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Opiate masking of stress-induced hypervigilance: The cause of delayed symptom presentation in PTSD

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14. ABSTRACT
The goal of this concept award was to establish different ways of eliciting persistent exaggerated startle responses in rats and to determine whether manipulation of endogenous opiate receptors can disrupt those processes. To date, we have tested 2 different means by which to increase startle reactivity: inescapable stress and avoidable stress. A single day versus 3 days of inescapable stress was compared. Three days of inescapable stress caused a delayed increase in startle reactivity, but administration of naloxone, an opiate antagonist, did not cause any appreciable change in the startle following inescapable stress. Avoidable stress also caused a delayed increase in startle reactivity, but the delay was substantially longer than in the inescapable stress paradigm and lasted longer. The appearance of an exaggerated startle response several days following avoidable stress suggests that the appearance of startle exaggeration days following stressor exposure is not likely caused by a specific pain-dependent mechanism as much as some type of coping process.

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Introduction

Post-traumatic stress disorder is a multi-symptom psychological disorder that includes, as one possible symptom, an exaggerated startle response (Butler et al. 1990;Yehuda et al. 1998;Grillon and Morgan, III 1999;Orr et al. 2002). As has been reported in longitudinal human studies, the change in startle reactivity occurs over a period of time following the associated trauma (Shalev et al. 1998). Increases in startle magnitudes can be elicited in rats by exposing them to inescapable shock, but, like the disorder, the change in this reflex response does not occur for a few days (Servatius et al. 1994;Servatius et al. 1995;Beck et al. 2002;Manion et al. 2007). In contrast, startle response magnitude can be elicited within several minutes pharmacologically using several compounds, most notably corticotrophin-releasing hormone (CRH)(Swerdlow et al. 1989;Lee et al. 1994;Risbrough et al. 2003;Servatius et al. 2005). CRH is a key element for the stress response as it is involved in communicating between centers of the brain that organize the autonomic and endocrine responses (Takahashi 2001;Rivier et al. 2003), and it is elevated in rats in the for several hours following shock exposure (Servatius et al. 2000). Given the discrepancy in the timing of stress-enhanced startle reactivity and CRH-enhanced startle reactivity, we hypothesized that there may be an additional physiological response to the stressor that overrides and masks the exaggerated startle that should be evident shortly after CRH is elevated. Likely candidates such a masking role are the endogenous opiates. In contrast, if it is shown that a delayed-expression exaggerated startle response can be elicited after exposure to a predictable and controllable stressor, then we would have to consider an alternative to the masking agent hypothesis.

Experiment 1: Inescapable stressor effects on startle reactivity

There has been some discussion in the literature as to whether there is a sufficient "dose" of stressor exposure that will cause lasting behavioral changes. Past results have been mixed with respect to an emergent enhanced startle response. Therefore, we tested whether a significant difference in the presentation of an exaggerated startle response could be elicited by either a single 2-h exposure to periodic inescapable shocks versus 3 2-h sessions of periodic inescapable shock.

Stressor Exposure Procedures: Rats are restrained in commercially purchased Plexiglas rodent restraint tubes. A tail-clip with exposed wire on the inside is attached securely to the tail (about 2-4 cm from the base of the tail – depending on the size of the rat). Conductive gel is administered to the tail on the location where the clip will be placed to minimize any tissue damage from the repeated shocks. Over approximately a 2 h period of time, up to a maximum of 40 scrambled shocks (2 mA, 3 sec in duration), are delivered to the tail. The shock cycles such that current is delivered 166 ms of every 200 ms. This either occurred on a single day or was repeated over 3 consecutive days.

Acoustic Startle Responses: Rats are placed in conditioning boxes in a light restrainer (for which they are habituated to prior to any stimulus presentation). The restrainer sits on an accelerometer which serves to transduce weight-shifts that occur in the restrainer in response to brief (100 ms) stimuli with sharp rise/fall characteristics (5 ms) at a frequency of 1000 Hz. A 102 dB stimulus intensity was used because it generally elicits 95-100% responding but is not too loud as to cause ceiling effects for the measured startle magnitudes.

Acoustic Startle Response Measures: Acoustic startle responses are measured by rectifying the signal and dividing those values by each rat's body weight. This correction is important for stressed rats will exhibit an attenuated growth curve following stressor exposure. Baseline is determined from the 200 ms of signal that precedes sound onset. Six times the standard deviation of the baseline period signal is used as the threshold for detecting significant deviations in body movement occurring 5-125 ms following stimulus onset. For those trials where the signal does not exceed the threshold criterion within

the specific window, an "NA" was recorded and the trial was not used in the calculation of the mean startle magnitude.

Procedure: All rats were pre-tested to obtain mean startle response magnitudes prior to stressor exposure. Rats were then matched based on those mean startle values and one of each pair was randomly assigned to either receive inescapable tailshock or serve as a homecage control. Tailshock began between 0700 and 0800 h (within the first hour of the daily 12 h light phase). Subsequent startle testing was conducted between the hours of 0730 and 1130 1, 4, 7, and 11 days after stressor exposure. Body weights were measured following each startle test. The same procedures were repeated a second time with the stressor manipulation being 3 days of consecutive tailshock.

Results: As shown in Figure 1, those rats that were exposed to 1 session of inescapable tailshock did not exhibit any significant increase in startle reactivity above that displayed by the homecage controls or themselves (during the pretest). However, also shown in Figure 1 is evidence that the shock did cause a significant change in the physiology of the shock-exposed rats. Body weight differences were evident from the first post-stress startle test to the last (post stress day 11).

A subsequent experiment extended the shock period to 3 days. As is evident in Figure 2, startle magnitudes significantly increased in rats exposed to 3 days of tailshock. This was confirmed by a significant Stress x Day interaction, $F(4,776)=7.6$, $p<.001$. The increase in startle reactivity occurred 4 days following the last session of shock exposure and it gradually decreased over subsequent startle testing sessions. These behavioral changes were coupled by significant differences in body weight across all post-stress assessments.

Conclusion: These data suggested a 3-day exposure would be necessary to create the delayed-presentation, enhanced startle reaction. Despite there being similar effects of 1 and 3 shock-exposure sessions in attenuating the stressed rats' growth curves, apparently a different mechanism is the cause of the increased startle magnitudes observed 4 days following 3 days of tailshock.

Experiment 2: Naloxone effects on startle reactivity

Before we administered the opiate antagonist naloxone to rats subjected to tailshock, we conducted an experiment where we tested

whether any acute or persistent changes in startle reactivity occurred from the 2 doses proposed for use.

Procedure: All rats (n=12) were pre-tested to obtain mean startle response magnitudes prior to stressor exposure. Rats were then matched based on those mean startle values, stratified in triplicates, and were randomly assigned to receive 1 or 10 mg/kg naloxone or saline vehicle. The rats were administered the drugs and subsequently tested for startle reactivity 2 h later (Day 1) and periodically thereafter over the same timeframe as followed for the stress experiments.

Results: No changes in startle reactivity were found following naloxone administration with respect both to baseline startle reactivity or vehicle controls. Still, as evidenced in Figure 3, the 10 mg/kg dose did show a trend to possibly cause an acute (albeit very moderate) elevation in startle reactivity.

Conclusion: These data suggested that an acute effect of naloxone could increase startle reactivity regardless of exposure to the tailshock. Hence, for the subsequent experiment treating rats with naloxone following tailshock, we decided it best to treat the rats at a time further removed from the startle testing.

Experiment 3: Post-stress opiate receptor blocking and startle reactivity

Once we established an enhanced startle could be achieved with a 3-day tailshock regimen and naloxone had a non-significant increase in startle reactivity, our goal was to determine whether the delay in the presentation of the enhanced startle could be reduced by blocking opiate receptors following exposure to the stressor. In other words, we were testing whether opiate antagonist naloxone would reveal a hypothesized masked hypervigilant state the day after the last session of tailshock. Because of the possible acute effect of naloxone on startle reactivity, we administered the opiate antagonist at the end of each day of tailshock. If there is an opiate-dependent mechanism involved in masking the acute hypervigilance, then disrupting those processes following each tailshock exposure should block the development of any masking effect due to endogenous opiates.

Procedure: All rats were pre-tested to obtain mean startle response magnitudes prior to stressor exposure. Rats were then matched based on those mean startle values and one of each pair was randomly assigned to either receive inescapable tailshock or serve as a homecage control. As in the previous studies, tailshock began between

0700 and 0800 h on 3 consecutive days. Immediately following each session, naloxone (10 mg/kg) or saline vehicle was administered systemically (i.p.). Subsequent startle testing was conducted between the hours of 0730 and 1130 1, 4, 7, and 11 days after stressor exposure. Body weights were measured following each startle test.

Results: Startle magnitudes changed as a function of Day, $F(3,60)=3.1$, $p < .05$ and Trial Block, $F(9,180)=12.8$, $p < .001$, as repeated testing occurred following stressor exposure. As shown in Figure 4, mean startle magnitudes on post-stress day 4 were significantly different than those on post-stress day 7. Within session mean startle magnitudes differed from block 1 from all other trial blocks (data not shown). This is suggestive of a general pattern of within-session habituation across all groups. Unexpectedly, there was a trend toward the factor Stress being associated with lower startle magnitudes, $F(1,20)=3.7$, $p < .06$. There was no significant effect of naloxone administration on mean startle magnitudes.

Conclusion: Despite past experiments showing enhanced startle reactivity could be elicited by a 3-day intermittent tailshock protocol, there was no sign of an increase in startle responding following inescapable shock in this experiment. In fact, the main effect trend was a significant difference due to stressor exposure that was in the opposite direction as previously observed. Therefore, it was impossible to evaluate an unmasking hypothesis when there was no delayed presentation of an enhanced startle response.

Experiment 4: A different approach to startle enhancements associated with stress

The inescapable stress model of enhanced startle reactivity is largely based on the concept that PTSD-like startle hyper-reactivity is a product of a single traumatic event, but, there are other models that may track the progression of an anxiety state in rats – namely the development of avoidant behavior. Avoidance is a common symptom to all anxiety disorders, including PTSD, and the presentation of increased avoidant behavior has been found to track the general worsening of PTSD symptoms (Karamustafalioglu et al. 2006;O'Donnell et al. 2007). Given that increased avoidant behavior is associated with PTSD symptoms, we hypothesized that a procedure which allows for a slow, methodical progression of increasing avoidant behavior may cause an increase in startle reactivity.

There are various forms of active avoidance that can be modeled in rats, but the desire to track the development of increased avoidant

behavior over time led us to adopt distinct lever-press avoidance as our active avoidance procedure. Lever-press avoidance has been utilized for decades to study learning, but it also has a history as a prominent model of anxiety (Pearl 1963;D'Amato and Fazzaro 1966;Hurwitz and Dillow 1968;Gilbert 1971;Dillow et al. 1972;Berger and Brush 1975). Derived initially from the 2-factor theory of threat/fear motivation and learned avoidance (Mowrer 1939a;Mowrer 1939b;Mowrer and Lamoreaux 1942;Mowrer and Lamoreaux 1946), the general premise of this approach is that a learned fear of signals is sufficient to support avoidant behavior without requiring a continued re-exposure to the actual noxious stimulus or event. To our knowledge, nobody has tracked how startle reactivity may change over time as a product of learned lever-press avoidance.

There are 3 possible periods of time startle reactivity may show changes as a function of acquiring lever-press avoidance and each would have associated with it a different theory of how the learning procedure was affecting general sensory reactivity. First, based on the above inescapable shock model, one could hypothesize that startle reactivity should be increased within days of the first few training trials, when the rats experience the most shock. Second, if the development of avoidant behavior follows the trajectory of developing anxiety, then one could hypothesize that startle reactivity should increase over acquisition. Yet, there is also a third option. That is, startle reactivity could increase if the association between the signals and the consequence becomes less certain. In this third possibility, startle reactivity could be increased if there is a change in the relationship between the signals that represent threat and the consequences following acquisition (such as conducting extinction trials). Importantly, these 3 timeframes also have associated with them different possible physiological correlates. For instance, only in early acquisition, when shock exposure is highest would we expect a possible endogenous opiate mechanism to be involved. If startle reactivity is increased during asymptotic performance or during extinction trials, then the likelihood that a direct-masking opiate mechanism may be involved in avoidance-related enhanced hypervigilance is substantially reduced.

Another consideration for this approach is that only certain animals may be affected in a way that increases their overall startle reactivity. It is well documented that approximately 10% of those people who experience a significant trauma develop PTSD; therefore, there has been recent interest in identifying vulnerability factors. We previously showed that Wistar-Kyoto (WKY) rats learn active avoidance generally quicker and to a higher asymptotic performance

level than Sprague Dawley (SD) rats (Servatius et al. 2008). In that same study, we also showed WKY rats extinguish the avoidance response much slower than SD rats, suggesting that they are resistant to associating learned warning signals in their environment with a lack of threat. This strain difference in both acquiring the avoidant behavior and resistance to extinguish it may be a sign of anxiety vulnerability that could also be reflected in a change in startle reactivity.

We hypothesized that any startle reactivity change that would be caused by exposure to shocks would be most evident in acquisition in SD rats. SD rats generally do not learn the response as well; hence, they would receive more shocks. In contrast, any observation of enhanced startle that is due to the learning of avoidant behavior or to the change in learned avoidance contingencies should be found in WKY rats. WKY rats become more avoidant than SD rats and are resistant to stop presenting the avoidant behavior once acquired.

Lever-press avoidance: Each rat is placed in a standard 30cm x 25cm x 30cm dimly lit (14W house-light) operant conditioning chamber that is contained in a sound-attenuating box fitted with a fan for air-circulation (Coulbourn Instruments). One side of the chamber has a lever (10.5 cm above the floor-bars), a white light (20.5 cm above the floor-bars), and a speaker (26 cm above the floor-bars). Each of the 20 trials (per session), begins with the presentation of the warning signal (1000 Hz tone). After 60 s of warning signal, 0.5 s intermittent shocks (1-2 mA) are delivered to the grid floor every 3 s until the lever is pressed (an escape response). The warning signal is presented throughout this time. If the lever is pressed during the initial 1min of warning signal, the shocks are avoided for that trial (an avoidance response). After the lever is pressed (either an escape or avoidance response) a 3 min inter-trial interval (ITI) occurs. The ITI period is explicitly distinguished by a 0.5Hz flashing light. During the 3 min ITI, no shocks are ever presented. Extinction sessions involve the removal of both the shock and the ITI signal.

Startle Sensitivity/Responsivity Assessment: Unlike the protocol described above, commonly used to assess differences in within session habituation and sensitization, a multi-stimulus intensity protocol was used in order to assess any changes in the threshold to elicit an acoustic startle response (sensitivity) or the magnitude of those elicited responses (responsivity). The stimuli had the same duration and rise-fall characteristics as the above experiments. The three different stimulus intensities (82, 92, 102 dBA) were presented 8 times in a pseudorandom order such that the same intensity was never presented consecutively. These 3 intensities were shown in the past to

elicit startle responses in 10-20, 50-60, and 90-100% of trials, respectively. Therefore, any changes in stimulus sensitivity would be reflected in a change in the parentage of responses elicited.

Procedure: Prior to avoidance training rats of both strains were tested to obtain a baseline level of startle reactivity. Rats within each strain were matched, stratified, and randomly assigned to either the homecage-control or avoidance-learning condition. The following week, avoidance training commenced. Avoidance learning sessions occurred 3 days a week, with at least one day separating consecutive avoidance learning sessions. Startle testing occurred on one of the non-avoidance training days, once each week.

Results: Lever-press avoidance behavior was acquired in both strains. As shown in Figure 5, the acquisition patterns were similar, as reflected in a main effect of Day, $F(9,135)=25.6$, $p<.001$, but the WKY rats attained a higher asymptotic level of performance. Extinction followed the same pattern. WKY rats appeared to exhibit much slower extinction of the lever-press response, but only a main effect of Day, $F(9,135)=14.1$, $p<.001$, was significant. Analysis over both acquisition and extinction did almost yield a significant effect of Strain, $F(1,15)=4.0$, $p<.06$.

Startle sensitivity measures mildly changed during the avoidance training period in both strains. As shown in Figure 6, the number of startles elicited increased in the rats trained to perform the lever-press avoidance response. Following the first acquisition session (denoted as A1), those rats exposed to the avoidance training exhibit more startles to the mid-intensity stimulus. For WKY rats, this is still apparent after 4 days of acquisition. These impressions were confirmed by 2 significant interactions. A Strain x Stimulus Intensity interaction, $F(2, 60)=7.4$, $p<.001$, suggested that the 2 strains responded differently to the 3 intensities. A Condition x Intensity x Day interaction, $F(6, 180)=2.9$, $p<.01$, suggested the experience of avoidance learning differentially affected how the rats responded to each stimulus intensity and that effect was different depending on the test day. As rats of both strains attained asymptotic performance, no more differences in startle sensitivity were apparent between the avoidance and home-cage control condition of either strain. This trend continued throughout extinction with the avoidance condition failing to differ from those of the home-cage controls within both strains.

Because all subjects were initially matched, within strain, on startle responsivity, both between and within-subject analyses were conducted for this variable. For simplicity and the most appropriate

representation of responsiveness, only the data from the high-intensity trials were analyzed. As shown in Figure 7, there was a clear difference in startle magnitudes between the strains. This was confirmed by a main effect of Strain, $F(1,30)=49.0$, $p<.001$. An additional Condition x Day interaction, $F(11,330)=1.9$, $p<.05$, suggested that changes in startle magnitude occurred across the testing period and those changes were avoidance-experience dependent. Significant Strain x Condition x Day interactions were only evident during the extinction period, $F(2,60)=3.3$, $p<.05$. Between-group differences in startle magnitudes were evident following the 6^{th} and 9^{th} extinction sessions in both SD and WKY rats. Comparing session means to that of the baseline, we found within-group differences in both strains in those exposed to avoidance training following the 9^{th} extinction session.

As with the inescapable stress protocols, active avoidance behavior acquisition affected the growth rate observed in both strains. As can be observed in Figure 8, the effect upon each strain was different depending on the phase of the experiment. For instance, during acquisition, when exposure to shocks occurred periodically, the growth curves for the SD rats diverged more than those of WKY rats. These impressions were confirmed by Strain x Condition, $F(1, 30)=6.5$, $p<.02$, and Condition x Day, $F(3,90)=2.9$, $p<.05$, interactions. Through extinction sessions, a significant Strain x Condition x Day interaction was evident, $F(2,60)=3.6$, $p<.05$. Examining the figure, this interaction reflects the beginning of the convergence of the SD growth curves and the first signs of divergence in the WKY curves. Interestingly, following extinction, a Strain x Condition x Day interaction is still apparent. Yet, as can be seen in the figure, the cause of this interaction has shifted from the SD rats showing an avoidance-dependent divergence in growth to the WKY rats exhibiting an avoidance-dependent divergence in growth.

Conclusion: These data provide an interesting example of how startle reactivity can be enhanced by prior exposure to an escapable and avoidable stressor. Moreover, as was observed following inescapable stress, the presentation of enhanced startle reactivity did not occur proximal to any period of significant shock exposure. Following this change in behavior the differences in the physiology of the 2 rat strains became apparent, as reflected in a switch in body weight differences within each strain from their respective controls. This finding is of upmost importance for 2 reasons. First, it shows that inescapable and uncontrollable stress is not necessary to increase startle reactivity, and yet, the appearance of the startle enhancement is still delayed. Second, these features are suggestive that a

mechanism not specifically triggered by the shock is causing the startle response to increase over time. Therefore, the concept that the delayed presentation of enhanced startle responding following a significant trauma is due to the startle-enhancing mechanisms being masked by other physiological responses for a period of time following the trauma is not supported.

The additional finding that these 2 strains exhibit a very different pattern of growth, through and following avoidance learning, is suggestive that the result of avoidant behavior acquisition has a short-term effect on SD rats, but a long-term effect on WKY rats. Further work will need to address whether this difference is due to the actual learning process or if it is a function of shock exposure.

Key Research Accomplishments

1. We demonstrated that 1 day of inescapable tailshock is not sufficient to increase startle reactivity in SD rats.
2. We demonstrated that 3 days of inescapable tailshock are sufficient to increase startle reactivity in SD rats.
3. We demonstrated that there is a dosage range where acute naloxone does not appreciably influence startle reactivity.
4. We demonstrated that naloxone does not affect startle reactivity following inescapable tailshock.
5. We demonstrated that inescapable stress is not necessary to increase startle reactivity. Both SD and WKY rats will exhibit increased startle reactivity several days following any exposure to escapable/avoidable shock.

Reportable Outcomes

The work supported herein was presented at the Military Health Research forum August 31-September 3, 2009 in Kansas City, Missouri. The data derived from this funding has produced additional grant proposals to the Department of Veterans Affairs and are a portion of the doctoral thesis of Thomas Ricart.

Conclusion

At this time, it appears the data derived fails to support an opiate-masking hypothesis for the development of delayed enhanced startle responding following exposure to a stressor. The fact that enhanced startle could be elicited in rats exposed to shocks they learned to escape and later avoid provides further evidence that the increased sensory reactivity experienced by the rats is not purely due to exposure above some type of threshold level of shock. Thus, the difference between 1 and 3-day inescapable shock on startle reactivity may not be due to the total number of shocks as much as it relates generally to more episodes (sessions) with experiencing some minimal level of stress and trying to cope with that stress. A test of this hypothesis could occur by reducing the number of shocks presented over the 3-day period to match that of a single session. If this type of procedure was shown to also elicit enhanced startle reactivity, when taken with the avoidance data, one conclusion that could be drawn is that the enhanced startle occurs as a lingering result of coping with repeated stressful situations. However, there is also the possibility that the increased startle reactivity is occurring because the stressed rats responding to the change in testing contingencies. In this case, we would be suggesting that anxiety comes from the rats being unsure about their environment. The addition of yoked-controls to the avoidance learning situation could account for this possibility. In both cases, differences in the ability to cope with a stressor would then have to be viewed as a critical factor in the development of subsequent abnormal behavior associated with clinical anxiety.

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SUPPORTING DATA

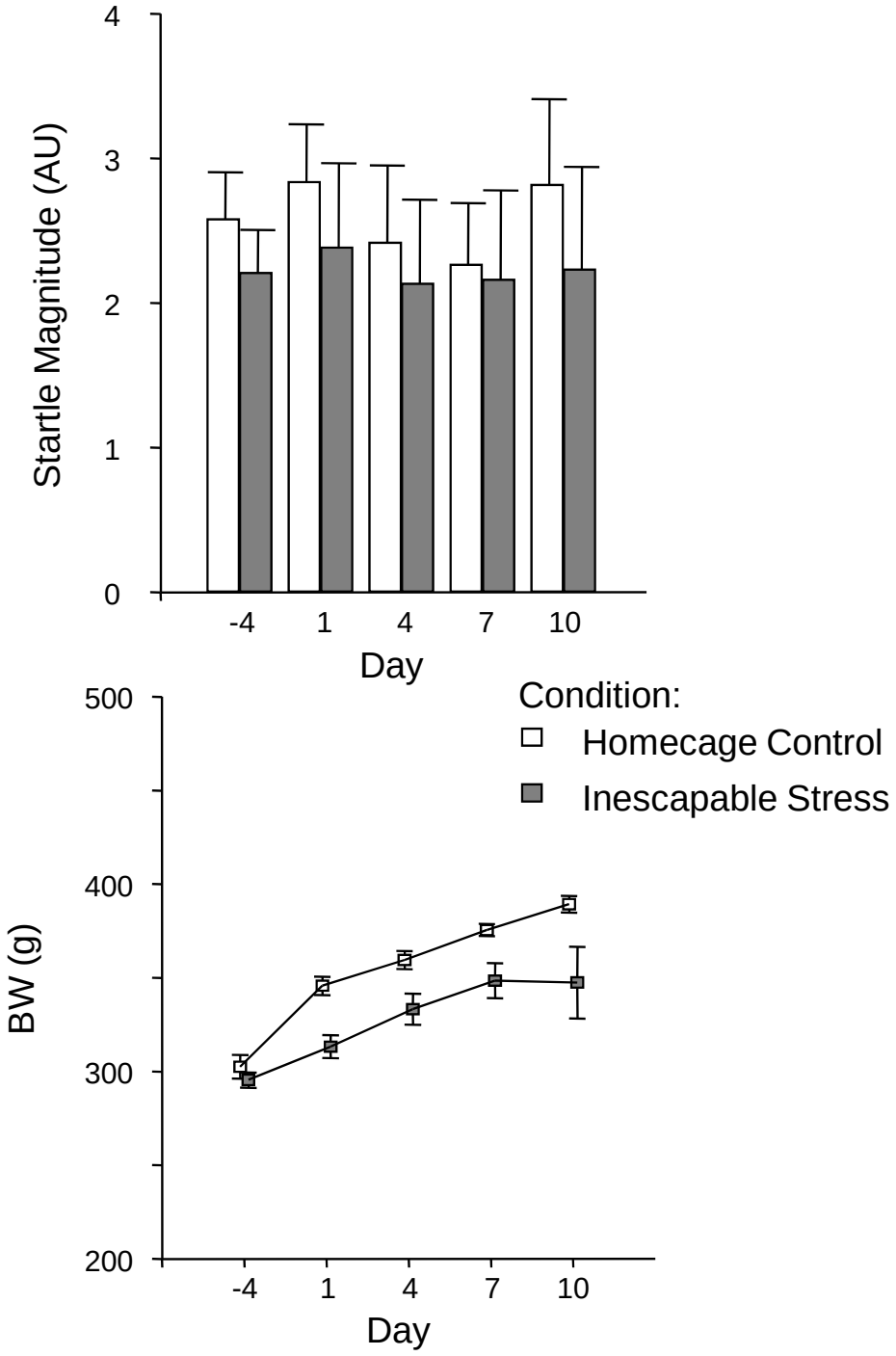

Figure 1: Mean startle response magnitudes and body weights for rats exposed to a single session of inescapable tailshock or served as homecage controls (n=4/group) .

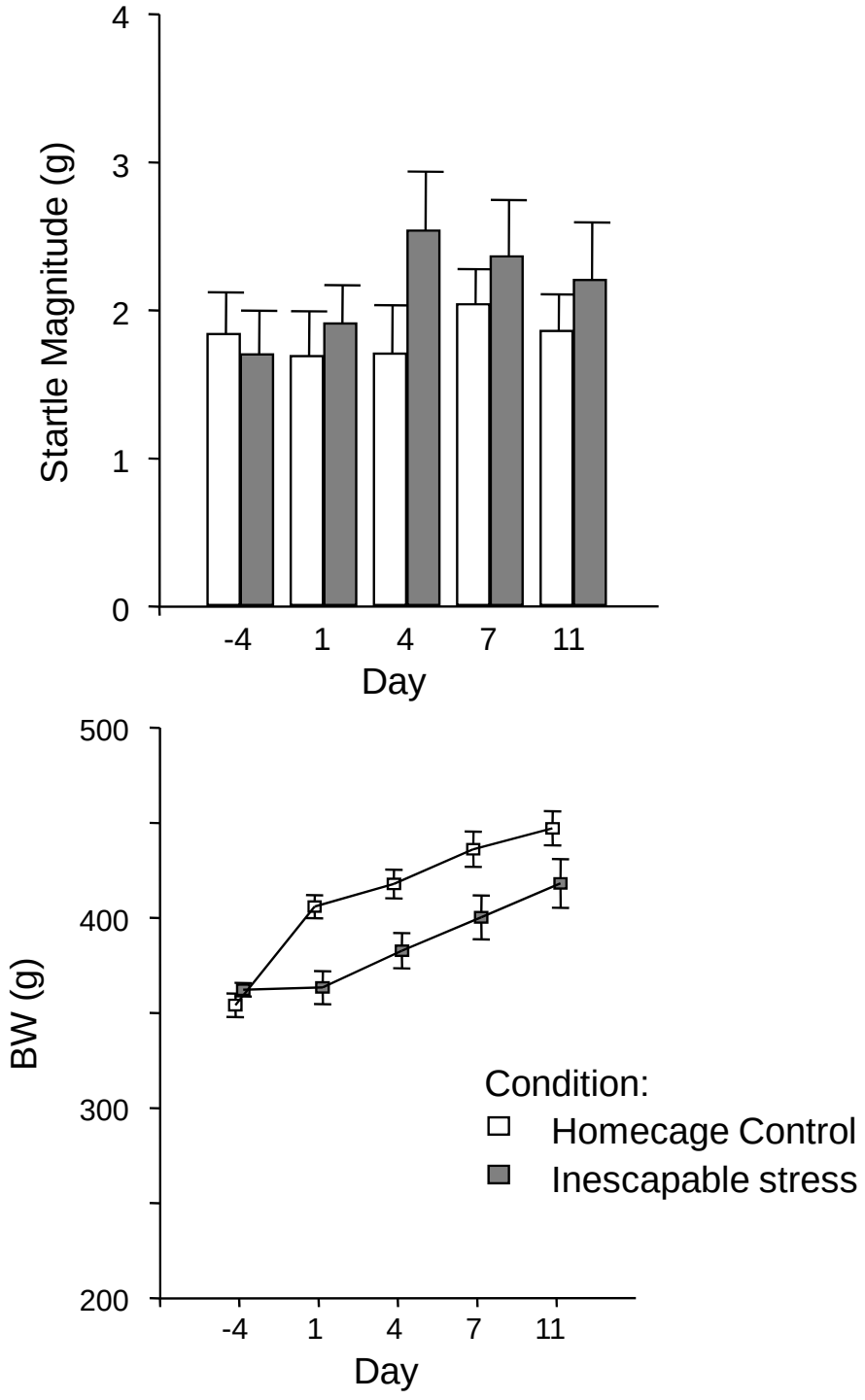

Figure 2: Mean startle response magnitudes and body weights for rats exposed to 3 sessions of inescapable tailshock or served as homecage controls(n=8/group).

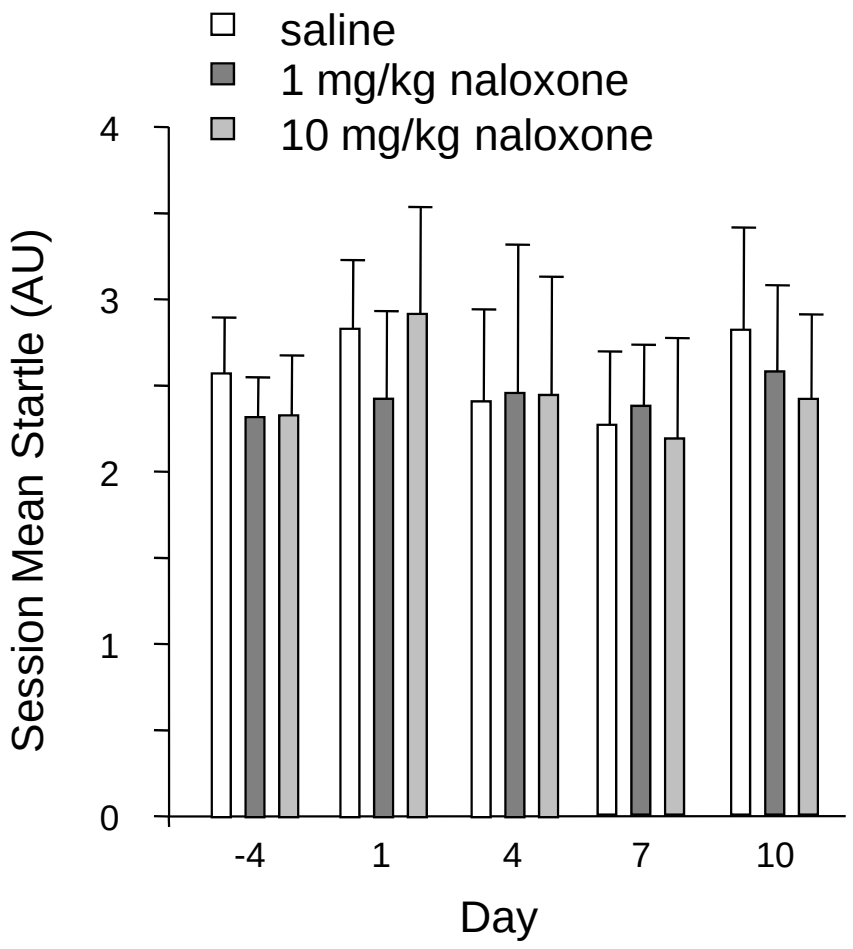

Figure 3: Mean startle response magnitudes for rats administered 1 of 2 possible doses of naloxone 30 min prior to startle testing (n=4/group).

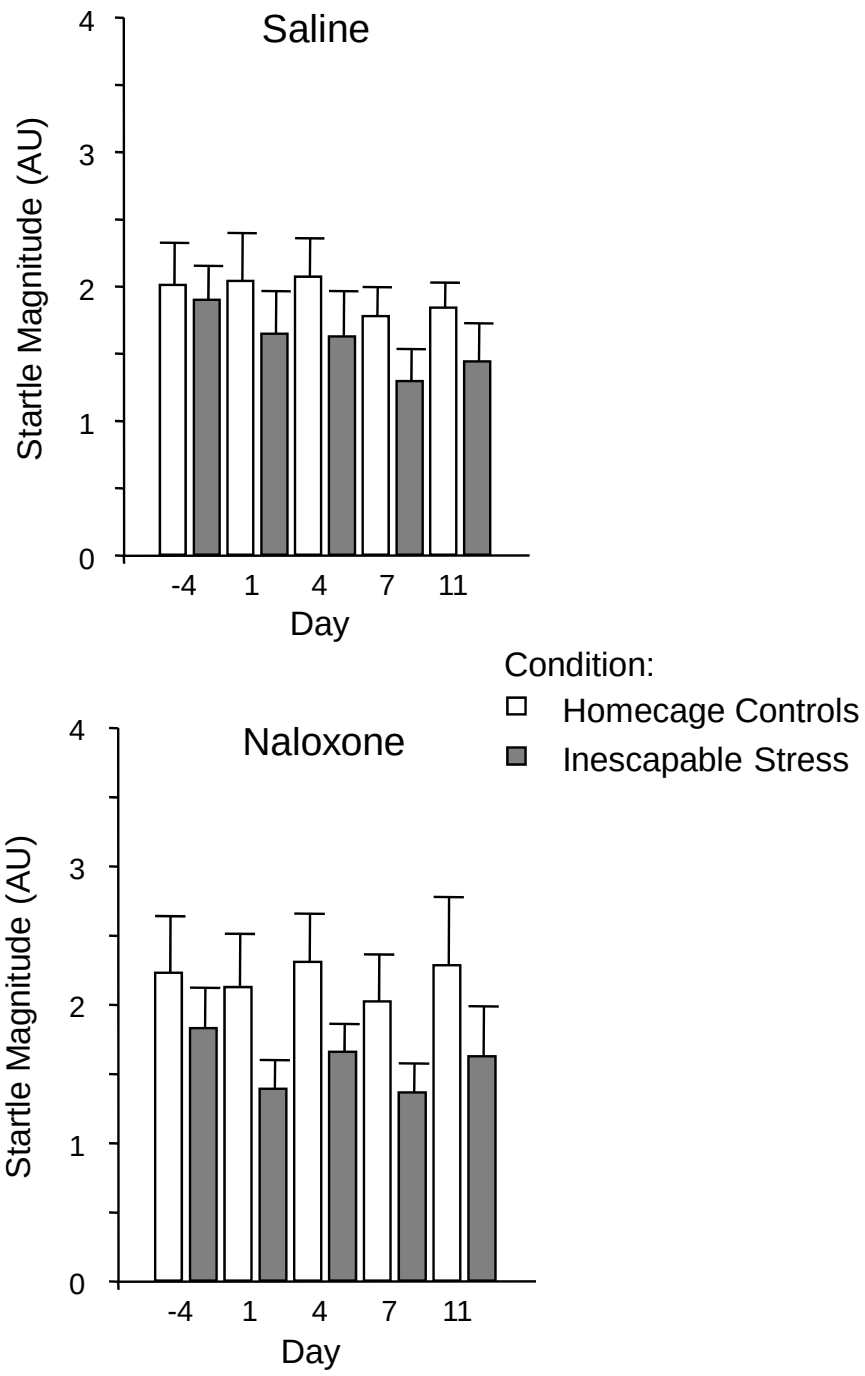

Figure 4: Mean startle response magnitudes for rats administered 10 mg/kg naloxone (bottom) or saline vehicle (top) following each 2 h session of shock or at the same time in the homecage (n=8/group).

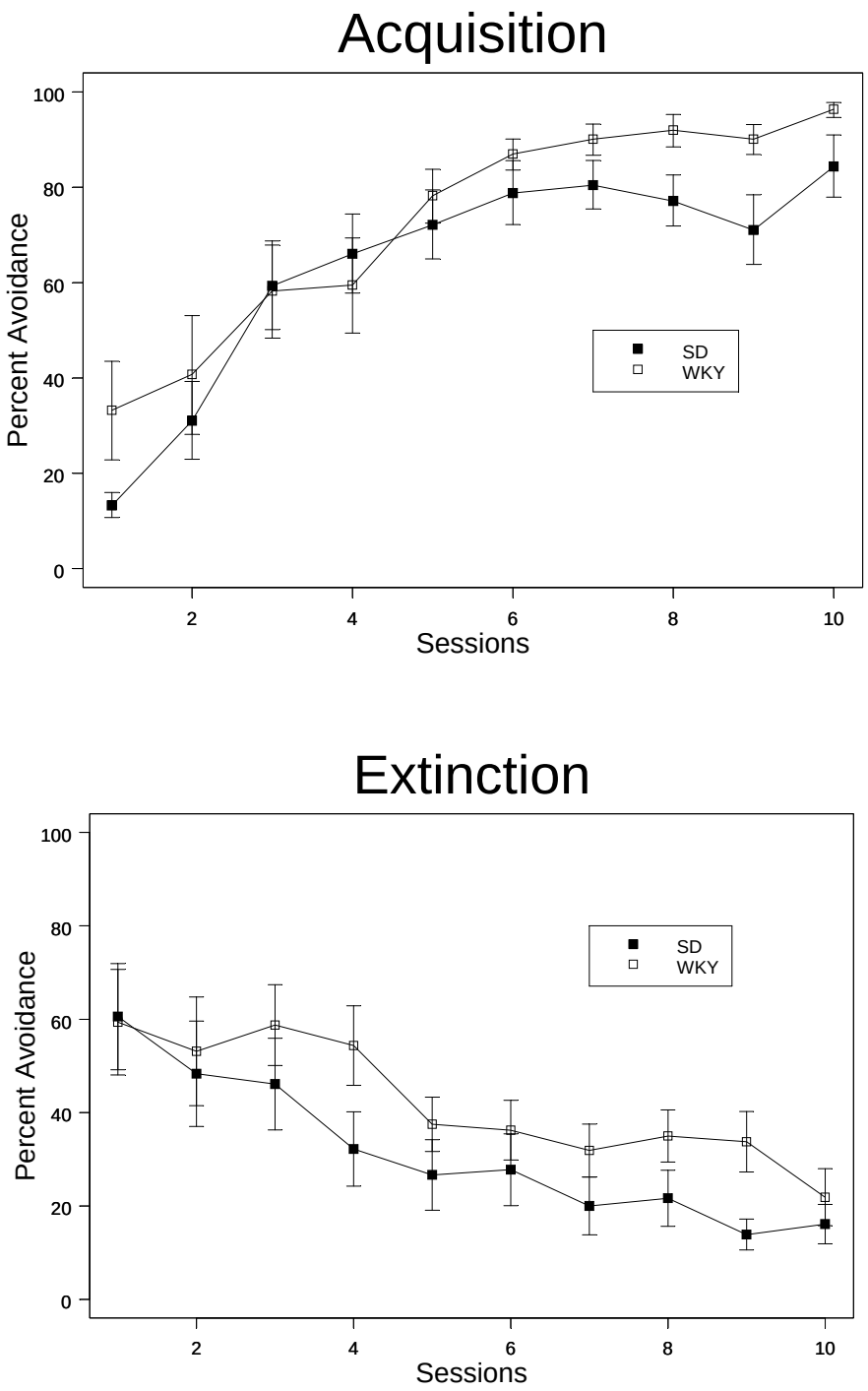

Figure 5: Sprague Dawley (SD) and Wistar-Kyoto (WKY) rats were trained over 10 sessions to avoid footshock (top). Subsequent sessions had the shock absent to assess the rate by which the behavior is extinguished (bottom).

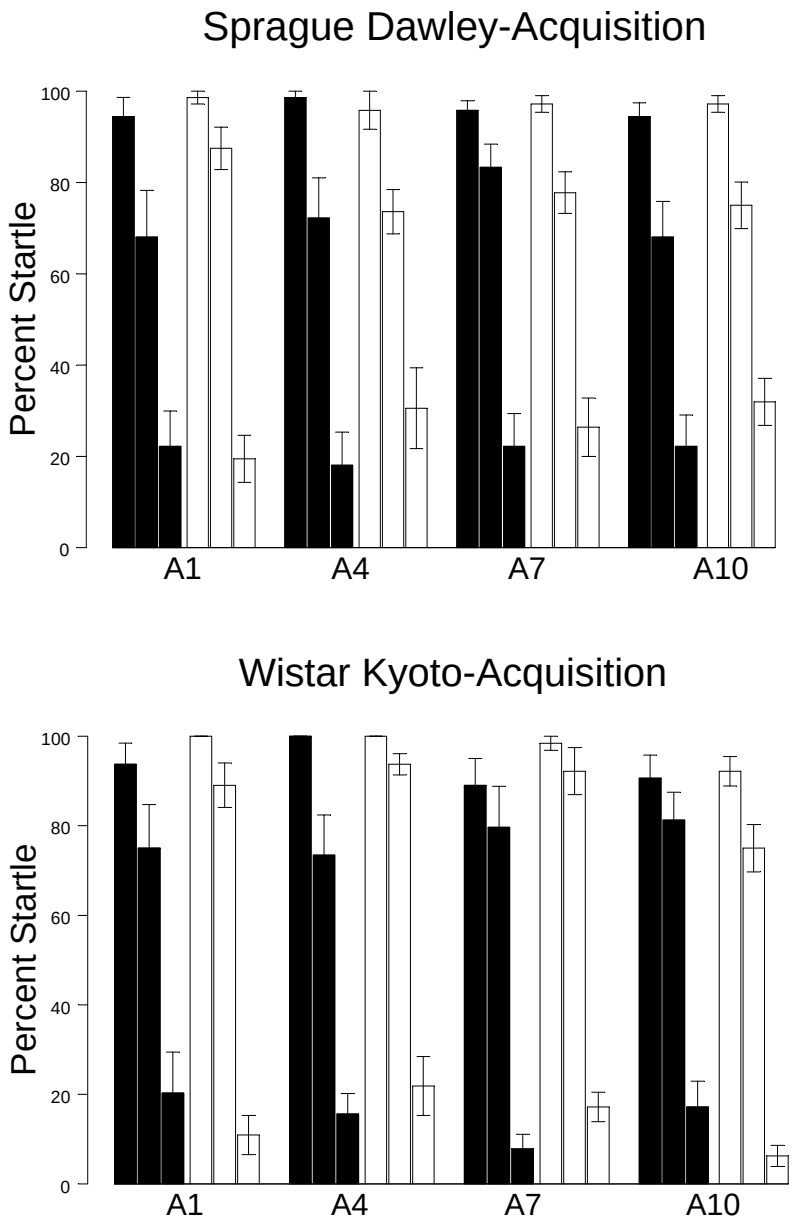

Figure 6: The percent of trials for which a startle response was elicited at 3 different stimulus intensities in rats trained in avoidance behavior (open) versus homecage control (darkened). For each session, the columns represent 102, 92, and 82 dBA, moving from left to right.

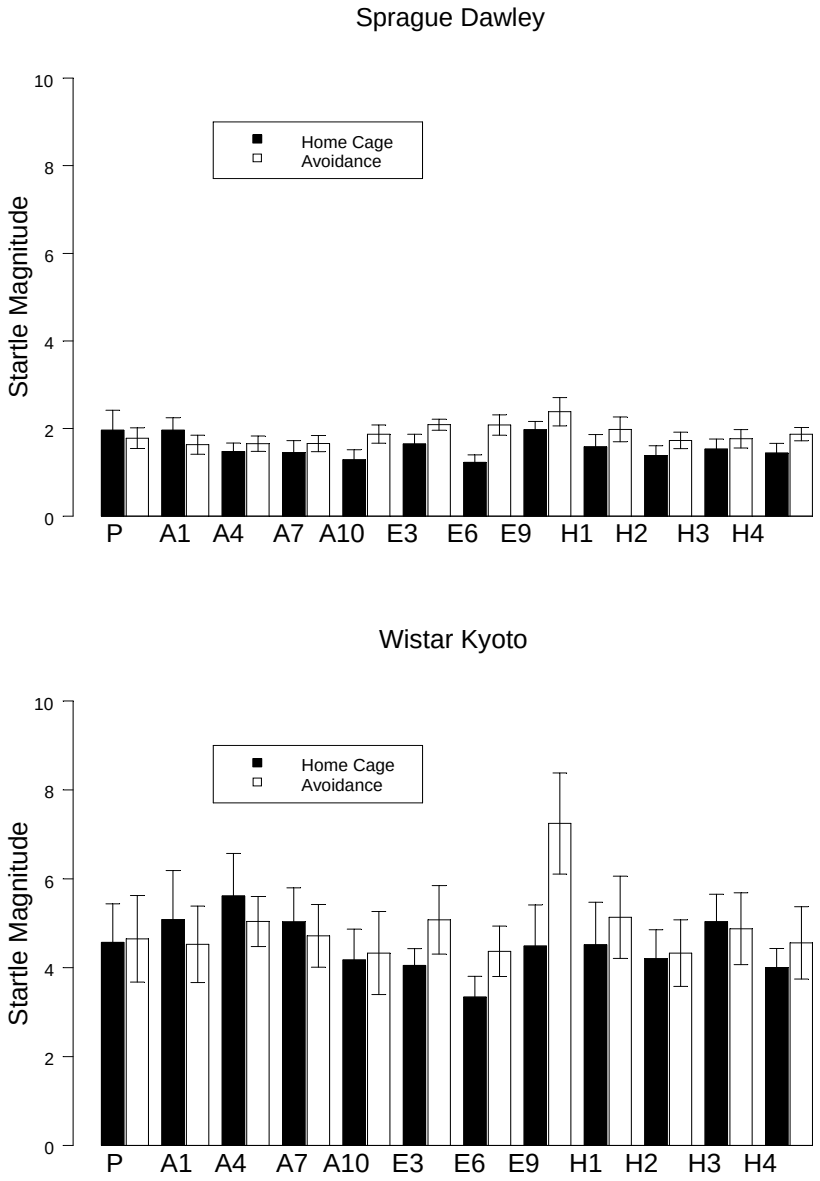

Figure 7: Mean startle response magnitudes for rats either exposed to avoidance learning (open) or served as homecage controls (filled). Testing began with a pretest (P) from which rats were matched prior to group assignment. Subsequent startle testing occurred weekly during 4 weeks of avoidance training (A), 3 weeks of extinction (E), and the last 4 weeks remaining in the homecage (H).

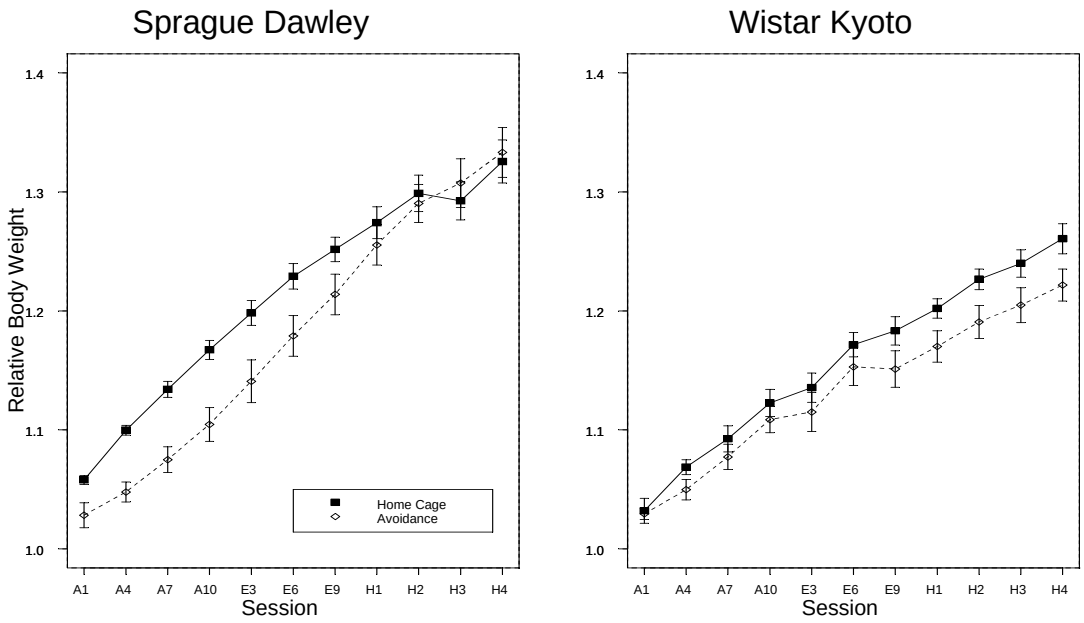

Figure 8: Shown are the mean body weights for each rat strain exposed to avoidance learning (open) or served as homecage controls (filled). All rats had their bodyweights measured following each startle test during avoidance training (A), extinction (E), and the last 4 weeks staying in the homecage (H).