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Blunted Hypothalamo-pituitary Adrenal Axis Response to Predator Odor Predicts High Stress Reactivity

Annie M. Whitaker and Nicholas W. Gilpin

Department of Physiology, Louisiana State University Health Sciences Center, New Orleans, LA

Abstract

Individuals with trauma- and stress-related disorders exhibit increases in avoidance of trauma-related stimuli, heightened anxiety and altered neuroendocrine stress responses. Our laboratory uses a rodent model of stress that mimics the avoidance symptom cluster associated with stress-related disorders. Animals are classified as 'Avoiders' or Non-Avoiders' post-stress based on avoidance of predator-odor paired context. Utilizing this model, we are able to examine subpopulation differences in stress reactivity. Here, we used this predator odor model of stress to examine differences in anxiety-like behavior and hypothalamo-pituitary adrenal (HPA) axis function in animals that avoid a predator-paired context relative to those that do not. Rats were exposed to predator odor stress paired with a context and tested for avoidance (24 hours and 11 days), anxiety-like behavior (48 hours and 5 days) and HPA activation following stress. Control animals were exposed to room air. Predator odor stress produced avoidance in approximately 65% of the animals at 24 hours that persisted 11 days post-stress. Both Avoiders and Non-Avoiders exhibited heightened anxiety-like behavior at 48 hours and 5 days post-stress when compared to unstressed Controls. Non-Avoiders exhibited significant increases in circulating adrenocorticotropin hormone (ACTH) and corticosterone (CORT) concentrations immediately following predator odor stress compared to Controls and this response was significantly attenuated in Avoiders. There was an inverse correlation between circulating ACTH/CORT concentrations and avoidance, indicating that lower levels of ACTH/CORT predicted higher levels of avoidance. These results suggest that stress effects on HPA stress axis activation predict long-term avoidance of stress-paired stimuli, and builds on previous data showing the utility of this model for exploring the neurobiological mechanisms of trauma- and stress-related disorders.

Keywords

Stress; HPA axis; paraventricular nucleus; anxiety; avoidance

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Mail Proofs and Correspondence to: Nicholas W. Gilpin, Ph.D. Department of Physiology, LSU Health Sciences Center, 1901 Perdido Street, New Orleans, LA 70112, Phone: 504.568.6182, Fax: 504.568.6158, ngilpi@lsuhsc.edu.

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1. Introduction

Traumatic stress disorders (e.g., post-traumatic stress disorder; PTSD) are debilitating disorders that affect 7.7 million Americans (20). Although there are several animal models of stress, few effectively capture the multi-faced behavioral and neuroendocrine disturbances, and the individual variability that define stress-related disorders in humans (26). Different types of stressors such as footshock or restraint stress produce varying degrees of enhanced anxiety-like behavior and activation of endocrine stress systems (2, 3, 13, 26). However, most current models typically study mean group stress effects without examining individual differences in stress reactivity. Our laboratory uses predator odor exposure (bobcat urine), a rodent model of stress that produces significant physiological and behavioral alterations including endocrine disturbances, heightened anxiety-like behavior and exaggerated startle reactivity (28). Predator odor stress produces increases in anxiety-like behavior, hyperarousal, fear conditioning, and resistance to fear extinction, and these effects are long-lasting, similar to what is seen in humans (6, 7, 24). The conditioned place aversion paradigm utilized in these studies provides a powerful tool for discerning subpopulations that respond differently to stress. This model produces persistent avoidance in a subpopulation of animals that continues for at least six weeks (10). Previously, we have shown that animals that exhibit avoidance of trauma-related environments also show long-term increases in alcohol consumption (10). Moreover, avoidance behavior is predictive of brain region-specific neuronal activation patterns upon re-exposure to predator odor-paired context (10). These data demonstrate that the utility of this predator odor stress model for examining the behavioral and neural adaptations associated with high and low stress reactivity.

Dysregulation of the hypothalamo-pituitary adrenal (HPA) axis has been implicated in the development of stress-related disorders and other psychiatric disorders (7, 23). The neuroendocrine HPA axis is activated during stress. Corticotropin releasing factor (CRF), a peptide hormone secreted from the paraventricular nucleus (PVN) of the hypothalamus, initiates the stress response and subsequent release of adrenocorticotropin releasing hormone (ACTH) and cortisol (corticosterone [CORT] in rodents) (15, 16). Stress-related disorders are associated with glucocorticoid abnormalities and HPA dysfunction. Studies by Yehuda et al. demonstrated that individuals with lower levels of circulating cortisol immediately after stress are more likely to subsequently develop PTSD (7, 27) and urinary cortisol levels are lower in trauma survivors with PTSD relative to those without (19). These data suggest that stress-induced hypo-cortisolemia in individuals with stress-related disorders may mediate post-stress behavioral dysregulation. In the present study, we examined the effects of predator odor stress on anxiety-like behavior and HPA activation in animals that exhibit persistent avoidance of a predator odor-paired context following stress. We hypothesized that animals with blunted ACTH and CORT responses to predator odor stress would subsequently exhibit higher avoidance of predator odor-paired context and higher anxiety-like behavior.

2. Methods

2.1 General Methods

2.1.1 Animals—Specific-pathogen free adult male Wistar rats (Charles River, Kingston, NY) initially weighing 200-250 g at the time of arrival were housed in groups of two and fed a standard rat diet (Purina Rat Chow, Ralston Purina, St. Louis, MO) *ad libitum* except during experimental procedures. Rats were exposed to a 12h light/12h dark cycle (lights off at 8pm). Animal procedures were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center (LSUHSC) and were in accordance with the National Institute of Health guidelines. Rats were given one week to acclimate to their surroundings and were handled daily prior to initiation of surgical of experimental procedures.

2.1.2 Surgical Procedures—On the day of surgery, rats were anesthetized with isoflurane and implanted with a silastic catheter into the right external jugular vein using sterile surgical procedures as previously described by our laboratory (14). The catheter consisted of silastic tubing (0.025 inch inner diameter, 0.047 inch outer diameter; Dow Corning, Midland, MI,) connected to a metal guide cannula (22 gauge; Plastics One, Roanoke, VA) bent at a right angle. Catheters were flushed with heparinized saline (0.2 ml), routed to the back of the rat and sealed with a plastic cap and metal cannula cover. This catheter design allows for serial blood sampling over an extended period of time. Starting at 4 days post-surgery, catheters were flushed once daily with cefazolin (20 mg i.v.; Sigma-Aldrich, St. Louis, MO) in heparinized saline (20 U/ml, 0.1 ml total volume). Rats were allowed to recover for 1 week following surgery before initiating experimental procedures to allow stress hormone levels to return to baseline.

2.1.3 Conditioned Place Aversion—To index rats for avoidance, animals underwent a classical 4-day conditioned place aversion (CPA) paradigm previously described by our laboratory (10). Briefly, on the first day rats underwent a 5 min video-recorded pre-test to explore two conditioning chambers with distinct tactile (circles vs. rods floor grids) and visual (stripes vs. circles) cues. Rats were counter-balanced for preferred chamber and conditioning chambers assigned in an unbiased procedure. On the second day, rats were placed in one chamber without odor (neutral environment) for 15 min. On the third day, rats were placed in the opposite context for 15 min with a urine-soaked sponge (bobcat urine) placed under the floor of the chamber (predator odor environment). On the fourth day, rats underwent a 5 min video-recorded post-test to explore the two conditioning chambers. Control animals were treated identically but not exposed to predator odor. Avoidance was calculated as a difference score between post-conditioning time spent in odor-paired context and pre-conditioning time spent in odor-paired context. Rats that display a >10 sec decrease in time spent in odor-paired chamber were classified as 'Avoiders'. Rats that display a <10 sec decrease in time spent in odor-paired chamber were classified as 'Non-Avoiders'. This group division criterion was selected to allow for the use of a single criterion across studies that is unaffected by sample distribution (i.e., two rats with the same score will always be in the same group, regardless of scores for other rats in the sample); and also because early

studies indicated that the 10-s cut-off criterion provides divergent and persistent avoidance scores that are predictive of brain and behavior changes (10).

2.1.4 Elevated Plus Maze—We utilized the elevated plus maze (EPM) test to examine the effects of predator odor exposure on anxiety-like behavior as previously described by our laboratory (10). Our EPM is a black Plexiglas apparatus consisting of two closed arms (50 cm long; 10 cm wide; walls 40-cm high) and two open arms (50 cm long; 10 cm wide) attached to metal legs elevating the maze 50 cm above the ground. The maze was placed in the center of the room and each open arm had similar levels of illumination. The test was initiated by placing the animal placed in the center of the EPM facing the open arm. Behavior was video-recorded for 5 min. The observer was not present in the room during testing. The EPM was cleaned thoroughly between subjects. A blinded observer determined the duration of time spent in the open and closed arm and arm entries. One entry was defined as all four paws entering the open/closed arm. Data are presented as % time spent in the open arm.

2.1.5 Open Field Test—Because the EPM cannot be repeated, we utilized the open field test as an additional measurement of anxiety-like behavior to examine the persistence of anxiety-like behavior observed in the EPM. Our open field box consists of a black Plexiglas arena (72cm × 72cm × 36cm) with white lines dividing the floor into 25 squares (5 × 5 squares with grid lines). The 16 squares adjacent to the outer perimeter of the box represent the wall (periphery) of the box and the 9 remaining squares represent the center. The apparatus was placed in the center of the room and each area of the box has similar levels of illumination. The test was initiated by placing the animal in the center of the open field box and behavior was video-recorded for 5 min. The observer was not present in the room during testing. The open field box was cleaned thoroughly between subjects. A blind observer determined the duration of time spent near the wall and in the center of the open field box. Data are presented as % time spent in the center of the open field box.

2.2 Experimental Protocols

All animals were subjected to one week of handling procedures prior to initiating the experimental protocols. Animals were conscious and unrestrained throughout the duration of the experiments.

2.2.1 Experiment I

Effects of predator odor stress on avoidance and anxiety-like behaviors: The aim of this study was to characterize the effects of predator odor stress on avoidance and anxiety-like behavior. Animals were subjected to a CPA paradigm as described above. Animals were indexed for avoidance 24 hours post-stress and classified as ‘Avoiders’ or ‘Non-Avoiders’. Forty-eight hours post-stress animals were tested for anxiety-like behavior using the EPM test. Animals were tested again for anxiety-like behavior five days post-stress utilizing the open field test. In order to confirm past results showing that avoidance was persistent (10), animals were re-tested for avoidant behavior 11 days post-stress. Animals were sacrificed under light isoflurane anesthesia by decapitation 12 days post-stress. The PVN was isolated from coronal brain slices (500µm) using a 14 gauge neuropunch for subsequent

determination of CRF content with radioimmunoassay (RIA) (see below). The brain punches were stored at -80°C until analysis.

2.2.2 Experiment II

Time course of predator odor stress on circulating ACTH/CORT: The aim of this study was to examine whether predator odor exposure produced activation of the HPA axis. Chronically-catheterized rats were acclimated to the sounds of the laboratory with white noise machines and experimental cages for 3 hours daily for one week prior to initiation of experimental procedures, and blood samples were collected (without handling rats) to obtain an accurate reflection of baseline ACTH and CORT concentrations. On the day of the experiment, the metal port of the catheters was connected to Tygon medical microbore tubing (0.020 I.D. × 0.060 I.D. × 0.020 I.D. (Merdock Industrial; Akron, OH) filled with normal saline and routed through a tether for protection. Animals were allowed 3 hours to acclimate to the room before the baseline sample was obtained. This acclimation period allowed for any stress effects from handling the animals to return to baseline. A blood sample (0.2 ml) was obtained (without handling the rats) at baseline for determination of circulating ACTH and CORT concentrations. After obtaining the blood sample, the catheter was flushed with normal saline (0.2 ml). After obtaining the baseline blood sample from all animals, a urine-soaked sponge was placed at the top of the cage for 10 min. The sponge was removed after 10 min and blood samples (0.2 ml) were obtained at 30, 60, 90, and 120 min post-stress and replaced with an equal volume of saline (0.2 ml). At the completion of the experiment, catheters were flushed with cefazolin, sealed and capped and rats were returned to their home cages.

2.2.3 Experiment III

Effects of predator odor stress on circulating ACTH/CORT in Avoider and Non-Avoider rats: The aim of this study was to examine the effects of predator odor stress on circulating ACTH and CORT concentrations in animals that exhibit persistent avoidance following stress. Animals underwent the 4-day conditioned place aversion paradigm as described above. On the second day, catheters were connected to Tygon medical microbore tubing (0.020 I.D. × 0.060 I.D. × 0.020 I.D. (Merdock Industrial; Akron, OH) filled with normal saline and routed through a tether for protection. Rats were then placed in one of the two distinct chambers without odor (neutral environment) for 15 min. At the completion of the neutral conditioning, a blood sample (0.2 ml) was obtained without removing animals from the chamber. The catheters were flushed with cefazolin, sealed and capped and rats were returned to their home cages. On the third day, catheters were connected to tubing for blood sampling as described above. Rats were placed in the opposite context for 15 min with a urine-soaked sponge (bobcat urine) placed under the floor of the chamber (predator odor environment). At the completion of the odor conditioning, a blood sample (0.2 ml) was obtained without removing animals from the chamber. The catheters were flushed with cefazolin, sealed and capped and rats were returned to their home cages. On the fourth day, animals were indexed for avoidance. While we started the study with 16 animals, one animal had a problem with the catheter and was excluded from the study. Also, data were not included for assay values that were not within range of the standard curve.

2.3 Analytical procedures

2.3.1 Blood sample collection—Blood samples (0.2 ml) were collected from the jugular catheter into chilled syringes. Blood samples were placed in tubes containing 10 μ L/ml of EDTA (Sigma, St. Louis, MO) and centrifuged for 10 min at 10,000 rpm and 4°C. Plasma was collected and stored at -80°C until analysis.

2.3.2 ACTH and CORT—Circulating ACTH concentrations were determined in plasma using a commercially available enzyme-linked immunosorbent assay (EIA) (MP Bioproducts, St. Paul, MN). The range of detection for the ACTH EIA was 0.22-500 pg/ml. Circulating CORT concentrations were determined using a commercially available RIA (MP Biomedicals, Solon, OH). The minimum detectable concentration of CORT by the assay was 7.7 ng/ml.

2.3.3. Brain tissue collection—Rats were sacrificed at 12 days post-stress for analysis of PVN CRF content. Briefly, rats were anesthetized with isoflurane, decapitated and the whole brains immediately excised and flash-frozen in 2-methyl butane (Sigma Aldrich; St. Louis, MO). Brains were sliced coronally in a cryostat and the PVN isolated from 500 μ m sections with a 14 gauge neuropunch with a rat brain atlas as a reference. Brain punches were stored at -80° C until the day of analysis.

2.3.4 Corticotropin releasing factor—PVN CRF content was determined by RIA in extracted samples (Phoenix Pharmaceuticals; Burlingame, CA). Briefly, tissue punches were homogenized in 0.01M PBS containing EDTA, Aprotinin (Sigma, St. Louis, MO) and 12.5% acetic acid. Lyophilized supernatant was stored at -80° C until extraction. The lyophilized powder was acidified with 1% trifluoroacetic acid in water (buffer A) and centrifuged for 10 min at 10,000 rpm and 4°C. C-18 SEP-Columns (Waters Corporation, Milford, MA) were washed with 1 ml buffer B (60% acetonitrile + 40% buffer A) followed by 3 washes with 3 ml buffer A. The acidified sample was loaded onto the pre-treated column and washed three times with buffer A. The peptides were eluted with 3 ml of buffer B and evaporated to dryness using a Speed Vac concentrator and condensation trap. The residue was stored at -80°C until analysis. On the day of the assay, the residue was reconstituted using the buffer provided by a rat-specific RIA kit (Phoenix Pharmaceuticals, Burlingame, CA). Values were normalized to total protein measured in the reconstituted sample using a BCA protein assay kit (Thermo Scientific, USA). The range of detection of the assay was 10-1280 pg/ml.

2.4 Statistical Analysis

Data are shown as mean $\pm$ SEM with the number of animals indicated in the figure legends. In Experiment 1, anxiety-like behavior and CRF content in Avoiders and Non-Avoiders were analyzed with one-way ANOVA where stress history was the treatment factor. In Experiment 2, differences in ACTH and CORT concentrations in Control and stress animals over time were determined by two-way RM ANOVA where stress history was the between-subjects factor and time point post-stress was the within-subjects factor. In Experiment 3, differences in ACTH and CORT concentrations in Avoiders and Non-Avoiders were determined by two-way RM ANOVA where stress history was the between-subjects factor

and context environment was the within-subjects factor. Post hoc analysis with Neuman–Keuls test was used when appropriate. Pearson's correlations were used to determine relationships between behavioral and endocrine outcomes. Statistical significance was set as $p < 0.05$.

3. Results

3.3.1 Predator odor stress produces persistent avoidance and increases anxiety-like behavior

Twenty-four hours following predator odor stress, animals were indexed for avoidance. Figure 1 shows that Avoiders exhibit significantly greater avoidance of the predator odor-paired context at both 24 hours and 11 days post-stress $F_{(1, 15)} = 21.213$, $p < 0.001$ when compared to Non-Avoiders, confirming previous reports that avoidance is persistent (10).

We tested anxiety-like behavior in Non-Avoiders and Avoiders post-stress. As shown in Figure 2A, a one-way ANOVA revealed a main effect of group $F_{(2, 21)} = 17.60$ $p < 0.001$ on the percent time spent in open arms of the EPM at 48 hours post-stress. Post-hoc analysis revealed a significant decrease in percent of time spent in open arms in both Avoiders (3 ± 3 vs $21 \pm 6\%$; $p < 0.001$) and Non-Avoiders ($3 \pm 3\%$ vs $21 \pm 6\%$; $p < 0.001$) relative to unstressed Controls. Because we observed enhanced anxiety-like behavior 48 hours following predator odor stress, we examined whether this behavior was persistent in the open field test. A one-way ANOVA revealed a main effect of group $F_{(2, 21)} = 7.97$ $p = 0.003$ on time spent in the center of the open field test at 5 days post-stress. Post-hoc analysis revealed a significant decrease in the time spent in the center of the open field in both Avoiders ($4 \pm 1\%$ vs $7 \pm 1\%$; $p = 0.002$) and Non-Avoiders ($3 \pm 1\%$ vs $7 \pm 1\%$; $p = 0.38$) relative to unstressed Controls (Figure 2B). These results suggest that predator odor exposure increases anxiety-like behavior in rats up to 5 days post-odor exposure.

3.3.2 Predator odor stress increases circulating ACTH and CORT concentrations

To determine the effect of predator odor stress on circulating ACTH and CORT concentrations, pre- and post-odor hormone concentrations were compared. As shown in Figure 3A, there were no differences ($p = 0.630$) in baseline concentrations of circulating ACTH in Control and predator odor-exposed animals (13 ± 7 pg/ml vs. 45 ± 50 pg/ml). A two-way RM ANOVA yielded a marginal effect of group $F_{(1, 20)} = 5.306$, $p = 0.069$ and a main effect of time $F_{(4, 20)} = 3.401$, $p = 0.028$ post-stress that were clearly attributable to increased ACTH in stressed rats over time (Figure 3A). There were no differences ($p = 0.511$) in baseline concentrations of circulating CORT between Control and predator odor exposed animals (105 ± 57 ng/ml vs. 217 ± 88 ng/ml; Figure 3B). A two-way RM ANOVA yielded a main effect of group $F_{(1, 20)} = 8.55$, $p = 0.033$ on circulating CORT concentrations demonstrating that predator odor exposure induces activation of the HPA stress response (Figure 3B).

3.3.3. Blunted HPA response to stress in animals that avoid predator odor-paired context

In Experiment II, predator odor stress produced a significant increase in circulating ACTH and CORT levels. The goal of this experiment was to examine individual differences in the

stress response in Non-Avoiders and Avoiders. A two-way RM ANOVA yielded a significant effect of time $F_{(2, 19)} = 19.19$, $p < 0.001$ and stress $\times$ time $F_{(2, 19)} = 6.17$, $p < 0.05$ on circulating ACTH concentrations. As shown in Figure 4A, there were no differences in baseline concentrations of ACTH (27 ± 12 pg/ml vs. 50 ± 21 pg/ml; $p = 0.294$) between Non-Avoiders and Avoiders. Additionally, no differences in circulating ACTH were noted following exposure to room air in the conditioning apparatus (79 ± 33 pg/ml vs. 98 ± 18 pg/ml; $p = 0.446$). Predator odor stress produced a significant increase ($p < 0.001$) in ACTH in Non-Avoiders compared to baseline values (226 ± 34 pg/ml vs. 27 ± 12 pg/ml). Post-hoc analysis revealed that the ACTH response to predator odor stress was significantly ($p = 0.002$) attenuated in Avoiders when compared to Non-Avoiders (116 ± 8 pg/ml vs. 226 ± 34 pg/ml).

A two-way RM ANOVA yielded a significant effect of time $F_{(2, 26)} = 58.22$, $p < 0.001$ and stress $\times$ time $F_{(2, 26)} = 4.07$, $p < 0.05$ on circulating CORT concentrations (Figure 4B). There were no differences in baseline values of CORT (91 ± 37 ng/ml vs. 74 ± 18 ng/ml; $p = 0.810$). There was a significant increase in circulating CORT concentrations in Non-Avoiders (333 ± 80 ng/ml vs. 91 ± 37 ng/ml; $p < 0.001$) and Avoiders (427 ± 33 ng/ml vs. 74 ± 18 ng/ml; $p < 0.001$) following exposure to room air in the conditioning apparatus when compared to baseline values (Figure 4B). Predator odor stress produced a significant increase ($p < 0.001$) in CORT concentrations in Non-Avoiders (586 ± 58 ng/ml vs. 91 ± 37 ng/ml) and Avoiders (425 ± 114 ng/ml vs. 74 ± 18 ng/ml) relative to baseline and in Non-Avoiders (586 ± 58 ng/ml vs. 333 ± 80 ng/ml; $p < 0.001$) relative to exposure to room air. Post-hoc analysis revealed that the CORT response to predator odor stress was significantly ($p = 0.002$) attenuated in Avoiders when compared to Non-Avoiders (445 ± 67 ng/ml vs. 586 ± 58 ng/ml; respectively).

We also examined associations between circulating HPA markers and avoidance of the predator odor stress-paired context. Figure 4C indicates that circulating ACTH concentrations were negatively correlated with avoidance ($r^2 = 0.32$; $p = 0.055$). Figure 4D indicates that circulating CORT concentrations were significantly negatively correlated with avoidance ($r^2 = 0.31$; $p = 0.024$). Animals with lower circulating levels of ACTH and CORT HPA markers were more likely to be Avoiders than Non-Avoiders.

3.3.4. Predator odor stress increases CRF content in the PVN

The goal of this experiment was to test the effect of predator odor stress on CRF content in the PVN of Avoiders. PVN CRF content was measured 12 days post-stress. As shown in Figure 5, a one-way ANOVA revealed a significant effect of group (stress history) $F_{(2, 18)} = 3.510$, $p < 0.05$. Post-hoc analysis reveals a significant increase ($p < 0.05$) in PVN CRF content in Avoiders (40 ± 6 pg/mg protein vs. 20 ± 2 pg/mg protein) when compared to unstressed Controls. A trend towards elevated CRF (38 ± 8 pg/mg protein vs. 20 ± 2 pg/mg protein; $p = 0.10$) was noted in Non-Avoider animals when compared to unstressed Controls; however, this was not statistically significant. No differences were noted between Non-Avoiders and Avoiders.

4. Discussion

The present study examined the effects of predator odor stress on anxiety-like behavior and HPA activation in animals that exhibit persistent avoidance of stress-related stimuli. In agreement with findings from the literature, these results demonstrate that predator odor exposure increases anxiety-like behavior as reflected by decreased time spent in the open arm of the EPM and decreased time in the center of the open field test (6, 17). Predator odor stress also produced significant increases in circulating ACTH and CORT concentrations. Because of the role of HPA activation in mediating physiologic stress responses, we examined whether predator odor exposure produced differential HPA activation in Avoiders and Non-Avoiders. Avoiders showed a markedly attenuated ACTH and CORT response to predator odor stress. Interestingly, blunted ACTH/CORT levels were predictive of avoidance, suggesting that HPA dysregulation may play a role in the subsequent emergence of stress-related symptoms.

Animal models of stress should account for between-subject variability in behavioral and neuroendocrine aspects of the stress response. These studies utilized predator odor stress to examine behavioral and neuroendocrine adaptations associated with stress reactivity. Predator odor was selected as a model of stress because it excludes pain as a confounding variable and allows for examination of variable behavioral responses to stress across animals. While the current studies demonstrate predator odor produces avoidance behavior in a subpopulation of animals, it is possible that this response is not unique to bobcat urine and that other volatile compounds may produce a similar response. Exposure to urine of different predator species, but not of herbivores, has been shown to induce defensive behaviors in rats, but in these studies only bobcat urine was tested (11). One limitation of this study is the lack of an aversive control, or one that uses an aversive odor other than predator odor to examine avoidance.

Rodent olfactory sensory neurons respond to 2-phenylethylamine found in predator odor (17). This compound binds to trace amine-associated receptor-4 located on rodent olfactory bulbs and produces innate behavioral responses (17). Urinary concentrations of 2-phenylethylamine varies between species with carnivores having the highest concentrations ($\sim 50 \mu\text{M}$) and other species $>1 \mu\text{M}$. (17). Ferrero et al. demonstrated that TAAR4 is important for detecting predator odors (12). Increased TAAR4 activity is detected in HEK293 cells transfected with TAAR4 incubated with bobcat urine and mountain lion urine (12). Interestingly, this response is specific to carnivore urine as TAAR4 activity is not affected by mouse, human or rat urine, or the absence of urine, suggesting that TAAR4 activation is required for avoidance behaviors (12). Additionally, these studies demonstrate that 2-phenylethylamine is key in mediating avoidance to predator odor (17). Lion urine produces significant avoidance of a paired context, but this avoidance is abolished if the urine is depleted of 2-phenylethylamine (12). It is possible that there may be differences in TAAR4 expression or activity in Avoiders vs. Non-Avoiders, identifying a potential area of future study. Utilizing predator odor stress, our published findings demonstrate that Avoiders exhibit avoidance up to 6 weeks post-odor exposure (10). Here, we show that avoidance persists at least 11 days post-stress, which agrees with previously published findings from our lab that avoidance persists up to 6 weeks in Avoiders (15).

Stress increases anxiety in humans, but although some individuals with stress-related disorders also display heightened anxiety, high anxiety is not necessary for diagnosis (DSM-5). Here, we report that predator odor increased anxiety-like behavior at 48 hours and 5 days post-stress, although we did not observe differences between Avoiders and Non-Avoiders. These data suggest that predator odor is a powerful aversive stimulus that produces similar increases in anxiety-like behavior in all stressed rats. This is in agreement with previous reports that predator odor increases anxiety-like behavior (1, 5) and startle reactivity in rats (1, 22). Other types of stress (e.g., restraint stress or footshock) also produce increases in anxiety-like behavior as measured by the EPM and open field test (3, 13).

One potential mediator of stress-induced behaviors, is an abnormal HPA stress response to the traumatic event (7). HPA disturbances during stress are well documented; however, the direction and magnitude of HPA changes are influenced by several factors including time of measurement (pre-stress baseline, post-stress, post-stress baseline) and the type of sample (plasma, cerebral spinal fluid, urine). Individuals that develop PTSD have blunted cortisol levels at the time of trauma (8, 9). Delahanty et. al. showed that low initial post-trauma urinary cortisol levels following motor vehicle accidents predict subsequent development of stress-related symptoms (9). Similar findings have been reported in children following child trauma with low cortisol levels predictive of the development of symptoms (8). Yehuda et. al. has extensively characterized basal HPA axis function and HPA axis reactivity in individuals with PTSD. These studies have found lower plasma basal cortisol concentrations in the weeks following the trauma, increased lymphocyte glucocorticoid receptor expression, and enhanced negative feedback as measured by increased suppression of cortisol following dexamethasone administration (33). Additionally, high levels of CRF in cerebral spinal fluid and low gene expression of the glucocorticoid receptor co-chaperone, FKBP5, in peripheral blood mononucleocyte cells have been noted in combat veterans (32). FKBP5 is a gene encoding FKBP51, a binding protein that is part of the glucocorticoid receptor complex that regulates glucocorticoid sensitivity and negative feedback (4). Binding of FKBP51 to HSP90 of the glucocorticoid receptor complex prevents nuclear translocation and transcription of target genes (4). Collectively, these data suggest that the HPA axis is hypoactive in individuals with PTSD.

In the present study, we examined circulating ACTH and CORT at baseline and following predator odor stress. The animals underwent significant handling and habituation protocols prior to initiating the experiments. While there was no significant difference in circulating baseline values of these hormones, and in spite of our habituation/acclimation procedures, predator odor-exposed animals did exhibit slightly higher baseline ACTH/CORT values. Interestingly, Avoiders exhibit blunted ACTH/CORT concentrations immediately following predator odor stress. Other pre-clinical studies utilizing Lewis rats report that blunted HPA axis response to stress increases the development of stress-related symptoms such as hyperarousal (8). Lewis rats exhibit exaggerated stress-induced anxiety-like behavior measured by the EPM 7 days post-stress and lower CORT concentrations relative to other strains of rats following predator stress (8). In contrast, Cohen and Zohar utilized behavioral tests to show that, “maladapted” animals, show increased circulating CORT levels measured in trunk blood 7-days post-stress (7, 9). Discrepancy among these studies is potentially the

result of differences in timing (7 days vs immediately following predator odor stress). Based on our ACTH/CORT results and the clinical literature, we hypothesized that Avoiders might have lower post-stress paraventricular CRF content than Non-Avoiders and unstressed Controls. At 12 days post-stress, we observed significantly higher CRF content in PVN of Avoiders relative to Controls, but no difference between Avoiders and Non-Avoiders. It is important to note that we measured total CRF peptide content in PVN 12 days post-stress, which does not reflect PVN CRF peptide content at the time of stress. It is possible that at the time of stress, Avoiders have lower CRF content and/or release in the PVN, but this hypothesis remains to be tested. Alternatively, predator odor stress may modulate the activity of noradrenergic inputs to PVN, which would also alter CRF release in PVN.

Impaired HPA function can impact behaviors in rodents other than those tested here, for example helpless-like and dysphoric-like behaviors. For example, mice genetically bred to have reduced glucocorticoid receptor expression displayed increased helplessness measured in a shuttle box test following exposure to inescapable footshocks (21). Other studies report that HPA hyposensitivity contributes to stress-induced behavioral abnormalities following social defeat stress (4). Mice that had low morning cortisol levels displayed increased dysphoria-like behavior as measured by decreases in immobility as well as increases in defecation during a 5 min tail suspension test when compared to high morning cortisol mice (4). These data are supported by studies by Krishnan et. al. showing that lower plasma corticosterone concentrations are positively correlated with anhedonia-like behavior and increases in social avoidance (18). Conversely, higher glucocorticoid levels may confer stress resilience; for example, higher levels of maternal care in rats are associated with epigenetic changes and higher glucocorticoid receptor expression and greater feedback inhibition associated with stress resilience in adulthood (25). Collectively, these data identify potential factors that may confer susceptibility and/or resilience to psychiatric illnesses following exposure to stress.

In the present study, we report that blunted HPA activation predicts avoidance. We observed an inverse relationship between ACTH/CORT and avoidance following predator odor stress. Animals that exhibit a blunted ACTH/CORT response to predator odor stress were more likely to be subsequently classified as Avoiders in comparison to those with a normal HPA response. These preclinical findings identify a potential mechanism for the behavioral dysregulation, particularly avoidance, associated with stress.

Conclusions

The results from these studies indicate that blunted HPA axis activation is predictive of high avoidance following predator odor exposure. Predator odor stress produced heightened anxiety-like behavior in both Avoider and Non-Avoider subpopulations suggesting that both groups perceived the odor. Avoiders exhibited lower circulating ACTH and CORT concentrations following stress when compared to Non-Avoiders. Future investigations aimed at understanding the mechanisms contributing to the blunted HPA stress response in animals that avoid a predator odor-paired context will inform the development of therapeutic strategies for the treatment of individuals with trauma and stress-related disorders.

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Highlights

- Predator odor exposure increases anxiety-like behavior.
- Predator odor stress increases circulating ACTH and CORT concentrations.
- Animals are divided into subpopulations based on stress-reactivity.
- Avoiders show a markedly attenuated ACTH and CORT response to predator odor stress.
- The blunted ACTH/CORT levels were predictive of avoidance.

Figure 1.

Avoidance (time pre-conditioning test to post-conditioning test time spent in predator odor-paired chamber). Rats were classified as Non-Avoiders (black striped bar; n=13) or Avoiders (black bar n=25) based on avoidance scores calculated at 24 hours post-odor exposure. In Experiment 1, animals were tested again for avoidance at 11 days post-stress prior to sacrifice. Data are shown as mean $\pm$ SEM. * indicates $p < 0.05$ vs. Non-Avoiders.

Figure 2.

(A) Anxiety-like behavior (% time in open arm) measured 48 hours post-stress in Controls (white bar; $n = 6$), Non-Avoiders (black striped bar; $n = 4$) and Avoiders (black bar; $n = 13$) using the elevated plus maze test. (B) Anxiety-like behavior (% of time in center) measured 5 days post-stress in Controls (white bar; $n = 6$), Non-Avoiders (black striped bar; $n = 4$) and Avoiders (black bar; $n = 13$) using the open field test. Data are shown as mean $\pm$ SEM. * indicates $p < 0.05$ vs. Control animals.

Figure 3.

(A) Circulating adrenocorticotropin hormone (ACTH) concentrations (pg/ml) measured at baseline, 30, 60, 90 and 120 min post-stress in Control (white circles; $n = 4$) and odor-exposed animals (black circles; $n = 4$). (B) Circulating corticosterone (CORT) concentrations (ng/ml) measured at baseline, 30, 60, 90 and 120 min post-stress in Control (white circles; $n = 4$) and odor-exposed animals (black circles; $n = 4$). Data are shown as mean $\pm$ SEM. # indicates $p < 0.05$ vs. Control animals.

Figure 4.

(A) Circulating adrenocorticotropin hormone (ACTH) concentrations (pg/ml) measured at baseline, following neutral conditioning and following predator-odor conditioning in Non-Avoiders (gray circles; $n = 6$) and Avoiders (black circles; $n = 6$). (B) Circulating corticosterone (CORT) concentrations (ng/ml) measured at baseline, following neutral conditioning and following predator-odor conditioning in Non-Avoiders (gray circles; $n = 7$) and Avoiders (black circles; $n = 8$). (C) Scatter plot for individual rats ($n = 12$) show change in circulating ACTH concentrations post-odor exposure versus change in preference for predator-paired context. Rats with lower ACTH concentrations exhibited high avoidance of the predator-paired context 24 hours post-odor exposure. (D) Scatter plot for individual rats ($n = 15$) show change in circulating CORT concentrations post-odor exposure versus change in preference for predator-paired context. Rats with lower CORT concentrations exhibited high avoidance of the predator-paired context 24 hours post-odor exposure. Data are shown as mean $\pm$ SEM. * indicates $p < 0.05$ vs. treatment-matched baseline values. # indicates $p < 0.05$ vs. Non-Avoiders.

Figure 5. Corticotropin releasing factor (CRF) measured in the PVN 12 days post-stress in Controls (white bar; $n = 6$), Non-Avoiders (striped bar; $n = 4$) and Avoiders (black bar; $n = 13$). Data are shown as mean $\pm$ SEM. * indicates $p < 0.05$ vs. treatment-matched baseline values.