

# Phasic and Sustained Fear are Pharmacologically Dissociable in Rats

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Previous findings suggest differences in the neuroanatomical substrates of short- (seconds) vs longer-duration (minutes) fear responses. We now report that phasic and sustained fear can also be differentiated pharmacologically, based on their response to several treatments that either are or are not clinically effective anxiolytics. For these experiments, short- or long-duration clicker stimuli were paired with footshock. Acoustic startle amplitude was later measured in the absence of the clicker, or within seconds (phasic fear) or minutes (sustained fear) of its onset. Before testing, rats received a single injection of vehicle, the benzodiazepine chlordiazepoxide, the 5HT<sub>1A</sub> agonist and dopamine D2 antagonist buspirone, the selective serotonin reuptake inhibitor fluoxetine, or a 3-week treatment with either vehicle or fluoxetine. Chlordiazepoxide blocked sustained, but not phasic startle increases. Acute buspirone, which is not anxiolytic in human beings, did not affect sustained startle increases, but did disrupt phasic increases. Chronic fluoxetine blocked sustained startle increases and unreliably reduced phasic increases; acute fluoxetine affected neither. The results indicate that phasic and sustained fear responses can be pharmacologically dissociated, further validating this distinction, and suggest that sustained startle increases may be especially useful as anxiety models and anxiolytic screens.

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## INTRODUCTION

A major aim of psychiatric research is to better understand the neural mechanisms of psychiatric disorders, including anxiety. Because the physiological symptoms of healthy fear and clinical anxiety are highly similar, and because it is generally believed that clinical anxiety reflects maladaptive activity within fear circuitry, experimentally induced fear in healthy controls and in research animals has been used extensively to pursue this goal.

We have used changes in the amplitude of the acoustic startle reflex as a fear measure and anxiety surrogate (Davis, 1986), and have described the neural circuitry that mediates this effect (cf, Davis, 2006). When evoked by brief presentations of stimuli previously paired with shock, fear-potentiated startle is mediated by direct and indirect projections from the medial central nucleus of the amygdala (CeA) to the primary startle reflex pathway in the pontine reticular formation (Hitchcock and Davis, 1991; Rosen *et al*,

1991; Meloni and Davis, 1999; Zhao and Davis, 2004). However, when evoked by other treatments, including intracerebroventricular infusions of the stress-related peptide corticotropin-releasing factor (Lee and Davis, 1997), by startle testing in illuminated vs darkened test chambers (Walker and Davis, 1997b), or by repeated footshock stress (Gewirtz *et al*, 1998), such increases appear to be mediated not by the medial CeA, but instead by a more rostral extension of the extended amygdala known as the bed nucleus of the stria terminalis (BNST).

In reviewing these and possibly related findings from other laboratories (eg, Hammack *et al*, 2004; Sullivan *et al*, 2004; Waddell *et al*, 2006), we have noted that CeA manipulations more consistently disrupt short-duration, rapid-onset/offset fear responses to distinct and imminent threats (phasic fear), whereas BNST manipulations more consistently disrupt longer-duration responses to more sustained stimuli (cf, Walker *et al*, 2009b).

To explicitly compare the neurobiological substrates of phasic vs sustained startle increases, and to further evaluate the validity of this distinction, we developed a modified conditioned fear-potentiated startle paradigm in which clicker stimuli of variable duration (from 3 s to 8 min) are paired with co-terminating footshocks. During testing, rats are presented with startle-eliciting noise bursts delivered within seconds (for phasic fear testing) or minutes (for sustained fear testing) of

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conditioned stimulus (CS) onset. In this study, we used this paradigm to compare the effect on phasic and sustained fear of several pharmacological treatments that are clinically useful for anxiety reduction (ie, acute chlordiazepoxide, chronic fluoxetine) and others that are not (ie, acute buspirone, acute fluoxetine). We discuss the results with respect to their implications for the validity of the phasic *vs* sustained fear distinction, and for the utility of phasic *vs* sustained fear procedures as anxiety models and anxiolytic screens.

## MATERIALS AND METHODS

### Animals

Male Sprague–Dawley rats (200–250 g at arrival; Charles River, Raleigh, NC) were housed four per cage on a 12-h light–dark cycle in a temperature- and humidity-controlled room with food and water freely available. Behavioral procedures began approximately 1 week after arrival, and were conducted in accordance with USDA, NIH, and Emory University guidelines.

### Apparatus

Rats were trained and tested in 8 × 15 × 15 cm Plexiglas and wire mesh cages with four 6.0-mm diameter stainless-steel floorbars, located within a sound-attenuated behavior chamber. Startle responses were evoked by 50-ms (95 dB) white-noise bursts generated by a computer sound file, amplified by a Radio Shack amplifier (Tandy, Fort Worth, TX), and delivered through Radio Shack Supertweeter speakers located in front of the cage. The same speakers delivered background noise (60 dB, 1–20 kHz) provided by an ACO Pacific (Belmont, CA, USA) noise generator. All sound level measurements were made from the center of the cage.

Startle amplitude and shock reactivity were quantified using a PCB Piezotronics (Depew, NY, USA) accelerometer affixed to the bottom of the cage. The accelerometer produces a voltage output proportional to the velocity of cage movement (eg, produced by the rats' startle response), which was integrated by a PCB Piezotronics signal conditioner and digitized by a GW Instruments (Somerville, MA) InstruNet device. Startle amplitude was defined as the maximum peak-to-peak voltage during the first 200 ms after each noise burst. Shock responses were similarly quantified, using a 500-ms window concurrent with shock delivery.

The CS was a 70-dB, 60-Hz clicker stimulus delivered through speakers located behind each chamber. The unconditioned stimulus was a 0.5-s, 0.35-mA footshock delivered through the floor bars. The sequencing of all stimuli was controlled by a desktop computer using custom-designed software (The Experimenter; Glass Bead Software, New Haven, CT).

### Behavioral Procedures

*Experimental sequence.* Rats received two acclimation sessions (days 1 and 2), followed by a pre-conditioning test for sustained or phasic fear (day 3), followed by three conditioning sessions (days 4–6), and followed 48 h later by a post-conditioning test. Thus, some rats were tested for sustained and others for phasic fear, but all rats received the

same conditioning procedure. These procedures are described below, and presented graphically in Figure 1.

*Acclimation.* Rats were placed into the test cage and, after 5 min, presented with the first of 48 startle-eliciting white-noise bursts (inter-stimulus interval (ISI) = 30 s).

*Pre-conditioning sustained fear test.* Rats were placed into the test cage and, after 5 min, presented with the first of 32 startle-eliciting noise bursts (ISI = 30 s). The first 16 were presented in the absence, and the next 16 in the presence of a 60-Hz clicker stimulus.

*Pre-conditioning phasic fear test.* Rats were placed into the test cage and, after 5 min, presented with the first of 75 startle-eliciting noise bursts (ISI = 30 s). Thirty of the final 60 were presented 3.2 s after the onset of a 3.7-s clicker stimulus and another 30 (intermixed) were presented in its absence.

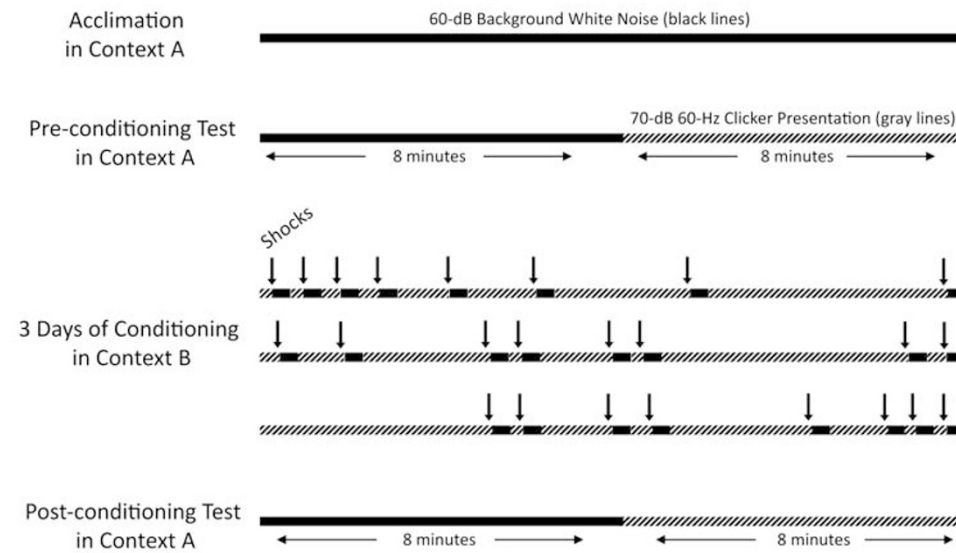
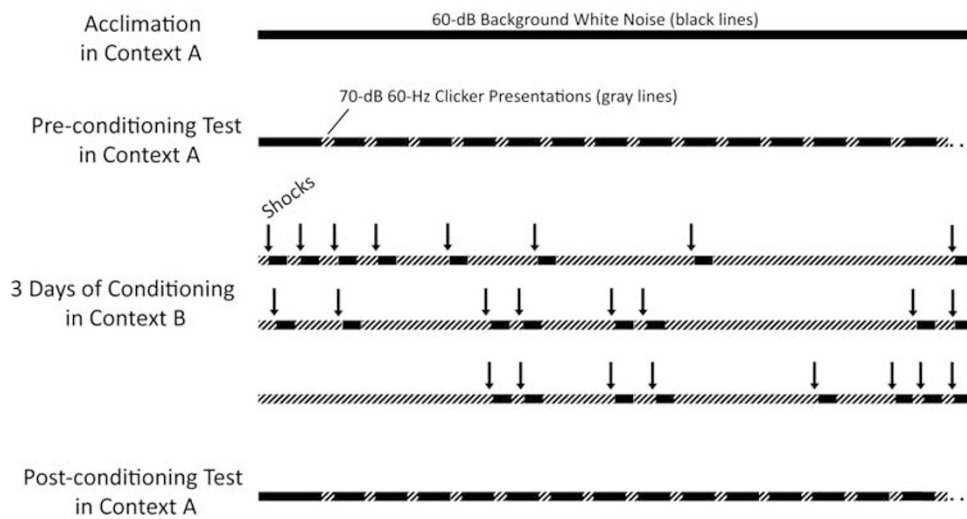
*Fear conditioning.* On each conditioning day, rats received eight presentations of variable-duration clicker stimuli (3-, 10-, and 20-s; 1-, 2-, 4-, 6-, and 8-min), each co-terminating with footshock. The first CS of each session occurred 5 min after the rat was placed into the conditioning chamber. The interval between offset of one CS and onset of the next was 3 min. During the first conditioning session, the clicker stimuli were presented in the order of increasing duration. During the second and third, they were sequenced randomly.

*Post-conditioning phasic and sustained fear tests.* Rats were tested after conditioning, using procedures identical to those described for the pre-conditioning tests.

*Context manipulations.* During conditioning, a cotton gauze pad wetted with 0.4 ml of 70% ethanol solution was placed in front of the test cage. A fluorescent light placed behind the cage (150 lx as measured from the middle of the cage) provided constant illumination. During testing, to minimize context-potentiated startle, the cage was dark and no explicit olfactory stimuli were introduced. In addition, two 5-cm chains hung from the top of the test cage and a sandpaper insert was placed over the floorbars. We have previously found similar changes to be effective in producing discriminable contexts (McNish *et al*, 1997).

### Statistical Analyses

*Sustained fear.* Each rat tested for sustained fear received a sustained fear-potentiated startle score. Because our analysis of the control data set indicated that the first startle response after CS onset was markedly higher than all those that followed, and that sustained fear diminished with time, becoming unreliable after approximately the fourth minute of CS presentation (see Figure 2), we calculated a sustained fear-potentiated startle score by dividing mean startle amplitude during the first 4 min of the CS (beginning with the second CS test trial) by the mean startle amplitude during the last 4 min of the pre-CS period (see Figure 2). For presentation purposes, these ratios were converted to percent change scores.

**a Sustained Fear Procedure****b Phasic Fear Procedure**

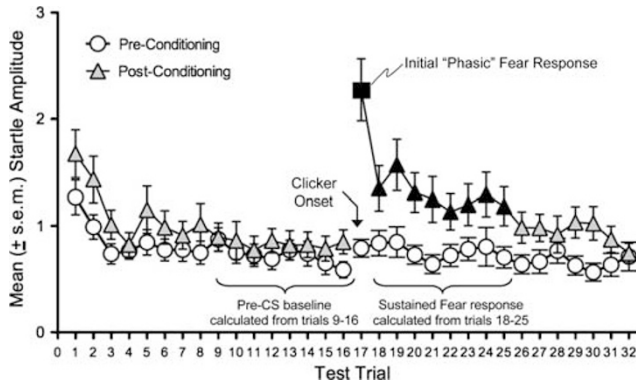
**Figure 1** Behavioral procedures and timeline. Acclimation, a pre-conditioning test, each of three conditioning sessions in which clicker stimuli (gray) of variable duration were paired with co-terminating footshock (arrows), and a post-conditioning test, took place on separate days. With the exception of testing, the sustained and phasic fear procedures were identical. For sustained fear testing, startle was measured before and then during presentation of an 8-min clicker stimulus. For phasic fear testing, startle was measured in the presence and in the absence, on intermixed test trials, of 3.7-s clicker stimuli. Session and event lengths are not drawn to scale. For a detailed description, see the Materials and Methods section. Panel (a) Sustained Fear Procedure. Panel (b) Phasic Fear Procedure.

**Phasic fear measure 1.** Each rat tested for phasic fear received a phasic fear-potentiated startle score, defined as the ratio between the mean startle amplitude of all CS test trials and the mean startle amplitude of all intermixed non-CS test trials. For presentation purposes, these ratios were converted to percent change scores.

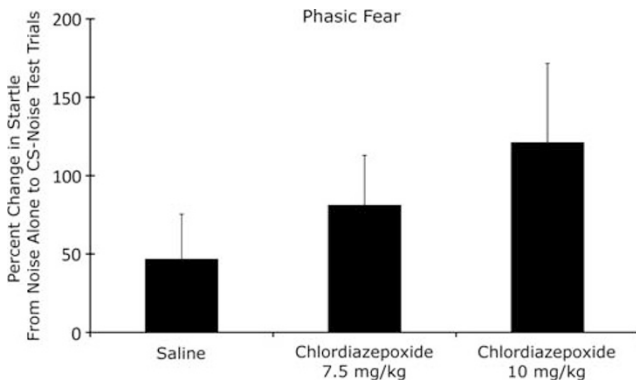
**Phasic fear measure 2.** An additional measure of phasic fear was computed for rats that received sustained fear testing. This was defined as the ratio between startle amplitude to the very first noise burst after CS onset (ie, on trial 17 that occurred 19.2 s after CS onset) and the pre-CS baseline (ie, trials 9–16).

For all measures, we used ratio rather than absolute difference (ie, startle in the presence of clicker–startle in

the absence of clicker), because we have previously found that when fear levels remain constant, ratio, but not difference scores remain stable as well (Walker and Davis, 2002b). We also note that our use of startle responses elicited 19.2 s or less as opposed to 49.2 s or more, for phasic and sustained fear respectively, is somewhat arbitrary (as would almost certainly be true for any specific time point), as the transition from one to the other is most likely gradual rather than abrupt. Our aim here was to have one set of measures that was *more* phasic and *less* sustained than the other, but we recognize that each may have elements of both. As will be seen from the results that follow, the data obtained with these scoring methods generally appear to confirm their validity.



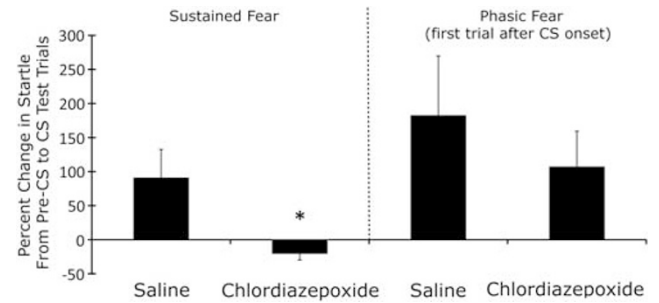
**Figure 2** The trial-by-trial raw startle data during conditioning. The raw startle data for all control rats used in these studies is plotted for both the pre- (open circles) and post- (filled square and triangles) conditioning test sessions. Startle responses were evoked every 30 s during the 8 min before CS onset (trials 1–16) and for the 8 min during which the CS was presented (trials 17–32). Before conditioning, there was little if any effect of the 60-Hz clicker stimulus on startle. After conditioning, potentiation by the clicker CS was clearly evident and especially pronounced on the first test trial after CS onset (ie, trial 17, filled square), which we consider more akin to phasic than sustained fear. Potentiation dropped precipitously from the first to the second CS test trial and more gradually thereafter, becoming statistically unreliable approximately halfway through the 8 min CS.  $\blacktriangle = p < 0.05$  for each individual trial vs pre-CS baseline. The percent change scores illustrated in the following figures are based on the ratio (mean startle amplitude across CS test trials 18–25/mean startle amplitude across pre-CS test trials 9–16) for sustained fear, and (mean startle amplitude on trial 17/mean startle amplitude across pre-CS test trials 9–16) for phasic fear measure 2.



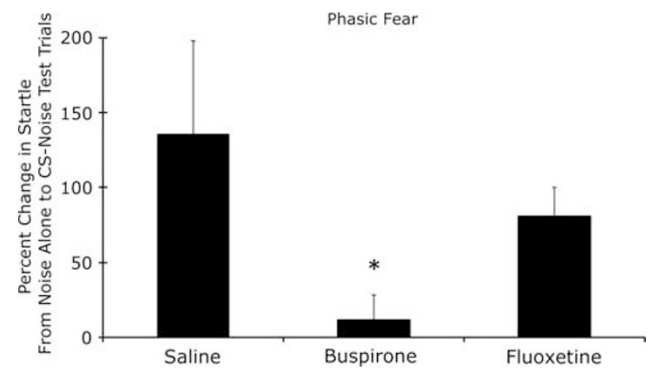
**Figure 3** Pre-test chlordiazepoxide did not disrupt phasic startle potentiation to 3.7-sec clicker stimuli.

**Exclusion criteria.** Fear conditioning requires that rats perceive the aversive stimulus. Therefore, we excluded from further analysis the data obtained from rats with footshock reactions of 1.0 or less (the mean of all rats was 3.5) on 12 or more of the 24 conditioning trials. The potentiation data from control rats validated this criterion. Thus, for sustained fear, the mean ( $\pm$  SEM) fear-potentiated startle score of rats exceeding this threshold was  $83.1 \pm 19.5\%$  ( $N = 55$ ) vs  $11.5 \pm 16.4\%$  for those that did not ( $N = 12$ ). For the phasic fear experiments, the mean fear-potentiated startle score of shock-responsive rats was  $107.7 \pm 38.9\%$  ( $N = 21$ ) vs  $-26 \pm 23.4\%$  ( $N = 3$ ) for excluded rats.

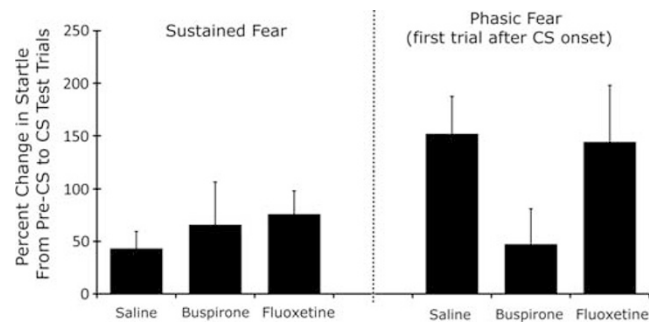
Also, because meaningful ratios cannot be calculated for rats that do not show a baseline startle response, the



**Figure 4** Pre-test chlordiazepoxide (10 mg/kg, i.p.) blocked sustained startle potentiation, but not the initial potentiation to the first startle stimulus after CS onset.  $* = p < 0.05$  vs saline.



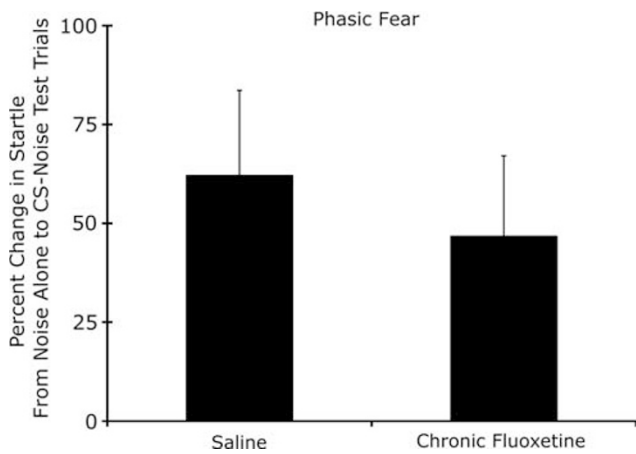
**Figure 5** Pre-test buspirone (5 mg/kg, s.c.), but not fluoxetine (10 mg/kg, i.p.), disrupted startle increases to 3.7-second clicker presentations. High variance in the saline group, and the modest difference versus the fluoxetine group, was largely attributable to a single outlier with a fear-potentiated startle score of 826%. Without this rat, the mean ( $\pm$  s.e.m.) for the saline group was  $71.8 \pm 12.9$ .  $* = p < 0.05$  vs saline (with outlier included).



**Figure 6** Neither buspirone nor fluoxetine (single injection) disrupted sustained startle increases. The lower level of phasic potentiation in the buspirone compared to saline group was roughly comparable to that seen in Experiment 4, but did not reach significance in this experiment when corrected for multiple comparisons.

data from rats with a mean accelerometer output of  $\leq 0.1$  (ie, what we observe when cage output is sampled in non-startled rats) on baseline test trials were also excluded. Only two rats failed to meet this criterion. Both had received chlordiazepoxide before sustained fear testing.

**Inferential statistics.** The primary analyses were between-group comparisons of fear-potentiated scores. Because D'Agostino and Pearson omnibus normality tests indicated significant deviations from normality for both the sustained



**Figure 7** Chronic fluoxetine (10 mg/kg, p.o., for 21 days beginning approximately 3 hours after the final conditioning session) did not significantly affect startle amplitude increases to phasically-presented fear stimuli.

( $K2 = 51.78$ ) and phasic fear ( $K2 = 45.53$ ) data sets, and because Grubb's test identified several outliers, between-group differences were evaluated using distribution-free (non-parametric) Mann-Whitney or Kruskal-Wallis tests, and also, to establish statistical robustness, by using *t*-tests and ANOVA on log-transformed scores (see Keene (1995)). Follow-up comparisons were made using Dunn's (non-parametric) or Dunnett's *t*-test (parametric) for multiple comparisons with a control. Other analyses intended to address specific questions or issues are included where relevant. For all tests, the criterion for significance was 0.05 (two-tailed).

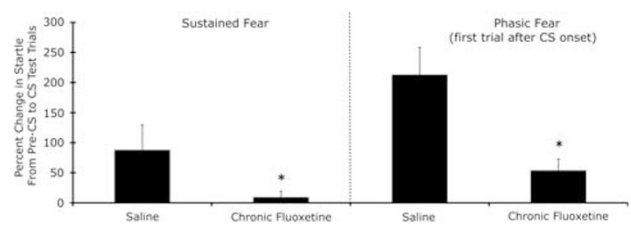
### Procedures Specific to Individual Experiments

**Experiment 1: No-shock control.** Because the sustained fear procedure is new, we wished to determine if the clicker-induced startle changes observed in control rats were indeed due to conditioning. To this end, 12 rats underwent the sustained fear procedure exactly as described above, but without shock administration during clicker presentations.

**Experiment 2: Acute benzodiazepine effect on phasic fear-potentiated startle.** At 10 min before the post-conditioning test, rats received an intra-peritoneal (i.p.) injection of either saline ( $N = 5$ ), 7.5 mg/kg chlordiazepoxide ( $N = 6$ ), or 10 mg/kg chlordiazepoxide ( $N = 5$ ). Chlordiazepoxide was obtained from Sigma-Aldrich Chemical (St Louis, MO).

For this and all other acute drug administration experiments, rats received the same drug and dose before the pre-conditioning test, which allowed us to evaluate drug effects on unconditioned startle amplitude. The sole exception was rats that received 7.5 mg/kg chlordiazepoxide before the post-conditioning test, but 2.5 mg/kg before the pre-conditioning test (we had anticipated using 2.5 mg/kg before both, but increased to the higher dose based on the initial potentiation data from rats that received 10 mg/kg chlordiazepoxide).

For all experiments, drugs were administered at 0.1 ml saline/100 g body weight.



**Figure 8** Chronic fluoxetine (10 mg/kg, p.o., for 21 days beginning approximately 3 hours after the final conditioning session) blocked the sustained increase in startle and significantly attenuated the phasic increase (i.e., the first startle probe after CS onset). \* =  $p < 0.05$  vs saline.

**Experiment 3: Acute benzodiazepine effects on sustained fear-potentiated startle.** At 10 min before the pre- and post-conditioning tests, rats received an i.p. injection of saline ( $N = 12$ ) or chlordiazepoxide ( $N = 11$ ; 10 mg/kg).

**Experiment 4: Acute buspirone and fluoxetine effects on phasic fear-potentiated startle.** At 10 min before the pre- and post-conditioning tests, rats received a subcutaneous (s.c.) injection of buspirone ( $N = 7$ ; 5 mg/kg; Sigma-Aldrich Chemical), an i.p. injection of fluoxetine ( $N = 16$ ; 10 mg/kg; Spectrum Chemical, Gardena, CA), or saline ( $N = 6$ , i.p.;  $N = 7$ , s.c.).

**Experiment 5: Acute buspirone and fluoxetine effects on sustained fear-potentiated startle.** At 10 min before the pre- and post-conditioning tests, rats received buspirone ( $N = 13$ ; 5 mg/kg, s.c.), fluoxetine ( $N = 22$ ; 10 mg/kg, i.p.), or saline ( $N = 13$ , i.p.;  $N = 12$ , s.c.).

**Experiment 6: Chronic fluoxetine effects on phasic fear-potentiated startle.** Rats received the first of 21 treatments of either saline ( $N = 8$ ) or fluoxetine ( $N = 7$ ; 10 mg/kg) by oral gavage approximately 3 h after the final conditioning session and for each of the following 20 days. The post-conditioning test was conducted approximately 24 h after the final injection.

**Experiment 7: Chronic fluoxetine effects on sustained fear-potentiated startle.** Rats received the first of 21 treatments of saline ( $N = 18$ ) or fluoxetine ( $N = 20$ ; 10 mg/kg) by oral gavage approximately 3 h after the final conditioning session and for each of the following 20 days. The post-conditioning test was conducted approximately 24 h after the final injection.

## RESULTS

### Sustained Fear in Control Animals

Before presenting results from the individual experiments, we first provide a descriptive account of sustained fear in control rats, as we have not previously presented results from this paradigm. Figure 2 illustrates the trial-by-trial data pooled from all control rats used in the sustained fear experiments. As shown in this figure, startle amplitude mostly habituated to a stable baseline within the first few trials of the pre-CS conditioning phase and remained relatively stable thereafter. Introduction of the clicker

stimulus, between trials 16 and 17, had little if any effect on startle before conditioning (open circles), but caused a marked enhancement of startle after conditioning (filled square and triangles). This enhancement was especially pronounced on the first trial after CS onset (trial 17), which we have scored independently as an ancillary measure of *phasic* fear (ie, phasic fear measure 2). By the fifth minute after CS onset, potentiation began to wane and was no longer statistically reliable.

Figure 2 also shows a modest increase in pre-CS startle amplitude from the pre- to post-conditioning test. Although significant overall ANOVA on the first 16 trials that preceded CS onset indicated a main effect of Session (ie, pre- vs post-conditioning),  $F(1, 51) = 8.54$ ,  $p < 0.05$ , the increase was only observed in rats from Experiment 7—that is, the group that had a 25-day delay interposed between the two tests (see also Table 1). As such, we believe this increase most likely reflects weight gain or something associated with the daily gavage procedure, as opposed, for example, to a generalized context fear response.

The figures that follow show percent change (ie, from non-CS or pre-CS test trials to CS test trials) scores only. The absolute startle amplitudes from which these change scores are derived are provided in Table 1.

*Experiment 1: Clicker-induced startle changes require conditioning.* During testing, rats that did not receive clicker-shock pairings did not show either phasic or sustained startle increases (see Table 1).

*Experiment 2: Pre-test chlordiazepoxide did not disrupt phasic startle potentiation.* Phasic fear potentiation was not reduced, but was nominally (although not significantly) greater in the chlordiazepoxide compared with saline groups (Figure 3) based on ANOVA. Baseline startle was reduced, however, as indicated by a significant Group effect,  $F(2, 13) = 4.40$ . A Dunnett's *t*-test indicated a significant baseline difference between the vehicle and 10 mg/kg group,  $q(9) = 2.95$ , but not between the 0 and 7.5 mg/kg group (see also Table 1).

*Experiment 3: Pre-test chlordiazepoxide blocked the sustained, but not initial component of startle potentiation.* As shown in Figure 4 (and see also Table 1), acute pre-test chlordiazepoxide administration blocked startle increases to the long-duration clicker CS. The disruption was statistically robust, being detected by parametric,  $t(21) = 3.15$ , as well as non-parametric,  $U = 23$ , analyses. When limited to the first startle response after CS onset (phasic fear measure 2), a between-group difference was not found using either analysis. There were no between-group differences in baseline startle.

*Experiment 4: Pre-test buspirone, but not fluoxetine (single injection) blocked phasic startle potentiation.* ANOVA on the log-transformed scores indicated a significant Treatment effect,  $F(2, 33) = 4.25$ , which was due to the difference between the saline and buspirone groups,  $q(18) = 2.87$  (Dunnett's *t*-test). Non-parametric analyses

yielded essentially the same results. Thus, a Kruskal–Wallis comparison also indicated significant between-group differences,  $H = 7.74$ , with Dunn's multiple comparison test indicating a significant difference between the saline and buspirone groups (rank sum difference = 13.21), but not between the saline and acute fluoxetine groups. These results are shown in Figure 5. ANOVA also indicated a significant Group effect on baseline startle,  $F(2, 33) = 4.80$ . Dunnett's *t*-test indicated a significant difference between the saline and buspirone groups,  $q(18) = 3.09$ , but not between the saline and fluoxetine groups (see Table 1).

*Experiment 5: Neither buspirone nor acute fluoxetine blocked sustained startle potentiation.* These results are shown in Figure 6 (and see also Table 1). As in the preceding experiment, phasic potentiation was markedly lower in the buspirone compared with saline groups (ie, using the first startle response after CS onset as a supplemental phasic fear measure)—in this case,  $47 \pm 34$  vs  $152 \pm 35\%$ , respectively—but this was not statistically significant using either ANOVA or Kruskal–Wallis analyses. The between-group difference with respect to baseline startle was also comparable to the preceding experiment and this was significant,  $F(2, 57) = 4.91$ . As before, this was due to a higher baseline in the buspirone group compared with saline group,  $q(18) = 2.47$ , Dunnett's *t*-test, which we have found previously (Kehne et al, 1988).

*Experiment 6: Chronic fluoxetine does not affect startle increases to phasically presented fear stimuli.* Neither Mann–Whitney nor *t*-test analyses indicated significant between-group differences ( $p > 0.05$  for both). Baseline startle was similarly unaffected. These results are shown in Figure 7 (and see also Table 1).

*Experiment 7: Chronic fluoxetine disrupts startle increases to a sustained fear stimulus.* As shown in Figure 8 and confirmed statistically by an independent-samples *t*-test on the log-transformed scores,  $t(36) = 2.69$ , as well as Mann–Whitney analysis of percent change scores ( $U = 99$ ), chronic fluoxetine significantly disrupted startle increases to the sustained fear stimulus. Fluoxetine also significantly reduced fear-potentiated startle to the first startle stimulus after CS onset (phasic fear measure 2),  $t(36) = 1.71$  and  $U = 125$ . Baseline startle was significantly greater in the fluoxetine group,  $t(36) = 2.27$ .

## DISCUSSION

We evaluated the effects on phasic and sustained startle increases of several pharmacological treatments that either are or are not clinically effective for anxiety reduction. Phasic and sustained startle increases responded differently, and in opposite directions to several of these treatments, lending support to the validity of the distinction. Moreover, the specific pattern of results suggested that sustained startle increases may have greater predictive validity, and might therefore be more useful, as an animal model of clinical anxiety, than phasic startle increases. The results obtained with each compound are discussed in turn below.



**Table 1** Mean Startle Amplitude ( $\pm$  SEM) in Arbitrary Units on a Linear Scale, Together with Log-Transformed FPS Scores

	Pre-conditioning test					Post-conditioning test				
	Startle without clicker	Startle with clicker (phasic)	Startle with clicker (sustained)	Log-transformed phasic FPS <sup>a</sup>	Log-transformed sustained FPS	Startle without clicker	Startle with clicker (phasic)	Startle with clicker (sustained)	Log-transformed phasic FPS <sup>a</sup>	Log-transformed sustained FPS
<i>Sustained fear tests<sup>a</sup></i>										
Experiment 1										
No shock control	0.64 $\pm$ 0.09	0.61 $\pm$ 0.10	0.76 $\pm$ 0.11	-0.03 $\pm$ 0.08	0.08 $\pm$ 0.05	0.56 $\pm$ 0.18	0.47 $\pm$ 0.08	0.49 $\pm$ 0.07	-0.05 $\pm$ 0.06	-0.05 $\pm$ 0.06
Experiment 3										
Acute saline	0.75 $\pm$ 0.19	0.69 $\pm$ 0.13	0.83 $\pm$ 0.24	0.01 $\pm$ 0.06	-0.03 $\pm$ 0.05	0.58 $\pm$ 0.16	1.66 $\pm$ 0.72	0.74 $\pm$ 0.19	0.25 $\pm$ 0.14	0.19 $\pm$ 0.09
Acute chlordiazepoxide	0.79 $\pm$ 0.32	0.52 $\pm$ 0.14	1.0 $\pm$ 0.33	-0.08 $\pm$ 0.15	0.16 $\pm$ 0.08	0.80 $\pm$ 0.29	1.72 $\pm$ 0.86	0.58 $\pm$ 0.27	0.22 $\pm$ 0.09	-0.13 $\pm$ 0.05
Experiment 5										
Acute saline	0.73 $\pm$ 0.12	0.88 $\pm$ 0.15	0.79 $\pm$ 0.13	0.05 $\pm$ 0.07	0.02 $\pm$ 0.04	0.80 $\pm$ 0.13	1.74 $\pm$ 0.35	1.07 $\pm$ 0.20	0.29 $\pm$ 0.07	0.10 $\pm$ 0.05
Acute buspirone	1.39 $\pm$ 0.24	1.62 $\pm$ 0.30	1.43 $\pm$ 0.27	0.06 $\pm$ 0.06	-0.01 $\pm$ 0.09	1.40 $\pm$ 0.31	1.34 $\pm$ 0.23	1.73 $\pm$ 0.32	0.05 $\pm$ 0.10	0.12 $\pm$ 0.08
Acute fluoxetine	0.80 $\pm$ 0.16	0.94 $\pm$ 0.17	0.81 $\pm$ 0.11	0.06 $\pm$ 0.08	0.02 $\pm$ 0.05	0.63 $\pm$ 0.11	1.25 $\pm$ 0.26	0.97 $\pm$ 0.18	0.21 $\pm$ 0.09	0.18 $\pm$ 0.06
Experiment 7										
Chronic saline	0.70 $\pm$ 0.13	0.78 $\pm$ 0.13	0.73 $\pm$ 0.10	0.04 $\pm$ 0.06	0.01 $\pm$ 0.05	1.1 $\pm$ 0.21	3.09 $\pm$ 0.58	1.70 $\pm$ 0.35	0.42 $\pm$ 0.06	0.17 $\pm$ 0.06
Chronic fluoxetine	0.64 $\pm$ 0.10	0.67 $\pm$ 0.12	0.69 $\pm$ 0.12	-0.01 $\pm$ 0.08	0.00 $\pm$ 0.07	2.20 $\pm$ 0.43	2.99 $\pm$ 0.58	2.40 $\pm$ 0.58	0.13 $\pm$ 0.05	0.01 $\pm$ 0.04
<i>Phasic fear tests</i>										
Experiment 2										
Acute saline	0.68 $\pm$ 0.12	0.72 $\pm$ 0.21	N/A	-0.01 $\pm$ 0.11	N/A	0.55 $\pm$ 0.11	0.76 $\pm$ 0.16	N/A	0.14 $\pm$ 0.10	N/A
Acute chlordiazepoxide (7.5 mg/kg) <sup>b</sup>	0.57 $\pm$ 0.13	0.56 $\pm$ 0.14	N/A	-0.01 $\pm$ 0.05	N/A	0.39 $\pm$ 0.13	0.71 $\pm$ 0.25	N/A	0.24 $\pm$ 0.08	N/A
Acute chlordiazepoxide (10 mg/kg)	0.34 $\pm$ 0.07	0.40 $\pm$ 0.07	N/A	0.08 $\pm$ 0.05	N/A	0.20 $\pm$ 0.04	0.36 $\pm$ 0.04	N/A	0.29 $\pm$ 0.11	N/A
Experiment 4										
Acute saline	0.62 $\pm$ 0.12	0.55 $\pm$ 0.11	N/A	-0.03 $\pm$ 0.05	N/A	0.50 $\pm$ 0.08	1.16 $\pm$ 0.36	N/A	0.29 $\pm$ 0.07	N/A
Acute buspirone	0.81 $\pm$ 0.24	0.79 $\pm$ 0.29	N/A	-0.04 $\pm$ 0.09	N/A	1.12 $\pm$ 0.24	1.15 $\pm$ 0.24	N/A	0.02 $\pm$ 0.07	N/A
Acute fluoxetine	0.61 $\pm$ 0.07	0.55 $\pm$ 0.15	N/A	-0.03 $\pm$ 0.07	N/A	0.76 $\pm$ 0.12	1.43 $\pm$ 0.31	N/A	0.23 $\pm$ 0.04	N/A
Experiment 6										
Chronic saline	1.26 $\pm$ 0.29	1.29 $\pm$ 0.47	N/A	-0.04 $\pm$ 0.06	N/A	1.42 $\pm$ 0.35	2.12 $\pm$ 0.45	N/A	0.19 $\pm$ 0.06	N/A
Chronic fluoxetine	1.09 $\pm$ 0.29	1.07 $\pm$ 0.28	N/A	-0.03 $\pm$ 0.04	N/A	1.82 $\pm$ 0.37	2.76 $\pm$ 0.69	N/A	0.14 $\pm$ 0.07	N/A

Abbreviations: FPS, fear-potentiated startle; NA, not applicable.

<sup>a</sup>For the sustained fear tests, startle potentiation during the first test trial after CS onset was used as a supplementary phasic fear measure.

<sup>b</sup>These rats received 2.5 mg/kg before the pre-conditioning test, but 7.5 mg/kg before the post-conditioning test.

For many years, benzodiazepines have been the drug of choice for anxiety reduction, being partly supplanted more recently by monoamine reuptake inhibitors because of the greater potential for dependency and abuse with benzodiazepines. It is perhaps surprising then that the effect of benzodiazepines on phasic startle potentiation has been inconsistent, at least in human beings, with positive (Patrick *et al*, 1996; Bitsios *et al*, 1999; Riba *et al*, 2001; Graham *et al*, 2005) as well as negative (Baas *et al*, 2002; Scaife *et al*, 2005; Grillon *et al*, 2006) results being reported, and suggestions that the positive effects that have been reported were secondary to sedative effects (Baas *et al*, 2002)—a problem that may be especially pronounced when using absolute difference scores (ie, from trials without to those with the fear stimulus) rather than percent change scores (Grillon and Baas, 2002; Walker and Davis, 2002b).

Using percent change scores, we found that the benzodiazepine chlordiazepoxide did block sustained startle potentiation, but at the same dose (10 mg/kg) did not significantly influence phasic startle potentiation using either a standard test for phasic startle potentiation (Experiment 2), or the first trial of sustained fear testing as an alternative measure (Experiment 3). It is possible of course that a higher dose *might* have been effective. Doses above 10 mg/kg are generally avoided, however, due to the emergence of 'nonspecific' behavioral effects. In fact, even at 10 mg/kg, baseline startle was reduced by approximately 50% in Experiment 2, which makes the preservation of phasic startle potentiation all the more remarkable. We do not believe that phasic startle potentiation is immune to benzodiazepine administration. Indeed, we ourselves, using a different protocol for phasic fear training (2 days of training with 10 pairings of a 3.7-s light and co-terminating footshock per day), have previously observed such effects (Davis, 1979; Walker and Davis, 2002a). However, these results, in which phasic and sustained startle potentiation data were measured after identical training and, in Experiment 3, in the same rats in the same test session in response to the same CS, seem especially compelling in showing a *differential* sensitivity. Indirect evidence for a greater sensitivity can be found in several other studies. For example, Guscott *et al* (2000) reported a statistically significant disruption of fear-potentiated startle to a 3.7-s CS by 10 but not 3 mg/kg chlordiazepoxide in rats trained and tested in different contexts, but significant effects on potentiated startle to the training context itself (sustained fear) at doses as low as 1 mg/kg (ie, 10-fold difference). In human beings, Grillon *et al* (2006) reported that startle increases to an 8-s CS (phasic fear) were not affected by the benzodiazepine alprazolam, whereas startle increases to the experimental context (sustained fear) during the same test session were significantly reduced.

It is possible that phasic startle increases are simply more robust than sustained startle increases, and therefore less sensitive to disruption by any means (ie, a quantitative rather than qualitative difference). However, this explanation would not account for the opposite pattern of results (ie, a disruption of phasic but not sustained fear) that we observed for buspirone, which we turn to next.

Buspirone, known primarily as a 5HT<sub>1A</sub> partial agonist and dopamine D2 antagonist, potently disrupts phasic startle increases in rats when administered shortly before

testing (Kehne *et al*, 1988; Mansbach and Geyer, 1988). In human beings, however, acute administration is *not* anxiolytic, and in a non-human primate model, does not reduce phasic fear (Winslow *et al*, 2007). For clinical anxiolysis, chronic administration is required (Jacobson *et al*, 1985; Goa and Ward, 1986; Goodman, 2004), suggesting that the mechanism of action for effects on phasic startle potentiation in rats (which may not involve serotonin—Davis *et al* (1988)) and for clinical efficacy in human beings may be different. In Experiment 4, we replicated the frequently reported effect observed in rats and, in Experiment 5, observed a quantitatively similar difference. In both experiments, an effect on baseline startle was also observed. These baseline effects frequently accompany (Mansbach and Geyer, 1988; Walker and Davis, 1997a) but are not required (Kehne *et al*, 1988; Melia and Davis, 1991) for buspirone effects on phasic fear.

In contrast, we saw no evidence for a disruption of sustained fear (Experiment 5). As always, it is conceivable that a higher dose might have been effective. However, we previously found that doses as low as 1.25 mg/kg (*vs* the 5 mg/kg used here) markedly disrupt phasic startle potentiation and that doses half that used here completely abolish the effect (Kehne *et al*, 1988). Note also that the effect on baseline startle provides a positive control for drug activity. Thus, we believe it unlikely that the failure to disrupt sustained startle potentiation was due to insufficient dosing. Thus, the effect of acute buspirone on sustained startle potentiation appears to reflect more accurately the effect of acute buspirone on clinical anxiety (no effect), than the effect of acute buspirone on phasic startle potentiation.

We should note that buspirone *does* disrupt light-enhanced startle, which is also a sustained increase in startle, albeit to an unconditioned stimulus (Walker and Davis, 1997a,b). Assuming that light-enhanced startle reflects anxiety, which we do, the results from that and this study suggest there may be more than one type of sustained fear (perhaps, for example, conditioned *vs* unconditioned) with different neural substrates or sensitivity to anxiolytic compounds.

As mentioned previously, chronic monoamine reuptake inhibitors have largely supplanted the use of benzodiazepines for anxiety reduction. Here, we evaluated the effect of acute and also chronic fluoxetine on phasic as well as sustained startle increases. Acute fluoxetine, which does not reduce anxiety in human beings, had no effect on phasic or sustained startle increases. In marked contrast, chronic fluoxetine did block sustained, but not phasic, increases. As indicated in Table 1, chronic fluoxetine also increased baseline startle responses in Experiment 7 and, to a lesser degree, in Experiment 6. These increases on baseline startle amplitude might be a result of actions in the spinal cord. Previous studies indicate that intrathecal administration of serotonin and other serotonin agonists increases startle amplitude, most likely by activating 5-HT<sub>1A</sub> receptors on spinal motor neurons (Davis *et al*, 1980a,b; Commissaris and Davis, 1982). It is possible that fluoxetine is increasing startle through a similar mechanism in this study. Whatever the mechanism, we considered the possibility that these baseline increases could have occluded further fear-induced increases, without affecting fear itself. However, for several reasons, we do not believe this likely. First, the same rats



*did* show potentiation to the first startle probe after CS onset (phasic fear) indicating that startle was not at a ceiling and that further increases were indeed possible. Moreover, we have found in other experiments that phasic startle increases are unaffected by much larger baseline elevations brought about by i.c.v. CRF infusions or systemic strychnine injections (Walker and Davis, 2002b). Finally, we found no evidence of a correlation between the effect on baseline startle and the effect on startle potentiation. For these analyses, and in keeping with the other analyses reported herein, we again used parametric (Pearson's) as well as non-parametric (Spearman's) techniques to correlate the baseline increase (expressed as the ratio between the post-conditioning pre-CS baseline and the pre-conditioning pre-CS baseline, or the log transformation of that ratio) with our ratio and log-transformed measures of sustained fear. Neither analysis (conducted on data from fluoxetine-treated rats only) found evidence for a relationship between these variables (Spearman's  $r = -0.113$ ,  $p = 0.64$ ; Pearson's  $r = -0.11$ ,  $p = 0.65$ ). Overall then, we are confident that the abolition of sustained startle potentiation in Experiment 7 was not an artifact of the baseline startle increase.

As noted previously, the effect of chronic fluoxetine on sustained startle increases was in contrast to the effect on phasic startle increases, which were more variable. On the basis of the explicit test of phasic fear (Experiment 6), in which startle was elicited 3.2 s after CS onset, there was no disruption at all. However, when startle was elicited 19 s after CS onset (ie, to the first probe after onset of the sustained fear stimulus in Experiment 7), startle was significantly reduced but not abolished (as was the more sustained component of potentiation in these same rats).

Disruptions of sustained fear (ie, to context CSs) by chronic (Li *et al*, 2001) or sub-chronic (Santos *et al*, 2006) selective serotonin reuptake inhibitor (SSRI) administration have previously been reported. In one study (Burghardt *et al*, 2004), chronic administration of the SSRI citalopram beginning before training also disrupted freezing to a relatively short 20-s fear stimulus. Because freezing is generally found to persist well beyond CS offset, even when elicited by phasically presented fear stimuli (Quinn *et al*, 2002), it is likely that the fear response in that study was more sustained than phasic. In human beings, Grillon *et al* (2009a) found that chronic citalopram administration had no effect on startle potentiation to an 8-s fear stimulus, but did reduce the startle potentiation that occurred between stimulus presentations (a more sustained increase which may have reflected context fear). Thus, those results may reflect the same underlying time-dependent dissociation that we have observed in rats.

In interpreting the pattern of results observed across our experiments, it is perhaps relevant that phasic fear response is, in most cases, the stronger response. This could reflect the fact that, during conditioning, a greater number of shocks were experienced by the rats during the early part of the 8-min CS. For example, using these training procedures for sustained fear, half of all footshocks occurred during the first minute of CS presentation (ie, at 3, 10, 20, and 60 s after CS onset). In developing this paradigm, we explored a number of different conditioning protocols, including one in which the ordering of shocks was reversed (ie, a mirror image of the shock schedule used here in which shock density was

greater towards the end of the CS). Invariably however, we find that startle potentiation immediately following CS onset is greater than potentiation at later times after CS onset.

In any case, and as noted earlier, the greater magnitude of phasic compared to sustained startle potentiation does allow for the possibility that chlordiazepoxide and chronic fluoxetine disrupted sustained, but not phasic potentiation, simply because sustained startle potentiation was the weaker of the two responses. This makes the results obtained with buspirone—which disrupted the stronger phasic response and not the weaker sustained response—all the more important and highly suggestive of the possibility that phasic and sustained fear are fundamentally, not just quantitatively, different.

To our knowledge, these are the first explicit comparisons of drug effects on short- vs longer-duration fear responses. On the basis of the compounds tested here, the results suggest that sustained fear paradigms may have greater predictive validity, and that the sustained fear itself may be more homologous to at least some types of clinical anxiety than phasic fear. In this regard, other findings by Grillon *et al* (2009b) are also relevant. In particular, they observed that startle potentiation to short-duration stimuli that have been paired with shock are no greater in post-traumatic stress (Grillon *et al*, 2009b) and panic disorder patients (Grillon *et al*, 2008) than in healthy controls, but that the startle responses that occur *between* stimulus presentations, which as noted earlier may reflect a more sustained type of anxiety to the less-predictive threat context, are greater (cf, Davis *et al*, 2010). These very interesting results lend support to the view that drugs that reduce sustained startle increases may be more clinically efficacious than those that preferentially reduce phasic startle increases.

The search for such compounds (ie, drugs that reduce sustained startle increases) may be aided by evidence that sustained fear, including fear responses to static contexts, is especially dependent on the BNST (eg, Walker and Davis, 1997b; Hammack *et al*, 2004; Sullivan *et al*, 2004; Waddell *et al*, 2006), whereas phasic fear responses are more dependent on the medial division of the CeA (cf, Walker *et al*, 2009b). Evidence supporting this view is derived primarily from lesion and inactivation studies, but is consistent with the results of unit recording and imaging studies in rats (Quirk *et al*, 1995) and human beings (Phelps *et al*, 2001), which have indicated only a transient activation of the amygdala by threat stimuli, but perhaps a more sustained activation of the BNST, the latter which may be exaggerated in subjects with high trait anxiety (Somerville *et al*, 2010). Perhaps, also relevant are recent results from Klumpers *et al* (2010), who reported a significant time-dependent correlation between amygdala activation and startle potentiation in healthy human beings.

One notable feature of the BNST as well as the *lateral* CeA (which projects to the BNST), which distinguishes both from the medial CeA, is the abundance of so many different neuropeptide-positive cells and terminals (Roberts *et al*, 1982; Woodhams *et al*, 1983; Cassell *et al*, 1986; Ju *et al*, 1989; Shimada *et al*, 1989; Walter *et al*, 1991; Gray and Magnuson, 1992; Arluison *et al*, 1994). Because peptides often act for long periods of time, as we have found from local BNST infusions of either CRF (Liang *et al*, 1992) or CGRP (Sink *et al*, 2011), we believe that accelerated

development of small molecule ligands for these receptors, and their evaluation in anxiety models such as the one used here, may be a prudent strategy for the development of new anxiolytic compounds with novel mechanisms of action. We have found, for example, that oral administration of the non-peptide CRF-R1 antagonist GSK876008 disrupts sustained, but not phasic startle increases to conditioned fear stimuli (Walker *et al*, 2009a, b), and also startle increases evoked directly by calcitonin gene-related peptide (CGRP) infusions into the BNST (Sink *et al* (2011) and unpublished observations), where receptors for both peptides are abundant (Skofitsch and Jacobowitz, 1985; Kruger *et al*, 1988; Chalmers *et al*, 1995; Christopoulos *et al*, 1995). We have also found that intra-BNST CGRP infusions increase anxiety measures in the plus maze, and that intra-BNST infusions of a CGRP antagonist decrease sustained startle increases produced by the predator odor 2,5-dihydro-2,4,5-trimethylthiazoline (Sink *et al*, 2011).

It is also worth noting that the BNST expresses several types of serotonin receptors, is densely innervated by serotonergic afferents (Freedman and Shi, 2001; Hammack *et al*, 2009) that in many cases project to CRF-positive BNST neurons (Phelix *et al*, 1992), and is especially enriched in serotonin transporters (Smith *et al*, 1999). These findings suggest that the BNST may be one site of action for SSRI-mediated anxiolysis. The recent observation that anxious temperament in monkeys is correlated with serotonin transporter availability in the BNST is consistent with this view (Oler *et al*, 2009).

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#### DISCLOSURE

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