of suppression is faster for Group 11kHz than for Group 1kHz. In order to quantify more precisely the evolution of the suppression pattern with training, we constructed a learning curve for each group by calculating a superposition index (η^2) between the curve of each week and the curve of the last week of training taken as a reference (Figure 5, see also Methods, Data analysis). This compares for each subject its weekly temporal pattern of suppression to its temporal pattern on week 6, taking into account that the shape of the temporal behavior of each subject is likely to be unique (Brown et al., 1992). This learning curve therefore tends to 1.0 if the temporal curve taken as a reference is close to the final temporal pattern. The observation of the learning curves for the two groups suggests that initial levels of superposition were comparable between the two groups, but the evolution of the temporal pattern toward a final pattern as measured by the superposition index was more rapid in Group 11kHz.

A two-way ANOVA (with factors Group and Week) revealed that groups differed significantly (Group: $F(1, 10) = 9.32$, $p = 0.01$), that there was an evolution of the superposition index over weeks (Week: $F(4, 40) = 11.76$, $p < 0.001$), and that the two learning curves did not have the same slope (Group x Week interaction: $F(4, 40) = 4.15$, $p = 0.007$). Indeed, in Group 11kHz, the superposition index evolved 2.5 times faster than in Group 1kHz (slope of the regression line of 0.046 for Group 11kHz and 0.017 for Group 1kHz, $r = 0.91$ and $r = 0.92$ for Group 1kHz and 11 kHz, respectively).

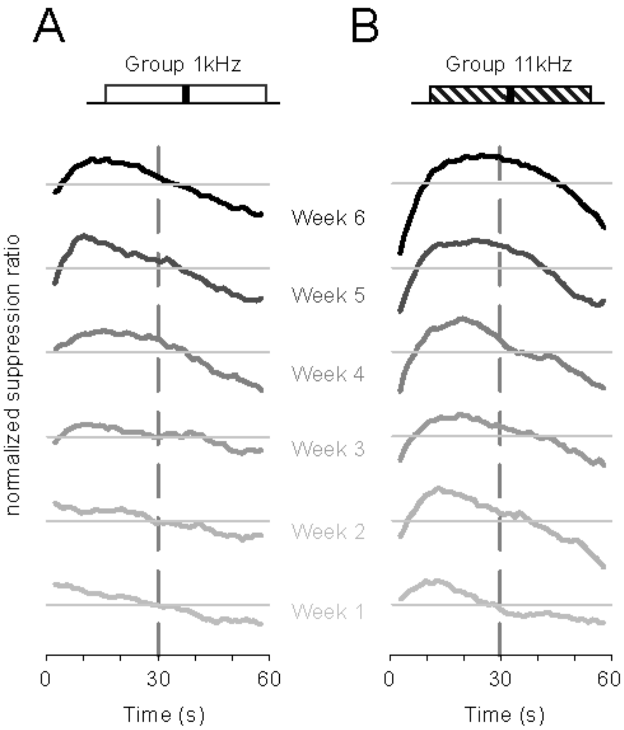

Figure 4. Mean smoothed normalized suppression ratio curves during the probe trials, across the 6 weeks of PI conditioning. The suppression ratio was calculated for Groups 1kHz (A) and 11kHz (B) for each rat (n = 6 per group) in each session, and averaged over 5 sessions (thus giving one curve per week). Each week curve has been normalized as explained in the methods, the thin horizontal line representing the value 1, the mean normalized suppression level.

Further post-hoc comparisons showed that whereas the superposition indexes were similar in the two groups for week 1, $t(10) = 1.51$, $p = 0.16$, they were significantly different at week 5, $t(10) = -3.81$, $p = 0.004$. Moreover, in Group 1kHz, the superposition index at week 5 was only 0.61, suggesting that there was still evolution of the curve's shape between week 5 and week 6.

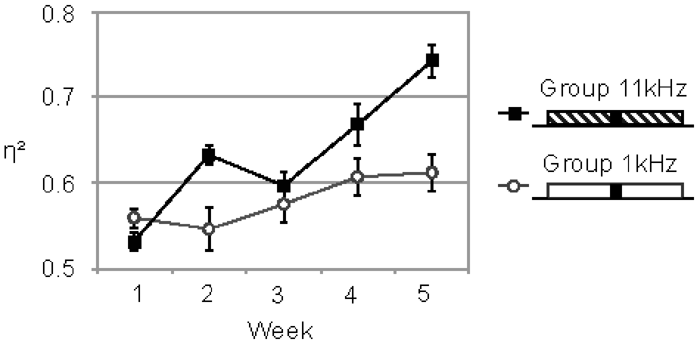

Figure 5. Learning curve (mean ± SEM). Evolution of the superposition index (η^2) in the two experimental groups (n = 6 per group).

Group 11kHz peak time of suppression evolves toward 30 s with repeated training. The peak time (time of maximum suppression) measured during probe trials may be interpreted as an estimation of the expected time of US arrival. Peak times were determined by fitting the curves of each week for each rat. However, over the course of training, a number of animals did not show a measurable pattern of temporal control, with suppression decreasing monotonically throughout the CS presentation, or following a pattern that could not be fitted by a peak (see exclusion criteria in Methods, Data Analysis and Figure 2). Overall, the proportion of rats for which data could be fitted each week was higher for Group 11kHz than for Group 1kHz (see Table 1), and the group mean number of total fitted curves for each rat over the 6 weeks was significantly higher in Group 11kHz than in Group 1kHz, $t(10) = 2.6$, $p = 0.03$. On the last week, where all animals of Group 11kHz showed good temporal control, with well-defined bell-shaped suppression curves (Figure 6B), only three out of the six animals of Group 1kHz could be defined as good performers (Figure 6A).

Table 1
Percentage of the rats that could be fitted each week

	Group 1kHz (*n* = 6)	Group 11kHz (*n* = 6)
Week 1	0%	50%
Week 2	50%	100%
Week 3	33%	66%
Week 4	83%	100%
Week 5	66%	100%
Week 6	50%	100%

The evolution of the peak times with training was therefore analyzed only for Group 11kHz for which a sufficient number (minimum 50%) of curves could be fitted each week (Table 1). Over training, the peak times tended to evolve toward 30 s, the programmed time of shock arrival (Figure 7A). However, as the peak times could not be determined for every animal in all 6 weeks because of the selection procedure, statistical analysis could not be performed on these data. Only two animals of Group 11kHz had curves that could be fitted for all 6 weeks. The progression of their peak times with training is shown in Figure 7B, and a statistical analysis confirmed a significant change in peak times across weeks, $F(5, 5) = 4.92$, $p = 0.05$.

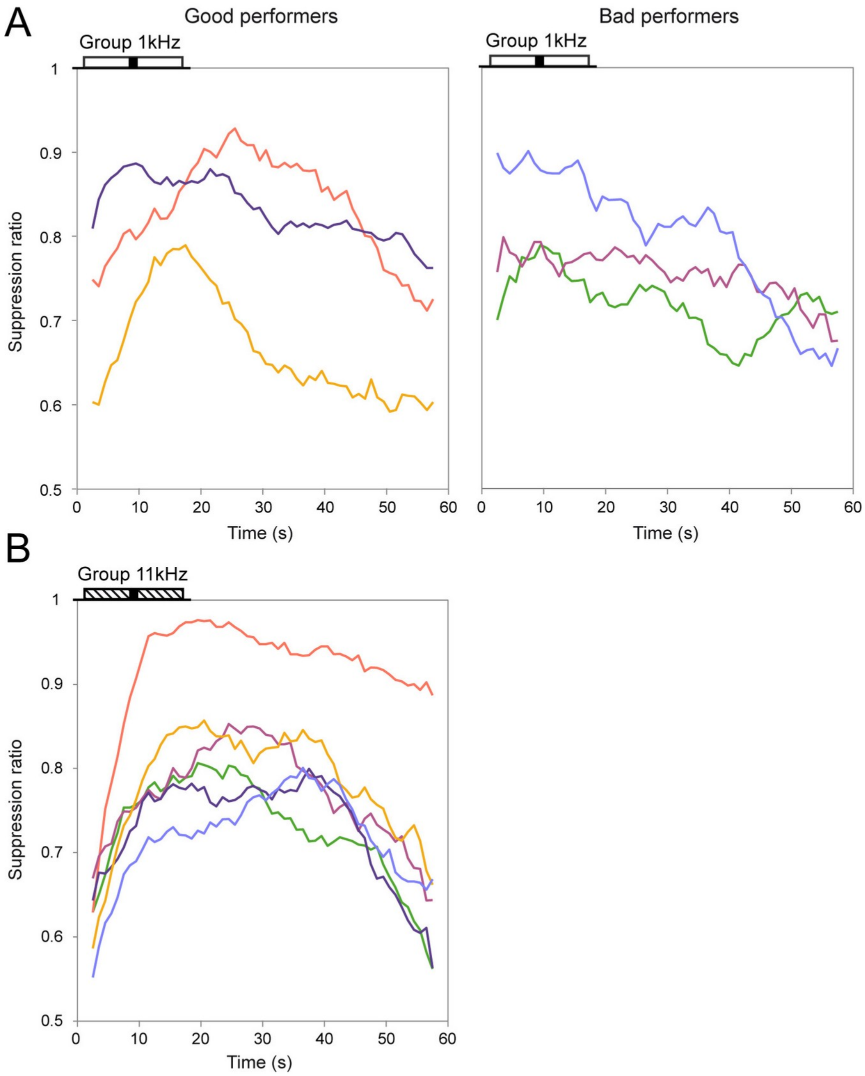

Figure 6. A, B. Smoothed suppression ratio curves averaged over probe trials for each rat of Group 1kHz (A) and Group 11kHz (B). Rats are identified as good performers (curves that can be fitted) and bad performers.

Experiment 2

The discrimination between two durations is influenced by assignment of tone frequency. In Experiment 2, the rats were trained to discriminate between 10-s and 30-s time intervals, with each CS-US interval signaled by either the 1-kHz or 11-kHz tone CS depending on the group (see Methods and Figure 1). The evolution of the suppression curves in response to each tone CS during probe trials over the 6 weeks is shown in Figure 8. It can be seen that, for both groups, a temporally shaped response pattern gradually emerged over weeks, resulting in a peak appearing progressively and evolving toward the time of the shock arrival associated with the tone frequency presented. However, the response pattern differed depending on the tone frequency presented for a given CS-US interval. When a shock delivered at 30 s was predicted by the 1-kHz tone (Figure 8A), the peak was less well defined than when it was predicted by the 11-kHz tone (Figure 8B). A mixed ANOVA with factors Group, Bin, Week and Duration confirmed that there was an overall temporal pattern of behavior (Bin effect: *F*(59, 531) = 30.06, $p < 0.001$) that evolved over weeks (Bin x Week: *F*(295, 2655) = 2.29, $p < 0.001$), with a discrimination of the two durations (Bin x Duration: *F*(59, 531) = 7.03, $p < 0.001$). It was also confirmed that the two groups were different in terms of evolution of the temporal behavior (Group x Bin x Week: *F*(295, 2655) = 1.35, $p < 0.001$) and in terms of discrimination of the two durations (Group x Bin x Duration: *F*(59, 531) = 1.34, $p = 0.05$).

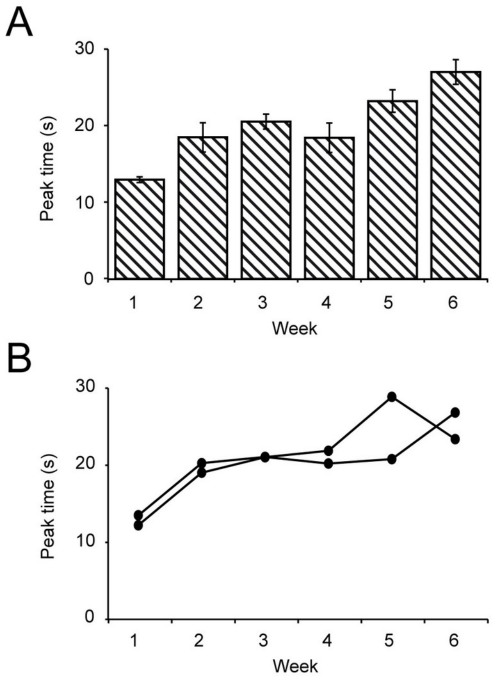

Figure 7. A. Peak times (mean ± SEM) for Group 11kHz ($n = 6$) across the 6 weeks of PI conditioning. B. Peak time is plotted for the two rats for which data could be fitted for every week of PI training.

The suppression curves on the last week (week 6) of PI conditioning were fitted to determine their peak (see Method, Data Analysis). Data for 3 of 5 rats and 4 of 6 rats were successfully fitted for both 10-s and 30-s trials in Groups 30s@1kHz and 30s@11kHz, respectively. The peak times determined in both groups are presented in Figure 9, and suggest that the discrimination between the two durations was better for Group 30s@11kHz than for Group 30s@1kHz. Within group comparison of these peak times by a paired Student's *t*-test confirmed significant differential temporal behavior for Group 30s@11kHz, $t(3) = 3.27$, $p = 0.05$, but not for Group 30s@1kHz ($t < 1$).

The scalar property is better approximated when the 30-s US time arrival is associated with the 11-kHz tone. The scalar property implies a directly proportional relationship between peak time and variability (Gibbon & Church, 1990; Gibbon, Church, & Meck, 1984). In other words, when comparing the temporal curves for two durations, a multiplicative transform (normalization of the time axis) should yield better superposition than no transform or an additive transform (translation of the untransformed time axis). In the discrimination paradigm, we calculated the superposition index η^2 between the group mean suppression curve for the 10-s interval and the group mean suppression curve for the 30-s interval, using either a multiplicative or an additive transform or no transform. For the multiplicative transform, the time axis for each function was normalized to a range of 1–20 bins by averaging the values of the 60-bins 30-s curves by 3-s bins. Then, the suppression ratio (y axis) of both 10-s and 30-s curves was normalized by expressing response rate relative to the mean rate. For the additive transform, the time axis remained untransformed but was translated by a constant (subtraction of 20 s for the 30-s function), and the y axis of the original suppression functions was normalized to mean ratio. When not transformed, the curves superposition was measured on bins 1 to 20, and on curves normalized on y axis to mean ratio, as for the other comparisons. Table 2 summarizes the calculated η^2 for each comparison for each frequency/duration combination. An example of the superposition resulting from these transformations is shown Figure 10. The results confirmed that in every case, the superposition was better following a multiplicative transformation, thus showing that the temporal function of the suppression curves followed the scalar property. Using this multiplicative transformation, it can be observed that the superposition index was higher when the 30-s intervals were associated with the 11-kHz tone CS rather than with the 1-kHz one. This result is consistent with superior temporal control of suppression for the 30s-11kHz combination.

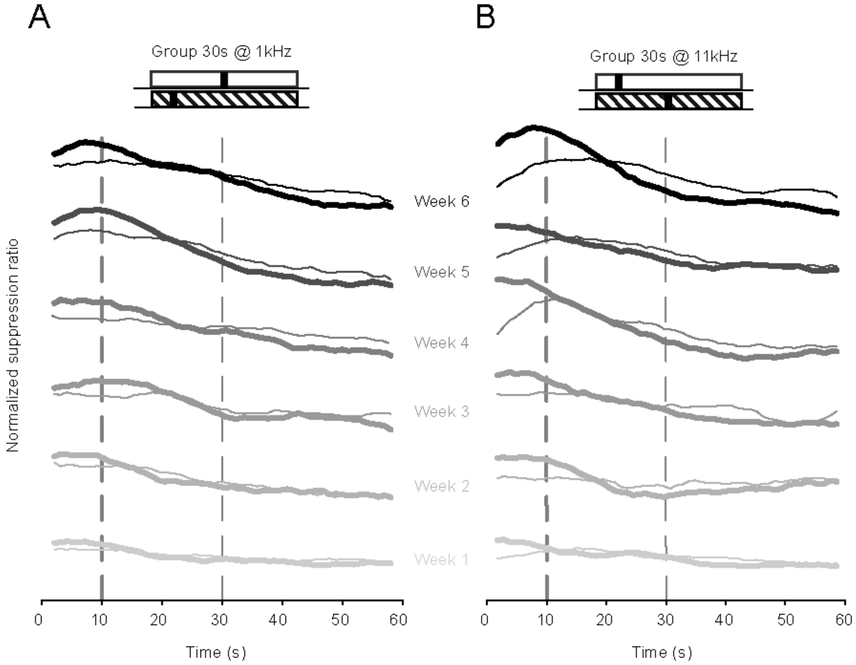

Figure 8. Mean smoothed normalized suppression ratio curves during the probe trials, across the 6 weeks of PI conditioning for the two groups during discriminative auditory fear conditioning. The suppression ratio was calculated and normalized for each week in Group 30s@1kHz (*n* = 5) and in Group 30s@11kHz (*n* = 6). Probe trials with the tone frequency predicting a shock arrival at 30 s are represented by a thin curve, those with the frequency predicting a shock arrival at 10 s by a bold curve. The corresponding times of US arrival are represented as vertical dotted lines.

Table 2

Superposition index η^2 *of the 10-s versus 30-s interval suppression curves in both discrimination groups, using either no transform, multiplicative (rescaling) or additive (sliding) transforms*

	30 s – 1 kHz			30 s – 11 kHz		
	None	Multiplicative	Additive	None	Multiplicative	Additive
10 s – 1 kHz	0.69	0.71	0.66	<0.73>	<0.79>	<0.59>
10 s – 11 kHz	<0.64>	<0.75>	<0.68>	0.60	0.79	0.58

Note. Numbers in brackets indicate within group comparisons, no bracket indicates within frequency comparison (i.e. between groups).

Discussion

The results indicate that the frequency of the tone used as a CS in an auditory Pavlovian fear conditioning can influence the temporal pattern of fear expression as assessed through conditioned suppression of lever pressing for food, even though it did not appear to modify the initial learning of the CS-US association as assessed through the absolute amplitude of suppression. Compared to animals trained with an 11-kHz tone, those trained with a 1-kHz tone showed a more variable and less accurate temporal pattern of conditioned

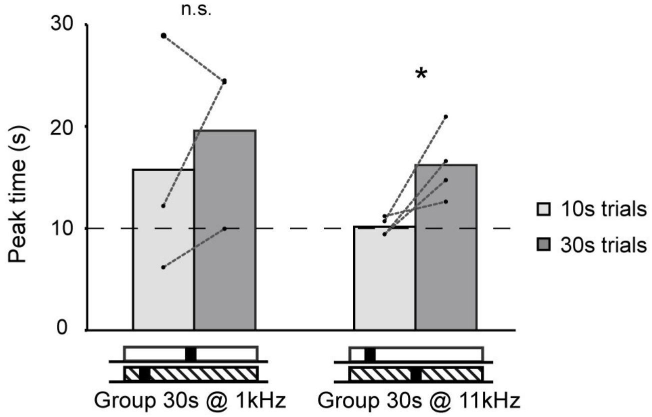

Figure 9. Peak times (mean ± SEM) for Group 30s@1kHz and Group 30s@11kHz. In each group, the peak times were determined by fitting the curves of each week. For 3 rats for Group 30s@1kHz and 4 rats for Group 30s@11kHz the peak time could be determined for both 10-s and 30-s trials (see Method, Data Analysis). The data for each rat in each group are represented as individual lines. n.s.: non-significant. * $p < 0.05$.

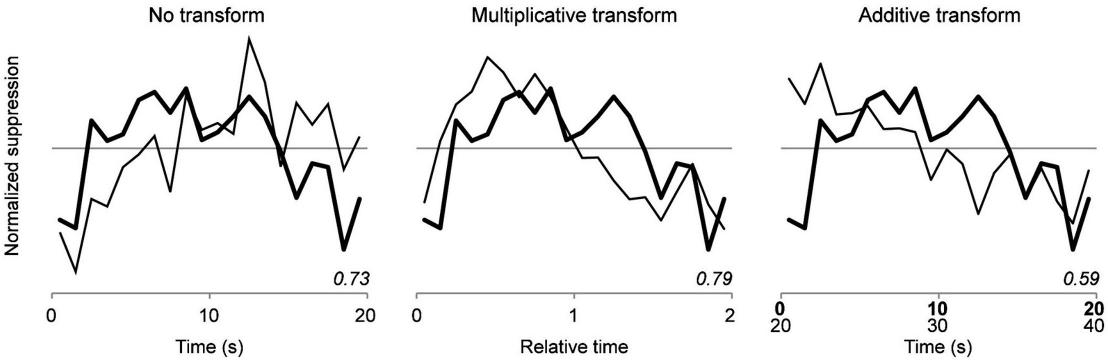

Figure 10. Superposition of the normalized suppression curves obtained for Group 30s@11kHz, in response to the CS signaling either 10 s (bold curve) or 30 s (thin curve) following either no transformation of the curves (left panel), a multiplicative transformation of the 30-s curve (middle panel), or an additive transformation of the 30-s curve (right panel). For the additive transform, the lower numbers on the x axis refer to the 30-s curve, while the upper numbers refer to the 10-s curve. Numbers in italic are the corresponding η^2.

response, and a slower development of this pattern with repeated training. We also showed that the frequencies of the tones can impact the expression of a discrimination between two durations, with poorer discriminative temporal control expressed when the low frequency was associated with the long duration.

In the Learning of a Single Duration (Experiment 1), the Frequency of the Tone-CS Matters

In the present study, the animals received 320 presentations of CS-US pairings during training over eight weeks. The data of Experiment 1 suggest that the difference in acquisition of temporal control between the low and the high tone frequencies might be due, at least in part, to a greater proportion of poor performers in the case of a 1-kHz tone. We do not know however whether the poor temporal control of behavior for animals trained with a 1-kHz tone reflects slower acquisition toward an equivalent terminal temporal pattern, which might have been reached with more training, or whether it is a consequence of some inherent characteristics of the low frequency in a fear conditioning paradigm. In any case, our results clearly demonstrate poorer temporal control of fear expression with a 1-kHz tone-CS, as compared to an 11-kHz tone-CS.

The possible reasons for such differential behavior can only be speculative at this point. It can be suggested that the asymmetry may be due to the fact that high frequencies are better perceived than low frequencies. Indeed, the behavioral detection threshold of an 11-kHz tone has been shown to be lower than that of a 1-kHz tone (2 dB and 23 dB, respectively; Kelly & Masterton, 1977). Zheng et al. (1992) confirmed these results by quantifying the response of single cochlear nerve fiber: they measured a response threshold at about 40 dB for a 1-kHz tone and 22 dB for an 11-kHz tone. Moreover, when considering the best frequency of responding of cochlear fibers, Zheng et al. reported that there are few fibers responding at 1 kHz, and that 11 kHz is among the frequencies to which the maximum number of fibers responds. As a consequence, the response in the auditory cortex will be smaller for 1-kHz than for 11-kHz tones.

Therefore, it could be argued that in the present study, although the tones were presented at intensities well above their detection thresholds, perceived loudness may have differed between the 1-kHz tone and the 11-kHz tone, thus resulting in different signal/noise ratios and acquisition performances.

However, in our experiment, rats exhibited statistically indistinguishable levels of unconditioned response to the two tone frequencies, as well as similar acquisition of conditioned suppression, whether the CS was associated with the low or the high frequency stimulus. This was presumably due to the fact that the intensity chosen (80 dB) was at least 15 dB above the background noise. Accordingly, previous studies have shown that both 1-kHz and 11-kHz 80-dB tones are perceived by rats (Borg, 1982; Kelly & Masterton, 1977; Zheng et al., 1992) and activate cells in the lateral amygdala, the critical neural structure involved in fear learning (Bordi & LeDoux, 1992; Bordi, LeDoux, Clugnet, & Pavlides, 1993; LeDoux, 2000). Thus, rats were able to hear the 1-kHz tone and to associate it with the shock-US. Although a difference in salience may exist between the two tones, this difference was not sufficient to elicit a detectable difference in the level of suppression. It remains possible that a lower signal to noise ratio may have rendered the timing of the CS-US interval more difficult.

In Duration Discrimination (Experiment 2), the Frequency-Duration Combination Matters

The results obtained in the present study show an asymmetry in discrimination, with poorer temporal discrimination in the group where the 1-kHz tone was paired with the long interval, *i.e.* Group 30s@1kHz.

Asymmetry in learning due to the frequency of a tone CS has only been described previously (Andrews et al., 1995; Bang et al., 2008) in a discriminative Pavlovian conditioning procedure in rats in which two different tone frequencies (2.8 *vs.* 9 kHz; and 4 *vs.* 19 kHz, respectively) signaled either a reinforced (electrical shock) or non-reinforced trial. The results of these studies consistently showed asymmetrical results, with a better discrimination of the two CSs observed when the higher frequency was associated with the reinforced stimulus.

In line with the above studies, it could be argued that, in our study, the strength of associative learning linking the tone with the shock was lower for the 1-kHz tone than for the 11-kHz tone. Several arguments allow us to assume this is unlikely, and that in the present study tone frequency modulated temporal learning rather than associative learning. Indeed, no significant differences were observed in the suppression ratios of the two groups during the initial FI learning phase (80 CS-US pairings), whereas a clear difference in the temporal pattern of the suppression ratios could be seen at week 6 of PI training (see Figure 4 and 6), as well as a difference in the evolution of the patterns of suppression (Figure 4). Such results might be explained by the fact that temporal control for learned long durations is less precise than that for short durations in absolute time, in accordance with scalar rule, and it is well known that the strength of the conditioned response is greater when the CS-US interval is short (Balsam et al., 2010; Delamater & Holland, 2008; Stein, Sidman, & Brady, 1958). The results of Experiment 1 showed that for a 30-s CS-US interval, the temporal pattern of conditioned responses for the 1-kHz tone was less precise than that for the 11-kHz tone. Therefore, pairing the lower frequency with the longer duration is the combination that is likely to yield greater noise or poorer precision in the response on the discrimination task, thus resulting in the poorer discrimination performance for Group 30s@1kHz compared to Group 30s@11kHz in Experiment 2. But the present results suggest that effects of frequency and duration were not simply additive, as temporal control of behavior appeared to be superior for both frequencies (hence both durations) in Group 30s@11kHz compared to Group 30s@1kHz. This result represents a more complex effect of tone frequency upon conditioned responding than has been reported heretofore, and is discussed further below.

The Peak Time for Suppression is Anticipatory and Migrates with Training Toward the US Arrival Time (Experiment 1)

In both experiments of the present study, we observed that rats tended to show an anticipatory response in that peak time was systematically earlier than the programmed time of reinforcement (Figure 7A*)*. A tentative explanation for this trend could be that the temporal pattern of the conditioned response observed in our experiments reflects a combination of two behaviors, with different time courses: one, an immediate suppression response, triggered by the tone onset and independent of the CS-US interval, the other response being specifically related to temporal processing and showing accurate temporal estimation of US arrival. The coexistence of multiple conditioned responses with different time courses has been reported in appetitive and aversive Pavlovian conditioning situations (Bermudez, Göbel, & Schultz, 2012; Holland, 1980; Miles, Davis, & Walker, 2011; Shionoya et al., 2013). These studies reported responses to CS onset that were independent of the time of reinforcer arrival. In particular, in the aversive situation, Miles et al. (2011) suggested different types – phasic and sustained - of conditioned fear, occurring at the beginning of the CS and later on, respectively. Moreover, in the context of conditioned lick suppression, Jozefowiez et al. (2011) demonstrated the existence of two components in the temporal pattern of the conditioned suppression response: a conditioned component due to the CS-US pairing, and an initial CS-onset-triggered component due to mere exposure to a shock-US. We therefore propose the following hypothesis which could explain the pattern of behavior observed in our study: the temporal pattern of suppression behavior is the result of the sum of two functions: a decreasing exponential reflecting the fearful, non-temporally based reaction to the onset of the CS, the characteristics of which may be sensory- and/or species-specific, and a Gaussian function resulting from the temporal conditioning and exhibiting a peak centered on the US arrival time. The resulting envelope is a scallop-shaped curve with an earlier peak compared to the peak of the Gaussian curve (Figure 11).

Such a model could also explain the progressive apparent drift in anticipatory responses with repeated training that has been previously reported (Balsam, Drew, & Yang, 2002; Delamater & Holland, 2008; Ellison, 1964) and that we have also observed here. Indeed, for Group 11kHz (Experiment 1), we observed that the peak time evolved across weeks drifting progressively toward the actual time of US arrival. A drift in peak time is *a priori* not consistent with current hypotheses suggesting that the association between events and the timing of these events are encoded simultaneously (Balsam &

Gallistel, 2009). However, in the model proposed above, the temporal behavior observed in our experiments reflects a combination of two response patterns that may evolve with training the following way: a decrease in magnitude of the initial response to the CS with training would reduce the weight of the decreasing exponential on the observed curve, while an increase in the amplitude of the stable peak of the Gaussian function with training would increase its weight on the curve. Either of these modifications, as well as their combination would result in an apparent drift of the peak time toward a more accurate adaptive value (Figure 11). This hypothesis suggests that (a) the displacement of the peak time toward the programmed time of the US as observed in the present study in Group 11kHz, and in other studies, would not be the representation of temporal estimation *per se,* but only of the complex conditioned response, and that (b) the CS-US interval duration may have been learned at the very beginning of fear conditioning, in agreement with other studies (Davis et al., 1989; Dìaz-Mataix et al., 2013; Drew et al., 2005; Boulanger Bertolus et al., 2014; Ohyama, Nores, Medina, Riusech, & Mauk, 2006; Shionoya et al., 2013). Further experiments should evaluate this hypothesis of combined processes underlying temporal behavior in the case of Pavlovian conditioning in the long duration range.

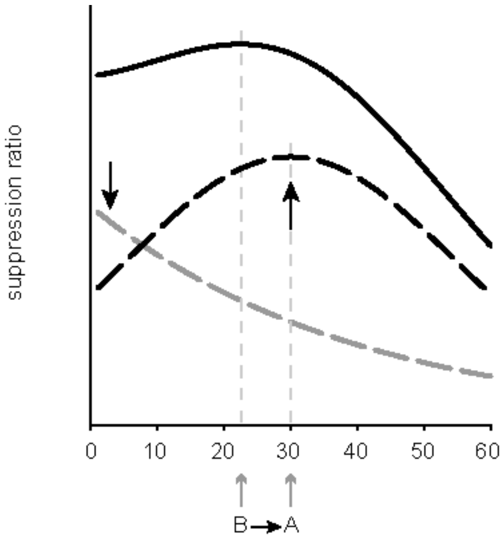

Figure 11. Explanatory model for the evolution of the peak time over training. The temporal behavior observed during the experiment (black curve) is the result of the sum of two curves: a decreasing exponential (hatched grey curve) reflecting the animal's reaction to the CS onset, and a Gaussian curve (hatched black curve) resulting from the temporal conditioning and exhibiting a peak centered on the accurately expected US arrival time (A). The combination of these two curves results in an apparently early peak time (B) compared to the US arrival time. The decrease of the Y-intercept and slope of the exponential function as well as the increase of the peak of the Gaussian curve (black arrows) as training proceeds lead to the progressive drifting of B toward A as observed in our results.

The Potential Role of Differential Affective Value of the Tones (Experiment 1&2)

It can be hypothesized that the tones used in the present study have some inherent characteristics that differ and contribute to the observed differences in behavior. That is, the low frequency 1-kHz tone may have a stronger affective value than the 11-kHz tone. This emotional value of the 1-kHz tone would be ethologically consistent with the fact that rats' predators tend to vocalize below 4 kHz (cats & humans: Qin, Wang, & Sato, 2008; canids: Tembrock, 1976; large birds: Fletcher, 2004). In the hypothetical model presented above, a stronger emotional value associated with the 1-kHz CS would increase the amplitude of response at tone onset, i.e., the Y-intercept, and/or decrease the decaying slope of the exponential function, and would thus promote a peak anticipating the US arrival time. This hypothesis could explain our observation that the 11kHz-10s/1kHz-30s combination yielded poorer discrimination of the durations than the reverse case. That is, in the 11kHz-10s/1kHz-30s case, the 1-kHz-tone signaled interval, with a stronger emotional value and thus a higher initial function level, would lead to an earlier peak responding compared to the programmed time of the shock, and thus the two resulting suppression curves would tend to be closer to each other (Figure 12A). In the reverse situation (1kHz-10s/11kHz-30s), the same reasoning leads to suppression curves that are more distinct (with the 1-kHz conditioned response showing an earlier peak), thus leading to a better apparent temporal discrimination, as observed in the present study (Figure 12B).

Conclusion

A hypothesis that is implicitly assumed in the timing literature is that stimuli within the same sensory domain, when used as CSs, have the same potential to signal delays to important events, and to exert temporal control of behavior based on those delays. The present study demonstrates that this principle of equipotentiality in classical conditioning may not hold at the level of observed response patterns. In effect, our study highlights the fact that temporal behavior may depend on characteristics of the stimulus apart from sensory domain, parameters that are often not fully controlled in behavioral experiments and that may be of importance for comparison purpose in the timing literature. Further, we suggest that the responses measured in temporal studies using Pavlovian procedures may result from the combination of several response systems of which only one component may relate to prospective timing.

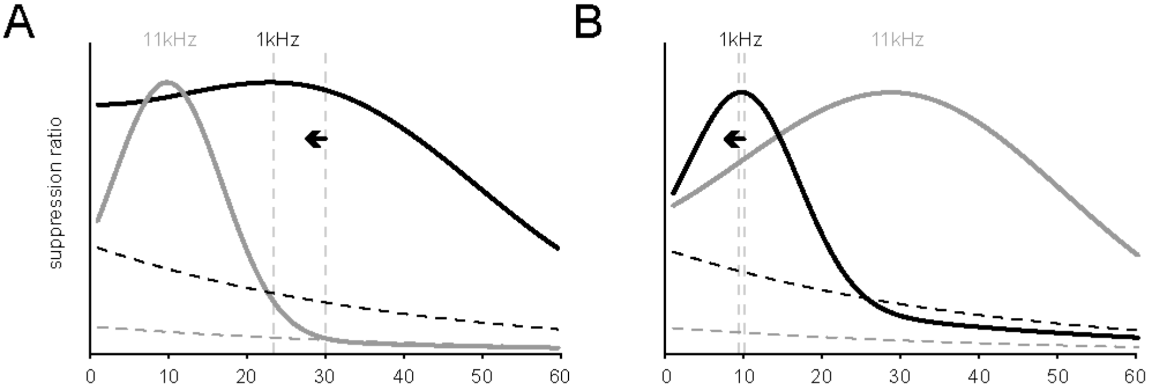

Figure 12. Hypothetical model for the asymmetry in discrimination. If the 1-kHz tone has a higher emotional value, the non-temporal component (dashed curves) would contribute more strongly to the resulting composite curve (solid curves, see model Figure 11 and Methods, Behavioral analysis). Thus the response pattern is apparently anticipatory, indicated as a

shift (black arrow) of the peak of the initial 1-kHz 30-s curve (black curve) toward the left. This process would increase the proximity of the two observed functions in the case of the 11kHz-10s/1kHz-30s combination (A), but increase the difference between the two curves in the alternative combination (B).

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