

Towards chronic contextual conditioning in rats: The effects of different numbers of unpaired tone-shock presentations on freezing time and startle

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Contextual conditioning in rats is typically quantified using freezing time or startle amplitude. In this study, we combined both anxiety measures in one procedure and systematically examined the effect of training with 0, 5, 10 or 15 unpaired tone-shock (0.8 mA – 250 ms) presentations on the expression of contextual conditioning in a chronic protocol with two training and testing days. Such a chronic procedure may be valuable as a chronic anxiety model. Training with 5, 10 or 15 explicitly unpaired shocks resulted in significant contextual freezing. There was no significant increase in freezing time from post-test 1 to post-test 2 and there were no differences between the three shocked groups, implying that the different numbers of shocks did not affect the degree of contextual freezing, probably because the ceiling freezing value had already been reached. Surprisingly, we observed no potentiated startle in the conditioned context. To summarize, our protocol produced consistent contextual freezing over two testing days.

Key words: contextual conditioning, freezing, startle amplitude, shocks, chronic protocol, rat

INTRODUCTION

In classical fear conditioning, a neutral stimulus (a cue, e.g. a tone) is paired with an aversive stimulus (usually footshock). The tone, through its relationship with the shock, acquires aversive properties and subsequently produces fear responses. Conditioned emotional responses are also elicited by placing an animal in a chamber in which an aversive event has previously been experienced. Here, the conditioned emotional responses are elicited not by a cue that was explicitly paired with the shock, but instead by the various contextual stimuli that were present in the chamber when the shock occurred and are still there when the animal is returned to the chamber (Phillips and LeDoux 1992, McNish et al. 1997). It has been suggested that contextual conditioning is enhanced by manipulations that increase the unpredictability of the shock: when presented alone or unpaired with a cue, they

produce greater contextual conditioning than shocks that are signaled through cue–shock pairing (Grillon and Davis 1997, Alvarez et al. 2008). In this study, we will focus on rats trained with explicitly unpaired tone-shock presentations, as this may form the optimal contrasting condition for a cued fear conditioning group trained with paired tone-shock presentations in future experiments.

Freezing time and startle amplitude are the most commonly used measures to quantify conditioned contextual anxiety (Grillon 2002). These responses to the context can be compared before (‘pre-test’) and after (‘post-test’) conditioning (Vansteenwegen et al. 2008). Freezing is defined as the total absence of movements of the body and whiskers with the exception of movements necessary for respiration (Fanselow 1982). After reintroduction into the conditioned context, the time the animals spend freezing is measured. Fear-potentiated startle refers to the increase of the amplitude of the startle reflex to a startle stimulus (e.g. a brief loud noise) elicited by the anticipation of an aversive stimulus in the conditioned context (Grillon 2002). Usually, only one of both measures is used, in our

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Received 28 March, accepted 05 September 2011

study however, freezing and startle were combined in one protocol.

In a previous experiment, we found that conditioning with ten 0.8 mA – 250 ms shocks (explicitly unpaired with tones) produced significant contextual conditioning, as measured with both freezing and startle (Luyten et al. 2011a). The goal of the present study was to further optimize this contextual conditioning protocol, i.e. maximize the increase of freezing time and startle amplitude in the conditioned context. We therefore systematically investigated the effect of the number of unpaired tone-shock presentations (0, 5, 10 or 15). Furthermore, we wanted to examine the effect of adding an extra training and testing day to create a more chronic protocol, which might be valuable with regard to the use of this conditioning procedure as a model of chronic anxiety (Luyten et al. 2011b).

METHODS

Subjects

Thirty-six male Wistar rats (250–280 g) were housed in groups of three with food and water *ad libitum* available. They were maintained on a 12-h light–dark cycle (lights on at 07:00 AM) with a room temperature of $\pm 21^\circ\text{C}$. The experimental protocol was approved by the animal ethics committee of the Katholieke Universiteit Leuven.

Equipment

During the experiment, the rats were placed into an acrylic cylindrical rat holder (7.6 cm inner diameter, 14.3 cm length) with a grid floor, which was firmly placed on a response platform by four thumb screws and located inside a ventilated sound-attenuating chamber (Med Associates, St. Albans, VT, USA). The grid floor consisted of nine 3-mm-diameter stainless steel bars spaced 9 mm apart, through which footshocks could be delivered (Med Associates). A red light bulb (3.8 W) was continuously on. The freezing behavior of the animals was recorded by a camera (DCR-SR55E Super NightShot Plus, Sony, Tokyo, Japan) positioned in front of the rat holder. Freezing was timed from the video recordings by a blinded observer. In addition, the startle reaction of the rats generated a pressure on the response platform and analogue signals were amplified, digitized, and processed by software (Startle reflex, version 5.95, Med

Associates) provided by the manufacturer of the equipment. The presentation and sequencing of the acoustic stimuli and footshocks were controlled by the same software. One of two loudspeakers, both located 7 cm behind the rat holder, was used to deliver a continuous white background noise (55 dB), the other speaker delivered the startle (white noise, 100 dB, 50 ms) and tone stimuli [4000 Hz, 75 dB, 10 s (5 ms rise/fall)]. The amplitude of the startle response was defined as the first peak accelerometer voltage that occurred during the first 400 ms after onset of the startle stimulus (or footshock), as measured on an arbitrary scale ranging from 0 to 2047. The startle platform and loudspeakers were calibrated before the experiment.

Behavioral procedure

In total, there were 7 experimentation days: habituation 1 and 2, pre-test, training 1, post-test 1, training 2 and post-test 2. Figure 1 shows the experimental design.

On the first two habituation days, the animals were placed in the chamber and after 5 min of acclimation they received a total of 30 startle stimuli at a fixed inter-trial interval (ITI) of 30 s. After these 30 startle stimuli, 3 tone trials were presented. These 3 tone-alone trials were added to reduce the novelty effect of the tones during the subsequent pre-test. The rats then remained undisturbed for 2–3 min. Each habituation session was 25 min in duration. These sessions were designed to produce some habituation and stabilization of the startle response, which has been proven useful to include before experimental manipulations (Zhao and Davis 2004).

Three days later, the pre-test was carried out to obtain baseline freezing and startle measurements. The animals were placed into the chamber, and freezing was timed during the 5 min of acclimation. Next, the rats were presented with 30 startle stimuli (noise bursts) at a 30 s ITI. Half of the startle stimuli were presented in the presence of a tone (15 tone-noise trials), the other half in the absence of a tone (15 noise-alone trials). On tone-noise trials, the startle and tone stimuli co-terminated. Both trial types were presented in a balanced, irregular order, with a maximum of 2 subsequent noise-alone or tone-noise trials. Afterwards, the rats remained undisturbed for 2.5 min. Each test session lasted 25 min. Based upon their mean startle amplitude across the 15 noise-alone trials during the pre-test, the rats were matched in 4 equivalent groups (0 SHOCKS, 5 SHOCKS, 10 SHOCKS and 15 SHOCKS groups).

Twenty-four hours later, the animals were conditioned to the context of the chamber during the first training session. The animals were placed in the cage and 5 min of acclimation later, each rat received explicitly unpaired presentations of 0.8-mA, 250-ms footshocks and tones (4 groups: 0, 5, 10 or 15 shocks and the same amount of tones) with a variable ITI of 45–65 s. First, a shock was administered, 45–65 s later followed by a tone, 45–65 s later followed by a shock, etc. Each animal was removed 2–4 min after the last tone and returned to its home cage. The session lasted approximately 16 min in the 5 SHOCKS group, 27 min in the 10 SHOCKS groups and 38 min in the 15 SHOCKS group. In the 0 SHOCKS group, animals were matched (based upon their mean startle amplitude across the 15 noise-alone trials during the pre-test) in 3 subgroups with sessions lasting 16, 27 or 38 min.

The following day, the animals were tested for contextual freezing and fear-potentiated startle in the chamber during post-test 1. This test session was identical to the pre-test. On the next day, a second training session, identical to training 1 was carried out. One day later, the animals were tested again during post-test 2. This session was identical to the pre-test and post-test 1.

Statistical analyses

Repeated measures ANOVAs were used to assess the contextual conditioning potential of the different numbers of unpaired shock-tone presentations. The analyses were conducted on both freezing time and startle amplitude on noise-alone trials with Session (pre-test, post-test 1 and post-test 2) and Group (0 SHOCKS, 5 SHOCKS, 10 SHOCKS and 15 SHOCKS) as factors. To meet ANOVA assumptions (normal distribution and homogeneity of variances), freezing data were transformed using a natural logarithm of the time in $s + 1$ ($\ln+1$). Additionally, a Greenhouse-Geisser correction for sphericity was applied for the ANOVA of transformed freezing time. Next, a repeated measures ANOVA of startle amplitude on noise-alone trials with Session (habituation 1, habituation 2 and pre-test) as factor, was carried out to examine the evolution of the startle amplitudes during these 3 sessions. For this analysis, the 4 groups were pooled because at this point in time all groups were still treated identically. Finally, a repeated measures ANOVA was carried out on startle amplitude with Trial Type (noise-alone and tone-noise

Habituation 1	
accl	30 startle stimuli + 3 tones
Habituation 2	
accl	30 startle stimuli + 3 tones
<i>weekend</i>	
Pre-test	
<i>behavioral baseline measurements</i>	
accl	15 noise-alone and 15 tone-noise trials
Training 1	
<i>conditioning to context</i>	
accl	explicitly unpaired shocks (0.8 mA - 250 ms) and tones (0, 5, 10 or 15)
Post-test 1	
<i>behavioral measurements of contextual anxiety</i>	
accl	15 noise-alone and 15 tone-noise trials
Training 2	
<i>conditioning to context</i>	
accl	explicitly unpaired shocks (0.8 mA - 250 ms) and tones (0, 5, 10 or 15)
Post-test 2	
<i>behavioral measurements of contextual anxiety</i>	
accl	15 noise-alone and 15 tone-noise trials

Fig. 1. Experimental design. $n=9$ per group. (accl) acclimation phase.

trials), Session (pre-test, post-test 1 and post-test 2) and Group (0 SHOCKS, 5 SHOCKS, 10 SHOCKS and 15 SHOCKS) as factors. Tukey's *post-hoc* tests were carried out and only relevant differences (i.e. comparisons within sessions or within groups) are listed in the text or indicated on figures. All statistical analyses were carried out using Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA), with the significance level set at $P<0.05$.

RESULTS

The ANOVA conducted on transformed freezing time (Fig. 2A) showed main effects of Group ($F_{3,32}=35.62$, $P<0.0001$) and Session ($F_{2,64}=88.87$, $P<0.0001$), and a significant Group \times Session interaction ($F_{6,64}=7.99$, $P<0.0001$). Tukey's *post-hoc* tests revealed that there was no difference between the 4 groups on the pre-test day, indicating that the groups had equivalent baseline freezing levels. On both post-tests, the 3 shocked groups (5 SHOCKS, 10 SHOCKS and 15 SHOCKS groups) froze significantly longer than on their respective pre-test.

Moreover, the 3 shocked groups froze significantly longer than the non-shocked control (0 SHOCKS group) on both post-test 1 and post-test 2 (all P s<0.0002). Within each shocked group, there was no significant increase of freezing time from post-test 1 to 2, probably because the ceiling value (absolute maximum would be 300 s, or 5.71 after transformation) had already been reached. Likewise, there were no differences between the 3 shocked groups on both post-tests 1 and 2 (all P s>0.99). In the 0 SHOCKS group, there was no difference between the 3 sessions, indicating that freezing stayed at a baseline level in this control group. In summary, training with 5, 10 or 15 explicitly unpaired shock-tone presentations resulted in significant contextual conditioning, producing similar levels of freezing, on post-tests 1 and 2.

The ANOVA conducted on startle amplitude on noise-alone trials (Fig. 2B) showed no main effects of Session ($F_{2,64}=0.89$, $P=0.42$) or Group ($F_{3,32}=0.15$,

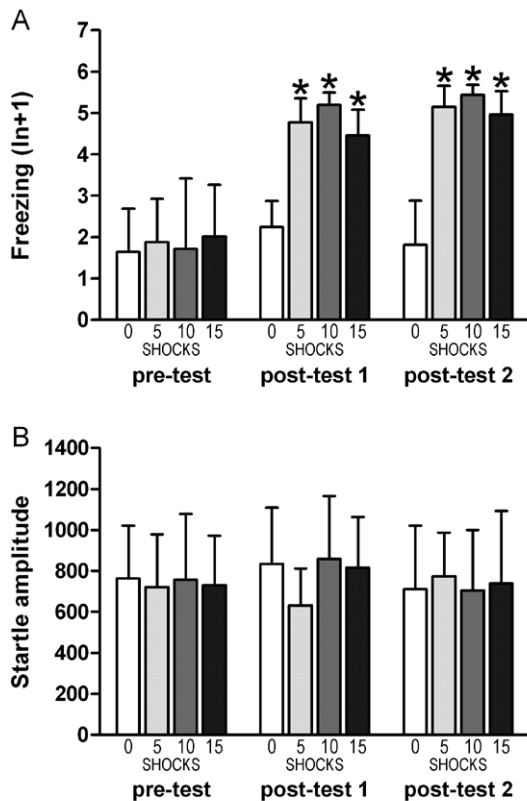


Fig. 2. Effects of contextual conditioning on (A) transformed freezing time and (B) startle amplitude on noise-alone trials. Means \pm SD ($n=9$ per group) during pre-test, post-tests 1 and 2 are shown. *Different from the pre-test of this group and different from the 0 SHOCKS group during this session (Tukey's *post-hoc* tests: all P s<0.0002).

$P=0.93$) and no Group \times Session interaction ($F_{6,64}=1.32$, $P=0.26$). Tukey's *post-hoc* tests did not reveal any differences within groups or sessions. Thus, the startle amplitude on noise-alone trials was no adequate measurement of contextual anxiety in this experiment.

Since this seemed discrepant with the findings of our previous study, we took a closer look at the evolution of the startle amplitudes on noise-alone trials during habituation 1 (mean \pm SD of all 36 rats: 662 ± 324), habituation 2 (639 ± 233) and the pre-test (743 ± 259). This ANOVA showed a significant effect of Session ($F_{2,70}=3.23$; $P=0.045$), with startle amplitudes on the pre-test being significantly higher than on habituation 2 (Tukey's *post-hoc* test, $P=0.047$), which is the opposite of what we expected.

Finally, Table I shows the startle amplitude data on noise-alone and tone-noise trials in each group during the 3 test sessions (pre-test, post-test 1 and post-test 2). The ANOVA conducted on these data revealed a main effect of Trial Type ($F_{1,32}=10.99$, $P=0.002$), but no other main effects of Group or Session and no interactions between these factors. More specifically, when pooling all 4 groups and the 3 test sessions, the startle amplitudes on noise-alone trials (mean = 753) were significantly higher than on tone-noise trials (mean = 696) (Tukey's *post-hoc* test, $P=0.002$).

DISCUSSION

The present study systematically examined the effect of training with 0, 5, 10 or 15 unpaired tone-shock (0.8 mA – 250 ms) presentations on the expression of contextual conditioning in a protocol with two training and two post-testing days, as measured with both freezing time and startle amplitude. Training with 5, 10 or 15 explicitly unpaired shocks resulted in significant contextual freezing. There was no significant increase in freezing time from post-test 1 to post-test 2 and there were no differences in freezing time between the 3 shocked groups, implicating that the ceiling freezing value was already reached after one training session with 5 shocks. Surprisingly, we observed no differences in startle amplitude between the groups.

In a preceding study, we did find significantly elevated startle amplitudes in the conditioned context for rats trained with 10 shocks (Luyten et al. 2011a). However, in these previous experiments we used a slightly different protocol with a single habituation session, 24 hours before the pre-test. In the present experiment, we

Table I

Startle amplitude on noise-alone and tone-noise trials				
Group	Trial type	Pre-test	Post-test 1	Post-test 2
0 shocks	Noise-alone	764 ± 256	834 ± 274	711 ± 310
	Tone-noise	712 ± 235	747 ± 268	581 ± 302
5 shocks	Noise-alone	722 ± 257	632 ± 180	772 ± 214
	Tone-noise	638 ± 218	637 ± 187	702 ± 140
10 shocks	Noise-alone	756 ± 321	858 ± 307	705 ± 296
	Tone-noise	715 ± 293	782 ± 275	595 ± 242
15 shocks	Noise-alone	730 ± 242	818 ± 246	739 ± 353
	Tone-noise	709 ± 195	765 ± 190	770 ± 348

Means ± SD ($n=9$ per group) during pre-test, post-tests 1 and 2 are shown

observed that the startle amplitude on the pre-test was significantly higher than on habituation 2, whereas habituation was expected to produce lower startle amplitudes (Zhao and Davis 2004). We assume that these higher startle response levels on the pre-test might have interfered with the conditioning effects. However, to be sure that the altered habituation procedure caused the disruption of previously found significant effects on startle, a new experiment, specifically designed to answer this question, should be carried out.

In this study, we used a protocol with combined measurements of freezing time and startle amplitude to assess conditioning to the context. An advantage of the latter measurement is that it is under control of the experimenters. Startle-evoking stimuli can be presented at any given time during the experiment, functioning as a probe, assessing changes in emotional reactivity. In addition, although startle potentiation was first described in animals, the same effect has been found in humans, which is a major advantage with regard to translational research (Grillon 2002). However, this startle procedure may also have a disadvantage, i.e. the loud startle-evoking stimulus itself (white noise, 100 dB, 50 ms) may be aversive. Similar loud noises may even function as an unconditioned stimulus and startle probes have been rated as very unpleasant by human

subjects (Borszcz et al. 1989, Bradley et al. 1993). A few years ago, Lissek and colleagues (2005) suggested that low intensity airpuffs to the forehead may be an efficacious and less aversive alternative to white noise startle probes in human research measuring the eye-blink reflex. However, to date, most rodent studies still use acoustic startle probes.

Although we cannot rule out an effect of potentially aversive startle probes in the present study, it is noteworthy in this respect that the presentation of 2×30 startle probes during the habituation sessions did not seem to result in substantial conditioning to the context on the pre-test, as the freezing levels during the acclimation phase of the pre-test were still very low (average of 13 s of freezing during this 5-min phase). Nevertheless, the exposure to multiple unpleasant events during the habituation session might partially explain the slight increase in startle amplitude on noise-alone trials from habituation 2 to pre-test.

Furthermore, during pre-test, post-test 1 and 2, the context in fact involved both predicted and unpredicted potentially aversive events (startle probe predicted by a tone on tone-noise trials and unpredicted on noise-alone trials). It has been described that unpredictable aversive stimuli may produce more incapacitating cognitive, behavioral and somatic effects than predictable

aversive stimuli (Grillon 2002). In line with these findings, we observed that the startle amplitudes on predictable tone-noise trials were significantly lower than on unpredictable noise-alone trials.

In addition, we cannot rule out that the tone may have functioned as a prepulse, which may result in a prepulse inhibition-like or prepulse facilitation-like effect, even though there are some clear methodological differences between our study and a typical prepulse inhibition experiment. Prepulse inhibition is defined as the reduction in startle response produced by a low intensity stimulus (the prepulse) preceding a high intensity, startle-evoking stimulus. In a typical prepulse inhibition experiment, the prepulse is very short (e.g. 20 ms) with a 30–100 ms interval between the prepulse offset and startle probe onset (Koch and Schnitzler 1997, Jones and Shannon 2000). In comparison, our study used co-terminating 10-s tones and 50-ms startle stimuli. Prepulse facilitation is the increase in startle response after a prepulse with a longer interval (>500 ms) or with a longer prepulse. Davis and colleagues observed prepulse inhibition effects using short light cues (50–200 ms) and facilitation with longer light cues (51.2 s). In their study the prepulse and startle stimulus co-terminated. Note that a visual prepulse of 3.2 s or 12.8 s (duration comparable to our 10-s tone) appeared to result in an equilibrium between inhibition and facilitation (Davis et al. 1989).

Although we cannot rule out any effects of predicted versus unpredicted startle probes nor of possible prepulse-like effects of the tones, this is not a major problem, as the between-group and within-group comparisons effectively factor out these potential confounds.

During the training sessions, rats in the shocked groups (5 SHOCKS, 10 SHOCKS and 15 SHOCKS) are exposed to unpredictable shocks that are explicitly unpaired with tones. Rescorla stated that the tone then provides information that the shock will not occur and as a consequence, this tone becomes a safety signal or conditioned inhibitor (Rescorla 1969). Thus, theoretically, the rats can learn that the presence of the tone predicts the absence of the shock and will subsequently treat the tone as a safety signal (Gleitman and Landau 1994). However, in practice, safety signals are acquired more slowly than danger signals or are not established at all (Candido et al. 2004, Orman and Stewart 2007, Marschner et al. 2008). In the present study, there was no evidence for a conditioned inhibitory role for the tone. Although we observed signifi-

cantly lower startle amplitudes on tone-noise trials than on noise-alone trials, this was already the case during the pre-test, i.e. before the first training session. On the other hand, we cannot ignore the possibility that the rats considered the tones as part of the aversive context, which may result in equally high startle amplitudes on noise-alone and tone-noise trials. However, it was not within the scope of this experiment to investigate the possible effects of the unpaired tones. Moreover, in a prior study we already showed that training with explicitly unpaired tones and shocks or training with shocks alone may result in equivalent contextual conditioning (Luyten et al. 2011a).

In the current experiment, we primarily wanted to systematically examine the effect of different numbers of training trials on the expression of contextual conditioning. We are, to our knowledge, the first to report on the effects of different numbers of explicitly unpaired cue-shock presentations on contextual anxiety as measured with freezing and startle amplitude. Some authors have used the combination of startle and freezing measurements, but they trained their animals with shocks alone or cue-shock pairings (McNish et al. 1997, Jones et al. 2005), not with unpaired cue-shock presentations like in our study. In addition, there is some literature describing the effects of different numbers of shocks in procedures with shocks alone or cue-shock pairings. Generally, contextual freezing after training with shocks alone appears to be proportional to the number of shocks an animal received (Rau and Fanselow 2009, Woodcock and Richardson 2000). Likewise, most studies using explicit cue-shock pairings for cued fear conditioning found a positive linear relationship between the number of pairings and subsequent freezing to the discrete cue (Richmond et al. 1998, Laxmi et al. 2003). Furthermore, our results are consistent with the finding that conditioned freezing is typically asymptotic after 3–5 shocks, reaching a ceiling level of performance (Rau and Fanselow 2009). We found no records describing the effects of number of shocks on contextual anxiety as measured with startle amplitude. In the present experiment, we found no effects of conditioning with different numbers of shocks on startle amplitude.

In the current study, we also sought to take a first step towards a chronic conditioning procedure, i.e. with more than one training and testing day. Although chronic conditioning protocols have already been described in the literature, most of these are cued conditioning procedures (training with cue-shock pairings) or ‘classical’ contex-

tual conditioning procedures (training with shocks alone or with cue-shock pairings; Fanselow and Helmstetter 1988, Kim and Davis 1993, Gewirtz et al. 1997, 1998, Fendt 2001). To our knowledge, this is the first report of a chronic contextual conditioning procedure using explicitly unpaired cue-shock presentations during training. Moreover, in our study we found consistent contextual freezing during both post-tests, implying that our protocol produced reliable contextual anxiety over two testing days. Future experiments should investigate whether this procedure can be extended to a truly chronic protocol with even more training and testing days.

CONCLUSION

The present study systematically examined the effect of training with 0, 5, 10 or 15 unpaired toneshock (0.8 mA – 250 ms) presentations on the expression of contextual conditioning. At the same time, we took the first steps towards a chronic contextual conditioning protocol. Training with 5, 10 or 15 explicitly unpaired shocks resulted in consistent contextual freezing on both post-tests. Unexpectedly, we observed no differences in startle amplitude between the groups.

ACKNOWLEDGEMENTS

We acknowledge the financial support of the Research Foundation - Flanders (FWO) (project G.0729.09N) and the K.U.Leuven Research Fund. L. Luyten is a doctoral fellow and K. van Kuyck a postdoctoral fellow of the FWO.

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