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Behavioral and pharmacological validation of an integrated fear-potentiated startle and prepulse inhibition paradigm

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Abstract

Fear-potentiated startle (FPS) and prepulse inhibition (PPI) of acoustic startle are two widely used paradigms specifically designed to capture the impact of negative emotion (e.g. fear) and preattentive function on startle response. Currently, there is no single paradigm that incorporates both FPS and PPI, making it impossible to examine the potential interactions between fear and attention in the regulation of startle response. In this study, we developed an integrated FPS and PPI test protocol and validated it with psychoactive drugs. In Experiment 1, male Sprague-Dawley rats were randomly assigned to one of five groups, receiving either Light -Shock conditioning trials, non-overlapping Lights and Shocks, Light alone, Shock alone, or no Light and Shock. They were then tested for startle response and PPI concurrently, under the Light or No Light. FPS was observed only in rats subjected to fear conditioning, whereas all rats showed PPI and startle habituation. Experiment 2 used this paradigm and demonstrated a dissociative effect between diazepam (an anxiolytic drug) and phencyclidine (a nonselective NMDA receptor antagonist) on FPS and PPI. Diazepam suppressed both FPS and PPI, while PCP selectively disrupted PPI but not FPS. The diazepam's anxiolytic effect on FPS was further confirmed in the elevated plus maze test. Together, our findings indicate that our paradigm combines FPS and PPI into a single paradigm, and that is useful to examine potential interactions between multiple psychological processes, to identify the common neural substrates and to screen new drugs with multiple psychoactive effects.

Keywords

fear-potentiated startle; prepulse inhibition of acoustic startle; phencyclidine; diazepam; rat

1. Introduction

The startle response is an innate motoric response to a sudden and intense stimulus. It protects the integrity of the body and facilitates escape from potential danger. Startle can be elicited by acoustic, tactile and visual stimuli in a variety of animal species and in humans,

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although the most commonly investigated phenomenon is acoustic startle response. Because the startle response magnitude can be modulated by a variety of psychological variables, such as fear, anxiety, learning, memory and attention, it becomes a valuable behavioral tool to assess neurobiological mechanisms of psychological functions. Fear-potentiated startle (FPS) and prepulse inhibition (PPI) are two exemplar paradigms that serve this purpose [1].

Fear-potentiated startle refers to a phenomenon that the magnitude of the acoustic startle reflex is augmented in the presence of a cue (e.g., a light) that has previously been paired with a shock [2]. In the test, a neutral stimulus (termed conditioned stimulus, CS) is first paired with a shock, and then the animal's startle reflex is compared in either the presence or the absence of the CS. FPS is operationally defined by elevated startle amplitude in the presence of the CS and is supposed to measure a central state of fear. This paradigm is thus often used to study the neurobiology of conditioned fear and for the identification of potential anxiolytic drugs [3]. For example, using FPS, Davis and his colleagues suggested that the central and basolateral amygdala play a critical role in the mediation of fear learning and memory, as lesions of the central, or the basolateral nucleus of the amygdala block the impacts of conditioned fear on the startle response [4]. Psychopharmacological studies document that anxiolytic drugs that reduce overall excitability of the CNS, such as ethanol or diazepam, but not other classes of psychoactive drugs, have a common effect of attenuating the CS-elicited enhancement of startle response and having no effect on the baseline startle magnitude [4–7]. This feature has been used to identify potential anxiolytic drugs.

PPI is developed to study the sensorimotor gating ability, a preattentive process that is involved in early stages of information filtering [8]. PPI is observed when the amplitude of startle reflex elicited by an intense startling stimulus (e.g., 120 dB white noise) is reduced when the startle stimulus is immediately preceded by a weak prepulse (e.g. 80 dB white noise). PPI has often been used to study attention deficits associated with severe mental disorders such as schizophrenia and to screen potential antipsychotic drugs. For example, it has been widely used as a translational model of schizophrenia [9, 10], as animals treated with psychotomimetic drugs, such as amphetamine (a potent psychostimulant targeting monoamine transporters), quinpirole (a $D_{2/3}$ agonist), PCP (a nonselective NMDA antagonist) or MK-801 (a nonselective NMDA antagonist) also exhibit PPI deficits [11–15]. Further, it has also been successfully used to screen chemical compounds with potential antipsychotic activity [16, 17], to rank order clinical potency of approved antipsychotics [9], and to differentiate atypical from typical antipsychotics [18].

Because each paradigm serves relatively independent purposes, FPS and PPI are often used separately. To the best of our knowledge, there is no paradigm that incorporates the fear-potentiated component with the PPI component. However, such a combined paradigm would have some advantages. For example, it would allow an examination of interactive effects of fear and attention on the regulation of startle response, while a single paradigm is unable to do. Furthermore, the integrated paradigm is useful for identifying the shared neural substrates underlying conditioned fear (as measured in FPS) and attentional filtering ability (as measured in PPI). For instance, lesions of the basolateral amygdala is found to disrupt FPS and PPI in separate tests [4, 19]. The new paradigm would be able to corroborate this

finding in a single test. Finally, such an integrated paradigm would be efficiently used to identify novel compounds possessing dual anxiolytic and antipsychotic property in a single test. It also better serves as a behavioral model of severe mental disorders because most disorders have abnormal functions in multiple domains. In the present study, we report development of an integrated FPS and PPI test protocol by demonstrating the effectiveness of this paradigm in recording conditioned fear and sensorimotor gating ability simultaneously. In addition, we provide pharmacological validation showing that two psychoactive drugs (PCP and diazepam) maintain their selective and dissociative effects on FPS and PPI.

2. Materials and Methods

2.1 Subjects

Male adult Sprague-Dawley rats (~ 2–3 months old, Charles River, Portage, MI) were housed two per cage (30.48 cm × 29.21 cm × 17.78 cm), with food and water available *ad libitum*. The colony was under a 12/12h light/k cycle (lights on from 7:00 am to 7:00 pm), with temperature maintained at 22±1° and humidity around 32%. Experiments were conducted during the light portion of the cycle. All procedures were approved by the animal care committees at the University of Nebraska-Lincoln.

2.2 Startle and elevated plus maze apparatus

Six startle monitor systems (Kinder Scientific, Julian, CA) controlled by a PC were used. They were housed in compact sound attenuation cabinets (35.56 cm wide × 27.62 cm deep × 49.53 cm high). A speaker (diameter: 11 cm) mounted on the cabinet's ceiling was used to generate acoustic stimuli. During tests, rats were placed in a restrainer (17.2 cm long × 9.0 cm wide) with an adjustable ceiling positioned atop the box, providing only limited restraint while prohibiting ambulation. The startle response was measured by a piezoelectric sensing platform on the floor in a time window of 100 ms, beginning at the onset of the startle-eliciting stimulus (pulse). The peak value within the record window indexes the magnitude of the startle response. The CS (light) was delivered by an E light Bulb (18V, 6W, Eiko 40717) mounted on the cabinet's ceiling.

The EPM consisted of two open arms (50 cm × 10 cm), two enclosed arms (50 cm × 10 cm) and a central platform (10 cm × 10 cm) made of black Plexiglas. Each arm was supported by a sturdy plastic leg and was elevated 70 cm above the floor. The two enclosed arms had high walls (40 cm in height), while the two open arms had raised edges (1.0 cm in height) along each side and end to decrease the possibility of falling during drug testing.

2.3 Drugs

Diazepam (Sigma-Aldrich, St. Louis, MO) was dissolved in 30% N,N-Dimethylformamide (DMF, Sigma-Aldrich). Phencyclidine hydrochloride (PCP, a gift from NIDA Chemical Synthesis and Drug Supply Program [RTI, Research Triangle Park, NC]) was dissolved in 0.9% saline. Diazepam was injected subcutaneously (s.c.) and PCP intraperitoneally (i.p.).

2.4 Experiment 1: Behavioral validation of an integrated fear-potentiated startle and prepulse inhibition paradigm

Experiment 1 systematically evaluated the effectiveness of the integrated FPS and PPI paradigm to simultaneously record conditioned fear and sensorimotor gating ability. Forty adult male rats were randomly assigned to five groups (n = 8/group): the CS+, the CS−, the Light-only, the Shock-only, or no-Light-Shock group. They only differed on the fear conditioning day. The overall experimental procedure consisted of the following four phases: Baseline tests of startle and PPI, Fear conditioning, Post-conditioning retests of startle and PPI, and Fear-potentiated startle and PPI test (Fig. 1).

Baseline tests of startle and PPI (Day 1 and Day 2)—Rats were first habituated to the startle chambers for 20 min under 70 dB background noise (Day 0), then they were tested for their baseline startle response and PPI daily for 2 days. Each daily session started with a 5-min period acclimatization with 70 dB background noise, followed by four mixed trial types: PULSE ALONE trials (105 dB white nose, 40 ms), and three types of PREPULSE+PULSE trials (a 20 ms 75, 78, or 82 dB prepulse, followed 100 ms later by the PULSE). Each trial type occurred 10 times, and a total of 40 trials occurred in a pseudorandom order. At the beginning and end of each session, 4 additional PULSE ALONE trials were added to examine startle response habituation and they were not used in the calculation of PPI. The inter-trial interval ranges from 25 to 35 s (average 30 s). Startle magnitude was defined as the maximum force (measured in Newtons) applied by the rat to the startle apparatus recorded over a period of 100 ms beginning at the onset of the PULSE stimulus.

Percent prepulse inhibition (% PPI) for each acoustic prepulse trial type was calculated using the standard PPI equation: [100−(mean PREPULSE+PULSE response/mean PULSE response) × 100]. Percent startle habituation (% habituation) was calculated as: 100−(averaged startle magnitude for the final 4 PULSE trials/averaged startle magnitude for the first 4 PULSE trials) × 100, i.e., the higher the % value, the greater the habituation.

Fear conditioning (Day 3)—Fear conditioning session was conducted in the same cabinets where the rats were tested for the startle and PPI, with the same background noise (70 dB). Rats in the CS+ group received 10 CS (3.7 s light) – US (0.6 mA, 0.5 s footshock) pairing trials, with an average inter-trial interval of 4 min (3 min to 5 min). The CS− group received the same number of light and footshock trials, but they were presented randomly and never overlapped. The Light-only group received 10 light presentations with no shock, while the Shock-only group received 10 footshocks but no light. Finally, the no-Light-Shock group was not exposed to any light or footshock.

Post-conditioning retests of startle and PPI (Day 4 and Day 5)—Rats were tested again for startle response and PPI using the same procedure as used in the baseline tests for 2 days. Changes from the baseline tests could potentially reveal the impact of contextual fear on startle response and PPI.

Fear-potentiated startle and PPI test (Day 6)—The test procedure was similar to that used in the baseline startle response and PPI test with the exception that the CS (3.2 s) was presented in 40 trials (10 PULSE ALONE trials and 10 trials of each PREPULSE+PULSE type), while in another 40 trials, no CS was presented. In the CS presented trials, the CS was initiated 3.2 s before the onset of the PULSE (40 ms), and co-terminated with the PULSE. These 80 trials were intermixed and presented in a random fashion, with an average inter-trial interval of 30 s. The magnitude of startle response (mean of the 10 PULSE ALONE trials) and PPI under both CS and No CS conditions were recorded and calculated separately.

2.5 Experiment 2: Pharmacological validation of an integrated fear-potentiated startle and prepulse inhibition paradigm

This experiment validated this integrated fear-potentiated startle and prepulse inhibition paradigm using pharmacological tools. Thirty adult male SD rats were randomly assigned to one of the three groups (n = 10/group): Diazepam (2.0 mg/kg), PCP (1.5 mg/kg), and vehicle control (5 on 30% DMF and 5 on 0.9% saline). Rats were first handled for five consective days. On the last day of handling, they were injected with either 30% DMF (i.p.) or saline (s.c.) based on the solvent that they were given on the test to habituate them to the injection procedure. The FPS and PPI procedure was identical to that of Experiment 1, consisting of the 4 phases: Baseline tests of startle and PPI, Fear conditioning, Post-conditioning retests of startle and PPI, and Fear-potentiated startle and PPI test. Diazepam, PCP or vehicle was administered 10 min before the final FPS and PPI test.

Eight days after the test, all rats were tested in the EPM (averaged light intensity for open arms: 83 lux; averaged light intensity for close arms: 9 lux) in a separate room. On the test day, rats were first brought to the experimental room and habituated to the test environment for at least 30 min. Each rat was injected with diazepam, PCP, or vehicle first. Thirty min later, they were placed in the center of the maze facing an open arm and allowed to freely explore the maze for 5 min. All experimental sessions were recorded by a video camera. Time in open arm and close arm was obtained using Viewer II (Biobserve), and percent (% open arm time) in open arms was calculated as: 100 × time spent in the open arms/total time. An entry to an arm was defined as the all four feet entering the arm and % open arm entry was calculated as: 100 × number of entries to the open arms/number of entries to all arms. Three rats from the diazepam group fell off the maze, their data were excluded for further analysis.

2.6 Statistical analysis

All data are expressed as mean ± SEM. Percent PPI data on different test days are presented separately. Percent startle habituation on the two baseline test days before conditioning and two days after conditioning are combined and averaged and presented separately from those on the final test day. In all experiments, there were highly signficiant prepulse intensity effects (75, 78 and 82 dB Prepulse levels). For clarity, only averaged % PPI is reported in the text. Averaged % PPI and the startle response data were analyzed using repeated measures ANOVAs with group (CS+, CS−, Light-only, Shock-only, no-Light-Shock) (Experiment 1) and drug (PCP, Diazepam, Vehicle) (Experiment 2) as between-group factors, and test day or

test condition (CS vs. No CS) as within-subjects factors followed by post hoc Bonferroni test. Percent changes of startle magnitude and averaged % PPI between the CS and No CS conditions are additionally analyzed using nonparametric tests if normal distrubtion was violated. Percent startle habituation was analyzed by one-way ANOVA. For the EPM test, one-way ANOVA was conducted on % open arm time and % open arm entries separately. If the sphericity assumption was not valid, then Greenhouse-Geisser correction would be used. Statistical significance is defined as $p < 0.05$. All data were analyzed using SPSS (v. 22).

3. Results

3.1 Experiment 1: Behavioral validation of an integrated fear-potentiated startle and prepulse inhibition paradigm

3.1.1 Baseline startle response and PPI—The five groups did not differ on the magnitude of baseline startle response on both days. Repeated measures ANOVA showed no significant main effect of day, $F(1, 35) = 0.563$, $p = 0.458$, group, $F(4, 35) = 1.099$, $p = 0.372$, nor the day × group interaction, $F(4, 35) = 0.227$, $p = 0.922$ (Fig. 2A). The five groups also did not show any significant group difference on the averaged PPIs, as the main effect of group, $F(4, 35) = 0.986$, $p = 0.428$, day, $F(1, 35) = 2.141$, $p = 0.152$, and the day × group interaction, $F(4, 35) = 0.319$, $p = 0.863$ were not significant (Fig. 2B).

3.1.2 Changes in startle and PPI responses from Day 2 (pre-conditioning) to Day 4 (post-conditioning)—Comparisons between the startle responses of the five groups one day before the fear conditioning (Day 2) and one day after fear conditioning (Day 4) were made in an attempt to reveal potential contexutal fear on baseline startle response. Repeated measues ANOVA found no significant main effect of group, $F(4, 35) = 1.014$, $p = 0.413$, day, $F(1, 35) = 0.801$, $p = 0.377$, or test day × group interaction, $F(4, 35) = 1.226$, $p = 0.318$. The main effect of day was also not significant, $p = 0.377$ (Fig. 2C). Similarly, the five groups were compared for their PPIs one day before conditioning and one day after conditioning. Once again, there was no significant main effect of group, $F(4, 35) = 1.141$, $p = 0.353$, day, $F(1, 35) = 3.322$, $p = 0.077$, or test day × group interaction, $F(4, 35) = 1.637$, $p = 0.187$ (Fig. 2D).

3.1.3 Fear-potentiated startle and PPI test (Day 6)—On the fear-potentiated startle test day, only rats that were subjected to the light-footshock conditioning (the CS+ group) showed a higher startle response under the CS (light) condition compared to the No CS (dark) condition, indicating that only this group exhibited fear-potentiated startle. Repeated measures ANOVA revealed a condition (Light vs. No Light) × group interaction, $F(4, 35) = 3.023$, $p = 0.031$. Paired samples test showed that the CS+ group exhibited a significantly higher startle response under the CS condition than under the No CS (dark) condition, $p = 0.004$ (Fig. 3A). Other groups did not show any significant effect of condition, all $p > 0.289$.

Because data of the CS− group under the CS condition were not normally distributed, to further determine the effect of CS on startle response, we calculated the percent change of startle under the CS condition compared to the no CS condition using the following formula: [(startle magnitude under the CS condition - startle magnitude under the No CS condition)/ startle magnitude under the No CS condition] × 100 (see Fig. 3B). We tested group

differences using the nonparametric Kruskal-Wallis test. This analysis shows that the five groups were still significantly different from each other, $p = 0.034$. Further pairwise-comparison shows that only the CS+ group had higher percent increase in startle response under the CS condition compared to the other four groups (all $p < 0.045$, unadjusted sig.). We also did the paired-samples t-tests and found that only the CS+ group showed an increase of startle magnitude (noted by positive value of percent change of startle magnitude compared to zero), $t(7) = 3.225$, $p = 0.014$.

Although there was no group difference in PPI under either the CS or No CS condition, PPI was significantly lower under the CS (light) condition than under the No CS (dark) condition, suggesting that presence of a visual stimulus interfered with attentional process to an auditory stimulus. Three-way ANOVA confirmed this observation. There was a main effect of CS condition, $F(1, 35) = 4.495$, $p = 0.041$, but no main effect of group, $F(4, 35) = 2.345$, $p = 0.074$, or condition × group interaction, $F(4, 35) = 2.571$, $p = 0.055$, and the observed power was 0.663 (Fig. 3C).

Because the averaged PPI data of the Light group under the No CS condition were not normally distributed, in order to determine whether the presence of the CS affected the PPI, we calculated the percent change of averaged PPI using this formula: [(averaged PPI under the CS condition − averaged PPI under the No CS condition)/averaged PPI under the No CS condition] × 100 (Fig. 3D). This transformed dataset from the five groups were normally distributed. One-way ANOVA shows the group difference was significant, $F(4,35) = 2.842$, $p = 0.039$, and that the Light group was different from the CS+ group, $p = 0.020$. Five separate paired-sample t-tests were conducted, and only the Light group showed a decrease of averaged PPI, $t(7) = -4.256$, $p = 0.004$.

3.1.4 Startle response habituation—There appear to be a lot of individual variances in % startle habituation. However, all five groups, as a whole showed a habituation effect (i.e. an decrease in averaged startle magnitude from the first 4 trials at the beginning of each session to the final 4 trials at the end of each session) and they did not differ from each other on the 4 test days prior to and afer fear conditioning (Fig. 4A) (one-way ANOVA, $F(4, 35) = 1.967$, $p = 0.121$). The five groups also did not differ on % startle response habituation on the CS test day, as one-way ANOVA showed the group effect was not significant, $F(4, 35) = 2.243$, $p = 0.084$, and the observed power was 0.595 (Fig. 4B).

3.2 Experiment 2: Pharmacological validation of an integrated fear-potentiated startle and prepulse inhibition paradigm

3.2.1 Baseline startle response and PPI—As expected, the three groups did not differ on the magnitude of startle response on both days, as repeated measures ANOVA showed no significant main effect of day, $F(1, 27) = 0.087$, $p = 0.770$, group, $F(2, 27) = 1.410$, $p = 0.262$, nor the day × group interaction, $F(2, 27) = 1.605$, $p = 0.219$ (Fig. 5A). They also did not differ on the averaged PPIs, as the main effect of group, $F(2, 27) = 1.762$, $p = 0.191$, and day × group interaction, $F(2, 27) = 0.110$, $p = 0.896$ were not significant (Fig. 5B). However, the main effect of day was significant, $F(1, 27) = 6.671$, $p = 0.016$, showing an improvement of PPI over time, a finding consistent with our previous reports [15, 20].

3.2.2 Changes in startle and PPI responses from Day 2 (pre-conditioning) to Day 4 (post-conditioning)—To examine whether fear conditioning altered startle response and PPI perfromance, repeated measues ANOVA was used to compare startle and PPI on Day 2 and Day 4. On the startle response, no significant main effect of day, $F(1, 27) = 1.082$, $p = 0.308$, group, $F(2, 27) = 2.054$, $p = 0.148$, or test day × group interaction, $F(2, 27) = 0.460$, $p = 0.636$ was found (Fig. 5C). Similarly, for the PPI performance, there was no significant main effect of group, $F(2, 27) = 3.009$, $p = 0.066$ (the observed power was 0.535), day, $F(1, 27) = 3.040$, $p = 0.093$, nor day × group interaction, $F(2, 27) = 0.033$, $p = 0.967$ (Fig. 5D).

3.2.3 Fear-potentiated startle and PPI test (Day 6)—On the fear-potentiated startle test day, while the PCP and control groups all showed enhanced startle response under the CS (light) condition relative to the No CS (dark) condition, this fear-potentiated startle was abolished by diazepam treatment. A CS condition × group repeated measures ANOVA revealed a main effect of CS condition (CS vs. No CS), $F(1, 27) = 44.627$, $p < 0.001$, and the significant group × CS condition interaction, $F(2, 27) = 6.207$, $p = 0.006$, but no main effect of group, $F(2, 27) = 2.100$, $p = 0.142$. Further comparisons revealed that the PCP and the vehicle control groups expressed enhanced startle response under the CS condition in comparison to the No CS condition, $p < .001$, while the diazepam group did not, $p = 0.296$ (Fig. 6A).

On the PPI measures, both the diazepam and PCP groups showed lowered PPI compared to the control group. Once again, the averaged PPI was lower in the CS trials than in the No CS trials. Repeated measures ANOVA found a main effect of group, $F(2, 27) = 4.822$, $p = 0.016$, and CS condition, $F(1, 27) = 8.039$, $p = 0.009$, but no group × condition interaction, $F(2, 27) = 0.587$, $p = 0.563$. Post hoc analysis revealed that the PCP group had significantly lower PPI compared to the control group, $p = 0.026$. The diazepam group also showed a reduction of PPI, $p = 0.039$ (Fig. 6B).

3.2.4 Startle response habituation—Like what is seen in Experiment 1, large individual variances in % startle habituation were observed. The groups were compared on the averaged four baseline test startle response hibitation, and they did not differ from each other on this measure (Fig. 7A) ($F(2, 27) = 0.866$, $p = 0.432$). They also did not differ on the CS test day ($F(2, 27) = 0.332$, $p = 0.721$) (Fig. 7B).

3.2.5 Elevated Plus Maze—To confirm the anxiolytic effect of diazepam, rats were tested in the classic EPM test. Diazepam appeared to have increased time spent on the open arms. One-way ANOVA showed that the three groups differed on the percent time spent on the open arms, $F(2, 24) = 5.436$, $p = 0.011$. Post hoc analyses found that the diazepam group spent relatively more time in the open arms than the vehicle group and PCP group, $p = 0.012$ (vs. PCP group) and $p = 0.047$ (vs. Control group) (Fig. 8A). Furthermore, the three groups also differed on % open arm entries, $F(2, 27) = 6.544$, $p = 0.005$, with the diazepam group showing higher % open arm entries than the other two groups, $p = 0.010$ (vs. PCP group) and $p = 0.017$ (vs. Control group). These finding confirmed the anxiolytic effect of diazepam (Fig. 8B).

4. Discussion

Here we introduce a new protocol that successfully and simultaneously records PPI and FPS and provides pharmacological validation of such a protocol. Specifically, in Experiment 1, we showed that the enhanced startle response under the Light condition relative to the no Light condition reflects conditioned fear, as only rats that were subject to the CS-US conditioning showed this enhanced startle elicited by Light, but not those who only experienced the CS alone, or the US alone, or non-paired CS and US or not experienced the CS or US at all. More importantly, all rats displayed PPI and the overall magnitude was no different from what have reported in the literature (40–60%) and reported by us [15, 20, 21]. Experiment 2 used this paradigm and revealed some known psychotropic effects of diazepam and PCP: diazepam suppressed FPS, while PCP selectively disrupted PPI but not FPS. The diazepam EPM data were consistent with the effect of diazepam on negative emotions (e.g. fear or anxiety). Moreover, this new paradigm reveals a process pertaining to an across-modality interference on attentional processing, as PPI was found to be significantly lower under the CS (Light) condition than under the No CS (dark) condition. In addition, our paradigm reveals a subtle yet significant suppressive effect of diazepam on PPI. Overall, this study illustrates the power of an integrated FPS and PPI paradigm in detecting multiple psychological processes and their potential interactions. It is useful for screening new compounds with multiple psychoactive effects and for the study of neurobiology of negative emotions and attention.

One unique aspect of our new paradigm is that its FPS component follows almost the exact same procedure as typically employed in a FPS alone experiment [3, 22]. This feature allows easy comparison and validation of FPS as measured in this study with that in FPS alone studies. First, animals are subject to a Pavlovian fear conditioning procedure involving 10 pairings of a 3.7 sec light with a 0.5 sec electric footshock of moderate intensity (0.6 mA) presented 3.2 sec after the light onset. Three days after conditioning, the animals undergo a test in which acoustic startle stimuli are presented during the presentation of the light (CS trials) or in the absence of the light (No CS trials) and the differences in startle magnitude between CS and No CS trials provide the operational measure for conditioned fear [1, 23]. We are confident that FPS measures conditioned fear because only the conditioned group (the CS+ group) showed the significant differences in startle magnitude between CS and No CS trials. The Light itself did not elicit startle response, and the startle-eliciting stimulus (i.e. PULSE) was never paired with a shock. Furthermore, the shock itself or separated Light and shock presentation did not cause FPS. Therefore, inclusion of 5 experimental conditions allows us to determine unequivocally that the temporally associated light and shock is responsible for the elevated startle amplitude in the presence of the light previously paired with shock. This feature of our new paradigm ensures an easy comparison of results from the present study to those in the literature. Indeed, the revealed suppressive effect of diazepam on the expression of FPS is consistent with the known anxiolytic effect of diazepam in a variety of behavioral tests of anxiety [24–27]. We recently conducted a separate experiment to examine how diazepam administered prior to the fear conditioning session would affect the acquisition of conditioned fear as measured in FPS. Our results showed that diazepam did not affect neither FPS nor PPI when given 10 min prior to the

conditioning session (Figure 9), as opposed to the test session (in the present study). Together with the present study, these findings suggest that this new paradigm is not only capable of detecting anxiolytic effect of a drug, but also useful to identify the specific processes that are affected by various pharmacological manipulations.

In both experiments, we observed that averaged PPI was significantly lower under the CS (light) condition than under the No CS (dark) condition (Fig. 3B and 6B). We interpreted this finding as reflecting an across-modality interference (visual to auditory) on attention to sensory processing, as if the presence of a visual stimulus interfered with attentional processing of an auditory stimulus. This interference appears in all experimental conditions even when the light was not paired with shock or simply appeared for the first time, indicating that the main source of this interference comes from attentional modulation of auditory sensory processing by a visual stimulus. It is possible that the onset of light might disrupt attention (e.g. alerting and/or orienting) to process the prepulse, thus interferes with its ability to reduce startle response. Another source, although not apparent, may come from the modulation of attention by negative emotion (e.g. conditioned fear) [28]. The light, after becoming a CS due to its pairing with shock, may activate a central state of fear. Evidence suggests that negative emotion can alter attention [29, 30]. For example, it has been reported that PPI is enhanced when the prepulse is emotionally salient rather than neutral stimulus in humans [31]. In rats, following the prepulse becoming fear conditioned or fear-extinction conditioned, its strength in inhibiting the startle reflex is enhanced, resulting an enhanced PPI [32]. These studies suggest that when the prepulse becomes biologically significant, more attention resource is directed toward processing its occurrence. The present finding that a fear-conditioned stimulus could interfere with PPI provides another example that emotional learning (fear conditioning) indeed top-down modulates sensory gating. However, we think the impact of this source on PPI is relatively small. This is because we did not observe a larger decrease in PPI in the CS+ group than in other groups, which should be expected if the main source of Light impact is from conditioned fear. Regardless of the exact sources, the mere observation of this across-modality interference on PPI performance is an interesting finding. We are not aware of any similar finding in the literature. If replicated and confirmed in the future studies, it could be used as a behavioral measure of conflict in sensory processing.

In the present study, we also compared changes in startle magnitude and PPI before and after fear conditioning in an attempt to reveal the potential impact of putative contextual fear on startle response and PPI. We hypothesized that rats might develop a contextual fear to the testing environment, in addition to acquiring a conditioned fear to the CS. If there were a contextual fear, we would expect to see higher startle responses one day after the conditioning relative to one day before. Our results did not support this hypothesis. The lack of contextual fear might be due to the lack of salient environmental cues and loss of novelty of the fear conditioning environment. In our studies, rats were habituated to the environment for 3 days. The startle chamber does not contain any distinct sensory cues. On the conditioning day, the CS was presented in this familiar environment. Previous work has shown that under this testing procedure, the animal is better able to learn the CS-US association because the context is not as accurate a predictor of shock as the CS[33] and conditioning to the explicit cues is easier than conditioning to the experimental context [34].

The second reason that we failed to observe contextual fear might be that our startle response is not as sensitive as freezing in detecting contextual fear.

The disruptive effect of PCP on PPI and the decreasing effect of diazepam on FPS are two well documented findings in the literature [15, 20, 26, 35–37]. The fact that we observed both effects in our new paradigm suggests that this paradigm is just as good as the PPI alone or FPS alone paradigm. The PPI-disruptive effect of diazepam was a little bit surprising, given that benzodiazepines are not known for their effect on PPI [38]. However, such a finding does exist. For example, Depoortere et al. (1997) examined three doses of diazepam (1.0, 3.0 and 10.0 mg/kg) and found that diazepam at the highest dose reduced PPI [39]. Silva et al. (2005) also reported a similar finding with 4.0 mg/kg diazepam [40]. Other studies in the literature either report that diazepam does not affect PPI [41] or improve it [42]. Taken together, it appears that diazepam's effect on PPI may depend on specific experimental conditions (e.g. number of trials, intensity of prepulse, animal strains, etc.) and remains inconclusive. Therefore, caution needs to be exercised when the PPI effect of diazepam is evaluated.

Our new integrated FPS and PPI testing protocol has important applications for behavioral neuroscience and pharmacological research. For basic behavioral neuroscience research, it has an advantage in the study of various psychological processes involved in Pavlovian conditioning (e.g. acquisition, consolidation, reconsolidation, retrieval, etc.) and attention regulation of sensory processing, as well as their interactions. This paradigm could also be easily used to study experimental factors that influence FPS and PPI and evaluate animal models of neuropsychiatric disorders with a fear and attention deficit component (e.g. posttraumatic stress disorder, schizophrenia, etc.). One other use of this paradigm is to explore effective behavioral techniques that could suppress fear and improve attention function. This work would expand our knowledge about the factors that control human fear and attention, and may help develop more effective techniques for the suppression of pathological fear in humans and improvement of cognition. From the neurobiological perspective, this integrated paradigm could be used to identify neurochemical and neuroanatomical substrates that mediate basal startle, FPS and PPI, as well as their interactions. The glutamatergic, dopaminergic and GABAergic neurotransmission in the amygdala-striatal network may play an important role in this regard [1, 4, 29, 43]. Pharmacologically, our new paradigm could be used to quickly screen chemical compounds with dual anxiolytic and cognitive-improving properties and identify relevant neurochemical mechanisms [44]. It could also be used to evaluate potential side effects of a drug on emotion regulation and attention. Diazepam is an example: although it suppresses conditioned fear, it causes a slight PPI deficit.

In conclusion, the present study shows that our integrated paradigm is capable of studying FPS and PPI simultaneously. In addition, it could be used to study an across-modality interference of PPI. This new paradigm is also capable of distinguishing different classes of psychoactive drugs and could be useful for the examination of potential interactions between multiple psychological processes, to identify the common neural substrates and to screen new drugs with multiple psychoactive effects.

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Highlights

1. Fear-potentiated startle and prepulse inhibition were shown in a single paradigm.
2. Prepulse inhibition of acoustic startle was lower in the presence of a light.
3. This integrated paradigm was validated by diazepam and phencyclidine.

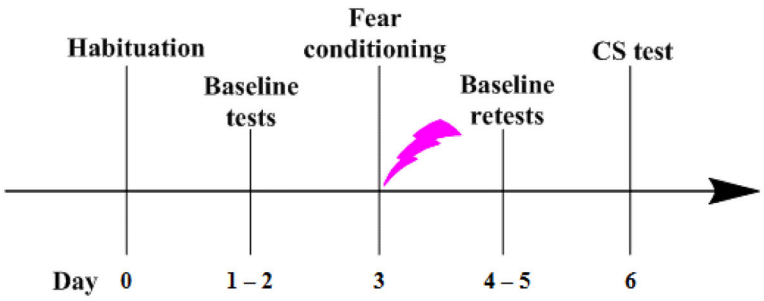

Fig. 1.
A schematic depiction of the experimental procedures used in Experiments 1 and 2.

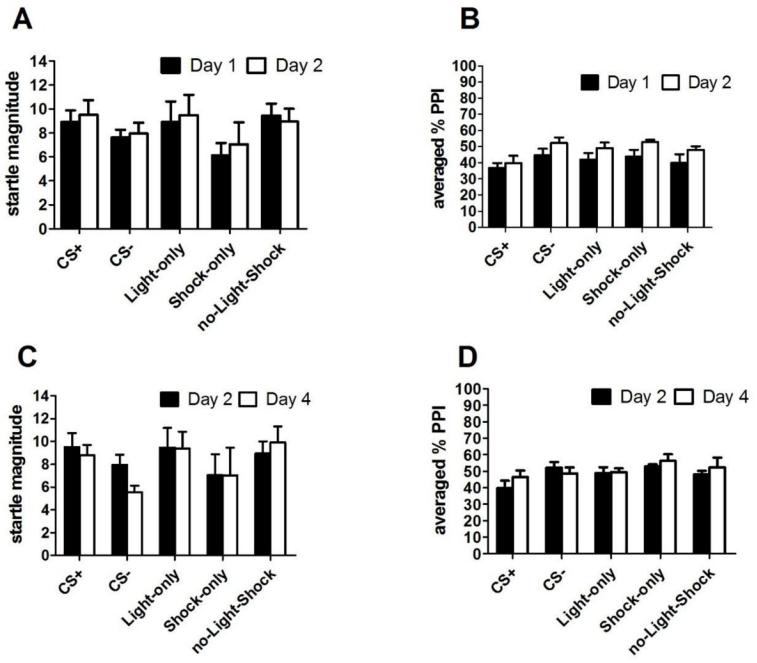

Fig. 2.

Baseline startle response and PPI. The basal startle response (**A**) and averaged PPI (**B**) before conditioning (Day 1 and Day 2) did not differ among the five experimental groups. Comparison of the startle response (**C**) and averaged PPI (**D**) one day before (Day 2) and one day after conditioning (Day 4) also did not reveal any group difference.

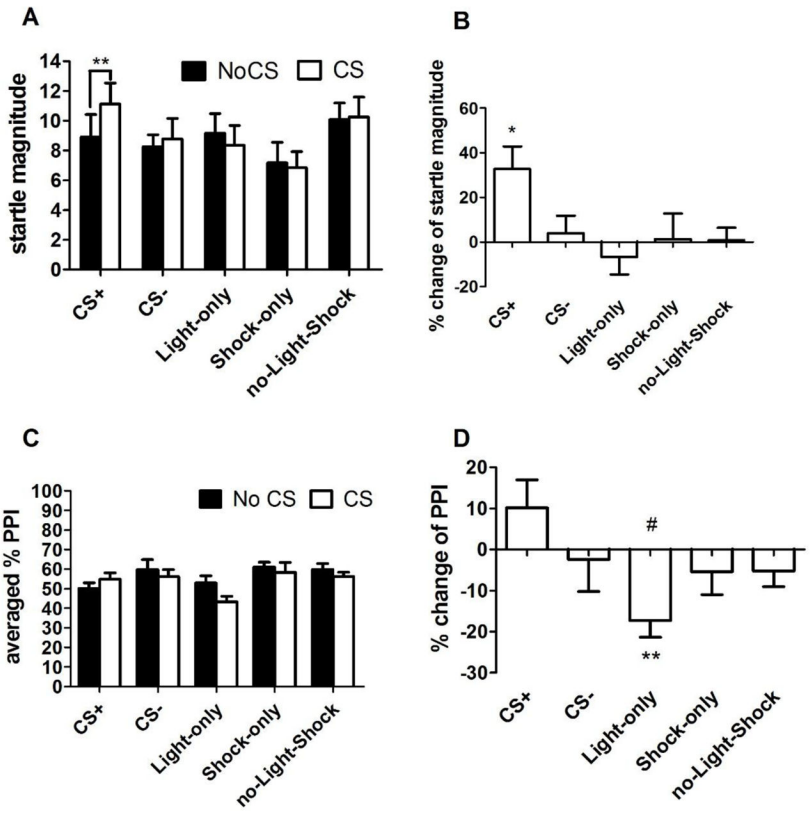

Fig. 3.

The fear-potentiated startle and PPI tested under the CS (Light) and no CS (dark, no Light) condition. The fear-potentiated startle (i.e., a significant difference in startle response under the CS versus no CS condition) (**A & B**) was observed only in the CS+ group. Averaged PPI was higher under the No CS condition than under the CS condition (**C**). However, the percent change of averaged PPI was only shown significant in the Light group (**D**). #$p < 0.05$ in comparison to the CS+ group. *$p < 0.05$; **$p < 0.01$.

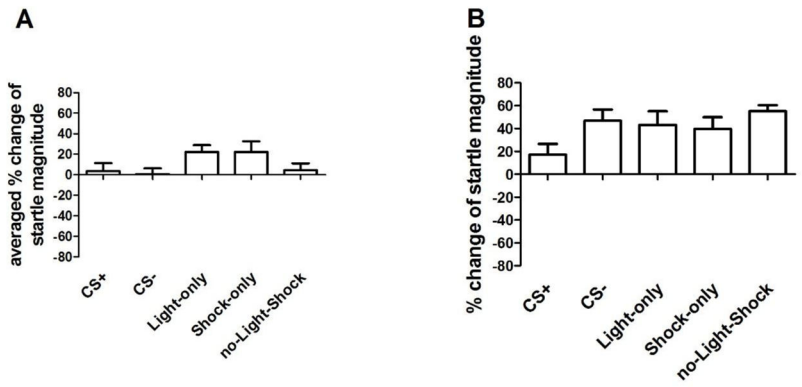

Fig. 4.

Habituation of startle response (percent change from the last 4 PULSE trials to the first 4 PULSE trials). The averaged percent change of startle magnitude from the four baseline tests did not differ among the five groups (**A**). They also did not differ on this measure the final fear-potentiated startle and PPI test (**B**).

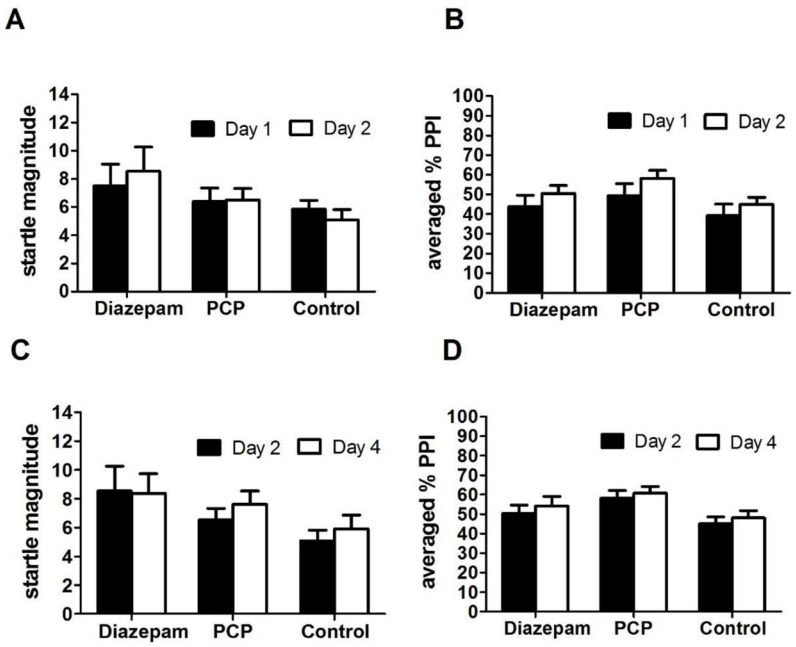

Fig. 5.

Baseline startle response and PPI. The basal startle response (**A**) and averaged PPI (**B**) before conditioning (Day 1 and Day 2) did not differ among the three experimental groups. Comparison of the startle response (**C**) and averaged PPI (**D**) one day before (Day 2) and one day after conditioning (Day 4) also did not reveal any group difference.

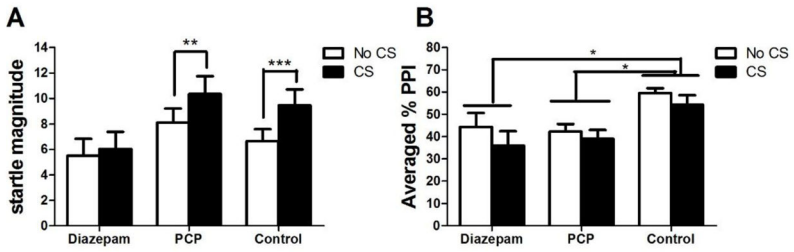

Fig. 6.

The fear-potentiated startle and PPI tested under the CS (Light) and no CS (dark, no Light) condition. The fear-potentiated startle (i.e., a significant difference in startle response under the CS versus no CS condition) (**A**) was observed in the PCP and control groups, but not in the diazepam group. Averaged PPI was significantly lower in the PCP and diazepam groups compared to the control group. *p < 0.05, **p < 0.01, ***p <0.001.

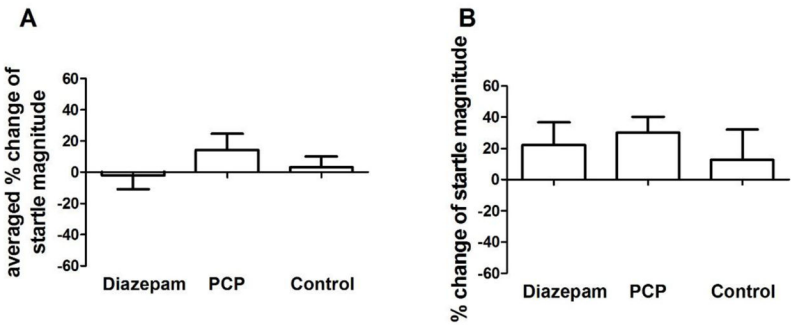

Fig. 7.

Habituation of startle response (percent change from the last 4 PULSE trials to the first 4 PULSE trials). The averaged percent change of startle magnitude from the four baseline tests did not differ among the three groups (**A**). They also did not differ on this measure the final fear-potentiated startle and PPI test (**B**).

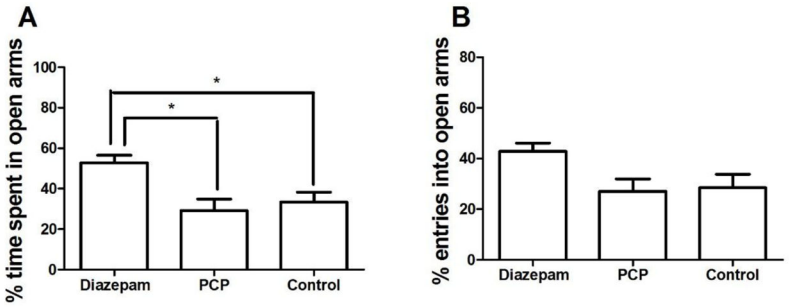

Fig. 8.

Percentage of time spent in the open arms (**A**) and percentage of open arm entries (**B**) by the three drug groups. Only the diazepam group showed a significant increase in the percentage of time spent in the open arms, * $p < 0.05$.

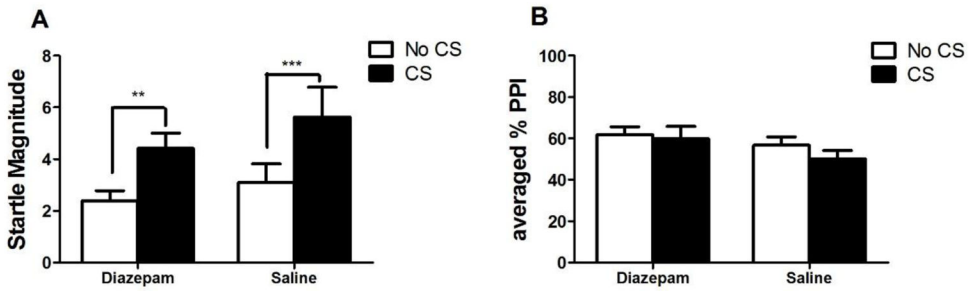

Fig. 9.

The fear-potentiated startle and PPI tested under the CS (Light) and no CS (dark, no Light) condition. Diazepam (2.0 mg/kg, i.p.) was administered 10 min prior to the fear conditioning session. The fear-potentiated startle (**A**) and averaged PPI (**B**) were not affected by diazepam treatment. **p < 0.01, ***p <0.001.