

**Corticosterone as a Predictor of Long-term Outcomes of Fear Attenuation Treatment**

by

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## Abstract

Blunted adrenal corticosteroid levels and dysregulation of the Hypothalamic Pituitary Adrenal (HPA) Axis are known risk factors for developing PTSD (Delahanty et al., 2000; McFarlane et al., 1997). Although other risk factors for PTSD and disorders involving conditioned fear exist, Kimble et al. (in preparation) have suggested that CS preexposure may serve as a protective measure against fear development and relapse of conditioned fear in an animal model. Although the mechanisms are not entirely clear, research has suggested that preexposure may change the activity of the HPA axis. For example, rats repeatedly exposed to a cue prior to fear conditioning exhibit more HPA activation than animals that were not preexposed to the cue, as evidenced by elevated heart rate and blood pressure (e.g., Zhang et al., 2004). Here, we attempted to determine whether CS preexposure affects HPA axis activity as measured through circulating corticosterone (Experiment 1) and if depressing the HPA axis through dexamethasone alters the CS preexposure effect.

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## **Introduction**

Posttraumatic stress disorder (PTSD) is a growing mental health concern worldwide. The National Institutes of Health (2009) estimate that over 7.7 million Americans suffer from PTSD in a given year. This translates to a lifetime incidence rate of PTSD of about 7% (Kessler, Berglund, Demler, Jin, Merikangas, & Walters, 2005). PTSD can occur after either direct or indirect exposure to a traumatic life event (American Psychiatric Association, 2013). Although PTSD was initially brought to the forefront of public attention due to the incidence of the disorder in war veterans, it can result from a variety of traumatic incidents, including witnessing death, threat of death, and experience or threat of serious injury or sexual abuse.

PTSD is characterized by the presence of symptoms from each of four different clusters, the first of which includes persistent, intrusive re-experience of the traumatic event. These intrusive re-experiences can consist of recurrent memories, traumatic nightmares, flashbacks, or increased stress or reactivity after re-exposure to trauma-related stimuli. In addition to persistent reminders, sufferers of PTSD also experience negative alterations in cognitions and mood. These alterations range from the inability to recall key features of the traumatic event to persistent negative emotions, expectations, and beliefs about oneself or others. For example, individuals with PTSD may blame themselves or others for causing the traumatic event or the consequences of the traumatic event, have persistent negative emotions related to the trauma, feel disinterested in activities they used to enjoy, feel alienated from others, or exhibit a persistent inability to experience positive emotions (American Psychiatric Association, 2013).

Behaviorally, patients with PTSD exhibit avoidance of trauma-related stimuli, either by physically avoiding external reminders of the trauma or by failing to think about, or express feelings about the trauma. Individuals with PTSD also exhibit alterations in physiological arousal and reactivity. These alterations might be expressed through changes in behavior (irritability, aggressiveness, self-destructiveness, or recklessness), hypervigilance (being “on-edge” at all times), exaggerated startle responses, problems in concentration, or sleep disturbances. For diagnostic purposes, cognitive and behavioral symptoms must persist for more than one month, result in distress or functional impairment, and not be the result of substance use or another medical condition. In addition to the above mentioned diagnostic criteria, sufferers of PTSD can often experience high levels of depersonalization (decreased self-awareness or feelings of watching oneself without being in control of one’s situation) or derealization (alteration in the perception or experience of the external world; American Psychiatric Association, 2013).

Although an estimated 40-60% of the population has experienced a traumatic event in their lifetime (e.g., Kessler et al., 1995; Norris, 1992), only a portion of them will develop PTSD (Feeny & Foa, 2005). Recently, it has been suggested that some psychological, physiological and situational factors may increase the likelihood of developing PTSD after a traumatic event, whereas other physiological factors may promote resiliency against developing PTSD under similar circumstances (e.g., Brewin et al., 2000; Zoladz, & Diamond, 2013). For example, the type of stressor (e.g., rape, natural disaster) may dictate whether an individual develops PTSD (e.g., Shalev & Freedman, 2005). This is most likely related to the fact that certain types of traumas may be viewed as being more intense than others (e.g., exposure to terrorist attacks vs. motor vehicle accidents; Shalev & Freedman, 2005). According to the *dose-response model of PTSD susceptibility*, the incidence and magnitude of PTSD symptoms intensify as the severity of

the trauma increases (March, 1993; see also Mollica et al., 1998; Pynoos, et al., 1993; Shore et al., 1986; Snow et al., 1988; Sutker, et al., 1993). Other trauma-related factors that are especially relevant in subsequent development of PTSD symptoms include the degree of controllability, predictability, and perceived threat of the traumatic experience. As control and predictability decrease, the severity of symptoms increases (Foa, Zinbarg, & Rothbaum, 1992). Conversely, as perceived threat increases, prevalence of PTSD symptoms increases.

Females (humans and other species, including rats) seem to be more susceptible to PTSD, independent of trauma type. This may be related to the individual's hormonal state. For example, women exposed to trauma in the luteal phase of their menstrual cycles (high progesterone and estradiol) appear to exhibit more intense emotional memories than those exposed to trauma when hormone levels were lower (Bryant et al., 2011). Estradiol levels also appear to be associated with the rate and effectiveness of extinction of emotional memories in women, as well as in rodents (Glover et al., 2012; Graham & Milad, 2013, Zeidan et al., 2011). Nonetheless, the role of sex as a factor in the development of PTSD (in humans) is not universally accepted (see Yehuda, 2002; Pratchett et al., 2010), as some argue that such findings could be related to the types and frequency of the traumatic events that women are exposed compared with men. For example, women are more likely than men to be the victims of certain types of traumas such as rape and domestic violence (Coker et al., 2002). Other demographic variables that may affect susceptibility to PTSD may include age, ethnicity, and education level. Specifically, being of younger age, being part of a minority group, and having less education constitute risk factors for developing PTSD (Brewin, Andrews, & Valentine, 2000; Zoladz, & Diamond, 2013).

Physiological arousal and activation of the hypothalamic pituitary adrenal (HPA) axis at the time of trauma may also be related to the subsequent development of PTSD. Various



physiological indicators measured shortly after exposure to a traumatic event have been correlated with subsequent development of PTSD. For example, elevated peritrauma heart rate has repeatedly been shown to be a predictor of PTSD (Bryant, Harvey, Guthrie, and Moulds, 2000, 2003; Shalev et al., 1998; Shalev & Freeman, 2005; Zatzick et al., 2005). Shalev et al. (1998) measured heart rates of trauma survivors presenting in an emergency room of a general hospital and observed that elevated heart rates predicted subsequent development of PTSD at a four-month assessment (see also Shalev & Freeman, 2005). Bryant et al. (2000) assessed heart rates of individuals treated for motor vehicle accident-related injuries, and observed that those individuals who had higher heart rates at the time of hospital discharge were more likely to exhibit PTSD two years posttrauma than those who had lower heart rates. Zoldaz and Diamond (2013) suggested that large sympathetic responses to trauma may serve to facilitate the development of PTSD by enhancing the consolidation of the traumatic memory through excessive adrenergic activity at the time of trauma, which should in turn result in more robust memories for the traumatic event.

Other studies have provided evidence for a role of the HPA axis in the development of PTSD by examining the levels of circulating cortisol in individuals exhibiting PTSD. More specifically, these studies have suggested that a blunted adrenal corticosteroid response following trauma may contribute to the development of PTSD. For example, when studying circulating blood cortisol levels throughout the course of a day, Yehuda, Teicher, Trestman, Levengood, and Seiver (1998) found lower cortisol levels, particularly in the evening and early morning, in combat veterans with PTSD compared to healthy controls or depressed subjects. Neylan et al. (2005) similarly found that the incidence of PTSD symptoms was correlated with lower baseline cortisol levels in police officers with risk factors with PTSD (i.e., exposure to a

potentially traumatic event). Goenjian, et al. (1996) also found significantly lower baseline salivary cortisol levels and increased cortisol suppression (increased negative feedback inhibition) following the administration of the synthetic glucocorticoid dexamethasone in subjects experiencing more PTSD-like symptoms five years following traumatic experience of an earthquake. However, although many studies have found blunted cortisol levels in patients with chronic PTSD, other studies have found evidence for a blunted cortisol response shortly after trauma. For example, Delahanty et al. (2000) collected urine samples 15 h after individuals experienced motor vehicle accidents and then assessed the development of PTSD symptomatology in these individuals 1 month later. Individuals who had lower urinary cortisol levels following the trauma were more likely to develop PTSD than trauma-exposed individuals who had higher urinary cortisol levels shortly after the accident.

The blunted cortisol responses that correlate with the development of PTSD are thought to be due to an increased concentration and responsiveness of glucocorticoid receptors (Yehuda, Giller, Levengood, Southwick & Sieve, 1995), increased sensitivity of HPA negative feedback inhibition (Yehuda et al., 1996), and progressive sensitization of the entire HPA axis (Yehuda 1997, Yehuda 1998; Yehuda, et al., 1998) following trauma. One explanation for the increased concentration and responsiveness of glucocorticoid receptors is that certain individuals who are exposed to trauma have a heightened initial HPA activation in response to the trauma, producing an excess of stress hormones, including cortisol. As a result of this increased presence of cortisol, there is up-regulation of glucocorticoid receptors (Shaffer, & Giller, 1991; Yehuda et al., 1995). Epigenetic factors may also play a role in the development of PTSD (For a review of this in animal models, see Seckl & Meany, 2006). For example, even in the absence of trauma, low cortisol levels have been observed in the children of Holocaust survivors with PTSD (Yehuda,

Halligan, & Bierer, 2002; Yehuda, Schmeidler, Giller, Siever, & Binder-Brynes, 1998; Yehuda, Schmeidler, Wainberg, Binder-Brynes, & Duvdevani, 1998). Certain genetic polymorphisms have also been found to be related to the physiological effects of PTSD. For example, Bachmann et al. (2005) observed increased glucocorticoid receptor (GR) sensitivity in a subset of PTSD patients who possessed the GG BclI genotype (as compared to GC and CC genotypes). Other reports have similarly shown enhanced GR sensitivity in individuals who are GG carriers of the BclI polymorphism in the absence of a psychiatric disorder (*cf.*, Mehta & Binder, 2012; see Panarelli et al., 1998). This finding supports the idea that certain individuals may be biologically predisposed to developing a psychological disorder, but only do so if they are exposed to a stressful event. In addition to some individuals being more susceptible to developing PTSD than others based on genetic factors, previous experience with trauma may alter an individual's biochemistry, thus altering an individual's physiological response to future stressors.

Prior experience with trauma may increase the likelihood of developing PTSD. For example, Resnick et al. (Resnick, Yehuda, Pittman, & Foy, 1995) measured plasma cortisol levels of rape victims within 51 h of assault and observed that those women who had a history of previous assault were more likely to later develop PTSD relative to those who had never been raped. Moreover, women with a prior history of rape had significantly lower cortisol levels than women who had never experienced such a trauma. Similarly, Delahanty et al. (2003) observed that, in motor vehicle accident victims, a prior history of trauma and low levels of urinary cortisol were correlated with PTSD symptoms at a one-month follow-up assessment. Consistent with these clinical observations, laboratory animal studies suggest that previous exposure to stress can alter the HPA axis and affect response to subsequent stressful events in rats (Liu et al., 1997; Meany, et al., 1989; Meany et al., 1991).

People experiencing PTSD are exposed to many different stressors and have widely different previous experiences and immediate responses to those stressors. Thus, animal models are essential to the investigation of human psychological disorders such as PTSD because they allow for the use of subjects with a controlled genetic and environmental history. Such control enables experimenters to draw more precise conclusions about the etiology of symptoms and the efficacy of treatment (Laborda, Miguez, Polack, & Miller, 2012; Mineka & Oehlberg, 2008; Yehuda & Antelman, 1993). Additionally, animal models can provide the benefit of having larger samples, which can help to increase the statistical power of experimental studies (Yehuda & Antelman, 1993). Animal studies designed with translational research in mind provide a first-step in the development of clinical procedures to address pathological fear. One of the most common animal models of PTSD uses the paradigm of *Pavlovian fear conditioning* (Pavlov, 1927). In Pavlovian fear conditioning, a cue (e.g., a tone) is paired with an unconditioned stimulus (US; e.g., a shock) that naturally elicits a measurable fear response. After repeated pairings with the unconditioned stimulus (US), presentation of the cue (now called the conditioned stimulus, CS, or stressor) alone results in a conditioned response of fear. Exposure to the cue after fear conditioning training can be used to model exposure to trauma-related stimuli in PTSD. As in PTSD, subjects will often attempt to avoid exposure to the cue and will display a number of responses consistent with a heightened physiological arousal associated with the cue. Fear can be measured in animal models through various methods, such as behavioral change (e.g., freezing in rodents), or physiological indicators of preparatory and defensive responses.

Conditioned fear responses can be “treated” or attenuated using a procedure commonly known as *extinction* (Pavlov, 1927), in which the subject is repeatedly exposed to the cue in the absence of the US. Extinction treatments have gained the most evidence in the clinical literature

for their efficacy, their efficiency, and the ease with which clients can learn to use them (Foa & Rothbaum, 2001). Furthermore, extinction is most easily modeled using animals due to its direct impact on observable behavior. However, despite the effectiveness of extinction, it is prone to relapse (e.g., Rachman, 1989).

*Relapse of PTSD symptoms* consists of the return of symptoms that had been previously reduced by treatment. Relapse can include any measurable increase of cognitive or behavioral symptoms since an initial posttreatment test. In the worst cases of relapse, symptoms can return to pretreatment levels. Relapse can be assessed at a follow-up assessment within the context of a therapeutic session, or it can occur outside of therapy when individuals are exposed to reminders of the traumatic event. For example, the effects of treatment can be profound during the context of a therapy session, but can fail to transfer to a novel context (e.g., Mineka, Mystkowski, Hladek, & Rodriguez, 1999; Mystkowski, Craske, & Echiverri, 2002; Rodriguez, Craske, Mineka, & Hladek, 1999). This phenomenon is known as *renewal* and it occurs whenever the physical context in which trauma-related stimuli are subsequently encountered (i.e., the test phase/assessment) is different from the context in which the traumatic association was treated (Bouton & King, 1983; Bouton & Peck, 1989). For example, in the ABA renewal design, experimental subjects acquire a fear association in Context A, undergo extinction in Context B, and then are tested in the context of fear acquisition (Context A). In this design, subjects' responding tends to be consistent with the association learned first (e.g., fear response) because the test context evokes memories of original learning. Similarly, in ABC renewal, subjects acquire a fear association in Context A, undergo extinction in Context B, and are tested in a new context (Context C). Again, renewal can occur if the new context is dissimilar to the extinction context. The ABA and ABC renewal designs can represent the unfortunate scenarios that can

easily occur throughout the process of therapy. For example, an individual undergoes a traumatic experience in particular context, undergoes therapy in a new context and then, once therapy is over, is exposed to the same context in which the trauma occurred (ABA renewal) or a novel context which is more similar to than the traumatic context than the therapeutic context (ABC renewal). AAC renewal can also occur when treatment is conducted in the context of acquisition and individuals are presented with trauma-related events in a novel context, although this type of renewal is typically weaker than the other two (Laborda, Witnauer, & Miller, 2011).

Temporal context can also play a role in determining whether relapse occurs (Bouton, 1993, 2004). More specifically, the effects of treatment can diminish with time, a phenomenon referred to as *spontaneous recovery* (cf. Pavlov, 1927; Rescorla, 2004). In clinical settings, patients who are exposed to stimuli associated with the traumatic event some time after treatment has concluded (e.g., weeks, months, years) may recover some of the initial fear response that they exhibited prior to treatment. Spontaneous recovery of PTSD symptoms would include any increase of symptoms when exposure to trauma-related stimuli is delayed after training.

Importantly, other learning procedures can be used to prevent the development of fear responses. For example, repeated presentations of the cue alone prior to conditioning result in reductions in subsequently-acquired fear (Lubow & Moore, 1959). This cue preexposure can be thought of as analogous to a having previous “successful” (non-stressful) experience with a cue, and has been proposed as a potential prophylactic treatment to prevent the development of maladaptive fear responses (Bouton & Nelson, 1998). Although some studies have suggested that latent inhibition is highly dependent on the physical context (Gordon & Weaver, 1989; Hall & Channell, 1985; Hall & Minor, 1984; Kaye, Preston, Szabo, Druiff, & Mackintosh, 1987; Lovibond, Preston, & Mackintosh, 1984), more recent studies have suggested that release from

latent inhibition can be reduced under certain preparations. For example, Powell, Escobar, and Kimble (2013), observed that the time of preexposure relative to conditioning is important in determining whether the effects of preexposure are maintained. Specifically, Powell et al. observed that when preexposure occurs immediately (12 minutes) before fear conditioning, responding (suppression of lever presses) at a 72-h retention test is attenuated relative to control subjects who did not receive preexposure. However, when conditioning is delayed relative to preexposure (24 h after preexposure), preexposed subjects perform similarly to control subjects. Kimble, Escobar, and Sauer (in preparation) attempted to determine the underlying mechanisms of preexposure that occurs immediately prior to conditioning. They manipulated the interval between conditioning and test (either 72 h or 7 d) when conditioning followed preexposure immediately or after a delay and observed that, regardless of the retention interval (the interval between conditioning and test), less fear was observed in the immediate groups than the delayed groups. Kimble et al. further attempted to examine the advantages of preexposing subjects immediately before conditioning by conducting conditioning in either the same or different physical context than that of preexposure. Although more fear was observed at test when preexposure was conducted in a different context than that of conditioning, immediate latent inhibition groups displayed less freezing at test than did delayed latent inhibition groups. Moreover, immediate latent inhibition conducted in a different context than conditioning resulted in responding that was equivalent to delayed latent inhibition conducted in the same context. Thus, it seems that the effects of CS preexposure on the inhibition of fear responding can be quite robust under certain conditions (such as when it is conducted immediately prior to conditioning).

Kimble, Escobar, and Dunaway (in preparation) attempted to determine whether CS preexposure provides resilience against the development of conditioned fear by combining CS preexposure and extinction. They observed that providing preexposure immediately prior to conditioning then extinguishing fear provided protection against relapse of conditioned fear relative to control subjects who did not receive preexposure. Stated differently, providing preexposure to the CS prior to conditioning and extinction enhanced the long-term effectiveness of the extinction treatment. Moreover, Kimble et al. observed this effect with as little as three preexposure trials prior to conditioning. In other words, CS preexposure attenuated spontaneous recovery as long as such preexposure occurred shortly before conditioning, regardless of whether there were many or few CS preexposure presentations (but see Leung & Westbrook, 2010; Rosenberg, Holmes, Harris, & Westbrook, 2011).

As described above, blunted adrenal corticosteroid responses to trauma are known risk factors for developing PTSD (Delahanty et al., 2000; McFarlane et al., 1997). Individuals experiencing trauma typically exhibit increased activity of the HPA Axis, resulting in increased epinephrine and norepinephrine activity, which in turn increase heart rate, blood pressure, and respiration rate. Furthermore, increased HPA activity increases cortisol levels, which leads to increased blood glucose levels and prepares the body for the “fight or flight” response. Thus, activation of the HPA axis could serve as a protective measure against developing PTSD. Previous research suggests that preexposure to a cue that will later be associated with a fear-inducing outcome changes the activity of the HPA axis. For example, Zhang, Murphy, & Feldon (2004) repeatedly preexposed rat subjects to a tone stimulus prior to pairing it with a mild footshock. Rats that were preexposed to the tone displayed higher blood pressure than nonpreexposed animals during assessment of contextual fear. These preexposed rats also had



higher heart rates, but lower freezing response during a test of cued conditioning than nonpreexposed animals. To date, however, no studies have measured the corticosteroid responses in preexposed subjects, nor have any previous studies measured whether elevated HPA activity is related to the resiliency against fear development provided by stimulus preexposure. To date, the only study that has directly investigated the effects of corticosteroids on latent inhibition reported that exogenous corticosterone administration disrupted latent inhibition (Shalev, Feldon, & Weiner, 1998). However, this study did not investigate the effects of preexposure on corticosteroid levels. These experiments therefore attempted to determine whether preexposure to conditioned stimuli change HPA axis activity levels, as measured through circulating corticosterone as well as corticosterone suppression.

## Experiment 1

Human subjects with PTSD appear to exhibit blunted adrenal corticosteroid responses to trauma. In this study, we attempted to elucidate the relationship between trauma exposure and corticosterone response in an animal model of acquired fear. Furthermore, we attempted to determine whether preexposure to a trauma-related stimulus influences levels of corticosterone prior to undergoing a traumatic event. Although our primary focus in this study was to compare the endogenous corticosterone levels of subjects that have undergone CS preexposure to control subjects that have not undergone preexposure, we also included a comparison of preexposed subjects to subjects with artificially high levels of corticosterone. This positive control group received corticosterone injections (and no preexposure) to control for physiological arousal prior to CS-US pairings. Subjects were sacrificed and corticosterone levels were measured after the preexposure phase, after the conditioning phase, and after a test of conditioned fear in order to measure corticosterone changes across phases.

**Subjects.** The subjects were 56 young adult, male, Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), randomly assigned to one of three conditions, Preexposure (PE,  $n=19$ ), Control (Control;  $n=18$ ), or Corticosterone (Cort;  $n=19$ ). Subjects were group-housed (group size: 2-4 rats) in standard plastic cages with wire lids in a vivarium maintained on a 12 hr light/12 hr dark cycle (lights on at 6:00 am). All experimental manipulations occurred during the light portion of the cycle. Access to water was gradually restricted to 30 min/day over the week before initiation of the first experimental session, and was given approximately one hour after

completion of daily experimental sessions, or at the equivalent time during retention intervals (food was available *ad libitum*).

**Apparatus.** The apparatus consisted of eight Med Associates standard rat operant chambers, each measuring 30.5 x 24.1 x 21.0 cm (l x w x h). The side walls of the chamber were made of aluminum sheet metal, and the front and back walls and the ceiling of the chamber were made of clear polycarbonate. The floor was constructed of 4.8 mm stainless steel rods, spaced 1.6 cm center-to-center. These rods could be electrified with a scrambled footshock.

A response lever was protracted on a side wall of the operant chambers; depressing this lever led to the delivery of a 0.05 mL water reinforcer. The reinforcer was delivered into a cup located inside a niche (5.1 x 5.1 x 5.1 cm, l x w x h), which opened 1.5 cm above the grid floor, and which was located to the right of the lever. Infrared photobeams were used to detect head entries into this niche.

All chambers were equipped with a speaker that was capable of producing a 1000 Hz tone that served as the CS. The volume of this tone was set to 80 dB (A-scale). Each chamber was housed in a melamine sound attenuation cubicle. All sound attenuation cubicles were equipped with an exhaust fan, which provided a constant, 70 dB (A-scale), background noise. The US consisted of delivery of a 0.60 mA, 0.5 s, footshock.

**Drug Administration.** Animals in the Cort group received a single 25 mg/kg dose of corticosterone (CORT; 98.5%, Sigma-Aldrich Inc., St. Louis, MO), dissolved in one mL of a vehicle containing 15% sesame oil, 50% polyethylene glycol, and 35% saline via intraperitoneal injection (Akirav et al., 2004) one hour prior to the preexposure phase of training. Preexposure and control subjects received equivalent vehicle injections.

**Behavioral Procedures.** All subjects were trained to press the lever to obtain a water reinforcer using an autoshaping procedure. Each shaping session lasted 60 minutes. On Day 1, water reinforcement was delivered on a concurrent Fixed Time 5 min (FT-5'), Fixed Ratio 1 (FR-1) schedule. On Days 2 and 3, reinforcement was delivered on a FR-1 schedule. On Day 4, reinforcement was delivered on a Variable Interval 20 s (VI-20'') schedule. Animals that did not produce at least 150 responses on Day 4 were given an extra 30 min VI-20'' session after completion of their scheduled session for that day.

On Day 5, subjects in the PE condition received 36 presentations of a tone CS. These stimuli were presented with an intertrial interval of 2 ( $\pm$  1 min). Water reinforcement for lever pressing was available on a VI-20'' schedule during this and all subsequent sessions. Subjects in the Control and Cort conditions received equivalent context exposure. Because we expected that the PE treatment would increase basal levels of corticosterone, the Cort group served as a positive control that received no PE treatment, but received an acute (25 mg/kg) dose of corticosterone delivered via intraperitoneal (i.p.) injection one hour before the preexposure phase. To equate for handling and injection cues, subjects in the PE and Control groups received an equivalent i.p. injection of vehicle (15% sesame oil, 50% polyethylene glycol, and 35% saline). After completing the PE treatment (or context exposure), animals were returned to their home cages for 10 min, and brought back to the operant chambers to begin the conditioning phase. During conditioning, all subjects received six pairings of the tone CS and the shock, with an interval between pairings of 5 ( $\pm$ 2) min. All shock presentations were delivered at the offset of the tone.

All subjects received restabilization training for lever pressing on Days 6 and 7, during which water was available on a VI-20" schedule, but no stimuli were presented. Restabilization training sessions were 60 min in duration.

All subjects were tested for conditioned responding to the tone CS on Day 8. Testing consisted of 12 presentations of the CS with a 4.5 min intertrial interval.

**Physiological Procedures.** At the end of each session (see Table 1), subjects from each condition (PE, Control, or Cort) were euthanized approximately 5 minutes after the end of the behavioral procedure. Animals were euthanized via carbon dioxide overdose, followed immediately by decapitation to collect trunk blood. Blood samples were centrifuged at 2000 rpm (3678 RCF) for 10 min at 4°C. Serum was collected and stored at -20 °C. All corticosterone assays were performed with Corticosterone ELISA kits (Enzo Life Sciences, Inc., Farmingdale, NY, USA) following manufacturer's directions. Samples from each subject were assayed in three replicates from 10- $\mu$ L aliquots.

**Data Analysis.** Fear was assessed through the suppression of responding in the presence of the tone CS (Annau & Kamin, 1961). Suppression ratios were calculated by comparing the number of lever presses during the 30-s CS presentation against the number of lever presses during a 90-s baseline period using the formula  $A/[A+(B\div 3)]$ , where A represents responding during the CS period and B represents responding during the baseline period.

Responding during the conditioning phase was analyzed using a survival analysis. This method was used because the number of conditioning trials in this preparation resulted in complete response suppression in all groups, regardless of preexposure status. Because the subjects completely stopped leverpressing by the end of the conditioning phase (during both the CS and baseline periods), suppression ratios could not be calculated (the fraction would need to

be divided by zero). Therefore, the cumulative percentage of animals that continued to leverpress through the session was assessed using a response survival analysis, during which behavior was described as not surviving if the animal ceased to leverpress during a trial and did not produce any more responses during subsequent trials.

Corticosterone concentrations were calculated from optical density readings using an online software package from Arbor Assays (Ann Arbor, MI; available at [www.myassays.com/arbor-assays-cortisol-enzyme-immunoassay-kit.assay](http://www.myassays.com/arbor-assays-cortisol-enzyme-immunoassay-kit.assay)). The average concentrations were analyzed with analyses of covariance (ANCOVAs), which included body weight as a continuous variable since levels of circulating Cort can potentially be affected by body weight. In all ANCOVAs, the covariate was not linearly related to the dependent variables.

Corticosterone concentrations were determined for each sample using the average of three replicates, unless one of the replicates was more than .02 OD different than the other two scores, in which case that replicate was dropped and the remaining two replicates were averaged. During the conditioning phase, it was unclear whether four of the subjects (Group LI-Conditioning,  $n=2$ ; Group Control-Conditioning,  $n=2$ ) received shock due to an experimenter error, thus their data were excluded from all analyses. Two further subjects (Group Cort-Test,  $n=1$ ; Group Control-Test,  $n=1$ ) were excluded from the corticosterone analysis due to sample contamination. Additionally, the physiological data from five subjects was not within the range of corticosterone expression that is biologically possible, and thus these subjects' data were either re-analyzed in a subsequent assay (Group Cort-Conditioning,  $n=1$ ; Group Control-Test,  $n=1$ ) or excluded from analysis (Group Cort-Conditioning,  $n=1$ ; Group Control-Preexposure,  $n=1$ ; Group LI-Test,  $n=1$ ). When appropriate, significant outliers were also excluded from analyses using a Grubb's test for outliers (Grubb's 1950). For all significant effects reported  $p <$

.05. Effect sizes are reported as partial eta-squared ( $\eta_p^2$ ), which represents the proportion of the variance of an effect excluding the other effects from the total nonerror variation.

### **Results and Discussion.**

**Conditioning.** A survival analysis of responding during the conditioning phase revealed a significant main effect of group,  $\chi^2(2) = 10.53$ . The LI group continued to respond for significantly longer than either the Control or Cort groups,  $\chi^2s(1) = 2.39$  and  $2.92$ , respectively. During the final conditioning trial, only 5.71% ( $n = 2$ ) of all subjects produced a minimum of one response. Response survival was 16.67% ( $n = 2$ ) in the LI group, and 0% ( $n = 0$ ) in both the Cort and Control groups (see Figure 1).

**Testing.** Suppression ratios were analyzed in blocks of three trials during testing. The first block of responding should provide the most direct measure of conditioned fear after preexposure and conditioning, and thus, it was analyzed to determine the extent to which preexposure served to attenuate fear. A one-way analysis of variance (ANOVA) conducted on the data from this first response block revealed an effect of group,  $F(2, 17) = 6.52$ ,  $\eta_p^2 = 0.43$  (see Figure 2). Planned comparisons revealed that this effect was due to responding in the LI group being higher than responding in both the Control and Cort groups,  $Fs(1, 17) = 9.68$  and  $9.64$ , respectively. There were no differences between the Control and Cort groups,  $F(1, 17) = 0.02$ . This finding suggests that providing preexposure to the CS reduced the observed conditioned fear acquired during the conditioning phase (i.e., the latent inhibition effect), but that artificially elevating corticosterone levels did not seem to have the same effect.

Extinction of the fear response was analyzed by looking at the remaining blocks of trials. A 3 (condition: LI vs. Control vs. Corticosterone; between-groups factor) x 4 (trial block; within-groups factor) ANOVA revealed a main effect of trial block,  $F(3, 48) = 8.68$ ,  $\eta_p^2 = 0.35$ , as well

as an interaction between condition and block,  $F(6, 48) = 2.62$ ,  $\eta_p^2 = 0.25$ (see Figure 2). This interaction is likely due to the LI group exhibiting consistent levels of fear through the test session, while the other two groups exhibited gradual extinction of the fear response as the number of test presentations of the CS increased.

**Corticosterone Analyses.** A 3 (condition: LI vs. Control vs. Corticosterone; between-groups factor) x 3 (phase: preexposure, conditioning, test; between-groups factor) ANCOVA (with weight as a covariate) of serum corticosterone concentrations revealed a main effect of condition,  $F(2, 34) = 3.61$ ,  $\eta_p^2 = 0.18$ . As evidenced by an inspection of Figure 3, corticosterone levels were higher than LI or Control groups in the preexposure and conditioning phases; however, all groups had similar corticosterone concentrations during the test phase. Pairwise comparisons confirmed that, in the preexposure phase, corticosterone levels differed between the Cort and LI groups,  $F(1, 34) = 5.59$ , but not between the Control and LI groups, or between the Control group and the Cort group,  $F_s < 1.60$ . The lack of differences between the Control and Cort group during the preexposure phase was likely driven by one subject whose score was approximately two standard deviations above the mean for its group, but which did not meet the statistical criterion for exclusion from the analyses. During the conditioning phase, corticosterone concentrations were higher in the Cort group than either the Control or LI groups,  $F_s(1, 34) = 4.95$ , and 5.36, respectively, while these latter two groups expressed equivalent levels of corticosterone,  $F(1, 34) < 1$ .

The high levels of circulating corticosterone observed in the Cort subjects assessed after preexposure were to be expected, given the high dose of exogenous corticosterone administered to these subjects prior to the preexposure phase. However, the lack of difference in corticosterone levels between the LI and Control groups suggests that CS preexposure did not



cause an immediate increase in circulating endogenous corticosterone. During the conditioning phase, it would appear that the corticosterone administered prior to the preexposure phase had not been entirely eliminated by the Cort subjects. The lack of difference between the corticosterone levels of the Control and LI groups during the conditioning phase, however, suggests that exposing subjects to the CS prior to conditioning did not influence corticosterone levels during the acquisition of conditioned fear. Interestingly, during the test phase, corticosterone levels did not vary across groups (all  $F_s < 0.27$ ), despite the differences in fear levels observed in the behavioral measure. Taken together, these results suggest that long-term attenuation of conditioned fear in preexposed subjects is *not* due to increased corticosterone activity during or as a result of preexposure to the CS.

## Experiment 2

Studies of humans with PTSD have yielded characteristically variable data. Indeed, due to the variability in basal urinary and blood cortisol measurements, some have suggested that there may be no static hypo or hypercortisolism in PTSD, but a tendency for the HPA axis to ‘hyperregulate’ itself (e.g., Mason et al., 2002). As a result, some have suggested the use of cortisol challenge procedures to provide more consistent findings (*cf.* Stam, 2007). Cortisol challenges work on the negative feedback mechanism of the HPA axis. When synthetic glucocorticoids such as dexamethasone (DEX) are administered, they bind to glucocorticoid receptors, which provides a signal to decrease glucocorticoid production. Thus, when glucocorticoid receptors are bound, less circulating cortisol is available in the bloodstream. Individuals with PTSD tend to have hypersuppression of cortisol in relation to individuals without PTSD in response to DEX administration and other cortisol challenge procedures (e.g., Yehuda et al., 1993). Thus, for Experiment 2, we conducted a dexamethasone challenge in rat subjects. Other rat studies have shown that dexamethasone administration suppresses the corticosterone response to stress caused by handling (e.g., Cole et al., 2000), maternal deprivation (e.g., Ogawa et al., 1994), and learned helplessness (e.g., Greenberg, Edwards, & Henn, 1989). If the preexposure effect is modulated by the effects of corticosterone, then presumably we should expect a decrease in the effectiveness of preexposure at promoting resilience to conditioned fear when subjects are administered dexamethasone.

**Subjects and apparatus.** The subjects were 32 male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), randomly assigned to one of four groups: Preexposure-

Dexamethasone (PE-DEX), Control-Dexamethasone (Control-DEX), Preexposure-Vehicle (PE-Veh), Control-Vehicle (Control-Veh;  $ns = 8$ ). Subjects were housed and maintained as described in Experiment 1. The experimental apparatus was the same used for Experiment 1.

**Drug Administration.** Two hours prior to preexposure, DEX animals received a single dose of 50  $\mu\text{g}/\text{kg}$  dexamethasone (DEX), dissolved in propylene glycol, administered via subcutaneous (SC) injection (Cole et al, 2000). Vehicle control animals received equivalent injections of the vehicle.

**Procedure.** All animals received either preexposure or control treatments as described in Experiment 1. Animals in the DEX conditions received SC injections of DEX two hours prior to the preexposure phase of training. The remaining subjects received vehicle only (Veh condition). This led to 4 conditions: Preexposure-Dexamethasone (PE-DEX), Control-Dexamethasone (Control-DEX), Preexposure-Vehicle (PE-Veh), and Control-Vehicle (Control-Veh;  $ns = 8$ ). All behavioral and physiological procedures were identical to those of Experiment 1, except that DEX rats were administered 50  $\mu\text{g}/\text{kg}$  dexamethasone SC 120 min prior to the preexposure session (the effects of DEX should peak at 120 min, and then rapidly decline; Cole, 2000).

**Results and Discussion.** As described for Experiment 1, conditioning data were analyzed with a response survival analysis, whereas test data were analyzed with suppression ratios, calculated as described in Experiment 1. During the final conditioning trial, 3.31% ( $n = 1$ ) of all subjects produced a minimum of one response. Response survival was 12.5% ( $n = 1$ ) in Group PE-DEX, and 0% ( $ns = 0$ ) in all other groups. There were no significant differences in responding across groups,  $\chi^2(3) = 6.62$ , suggesting that by the end of conditioning, all groups had acquired the CS-US association. However, a visual inspection of Figure 4, does suggest a trend towards longer response survival and thus slower acquisition of fear conditioning and decreased

fear response in the preexposed subjects compared to the nonpreexposed control subjects. This effect does not seem to depend on whether subjects received dexamethasone prior to preexposure.

Responding during the test phase was analyzed using blocks of three trials as described in Experiment 1. A 2 (condition: LI vs. Control; between-groups factor) x 2 (drug treatment: Dex vs Vehicle) x 4 (trial block; within-groups factor) ANOVA revealed main effects of condition,  $F(1, 18) = 12.83$ ,  $\eta_p^2 = 0.42$ , and block,  $F(3, 54) = 3.13$ ,  $\eta_p^2 = 0.15$ . No other main effects or interactions were observed, all  $F$ s  $< 3.45$  (see Figure 5). The main effect of condition was the result of lower levels of suppression (i.e., less fear) in the groups that received CS preexposure (LI condition) compared to their nonpreexposed counterparts (Control condition) throughout the test phase. Importantly, this latent inhibition effect did not interact with whether subjects received dexamethasone, suggesting that corticosterone levels did not influence whether preexposure resulted in attenuated fear.

## **General Discussion**

Individuals suffering from PTSD have been shown to exhibit a blunted cortisol response (e.g., Neylan et al., 2005; Yehuda et al., 1998) or dysregulation of the HPA axis (e.g., Shaffer & Giller, 1991). Additionally, results from animal studies have suggested that preexposure to a conditioned stimulus may affect activity of the HPA axis. For example, Zhang et al. (2004) reported that subjects receiving exposure to the conditioned stimulus prior to conditioning exhibited higher blood pressure and heart rates (both indicators of HPA axis activation) than nonpreexposed subjects during tests of conditioned fear. However, despite heightened HPA axis activity, these subjects displayed less fear at test than nonpreexposed subjects. Based on these findings, we hypothesized that HPA axis activity may play a role in the fear-attenuating effects of CS preexposure.

The timing of preexposure has a profound impact on whether its fear-attenuating effects are pervasive and/or subject to relapse. Previous research from our laboratory suggests that providing preexposure immediately prior to conditioning results in lower acquisition and/or expression of subsequently acquired fear, which is resistant to spontaneous recovery-mediated relapse (e.g., Powell et al., 2013). The present studies were designed to investigate whether the fear-reducing properties of immediate CS preexposure (i.e., immediate latent inhibition) may be mediated by activity of the HPA axis, specifically through the release of glucocorticoids during the preexposure phase of training. If this were the case, we should observe increased levels of corticosterone in subjects receiving preexposure to the CS, as compared to nonpreexposed control subjects, and a decrease in the immediate latent inhibition effect when corticosterone is

suppressed. However, Experiment 1 revealed that corticosterone levels remain relatively stable throughout the preexposure, conditioning, and test phases. Experiment 2 revealed that suppressing corticosterone through the use of dexamethasone does not have an impact on the immediate latent inhibition effect. This finding suggests that suppression of corticosterone did not diminish or alter the latent inhibition effect. Thus, our results do not support the hypothesis that the long-term fear-attenuating effects of immediate latent inhibition are mediated by the effects of circulating glucocorticoids.

Our finding that the latent inhibition effect is not mediated by corticosterone levels seem to contrast with Zhang et al.'s (2004) report of increased heart rate among preexposed subjects compared to nonpreexposed controls during a test of cued conditioning. Seemingly, if preexposure results in elevated heart rate, which is a consequence of HPA axis activation, one would expect to see other consequences of HPA axis activation, like increased glucocorticoid circulation. However, this did not appear to be the case in our study. Additionally, these findings do not fit easily into the observations that individuals who display symptoms of conditioned fear as a result of a traumatic event (e.g., individuals with PTSD) exhibit blunted glucocorticoid levels (Delahanty et al., 2000). Despite the dexamethasone-induced blunted corticosterone levels, PE-DEX subjects displayed less fear than their non-preexposed counterparts (Control-DEX). Moreover, DEX subjects displayed similar amounts of fear as the vehicle-control subjects. That is, that in our study, blunted corticosterone levels did not appear to increase the risk of developing conditioned fear.

Our observation of little to no involvement of HPA-axis activity in immediate latent inhibition is surprising, especially because both chronic and acute administration of endogenous corticosterone have been reported to disrupt latent inhibition (Shalev et al., 1998). In contrast to

Shalev et al., in our study, blunting corticosterone did not increase the latent inhibition effect (Experiment 2), nor did facilitating latent inhibition result in increased corticosterone levels (Experiment 1). However, it is possible that corticosterone only modulates the latent inhibition effect under certain circumstances or interacts with some other factor in order to do so. For example, it is possible that administration of corticosterone disrupts the latent inhibition effect, but decreasing corticosterone levels do not enhance or alter the latent inhibition effect.

Despite the elevated levels of corticosterone during the preexposure phase, Cort subjects responded similarly to the control groups at the onset of the test phase. Importantly, although corticosterone levels were manipulated in the Cort subjects prior to the preexposure phase (and were above normal levels throughout preexposure and conditioning), the behavior of these subjects showed signs of extinction towards the end of the test phase, suggesting that elevated corticosterone levels during fear acquisition may not be detrimental to treatment of conditioned fear. Unfortunately, because the main focus of this study was latent inhibition and not extinction of fear, we did not include a true extinction phase or a test of spontaneous recovery of fear. Future studies examining this effect could provide insight as to whether the manipulation of glucocorticoids may provide benefits during the extinction or treatment of conditioned fear. As Kimble et al. (in preparation) have demonstrated, combining CS preexposure and extinction can provide resilience against later relapse of conditioned fear. If glucocorticoids play any role in the retention of fear, it is possible that their role is during extinction instead of acquisition.

Another limitation of this study was that we only used one duration for the interval between the preexposure and conditioning phase. Although the immediate latent inhibition preparation used in our studies has been shown to be the most effective at maintaining the CS preexposure effect, it remains to be seen whether delayed latent inhibition (24+ h between

preexposure and conditioning) would yield a different pattern of corticosterone activity. Because delayed latent inhibition is effective in attenuating fear but not preventing relapse, it is possible that corticosterone levels are different after immediate and delayed CS preexposure (for example, Shalev et al.'s 2004 study included disruption of delayed latent inhibition after corticosterone administration).

An unavoidable consequence of using a short interval between the preexposure and conditioning phases is that the corticosterone administered in the Cort group (Experiment 1) had not been sufficiently eliminated at the completion of the preexposure phase, and thus the behavioral results of the Cort group in the conditioning phase are necessarily confounded. A future study would attempt to reduce this problem through the adjustment of the interval between these two phases. Another way that we might have reduced the exogenous corticosterone levels in the conditioning phase for Cort subjects would be to reduce the dose of CORT used. In this study, we used what is considered to be an acute dose. In some cases, similarly high doses of corticosterone have been shown to cause memory impairments in certain tasks, while lower doses may actually provide memory improvements (Joëls, 2006). Future studies, therefore should assess the effects of varying doses of corticosterone.

Despite these limitations, this study has provided some insight into the mechanisms of latent inhibition and the role of glucocorticoids. Future exploration of this topic may serve to further elucidate the conditions surrounding the acquisition, treatment, and retention of conditioned fear as well as the underlying mechanisms of psychological disorders such as PTSD.



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Table 1.

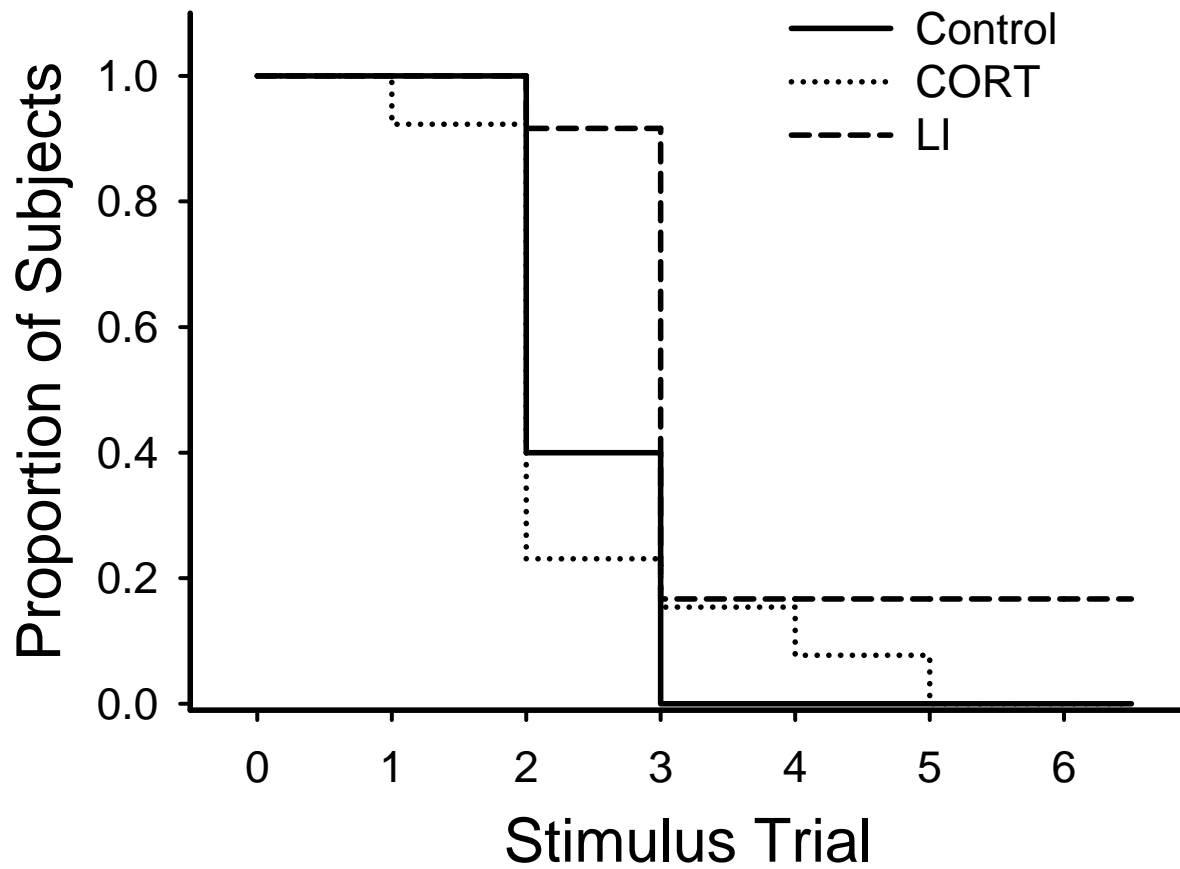
*Experimental Designs*

<b>Group</b>	<b>Preexposure</b>	<b>Interval</b>	<b>Conditioning</b>	<b>Retention Interval</b>	<b>Test</b>
<b>Experiment 1</b>					
PE	36 X-	12 min	6 X-US	48h	12 X-
Control	context	12 min	6 X-US	48h	12 X-
Corticosterone	*context	12 min	6 X-US	48h	12 X-
<b>Experiment 2</b>					
PE-DEX	**36 X-	12 min	6 X-US	48h	12 X-
Control-DEX	**context	12 min	6 X-US	48h	12 X-
PE-Veh	36 X-	12 min	6 X-US	48h	12 X-
Control-Veh	context	12 min	6 X-US	48h	12 X-

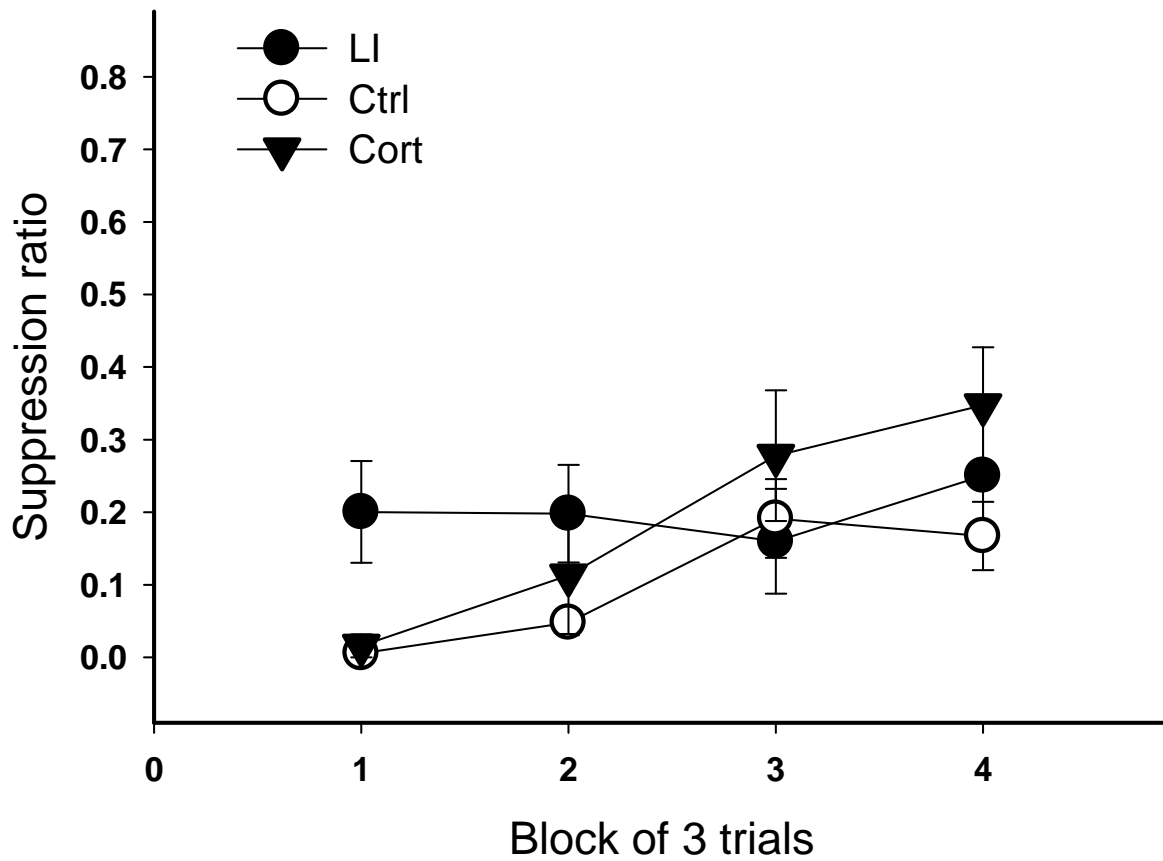
Note: 'X-' represents presentations of the tone CS; 'X-US' represents tone-shock pairings, where the tone CS is immediately followed by the shock US

\*Denotes corticosterone injection 60 min prior to session

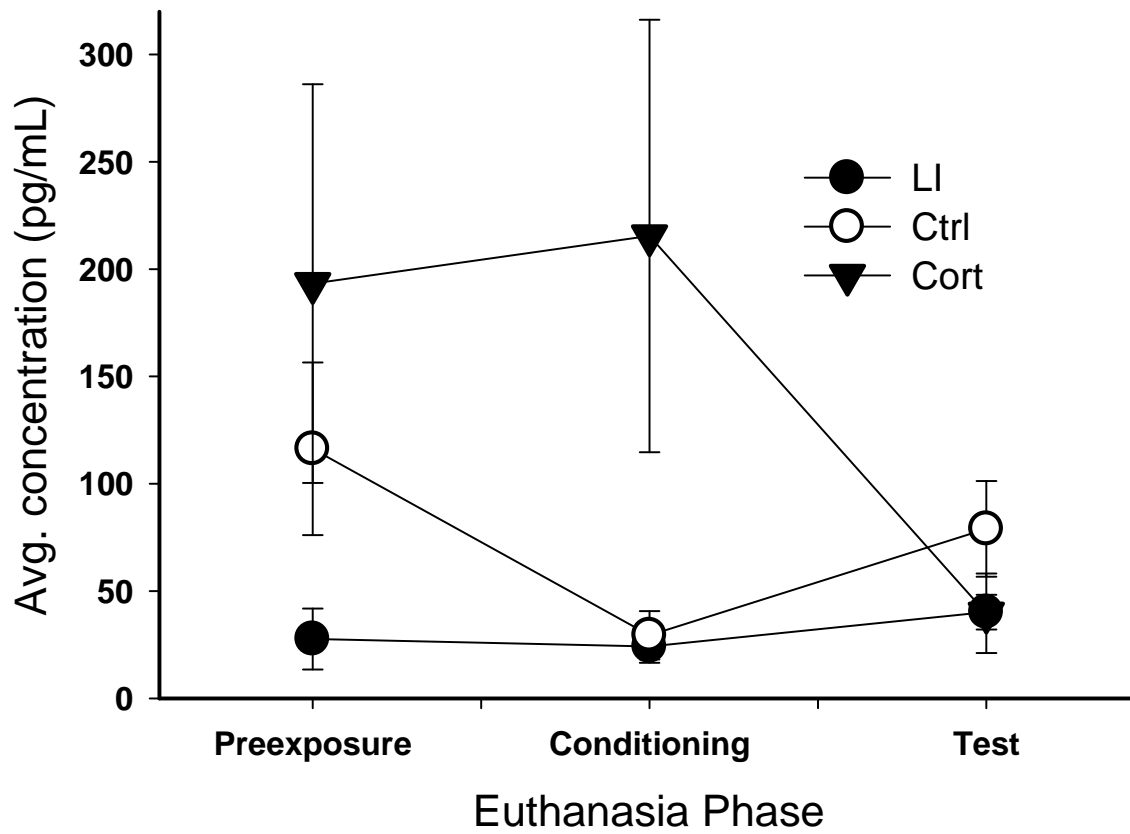
\*\*Denotes DEX injection 120 min prior to session



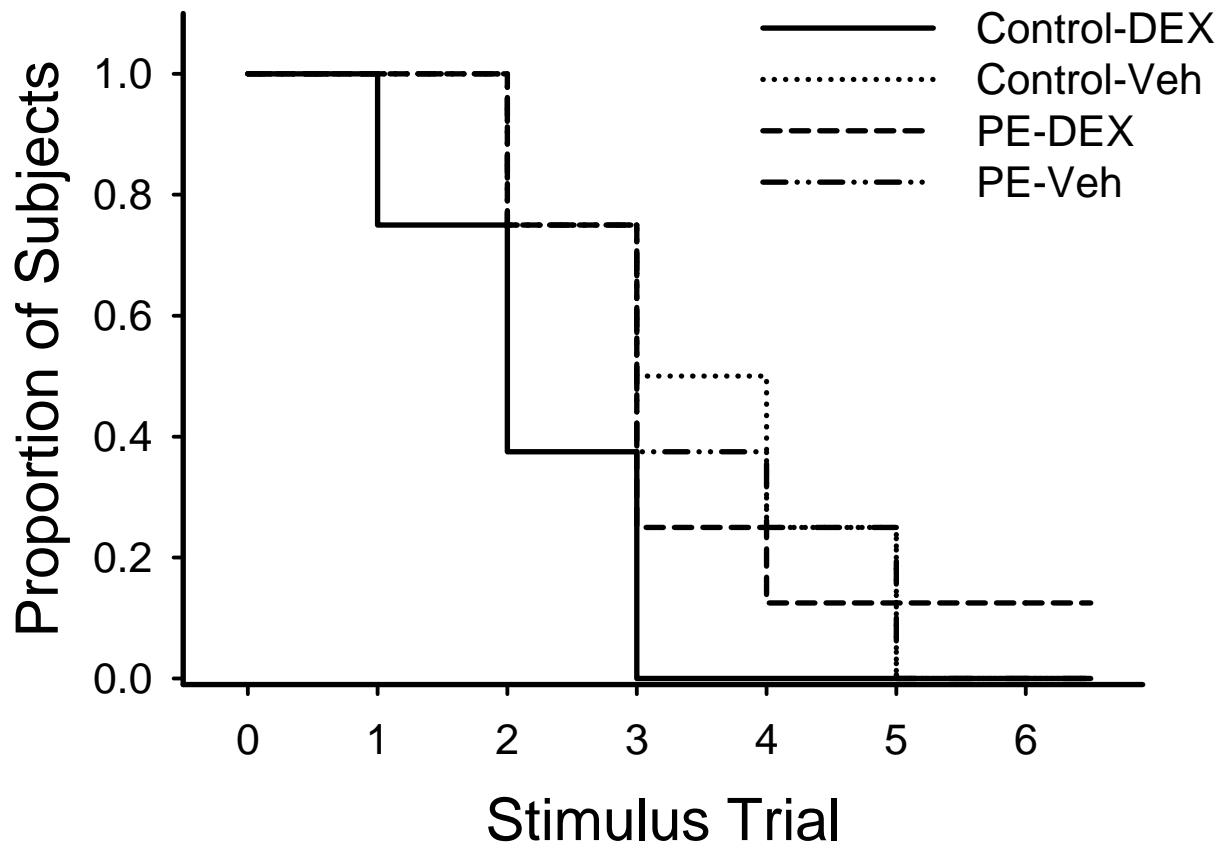
*Figure 1.* Response survival for Experiment 1. A stimulus trial represents one CS presentation during the Conditioning phase. Each descent in the graph represents the proportion of animals that ceased to leverpress during that trial and all subsequent trials, indicating complete suppression to the CS.



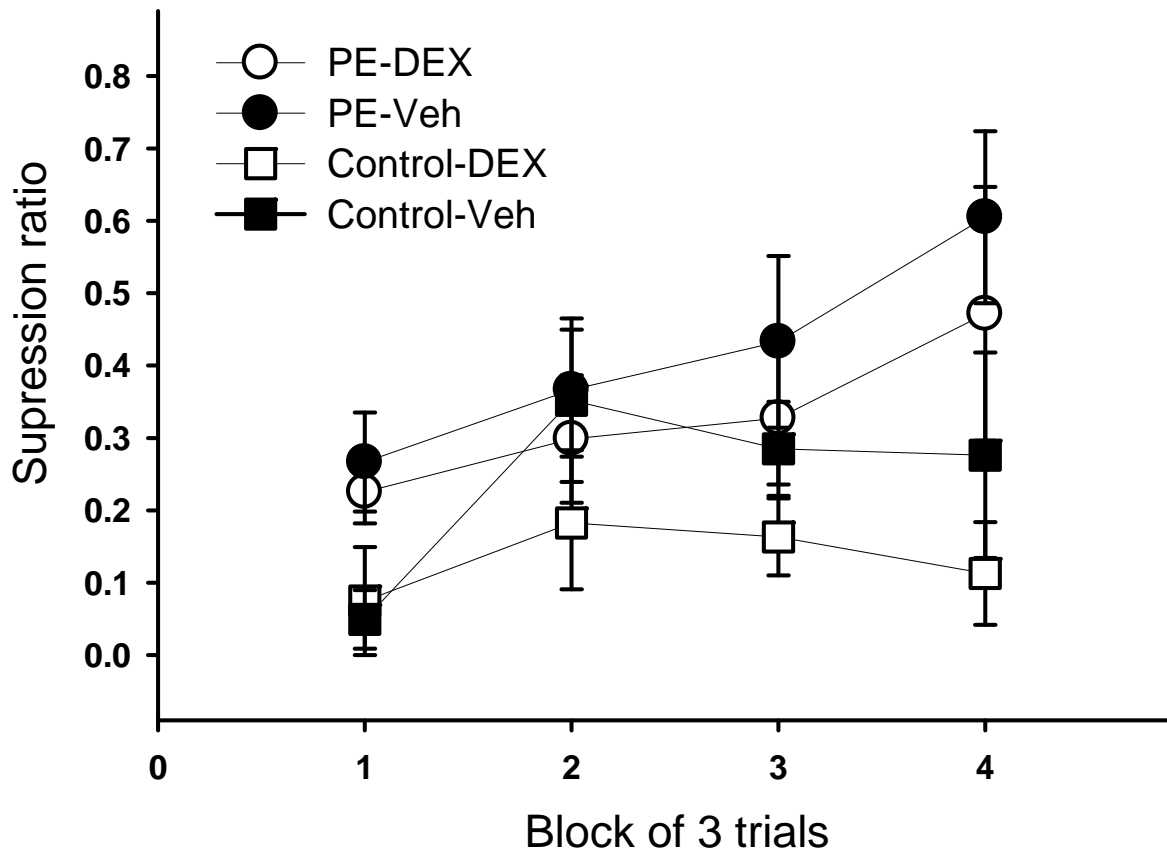
*Figure 2.* Test of conditioned response suppression in Experiment 1. This figure represents mean suppression ratios collected during blocks of test trials. Lower ratios reflect more suppression of lever pressing, which indicates more fear.



*Figure 3.* Serum corticosterone concentrations. This figure represents serum corticosterone levels after each phase of Experiment 1. Blood samples were collected immediately after preexposure, conditioning, and test phases.



*Figure 4.* Response survival for Experiment 2. A stimulus trial represents one CS presentation during the Conditioning phase. Each descent in the graph represents the proportion of animals that ceased to leverpress during that trial and all subsequent trials, indicating complete suppression to the CS.



*Figure 5.* Test of conditioned response suppression in Experiment 1. This figure represents mean suppression ratios collected during blocks of test trials. Lower ratios reflect more suppression of lever pressing, which indicates more fear.